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Template-Mediated Synthesis of Lariat RNA and DNA

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Nucleic acid "lariats" have been of great interest to the biological community since their discovery two decades ago as splicing intermediates in the biosynthesis of messenger RNA (lariat RNA introns). We report here the first synthesis of lariat DNA and RNA via template-mediated chemical ligation of Y-shaped oligonucleotides. The method allows for the synthesis of lariat DNA of any base composition as well as the more biologically relevant lariat RNA. Typically, branched precursors and complementary linear templates ("splints") were dissolved in an equimolar ratio at a total concentration of 10^{-4} M, and ligation was promoted by addition of cyanogen bromide in a pH 7.6 buffer. The template-directed cyclization was very efficient, since the amount of circularized lariat product observed in all cases was in the 40−60% range. The lariats were purified by polyacrylamide gel electrophoresis, and their structure and nucleotide composition confirmed by MALDI-TOF mass spectrometry. Thermal denaturation and circular dichroism studies of lariat:RNA and lariat:DNA duplexes were fully supportive of the isolated "lasso" structures. Further characterization was conducted by enzymatic degradation with spleen phosphodiesterase (a 3′-exonuclease) and the RNA lariat debranching enzyme, a specific 2′,5′-phosphodiesterase.

Introduction

A number of research efforts have been devoted to the synthesis of circular oligonucleotides. For practical purposes, chemists have been primarily interested in the structure and reactivity of circular DNA and RNA oligonucleotides smaller than those which occur in nature (e.g. supercoiled DNA, RNA viroids).[1,2] Duplex DNA can exist in circles as small as 125 base pairs;[3] however, realization of cyclic structures smaller than this is difficult owing to the rigidity of the double helix. In contrast, single-stranded circular DNAs and RNAs as small as two nucleotides have been reported owing to their innately more flexible structure.[4]

The synthesis of "lariat" oligoribonucleotides (circular RNA with a tail, Figure 1) remains a major challenge. Their discovery in 1983[5] raised significant interest as to their possible role in the splicing of precursor messenger RNA (pre-mRNA). In addition, lariat and branched nucleic acids offer many potential applications in nucleic acid biochemistry, particularly as tools for probing the substrate specificity of lariat debranching enzymes, and

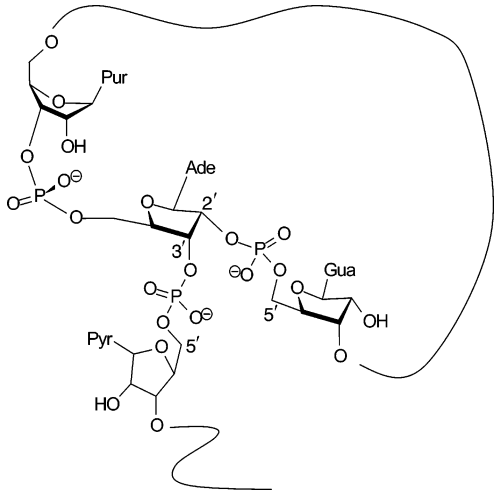

FIGURE 1. Chemical structure of lariat RNA.

for studying pre-mRNA splicing.[6,7] Unlike the synthesis of circular or branched oligonucleotides, lariat synthesis is highly complicated by the fact that additional protecting groups are typically required for the chemoselective introduction of a tail extension in the molecule. RNA "mini"-lariat syntheses have been reported;[8−10] nevertheless, the methodologies require the use of nonstandard

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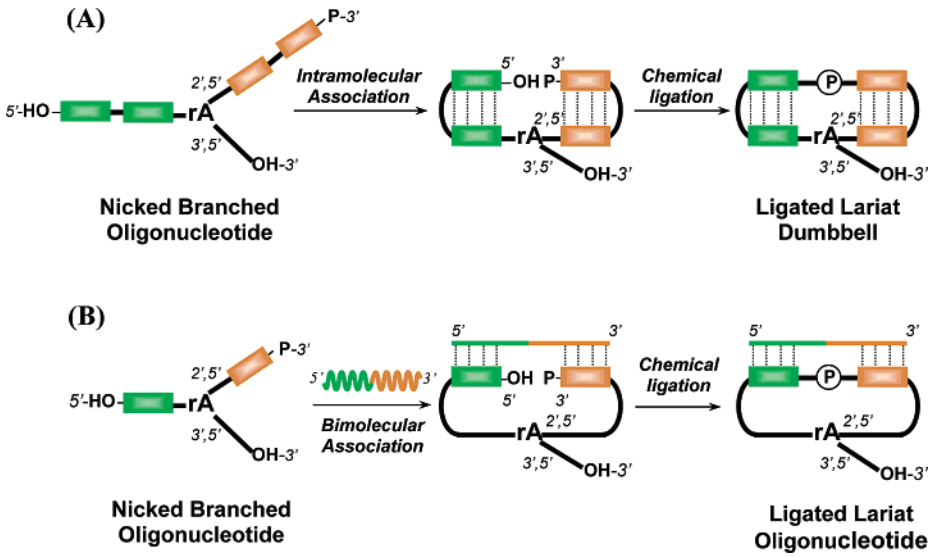

FIGURE 2. (A) Synthesis of lariat DNA via chemical ligation of a unimolecular dumbbell complex.[27] (B) Bimolecular association of branched nucleic acids with a complementary "splint" for the synthesis of lariat oligonucleotides (this work).

nucleoside building blocks, are extensively time-consuming (many chromatographic purification steps), and are restricted to the synthesis of small lariats (di-, tri-, tetra-, and pentameric loops) which are nonrepresentative of the naturally occurring structures. Furthermore, since the cyclization reactions were conducted in solution, high dilutions of reactant were requisite to prevent inevitable dimerization reactions and other high molecular weight polymers.

Given the wealth of information on the synthesis of circular nucleic acids, we imagined that these approaches could be exploited in the synthesis of DNA and RNA lariat oligonucleotides. The use of oligonucleotide templates has demonstrated considerable success for forming large ring structures by either chemical or enzymatic ligation means.[11−18] Fundamentally, a single stranded linear oligonucleotide (precursor circle) is designed to be effectively recognized by a complementary template strand (splint) such that it forms a stable hybrid complex. When the precursor is an oligonucleotide consisting of both a free phosphate and hydroxyl terminus, association renders the reactive moieties in a flanking alignment for efficient ligation.[19] Double,[11,15] triple-helical,[17,18,20−22] and four-stranded cytosine-rich complexes[23,24] have been used to assemble circular oligonucleotides; however in the latter two cases, their synthesis is limited to those precursors that contain homogeneous pyrimidine or purine tracts, or long tracts of cytosine residues. As such, the sequence differentiation within the circle itself is limited. In an alternative fashion, the templates may be included within the single strand itself (i.e. self-templating), resulting in the formation of a dumbbell-shaped (i.e. double-hairpin) complex, which are internally base-paired and closed off by two stabilizing hairpin loops.[25] To date, DNA dumbbells have been synthesized enzymatically by using T4 DNA ligase. More effectively, nicked junction ligation with purely chemical condensing reagents (e.g. EDC, cyanogen bromide) conveniently and inexpensively produces a circularized dumbbell without the many limitations inherent to enzymatic procedures (e.g. expense of DNA ligase).[13,14,26,27]

Recently we reported on the synthesis of a lariat DNA via a self-assembled dumbbell complex.[27] The starting precursor was a branched oligodeoxynucleotide containing both a phosphorylated and a hydroxylated terminus to effect a new phosphodiester bond, thereby cyclizing the structure (Figure 2A). This strategy affords regioisomerically pure lariat DNA molecules with reasonably high yields; however, it is limited to those sequences that contain self-complementarity within the loop structure. To potentially synthesize lariat molecules of any base sequence, we looked toward the template-mediated chemical ligation of branched oligonucleotides using an intermolecular template guide sequence or "splint". As we

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describe below, this approach allows us to incorporate virtually any nucleotide, thereby permitting us to efficiently synthesize lariats of any sequence composition as well as the more biologically relevant RNA lariat (Figure 2B).

