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A Novel Approach to the Synthesis of DNA and RNA Lariats

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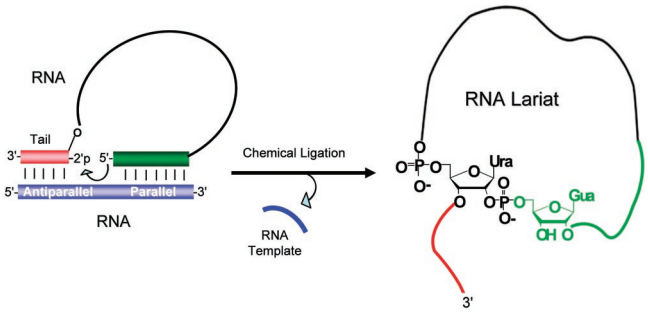

Current studies of lariat RNA structure and function are hindered by the lack of access to synthetic lariats. A novel approach to the synthesis of both DNA and RNA lariats is presented here. Noteworthy features of the methodology are the regiospecific formation of the 2′-5′-phosphodiester linkage, the unusual parallel stranded DNA/RNA hybrid (or parallel RNA/RNA duplex) that forms between an RNA template and a folded 22-nt DNA (or RNA) substrate, and the efficiency of the chemical ligation step at an adenosine branchpoint (50−80%). The DNA and RNA lariats were purified by polyacrylamide gel electrophoresis, and their structure and nucleotide composition were confirmed by MALDI-TOF mass spectrometry. Thermal denaturation as well as enzymatic and chemical hydrolysis fully supported the proposed lariat structures. Characterization of control parallel duplexes was conducted by gel shift assays and enzymatic degradation with RNase H. The successful synthesis of the lariat molecules described here will allow structural and biochemical studies aimed at better understanding the splicing and debranching mechanisms in which these unusual nucleic acids are involved.

Introduction

Lariats[1] are the byproduct of a biosynthetic pathway known as precursor messenger RNA (pre-mRNA) splicing.[2] In this process, the maturation of an mRNA strand is achieved through a series of transesterification reactions that ultimately lead to the excision of noncoding regions (introns) from the pre-mRNA in the form of these unusual RNA lariats. The branchpoint of these molecules consists of a highly conserved adenosine residue connected to "loop" and "tail" extensions through vicinal 2′-5′- and 3′-5′-phosphodiester linkages, respectively. Development of effective methods for the synthesis of lariat molecules is essential to understanding their biological role and probing the splicing and debranching mechanisms of RNA in further depth.[3−6] Recently Carriero and Damha demonstrated the inhibitory activity of synthetic branched RNA (bRNA) of the splicing pathway, providing insight into the mechanistic details of events of the spliceosomal assembly.[3] As well, synthetic bRNA has been used in potential antisense applications given that alternate splicing of pre-mRNA has been implicated in the mechanism of several human diseases.[7,8]

The synthesis of *circular* nucleic acids is particularly challenging, owing to the negative entropic change of the required

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(1) Wallace, J. C.; Edmonds, M. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 950−954.
(2) Padgett, R. A.; Konarska, M. M.; Grabowski, P. J.; Hardy, S. F.; Sharp, P. A. *Science* **1984**, *225*, 898−903.
(3) Carriero, S.; Damha, M. J. *Nucleic Acids Res.* **2003**, *31*, 6157−6167.
(4) Nam, K.; Hudson, R. H. E.; Chapman, K. B.; Ganeshan, K.; Damha, M. J.; Boeke, J. D. *J. Biol. Chem.* **1994**, *269*, 20613−20621.
(5) *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*; Cohen, J., Ed.; CRC Press: Boca Raton, FL, 1989.
(6) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543.
(7) Faustino, N. A.; Cooper, T. A. *Genes Dev.* **2003**, *17*, 419−437.
(8) Gendron, D.; Carriero, S.; Garneau, D.; Villemaire, J.; Klinck, R.; Abou, Elela, S.; Damha, M. J.; Chabot, B. *BMC Biotechnol.* **2006**, *6*, 1−12.

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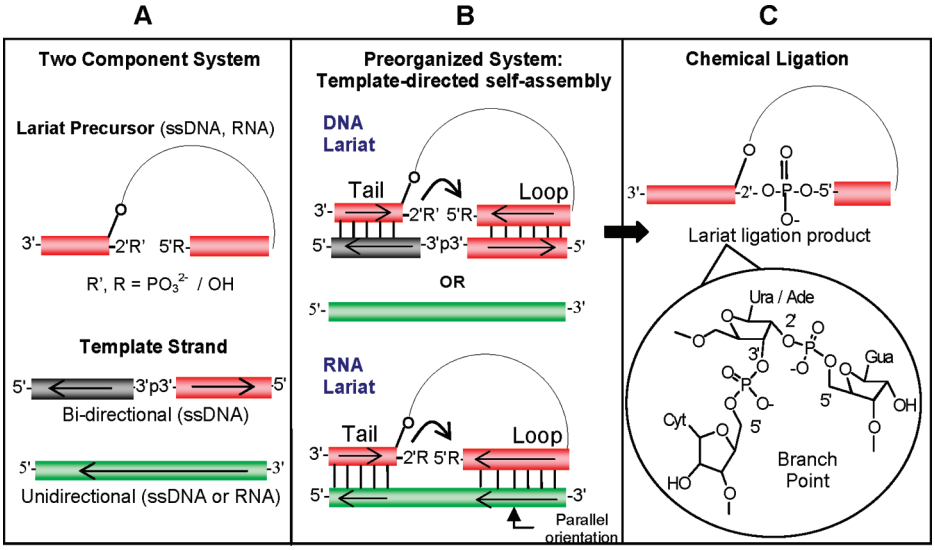
SCHEME 1. Template Directed Strategy[a]



[a] (A) The two component system involves the preorganization of a single strand oligonucleotide (ssON) precursor DNA or RNA by self-assembly with a template DNA or RNA strand. The two types of templates studied are the bidirectional DNA template which facilitates molecular recognition of the precursor ssON in a canonical fashion (antiparallel orientation) as well as a unidirectional RNA template that binds the ssON with partial parallel orientation. (B) The preorganization of the precursor ssON to the template is shown where R represents the two functional groups to be ligated in either 2′-OH (hydroxyl) and 5′-PO_3^{2-} (phosphate) or 2′-PO_3^{2-} (phosphate) and 5′-OH (hydroxyl). (C) Chemical ligation is carried out with cyanogen bromide or carbodiimide coupling (EDC) to afford the lariat product.

cyclization step. Intramolecular cyclization occurs readily when small circular oligonucleotides (2−14 nt) are prepared both in solution and on the solid phase.[9−16] For larger molecules, the entropic barrier posed by cyclization is greater and is overcome through the use of a DNA or RNA template that brings together the termini of a linear DNA or RNA oligonucleotide precursor. The termini are then joined via chemical[17,18] or enzymatic[19,20] ligation (Scheme 1).[21−24] Other template-directed methods construct cyclic oligomers through bimolecular triplex formation between the precursor and template strands.[25,26]

The synthesis of the more complex *lariat* structure usually requires chemically modified nucleotide precursors[27−29] and several purification steps and is generally limited in both length (2−5 nt) and scale (pmol−nmol). Elegant methodologies described by Silverman and Wang have demonstrated the use of deoxyribozymes, requiring the inclusion of activated functional groups or enzymatic ligations, in the synthesis of larger lariat molecules with fairly good yield.[30−32] Our group has recently reported on the chemical synthesis of medium sized DNA and RNA lariats through a template directed approach, whereby a *Y-shaped* precursor strand is preorganized and ligated through template-duplex formation.[33] In addition, an alternative method in the synthesis of lariats combined both self-assembly of the circular fragment through dumbbell-like interactions and template organization.[34] These methods were not regiospecific, and thus isomeric RNA lariat structures (differing in branchpoint sequence) were produced.[34] Nevertheless, the versatility of the template directed method for synthesis of RNA lariats is highly attractive, as sequence diversity, size, and incorporation of modified nucleotides may be possible.

