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Selection *in vitro* of novel ribozymes from a partially randomized U2 and U6 snRNA library

Thomas Tuschl[1,2,3], Phillip A.Sharp[1,3] and David P.Bartel[2,3]

[1]Center for Cancer Research and [1,2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139 and [2]Whitehead Institute for Biomedical Research, Cambridge, MA 02114, USA

[3]Corresponding authors
e-mail: dbartel@wi.mit.edu

Combinatorial libraries related to spliceosomal U2 and U6 snRNAs were tested for catalytic reactions typical of the splicing of nuclear pre-mRNAs. Ribozymes with four different activities were selected based on covalent bond formation to a substrate RNA. The first activity was reversible self-cleavage; ribozymes self-cleaved then ligated the 5′-hydroxyl group of the substrate oligonucleotide to their 2′,3′-cyclic phosphate intermediate. The second activity was 2′,5′-branch formation by the attack of a substrate 2′-hydroxyl group on the 5′-terminal triphosphate of the ribozyme transcript, releasing pyrophosphate. The third ribozyme activity was similar to reversible self-cleavage but was a three-step reaction. This ribozyme self-cleaved, then cleaved the substrate *in trans*, and then ligated the substrate 3′ cleavage product to its cyclic phosphate intermediate. This three-step pathway shares similarities with the pathway of tRNA splicing. The fourth activity was 2′,3′-branch formation; to form this unusual branch, a 2′-hydroxyl of the substrate attacked an internal phosphate of the ribozyme, releasing an oligonucleotide leaving group. The isolation of branching activities by the *in vitro* selection protocol was unanticipated and was due to surprising properties of reverse transcriptase, which can read through 2′,5′- or 2′,3′-branches and efficiently perform non-templated intramolecular jumps.

Keywords: *in vitro* selection/reverse transcriptase/ribozyme/RNA branch/snRNA

Introduction

Using iterative selection methods, molecules with unique activities can be isolated from large combinatorial libraries of nucleic acid sequences (Gold *et al*., 1995; Osborne and Ellington, 1997). This has provided a method to examine the functional abilities of RNA and other nucleic acid polymers. The isolation of new ribozymes from combinatorial libraries has extended the known catalytic repertoire of RNA far beyond what has been found in contemporary biology (Lorsch and Szostak, 1996; Williams and Bartel, 1996; Breaker, 1997). Exploration of the catalytic ability of RNA is of particular interest for evaluating theories of early evolution, which assert that key biocatalysts, currently composed of protein or ribonucleoprotein, were once composed of RNA (Joyce and Orgel, 1993; Lorsch and Szostak, 1994; Illangasekare *et al*., 1995; Ekland and Bartel, 1996; Zhang and Cech, 1997).

Previously, the starting libraries for the isolation of new ribozyme activities have either been pools of variants of a known ribozyme or large pools of random sequences (Beaudry and Joyce, 1992; Pan and Uhlenbeck, 1992b; Bartel and Szostak, 1993). Here, the type of library was different in that it was based on RNAs that reside within a catalytic ribonucleoprotein complex. A library built around the core sequences of spliceosomal RNAs U2 and U6 (Figure 1A) (Moore *et al*., 1993), served as the starting point for generating new RNA-processing ribozymes. Although not known to be catalytic in the absence of other spliceosomal components, the U2 and U6 small nuclear RNAs (snRNAs) are conjectured to be remnants from an era when all pre-mRNA splicing was catalyzed by RNA (Sharp, 1985; Cech, 1986). Thus, these snRNAs might retain some of the function or structure of the putative ancestral ribozyme, and an RNA pool based on their sequences may be enriched in RNA-processing ribozymes.

An *in vitro* selection experiment was designed to examine simultaneously $>10^{14}$ snRNA variants for their ability to promote RNA-processing reactions, including the type of transesterification reaction executed by spliceosomes. Four different activities were isolated. Even though the selection criteria were designed to be optimally satisfied by a reaction equivalent to the second step of splicing, none of the ribozymes promoted this reaction. Of the ribozymes isolated, the most notable were a ribozyme with a three-step reaction pathway similar to that of tRNA splicing and a ribozyme that promotes a previously undescribed and relatively difficult transesterification reaction.

Results

Design of the snRNA-based library and selection

Catalytically active RNA was isolated by an iterative process of selection and amplification from an RNA library of 3×10^{14} different 265 nt sequences (Figure 1B). The library contained partially randomized segments based on the core sequences of U2 and U6 (157 nt), fully randomized segments (80 nt), and invariant segments (28 nt). The snRNA segments were doped at 3%, so that on average five of the 157 positions differed from wild-type. The probability of finding the wild-type snRNA sequence was $(0.97)^{157}$, corresponding to 3×10^{12} molecules in the initial pool. Random-sequence elements were positioned at both ends of the snRNA region. A consensus 5′ exon sequence formed the 3′ end of each library molecule.

The library was incubated with a substrate that contained

a prototype 3′ splice site and a branch point sequence for hybridization to U2 (Figure 1C). The *in vitro* selection was designed to select for RNAs that catalyze a transesterification reaction modeled after the second step of splicing (exon ligation). Spliceosomal RNA interactions required for the second step (Umen and Guthrie, 1995) were provided by adding *in trans* a U5 stem–loop oligoribonucleotide and a 6 nt 5′ intron oligoribonucleotide. (Later analysis showed that these added oligoribonucleotides were not required by any of the isolated ribozymes.)

Selection of RNA-processing catalysts was designed to take advantage of (i) decreased gel mobility after incubation with substrate, (ii) acquisition of biotin that was covalently linked to the 3′ end of the substrate, (iii) acquisition of a reverse transcription primer-binding sequence, and (iv) specific priming by a PCR primer complementary to the anticipated ligated RNA sequences (Figure 2). These sequential selection steps should provide $>10^5$-fold enrichment for RNA products fulfilling all of the designed criteria. Ligation activity became clearly detectable in round five of the selection experiment. Although the selected ribozymes fulfilled the first three selection criteria and performed ligation reactions, they did this in unanticipated ways.

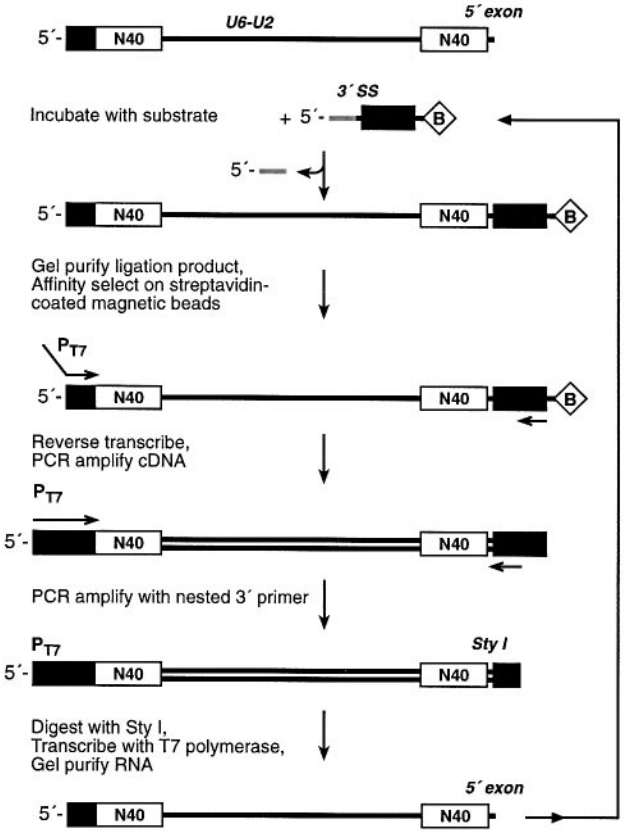


Fig. 2. *In vitro* selection scheme for ribozymes that are covalently linked to the 3′-exon sequence of the substrate. Abbreviations: P_{T7}, T7 RNA polymerase promoter; N40, 40 nt of random sequence; B, biotin.

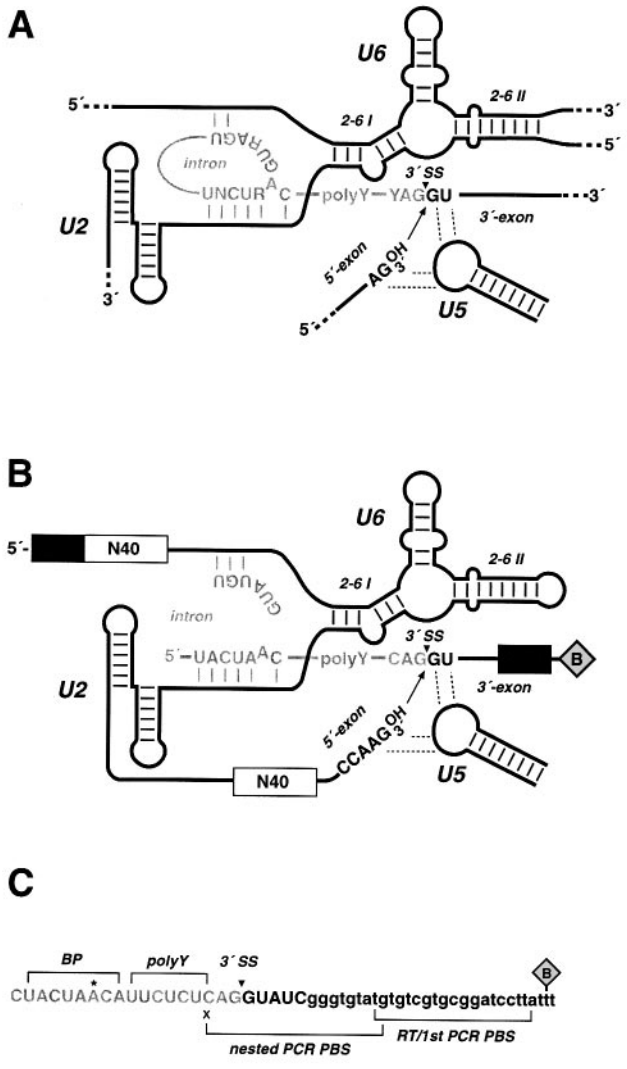
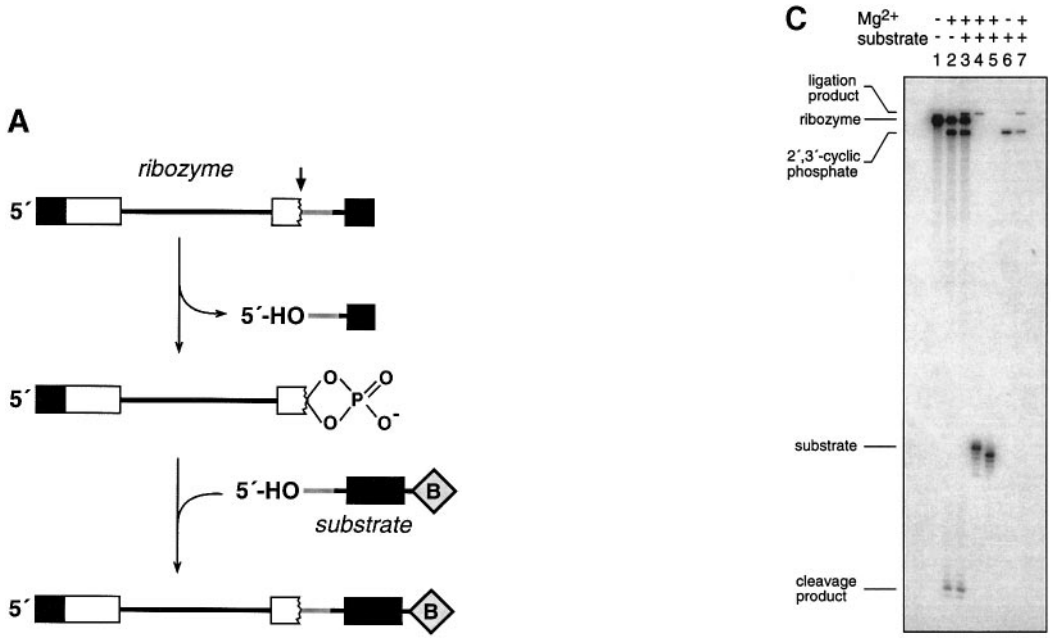