Results and Discussion

Experimental Design. Two template (or "splint") oligonucleotides (DNA **5** or RNA **9**) were constructed such that they were capable of forming an intermolecular duplex with the terminal regions of the 2′ and 5′ extensions of Y-shaped DNA (**4**) or RNA (**8**) nucleic acids (Figure 2B). Ideally, the lengths of the Y-shaped oligonucleotides should be sufficient to impart stable duplex formation with their complementary template oligonucleotides.[11,15,28−30] Effectively, this would align the reactive phosphate and hydroxyl junction for covalent ligation with the condensing reagent cyanogen bromide (CNBr), thus forming a circular oligonucleotide with a 3′-tail (Figure 2B). Preferably, the precursor would contain at least 6 + 6 nucleotides complementary to the DNA so as to meet this requirement.[11] Furthermore, the loop size has to exceed the number of nucleotides involved in base-pairing with the splint (>12-nt), to be sure that the ligation site is completely hybridized to it. If the splint is too long, it will hybridize all the way around the loop forming a distorted helix.[31,32] Loop sizes that are not at least twice as long as the splint have been shown to result in the construction of concatameric oligomers rather than circular ones.[11] Explicitly, the nicked Y-shaped precursor would preferentially bear a 3′-phosphate group and free 5′-hydroxyl, as well as the most productive contact at the internucleotide junction, namely 5′-T/3′-pT (see ref 27 and references therein). In addition, both the splint and complementary regions were designed to bear mirror sequence symmetry, thereby minimizing the number of aggregate products that could potentially form.

Branched lariat precursors contained a riboadenosine unit at the branchpoint, keeping in line with the natural lariat structure (Figure 1). A terminally phosphorylated branched DNA (Y-DNA lariat precursor) was synthesized by using the regiospecific and divergent synthetic methodology described previously for the synthesis of lariat-DNA dumbbell precursors.[27] Alternatively, a branched RNA molecule was synthesized by using the convergent solid-phase methodology and the adenosine 2′,3′-*O*-bisphosphoramidite branching monomer as developed by our laboratory.[33,34] Phosphate groups were introduced at the respective 3′-termini by using the chemical phosphorylation reagent 2-[2-(4,4′-dimethoxytrityloxy)ethylsulfonyl]-ethyl-(*β*-cyanoethyl)-(*N*,*N*-diisopropyl) phosphoramidite.[35]

Intermolecular Template-Mediated Synthesis of Lariat DNA. (a) General Considerations. DNA splint (**5**) and complementary DNA (**1**−**3**) as well as the branched DNA lariat precursor (**4**) were synthesized on an Applied Biosystems 381A synthesizer, using standard *β*-cyanoethyl phosphoramidite chemistry and commercially available reagents (Table 1).

TABLE 1. Sequence of Oligonucleotides Used in the Intermolecular Template-Mediated Synthesis of DNA and RNA Lariats[a]

Code	Sequence (5′ → 3′)	Designation
1	gcgttgt$_P$	Nicked 5′-DNA Control
2	^{HO}tgttgcg	Nicked 3′-DNA Control
3	gcgttgttgttgcg	Full Length DNA Control
4	^{HO}tgttgcgt$_{11}$A(2′5′ t$_{11}$gcgttgt$_P$; 3′5′ t$_{20}$)	Y-Shaped Lariat DNA Precursor
5	cgcaacaacaacgc	DNA Splint
6	GCGUUGt$_P$ ^{HO}tGUUGCG	Nicked 5′& 3′ DNA Control
7	GCGUUGttGUUGCG	Full-Length RNAControl
8	^{HO}tGUUGCGU$_{11}$A(2′5′GU$_{10}$GCGUUGt$_P$; 3′5′GU$_{10}$GCGUUGt$_P$)	Y-Shaped Lariat RNA Precursor
9	CGCAACAACAACGC	RNA Splint

[a] Notation: small caps = deoxynucleotide residues; A = riboadenosine; OH = 5′-terminal hydroxyl; P = 3′-terminal phosphate; underlined oligonucleotides represent those which are complementary to the templates **5** or **9**.

Oligonucleotides were purified by denaturing PAGE (12−20%, 7 M urea), desalted by size-exclusion chromatography, and their nucleotide composition was confirmed by MALDI-TOF-MS and gel mobility comparison to purified DNA standards. As mentioned, branched DNA was designed to contain terminal regions at the 2′- and 5′-extensions of the molecule that could associate with a DNA splint (**5**) by simple Watson−Crick complementarity. The complementary termini was comprised of 7 nucleotide stretches that could be recognized by the 14-nucleotide splint on each arm. Adjacent to the terminal portions were two undecanucleotide noncomplementary linkers appended to the rA branchpoint (**4**; Table 1). Additionally, the 3′-rA extension was a homopolymeric deoxythymidine oligonucleotide that could not hybridize to the DNA splint (**5**) in any manner, thereby excluding it from the loop structure (Figure 2B). It has previously been shown that the efficiency of circularization of an oligonucleotide correlates well with the secondary structure of the precursor oligomers, with hairpin-like structures possessing free termini favoring the circularization reaction.[11] Preorganization of the precursor into a more rigid hairpin-like entity minimizes the entropic penalty of hybridizing two freely rotating complementary sequences to one template in solution.[36] As such, the predicted secondary structure of the loop portion of the lariat precursor was analyzed by using the DNA MFOLD server and imposing certain reaction constraints.[37] Computer analysis revealed that the 37-mer loop would likely

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TABLE 2. Thermal Denaturation and Ligation Data for Nicked and Ligated DNA Oligonucleotides with Their Corresponding DNA Splint[a]

oligonucleotide	T_m (°C) TRIS[b]	T_m (°C) MES[c]
1	17.0	36.0
2	17.3	33.0
1 + **2**	19.1	36.5
4 (nicked)	12.0	39.0
3	51.0	–
4 (ligated) (45%)[d]	41.7	–

[a] T_m values are the average of 3 successive runs and are within ±0.5 °C. [b] 10 mM Tris-HCl, pH 7.5, 10 mM NaCl. [c] 0.25 M MES, pH 7.6, 20 mM $MgCl_2$. Dash = not determined. [d] The ligation yield (with **5** as template) was determined by densitometric quantitation of the circularized product band.

fold into one probable hairpin-like structure with a negative ΔG at the ligation temperature (4 °C), leaving both reactive 5′ and 3′ termini free to associate with the complementary splint (Supporting Information, Figure S1). The mini-stem portion of the hairpin appeared to be stabilized by two Watson−Crick hydrogen bonds between C_6/G_{33} and G_7/C_{32} while the loop closing base-pairs were stabilized by a noncanonical T_8/G_{31} wobble base-pair. Prior to assessing the efficiency of ligation, the thermal stability of **4** complexed with its template splint **5** was determined. Equimolar amounts of precursor and template were heated in the appropriate annealing buffer, and cooled slowly to allow intermolecular association. Under CNBr ligation conditions (i.e. 0.25 M MES buffer) the complex displayed a cooperative, monophasic transition with a respective T_m of 39 °C, indicating that the complex was indeed stable enough for ligation (Table 2). Appropriately, the stability of the complex was also measured in a buffer of lower ionic strength (i.e. 10 mM Tris-HCl, pH 7.5, 10 mM NaCl). As expected, the T_m of the complex decreases to 12 °C under these conditions. Comparatively, the thermal stability of the ternary complex between **5** and the nicked 5′- and 3′- DNA controls **1** and **2**, corresponding to the complementary regions of the Y-DNA precursor, was 7 °C higher than that of **4** (Table 2; Supporting Information, Figure S2).

