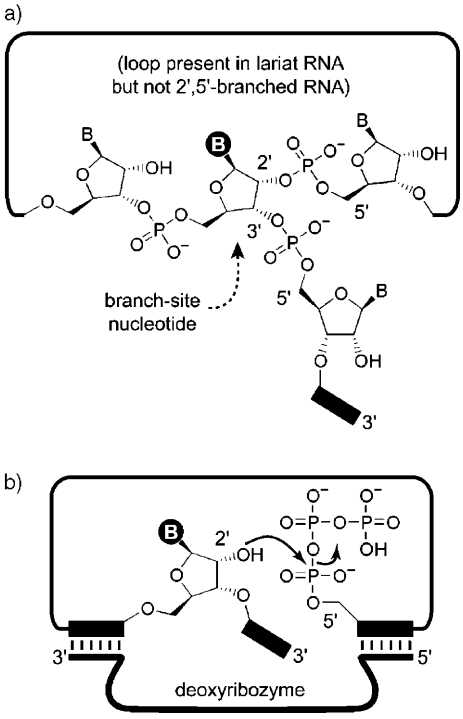

Figure 1. Lariat RNA and its synthesis by a deoxyribozyme (B = A, G, C, or U). a) Connectivity of lariat RNA and 2′,5′-branched RNA. The latter has the same branch-site nucleotide as a lariat but lacks the closed loop. The four illustrated nucleotides constitute the minimal part of a 2′,5′ -branched RNA. b) Deoxyribozyme-catalyzed synthesis of lariat RNA.

DNA Catalysis

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Efficient One-Step Synthesis of Biologically Related Lariat RNAs by a Deoxyribozyme**

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Lariat RNAs are intermediates in RNA splicing events catalyzed by group II introns and the spliceosome.[1,2] Lariats have a closed RNA loop with a single 2′–5′ linkage and a single-stranded oligonucleotide attached at the 3′-oxygen atom of the branch-site nucleotide (Figure 1a). Topologically, lariats are a subclass of 2′,5′-branched RNAs, which do not necessarily have the closed loop that is characteristic of lariats. Owing to their special topology, lariat RNAs are difficult to synthesize by any conventional chemical approach such as solid-phase synthesis,[3] and even the simpler 2′,5′-branched RNA core (without the closed loop of a lariat) is a significant challenge.[4] Recently we reported artificial deoxyribozymes (DNA enzymes)[5] that create branched RNAs in $>90\,\%$ yield by catalyzing the intermolecular reaction of an internal 2′-hydroxy group with a 5′-triphosphate.[6,7] Some of these deoxyribozymes are capable of synthesizing branched RNAs of wide sequence composition.[8,9] Lariat products can result from intramolecular branch formation events (that is, macrocyclizations with single linear RNA substrates), as illustrated in Figure 1b. Apart from using the frequently impractical biological splicing machinery itself, there is no method present for the synthesis of biologically relevant lariat RNAs, which are often several hundred nucleotides in length and have extensive secondary structure[10] that may interfere with loop formation. Indeed, most of our reported branch-forming deoxyribozymes such as 7S11, which is widely useful for branched RNA synthesis,[8] are not useful with biologically derived RNA sequences as substrates (lariat yield typically $<1\,\%$; data not shown). In contrast to these difficulties, we report herein that the 6BX22 deoxyribozyme can create two common classes of biological lariat RNAs efficiently in one step from readily available RNA substrates.

In previous efforts, we used in vitro selection[11] to identify many deoxyribozymes that synthesize branched RNA.[6–9,12]

