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# RNase H cleavage for processing of in vitro transcribed RNA for NMR studies and RNA ligation

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## ABSTRACT

**Large quantities of RNA for study by NMR and X-ray crystallography can be produced by transcription reactions in vitro using T7 bacteriophage RNA polymerase. A limitation on producing RNA with this polymerase has been the strong dependence of the yield of the transcription reaction on the sequence at the 5′ end of the RNA produced. We report a procedure for obtaining large quantities of enzymatically synthesized RNA from T7 RNA polymerase that has no dependence on the 5′ end sequence of the target RNA. Ribonuclease H has been shown previously (Inoue H, Hayase Y, Iwai S, Ohtsuka E, 1987, *FEBS Lett 215*:327–330) to cleave RNA site specifically using 2′-O-methyl RNA/DNA chimeras to direct the cleavage site. We show that 2′-O-methyl RNA nucleotides on the 5′-side of the DNA nucleotides in the chimera are not essential for site-specific cleavage. This allowed us to design the method such that the same 2′-O-methyl chimera may be used to process any RNA sequence. We have adapted this reaction to the cleavage of NMR-scale quantities of RNA at high yield. RNA is synthesized using T7 RNA polymerase with a 15-nt high-yielding leader sequence at the 5′ end, and then this sequence is cleaved off with the RNase H cleavage reaction. The cleaved RNA has 3′-hydroxyl and 5′-phosphate ends, so that the products can be used directly as substrates for ligation by T4 DNA ligase. We show that the cleavage reaction occurs efficiently in solution and on a solid streptavidin/agarose matrix. We report an example in which we are able to improve transcription yield by more than five-fold using this technique in the synthesis of a $^{15}N$ isotopically labeled hairpin found in the *Crithidia fasciculata* spliced leader RNA. We were able to obtain a 0.5-mM NMR sample from this inherently poorly transcribing sequence, while minimizing the amount of isotopically labeled rNTPs used to produce it. The NMR spectroscopic results are consistent with the predicted RNA secondary structure.**

**Keywords: *Crithidia fasciculata*; RNA processing; T7 RNA polymerase**

## INTRODUCTION

The bacteriophage protein T7 RNA polymerase has been used in in vitro transcription reactions utilizing a synthetic DNA template to generate large quantities of RNA (Milligan et al., 1987; Milligan & Uhlenbeck, 1989; Wyatt et al., 1991). Although other polymerases have been used to produce RNA, T7 RNA polymerase has been found to be the most amenable to large-scale (milligram) RNA synthesis and can be obtained readily in large quantities by overexpression and purification techniques (Davanloo et al., 1984; Grodberg & Dunn, 1988; Zawadzki & Gross, 1991). It has been shown that the first six nucleotides at the 5′ end of the RNA product are important in determining how efficiently the reaction will proceed. Typically, sequences at the 5′ end of the RNA must fit a [G(1)G/C(2)N(3)] consensus sequence in order to transcribe well (Milligan & Uhlenbeck, 1989). For this reason, RNAs used in various biophysical studies, and which are produced by T7 RNA polymerase, often contain modifications of their 5′ end sequences to maximize transcription yield, a compromise that sometimes must be avoided because of sequence effects on physical properties and structure. Another frequently encountered problem with T7 transcription is heterogeneity at the 3′-end, which can be a serious matter if samples are needed for crystallography or NMR, or if the 3′-end of the RNA is to be subsequently ligated to another RNA using a complementary bridging DNA oligonucleotide (Moore & Sharp, 1992).

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The method we describe can be used to solve both of these problems. A 5′-leader sequence can be incorporated in the DNA template, producing an efficient promoter and high transcription yield. Sequence-specific cleavage by RNase H with the aid of a chimeric RNA/DNA template removes the leader sequence. The method can also be used to produce RNAs with precisely defined 3′-ends. The cleavage reaction yields 3′-hydroxyl and 5′-phosphates, so the RNAs can be used as substrates for T4 DNA ligase-mediated ligation without further treatment. Our approach requires considerably smaller RNA leader sequences (~15 nt) than are needed for the ribozyme-mediated cleavage of Taira et al. (1991) (>50 nt), which has the added disadvantage for subsequent ligation of producing 3′-phosphate and 5′-hydroxyl ends.