Dissociation of the ternary complex displayed a single, cooperative transition, given that the hybridized portions were mirror images of one another. This destabilization in the looped complex is consistent with the proposed hairpin-like secondary structure, such that the DNA splint (**5**) has a more difficult time accommodating the complementary strands in the kinked portions of the stem-loop region. Quite possibly, the DNA splint (**5**) is not fully base-paired in this domain, and in effect, end-fraying is a likely consequence. Interestingly, when the nicked controls, **1** and **2**, were hybridized independently to the DNA splint, their respective melting temperatures were lower than their collectively hybridized value (Table 2). Previously, Agrawal's group has shown that when short, tandem oligonucleotides bind without a base separation between their binding sites to their target, they bind cooperatively, with a thermal stability greater than the average of the independent duplexes.[38] As such, the cooperative interactions between **1** and **2** are probably

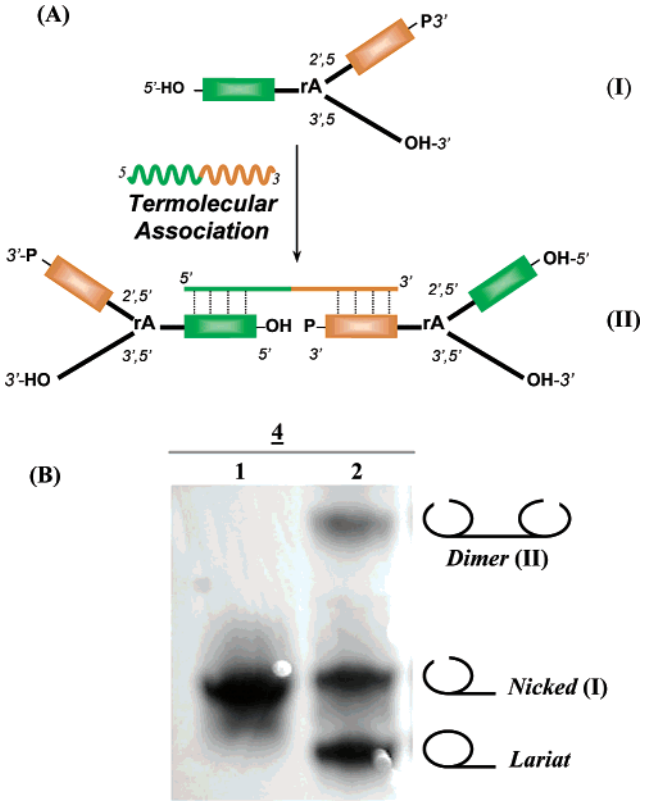

FIGURE 3. Intermolecular association and chemical ligation of branched Y-DNA **4**. (A) Competing termolecular association (2 template Y-DNAs + 1 splint DNA) produces dimeric Y-DNA hyperbranched species (**II**). (B) Analysis of the CNBr-mediated ligation of **4** in the presence of 1:1 complementary splint ***5***, using 12% denaturing PAGE (8.3 M urea). Lane 1: negative control **4**. Lane 2: CNBr ligation of **4** in the presence of template **5** (1:1).

driven by stacking interactions amid the terminal bases, resulting in a highly competent and productive contact.[30,39] Presumably, this analogy holds true for the nicked **4** complex, although supporting data regarding the independent associations of the 5′- and 2′-extensions to the template were impossible to obtain given the bimolecularity of the complex.

(b) Chemical Ligation of Y DNA Precursor 4. Chemical ligation was conducted with use of a DNA lariat precursor (**4**) to splint (**5**) ratio of 1:1 at a total nucleotide concentration of 10^{-4} M. Higher concentrations of splint to precursor (e.g. 10^{-3} M) have been shown to lead to the preferential formation of dimeric product species.[11] After heat denaturation, the duplex was left to associate slowly at 4 °C to promote juxtaposition of the terminal 5′-hydroxyl/3′-phosphate junction. Ligations were initiated by the addition of CNBr, and after 5 min, the condensation products were quantitatively precipitated from solution. PAGE analysis of the ligation mixture exposed a newly formed, major product band that migrated faster than its nicked precursor and was formed in approximately 45% yield (Figure 3B). A minor product band, which migrated near the top of the gel, was also evident (ca. 15%). As demonstrated in Figures 2B and 3A, the

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splint DNA **5** can associate with the branched lariat precursor **4** via two different binding modes. At high dilution, bimolecular association prevails to form the desired 57-mer circularized lariat structure consisting of a 37-nt loop and homopolymeric 20-nt stem. The resultant lariat structure likely exists in a more compact form such that it migrates through the cross-linked matrix at a faster rate than its analogous nicked precursor.

However, a competing reaction is the termolecular association of the splint DNA with two molecules of precursor, which aligns the junction in a head-to-tail assembly, resulting in the production of a dimeric, hyperbranched species containing 114 nucleotides (Figure 3A). Normally, such a reaction is prevalent at elevated concentrations of oligonucleotide precursors, suggesting that a higher dilution in our experiments (e.g. 10^{-5} M) might favor exclusive lariat formation. The suboptimal ligation efficiency, established by the amount of remaining nicked precursor (ca.40%), suggests that the terminus of the 2′-branch extension is not completely phosphorylated, owing to the divergent introduction of a terminal phosphate group in the vicinity of the solid support.

(c) Characterization of Lariat DNA. The three representative bands were excised from the gel and their nucleotide composition discriminated by negative MALDI-TOF mass spectrometry (see Materials and Methods). The molecular weight of the slowest moving band was indeed consistent with dimer formation, exposing a molecular weight of approximately 35 KDa. The respective molecular ions of the predominant product band and nicked precursor displayed a difference of 18 units, thereby confirming the condensation of the reactive phosphate/hydroxyl junction and consequent loss of a water molecule. Analysis of the thermal denaturation profiles for the sealed form of **4** (DNA lariat) and its open structure reinforced the covalent circularity of the molecule. In its ligated form, the complementary region of the circle is compelled to form a full 14-nt base-pair with the template **5**, thereupon exhibiting an enhanced thermal stability compared to its nicked counterpart. Indeed, an enhancement of ca. 30 °C results, indicating that a new phosphodiester bond between the nicked junctions had been properly accomplished (Table 2; Supporting Information, Figure S2). Nonetheless, the T_m of the closed-loop structure complexed to **5** was lower than that of the relevant full length linear 14-nt complement **3** bound to the identical template ($\Delta T_m = 9$ °C; Supporting Information, Figure S2). Furthermore, the transition appeared to be broader and less cooperative, signifying that full base-pairing between the splint **5** and complementary regions of the DNA lariat was lacking. The binding of the single stranded target to a circular complement imposes a topological constraint since duplexes are right-handed helices, and each turn of the helix (i.e. 10 base-pairs) requires that the strand pass through the circle once.[12,32,36] This requires that the circular portion first be threaded onto the end with the single-stranded target and then work its way down to the appropriate binding site. The rigidity of the loop probably causes the 14-nt template to be incompletely hybridized at its terminal regions resulting in significant end breathing and an attributable drop in the overall complex stability. This is supported by the CD studies described in the Supporting Information, Figure S3).

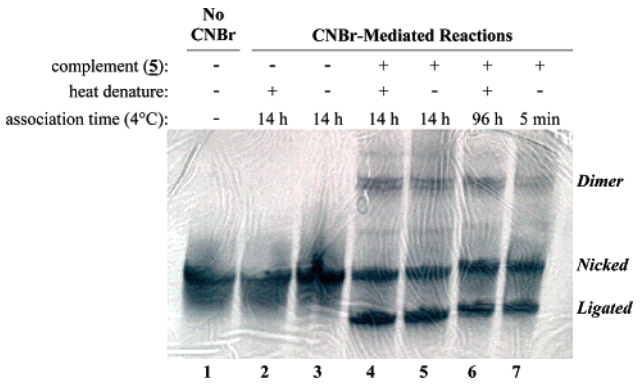

FIGURE 4. Assessment of CNBr-induced ligation of **4** under diverse reaction conditions: 12% denaturing gel (8.3 M urea); oligonucleotides were dissolved at a total concentration of 10^{-4} M precursor and complement in 0.25 M MES (pH 7.6), 20 mM $MgCl_2$ buffer.