Fig. 1. RNA interactions in the spliceosome and design of the snRNA-based sequence library. (**A**) RNA interactions prior to the second catalytic step (exon ligation) as recently reviewed (Umen and Guthrie, 1995). Intron sequence is depicted in gray. Conserved residues at the branch point and the splice sites of mammalian pre-mRNA are shown, and important base-pairing interactions (U2 branch site helix, U6 5′ splice site helix, U2–U6 helices I and II) and non-Watson–Crick interactions (U5 with 5′ and 3′ exon) are illustrated. (**B**) Design of an RNA library based on core U2 and U6 snRNA sequences. Partially mutagenized U2 and U6 snRNA elements were linked to each other at the end of helix 2–6 II by a tetranucleotide loop. The snRNA sequences were flanked by 40 nt of random sequence (N40) on either side. The 5′ exon sequence (CCAAG) was fused to the 3′ end of the pool and a constant primer-binding sequence was added to the 5′ end. The RNA pool was incubated in the presence of a biotinylated (B in diamond) RNA–DNA substrate containing the branch point recognition sequence (UACUAAC) and the 3′ splice site (3′ SS) followed by the reverse transcriptase primer-binding sequence (solid box). The potentially important U5 snRNA hairpin and a 6 nt 5′ intron RNA (GUAUGU) were also present during the incubation. Sequences from yeast were chosen for the 5′ intron oligonucleotide and the substrate branch site. (**C**) Ligation substrate. The 52 nt synthetic RNA–DNA chimeric molecule (RNA residues, uppercase letters; DNA residues, lowercase letters) contained a branch point-recognition sequence (BP; * indicates branch point adenosine) that is followed by a short polypyrimidine tract (polyY) and a consensus 3′ splice site (3′ SS). Intron sequence is depicted in gray, 3′ exon sequence in black. Most of the 3′ exon nucleotides were DNA residues that served as primer-binding sequences for reverse transcription–PCR. The 3′ residue of the nested PCR primer was a thymidine designed to be complementary to the product of '5′ exon' attack at the '3′ splice site' (the substrate residue that is mismatched with the nested PCR primer is indicated by an x). The substrate contained a biotinylated thymidine (B in diamond), used for affinity selection of ligated pool molecules.

Self-cleaving and substrate-ligating ribozymes

The first class of ribozymes to emerge performed the ligation to substrate in a two-step reaction (Figure 3A). Individual members of this class (Figure 3B) were obtained by cloning the PCR product after six rounds of selection.

Characterization of these ribozymes suggests a scheme of self-cleavage with formation of a 2′,3′-cyclic phosphate intermediate that is then attacked by the 5′-hydroxyl of the synthetic substrate. Self-cleavage of the transcribed nested PCR product required Mg^{2+} and occurred in the absence of the substrate (Figure 3C, lanes 1 and 2). The ligation product appeared in the presence of substrate (Figure 3C, lanes 3 and 4) and also formed when the long self-cleavage product was purified and then incubated with the substrate (Figure 3C, lane 7). The ligation was blocked when the substrate 5′-hydroxyl group was phosphorylated (Figure 3C, lane 5). These properties, together with the fact that the ligation product was



B

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     1         10        20        30        40        50        60        70        80        90
pool GGAAAUGCCCAACUGCUNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN--AUUGG-AACGAUACAGAGAAGAUUAGCAUGGCCCCUGCGCAAG
S1   GGAAAUGCCCAACUGCUAAAUUAAUGCAUAGUCUAACCGCGUUCUCGGCACUUCCACC-AUUGG-AACGAUACAGAGAAGUUUAGCAUUGCCCCUCCGCAAG
S9   GGAAAUGCCCAACUGCUGUGUAGUAGCCUGGCGGAAUUCGAUGCCCGCCUAGCACCG--AUUGG-AACGAUACAGGGAAGAUCAGCAUGGCCCCUGCGCAAG
S14  GGAAAUGCCCAACUGCUGUUUCAGUUUCAGGAGCGUAAGGUCAAUGGCUCCAGACAAC-AUUGG-AACGAUACAGAGAAGAUUAGCAUGGCCCCUGCGCCAG
S3   GGAAAUGCCCAACUGCUCGACUGGCCCAGCGACACGUCCUCCAAUAAACCAUUGGAG--AUUGG-AACGAUACAGAGAAGAUUAGCAAGGCCCCGGCGCAAG
S15  GGAAAUGCCCAACUGCUGUCCCGGUCCGACCGGAGGAAUGGAGAUAACGUGCAACUACAGUUGGGAACGAUACAGAGAAGAUUAGCAUGGCCUCUCCGCAAG

     100       110       120       130       140       150       160       170       180       190
pool GAUGACACGCAAAUUCGUGAAGCGGCACCGCUUCUCGGCCUUUUGGCUAA--GAUCAAGUGUAG-UAU-CUGUUCUUAUCAGUUUAAUAUCUGAUACGUCCU
S1   GAUGACACGCAAAUUCGUGAAGCGGCACCGCUUCUC-GCCUUUUGGCUAAGGGAUCAAGUGUAGGUAUUCUGUUCUUAUCAGUUUAAUAUCUGAUACGUCCU
S9   GAUGACACGCAAAUUCGUGA-GCGGCACCGCUUCUCGGCCUUUUGGCUAA--GAUCAAGUGUAG-UAU-CUGUUCUUAUCAGUUUACUAUCGGAUACGUCCU
S14  GAUGACACGCAAAUUCGUGAAGCGGCACCGCUUCUCGGCCUAUUGGCUAA--GAUCAAGAGUAG-UAU-AUGUUCUUAUCAGUUUAAUAUCUGAUACGUACU
S3   GAUGACACGCAAAUUCGUAAAGCGGCACCGCUUCUCGGCCUUUUG-CUAA--GAUCAAGUGUAG-UAU-CUGUUCUUAUCAGUUUAAUAUCUGAUACGUUCU
S15  GAUGAGACGCAAAUUCGUGAAACGGCACCGCUUCUCGGCCUUUUGGCUAA--GAUCAAGUGUAG-UAU-AUGUUCUUAUCAGUUUAAUAUCUGAUACGUCCU

      200       210       220       230       240       250       260
pool CUAUCCG-AGGACAAUAUA-UUAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCAAG
S1   CUAUCCGGAGGACAAUAUAAUUAGAUCGGAAAAGGAAGGA↓----------CUACUAACAUUCUCUAAGGUAUCGGGUGUAUG
S9   CUAUCCG-AGGGA↓----------CUACUAACAUUCUCUAAGGUAUCGGGUGUAUG
S14  CU-UCCG-AGGACAAUCUA-UUAAAAGCGAGAUUGGGGACUGUCUCGGCUCG↓----------CUACUAACAUUCUCUAAGGUAUCGGGUGUAUG
S3   CU-UCCG-AGGACAAUAUA-UUAAAAUUGGGAAUGCCUCGGUCAUA↓----------CUACUAACAUUCUCUAAGGUAUCGGGUGUAUG
S15  CUAUCCG-AGGACAAUAUA-UUAAAGAGCUCCCAUCCAGGGCC↓----------CUACUAACAUUCUCUAAGGUAUCGGGUGUAUG
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Fig. 3. Self-cleaving and substrate-ligating ribozymes. (**A**) Reaction scheme. Primer-binding sequences are depicted by black boxes, randomized regions by open boxes and doped U2/U6 snRNAs by a black line. The intron sequence present in the substrate is in gray. The cleavage site is indicated by a small arrow. B, biotin. (**B**) Sequence alignment of isolates of the reversibly self-cleaving ribozymes. The RNAs (S1–S15) are aligned relative to the pool from which they were selected. An arrow indicates the cleavage site. Sequences are ranked by the abundance of their sequence family within the sequences isolated (of the 14 isolates, seven were similar to S1, three were similar to S9, two were similar to S14; S3 and S15 were unique). The numbering refers to the pool sequence; dashes are used to accommodate deleted or inserted bases. Self-cleaving activity was confirmed for S1, S9 and S14. The length of the transcribed ribozymes prior to self-cleavage varies between 270 and 280 nt, except for S9 which is 240 nt. However, a variant clone of S9 with an additional 5′ T7 promoter and 5′ primer-binding sequence was found and when used as template for transcription yields RNAs of 277 and 240 nt in length. The longer variant was presumably enriched during selection because its mobility is closer to the 265 nt length marker used in the gel-purification of transcribed RNAs. (**C**) Reaction of the S14 self-cleaving and substrate-ligating ribozyme. All reactions were in selection buffer for 8 h at 30°C. Lanes 1–3, 6 and 7 included internally labeled ribozyme; lane 4, 3′-labeled substrate; lane 5, 5′-^{32}P-phosphorylated substrate.

efficiently reverse-transcribed during the selection, are reminiscent of the smaller natural ribozymes (e.g. hammerhead and hairpin ribozymes), which perform self-cleavage and ligation via a 2′,3′-cyclic phosphate. The accumulation of ligated product suggests a behavior more similar to that of the hairpin ribozyme, which does not favor the forward reaction (cleavage) over the reverse reaction (ligation) (Hegg and Fedor, 1995).

The self-cleavage rates of the selected ribozymes were two to three orders slower than those observed for the hammerhead or hairpin ribozymes (Hertel *et al*., 1994; Hegg and Fedor, 1995). The most efficient isolate analyzed, S14, had an observed rate constant for approach to equilibrium of ~0.002 min^{-1} at 500 nM ribozyme.