The method, outlined in Figure 1, is based in part on a procedure first described by Inoue et al. (1987). RNase H recognizes and binds to nucleic acids that are composed of RNA/DNA duplexes and catalyzes the hydrolysis of the phosphodiester backbone between the nucleotides of the RNA strand, leaving a 5′-phosphate and a 3′-hydroxyl (Berkower et al., 1973; Zawadzki & Gross, 1991). Inoue et al. (1987, 1990) showed that an RNA may be site specifically cleaved by RNase H if it is bound by an RNA/DNA chimera composed of four nucleotides of DNA flanked on both sides by 2′-O-methyl RNA. The RNA cleavage site is opposite the 5′-end of the DNA segment. We have exploited this cleavage reaction to circumvent the problems that T7 RNA polymerase has in transcribing low-yielding RNA sequences by transcribing an RNA with a 15-nt high-yielding 5′ end leader sequence (rLDR), which is cleaved off using the RNase H reaction described above. The product of the reaction is an RNA of the desired sequence.

We demonstrate that the 2′-O-methyl RNA on the 5′ side of the DNA of the chimera is not necessary for the reaction to proceed site specifically and efficiently. This allows for the same chimeric construct to be used in the production of any RNA sequence because the base pairing between the chimera and the transcribed RNA occurs only along the rLDR region. We show that this RNase H reaction works to greater than 90% yield and that it can be scaled up to NMR quantities of RNA. The reaction may be performed in solution as well as on a solid-state agarose bead matrix. In other work on a different sequence (Xu et al., 1996), we show that the products of the cleavage reaction can be used for ligation.

We present here a preliminary NMR study of an $^{15}N$ isotopically labeled RNA, found in the *Crithidia fasciculata* spliced leader RNA (SL RNA), that contains a poorly transcribing 5′ sequence. The RNase H cleavage reaction has allowed us to synthesize this NMR sample while maintaining the wild-type 5′ end sequence. Because of the high yield of the transcription reactions, an added benefit is that we minimize the amount of isotopically labeled nucleotides needed to obtain the sample.

## RESULTS

### Enhancement of transcription yield with a leader sequence

Transcription of the 30-nt hairpin of *C. fasciculata* with its wild-type sequence (5′-rGUUUCUGUACUUUAUU GGUAUAAGAAGCUU-3′, abbreviated r3lig) using T7 RNA polymerase at best gave a yield of 0.32 nmol of RNA per 1 mL of reaction after gel purification. Synthesis of an NMR sample of this RNA would require greater than 200 mL of transcription. However, addition of the 15-nt leader sequence rLDR (5′ GGGAUCA CACAAUAC 3′) to the 5′ end of the r3lig sequence increased the yield to an average of 10 nmol of RNA per 1 mL of transcription reaction after gel purification. The yield comparison between these two RNA molecules has further been quantitated by spiking small-scale transcription reactions with $\alpha$-$^{32}P$ UTP and using a

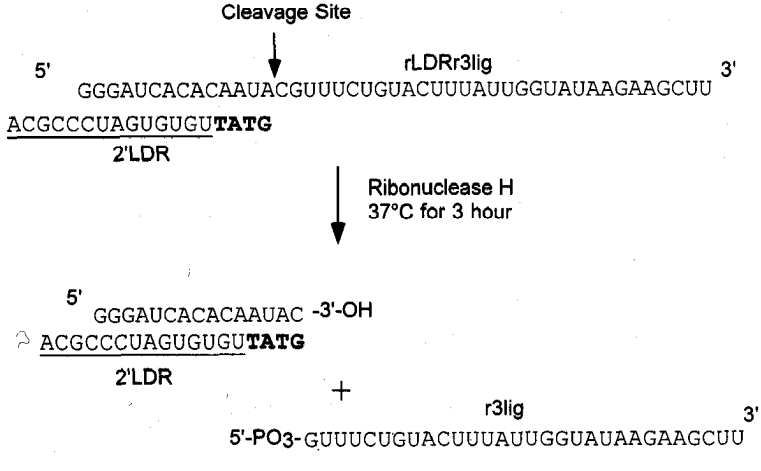


**FIGURE 1.** Solution RNase H cleavage. Diagram of RNase H cleavage of rLDRr3lig RNA by 2′LDR in solution. The 18-nt RNA/DNA chimera 2′LDR directs the site-specific cleavage of rLDRr3lig, leaving r3lig as the final product.