The capacity for forming the desired DNA lariat structure under diverse reaction conditions was also assessed by gel electrophoresis (Figure 4). The results unmistakably established that in the absence of complementary splint DNA **5**, none of the circularized product, or dimeric species for that matter, was formed (compare lanes 2 and 4). Moreover, production of the covalently closed circle did not require prior heat denaturation of the Y-DNA precursor−splint mixture before the addition of condensing reagent, suggesting that the precursor was in a freely associative state at the ligation temperature (lanes 4 and 5). Interestingly, the amount of time required for association of the complex prior to ligation was inconsequential as well. When the complex was left to associate over a 3-day period, the amount of circularized product was nearly identical with that obtained when the precursor and template were merely dissolved in ligation buffer and placed on ice with CNBr for the duration of the ligation time (5 min; lanes 6 and 7). Indeed, the complementary region of the loop appears to be in a pre-organized and accessible conformation for hybridization to its template splint. In addition, the amount of dimerization adduct formed by using an extended association time was slightly higher than that produced under kinetic conditions, suggesting that the pre-organized hairpin-like secondary structure loop opens up to allow for termolecular association with the splint. As such, hairpins with free 3′ and 5′ ends appear to be constructive structures for oligonucleotide circularization, and the extent of terminal ligation most probably reflects the equilibrium concentration of the hairpin structures under the initial reaction conditions. As shown in lanes 4−7, the dimer appears to migrate as two close moving bands. We do not have an explanation for this, but we suspect it may be due to the formation of both a dimer species (**II**) as well as a concatemer lariat (i.e., two interlocked lariats).

Further characterization of the closed-nature of the lariat was conducted by enzymatic degradation with the 3′-exonuclease, BSPDE (Figure 5). A radioactive α-[^{32}P]-ddAMP probe was introduced at the 3′-termini of both the nicked and ligated forms of **4** by way of the enzyme terminal deoxynucleotidyl transferase (TdT), such that the degradation pattern could be monitored electro-

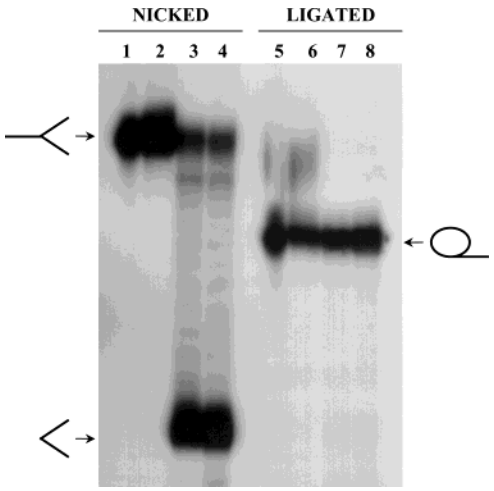

FIGURE 5. Characterization of the nicked and ligated forms of **4**, using the 3′-exonuclease, bovine spleen phosphodiesterase (BSPDE): 12% denaturing (8.3 M urea) gel. Lanes 1 and 5; negative controls (no buffer + no BSPDE). Lanes 2 and 6: Incubation with buffer only (0.1 M NAOAc, pH 6.5) at 37 °C for 2 h. Lanes 3 and 7: BSPDE + buffer (0.5 h). Lanes 4 and 8: BSPDE + buffer (2 h).

phoretically.[40] As described previously, the unligated loop structure bears a free 5′-hydroxyl terminus, which makes it an appropriate substrate for the enzyme's 5′-processivity. Indeed, the nicked form of **4** was readily degraded by the enzyme leading to the accumulation of one major product band that corresponded to the branched DNA lacking the 5′-extension (V-shaped DNA; lanes 3 and 4).[41,42] Contrarily, the ligated form of **4** was completely resistant to enzymatic cleavage, establishing that after ligation, the 5′-terminus of the molecule is integrated into the sugar−phosphate backbone of the DNA and is no longer accessible to the exonuclease.

Synthesis of Lariat RNA. (a) General Considerations. Encouraged by the successful ligation of the DNA lariat **4**, we were tempted to undertake RNA lariat synthesis using the same design. The optimal reaction constraints utilized for the synthesis of the DNA lariat (**4**) were maintained in this study, such as a 14-nt template bound to a 7 + 7 nucleotide complementary region. Furthermore, the favored secondary structure of the loop region was calculated by using the RNA MFOLD program; however, this time, reaction constraints such as temperature and ionic strength of the ligation buffer could not be input as these parameters were already defined (i.e. 1 M NaCl, $T = 37$ °C). Once again, computer analysis established that the 37-nt loop structure would likely fold into one probable hairpin-like motif ($\Delta G = -0.7$ kcal/mol), stabilized by two Watson−Crick interactions between C_6/G_{33} and G_7/C_{32} together with a wobble contact between U_8/G_{31} (Supporting Information, Figure S1). In addition, to ensure isomeric 3′,5′-phosphodiester bond purity during the ligation reaction, deoxythymidine residues were integrated at both the 5′ and 3′ terminal positions. Besides the very productive contact that these two pyrimidines make at the nicked junction, this was done to ensure chemical purity at the ligation site, since the nonenzymatic ligation may otherwise result in mixtures of 2′,5′ and 3′,5′ diesters when ribonucleotides are joined..[18,29,43,44]

(b) Synthesis of Y-RNA Precursor 8. The branched RNA lariat precursor (**8**; Table 1) was constructed by using our well-established protocols for the convergent synthesis of branched RNAs.[33,34,42,45] The 3′-phosphates were introduced by reacting a support-bound nucleoside (60 μmol/g, 1000 Å CPG) with the chemical phosphorylation reagent 2-[2-(4,4′-dimethoxytrityloxy)ethylsulfonyl]-ethyl-(β-cyanoethyl)-(*N*,*N*-diisopropyl) phosphoramidite prior to oligonucleotide assembly (see Experimental Section). Maintaining the natural structure (Figure 1), the RNA lariat precursor embodied a riboadenosine branchpoint, with the 5′-, 3′-, and 2′-branch junctions consisting of oligoribonucleotide appendages rather than deoxynucleotides as in the DNA lariat. The branching synthon utilized in this case was an adenosine 2′,3′-*O*-bis-phosphoramidite reagent, which was synthesized as described previously.[34,41] Furthermore, the sequence design incorporated a *G*-nucleotide at the 2′,5′ and 3′,5′ positions of the branch to permit efficient characterization of the lariat structure by hydrolysis with the RNA lariat debranching enzyme, a specific 2′,5′-phosphodiesterase.[6,46−49] Since two neighboring strands are tethered together via the branching agent, the oligonucleotide sequences found at the 2′ and 3′ positions of the branch junction are identical in base composition. The complementary regions in the 2′- and 5′-wings mirrored those sequences used in the DNA lariat study. As such, the 2′- and 5′-branch extensions would be able to associate with a complementary template splint so as to place the 3′-phosphate/5′-hydroxyl junction in the correct spatial geometry for effective ligation.