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

However, our procedure does not select for the particular ability to create lariats. One-step lariat formation is presumably more difficult than simple branch formation because the incipient RNA loop may clash sterically with the DNA structure, thereby inhibiting catalysis. We surveyed many of the branch-forming deoxyribozymes to determine their lariat-formation capabilities. One of these deoxyribozymes, 6BX22,[12] showed promise in this regard and was examined more carefully. The 6BX22 deoxyribozyme has a specific 39-nucleotide DNA enzyme region embedded between Watson–Crick binding arms, as shown schematically in Figure 1b. It was found to require Mn^{2+}; detectable RNA ligation activity was not observed with Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, or $[Co(NH_3)_6]^{3+}$ (all as chloride salts at concentrations in the range of 10 μM–10 mM; data not shown). The k_{obs} value for 6BX22-catalyzed branch formation is $\approx 0.07\ min^{-1}$ ($t_{1/2} \approx 10$ min) under standard incubation conditions (Mn^{2+} (20 mM), pH 7.5, 37 °C; see below). The background rate for the analogous DNA-templated reaction (the use of a DNA template that lacks an enzyme region between the DNA binding arms) is much lower; $k_{templated} \approx 4 \times 10^{-7}\ min^{-1}$ [6], which gives a rate enhancement ($k_{obs}/k_{templated}$) of 2×10^5.[13] Therefore, 6BX22 clearly "catalyzes" branch formation. If it merely templated the reaction by increasing the effective substrate concentration, then $k_{obs}/k_{templated}$ would be 1.[5a]

Before studying 6BX22-catalyzed lariat RNA synthesis in detail, we examined the substrate sequence generality of this deoxyribozyme for branch formation. This reaction is simpler than lariat formation because loop closure is not required. The selection procedure in which 6BX22 was identified[12] used RNA substrates that correspond to the conserved branch-site sequences of yeast spliceosomal substrates (Figure 2a), which are common models for understanding RNA splicing.[2] Systematic experiments revealed that sequence changes outside the conserved RNA elements are tolerated well by 6BX22, indicating that this DNA enzyme is general for branch formation with yeast spliceosomal substrates (Figure 2b). Most nucleotide changes within the conserved regions are also tolerated. For example, the branch-site nucleotide itself may be changed from A to C or U with nearly equivalent ligation efficiencies (Figure 2c). A branch-site G is accepted but with diminished yield.

We applied 6BX22 toward the synthesis of three specific representative biological lariat RNAs that are derived from yeast or mammalian spliceosomal substrates, but share no sequence elements outside of the small consensus region. The three RNAs are the 69-nt yeast YBL059W intron with a 51-nt lariat loop,[14] the 130-nt human β-globin IVS1 intron with a 94-nt loop,[15] and the 309-nt yeast actin (ACT1) intron with a 266-nt loop.[16] In each case, a linear 5′-triphosphate substrate was prepared by in vitro transcription with T7 RNA polymerase. By using the linear substrate, the small YBL059W lariat was readily synthesized by 6BX22 in one step and in high yield (Figure 3a). The larger β-globin and ACT1 lariats were also readily prepared (Figure 3b,c). The lariats were formed with k_{obs} values similar to those of the analogous branches, or in the case of β-globin, about sixfold slower (but still with a k_{obs} value that is preparatively useful). Compelling evidence for each lariat structure was provided by several