phosphorimager to analyze the purification gel (Fig. 2). These data show an approximate 13-fold increase in the molar yield of the RNA product of the combined sequence rLDRr3lig over r3lig RNA, after taking into account the difference in the number of uridines in the two RNAs.

### Yield and site specificity of the cleavage reaction

The rLDRr3lig RNA must be posttranscriptionally processed by RNase H to generate the final r3lig RNA. This reaction was attempted with two chimeras in solution, 2′SURROUND and 2′LDR (see Fig. 4), which differ in that the 2′SURROUND chimera contains 2′-O-methyl RNA flanking or surrounding both sides of the four DNA nucleotides, whereas the 2′LDR chimera has only 2′-O-methyl RNA on the 3′ side of the DNA (see sequences in the Material and methods). Figure 3 shows the typical large-scale cleavage yield obtained, illustrated for the 2′LDR chimera. More than 90% of the input oligomer is converted to a species that runs near the expected size of 30 nt on an ethidium-stained gel. Cleavage yield with the 2′SURROUND chimera was comparable.

For higher precision in identifying the site of cleavage, a 3′ labeled sample of rLDRr3lig was purified on a denaturing gel to remove 3′ end degeneracy, subjected to cleavage, and the products analyzed on a sequencing gel (Fig. 4). For both the 2′SURROUND and 2′LDR chimera-directed cleavage, there is a barely detectable level of a 31-nt product, in addition to the dominant 30-nt band. However, comparison with the partial T1 ribonuclease digestion band shows a similar level of minor contamination. We conclude that the presence of the $n + 1$ band is due not to lack of specificity in the RNase H cleavage site, but rather to residual $n + 1$ contamination of the starting oligomer. Hence, both chimeras were successful in directing the site-specific cleavage of RNase H. Because the 2′LDR chimera does not base pair to the RNA sequence on the 3′ side of the cleavage site, it may be used for processing any RNA sequence that has the rLDR leader sequence at its 5′-end. Because the 2′LDR chimera is not degraded in the processing reaction, it can be recovered and reused. All large-scale cleavage reactions were performed with the 2′LDR chimera.

### Cleavage on a solid-state matrix

The biotinylated chimera B2′LDR was complexed to a streptavidin/agarose bead matrix and was employed successfully to cleave RNA on this solid-phase support. After preparation of the beads and complexing of the B2′LDR chimera to the bead, two reactions were performed. In the first reaction, 5′ $^{32}$P end-labeled rLDRr3lig was incubated with the beads and cleaved with RNase H. After 3 h of the reaction, greater than 90% of the counts remained bound to the beads (Fig. 5), demonstrating that the 5′ end of rLDRr3lig remains bound to the beads after cleavage. In the second reaction, 3′ $^{32}$P end-labeled rLDRr3lig was also cleaved using the B2′LDR beads. After 3 h of reaction, 70–80% of the counts could be found in the supernatant (Fig. 5). As the reaction proceeds, the 3′ end of the rLDRr3lig is released into the supernatant. This 3′ end piece of the RNA was analyzed by sequencing and found to indeed