Although the two-step reaction produced ligation junctions with a single mismatch to the primer used in a nested PCR each selection cycle (Figure 2), the cDNAs were still amplified, probably requiring a few extra PCR cycles. As a consequence, the nested PCR products were not recognized by *Sty*I, and transcribed ribozymes carried a sequence complementary to the nested PCR primer at their 3′ end. The gel-mobility selection criterion only permitted the emergence of ribozymes that self-cleaved near their 3′ ends.

2′,5′-branch-forming ribozyme

Isolation and cloning of a second unanticipated ribozyme activity was achieved by repeating round six of the selection with 5′-phosphorylated substrate. Blocking the terminal 5′ hydroxyl of the substrate prevented the ligation to cyclic phosphate intermediates, hence suppressing the enrichment of the reversible self-cleaving ribozymes. All the isolates of the new activity were derivatives of one ancestral molecule which had accumulated various PCR errors and deletions in the 3′ random sequence element during selection. Among ten isolates, reaction rate constants varied between 0.0001 and 0.002 min^{-1}. The isolate with the fastest kinetics, B9 (Figure 4B), was used to characterize the new activity.

The new ribozyme appeared to catalyze the formation of a 2′,5′-branch utilizing the α-phosphate of the ribozyme 5′-triphosphate (Figure 4A). To test this conjecture, release of pyrophosphate was monitored by TLC analysis from [γ-^{32}P]-labeled ribozyme incubated in the presence of substrate (Figure 4C). As suspected, radioactivity was detected at the mobility of pyrophosphate. The formation of a 2′,5′-branch is similar to the first step of a splicing reaction. It is a one-step reaction in which pyrophosphate instead of a 5′ exon RNA is released during formation of the phosphodiester bond.

The branch point was mapped in the oligonucleotide substrate (RNA–DNA chimera) by using partial alkaline hydrolysis (Figure 4D). Alkaline hydrolysis is facilitated by the presence of a deprotonizable 2′-hydroxyl function that attacks the phosphodiester backbone. The branched residue, which has a blocked 2′-hydroxyl, was mapped by comparing bands from the hydrolysis ladders of branched and linear 5′-end-labeled substrate. The blocked hydroxyl group is at a guanosine within the CAGGU segment of the substrate.

Deletion analyses on the ribozyme and the substrate confined the range of sequences important for catalysis. A ribozyme of 76 nt (Figure 4B) was synthesized and tested for activity. The observed rate constant (0.002 min^{-1}) for branch formation at 1 μM ribozyme concentration was the same as that of the longer construct. Increasing the ribozyme concentration to 5 μM did not affect the reaction rate, indicating that 1 μM ribozyme is sufficient to saturate the substrate. Similarly, the reaction rate with a 33 nt minimized chimeric RNA–DNA substrate, UCUCUCAGGUAUCgggtgtatgtgtcgtgcgga, did not differ from that with the longer substrate. Branching with an all-RNA version of the substrate was much slower (1×10^{-5} min^{-1}).

The observation that further substrate and ribozyme deletions were inactive once they encroached on a region of Watson–Crick complementarity between substrate and ribozyme (data not shown) suggested that a base-paired helix forms between the 5′ end of the shortened substrate and the 3′ end of the shortened ribozyme (Figure 4B). The ribozyme and substrate were joined with a hairpin loop at the end of this helix (Figure 4B, lariat), thereby converting the intermolecular branching reaction to an intramolecular lariat formation (Figure 4E). Such lariats can be efficiently debranched with recombinant yeast debranching enzyme DBR1, which hydrolyses 2′,5′-branches with a strong preference for purines at the 2′-position (Nam *et al*., 1994) (Figure 4F).

The selection of a branched molecule was surprising because of the essential step of reverse transcription in the selection protocol (Figure 2). In order to generate a cDNA that could be amplified, reverse transcriptase had to traverse the branch in the template and also jump from the substrate 5′ end to the ribozyme 3′ end. To investigate the ability of reverse transcriptase to perform such a difficult cDNA synthesis, reverse transcription of unbranched template was compared with that of a branched template, in this case, the product of the 76 nt branching ribozyme (Figure 5). The linear substrate was reverse transcribed within 30 s, and N+1 blunt-end addition of an extra nucleotide was completed within the next 5 min. In the presence of a branch, reverse transcriptase stalled a few minutes at the branch site before reading through and stalled again at the end of the substrate. This stalled reverse transcriptase then performed a jump to the ribozyme 3′ end and reverse-transcribed the ribozyme strand. The amount of full-length cDNA relative to input primer DNA was 0.2% after 5 min and reached 10% after 1 h. Reverse transcription with AMV reverse transcriptase, which has an active RNase H, was also able to generate full-length cDNA, although at an ~10-fold reduced efficiency (data not shown).

The efficiency of branch read-through and template jumping was examined with a semi-quantitative reverse transcription–PCR, using linear RNA with 5′ and 3′ primer-binding sequences as an internal standard in a 1 h reverse transcription (data not shown). Reverse transcription of the long 220 nt branched ribozyme is ~200-fold less efficient than that of the linear standard. For the 76 nt branched ribozyme jumping appears more efficient; this RNA reverse transcribes ~10-fold less efficiently than does the standard. If substrate and ribozyme are not covalently linked so that intermolecular jumping must occur to form amplifiable cDNA, a PCR product can still be obtained, but at a 200- to 2000-fold reduced efficiency relative to the internal standard. Reverse transcription of

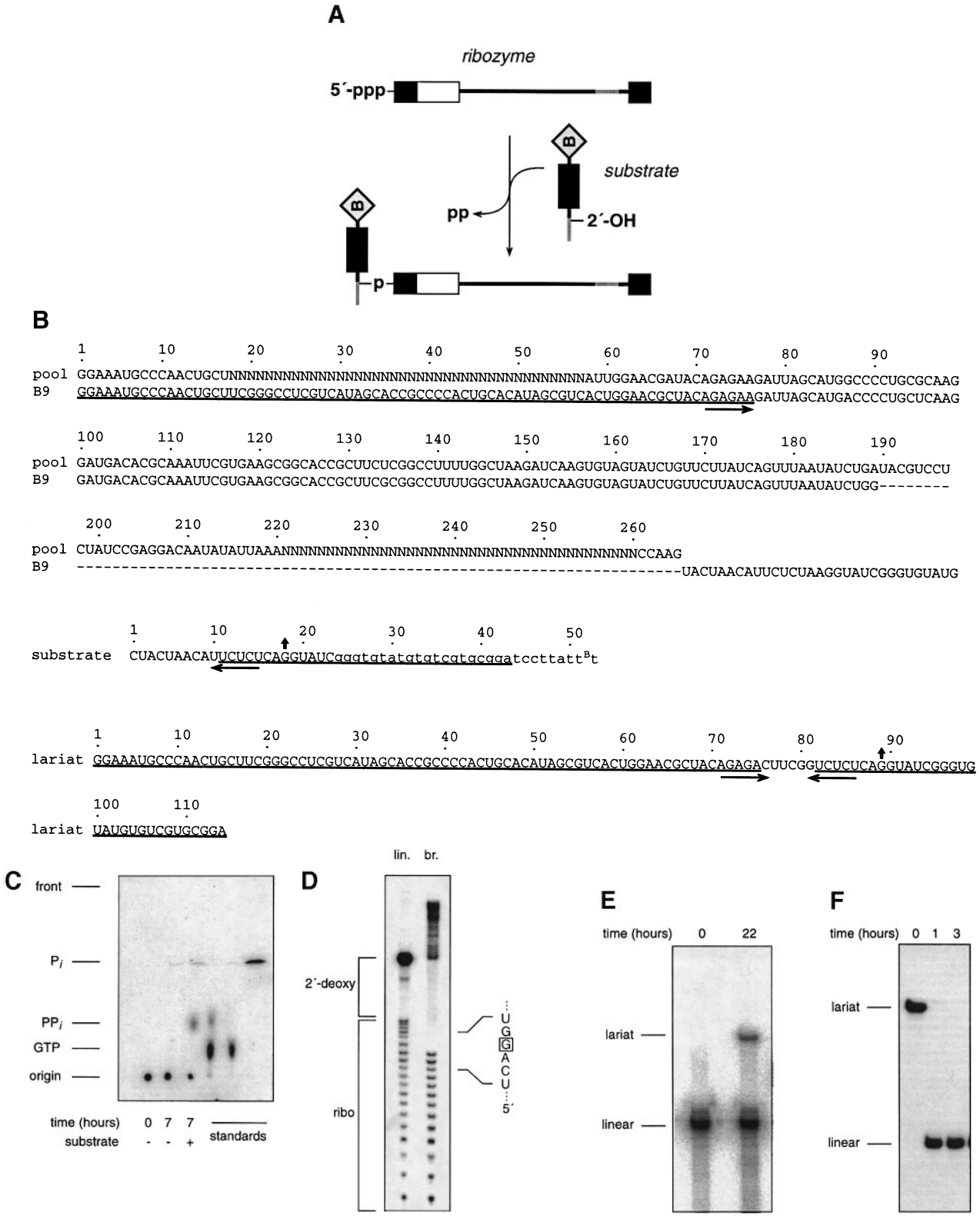

Fig. 4. 2′,5′-Branch-forming ribozyme. (**A**) Reaction scheme. Symbols: ppp, triphosphate; pp, pyrophosphate; p, internucleotide phosphodiester linkage; for other symbols, refer to Figure 3A. (**B**) Sequences of B9 RNA, the starting pool, the substrate oligonucleotide, and the lariat-forming construct. Underlined residues indicate the minimal active fragments as determined by truncation analysis. A potential base pairing interaction between the truncated 3′ end of the ribozyme and the 5′ end of the substrate is indicated by horizontal arrows; the branch point is marked by a short vertical arrow. Truncated ribozyme and substrate were connected by a C(UUCG)G stem-loop sequence at the end of the postulated helix. (**C**) Thin-layer chromatography (TLC) of the leaving group. γ-^{32}P-labeled ribozyme was incubated with or without substrate in selection buffer and samples were run on PEI cellulose with 0.5 M HCl. Standards (from right to left) were: inorganic ^{32}P-phosphate, [γ-^{32}P]GTP, ^{32}P-labeled pyrophosphate (generated by supplementing a T7 transcription reaction with [γ-^{32}P]GTP). (**D**) Mapping of the branch site. Partial alkaline hydrolysis was performed on linear (lin.) and 2′,5′-branched (br.) oligonucleotide substrate. Backbone hydrolysis requires the presence of a free 2′-hydroxyl group which was not available at the branch site or in the 2′-deoxy-half of the substrate. (**E**) Lariat-formation reaction by the 114 nt *cis*-construct (Figure 4D). Linear and lariat forms were separated on a 10% denaturing gel. (**F**) Debranching reaction of purified 114 nt lariat with recombinant yeast DBR1 debranching enzyme.