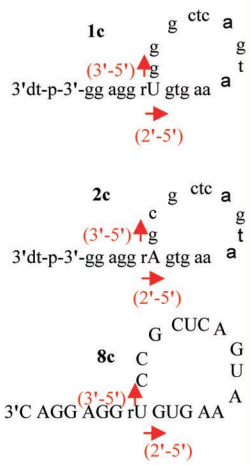
Herein we report a novel two-component template directed strategy in the synthesis of lariat nucleic acids. Briefly, the method comprises a short template strand that hybridizes to *linear* ssDNA or RNA precursors that are subsequently con-

(9) Capobianco, M. L.; Carcuro, A.; Tondelli, L.; Garbesi, A.; Bonora, G. M. *Nucleic Acids Res.* **1990**, *18*, 2661−2669.
(10) De Napoli, L.; Messere, A.; Montesarchio, D.; Piccialli, G.; Santacroce, C. *Nucleosides Nucleotides* **1993**, 12, 21−30.
(11) see review: Kool, E. T. *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 1−28.
(12) Micura, R. *Chem.—Eur. J.* **1999**, *5*, 2077−2082.
(13) Frieden, M.; Grandas, A.; Pedroso, E. *Chem. Commun.* **1999**, 1593−1594.
(14) Pedroso, E.; Escaja Frieden, M.; Grandas, A. *Solid-phase Synthesis of Circular Oligoucleotides. Methods in Molecular Biology*; Humana Press Inc: Totowa, NJ, 2005; Vol. 288, pp 101−125.
(15) Smietana, M.; Kool, E. T. *Angew. Chem., Int Ed.* **2002**, *41*, 3704−3707.
(16) Grimau, M. G.; Avino, A.; Gargallo, R.; Eritja, R. *Chem. Biodiversity* **2005**, *2*, 275−285.
(17) Rubin, E.; Rumney IV, S.; Wang, S.; Kool, E. T. *Nucleic Acids Res.* **1995**, *23*, 3547−3553.
(18) Dolinnaya, N. G.; Blumenfeld, M.; Merenkova, I. N.; Oretskaya, T. S.; Krynetskaya, N. F. et al. *Nucleic Acids Res.* **1993**, *21*, 5403−5407.
(19) Nilsson, M.; Malmgren, H.; Samiotaki, Kwiatkowski, M. M.; Chowdhary, B. P.; Landegren, U. *Science*. **1994**, *265*, 2085−2088.
(20) Li, Y.; Billen, L. P. *Biorg. Chem.* **2004**, *32*, 582−598.
(21) Ashley, G. W.; Kushlan, D. M. *Biochemistry* **1991**, *30*, 2927−2933.
(22) Chen, J.; Liu, D.; Lee, A. H. F.; Qi, J.; Chan, A. S. C.; Li, T. *Chem. Commun.* **2002**, 2686−2687.
(23) Liu, D.; Chen, J.; Lee, A. H. F.; Chow, L. M. C.; Chan, A. S. C.; Li, T. *Angew. Chem., Int. Ed.* **2003**, *42*, 797−799.
(24) Beaudry, D.; Perreault, J. P. *Nucleic Acids Res.* **1995**, *23*, 3064−3066.
(25) Prakash, G.; Kool, E. T. *J. Chem. Soc.* **1992**, *114*, 3523−3527.
(26) Selvasekaran, J.; Turnbull, K. D. *Nucleic Acids Res.* **1999**, *27*, 624−627.
(27) Sund, C.; Agback, P.; Chattopadhyaya, J. *Tetrahedron.* **1991**, *47*, 9659−9674.
(28) Sund, C.; Agback, P.; Chattopadhyaya, J. *Tetrahedron* **1993**, *49*, 649−668.
(29) Reese, C. B.; Song, Q. *Nucleic Acids Res.* **1999**, *27*, 2672−2681.
(30) Wang, Y.; Silverman, S. K. *RNA* **2006**, *12*, 313−321.
(31) Wang, Y.; Silverman, S. K. *Angew. Chem., Int. Ed.* **2005**, *44*, 5863−5866.
(32) Wang, Y.; Silverman, S. K. *J. Am. Chem. Soc.* **2003**, *125*, 6880−6881.
(33) Carriero, S.; Damha, M. *J. Org. Lett.* **2003**, *5*, 273−276.
(34) Carriero, S.; Damha, M. J. *J. Org. Chem.* **2003**, *68*, 8328−8338.

TABLE 1. Oligonucleotides Synthesized in This Study[a]

Sequences		
DNA Lariat precursor		
rU branch point	**1a**	5'-(PO_3^{2-})-gtg aaa tga ctc ggg rU gga gg-3'p-dt-3'
	1b	5'-gtg aaa tga ctc ggg rU-(2'PO_3^{2-}) gga gg-3'p-dt-3'
rA branch point	**2a**	5'-(PO_3^{2-})-gtg aaa tga ctc gcg rA gga gg-3'p-dt-3'
	2b	5'- gtg aaa tga ctc gcg rA-(2'PO_3^{2-}) gga gg-3'p-dt-3'
Template (DNA lariat synthesis)		
bidirectional (rU branch point)	**3**	5'-acc tcc a-3'-3'-cac tt-5'
unidirectional(rU branch point)	**4**	5'-ACC UCC ACA CUU-3'
bidirectional (rA branch point)	**5**	5'-acc tcc t-3'-3'-cac tt-5'
unidirectional (rA branch point)	**6**	5'-ACC UCC UCA CUU-3'
unidirectional (rA branch point)	**7**	5'-acc tcc tca ctt-3'
RNA lariat precursor		
rU branch point	**8**	5'- GUG AAA UGA CUC GCC rU GGA GGA C-3'
	8a	5'-(PO_3^{2-})- GUG AAA UGA CUC GCC rU GGA GGA C-3'
	8b	5'-GUG AAA UGA CUC GCC rU-(2'PO_3^{2-}) GGA GGA C-3'
Template (RNA lariat synthesis)		
bidirectional	**9**	5'-tcc tcc a-3'-3'-cac tt-5'
unidirectional	**10**	5'-GUC CUC CAC ACU U-3'
Strands Complementary to Templates		
bidirectional	**11a**	3'-tggaggu-5', 5'-gtgaa-3'
bidirectional	**11b**	3'-tggaggu-5'-5'-gtgaa-3'
antiparallel DNA	**12**	5'-aag tgt gga gga c-3'
parallel DNA	**13**	5'-cag gag gtg tga a-3'
antiparallel RNA	**14**	5'-AAG UGU GGA GGA C-3'
parallel RNA	**15**	5'-CAG GAG GUG UGA A -3'
unidirectional DNA	**16**	5'-gtc ctc cac act t-3'

Lariat Products



[a] Lowercase denotes deoxynucleotide residues; uppercase denotes ribonucleotide residues.

verted into the desired lariat structure through 2′−5′ phosphodiester bond formation at the branch point. Unlike our previous methods, the approach described below is regioselective and can be carried out in the μg−mg scale. Chemical ligation occurs efficiently through condensation with either cyanogen bromide in the case of DNA lariats or 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide (EDC) in the case of RNA lariats.

Results and Discussion

Synthesis of DNA Lariats. A. Design. In our approach, two portions of a single stranded oligonucleotide (ssON) are brought together through the use of an RNA or DNA template that preorganizes the ssON in the desired lasso-like configuration (Scheme 1). The terminal OH and activated phosphate moieties, now in close proximity, are ligated through the use of cyanogen bromide or a carbodiimide derivative as condensing reagents. The association of the template to the ssON's "tail" segment (Scheme 1B) is crucial in order to avoid any undesirable binding that may disrupt optimal lariat preorganization.[30−34] The DNA template chosen is "bidirectional" to ensure proper Watson−Crick base pairing in the intended (antiparallel) configuration (Scheme 1B). Here, an internal 3′−3′ phosphodiester bond within this otherwise unmodified DNA template ensures the required polarity reversal (e.g., templates **3** and **5**, Table 1). As the analogous "bidirectional" RNA template could not be synthesized from standard RNA monomers, the ssON/RNA association depicted in Scheme 1B assumes that a significant portion of the RNA strand hybridizes to the ssON in the parallel orientation. The successful synthesis of RNA lariats described below fully support the formation of such a novel chimeric [antiparallel]−[parallel] RNA/RNA duplex.

Other factors must be considered when optimizing assembly of the putative prelariat complex (Scheme 1B): (a) the sequence and length of the template and ssON lariat precursor; and (b) position and reactivity of the functional groups to be ligated. Thus, we first determined the optimal template composition and length as it directly impacts the conformation of the prelariat complex and the proximity of the phosphate and hydroxyl groups to be ligated. Past studies have determined a minimum length requirement for the association to the precursor ssON strand and the template corresponding to, at least, a full turn of a double helix.[18] The hybridization region is G/C rich, ensuring strong stabilization of the desired ssON/template complexes.