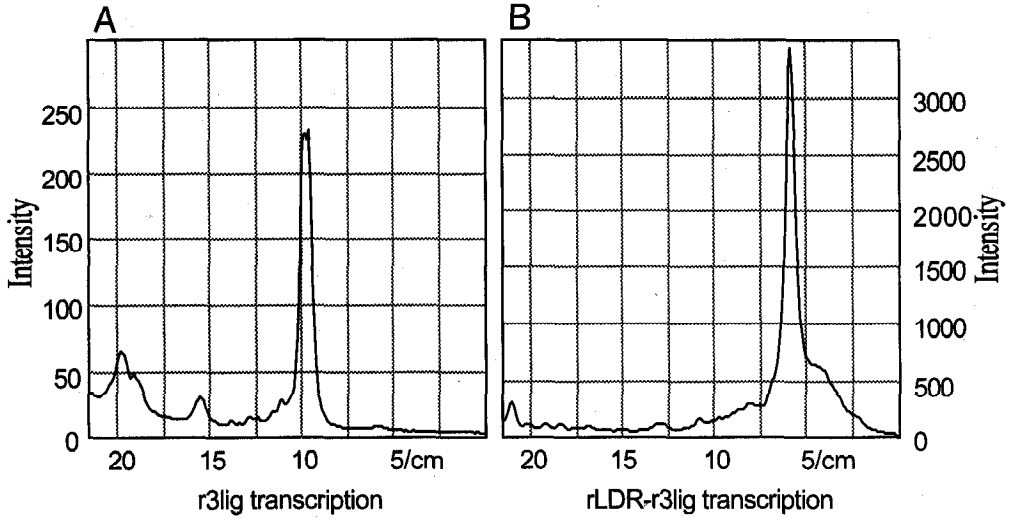


**FIGURE 2.** Transcription comparison: r3lig with rLDRr3lig. Phosphorimager data from a 15% denaturing polyacrylamide gel on which was loaded 20-$\mu$L transcription reactions spiked with $\alpha$ $^{32}$P rUTP (40 mM Tris-HCl, pH 8.3, 20 mM $MgCl_2$, 50 mg/mL PEG 8000, 5 mM DTT, 1 mM Spermidine, 0.01% NP-40, 200 nM DNA template, 4 mM each rNTP, 5 $\mu$Ci $\alpha$ $^{32}$P rUTP, and 0.1 mg/mL T7 RNA polymerase at 37 °C for 4 h). Yield comparison of (**A**) r3lig and (**B**) rLDRr3lig demonstrates the poor transcription yield of the r3lig RNA; note the difference in scale between A and B.

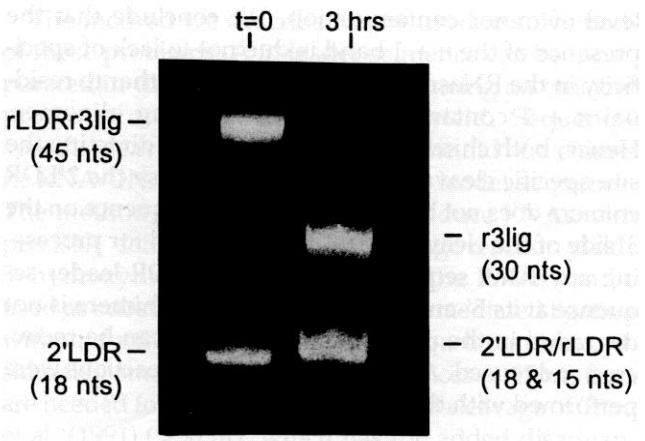


**FIGURE 3.** Large-scale cleavage. Ethidium-stained 20% PAGE of NMR scale cleavage of rLDRr3lig by 2′LDR. Comparison of the lane at 3 h with the starting material ($t = 0$) shows nearly complete conversion of the 45-nt transcript to the 30- and 15-nt products.

be the r3lig RNA, and no further purification was necessary after cleavage. The B2′LDR beads were also shown to be recyclable. By addition of denaturants at warm temperatures, the post cleavage 5′ RNA piece can be removed into the supernatant, and the beads may be used again.

The solid-state cleavage using B2 beads was not tested for the ability to scale up to NMR quantities of RNA, because the loading of B2′LDR that we could achieve on the beads would require a 10-fold increase in reaction volume, and a corresponding 10-fold increase in the amount of RNase H required for cleavage. This scale-up is too expensive with enzyme from commercial sources, although it should be quite feasible if an overproducing clone is employed.