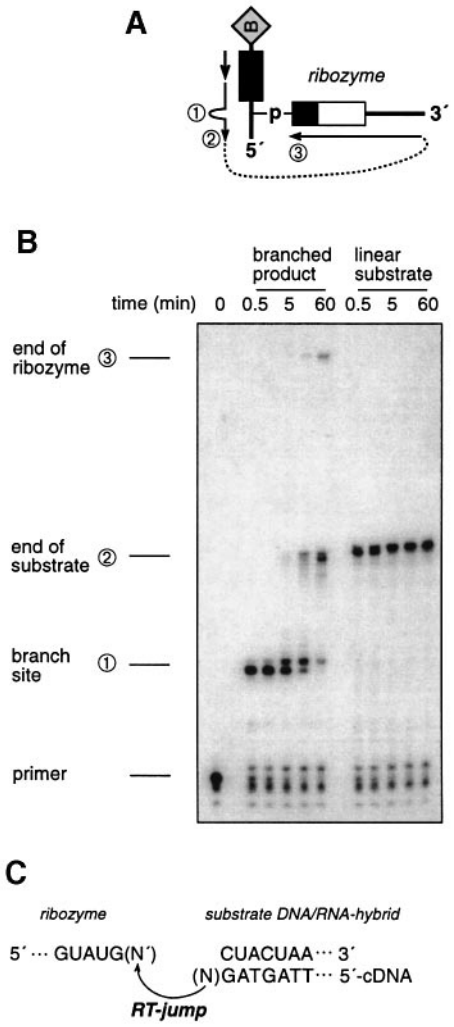

Fig. 5. Primer extension analysis of 2′,5′-branch. (**A**) Schematic presentation of the path of reverse transcriptase. Reverse transcriptase extends the primer (short arrow hybridized to the 3′ end of the substrate), reads through the branch (1), continues to the end of the substrate (2), jumps (dashed line) to the ribozyme 3′ end and continues to the 5′ end of the ribozyme (3). (**B**) Time course of primer extension. Primer extension was performed with a 5′-^{32}P-labeled primer on 2′,5′-branched product of the 76 nt ribozyme or on linear, unbranched control substrate for 0, 0.5, 1, 15 or 60 min. Products of the extension are indicated by numbers used in (A). (**C**) Illustration of nucleotide insertions between substrate and ribozyme sequences. During cDNA synthesis of 2′,5′-branches a one or two nucleotide insertion (indicated as N) is observed between the substrate and the ribozyme. This could arise from heterogeneity of the ribozyme run-off transcript usually observed by the addition of an untemplated nucleotide (N′) and/or by blunt-end nucleotide addition to the substrate–cDNA hybrid (N) by reverse transcriptase. Of the nine clones sequenced, six had a dC insertion on the cDNA strand, two had a dG, and one had a dCdC dinucleotide insertion.

branches in solution did not differ from that carried out on the streptavidin-coated magnetic beads (data not shown).

Nine cDNAs generated by reverse transcription of the RNA from the B9 branched product were cloned and sequenced. In reading through the branch, reverse transcriptase often skipped one or two residues at the branch site. The expected DNA sequence CAGGT (underline indicates branch site) was obtained once, and deletion of one (G) or two (AG) nucleotides occurred, each four times. This is consistent with the observed shorter mobility of the blunt-end product of the reverse transcribed branched substrate relative to that of the linear substrate (Figure 5B). No loss of ribozyme or substrate sequence was observed at the ribozyme–substrate junctions. Instead, an extra nucleotide (or two) was inserted between the substrate and the ribozyme sequence (Figure 5C). These insertions could be due to either the presence of N+1 T7 RNA polymerase transcripts (Milligan *et al*., 1987) of the ribozyme or by blunt-end nucleotide addition to the reverse-transcribed substrate (Patel and Preston, 1994) before the jump to the ribozyme 3′ end.

Second selection

Another selection was performed with a new, 10% doped snRNA library together with the previously used 3% doped starting pool. A total of 6×10^{14} different RNA sequences were present in the combined initial pool. The selection was performed using a further modified selection protocol that prevented the emergence of either of the previously described ligation activities, i.e. prior to the RNA ligation incubation step the substrate RNA was phosphorylated and the pool RNA was dephosphorylated.

After round eight of this selection two novel ribozyme activities were obtained. These RNAs could not be enriched with the standard selection protocol to the point where they were abundant in the sequence pool. Most likely, this was caused by the enrichment of inactive sequences that partially fulfilled the selection steps and amplified much better than did the catalysts. Nevertheless, the catalytically active sequences were cloned after additional enrichment through serial gel purifications.

Self-cleaving, substrate-cleaving and substrate fragment-ligating ribozyme

One of the isolated ribozymes performed the ligation to a portion of the substrate oligonucleotide in a three-step reaction (Figure 6A and B). Characterization of this ribozyme showed that it underwent self-cleavage with formation of a 2′,3′-cyclic phosphate and, in contrast to the earlier selected self-cleaving ribozymes, it also cleaved the substrate oligonucleotide *in trans*. By cleaving the 5′-phosphorylated substrate oligonucleotide, the ribozyme generated a biotinylated substrate fragment with a free 5′-hydroxyl group, which was then ligated to the ribozyme cyclic phosphate intermediate to complete the three-step splicing pathway (Figure 6A).

The multistep nature of the reaction was confirmed by examining pathway intermediates. The self-cleavage of the ribozyme occurred independently of the presence of substrate, and the isolated intermediate was able to support ligation (Figure 6C). This is consistent with the presence of a 2′,3′-cyclic phosphate after self-cleavage. This intermediate had the same gel mobility as did the starting pool RNA, suggesting that ribozymes which self-cleaved during the T7 RNA polymerase transcription reaction would have most easily survived the gel purification step that followed the transcription reaction of each selection cycle. The cleavage site in the substrate was determined by comparing the mobility of the cleavage fragment with a ladder from a partial alkaline hydrolysis of unreacted substrate (Figure

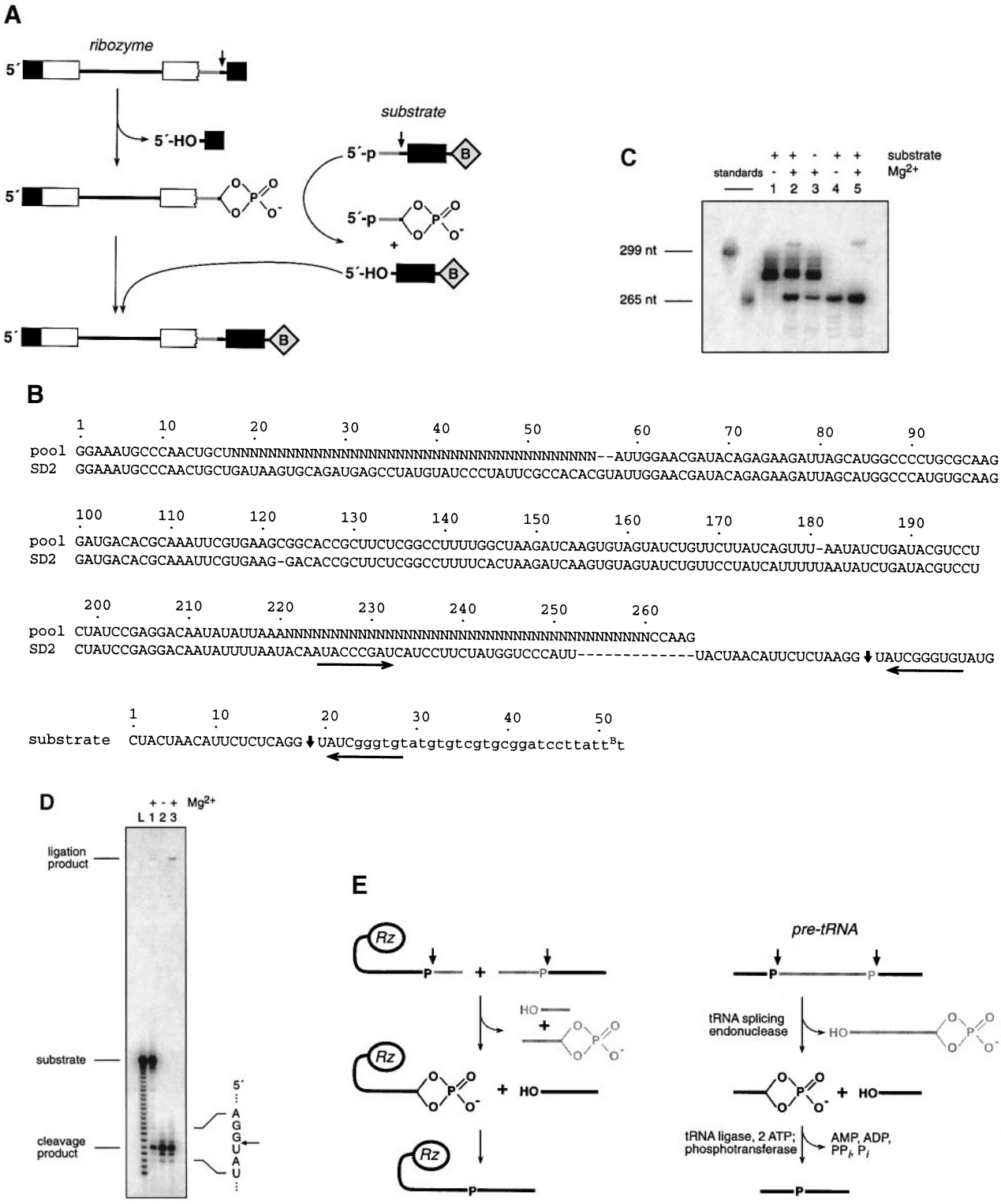

Fig. 6. Self-cleaving, substrate-cleaving and substrate fragment-ligating ribozyme. (**A**) Reaction scheme. For symbols, refer to Figure 3A. (**B**) Alignment of the SD2 sequence relative to the starting pool sequence. The cleavage sites in the ribozyme and the substrate are indicated by vertical arrows. Horizontal arrows indicate 9 nt complementary segments that may help position the *trans* cleaved substrate fragment for ligation to the cyclic phosphate intermediate. (**C**) Cleavage and ligation reactions with 5′-labeled ribozyme (lanes 1–3) or its isolated self-cleaved intermediate (lanes 4 and 5) stopped after 17 h and analyzed by denaturing PAGE. (**D**) Ligation reactions with 3′-^{32}P-labeled substrate (lane 1) or substrate fragment (lanes 2 and 3), stopped after 17 h and analyzed by denaturing PAGE. An alkaline hydrolysis ladder of the 3′-labeled substrate (lane L) was used to identify the cleavage site (arrow). (**E**) Similarities between a three-step cleavage and ligation reaction and a pre-tRNA splicing reaction. The selected ribozyme (Rz, left panel) catalyzes self- and *trans*-cleavage reactions generating 2′,3′-cyclic phosphate and releasing 5′-hydroxyl groups. Similar reactions are performed by the tRNA splicing endonuclease (right panel) to remove the intron.