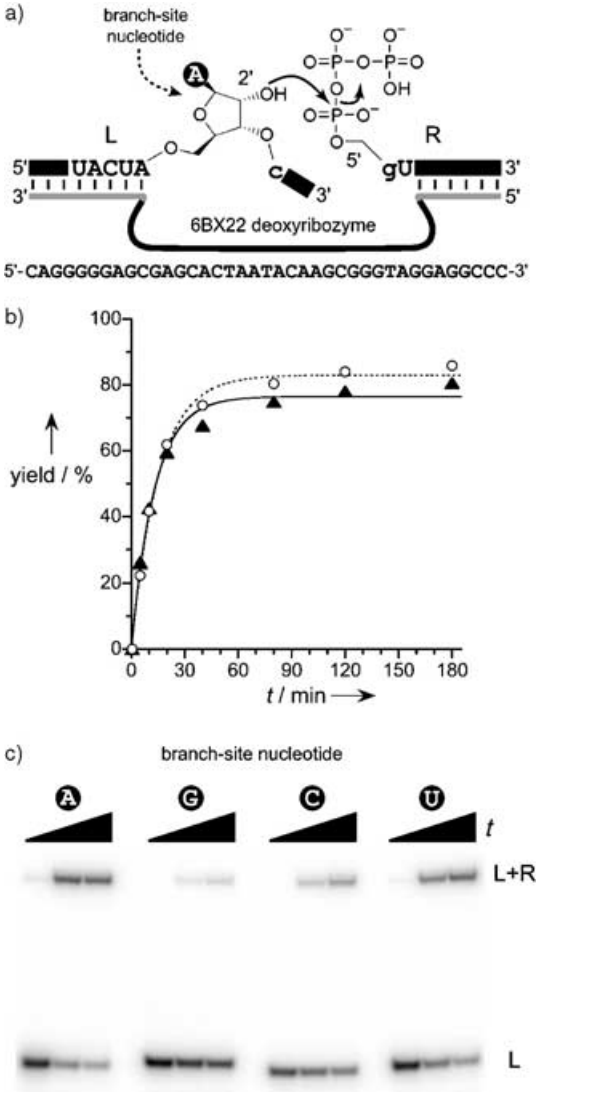

Figure 2. Branched RNA formation by the 6BX22 deoxyribozyme. a) With yeast spliceosomal substrate sequences; the conserved nucleotides are shown explicitly.[2] As demonstrated with the comprehensive experiments shown herein and in the Supporting Information, 6BX22 tolerates nucleotide changes at most of the conserved positions; the sites of tolerance are indicated with uppercase letters. The second nucleotide (U) of the right-hand substrate (R) prefers U, C, or A over G. The sequence of the enzyme region of 6BX22 (the 39 nucleotides not base-paired with the RNA substrates) is given below the structure. The Watson–Crick binding arms of the DNA are shown in light gray. See Experimental Section for full sequences of the left-hand (L) and right-hand (R) substrates. b) Demonstration of the generality of 6BX22 for its RNA substrate sequences; conditions: HEPES (*N*-(2-hydroxyethyl)-piperazine-*N*-(2-ethanesulfonic acid), 50 mM, pH 7.5), NaCl (150 mM), KCl (2 mM), $MnCl_2$ (20 mM), 37 °C. Original RNA sequences that correspond to the core nucleotides of the ACT1 RNA (▲); variant RNA sequences (○, see Experimental Section). For this experiment, $k_{obs} = 0.077 \pm 0.007\ min^{-1}$ (original sequences) and $0.066 \pm 0.004\ min^{-1}$ (variant sequences); errors are standard deviations from exponential curve fits. c) Demonstration of the generality of 6BX22 for branch-site nucleotides ($t = 0$, 0.5, and 1.5 h with 5′-^{32}P-radiolabeled L substrate; 20% PAGE). L+R product yields at $t = 1.5$ h: branch-site A, 71%; G, 5%; C, 33%; U, 64% ($k_{rel} = 1$, 0.085, 0.097, and 0.027, respectively; data not shown). In all cases, partial alkaline hydrolysis of the branched product[6] verified that the site of branching remained unchanged (data not shown).