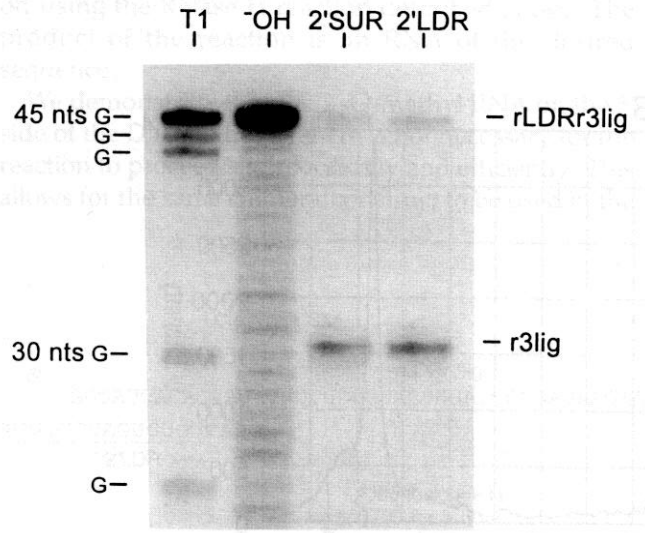


**FIGURE 4.** Site specificity of the cleavage. Site-specific cleavage of the 3′ end-labeled rLDRr3lig by RNase H. Lane 1, ⁻OH ladder; lane 2, T1 digestion; lane 3, 2′LDR-directed cleavage; lane 4, 2′SURROUND-directed cleavage. The product of the cleavage of the 45-nt transcript rLDRr3lig is the 3′ end-labeled 30-nt r3lig. Both the RNase H and the T1 digestion lane reveal comparable fractions of a minor contamination of a 31-nt product, judged to be due to a minor contaminant of a 46-nt transcript in the starting material.

### NMR sample preparation

Preparation of an NMR sample of r3lig required 30 mL of rLDRr3lig transcription, at an average yield of 5.4 nmol of RNA per mL of reaction after RNase H cleavage and PAGE purification. The 2′-O-methyl RNA/DNA chimera 2′LDR (Fig. 1) was used to direct the cleavage of the RNA in solution by RNase H and the reaction was followed by denaturing mini-gel until completion, taking an average of 3 h. After the final gel purification, a final yield of 75 nmol of r3lig RNA was obtained.

### NMR spectroscopy

Preliminary NMR spectroscopy demonstrates that the isotopically labeled nucleotides were incorporated into the sample and that the sample is adequately concentrated. The two-dimensional $^{1}H$-$^{15}N$ heteronuclear multiple quantum correlation (HMQC) spectrum (Fig. 6) shows clearly four A-U base pairs and two G-C base pairs. We do not detect the G7-U21 and G27-U2 base pairs at the temperature and buffer medium used in this experiment, but we have been able to observe corresponding resonances at colder temperatures and higher ionic strength buffers. We have not, however, been able to observe the U11-G17, A22-U6, and G1-C28 base pairs under any condition, probably because of fast solvent exchange due the hairpin loop opening, bulge loop opening, and helical end fraying respectively.

## DISCUSSION

We describe a method that permits the synthesis of RNA of any given sequence by template-directed transcription with T7 RNA polymerase, followed by cleavage with RNase H. The use of a leader sequence enhances transcription yield greatly, even for a target sequence, such as r3lig, that begins with G. We have shown that 2′-O-methyl RNA nucleotides need not be incorporated on the 5′ side of the DNA nucleotides in the chimera, which allows the use of one chimera for the cleavage of any RNA beginning with the rLDR sequence. The products of the reaction contain a 3′-hydroxyl and a 5′-phosphate, either of which could be used in a subsequent ligation reaction. In cases in which it is critical to remove 3′-end degeneracy, a longer RNA could be synthesized, and the unwanted 3′ portion cleaved at the desired sequence terminus using