(c) Hybridization of Y RNA Precursor (8) with DNA (5) and RNA (9) Splints. RNA lariat precursor **8** was annealed to either its complementary template DNA (**5**) or RNA (**9**) at a 1:1 stoichiometric ratio in ligation buffer (0.25 M MES). Complexes were left to associate overnight at 4 °C, and their thermal denaturation profiles were monitored at 260 nm. Under the condensation conditions studied, nicked **8** formed a much more stable hybrid complex with the RNA splint than its corresponding DNA template ($\Delta T_m = 22$ °C; Table 3). Suitably, a tandem oligonucleotide pair (**6**) mimicking the complementary regions of **8** was synthesized (Table 1), and their melting behavior in the presence of both splints **5** and **9** monitored for comparative purposes. The tandem RNAs (**6**) assembled into a more thermally stable complex with complementary RNAs (**9**) than the DNA counterpart (**5**) ($\Delta T_m = 16$ °C; Table 3), consistent with the general observation that RNA−RNA duplexes with mixed base

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TABLE 3. Thermal Denaturation and Ligation Data for Nicked and Ligated Intermolecular RNA Complements with Their Corresponding DNA and RNA Template Splints[a]

		T_m (°C)	
oligonucleotide	splint	TRIS[b]	MES[c]
6	**5**	<15	31.0
	9	23.0	47.1
8 (nicked)	**5**	<10	25.0
	9	–	47.0
7	**5**	44.0	–
	9	56.0	–
8 (ligated) (46%)[d]	**5**		–
8 (ligated) (35%)[d]	**9**	47.0	–

[a] T_m values are the average of 3 successive runs and are within ±0.5 °C. [b] 10 mM Tris, pH 7.5, 10 mM NaCl. [c] 0.25 M MES, pH 7.6, 20 mM $MgCl_2$. [d] The ligation yield was determined by densitometric quantitation of the circularized product band. Dash = not determined.

composition tend to be more energetically stabilized than DNA–RNA hybrids of identical sequence.[50,51] The more rigid nature of the RNA backbone, composed of C3′-*endo* "compact" nucleotide repeats, presumably pre-organizes the structure for favorable and specific binding to its complementary target.[36,52] A noticeable decrease in melting temperature is manifest when the DNA splint **5** is hybridized to the nicked RNA lariat **8** compared to its complex with the tandem RNAs **6** ($\Delta T_m = 6$ °C); however, this is not observed with the RNA splint **9** as the T_m for both complexes (**6**:**9** and **8**:**9**) is identical (47 °C). Even so, the helix-to-coil transition in the nicked loop structure is less cooperative than that in the tandem RNAs (not shown), suggesting that full complementarity within the binding domain was not achieved. Consistent with the data obtained for the DNA lariat, this likely stems from the secondary structure adopted by the loop portion of the lariat precursor and the difficulty imposed by accommodating the target stand in the stem-loop region. Since the T_m values of all complexes in MES buffer were deemed to be sufficiently stable, we proceeded with this system to conduct the synthesis of the RNA lariat.

(d) Efficiency of Template-Mediated Synthesis of Lariat RNA Is Influenced by Splint Structure. The RNA lariat precursor **8** was mixed with an equimolar amount of either DNA (**5**) or RNA splints (**9**) in MES ligation buffer at a total strand concentration of 10^{-4} M. Samples were heated briefly and allowed to cool slowly to the desired reaction temperature (4 °C). A major disadvantage of using the convergent strategy for branched RNA synthesis is that it introduces identical sequences at both the 2′- and 3′-extensions of the branch. Therefore, during strand association, the complementary splint could potentially hybridize and align either or both the 5′- and 2′-extensions or the 5′- and 3′-extensions (Figure 6). This would fold the loop into two probable structures, leading to the synthesis of desired 2′-lariat and unsought 3′-lariat RNA regioisomer following phosphodiester bond condensation.

Given that previous data have demonstrated a preferred stacking interaction between the branchpoint adenine and the base at the 2′-position,[53,54] we imagined that this factor may influence the ratio of folded 2′-loop structure leading to predominant ligation of the desired lariat. Analysis of the CNBr-mediated reaction products once again unveiled the presence of a distinct, faster moving oligonucleotide species, with a migration rate consistent to that of the DNA lariat described previously (Supporting Information, Figure S4). Such a lariat would contain a 37-nt loop and an 18-nt stem. A negligible amount of dimeric material, with an approximate molecular weight of 35 KDa (i.e. 110-nts), was also observed with both the DNA and RNA splints, formed by the termolecular association of two molecules of precursor with one copy of template. Densitometric quantitation of the resultant product bands indicated that the DNA splint was more effective at positioning the phosphate–hydroxyl junction for condensation, since the yield obtained was roughly 10% higher than that with the RNA splint (Supporting Information, Figure S4). This was surprising since the thermal melting profiles demonstrated that the RNA splint (**9**) formed the most stable hybrid duplex with the precursor under the ligation conditions ($\Delta T_m = 22$ °C).

As such, in this context it does not appear duplex stability is the dominating factor influencing ligation efficiency, and instead definition of the local stereogeometry at the nicked junction is of utmost importance. Unequivocally, duplex RNA exists as an A-form helix, and it is well established that the nucleotide repeats adopt a C3′-*endo* sugar pucker. Since the ligation junction contains two flanking deoxythymidine residues, RNA template binding likely forces the deoxyribose sugars to adopt an intermediary conformation between the normal DNA C2′-*endo* pucker and the C3′-*endo* conformation of the RNA-RNA duplex.[55] In this case, the 3′-phosphate would be placed in a pseudoequatorial position, thereby constraining it into a more sterically hindered orientation for interaction with the 5′-hydroxyl of the neighboring thymidine. Alternatively, when the DNA splint binds, the favorable C2′-*endo* local geometry is maintained, with some minor perturbations imposed by the adjacent ribonucleotide residues.[56] Essentially, this places the phosphate group in a pseudoaxial position, for constructive in-line attack by the adjacent 5′-hydroxyl moiety.[29]

Even though the DNA splint aligns the reactive interface into the proper spatial orientation, the cyclization yields obtained were still only moderately acceptable (Table 3). In contrast to the DNA lariat, the phosphate terminus during brached RNA synthesis was introduced as a first step, rather than at the end of the synthesis cycle, so theoretically, 100% of the 3′-termini should have been phosphorylated. Quite possibly, water molecules were consuming the activated phosphate groups and excess cyanogen bromide faster than they could react. To verify this, nicked **8** was ligated in the presence of

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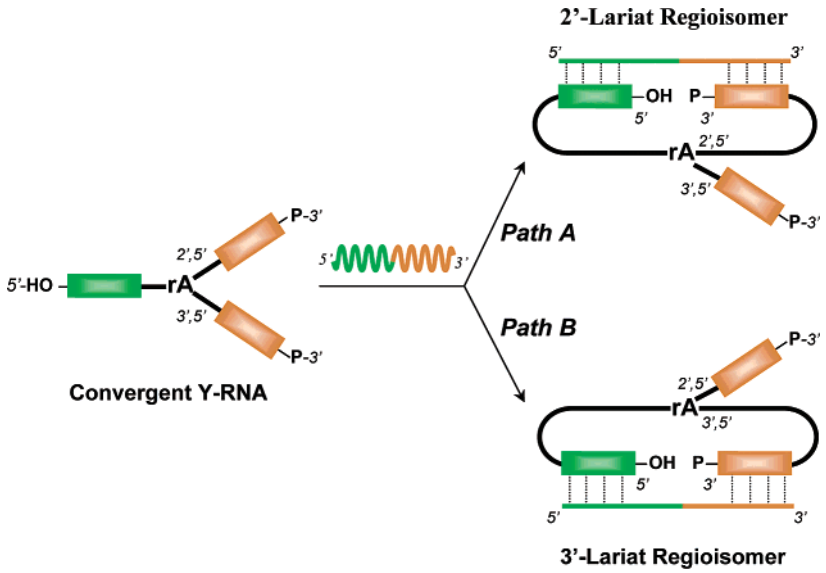