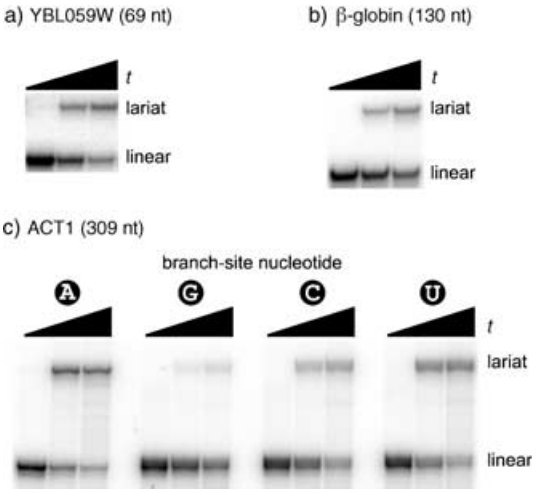

Figure 3. Formation of biologically related lariat RNAs with the 6BX22 deoxyribozyme. a) Synthesis of the 69-nt YBL059W lariat, which has a 51-nt loop (12% PAGE; $t = 0$, 0.5, and 1.5 h). Yield at $t = 1.5$ h: 52%; $k_{obs} = 1.1\ h^{-1}$ (data not shown). b) Synthesis of the 130-nt human β-globin IVS1 lariat, which has a 94-nt loop (8% PAGE; $t = 0$, 2, and 6 h). Yield at $t = 6$ h: 33%; $k_{obs} = 0.17\ h^{-1}$. At $t = 24$ h the yield was ≈50%, but some nonspecific RNA degradation was evident (data not shown). c) Synthesis of the 309-nt ACT1 lariat, which has a 266-nt loop; this was tested with mutants in which the branch-site nucleotide was changed as indicated (6% PAGE; $t = 0$, 0.5, and 1.5 h). Yields at $t = 1.5$ h: branch-site A, 72%; G, 9%; C, 48%; U, 70%. For branch-site A $k_{obs} = 2.8\ h^{-1}$ (data not shown). For the β-globin lariat, disruptor DNA oligonucleotides were required to sequester RNA secondary structure and enable binding of the DNA enzyme to the RNA substrates (Supporting Information). Without disruptors, the β-globin lariat yield was 0.3% at 6 h. A disruptor oligonucleotide was helpful but not absolutely required for ACT1 lariat synthesis, primarily by modestly enhancing the ligation rate (by less than twofold) and by suppressing nonspecific RNA degradation.

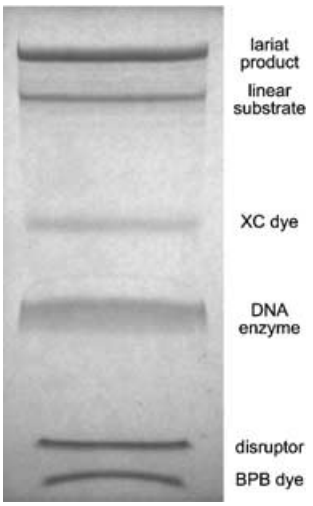

Figure 4. Preparative synthesis of the ACT1 lariat RNA; the image was recorded by UV shadowing of the 6% polyacrylamide gel.

biochemical assays in which the lariat RNA was cleaved with the 10–23 deoxyribozyme[17] and yeast debranching enzyme[18] to generate the predicted pattern of gel bands (Supporting Information). For the β-globin lariat, disruptor DNA oligonucleotides were required to allow the deoxyribozyme to bind productively with its RNA substrates (Supporting Information). For the largest ACT1 lariat, a disruptor helped to prevent RNA degradation, but it was not required for high lariat yield (data not shown).

The yeast ACT1 intron is a particularly common model system for studying spliceosomal RNA processing.[16,19] Artificial synthesis of such lariats without the natural splicing machinery will enable many biochemical experiments, because the sequence requirements of the spliceosome need not be obeyed. For example, the tolerance of 6BX22 toward changes of the branch-site nucleotide during branch formation (Figure 2c) is maintained for lariat synthesis (Figure 3c). Therefore, for the first time, biochemists have access to "real" spliceosomal lariats with mutations at their key branch-site nucleotides. To enable such experiments, preparative-scale (nanomole) synthesis of the ACT1 intron lariat was successfully carried out in high yield (Figure 4).

In summary, we have demonstrated that the 6BX22 deoxyribozyme catalyzes efficient and general one-step lariat synthesis with biological RNAs that are derived from commonly studied yeast and mammalian spliceosomal substrates. The resulting lariats are essentially impossible to synthesize by traditional organic and solid-phase synthesis methods. The only available approach until now has required the use of natural splicing enzymes, which have stringent sequence requirements that impose considerable limitations on the RNA sequences that may be used as substrates. Lariat RNA formation for the YBL059W, β-globin, and ACT1 intron substrates corresponds to the creation of rings with 307, 565, and 1597 atoms, respectively. These DNA-catalyzed macrocyclization reactions succeed without the use of protecting groups despite the presence of hundreds of competitive 2′-hydroxy nucleophiles. This demonstrates an extremely high level of site-selectivity. The reactions also produce very small amounts of side products such as RNA substrate oligomers that are created in large quantity with other deoxyribozymes that synthesize lariat RNAs in low yield.[6] The broadly useful lariat synthesis capacity of 6BX22 is unique in comparison with all of our other deoxyribozymes identified so far, including those such as 7S11, which are quite general for the formation of simpler branched RNAs.[8] The structural basis by which 6BX22—yet none of our other deoxyribozymes—readily tolerates a closed RNA loop in its catalytically active conformation requires further study, as does the mechanism by which 6BX22 achieves its $>10^5$-fold rate enhancement over mere templating. We continue to investigate these fundamental features of catalytic DNA, and we are using synthetic lariats created by 6BX22 to examine key biochemical aspects of RNA splicing.