To assess the feasibility of this strategy, the synthesis of a DNA lariat was attempted first through the use of 22-nt ssON precursors, namely **1a**, **1b** (rU branchpoint) and **2a**, **2b** (rA branchpoint) (Table 1). Furthermore, to assess the stability of the complexes formed, thermal denaturation (T_m) and circular dichroism studies of the following ssON/template combinations were performed: **1a:3**, **1b:4**, **2a:5**, and **2b:6** (Supporting Information). The thermal denaturation profiles for ssON (rU) **1a** or ssON (rA) **2a** upon hybridization to complementary DNA templates (**3** and **5**) showed poor cooperativity. By contrast, association to the RNA templates (**4** and **6**) provided stronger binding, and the resulting T_m curves were biphasic as may be anticipated for the proposed complexes **1a:4** and **2a:6** (Scheme 1B). Base pairing of the internal ribonucleotide unit in the ssONs was confirmed by comparing the melting curves (T_m) of complexes **2b:5** (rA/dT, T_m = 46, 80 °C) and **2b:3** (rA/dA mismatch, T_m = 42, 73 °C). Thermal denaturation profiles for the ssON **1a** and **2a** showed the same trends (Table 2). This suggests that RNA templates would provide more favorable

TABLE 2. Thermal Denaturation Experiments[a]

duplex	T_m[b] (°C)	% H[c]	duplex orientation[d]
DNA Lariat (rU)			
1a:3	n/a	10	A
1a:4	30, 65	11	P
1b:3	n/a	7	A
1b:4	48, 70	12	P
1c:4	31	16	P
DNA Lariat (rA)			
2a:5	41	7	A
2a:6	36, 72	18	P
2b:5	46, 80	5	A
2b:6	39, 66	7	P
2b:3	45, 76	12	A
2b:7	n/a	12	P
2c:6	28	10	P
RNA Lariat (rU)			
8a:9	40	20	A
8a:10	42, 73	9	P
8c:10	39	10	P
Controls			
11a:9	n/a	4	A
11a:10	n/a	7	A
11b:.9	41	13	A
11b:10	42	14	P
12:10	43	12	A
13:10	n/a	10	P
14:10	62	14	A
15:10	37	9	P

[a] Thermal denaturation experiments were conducted at a concentration of 2−4 μM in 10 mM Tris-HCl, pH 7.5, 10 mM NaCl buffer. [b]Melting temperatures (T_m) were calculated from the maximum of the first derivative of the melting curve. [c]% hyperchromicity was calculated as $[(A_f - A_o)/A_f]100$, where A_f represents final absorbance and A_o represents initial absorbance. All T_m experiments were repeated twice for reproducibility. [d]Duplex orientation at the loop region of ssON/template complex shown in Figure 1b; A = antiparallel; P = parallel.

TABLE 3. Chemical Ligation of 2b[a]

template	reaction	yield of **2c**, %
5 (bidirectional DNA)	**2b:5**	73
3 (bidirectional DNA; mismatch)	**2b:3**	64
6 (unidirectional RNA)	**2b:6**	81
7 (unidirectional DNA)	**2b:7**	0

[a] Chemical ligation reactions of **2b** as a function of template type. Yields of all four ligations were determined by densitometric measurements from the UV shadowing PAGE analyses.

TABLE 4. MALDI-TOF-MS Analysis

sequence	MW g/mol	experimental MW g/mol
precursor **1a**	6977.4	6978.1
precursor **1b**	6977.4	6977.5
rU DNA lariat **1c**	6959.4	6957.0
precursor **2a**	6960.4	6960.9
precursor **2b**	6960.4	6961.1
rA DNA lariat **2c**	6942.4	6940.6

hybridization conditions for ligation. Indeed, previous work has shown that A-like duplexes (like the ssON/RNA hybrids of this study) afford a better environment for condensing OH and phosphate groups in a template-directed ligation strategy.[34]

Previous studies have shown that ligation of 3′-phosphates to 5′-hydroxyl groups with CNBr occurred with greater success compared to ligation of 3′-hydroxyls to 5′-phosphate groups; this may be due to the increased flexibility at the primary 5′-position which results in exposure to hydrolysis and subsequent

TABLE 5. MALDI-MS TOF MS Analysis

sequence	theoretical MW g/mol	experimental MW g/mol
precursor **8a**	7489.5	7490.5
precursor **8b**	7489.5	7491.5
RNA lariat **8c**	7471.5	7472.9
8c + **8a**	7471.5, 7489.5	7479.3, 7492.7

deactivation of a 5′-phosphate group.[36] Therefore, two reactions were assessed to investigate functional group reactivity: potential internucleotide bond formation between a 5′-phosphate and a 2′-hydroxyl group (**1a:3**) and vice versa (**1b:3**). To ensure proper annealing, the lariat precursor (ssON **1b**) and bidirectional DNA template (**3**) strands were mixed in a 1:3 ratio in a buffer containing 250 mM MES-NEt_3 (pH 7.6). The concentration of the ssON lariat precursor was kept at 1×10^{-6} M.[20] Prior to ligation, the solutions were heated at 95 °C for 10 min and then allowed to cool to room temperature (16 h). Ligation involved activation of the phosphate group with a 5 M CNBr/MeCN solution for a duration of 5 min at 4 °C followed by quenching with cold ethanol. In agreement with previous reports, phosphodiester bond formation resulting from the 5′-hydroxyl/2′-phosphate combination, i.e., **1b:3**, was more successful (Figure 1). The assumed DNA lariat product observed, **1c**, was purified by polyacrylamide gel electrophoresis (PAGE; 24% acrylamide; 8.3 M urea) (Figure 1). The electrophoretic mobility of **1c** was less than that of its nicked linear precursor (**1b**). Upon gel excision, the product band was incubated at ambient temperature in aqueous media overnight, followed by desalting by size exclusion chromatography (Sephadex G-25). The final yield of **1c** was 15% determined by UV quantification of the isolated product.

To ascertain the optimal conditions, the synthesis of DNA lariat **1c** was carried out at various concentrations of the precursor strand **1b** (330 to 4.1 μM range), while maintaining the ratio of lariat precursor strand to template strand at 1:3. The results indicated that a narrow range of 33 to 8.2 μM was most favorable for cyclization, with 22 μM/**1b** being the optimal concentration (Figure 2). At this concentration, a ligation efficiency of 22% was observed. The potential for triplex formation as a result of the binding of two strands of the template to **1b** is possible since an excess of template is used. However, denaturation analysis monitoring at 284 nm (**1b:3**) gave no evidence of triple helix formation under these conditions (data not shown).[37]

Reaction of a mixture of **2b** and DNA template **5** under the optimal conditions (1:3 ratio; [**2b**] = 22 μM) afforded the DNA lariat **2c** (rA branchpoint) in yields greater than 70% as estimated by PAGE (see Supporting Information). The improved yields of **2c** versus **1c** were rationalized through a comparative analysis of thermal denaturation experiments conducted on the **1b:3** and **2b:5** pairs. A greater cooperative effect was observed for **2b:5** compared to **1b:3**. Furthermore, the thermal denaturation curve of **2b:5** showed a weak biphasic transition at 46 and 80 °C, which may be ascribed to the two hybridized regions of the duplex in the preorganized form (Supporting Information). No discernible sigmoidal transition was observed for **1b:3**. Thus,

(35) Mitra, D.; Damha, M. J. Manuscript in preparation.
(36) Dolinnaya, N. G.; Sokolova, N. I.; Ashirbekova, T. D.; Shabarova, Z. A. *Nucleic Acids Res.* **1991**, *19*, 3067−3072.
(37) Bandaru, R.; Hashimoto, H.; Switzer, C. *J. Org. Chem.* **1995**, *60*, 786−787.