6D, lanes L and 1). The site is located 3′ to the last riboG in the substrate sequence. An equivalent cleaved substrate fragment was prepared with RNase T1, which preferentially cleaves 3′ to guanosines, leaving a 3′-phosphate and a 5′-hydroxyl group. As anticipated by the scheme, ligation product was observed when a substrate fragment prepared in this manner was incubated with full-length ribozyme (Figure 6D, lane 3).

2′,3′-branch-forming ribozyme

The other ribozyme activity isolated in the second selection catalyzed the formation of a novel 2′,3′-branch near the 3′ end of the ribozyme (Figure 7A and B). This reaction did not depend on the presence of a terminal 5′ triphosphate, but instead was dependent upon cleavage of an internal phosphodiester bond during a concerted phosphodiester transfer reaction.

The 2′,3′-branch-forming ribozyme (Figure 7C) was inefficient and thus it was not possible to detect the released 3′-leaving group above the background of RNA cleaved by magnesium-induced hydrolysis. The presence of a 3′-leaving group could therefore only be shown indirectly. The 5′ or 3′ ends of the ribozyme and the substrate were labeled and each radioactive species was reacted with its non-labeled partner (Figure 7D). Only in the case of the 3′-radiolabeled ribozyme was a radiolabeled ligation product not observed, as expected if the 3′-radiolabeled end is released upon branch formation.

Another way of confirming the branch formation with concomitant release of an oligonucleotide is by varying the length of the leaving group. Although the location of the transesterified phosphodiester linkage was not specifically known, it was estimated from sequence analysis of reverse transcribed 2′,3′-branches that the leaving group was ~17 nt long. The 3′-end of the ribozyme was extended by 17 more nucleotides by transcribing it from the first PCR product rather than the nested PCR product (Figure 2). When this extended ribozyme was incubated with 3′- or 5′-radiolabeled substrate, the mobilities of the ligation products were identical to those obtained from the shorter ribozyme, showing that the 3′-end of the ribozyme was not retained in the ligated product (Figure 7E).

The putative 2′,3′-branch site could not be mapped by partial alkaline hydrolysis (the technique used to map the 2′,5′-branch; Figure 4C) because the linkage is highly base labile, with a half-life of ~1 min in 50 mM Na_2CO_3 at 90°C. Cleavage of a 2′,3′-phosphodiester bond would be expected to occur readily by the attack of an adjacent ribozyme 2′ oxygen (Figure 7B), forming a 2′,3′-cyclic phosphate and releasing the substrate 2′ oxygen. Hydrolysis of the putative 2′,3′-phosphodiester bond was much faster than that of a regular RNA 3′,5′-phosphodiester bond, as would be expected because a 2′-oxyanion is a better leaving group than the more basic 5′-oxyanion.

To identify the branch site in the substrate sequence, primer extension analysis was carried out on branched molecules (Figure 8). Due to limited material, a single 15 min reverse transcription was performed. Reverse transcription of the 2′,3′-branch generated several cDNA bands. As in the case of the 2′,5′-branch, reverse transcriptase stalled at the putative 2′,3′-branch site. At the branch site reverse transcriptase has two options, either to read through the branch to the end of the substrate or to switch the template strand from the substrate to the ribozyme and continue reverse transcription through the ribozyme. Both possible products appear with approximately equal intensity. The reverse transcriptase stall mapped to the uridine in the segment UCAGGU. Template switching at a 2′,3′-branch (Figure 8) appears more efficient than does read-through of a 2′,5′-branch and subsequent jump (Figure 5).

The branch site determination by primer extension was supplemented by sequence analysis of seven individual cDNA clones generated from reverse-transcribed 2′,3′-branches. In contrast to the observed irregular skipping of 1 or 2 nt at a 2′,5′-branch site, all analyzed cDNA clones from 2′,3′-branches had identical sequences around the branch site. The substrate cDNA sequence ended one nucleotide before the observed uridine block in the primer extension analysis. Perhaps reverse transcriptase could not use the branched nucleotide as a template and skipped it. It is also possible that more than one nucleotide was skipped, as was observed for some clones of reverse-transcribed 2′,5′-branches, or that an untemplated nucleotide was inserted before the template switch. Therefore the branch point and the 3′-leaving group was assigned only with ±1 nt accuracy (Figure 7C) (Lorsch *et al*., 1995).

Discussion

The potential of snRNA-related sequence variants to promote transesterification reactions has been investigated using an iterative selection–amplification protocol. The selection criteria was designed to be optimally satisfied by a reaction equivalent to the second step of splicing—a transesterification reaction resulting in the extension of one RNA by the 3′ fragment of a second RNA. A number of novel ribozymes that ligate RNAs through a variety of unanticipated reaction mechanisms was identified. Most of the ribozymes promoted a two-step reaction involving a 2′,3′-cyclic phosphate intermediate. Another ribozyme promoted a three-step reaction, also involving a 2′,3′-cyclic phosphate intermediate. The other two ribozymes promoted novel branching reactions; their enrichment in the *in vitro* selection protocol depended on unexpected properties of reverse transcriptase.

Self-cleaving ribozymes—two-step reaction

The selection of multiple self-cleaving and substrate-ligating ribozymes might reflect a vast number of reversibly self-cleaving ribozymes in RNA sequence space. Four types of such ribozymes have been isolated from nature (Symons, 1992), and many more, including RNA-cleaving DNA enzymes, have been isolated from combinatorial libraries (Pan and Uhlenbeck, 1992a; Breaker and Joyce, 1994, 1995; Williams *et al*., 1995; Faulhammer and Famulok, 1997; Geyer and Sen, 1997; Jayasena and Gold, 1997; Santoro and Joyce, 1997). Our isolation of self-cleaving ribozymes demonstrates that a scheme previously used to select active variants of a known self-cleaving motif (Berzal-Herranz *et al*., 1992) can also be used to generate entirely new motifs that perform reversible self-cleavage at a precise site within a target RNA sequence. The best characterized reversible self-cleaving RNA motif is the hammerhead motif. This motif would be expected

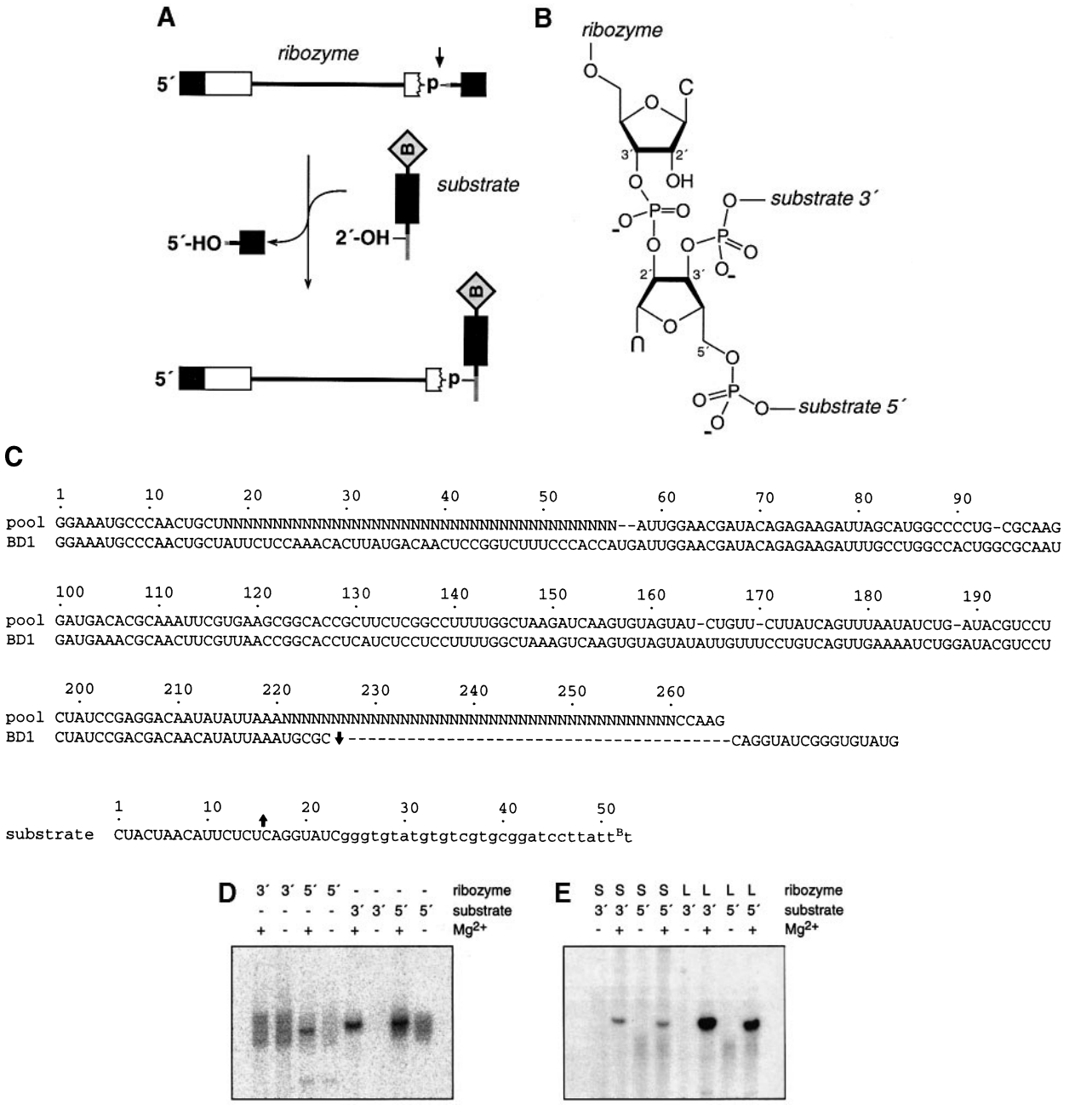

Fig. 7. 2′,3′-Branch-forming ribozyme. (**A**) Reaction scheme. The transesterified phosphodiester linkage (p) is indicated (arrow). For other symbols, refer to Figure 3A. (**B**) Structure of a 2′,3′-branch. (**C**) Alignment of the BD1 sequence relative to the starting pool sequence. Primer extension analysis of the branch product and sequence analysis of cloned cDNAs suggested with ±1 nt precision the indicated cleavage site in the ribozyme (down-facing arrow) and the substrate branch point (up-facing arrow). (**D**) 2′,3′-Branched ligation products of ribozyme and substrate end-labeled at the indicated terminus. The ligation products were eluted from a gel and run on a second gel (shown). The background smear is a consequence of the broad cut from the first gel. (**E**) Incubation products of short (S) or long (L) versions of 2′,3′-branch-forming ribozymes with 3′- or 5′-radiolabeled substrate. The small differences in the mobilities of branches formed with either 5′-radiolabeled or 3′-labeled substrate confirmed that the gel mobility of branches was very sensitive to length changes and thus a 17 nt difference in product length would have been readily apparent.

in one in every 10^7 to 10^8 arbitrary 265mers (Sabeti *et al.*, 1997). Though the abundance of the hammerhead motif in the pool used in this study would be lower because of the bias toward the snRNA sequences within the central 163 nt region, it is likely that this motif as well as numerous other known and unknown self-cleaving motifs were amply represented among the $>10^{14}$ sequences.