FIGURE 6. Convergently synthesized branched RNA can assemble into two lariat precursor structures with a complementary template giving rise to the desired 2′-lariat regioisomer and the unsought 3′-lariat regioisomer.

complementary DNA splint **5** under standard condensation conditions. After the usual 5-min incubation period at 4 °C, a small aliquot of ligated material was removed from the mixture, and the remaining RNA complex was treated with fresh CNBr for an additional 5 min. Analysis of both reaction mixtures demonstrated that the first incubation produced approximately 44% of ligated material, whereas the reaction with supplemental CNBr exhibited an appreciable intensification of lariat product (ca.. 58%). Clearly, this suggests that hydrolysis of the activated phosphate is the rationale behind the lower than anticipated yields (data not shown). This technique has successfully been used to intensify the amount of ligation product produced even in strictly unfavored duplexes.[29]

(e) Characterization of Lariat RNA. The anticipated RNA lariat product band was excised from a denaturing preparatory gel, extracted into water, and desalted by size-exclusion chromatography. The oligonucleotide composition was determined by MALDI-TOF-MS and yet again, the difference in molecular weight between the nicked and ligated forms of **8** was consistent with the condensation of the phosphate and hydroxyl groups. Furthermore, thermal melting analysis of the closed RNA lariat hybridized to its complementary RNA splint **9** displayed enhanced thermal stability compared to its nicked tandem RNAs **6** in 10 mM NaCl (Table 2; Supporting Information, Figure S5). The resultant T_m was also 9 °C lower than that of the full-length linear complementary region (**7**) complexed to the RNA splint, consistent with the results obtained for the DNA lariat. As described previously, this suggests that complementary RNA has a difficult time integrating itself within the circularized RNA loop structure for productive complex formation, resulting in a reduction in the overall hybrid stability.

Encouraged by the presence of only one new product band, concurring with the anomalous migratory rate of a circularized lasso species, we hoped that only one lariat regioisomer, either the 2′-cyclized or 3′-cyclized, had been produced. Although the two regioisomeric species would have indistinguishable molecular weights, it seemed feasible that they might adopt different topological structures, thereby permitting their resolution on a more highly cross-linked gel matrix (i.e. 24% acrylamide).[57−59] However, even under less porous denaturing PAGE conditions, the lariat product band did not separate into two distinct species. The same was true when the cyclization product was analyzed under nondenaturing gel conditions and by both anion-exchange and reverse-phase HPLC (data not shown).

To further characterize the lariat structure and determine if one regioisomer indeed predominated over another, an RNA debranching assay was performed. Debranching of the lariat product should proceed efficiently since the synthetic structure incorporated a wild-type *G*-nucleotide at the 2′-position of the adenosine. Purine-containing branches have been shown to be the preferred substrates for hydrolysis.[6,48,49,60] The individual regioisomeric ligation products could be easily distinguished by using this assay, since cleavage would result in completely different hydrolysis products (Figure 7A,B). Specific hydrolysis of the desired 2′-lariat ligation product at its 2′,5′-phosphodiester bond would convert it to a linearized RNA molecule consisting of 55 nucleotides (Figure 7A). On the other hand, treatment of the undesired 3′-lariat regioisomer with DBR would produce two digestion products: an 18 nucleotide product derived from the stem of the molecule and a 37 nucleotide circular RNA containing the riboadenosine branchsite (Figure 7B). The substrate lariat RNA **8** was incubated under debranching conditions in HeLa nuclear extract,[46] and the resultant hydrolysis products were visualized by staining of the gel with the commercially available Stains-All. EDTA (10 mM) was added to the assay mix

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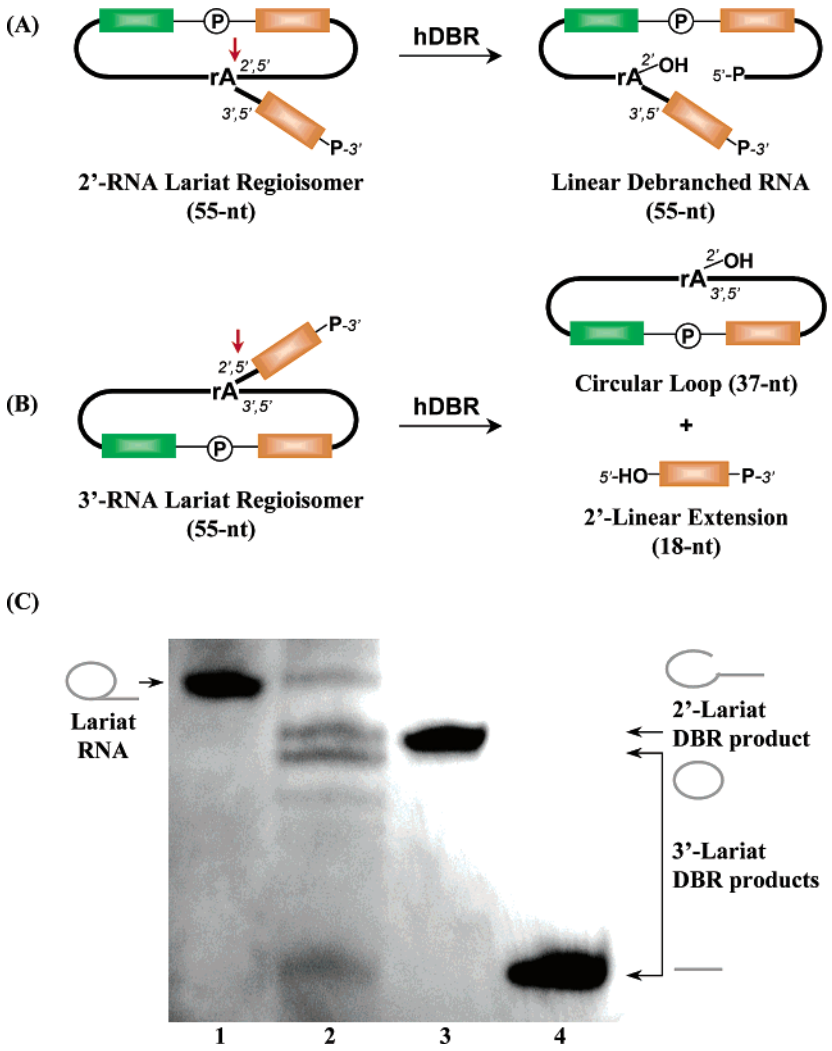

FIGURE 7. Schematic representation demonstrating hydrolysis of regioisomeric 2′- and 3′-lariat forms of **8**, using the HeLa debranching enzyme (hDBR) found in nuclear extract. The red arrow represents the DBR hydrolysis site. Panel A: Debranching of the 2′-lariat regioisomer. Panel B: Debranching of the 3′-lariat regioisomer. Panel C: 24% denaturing PAGE (7 M urea) demonstrating the debranching of ligated lariat RNA **8**. Lane 1: Ligated lariat RNA **8**. Lane 2: Lariat RNA **8** + 30% HeLa extract (debranching). Lane 3: 2′-Lariat debranching product 55-mer (5′-PGU$_{10}$GCGUUGttGUUGCG U$_{11}$AGU$_{10}$GCGUUGtP-3′). Lane 4: 3′-Lariat debranching product 18-mer (5′-PGU$_{10}$GCGUUGtP-3′).

as a general exonuclease inhibitor.[46] Debranching of the purified lariat RNA **8** resulted in the production of three new product species, two of which comigrated with appropriate oligonucleotide standards (Figure 7C). The most retained product band coincided with the full length linear 55-nt RNA; the debranching product of the desired 2′-lariat regioisomer (Lanes 2 and 3). On the other hand, another product band comigrated with the 18-nt linear control, one of the two debranching products of the 3′-lariat isomer (Lanes 2 and 4). An unidentified artifact, which migrated slightly faster than the linear 55-nt product, was also evident (Lane 2). Provided that both regioisomeric lariats were present in equivalent amounts, as determined by densitometry, this unknown oligonucleotide band was attributed to the circular 37-nt loop structure. Thus, it appeared that cyclization of the 2′-branch extension and the 3′-appendage occurred with equal efficiency, and it is likely that the base-stacking interaction between the adenine base at the branchpoint and the 2′,5′-linked guanine is too weak to drive circularization of one extension over the other in such a large loop structure.