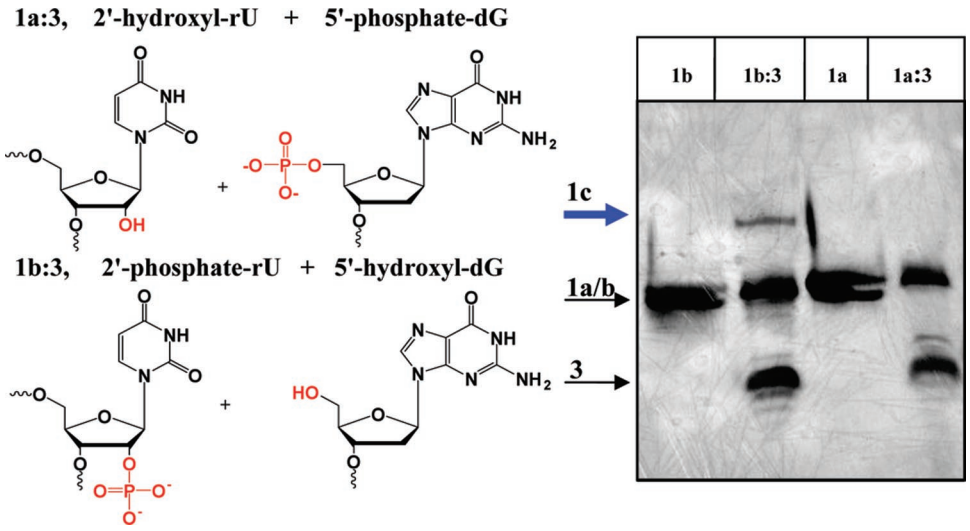


FIGURE 1. UV-shadowing, PAGE (24%, 8.3 M urea) (a) Branch point rU DNA Lariat Reaction: Lane 1, control precursor strand **1b**; Lane 2, represents the chemical ligation reaction of **1b:3** with CNBr, **1c**, DNA Lariat product formation is indicated by the arrow of slowest electrophoretic mobility (in blue); Lane 3, control precursor strand **1a**; Lane 4, represents the chemical ligation reaction of **1a:3** with CNBr.

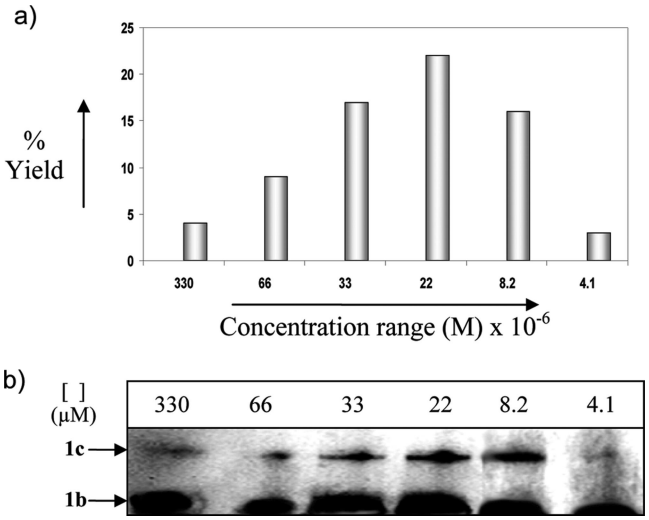


FIGURE 2. (a) Ligation efficiency of CNBr mediated chemical ligation of **1b:3** in 250 mM MES-NEt_3, 20 mM $MgCl_2$, pH 7.6. Concentration of **1b** is calculated as [**1b**]/nt residues and ranged from 330 to 4.1 μM. (b) 20% PAGE, 8.3 M urea Stains All depiction of chemical ligation reaction showing lariat ligation product with slower migration than that of the nicked precursor, **1b**. All % yields were calculated in relation to linear **1b** nicked starting material, quantified by densitometric measurements.

thermal melting studies of these systems appear to be good indicators of ligation performance.[38]

In efforts to better understand the effect of the template on ligation efficiency, **2b** was allowed to react with four different template strands (**3**, **5**, **6**, and **7**) under the same conditions. All reactions were analyzed by 20% PAGE and purified by standard desalting procedures described above. The ligation results are depicted in Table 3, and a summary of thermal melting transitions of substrate/template combinations is given in Table 2.

Stronger thermal melting for **2b:5** relative to the mismatched hybrid **2b:3** was observed, as expected, which may account for the 9% decrease in ligation yield in the latter. This result also suggests that branch rA/dT Watson−Crick pairing is necessary for obtaining the best yield of lariat product. Indeed, strength of binding is a definite factor in yield enhancement. For instance, reactions with the RNA templates (**2b:6**) gave the most stable hybrids (Table 2) and best yield (81%; Table 3). Finally, no T_m or ligation was observed for the **2b:7** pair, highlighting the importance of the bidirectional configuration of the DNA template (e.g., **3**). Thus, ligations are fairly influenced by both sequence type (DNA vs RNA) and orientation of the template.

Examinations of the global conformations of all preorganized ssON/template hybrids by circular dichroism (CD) analysis showed universally A-like conformations, even in cases where the B-form was expected, e.g., complexes **1b:3** and **2b:5**. Perhaps the unique bipolar configuration of the template, in these cases, facilitates the formation of the more stable A-like duplex conformation.

B. Synthesis of RNA Lariat Containing rU Branch Point, 8c. In a similar manner, the RNA lariat **8c** was attempted through chemical ligation of complexes **8b:9** and **8b:10**. However, in this case, CNBr afforded a poor yield of products, in line with previous reports attempting the ligation of RNA strands with this condensing reagent.[36] As a result, the water soluble 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) reagent was used instead.[39] Optimal conditions for substrate concentration of 22 μM dilution, previously established in the DNA lariat synthesis, were employed. Thus, substrates **8a** and **8b** were hybridized to template DNA **9** and RNA **10** in a 1:2 ratio, at a concentration of 22 μM, in the presence of 0.2 M EDC. Reactions were allowed to proceed for 1.5, 3.0, 4.5, and 6.0 days. Interestingly, RNA lariat formation was observed for all four duplex combinations (Figure 3), with the most favorable coupling conditions found for the **8b** (2′-phosphate/5′OH)**:10**

(38) Attempted synthesis of **1b:4** with the RNA template, showing biphasic thermal denaturation curve in the nicked form, resulted in a 30% yield of **1c**. The increase in product yield may be indicative of a stronger cooperative trend upon association to the RNA template. In general, however, the poor yields of **1c** observed vs **2c** may be the result of the inherent chemical ligation capability or efficiency of the CNBr reagent in the preferential formation rA internucleotide phosphodiester bonds vs that of rU.

(39) Dolinnaya, N. G.; Tsytovich, A. V.; Sergeev, V. N.; Orestskaya, T. S.; Shabarova, Z. A. *Nucleic Acids Res.* **1991**, *19*, 3073−3080.

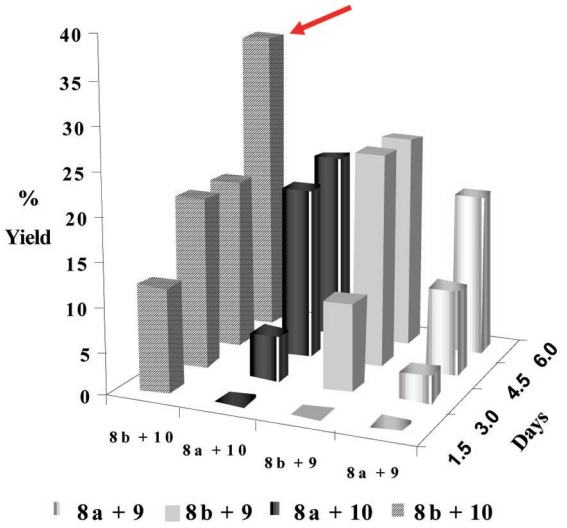


FIGURE 3. Ligation efficiency of **8a**,**b** with **9** and **10** in the presence of 0.2 M EDC (50 mM MES, 20 mM $MgCl_2$ pH 6.0). Reactions were analyzed by 20% PAGE, 8.3 M urea. The amounts of RNA lariat product **8c** were quantified by densitometry.

pair which assumes the formation of a parallel duplex (RNA:RNA) region (bottom of Scheme 1B; yield 36%, 6 days). The RNA lariat product had a faster mobility (PAGE and HPLC column) than its precursor strands **8a** or **8b** (Figure 4 and Supporting Information), consistent with several other examples of cyclic/linear oligoribonucleotide pairs.[13,20,25]

Since good yields of RNA lariat were obtained with the more readily available 5′-phosphate precursor (**8a**), further optimization experiments with this sequence were carried out. In a control experiment, **8a** was mixed with the EDC coupling reagent alone, and as expected, no lariat product was formed. In another set of experiments, **8a** was hybridized to **10** and subjected to increasing concentrations of EDC (0.5 to 2 M) (Figure 4a,b). At EDC concentrations above 0.7 M, bands of much slower electrophoretic mobility than the linear precursor **8a** were observed, suggesting the formation of side products.[40] A concentration of 0.5 M EDC, 22 μM **8a**, and 6 day coupling were found to be optimal, producing 51% (5.3 O.D., 172 μg) of the desired lariat structure (Figure 4b,c). These yields are similar to those previously reported;[34] however, the current method is regiospecific producing a single RNA lariat corresponding to the native branchpoint structure.