Self-cleaving ribozymes—three-step reaction

A more sophisticated reversible self-cleaving ribozyme was also isolated. This ribozyme performed ligation in a three-step pathway. The first cleavage event occurs intramolecularly, leaving a cyclic phosphate terminus at the ribozyme 3′-end. After release of the 3′ cleavage product, unprocessed substrate can bind to the ribozyme active site, presumably by looping out the ribozyme 2′,3′-cyclic phosphate end. After the second cleavage and release of the 5′ substrate fragment, the ribozyme 3′ end can swing back into the active site and ligate to the 3′ substrate fragment. The group I self-splicing ribozyme as well as engineered forms of other ribozymes are able to promote reactions with three or more steps (Zaug *et al.*,

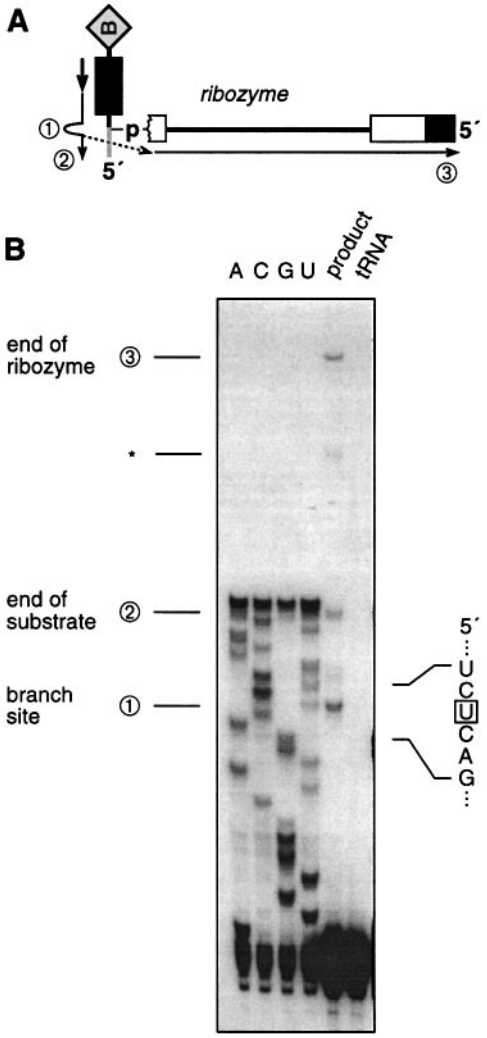

Fig. 8. Primer extension analysis of 2′,3′-branch. (**A**) Scheme for reverse transcription of the branch. To generate full-length cDNA, reverse transcriptase reads through the branch (1) and switches template (dashed arrow) to reach the 5′-end of the ribozyme (3). If no template switching occurs, reverse transcriptase can read to the end of the substrate (2). (**B**) A primer extension performed on branched ligation product and stopped after 15 min for PAGE analysis of the cDNA products. A strong stop, presumably at the branch site (1), was observed as well as cDNAs corresponding to read-through on the substrate strand (3) or template switching to the ribozyme RNA (2). A weak unidentified band (*) could possibly be formed by a jump from the 5′-end of the substrate into the ribozyme sequence. No cDNA bands were generated when carrier-tRNA alone (right lane) or unbranched ribozyme (data not shown) were reverse-transcribed. The reference lanes (A, C, G, U) show dideoxy reverse transcriptase sequencing of the substrate oligonucleotide using the labeled primer.

1983; Mueller *et al*., 1993; Ekland and Bartel, 1996). Nevertheless, the demonstration that an *in vitro* selection protocol can select directly for both the chemistry and the dynamic conformational rearrangements of multi-step reactions bodes well for the prospects of isolating other interesting complex activities.

In principle, the three-step cleavage–ligation reaction can generate the same product RNA as is generated by the *in vivo* RNA splicing processes, including RNA-catalyzed self-splicing, spliceosome-catalyzed pre-mRNA splicing, and protein-catalyzed splicing. Perhaps surprisingly, the three-step cleavage–ligation reaction of our ribozyme is most similar to the protein-catalyzed splicing (Figure 6E). In the protein-catalyzed pathway (recently reviewed by Phizicky and Greer, 1993; Belfort and Weiner, 1997; Lykke-Andersen *et al*., 1997) the precursor RNA (typically a pre-tRNA) is first cleaved at the two splice sites, leaving 2′,3′-cyclic phosphate and 5′-hydroxyl termini, and then the exons are ligated. Our selected ribozyme also performs two cleavage reactions and one ligation reaction. It might be possible to engineer the ribozyme so that it too acts on a unimolecular splicing precursor. Other important differences would remain: in the protein-catalyzed pathway, the endonuclease has two separate active sites that cleave the two splice sites, and ligation is catalyzed by a separate enzyme in an effectively irreversible, multi-step process. In the ribozyme pathway, we suspect that ligation is a single step, and all reactions are reversible and catalyzed by a single active site.

Branching ribozyme—2′,5′ branch

We have identified a 76 nt ribozyme which can efficiently catalyze formation of an RNA branch by the attack of a 2′-hydroxyl on the α-phosphate of a 5′ triphosphate. The ribozyme sequence includes the 5′ primer-binding sequence, 40 nt from a random-sequence domain, and 19 nt from U6 snRNA. Further studies will be necessary to identify the RNA residues and structure critical for this reaction. The rate of the most active branching isolate was 0.002 min^{-1} which compares reasonably well with rates of *in vitro* branch formation by the spliceosome and self-splicing introns, ranging from 0.0005 to 0.2 min^{-1} (Grabowski *et al*., 1984; Daniels *et al*., 1996). As with the standard spliceosomal reaction, the phosphate center of branch formation was 5′ to a G residue. However, the reaction differs from the spliceosomal reaction in that the branch nucleotide is a guanosine, not an adenosine, and the leaving group is a pyrophosphate, not an oligonucleotide.

2′,5′-Branch formation is also similar to the activity of RNA ligases previously isolated from random sequence (Bartel and Szostak, 1993; Ekland *et al*., 1995). These ligases promote the attack of a terminal 2′- or 3′-hydroxyl group onto the α-phosphate of the ribozyme 5′ triphosphate, forming linear RNAs with 2′,5′- or 3′,5′-phosphodiester linkages. The frequency of selection of such RNA ligase motifs suggests that they are relatively abundant in sequence space, suggesting that ribozymes that use internal 2′-hydroxyl groups to displace pyrophosphate from a 5′-triphosphate end might be similarly abundant. It is, however, not clear how steric hindrance at a more crowded branch site would restrict their abundance.

Branching ribozyme—2′,3′ branch

The 2′,3′-branch-forming ribozyme, like the spliceosome and the group II self-splicing intron, catalyzes a transesterification reaction in which an attacking 2′-hydroxyl nucleophile displaces an oligonucleotide leaving group. However, in contrast to splicing, the leaving group is a 5′-hydroxyl and not a 3′-hydroxyl group. Because the 5′-hydroxyl group is less acidic than is the 3′-hydroxyl group (a primary alcohol being less acidic than a secondary *cis* diol), the stabilization of the developing negative charge in the transition state of transesterification is more difficult for 2′,3′-branch formation than for 2′,5′-branch formation. This supports our view that the *in vitro* selection scheme

was sufficiently powerful to have isolated ribozymes with the ability to catalyze ordinary splicing reactions. The fact that naturally occurring ribozymes or RNPs do not use this transesterification mechanism (cleaving first at the 3′ splice site) might be a consequence of the more difficult reaction.

Novel properties of reverse transcriptase

When considering that reverse transcriptase is known to stall at a 2′,5′ branch in the template RNA (Ruskin *et al*., 1984), the isolation of branch-forming ribozymes was not anticipated. Examining the cDNA synthesis of these branch-forming ribozymes revealed unexpected properties of reverse transcriptase. With sufficient time, reverse transcriptase can quantitatively polymerize past a branch in the template and, at lower efficiency, perform a non-templated intramolecular jump. With the AMV polymerase these properties are detectable but less pronounced than with the RNase H-deficient MMLV enzyme, perhaps because the AMV RNase H degrades the template before the polymerase has traversed the branch or jumped to a new template strand. We suggest that these properties are general for retroviral reverse transcriptases and the non-templated jump could be one of the mechanisms by which viruses hijack cellular genes. It remains to be seen whether these properties are as pronounced when using other template sequences; branching ribozymes that provide a particularly good context for branch read-through and untemplated jumping would have had a selective advantage during our procedure. When approaching a 2′,3′ branch the reverse transcriptase must choose between two possible templates. Interestingly, the polymerase negotiated the 5′,3′ linkage and the 2′,3′ linkage at approximately equal rates.

Reverse transcription of a 2′,5′-branched RNA can be primed on two different template strands. We show read-through when priming synthesis on the 3′-linked strand. Others have detected read-through when priming on the 2′-linked strand (Vogel *et al*., 1997). When reverse transcriptase approaches a branched adenosine from the 2′-linked strand no skipping of template nucleotides occurs; instead, dA is incorporated opposite the branched adenosine (Vogel *et al*., 1997). It is unclear how the efficiencies for these two different processes compare because read-through from the 2′-linked strand was detected after PCR amplification.

Final comments

The optimal design of combinatorial libraries for accessing very rare activities has not been determined. During recent natural evolution, new protein function has arisen by modification of pre-existing structural motifs (Li and Graur, 1991). This suggests a possible strategy for increasing the probability of accessing new RNA function; biasing an RNA pool towards a core RNA structure may increase the abundance of very rare ribozyme activities (Jaeger, 1997). Alternatively, it could be argued that by sampling a larger diversity of structural architectures, random sequence RNA may offer maximal access to rare activities. Thus, it will be of interest to examine whether the doped snRNA-like domain or the random sequence segments contribute more to the catalytic motifs of our new ribozymes.