Conclusions

Overall, we have successfully demonstrated that lariat oligonucleotides of both DNA and RNA circular loop and stem structures can be synthesized using template-mediated methodologies. As this paper was being written, a report emerged concerning a de novo approach to synthesizing lariat RNA based on a deoxyribozymes isolated from random DNA pools that contained RNA ligase activity (i.e. DNA-dependent RNA ligase).[61,62] The deoxyribozymes selected created non-native 2′,5′-phosphodiester bonds from a linear RNA species with greater than 95% selectivity for the non-native bonds versus the

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native 3′,5′-linkage. Nonetheless, the precursor RNA substrates were synthesized via enzymatic T7 RNA polymerase transcription from a DNA template, and as such, yields of precursor are limited in this scope. Furthermore, the apparent yields of lariats observed, depending on the deoxyribozyme species used, were only in the 1−15% range. Our method provides a significant advantage since the precursor substrates are synthesized chemically, via well-established solid-phase methodologies, thereby allowing us to isolate an appreciable amount of precursor for larger scale production. In addition, the template-directed cyclization is clearly more efficient, since the amount of circularized lariat product observed in all cases was in the 40−60% range. Regardless, the results from the deoxyribozyme-mediated ligation are indeed of considerable mechanistic interest, given that nature's spliceosome is likely a ribozyme in itself.[63−65]

Our results reported herein validate the first examples of the synthesis of medium-sized DNA and RNA lariat oligonucleotides with well-established solid-phase phosphoramidite chemistry and commercially available reagents. Both the intramolecular dumbbell strategy previously reported[27] and the intermolecular splint/complement strategy described here are effective; however, only the latter allows for the introduction of any base composition within the loop and stem structures and is not limited to those molecules containing internal templates. Furthermore, it permitted the synthesis of a biologically relevant RNA lariat molecule containing the wild-type adenosine branchpoint and guanosine nucleotide at the 2′-appendage of the branch, which was a substrate for the human lariat debranching enzyme.

Experimental Section

Divergent Synthesis of the Branched DNA-Lariat Precursor 4. Syntheses were performed on an Applied Biosystems 381A oligonucleotide synthesizer, using standard phosphoramidite chemistry and reagents. Nucleoside derivatized CPG was prepared according to known methods.[66] All standard 3′,5′-DNAs [5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside-3′-*O*-(*β*-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidites] and inverted 5′,3′-DNA [3′-*O*-dimethoxytrityl-2′-deoxyribonucleoside-5′-*O*-(*β*-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite] were stored at −20 °C and dried in vacuo overnight prior to use. The branched precursor for the intermolecular ligation of lariat DNA was synthesized by using our previously published methodology for the regiospecific solid-phase synthesis of branched DNA.[34,67] The sequence was designed to be devoid of any intramolecular complementarity, and the 5′- and 2′-extensions were complementary to an intermolecular DNA or RNA template introduced during the chemical ligation step. Synthesis was conducted on a 1-*μ*mol scale with a low nucleoside loading of CPG (dT loading: 19 *μ*mol/g) to ensure ample extension from the branchpoint. The 3′,5′-linked linear strand was assembled by using the standard automated synthesis cycle utilizing 3′-DNA phosphoramidites (0.1 M in acetonitrile). Activation of the nucleoside phosphoramidites was conducted with either 1-H-tetrazole or 4,5-dicyanoimidazole (both at 0.5 M in CH_3CN). Coupling time for DNA monomers was 2 min. The branching nucleotide was introduced with 5′-O-DMT-N^6-benzoyl adenosine-3′-*O*-(*β*-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite (3′-RNA phosphoramidite; 0.15M in acetonitrile) at which point chain elongation was continued in the 3′ → 5′ direction followed by automated detritylation of the 5′-position of the last nucleotide. The terminal hydroxyl was "capped" with acetic anhydride on the synthesizer, washed thoroughly with CH_3CN, and dried with a stream of argon. The column containing the support bound oligonucleotide was removed from the synthesizer and fitted with a luer-lock syringe adapter. The phosphate *β*-cyanoethyl protecting groups were removed by pushing a solution of triethylamine/acetonitrile (4:6 v/v, 10 mL) through the column by syringe over a 90-min period. This converted the phosphotriester moieties to the more stable phosphodiesters, which withstand the conditions required to remove the branchpoint silyl-protecting group in the ensuing step. The CPG beads were washed extensively with CH_3CN (30 mL) and THF (30 mL). The 2′-TBDMS group was selectively removed by washing the CPG with a solution of TBAF (1 M in THF, 1 mL) for 10 min. Prolonged treatments with TBAF were discouraged as it has been shown to cleave the oligonucleotide from the solid support.[49] The support was washed sequentially with THF (50 mL) and CH_3CN (50 mL), reinstalled on the synthesizer, and dried by flushing argon through the column for 15 min. The branch was synthesized by extending the chain from the 2′-hydroxyl of the riboadenosine unit with inverted 5′-DNA phosphoramidites (3′-O-DMT-2′-deoxy-(*N*-protected ribonucleosides)-5′-*O*-(*β*-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidites; 0.10 M in acetonitrile; coupling time 2 min). Owing to the steric hindrance surrounding the 2′-position, the first phosphoramidite addition necessitated a higher concentration (0.3 M in acetonitrile) and coupling time (30 min).[68,69] All successive couplings utilized standard conditions. Introduction of the terminal 3′-phosphoryl group was conducted by reacting the 3′-hydroxyl of the branch extension with the chemical phosphorylation reagent, 2-[2-(4,4′-dimethoxytrityloxy)ethylsulfonyl]ethyl-(*β*-cyanoethyl)-(*N*,*N*-diisopropyl) phosphoramidite (0.10 M in acetonitrile); however, an extended coupling time was used (10 min) owing to the increased sterics of extending the chain in the vicinity of the solid support. The sequence was deprotected under standard conditions (3:1 NH_4OH/EtOH, 48 h, rt) and purified by denaturing PAGE (12%, 7 M urea) followed by desalting on Sephadex G-25 and characterization by MALDI-MS: $[M]_{calc} = 17502.31$, $[M - 3H + 2Li]_{observed} = 17513.61$. The overall isolated yield was 10.1 A_{260} units.