C. Characterization of DNA and RNA Lariats. In accordance with previous work, and the present study, gel migration patterns exhibited by cyclic DNA and RNA oligonucleotides (>25 nt) may be faster or slower relative to that of the linear counterparts.[13,17,20,21,24,26] Migratory patterns of cyclic oligonucleotides relative to their linear counterparts also fluctuate depending on their base sequence and conformation.[13,23,25] Therefore, thermal denaturation experiments and chemical and enzymatic assays were carried out in order to further characterize the lariat structures.

D. Characterization of DNA Lariats. T_m profiles of the ligated **1c** and **2c** versus nicked precursor strands **1b** and **2b** upon association to the RNA templates **4** and **6** showed enhanced cooperative *monophasic* transitions for the lariat species (Supporting Information). For the lariat complexes **1c:4** and **2c:6**, the T_m (%H) values are 31 °C (16%) and 28 °C (12%), respectively. By comparing these T_m (%H) values to those of the control duplex **11b:10** [T_m 42 °C (14%)], one concludes that the branchpoint junction in **1c** and **2c** destabilizes the duplex by approximately 10 °C. This is fully consistent with previous work that demonstrated a similar reduction in stability of branched RNA:RNA duplexes in comparison to the corresponding linear counterparts.[41]

Common characterization techniques used to confirm lariat formation include both debranching and base hydrolysis assays.[42−48] Debranching enzyme (Dbr1) substrate activity is based on hydrolysis of the 2′−5′ phosphodiester bond moiety in branched RNA (Scheme 1C).[42−45] Enzymatic digestion occurs only if the 2′−5′ linkage is cis to the vicinal 3′−5′ linkage, resulting in the formation of a linear RNA species. In addition, vicinal 2′−5′ and the 3′−5′ phosphodiester linkages at the branch point are rather unstable, resulting in cleavage under strong alkaline conditions.[46−48] Thus, as anticipated, the rU DNA lariat, **1c**, upon exposure to Dbr1, was converted to a linear product with migration patterns corresponding to the linear **1b** control, as evidenced by PAGE analysis (Figure 5a). Base-mediated hydrolysis of **1c** resulted in partial cleavage to generate a strand with the same mobility as that of **1b**. Interestingly, **2c** (rA branchpoint) was not found to be a substrate for Dbr1; however, it was cleaved to the linear form under basic conditions (Figure 5b). Finally, MALDI-TOF mass spectrometry of **1c** and **2c** and their linear precursors yielded the expected masses (see Results and Discussion).

E. Characterization of RNA Lariats. E.1. Radiolabeling Assays. The circular nature of lariat RNA **8c** was verified by radioactive ^{32}P-labeling experiments. Radiolabeling at the 5′-end of **8c** would not be feasible since its 5′-terminal position is tied as part of the 2′−5′ linkage. Thus, we expected **8c** to be resistant to reaction with γ-[^{32}P]-ATP and T4 polynucleotide kinase, a method commonly used to radiolabel the 5′-end of oligonucleotides. On the other hand, lariats carry a free 3′-termini which therefore should be recognized and labeled by ^{32}pCp and T4 RNA ligase.[49] Indeed, Figure 6a shows that lariat **8c** is not a substrate of T4 polynucleotide kinase, and only exclusive 3′-labeling with T4 RNA ligase is observed.

E.2. Nuclease Assays and MALDI MS. Further verification of the existence of the lariat species **8c** was carried out through digestion with Bovine spleen phosphodiesterase (BSPDE). This nuclease starts degrading an oligonucleotide in a stepwise manner from the 5′-terminus and liberates nucleoside 3′-phosphates as it reaches the 3′-end of the molecule. Thus, as shown in Figure 6b, the linear precursor **8** is clearly susceptible to cleavage upon exposure to BSPDE, while the lariat RNA (**8c**) is completely resistant.

Though debranching and base hydrolysis experiments did not

(40) Shabarova, Z. A.; Dolinnaya, N. G.; Drustsa, V. L.; Melnikova, N. P.; Purmal, A. A. *Nucleic Acids Res.* **1981**, *9*, 5747−5761.
(41) Ganeshan, K. Ph.D. Thesis, University of Toronto, Toronto, Ontario, Canada, 1995.
(42) Ruskin, B.; Green, M. R. *Science* **1985**, *229*, 135−140.
(43) Arenas, J.; Hurwitz, J. *J. Biol. Chem.* **1987**, *262*, 4274−4279.
(44) Nam, K.; Hudson, R. H. E.; Chapman, K. B.; Ganeshan, K.; Damha, M. J.; Boeke, J. D. *J. Biol. Chem.* **1994**, *269*, 20613−20621.
(45) Braich, R. S. Ph.D. Thesis, McGill University, Montreal, Quebec, Canada, 1999.
(46) Usher, D.A; McHale, A.H. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73* (4), 1149−1153.
(47) Rohatgi, R.; Bartel, D. P.; Szostak, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 3340−3344.
(48) Chu, Vi, T.; Adamidi, C.; Liu, Q.; Perlman, P. S.; Pyle, A. M. *EMBO* **2001**, *20*, 6866−6876.
(49) Coombes, C. E.; Boeke, J. D. *RNA* **2005**, *11*, 323−331.

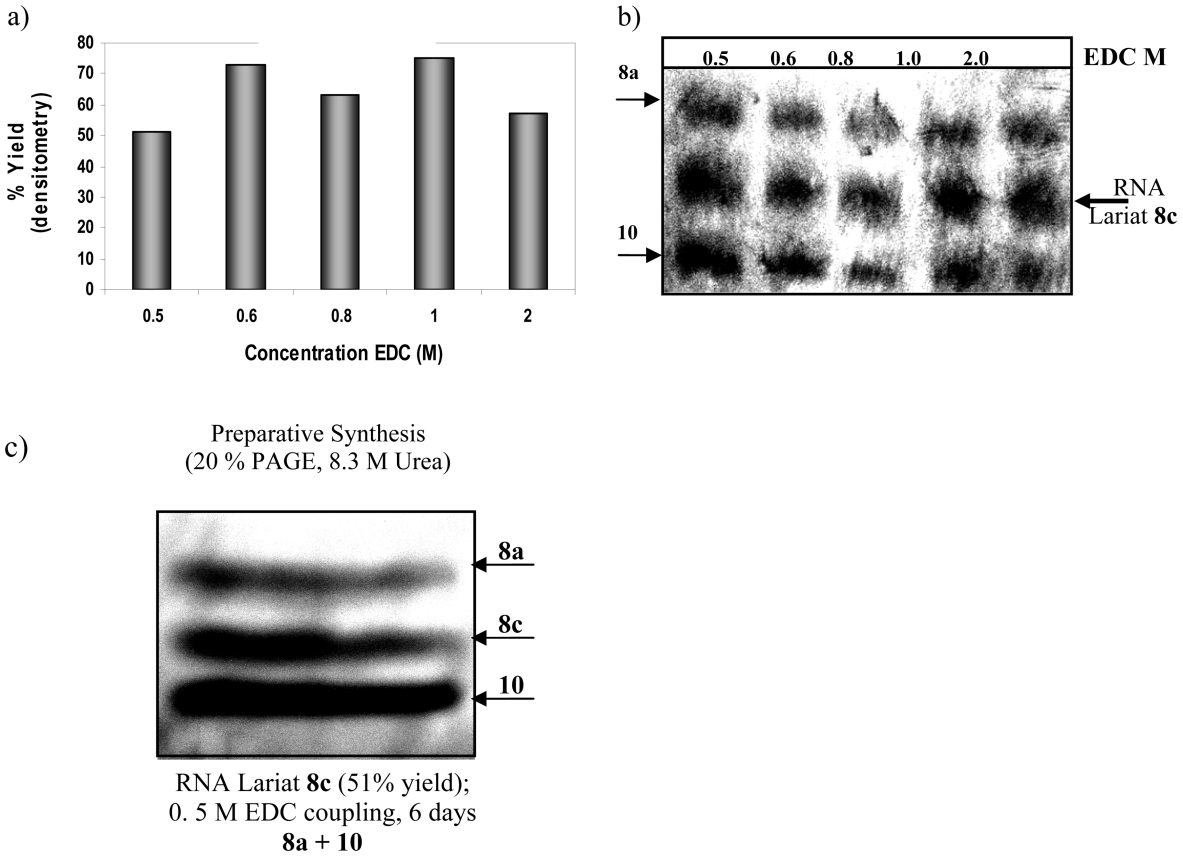


FIGURE 4. (a) Ligation efficiency of **8a** as a function of EDC concentration (from 0.5−2 M) in the presence of RNA template **10**. Reactions were conducted with a 22 μM of **8a** in an MES buffer (50 mM MES, 20 mM $MgCl_2$ pH 6.0). (b) Reactions were analyzed by 20% PAGE, 8.3 M. Chemical ligation products were quantified by densitometry. (c) Preparative 16% PAGE of **8a** + **10**, 7 day coupling at 0.5 M EDC; yield of lariat **8c** was 51% (densitometric measurements).

prove to be effective characterization methods, since no site-specific cleavage for the lariat molecule (**8c**) was observed in this case, extensive MALDI-TOF MS analysis was conducted to further confirm its structure. The MS spectra of a mixture of **8a** and **8c** showed two major peaks corresponding to their theoretical mass of 7489.5 and 7471.5 g/mol, respectively (see Results and Discussion). Their mass difference, 18 g/mol, due to water released in the condensation reaction, and all aforementioned characterization confirm the formation of the RNA lariat molecule.