The fact that we were able to isolate four different activities, including an activity for a reaction more difficult than the ordinary splicing reactions, raises the question of why we did not isolate a ribozyme that promotes the transesterification reaction of the second step of splicing. The absence of this activity among our isolates cannot be explained by the inability of RNA to catalyze this type of transesterification reaction; such a reaction is efficiently catalyzed by group I self-splicing ribozymes no larger than our pool molecules (Green and Szostak, 1992; Tanner and Cech, 1996). Of course, the chance that a full group I intron motif, one of the more complex of the known ribozyme motifs, would have resided in a pool with only 10^{14} different sequences was remote (Sabeti *et al*., 1997), and biasing the library sequences toward the U2 and U6 snRNAs further lowered the probability of accessing a group I motif. Biasing the library sequences toward the snRNAs presumably did increase the chance of accessing the motif of the ribozyme from which snRNAs are presumed to have descended. The observation that such a motif was not accessed could be construed to suggest that the spliceosomal reaction did not arise from an RNA-only reaction. We favor instead the idea that the human spliceosomal snRNAs have diverged so far from the putatively RNA-only ancestral catalyst that it is difficult to access the ancestral RNA activity with only 6×10^{14} minor variants.

Materials and methods

Pool construction

Two 265 nt RNA pools (Figure 1B) were constructed using the general strategy described previously (Ekland and Bartel, 1995). One pool was prepared with 3% mutagenesis of the snRNA sequences (i.e. at each doped position the ratio of the parental nucleotide to the other three nucleotides was 97:1:1:1), the other library with 10% mutagenesis. For the construction of each U2/U6 snRNA library, two degenerate oligodeoxynucleotides were synthesized (1 μmol scale) on Millipore 8909 Expedite Synthesizer using appropriate phosphoramidite mixtures at random or mutagenized positions of the snRNA sequences. The totally random and doped phosphoramidite mixtures were formulated to take into account the faster coupling rate of T. For example, the phosphoramidite concentrations for totally random couplings were 17 mM T, 21 mM A, 21 mM C, and 21 mM G. The degree of mutagenesis or randomness was verified by sequencing of randomly cloned DNA templates from the initial pools. The U6 oligodeoxynucleotide, GGAAATGCCCA-ACTGCT-$(N)_{40}$-ATTGGAACGATACAGAGAAGATTAGCATGGCCC-CTGCGCAAGGATGACACGCAAATTCGTGAAGC*GGCACC*AGTC-TTATCTC, contained partially randomized positions 30–94 of the 107 nt long human U6 snRNA sequence and the U2 oligodeoxynucleotide, CGTCAGGAAATG*GGCACC*GCTTCTCGGCCTTTTGGCTAAGATC-AAGTGTAGTATCTGTTCTTATCAGTTTAATATCTGATACGTCCTC-TATCCGAGGACAATATATTAAA-$(N)_{40}$-*CCAAGG*TTATCGGTAGAG, contained partially randomized positions 4–95 of the 188 nt long human U2 snRNA sequence (underlined sequences, constant primer-binding sequences; N, random positions; italic letters, *Sty*I and *Ban*I restriction sites). The degenerate oligonucleotides were amplified by large-scale PCR (Ekland and Bartel, 1995) using the corresponding primer pairs, except for the U6 DNA, where the T7 primer (TAC**TAATACGACT-CACTATA**GGAAATGCCCAACTGCT; bold, T7 promoter; underlined, primer-binding sequence) was used to append the T7 promoter. Amplified DNAs were digested with the restriction endonuclease *Ban*I (3 ml reactions, 100 μg DNA, 1000 U enzyme from NEB, 3 h). Digested DNA was heated to 60°C for 5 min, phenol-extracted, ethanol-precipitated and ligated using T4 DNA ligase (2 ml reaction, 50–100 μg cut DNA fragments, 40 000 U enzyme from NEB, 1.5 h, 22°C). Half of the ligation mix (3×10^{14} different sequences) was digested with restriction endonuclease *Sty*I (1.5 ml reaction, 500 U enzyme from NEB, 18 h) and then transcribed with T7 RNA polymerase (10 ml reaction, 65 000 U enzyme from USB, 8 mM GTP, 5 mM ATP or CTP, 2 mM UTP, 26 mM

$MgCl_2$, 1 mM spermidine, 5 mM DDT, 0.1% Triton X-100, 40 mM Tris pH 7.9, 1 h, 37°C) to yield the degenerate RNA pool. Gel-purified pool RNA was incubated with RQ1 DNase (Promega) to degrade template DNA.

Synthetic RNA

The substrate for exon ligation (Figure 1C) was synthesized by standard phosphoramidite chemistry using biotin-dT amidite (Glen Research). The first deprotection was performed in ethanolic ammonia (24 h at 60°C). Silyl groups were removed by incubation in TBAF/THF as described (Tuschl *et al.*, 1993). Synthesis of a DNA/RNA chimeric molecule instead of an all-RNA substrate resulted in higher yield due to better coupling efficiency of DNA over RNA amidites and reduced oligonucleotide degradation during ammonia deprotection. Primer extension experiments confirmed that this substrate can be efficiently reverse-transcribed when bound to streptavidin-coated magnetic beads. U5 snRNA hairpin (AAAUCUUUCGCCUUUUACUAAAGAUUU, positions 28–54 of human U5 snRNA) and 5′ intron fragment (GUAUGU, yeast consensus sequence) were also synthesized. All synthetic RNAs were gel-purified.

Selection and amplification

Ligation incubation. 0.5 μM Pool RNA, 1 μM biotinylated substrate, 1 μM U5 hairpin, 1 μM 5′ intron 6mer were incubated at 30°C in selection buffer (100 mM KCl, 25 mM $MgCl_2$, 0.25 mM EDTA, 30 mM Tris pH 7.6). Radiolabeled substrate or pool RNA was added to monitor ligation activity. The initial round of the first selection had 0.5 mg of 3% doped pool RNA. In the second selection, round one was initiated with 0.5 mg each of 3 and 10% doped pools in the presence of 5′-phosphorylated substrate. In subsequent rounds, the amount of input RNA was reduced by at least a factor of 10 and incubation times were reduced from 30 to 15–20 h. Following incubation, the total RNA was ethanol-precipitated.

Gel selection. RNA from the first large-scale incubation was run a short distance (~5 cm) on a 6% denaturing polyacrylamide gel (PAGE) to separate unreacted biotinylated substrate from pool and ligation product. Pool RNA and all higher mobility products were excised and electro-eluted (ELUTRAP, Schleicher and Schuell) in TBE buffer (45 mM Tris–borate, 20 mM EDTA). In subsequent rounds, gel purification was performed on 40 cm 5% gels to separate ligated pool RNAs from unreacted RNAs. A broad band at the mobility of the expected ligation product was excised and soaked overnight in 0.3 M NaCl and 10 μM reverse transcription primer as carrier. Gel-purified RNA was ethanol-precipitated and dissolved in water.

Biotin selection and selective reverse transcription. The electro-eluate from round one (1.5 ml) was heat-treated for 3 min at 80°C, cooled to room temperature, and supplemented with SSC buffer (75 mM NaCl, 10 mM Na citrate pH 7.2). Streptavidin-coated paramagnetic beads (Promega) of a binding capacity of 1 nmol biotinylated oligonucleotide were added. In the following rounds, the amount of streptavidin beads was reduced 5-fold and binding was performed in 0.2 ml of SSC buffer. Subsequent washes and reverse transcription on the beads was performed as described previously (Ekland and Bartel, 1995), using an RNase H-inactivated MMLV reverse transcriptase (Superscript II, Gibco-BRL) and the reverse transcription primer (TAAGGATCCGCACGACACA) which is complementary to the substrate sequence. The cDNA was recovered from the beads by base elution (150 mM KOH, 20 mM Tris base). Remaining RNA was completely hydrolyzed by a subsequent incubation for 10 min at 90°C, then titrated to pH 8–9 with HCl. Every second or third round, the recovered RNA product from the incubation was reverse-transcribed first and then bound to the streptavidin beads (Ekland and Bartel, 1995).

Selective amplification. Eluted cDNA was amplified by PCR with a hot start (80°C) using the T7 and reverse transcription primers (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, 0.2 mM dNTP each, 0.5 μM primer, 2.5 U/ml Taq polymerase). The PCR product was diluted 1000-fold to serve as template in a subsequent nested PCR using the T7 primer in combination with the nested 3′ primer (CATACACCCGATACCTT). Nested PCR was carried out at a total volume of 2 ml and was stopped before the appearance of undesired product bands. To minimize the amount of heteroduplex formation (Ekland and Bartel, 1995), one ml of the nested PCR was used as template for a single PCR amplification cycle done manually in an 8 ml volume. This PCR product was ethanol-precipitated and the pellet dissolved in 0.2 ml of *Sty*I digestion buffer. The restriction digestion was performed with 60 U *Sty*I (NEB) for 14 h, heat-inactivated at 65°C for 20 min, and the solution was used directly for T7 run-off transcription.

If ligation occurred by attack of the 5′ exon segment (CCAAG, Figure 1B) at the 3′ splice site (CUCAG*GU) the sequence in the ligation product would be changed to **CCAAG***GU (bold, sequence from the transferred 5′ exon; underlined, *Sty*I site). The *Sty*I digestion would restore the original 3′ end of the RNA pool (representing the 5′ exon). However, none of the isolated ribozymes promoted attack of the 5′ exon at the 3′ splice site and thus their ligated sequences were not susceptible to *Sty*I. T7 transcripts of these ribozymes retained the 3′-terminal sequence complementary to the nested PCR primer.

RNA preparation. Transcription was usually performed on a 1 ml scale using 0.1–0.2 ml of restricted PCR DNA under conditions described above for the pool construction. A trace amount of 5′-^{32}P-radiolabeled RNA from the initial pool was added 10 min after the transcription was started. It served as an internal length marker to isolate proper-sized RNA on 5% denaturing polyacrylamide gels. Electro-eluted RNA was treated with DNase RQ1 (Promega) in 0.1 ml using the recommended buffer with 4 units enzyme for 30 min at 37°C. The solution was phenol extracted three times and ethanol-precipitated. In the second selection, the RNA was dephosphorylated with alkaline phosphatase prior to each selection cycle (50 μl reaction, 1.5 nmol RNA, 50 U enzyme from Boehringer Mannheim, 30 min, 50°C). The solution was phenol-extracted three times and ethanol-precipitated.

After the appearance of ligation activity, the amplified cDNA from a first PCR was cloned (T-Vector kit, Novagen) and individual clones were tested and sequenced.