Convergent Synthesis of the Branched RNA-Lariat Precursor 8. 3′,5′-RNA phosphoramidite monomers [5′-*O*-dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilyl-(*N*-protected ribonucleoside)-3′-*O*-(*β*-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite] were stored at −20 °C and dried in vacuo overnight prior to use. Nucleoside-derivatized CPG was prepared according to known methods.[66] Syntheses were performed on an Applied Biosystems 381A oligonucleotide synthesizer, using RNA 3′-phosphoramidite monomer at a concentration of 0.15 M (in acetonitrile) and a coupling time of 8 min. The Y-shaped RNA-lariat precursor was synthesized by using the convergent (nonregiospecific) methodology for branched RNA synthesis on a 1-*μ*mol scale.[33,34] Briefly, the forked or Y-RNA structure was assembled in the normal 3′ → 5′ direction on a medium-density deoxythymidine functionalized support (60 *μ*mol/g) to ensure maximal coupling between adjacent strands once the bisphosphoramidite was introduced. Although lower nucleoside loadings are typically favored for the synthesis of long oligonucleotides (>40-mers), maximizing branching efficiency by using a higher CPG loading was key to ensuring a sufficient yield of lariat precursor. To compensate for this, a 1000 Å pore-sized support was used instead of the more common 500 Å. CPG functionalized with any nucleoside could have been used at this point, since the first step in chain assembly involved

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the incorporation of the commercial phosphorylation reagent, (2-[2-(4,4′-dimethoxytrityloxy)ethylsulfonyl]ethyl-(β-cyanoethyl)-(*N*,*N*-diisopropyl) phosphoramidite (0.10 M in acetonitrile), followed by strand elongation after detritylation of the DMT-$OCH_2CH_2SO_2-$ group.[35] Ammonia deprotection results in the concomitant cleavage of the support bound nucleoside and the bond between phosphorylation reagent and the second nucleotide added, in this case a 2′-deoxythymidine, thereby releasing two contiguous 3′-phosphate termini, at both the 2′,5′ and 3′,5′ extensions from the branch. The 2′-TBDMS groups of the ribose sugars were released by treatment with neat TEA-3HF (5 μL/crude A_{260} units, 24−48 h) and precipitation from *n*-butanol as previously described.[70] The branched RNA was purified by denaturing PAGE (12%, 7 M urea) and desalted on Sephadex G-25 and characterized by MALDI-MS: $[M]_{calc}$ = 17380.91, $[M - H]^-_{observed}$ = 17382.27. The isolated yield of branched lariat precursor was 17.5 A_{260} units.

Template-Mediated Chemical Ligation of Lariats. DNA and RNA nicked branched precursors (**4** and **8**) and their corresponding linear templates (**5** and **9**) were dissolved in an equimolar ratio (100 μM each) in 0.25 M 2-(*N*-morpholino)-ethane sulfonic acid (MES), 20 mM $MgCl_2$, pH 7.6 buffer. The samples were heated to 95 °C for 10 min, cooled slowly to rt, and left at 4 °C overnight. Reactions were placed on ice for 10 min at which time 1/10 volume of cyanogen bromide (CNBr; 5 M in CH_3CN) was added, and the mixtures left on ice for an additional 5 min. The ligations were terminated by precipitation with 2% $LiClO_4$ in acetone (1 mL), placed at −20 °C for 0.5−2 h, and the pellet was recovered by centrifugation at 14 000 rpm for 10 min. The supernatant was removed and the pellet dried. Ligated lariats were analyzed and purified by denaturing PAGE (12%, 8.3 M urea) and desalted on Sephadex G-25. Prior to gel analysis and purification, the samples were heat-treated (95 °C, 5 min). The yields of ligated circles were determined by densitometric analysis (UN-SCAN-IT Software) of the ligated lariat to unreacted precursor. Compounds were characterized by MALDI-MS. Lariat DNA **4**: MALDI-MS $[M]_{calc}$ = 17484.3, $[M - H]^-_{observed}$ = 17484.4; ligation yield (using DNA splint **5**) = 45%. Lariat RNA **8**: MALDI-MS $[M]_{calc}$ = 17362.9, $[M - 2H + Na]^-_{observed}$ = 17386.4; ligation yield (using DNA splint **5**) = 46% and 58% (ligation reaction conducted twice); ligation yield (using RNA splint **9**) = 35%.

Oligonucleotide Analysis and Purification. The oligonucleotides were analyzed and purified by denaturing polyacrylamide gel electrophoresis (12%, 7 or 8.3 M urea) and desalted by size exclusion chromatography on Sephadex G-25. The sequences were characterized by negative MALDI-TOF-MS, using 6-aza-2-thiothymine as a matrix and spermine/fucose as co-matrices as described elsewhere.[71] HPLC analyses of crude oligonucleotides were conducted on a Waters DEAE-5PW (75 × 7.5 mm) anion exchange column by using a linear gradient of 10−20% solvent B over 60 min (solvent A: deionized water; solvent B: 1 M $LiClO_4$; T = 60 °C).

Thermal Denaturation (T_m) Analysis. Thermal denaturation profiles were obtained on a Varian CARY 1 UV−vis spectrophotometer equipped with a multiple cell holder and a Peltier temperature controller. T_m measurements were conducted in 10 mM Tris-HCl (pH 7.5), 10 mM NaCl buffer or 0.25 M MES (pH 7.6), 20 mM $MgCl_2$ at a concentration of 4−10 μM of oligonucleotide single strands. Samples were heated for 10 min at 95 °C, cooled slowly to room temperature and cooled for 12−24 h at 4 °C prior to analysis. Spectra were acquired at 260 nm over a temperature range of 5−95 °C with a heating ramp of 0.5 °C/min. T_m values are the maxima of the first derivative plots of the absorbance versus temperature profiles.

3′-End Labeling of DNA Oligonucleotide Substrates with Terminal Deoxynucleotidyl Transferase (TdT). Linear and branched DNA molecules were radioactively labeled with 5′-[α-^{32}P]-dideoxyadenosine monophosphate at their free 3′-hydroxyl termini with use of the enzyme terminal deoxynucleotidyl transferase (TdT).[40] Reactions were conducted in 50 μL total volumes consisting of the following: DNA oligonucleotide substrate (10 pmol), 10 μL of 5× terminal deoxynucleotidyl transferase buffer (0.5 M sodium cocadylate, pH 7.2, 10 mM $CoCl_2$, 1 mM 2-mercaptoethanol), 17−20 pmol of [α-^{32}P]-ddATP (3000 Ci/mmol, 10 mCi/mL), 5 μL of terminal deoxynucleotidyl transferase enzyme (11 U/μL), and water to 50 μL total volume. Incubation at 37 °C for 2 h proceeded followed by deactivation of the enzyme at 65 °C for 10 min. Radiolabeled samples were purified by gel electrophoresis (16%, 7 M urea), then the gel bands were crushed and soaked in sterile water and desalted on NAP-5 columns.

Lariat Debranching with HeLa Nuclear Extract.[46] HeLa nuclear containing a specific 2′-phosphodiesterase activity (debranching) was a generous donation from Dr. Andrew MacMillan (University of Alberta). Radioactively labeled or unlabeled branched and lariat substrates (1−200 pmol) were dissolved in 7 μL of buffer comprised of 20 mM HEPES, pH 7.6, 100 mM KCl, 20% glycerol and 0.5 mM DTT and also containing 10 mM EDTA as a general 3′-exonuclease inhibitor.[46] HeLa nuclear extract (3 μL) was added and the reactions incubated at 30 °C for 0.5−1 h. The samples were either phenol/chloroform extracted prior to gel loading or directly loaded onto a denaturing gel (12−24%) that had been pre-run at 500 V for 15 min. Radioactively labeled substrates were visualized by autoradiography and nonradioactively labeled substrates were visualized by staining the gel for 16−24 h in Stains-All solution prepared according to manufacturer's protocols.

Characterization of Lariats by Digestion with Bovine Spleen Phosphodiesterase (3′-Exonuclease). Bovine spleen phosphodiesterase (BSPDE) was obtained as a lyophilized powder (10 U/mg) and diluted with sterile water to an activity of 0.1 U/μL. Oligonucleotides radiolabeled at the 3′-termini (100 $fmol^{-1}$ pmol) were dissolved in 48 μL of 0.1 M sodium acetate, pH 6.5, and 2 μL of BSPDE (0.2 U) was added. The reactions were incubated at 37 °C for 0.5−1 h and dried. The samples were redissolved in 5 μL of sterile water and 5 μL of gel loading dye, run on a 12−16% denaturing gel (7 M urea) and visualized by autoradiography.

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Supporting Information Available: Drawing of favored secondary structure of the loop region of Y-DNA/RNA precursors calculated with the DNA MFOLD program; thermal denaturation and circular dichroism curves of lariat and Y-precursors complexes with complementary DNA or RNA strands; gel electrophoresis analysis of lariat RNA synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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