E.3. Thermal Denaturation Studies. E.3.1. Study of parallel stranded duplexes. Parallel stranded duplexes have been well documented over the past several years.[50,51−61] Early work demonstrated parallel duplex formation in a series of hairpin, linear and modified oligonucleotide sequences.[50] In order to ascertain parallel stranded duplex formation resulting from the association of the lariat precursor and template **10** (Scheme 1B), thermal denaturation experiments, CD, gel-shift assay, and RNase H digestion assays were conducted on four control duplexes of the same base sequence, namely **12:10** (apDNA/RNA), **13:10** (pDNA/RNA), **14:10** (apRNA/RNA) and **15:10** (pRNA:RNA) [see Supporting Information]. Examination of the T_m profiles and CD spectra for these duplexes provided interesting results. Generally, all duplexes displayed strongly cooperative monophasic transitions (refer to Table 2). The exception was the pDNA+RNA pair (**13**+**10**), which exhibited a broad thermal melting profile and no observable melting temperature. The pRNA:RNA duplex showed sigmoidal melting with a T_m value of 37 °C, which is half the T_m value observed for the apRNA:RNA duplex control, and consistent with previous findings.[50] The CD spectra of the DNA:RNA hybrids (**12**:**10** and **13**:**10**) and pRNA:RNA (**15**:**10**) were characteristic of an A-like helix conformation. As expected, the apRNA:RNA control duplex, **14:10**, exhibited a standard A-form conformation with a 10 nm blue shift relative to **12:10**, **13:10** and **14:10**. Notably, the different T_m curves and global conformations of

(50) van de Sande, J. H.; Ramsing, N. B.; Germann, M. W.; Elhorst, W.; Kalisch, B. W.; Kitzing, E. V.; Pon, R. T.; Clegg, R. C.; Jovin, T. M. *Science*. **1988**, *241*, 551−557.
(51) Rippe, K.; Jovin, T. M. *Methods Enzymol.* **1992**, *211*, 199−220.
(52) Ramsing, N. B.; Rippe, K.; Jovin, T. M. *Biochemistry* **1989**, *28*, 9528−9535.
(53) Germann, M. W.; Vogel, H. J.; Pon. R. T.; van de Sande, J. H. *Biochemistry* **1989**, *28*, 6220−6228.
(54) Rentzeperis, D.; Rippe, K.; Jovin, T. M.; Marky, L. A. *J. Am. Chem. Soc.* **1992**, *114*, 5926−5928.
(55) Germann, M. W.; Kalisch, B. W.; Pon, R. T.; van de Sande, J. H. *Biochemistry* **1990**, *29*, 9426−9432.
(56) Lavelle, L.; Fresco, J. R. *Nucleic Acids Res.* **1995**, *23*, 2692−2705.
(57) Scaria, P. V.; Shafer, R. H. *Biochemistry* **1996**, *35*, 10985−10994.
(58) Bhaumik, S. R.; Kandala, V. R.; Govil, G.; Liu, K.; Miles, H. T. *Nucleic Acids Res.* **1995**, *23*, 4116−4121.
(59) Liu, K.; Miles, T.; Frazier, J.; Sasisekharan, V. *Biochemistry* **1993**, *32*, 11802−11809.
(60) Germann, M. W.; Aramini, J. M.; Kalisch, B. W.; van de Sande, J. H. *NNN* **2001**, *20*, 493−499.
(61) Cubero, E.; Avino, A.; de la Torre, B. G.; Frieden, M.; Eritja, R.; Luque, F. J.; Gonzalez, C.; Orozco, M. *J. Am. Chem. Soc*. **2002**, *124*, 3133−3142.

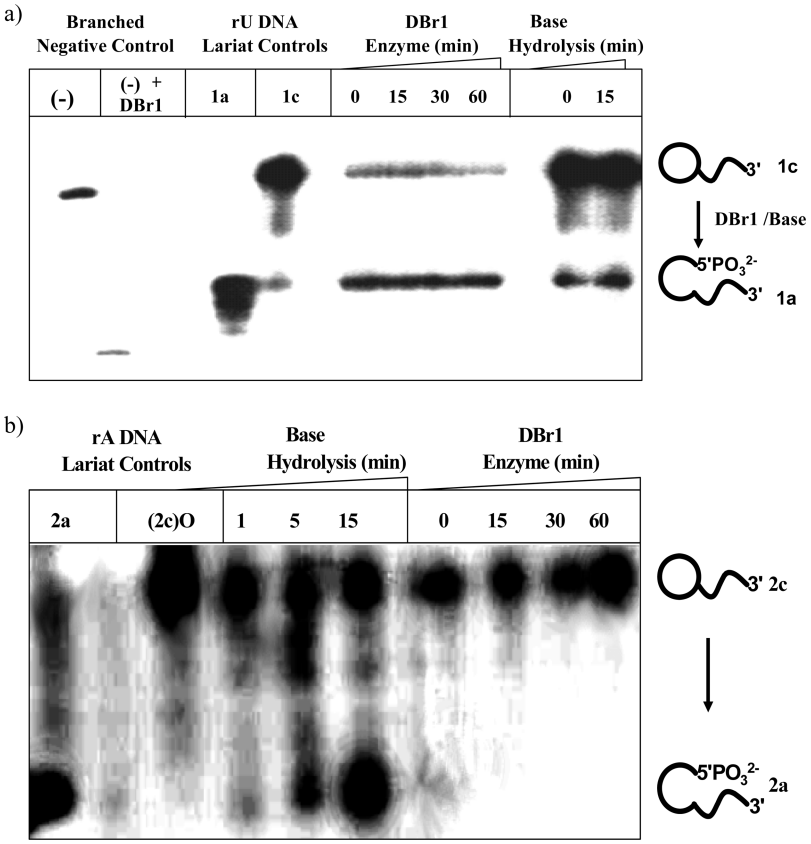


FIGURE 5. Basic and enzymatic cleavage of (a) DNA lariat **1c** and (b) **2c** into linear oligonucleotides. Enzymatic digestion: yeast debranching enzyme, Dbr1 at room temperature (0−60 min). Base hydrolysis: 100 mM Na_2CO_3, 90 °C (0−15 min).

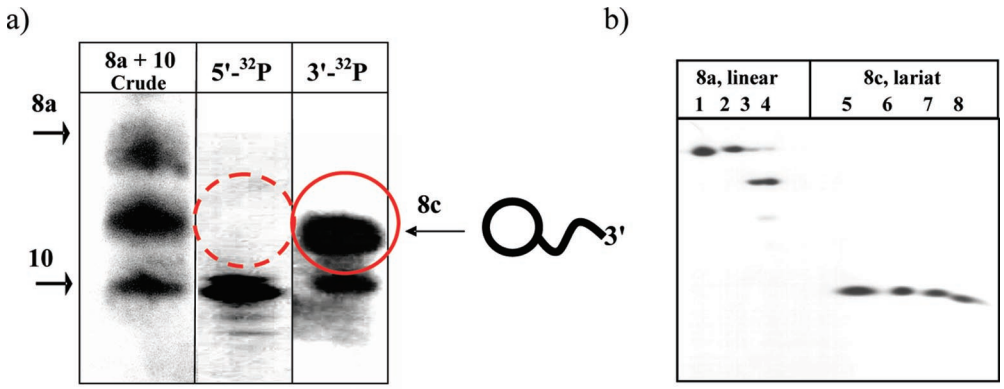


FIGURE 6. (a) Radiolabeling experiments: Lane 1, 20% PAGE analysis of a reaction of **8a** + **10** in the presence of EDC condensing reagent. The lariat product **8c** is the major band moving between **8a** and **10**. Lane 2, 5′-^{32}P radiolabeling of isolated RNA lariat species showing residual template **10** of faster electrophoretic mobility. No labeled lariat product (**8c**) was observed. Lane 3, reaction of mixture shown in lane 1 with labeled 3′-pCp in the presence of T4 RNA ligase afforded the 3′-labeled RNA lariat species **8c** and residual template **10**. (b) 12% PAGE of enzymatic digest of both **8a** and lariat RNA **8c** with bovine spleen phosphodiesterase (BSPDE). Lanes 1 and 5, negative controls (in water). Lanes 2 and 6, incubation with buffer (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).

the CD spectra of apRNA:RNA vs pRNA:RNA, provides strong evidence of their distinct antiparallel and parallel associations. Further support for these structures were obtained from native gel electrophoresis and RNase H assays described in the Supporting Information.