^{32}P-radiolabeling

5′-^{32}P-labeling of dephosphorylated or synthetic RNA or DNA was performed with polynucleotide kinase (NEB) and [γ-^{32}P]ATP. 3′-^{32}P-labeling of the chimeric RNA–DNA substrate was performed by primer extension using Sequenase, version 2.0 (Amersham), [α-^{32}P]TTP and oligodeoxynucleotide template (AAAATAAGGATCCGCACG; underlined bases hybridize to the substrate 3′-end). The γ-phosphate of the terminal triphosphate of 2′,5′-branch-forming ribozymes was labeled by transcription in the presence of [γ-^{32}P]GTP. 3′-^{32}P-labeling of 2′,3′-branch-forming ribozymes was performed with yeast poly(A) polymerase (Amersham) and [α-^{32}P]cordycepin triphosphate. RNA was internally labeled by transcription in the presence of [α-^{32}P]UTP. Radioactivity on gels was quantitated using a Fujix BAS 2000 PhosphorImager.

Characterization of 2′,5′-branch-forming ribozyme

TLC analysis of pyrophosphate release. Samples were spotted on PEI cellulose-coated glass plates (Merck, No. 5722) and developed with 0.5 M HCl. To improve the resolution, 1 μl of 10 mM carrier (Na_2HPO_4, $Na_4P_2O_7$, Na_4GTP) was spotted with each sample.

Mapping of the branch point by partial alkaline hydrolysis. 5′-^{32}P-labeled linear or branched substrate oligonucleotide was partially hydrolyzed by incubation in 50 mM Na_2CO_3 for 3 min at 90°C. After addition of an equal volume of 8 M urea/50 mM EDTA stop solution, the samples were run on a 20% sequencing gel.

Ribozyme 3′-deletion analysis. A ladder of ribozyme 3′-deletions was prepared by cordycepin (3′-deoxyadenosine) incorporation during transcription (25 μl reaction, 3 mM GTP, 1 mM ATP, 1 mM CTP, 1 mM UTP, 70 μM cordycepin triphosphate, 20 mM $MgCl_2$, 5 mM DDT, 40 mM Tris–HCl pH 8.0, 5 mg/ml nuclease-free BSA, 15 U T7 RNA polymerase from USB, 2 h, 37°C) (Axelrod and Kramer, 1985). The PCR DNA used as template for this transcription included an additional vector sequence (84 bp) beyond the 3′ nested PCR primer sequence. The reaction was stopped by the addition of 70 μl 15 mM EDTA and passed over a G-50 Sephadex spin column (Boehringer Mannheim). The flow-through was phenol-extracted, ethanol-precipitated, and redissolved in 20 μl water. The branching reaction was performed as a 10 μl reaction using 2 μl of cordycepin-terminated RNA ladder and 0.4 μM 5′-^{32}P-labeled substrate in selection buffer for 7 h at 30°C. Active deletion derivatives were identified by comparison with a marker ladder generated by partial T1 digestion of the branched product of full-length (220 nt) ribozyme and 5′-^{32}P-substrate. The reaction was stopped by the addition of 20 μl 8 M urea/50 mM EDTA and resolved on a 5% sequencing gel.

Preparation of substrate 3′-DNA-ladder for 3′-deletion analysis. Abortive polymerization was carried out on a truncated, 5′-^{32}P-labeled chimeric substrate (UCUCUCAGGUAUCgggtgtatg), which was pre-annealed to a 58 nt DNA template (GCTGAATGCGTCTGGCTAGAGTTGAAAT-AAGGATCCGCACGACACATACACCCGATAC; underlined, comple-

mentary sequence to truncated substrate) in Sequenase buffer (Amersham) and extended with Sequenase DNA polymerase (50 µl reaction, 1 µM substrate, 2 µM template, 190 µM each dNTP, 10 µM each ddNTP, 13 U enzyme, 5 min). The reaction was stopped by the addition of an equal volume of 8 M urea/50 mM EDTA stop solution as well as 1.5 equivalents of a 64 nt DNA (TCTCAGGTATCGGGTGTATGTG-TCGTGCGGATCCTTATTTCAACTCTAGCCAGACGCATTCAGC) complementary to the 58 nt template. The sample was incubated for 3 min at 90°C and run on a short 15% denaturing acrylamide gel. The ladder below the fully extended product was excised, eluted in 0.3 mM NaCl overnight, ethanol-precipitated and redissolved in 50 µl water. This gel purification step was not sufficient to remove completely the 58 nt DNA template, which remained partly associated with the longer extended substrate molecules. Complete removal was achieved by incubating the sample with a 250-fold molar excess of 52 nt full-length substrate pre-bound to streptavidin beads. Before the addition of the substrate on beads, the sample was denatured for 2 min at 90°C in the absence of salt. After the addition of beads, the solution was adjusted to a NaCl concentration of 0.3 M and was further incubated for 5 min at 55°C and 10 min at 37°C. The solution was removed from the magnetic beads and the purified oligonucleotide ladder recovered by ethanol-precipitation. The pellet was redissolved in water.

Substrate deletion analysis. 5′-Deletion analysis was performed by using a partial alkaline hydrolysis ladder of 3′-^{32}P-labeled substrate while 3′-deletion analysis was carried out by using the substrate 3′-DNA ladder described above. Branching reactions were performed by incubating a trace amount of radiolabeled ladder with 5 µM ribozyme in selection buffer for 15 h at 30°C. Samples were run with control incubations of the ladders in the absence of ribozyme on 10% sequencing gels. Intensity profiles were compared to determine the shortest active derivatives.

Primer extension analysis of 2′,5′-branch. Reverse transcription was performed using 5′-^{32}P-labeled reverse transcription primer (50 µl reaction, 200 nM branched or linear substrate, 100 nM primer, 20 mM Tris–HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 µg/µl BSA, 10 mM DTT, 0.56 mM dNTP each, 500 units Superscript II reverse transcriptase from Gibco-BRL, 48°C). Before the addition of reverse transcriptase, RNA template and primer were annealed for 3 min at 48°C in the reaction buffer. After the addition of reverse transcriptase, 5 µl aliquots were withdrawn at indicated time points and added to 15 µl 150 mM KOH–20 mM Tris base. The samples were incubated for 10 min at 90°C and then neutralized by the addition of 15 µl of 150 mM HCl. After the addition of 30 µl of 8 M urea–50 mM EDTA the samples were analyzed by 10% PAGE.

Lariat debranching. Debranching was performed using DBR1, the yeast debranching enzyme (Chapman and Boeke, 1991; Nam *et al*., 1994) (20 µl reaction, 10 nM lariat, 20 mM HEPES–KOH pH 7.6, 125 mM KCl, 0.5 mM $MgCl_2$, 1 mM DTT, 10% v/v glycerol, 10 units enzyme, 30°C). Before adding buffer and debranching enzyme, the RNA solution was heat-treated for 1 min at 90°C. At indicated time points, 3 µl aliquots were withdrawn and stopped by the addition of 8 µl 8 M urea. Samples were analyzed by 10% PAGE.

Semi-quantitative reverse transcription/PCR of 2′,5′-branches. A constant concentration (100 nM) of branched RNA was reverse-transcribed in the presence of varying concentrations (0.5–50 nM) of 294 nt linear standard (a selected, slowly self-cleaving RNA related to isolate S9) with 5′ and 3′ primer sites. Conditions for reverse transcription and PCR were as described in the selection protocol.

Isolation of three-step substrate-ligating and 2′,3′-branch-forming ribozymes

At round eight, two individual ligation product bands were detectable after incubation of pool RNA with radiolabeled substrate. The individual bands were excised and the ligation product was eluted from the gel in the presence of carrier tRNA. Then each sample was purified once more on a long sequencing gel, eluted, reverse-transcribed and PCR-amplified. The three-step substrate-ligating ribozyme could be cloned from a single PCR product. However, multiple first and nested PCR bands were obtained for the 2′,3′-branch-forming activity. The number of nested PCR bands was reduced by switching to a new nested PCR primer, CATACACCCGATACCTG, which was perfectly complementary to the substrate sequence but introduced a single mismatch (underlined) to the contaminating RNA that was generated from a previous regular nested PCR template. Individual nested PCR bands were isolated from low-melt 3% NuSieve agarose gels under non-denaturing conditions and rerun on 4% NuSieve gels. The re-amplified single bands were transcribed and tested for ligation activity. The PCR band displaying activity was cloned and individual clones were sequenced and tested for activity.

Primer extension analysis of 2′,3′-branch

Reverse transcription was performed as for the 2′,5′-branch but on a smaller scale (7.5 µl, 5–10 nM branch, 20 nM 5′-^{32}P-labeled reverse transcription primer, 10 µg carrier tRNA from yeast, 48°C, 15 min). The sample was ethanol-precipitated, dissolved in 10 µl of 8 M urea–50 mM EDTA stop solution, and heat-treated (2 min at 90°C) prior to loading on a 15% sequencing gel. To improve PAGE resolution, a large excess of a 64 nt cold competitor oligonucleotide complementary to a 40 nt segment of the chimeric substrate was added. The A, C, G and U reference ladders were processed as described for the primer extension product of the branched RNA.

Ribozyme reactions and kinetics

Ribozyme and substrate stock solutions were heat-treated in water for 1 min at 90°C followed by a 15 min incubation at 30°C. Selection buffer was added and the pre-incubation continued for another 15 min. Reactions were initiated by mixing of pre-incubated ribozyme and substrate solutions. Aliquots were removed from the reaction at indicated time points, quenched by the addition of 2 volumes 8 M urea–50 mM EDTA stop solution, and analyzed by denaturing PAGE.

Single-turnover kinetics of 2′,5′-branch formation. Trace (<10 nM) 5′-^{32}P-labeled substrate was incubated with 1 µM ribozyme in selection buffer at 30°C. The observed rate constants were determined by fitting the fraction of unreacted substrate to the equation of a single exponential decay as a function of time. Lariat formation was assayed in the same manner, except the reaction was started by the addition of selection buffer to heat-treated linear RNA. Lariat and linear RNA co-migrated on 5% denaturing gels but can be separated on 10% gels. Eight percent of the input RNA formed lariats in 20 h.

Self-cleaving, substrate-cleaving and substrate fragment-ligating ribozyme. To monitor self-cleavage and substrate fragment ligation, 5′-^{32}P-labeled ribozyme or self-cleaved intermediate (0.5 µM) was incubated with unlabeled substrate (0.75 µM) in selection buffer at 30°C. For determination of the substrate cleavage site, a trace amount of 3′-^{32}P-labeled substrate (<10 nM) was incubated with unlabeled ribozyme (0.5 µM) in selection buffer at 30°C. The substrate fragment was prepared by RNase T1 digestion [20 µl reaction, 4 pmol 3′-labeled substrate, 100 units RNase T1 (NEB), 50 mM Tris–HCl pH 7.5, 2 mM EDTA, 5 min, 37°C], followed by multiple phenol extractions and gel-purification.

2′,3′-Branch formation. A trace amount of ^{32}P-labeled substrate (<10 nM) was incubated with excess unlabeled ribozyme (1 µM) in selection buffer for 20 h at 30°C. When radiolabeled ribozyme was used, a trace amount of ribozyme was incubated with excess substrate (1 µM). Approximately 0.05% of product formation was observed.

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