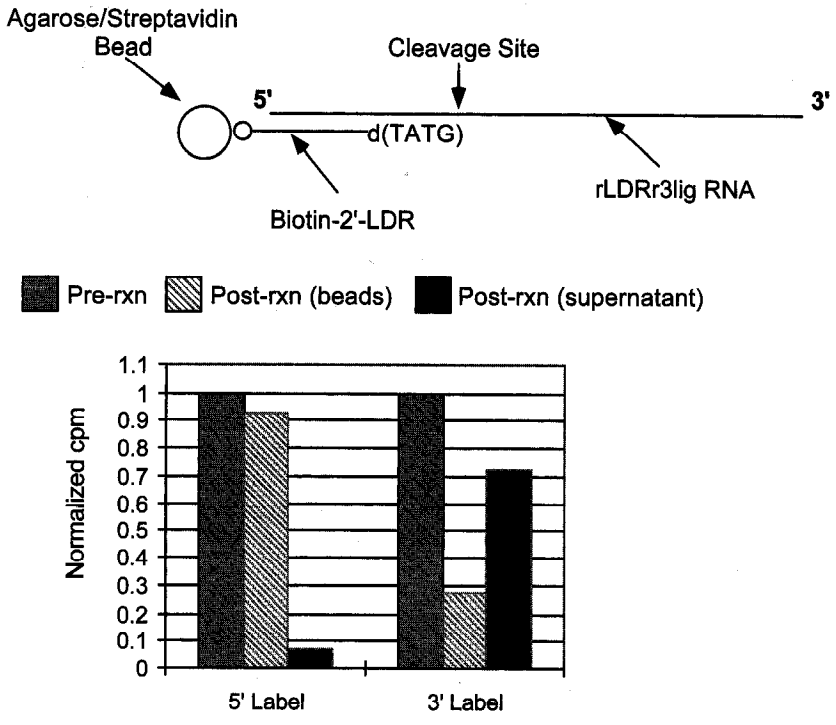


**FIGURE 5.** Solid-state RNase H cleavage. **A:** Diagram of rLDRr3lig RNA hybridized to a streptavidin-biotin-chimera (B2′LDR) column. **B:** Results of RNase H cleavage of rLDRr3lig RNA bound to B2′LDR column. After 3 h of reaction, supernatant was removed from the beads by centrifugation and the beads were rinsed. The 5′ end-labeled RNA remained bound to the beads, whereas the 3′ end-labeled RNA came off with the supernatant. Bar graph shows the fraction of counts found in the bead or supernatant fraction when either 5′- or 3′-labeled RNA is used.

an appropriately designed chimera. The cleavage reaction has been adapted to work efficiently on a reusable solid matrix, allowing an efficient method for small-scale RNA production. Large amounts of RNA can be processed posttranscriptionally more efficiently in solution than on a solid matrix because the latter requires a much larger volume and correspondingly increased amount of RNase H. Both the solution- and solid-phase methods of RNA production using this RNase H cleavage reaction permit the synthesis of RNA that has no 5′ end sequence dependence.

## MATERIALS AND METHODS

### Oligonucleotide synthesis

All DNA oligonucleotides used as templates for T7 RNA polymerase transcription reactions were synthesized on an Applied Biosystems 380B DNA synthesizer in 1-μmol quantities. The three 2′-O-methyl RNA/DNA chimeras were synthesized by the Keck Foundation Oligonucleotide Synthesis Facility at Yale University in 1-μmol quantities. All oligonucleotides were purified by electrophoresis on denaturing 15% polyacrylamide gels. The abbreviation, full name, and 5′ to 3′ sequence, respectively, of these chimeras are as follows (**RNA in bold is 2′-O-methyl**):

| | | |
|---|---|---|
| 2′SURROUND | 2′-O-$CH_3$-SURROUND | $_r$(**UAGUGUGU**)$_d$(TATG) $_r$(**CAAAG**) |
| 2′LDR | 2′-O-$CH_3$-LEADER | $_r$(**ACGCCCUAGUGUGU**)$_d$(TATG) |
| B2′LDR | Biotin-2′-O-$CH_3$-LEADER | Biotin-$_r$(**ACGCCCUAGUGUGU**)$_d$(TATG) |

### Enzymes

RNase H used in the cleavage reactions was obtained from Pharmacia (27-0894) at 1.9 units/μL where 1 unit is defined as able to catalyze the production of 1 nmol acid-soluble RNA nucleotide in 20 min at 37 °C. T4 DNA ligase used in the ligation reactions was obtained from New England Biolabs (202L) at 400 units/μL. T7 RNA polymerase was produced using published techniques (Davanloo et al., 1984; Grodberg & Dunn, 1988; Zawadzki & Gross, 1991).