E.3.2. T_m Denaturation Studies in Support of RNA Lariat Structure 8c. The thermal melting of lariat RNA precursor **8a** (5′-phosphate/2′-OH) with templates **9** (DNA) and **10** (RNA) paralleled the behavior observed for prelariat DNA ssONs described above. Melting of **8a** from the bidirectional DNA template (**9**) afforded a strong monophasic profile (T_m 40 °C), whereas melting of **8a:10** exhibited biphasic behavior (T_m 42 and 73 °C; Supporting Information). As expected, the CD spectra of **8a:9** (RNA/DNA) and **8a:10** (RNA/RNA) were characteristic of A-like and pure A-form duplexes, respectively.

Comparison of the melting behavior of prelariat **8a:10** duplex with the corresponding ligated **8c:10** hybrid provides evidence for the lariat structure. Thus, ligation of **8a:10** into the lariat complex (**8c:10**) converted its biphasic transition into a clear-cut monophasic transition of similar strength (T_m 39 °C) and profile to those of the pRNA/RNA control **15:10** (T_m 36 °C) (Supporting Information). Similarly, the pRNA:RNA duplex

control (**15:10**) displayed a CD signature that is nearly superimposable with that of the prelariat **8a:10** and lariat **8c:10** duplexes (Supporting Information).

Conclusions

DNA lariats possessing a rU and rA branchpoint as well as a rU RNA lariat were synthesized via the template directed strategy in 30−80% yield. The methodology employed is unique in that ligation at the branch point is regioselective, owing to the presence of a single site for ligation to occur. In addition, the versatility of this strategy is demonstrated by successful chemical ligation irrespective of sequence composition. While past reports show significant limitations to CNBr and EDC induced condensations of RNA, the present findings suggest the importance of conformation on reactivity. As a result, the nature of the template directly influences the ligation efficiency and the use of RNA templates dramatically changed the outcome of the ligation reactions. The distinctive preorganization of the nicked precursors shows novel partial parallel duplex assembly, as characterized by T_m, CD, native gel, and RNase H assays. EDC condensation reactions also proved to be more effective for RNA based ligations. As predicted, in both lariat DNA and RNA analogues, the nature of the functional groups showed greater reactivity for 2′-phosphate to 5′-hydroxyl.

The approach presented is a viable method for the synthesis of biologically relevant molecules. This is evidenced by the synthesis of DNA lariats possessing the naturally occurring riboadenosine branch point linked to a 2′-guanosine residue. The ability to easily integrate modifications or rN branch points (n = rA, rG, and rC) into the lariat structure may find several applications, including diagnostics and the elucidation of biological mechanisms (splicing and debranching).

Experimental Section

Synthesis of DNA Lariats 1c and 2c. A. Synthesis of Precursors: 1a and **2a**, possessing a 5′-terminal phosphate, were prepared according to general methods of solid-phase automated synthesis occurring in a 3′−5′ direction. Incorporation of the ribonucleotide inserts (rU and rA) was performed at a standard 0.15 M concentration for 600 s. Prior to phosphorylation at the 2′ position of rU and rA in the synthesis of **1b** and **2b**, the oligonucleotides were "capped" or protected at the 5′-terminus with acetic anhydride. The support was then rinsed with acetonitrile (ACN) and dried under argon. To prevent the intramolecular cleavage that can occur when a hydroxyl group is liberated in the presence of a vicinal phosphotriesters, they were first converted to the more stable phosphodiesters by exposure of the solid-support bound oligonucleotides to a mixture of triethylamine/acetonitrile (4:6 v/v, 10 mL via syringe over 90 min). The support was then washed with ACN (30 mL) and THF (30 mL). Deprotection of the 2′-TBDMS groups occurred when the support was exposed to a fresh solution of 1 M tetrabutylammonium fluoride (TBAF) in anhydrous THF for no longer then 10 min (longer exposure results in oligonucleotide cleavage from the support).[45] The support was washed a second time with THF (50 mL) and ACN (50 mL) to remove any residual TBAF and dried for 20 min with argon. Finally, phosphorylation at the free 2′-position of the ribo-branch point was conducted upon exposure to the 5′-phosphate ON reagent {(2-[2-(4,4′-dimethoxytrityloxy)ethylsulfonyl]-ethyl-(β-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite}. Coupling of the phosphate specialty amidites was achieved using a concentrated solution of ACN (0.3 M) with extended coupling times of 30 min. Coupling efficiency was directly monitored by detritylation. Procedures for isolation and purification of the oligomers were conducted via analytical 24% PAGE (8.3 M Urea). Samples were heated for 5 min prior to loading. PAGE purifications were run at low voltage, 200 V, to ensure separation of the 2′-phosphorylated sequences (**1b** and **2b**) from the non-phosphorylated controls, as they exhibit similar electrophoretic mobilities.

B. Chemical Ligation via Template-Directed Strategy, CNBr Ligation: The suitable nicked precursor strands and their complementary templates were combined in 1:3 ratios. The reactions were then evaporated to dryness and redissolved with various dilutions of buffer solution (250 mM MES-NEt_3, 20 mM $MgCl_2$, pH 7.6) at the concentrations indicated in the Results and Discussion section. Under optimal conditions, strand **1b** or **2b** (0.1 OD units) is mixed with strand **4** or **6**, respectively (0.3 ODs), and buffer to give a total reaction volume of 21 μL. The homogeneous mixture is hybridized under the standard annealing protocols where solutions are heated to 95 °C for 5 min to ensure complete denaturation, followed by slow cooling to room temperature for 3−4 h. Samples are then left for 2−24 h at 4 °C. The assembled preorganized system is then put on ice for 10 min. Ligation occurs by the addition of a one-tenth volume (5 μL) of a 5 M CNBr in anhydrous acetonitrile for 5 min. A one-tenth volume of CNBr was added again, as reports have shown that repeated aliquots enhance ligation efficiency by approximately 30%. This is due to the spontaneous hydrolysis of the reagent in buffer.[39] The reactions are then halted via ethanol precipitation using 3 times the volume of anhydrous EtOH and cooling to −78 °C for 2 h or 12 h at 4 °C. The samples are isolated as a pellet by centrifugation for 10 min at 14 000 rpm. Products were then excised, extracted in water for 12 h at room temperature and desalted by size exclusion chromatography on Sephadex G-25. Characterization by MALDI TOF-MS was carried out on all lariat starting material and products. Molecular weights (MW g/mol) can be found in Table 4. Please see Supporting Information for PAGE analysis of the chemical ligations based on template variation and CD characterization of the Y-branched products. Yield were obtained by densitometry (UN-SCAN-IT Software) through UV-shadowing of PAGE bands, calculating ligation efficiency based on the relative amount of residual precursor strand to product formation.

Synthesis of RNA Lariat 8c. A. Synthesis of Precursors: Oligonucleotide RNA precursor **8a** was prepared using the standard nucleic acid solid-phase protocols, and introduction of the 5′-phosphate was achieved as described above. **8b** was prepared with a novel modified nucleotide insert, 5′-*O*-(levulinyl)-2′-*O*-(4-monomethoxytrityl)uridine-3′-*O*-(*N*,*N*-diisopropyl)-β-cyanoethylphosphoramidite. This monomer was graciously donated by Jeremy Lackey of the Damha group.[62] Incorporation of the 5′-*O*-levulinyl 2′-MMT rU amidite required a coupling time of 30 min at a 0.1 M concentration in anhydrous ACN. Decyanoethylation as described above prior to removal of the internal 2′-*O*-MMT group was necessary to prevent internucleotide cleavage. Following 2′-MMT deprotection, the 2′-OH group released was phosphorylated as described previously for the DNA lariat precursors **1b** and **2b**. The sequence is then capped to prevent further reaction at the 2′-phosphodiester position. The 5′-*O*-levulinyl (Lv) group at the branchpoint rU was cleaved by flushing the support with 0.5 M $NH_2NH_2 \cdot H_2O$ in (3:2 v/v pyr/HOAc) for 30 min at room temperature.[63−65] Chain extension of **8b** was conducted following removal of the 5′-Lv group. The support is then washed with ACN and dried with argon on the synthesizer. The modified oligonucleotide was purified by analytical-PAGE, following procedures for DNA lariat precursors. The 2′-phosphorylated species move slightly faster than the 2′-underivatized control strand.