Experimental Section

Systematic variation of RNA substrate sequences (Figure 2): The original left-hand (L) RNA substrate sequence (which corresponds to that used in the selection procedure[12]) was 5′-GGAAGUCUCAUGUACUAACA-3′. The original right-hand (R) RNA substrate sequence was 5′-GUAUGUUCUAGCGCGGA-3′. Together these

sequences comprise the "core" of the ACT1 branch. For the experiment shown in Figure 2b, nearly all RNA substrate nucleotides in both L and R were changed by transversions (A↔C and G↔U). In L, changes were made to all nucleotides from the 5′ end through and including UACU. In R, changes were made to all nucleotides after 5′-GU through the 3′ end. In all cases, corresponding transversions were made to the DNA to maintain Watson–Crick complementarity. For the experiment shown in Figure 2c, the L substrate was varied only at the branch-site nucleotide position (UACUAAC). (For experiments with variations at the L substrate UACUA nucleotide and at the R substrate GU nucleotide, see the Supporting Information.) Variation of the 5′-triphosphorylated guanosine (5′-pppG) of the R substrate was not tested. The assays were performed with 5′-^{32}P-radiolabeled L substrate and L/deoxyribozyme/R = 1:3:6, under incubation conditions of HEPES (50 mM, pH 7.5), NaCl (150 mM), KCl (2 mM), and $MnCl_2$ (20 mM) at 37 °C.

Synthesis of the three lariat RNAs (Figure 3): Each linear RNA substrate was prepared by transcription in the presence of α-^{32}P-CTP to give internally ^{32}P-radiolabeled transcript. The assays were carried out under the incubation conditions above and a substrate/deoxyribozyme ratio of 1:2. In all cases, side products (presumably substrate oligomers[6]) were observed in very small amounts: < 3% yield for YBL059W and β-globin; < 0.1% yield for ACT1 (data not shown).

Preparative ACT1 lariat synthesis (Figure 4): A sample was prepared that contained 1.0 nmol linear substrate and 1.5 nmol deoxyribozyme plus 2.0 nmol disruptor DNA oligonucleotide (Supporting Information) in HEPES (5 mM, pH 7.5), NaCl (15 mM), and EDTA (ethylenediaminetetraacetic acid, 0.1 mM) in a volume of 150 μL. The sample was annealed by heating at 95 °C for 3 min and then cooling on ice for 5 min. The volume was increased to 200 μL: HEPES (50 mM, pH 7.5), NaCl (150 mM), KCl (2 mM), and $MnCl_2$ (20 mM); Mn^{2+} was added from an aqueous stock solution (1M). The 200-μL solution was incubated at 37 °C for 1.5 h and then mixed with 300 μL low-dye stop solution (80% formamide, 1 × TBE (tris(hydroxymethyl)aminomethane and boric acid, 89 mM each, pH 8.3), and xylene cyanol and bromophenol blue, 0.0025% each). The sample was resolved by 6% PAGE and visualized by UV shadowing; the lariat product was extracted and ethanol-precipitated as described.[7] The isolated yield of lariat RNA product (after gel extraction and ethanol precipitation) was 0.39 nmol starting from 1.0 nmol of linear substrate. This 39% yield (compared with the 70% yield observed at the analytical scale without PAGE purification; Figure 3c) reflects losses that are commonly observed during extraction and precipitation of large RNA species from polyacrylamide gels.

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