(62) Lackey, J. G.; Sabatino, D.; Damha, M. J. *Org. Lett.* **2007**, *9*, 789−792.
(63) Ogilvie, K. K.; Nemer, M. J. *Can. J. Chem.* **1980**, *58*, 1389−1397.
(64) Ogilvie, K. K.; Nemer, M. J.; Hakimelahi, G. H.; Proba, Z. A.; Lucas, M. *Tetrahedron Lett.* **1982**, *23*, 2615−2618.
(65) Nemer, M. Ph.D. Thesis, McGill University, Montreal, Canada, 1982 (Supervisor: K.K. Ogilvie).

B. Carbodiimide-Induced Coupling Using 1-Ethyl-3-[3-dimethylaminopropyl] Carbodiimide (EDC): The reactions are prepared by combining the appropriate precursor strands with their complementary template in a 1:2 ratio and dissolving in 50 mM MES, 20 mM $MgCl_2$ pH 6.0, according to the previously indicated concentrations studied (Figure 2, optimization). Under optimal conditions strand **8a** (0.1 OD units) is mixed with strand **10** (0.2 ODs) and buffer to give a total reaction volume of 14.5 μL. Following standard annealing protocol, the solution was incubated at (4 °C) overnight, before adding 0.5 M EDC (5.5 μL of a 1.8 M EDC/buffer solution) maintaining a pH of 6.0. The reaction was conducted in the absence of light and remained at 4 °C for 6 days. The sample was then quenched with cold, anhydrous EtOH (100 μL) and precipitated by cooling to −78 °C for 12h at 4 °C. The reaction mixture is centrifuged for 10 min at 14000 rpm and decanted. Purification and isolation of products are also conducted as described for DNA lariats. Yield after purification was 0.7 OD units. Characterization by MALDI-MS of precursor and RNA lariat product can be found in Table 5 and spectra in Supporting Information. Final yield was quantified by UV−vis spectroscopy. Characterization by MALDI-MS of precursor and RNA lariat product can be found in Table 5 and spectra in the Supporting Information. The control reactions conducted for EDC coupling are also depicted in the Supporting Information.

5′-End ^{32}P-Radiolabeling DNA and RNA Oligonucleotides. A solution containing 200 pmol of oligonucleotide possessing a free 5′-hydroxyl was added to 2 μL of 10 × polynucleotide kinase buffer (500 mM Tris·HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT, 100 mM spermidine (pH 7.0), 4 μL of 5′-[γ -^{32}P]-adenosine triphosphate, and 1 unit of T4 polynucleotide kinase, dissolved in 13 μL of water. The reaction is then incubated at 37 °C for 30 min and then evaporated to dryness. The samples were redissolved in 8 μL of formamide and purified by gel electrophoresis (16%, 7M urea). Excision of the product bands, extraction with 1 mL of water (incubation at 37 °C or 12 h), and desalting by Sephadex G-25 afford the desired labeled oligonucleotide. Please note that 5′-labeling was successfully conducted on sequences containing the reverse thymidine nucleotides (3′p-dT) at the 3′ terminus of the oligonucleotide.

3′-End Labeling of RNA Oligonucleotide Substrates. Two solutions were prepared separately prior to labeling of the RNA substrate. The preparation of buffer A entails the combination of 330 μL of DMSO (33%), 10 μL of 1 M Tris/HCl pH 7.8 (10 mM), and 660 μL of sterile H_2O to afford a 1 mL solution. Buffer B is prepared by combining 26 μL of DMSO (26%), 1.2 μL of 1 M Tris/HCl pH 7.8 (12 mM), 3 μL of 1 M MgC_2 (30 mM), 7.5 μL of 100 mM ATP (7.5 mM), and 62.3 μL in sterile ddH_2O for a total of a 100 μL solution. Thus, 200 pmol of RNA substrate, possessing a free 3′ terminus, was combined with 2 μL of buffer A incubated for 2 min at 95 °C to denature the sample. A 2 μL aliquot of buffer B was added to the sample and kept at 4 °C for 1 min upon which time 4 μL of ^{32}pCp (9.25 MBq) was added. A final 3.6 μL of T4 RNA ligase solution (10 U/ μL) was added to the substrate solution to begin the labeling reaction. The solution is incubated at 30 °C for 2 h followed by evaporation to dryness. Labeled samples are isolated and purified according to procedures outlined for 5′-radiolabeling.

Debranching Assays and Base Hydrolysis. Characterization of the asymmetric RNA lariats was performed through enzymatic digestions with the yeast *S. cerevisiae* debranching enzyme. This enzyme was graciously donated by Dr. Beate Schwer of Cornell University. The enzymatic activity results in the specific cleavage of 2′−5′ phosphodiester bonds in branched architectures. 2−20 pmol of radioactively labeled lariat was dissolved in 10 μL of Tris buffer pH 7.0 (50 mM Tris HCl, 0.5 mM $MnCl_2$, 2 mM DTT). Greater enzymatic activity was observed when reactions were conducted in the presence of Mn^{2+} divalent cation versus that of Mg^{2+}.[66] The assays are run from 0.5 to 1 h at room temperature upon activation by addition of 1 μL of debranching enzyme to each reaction. The negative control (−) was a branched RNA substrate. Digestions were terminated by adding loading buffer (98% deionized formamide containing 10 mM EDTA, 1 mg/mL bromophenol blue, and 1 mg/mL xylene cyanol). Base hydrolysis was conducted on 2−20 pmol of substrate. Samples were dissolved in an aqueous solution of 100 mM Na_2CO_3 and 0.1 mM EDTA, at 85 °C for 1−15 min. The reactions are then evaporated to dryness and dissolved in 5 μL of loading buffer. Both enzymatic digestion and hydrolysis products were analyzed by gel electrophoresis (16%, 7 M) and visualized using autoradiography.

Bovine Spleen Phosphodiesterase (BSPDE) -3′-Exonuclese Digest. The enzyme was purchased from Aldrich as a dry powder (10 U/mg). It was dissolved in sterile water to attain an activity of 0.1 U/L. 1 pmol of the 3′-radiolabeled RNA lariat substrate was dissolved in 48 μL of 0.1 M sodium acetate, pH 6.5 buffer, and 2 μL of BSPDE (0.2 U). The assays were run for 30 min and 2 h at 37 °C. Samples were then evaporated and dissolved in 5 μL of deionized formamide. Reactions were analyzed by 12% PAGE (7 M urea) and visualized by autoradiography.

RNase H Assays. All enzymatic assays were conducted at a temperature of 4 °C. Duplexes were prepared by combining 3 pmol of the DNA lariat substrate with 1 pmol of the RNA radiolabeled template or 3 pmol of the labeled RNA lariat substrate with 1 pmol of the DNA template. Each reaction was dissolved in 10 μL of 60 mM Tris-HCl (pH 7.8) containing 60 mM KCl and 2.5 mM $MgCl_2$. Samples were hybridized by heating to 90 °C for 10 min and slow cooling, after which time reactions were maintained at 4 °C for 12 h. Initiation of enzymatic activity is carried out by the addition of various concentrations of *E. coli* RNase HI. Assays were conducted with an excess of enzyme 1 μL/reaction − stock 10U/μL or 0.5 enzyme concentration 0.5 μL/reaction − stock 10U/μL, as indicated. Aliquots are removed at different time intervals and are quenched by adding an equal volume of loading buffer. Samples were heated to 95 °C for 5 min and loaded onto a 12% PAGE (7 M urea) for product analysis. The gels were visualized by autoradiography.

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Supporting Information Available: Characterization data including thermal denaturation, PAGE, and circular dichroism analysis. MALDI-TOF spectra of the RNA lariat **8c** are provided. Parallel duplex characterization through spectral techniques and RNase H digests is also provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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