### T7 RNA polymerase transcriptions

All RNA transcriptions utilized a bottom strand DNA template coding for the RNA plus a 5′ 17-nt T7 RNA polymerase promoter sequence. The top strand DNA template was complementary to the 17-nt promoter sequence. All reactions were conducted under identical conditions, except that the magnesium ion concentration was optimized independently for each reaction. $^{15}N$ isotopically labeled NTPs were obtained using published methods (Batey et al., 1992; Nikonowicz et al., 1992), modified as described below. The reaction conditions for the transcriptions were 40 mM Tris HCl, pH 8.3 at 20 °C, 5 mM DTT, 1 mM spermidine, 20 mM $MgCl_2$, 0.01% NP-40, 50 mg/mL PEG 8000, 2 mM in each rNTP, 200 nM DNA template, and 0.1 mg/mL T7 RNA polymerase. All reactions were carried out at 37 °C for 4–8 h. Products of the transcriptions were purified by 15% denaturing PAGE.

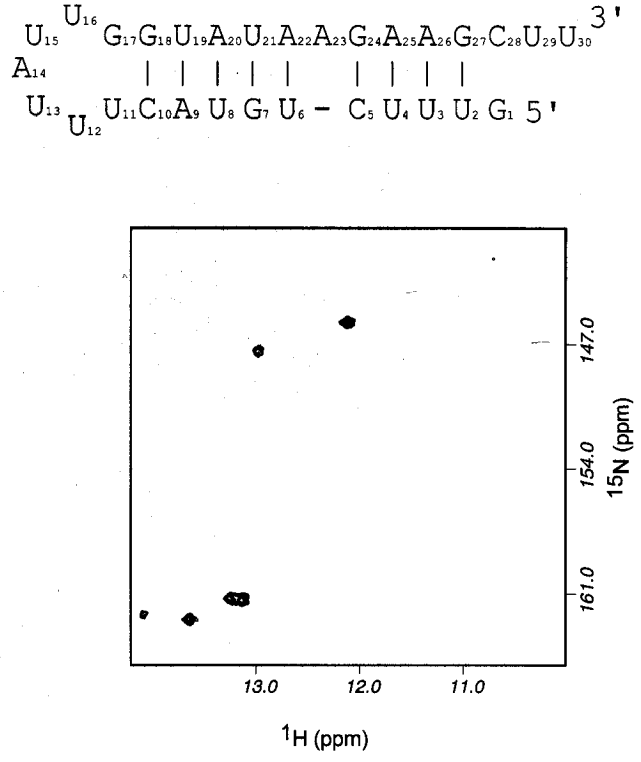


**FIGURE 6.** 2D $^{1}H$-$^{15}N$ HMQC of r3lig product from the RNase H cleavage. Two-dimensional $^{15}N$-$^{1}H$ HMQC spectra of r3lig obtained from the RNase H cleavage reaction. The four A-U pairs are grouped by $^{15}N$ chemical shift of the U imino nitrogen around 161 ppm, whereas the two G-C pairs have a corresponding $^{15}N$ chemical shift around 147 ppm.

Comparisons of transcription yields between r3lig and rLDRr3lig, shown in Figure 2, were conducted by analyzing 20-$\mu$L transcriptions spiked with 5 $\mu$Ci of $\alpha$-$^{32}$P-UTP, run on 15% denaturing gels, and quantitated by phosphorimager (Fuji Inc., Fujix 2000) analysis. Calculations of transcription yields for the body $\alpha$-$^{32}$P-UTP labeled RNAs included a correction factor for the number of uridines in the sequence.

### $^{15}$N NTP isolation and purification

We used the methods of Batey et al. (1992) and Nikonowicz et al. (1992), with modification of the method of isolation of nucleic acids from the cell extract. *Escherichia coli* cells were grown on a minimal media containing $^{15}N$ ammonium chloride as the only nitrogen source. The cells were harvested in the log phase of cell growth by centrifugation. The cell pellet was resuspended in a minimal volume (20 mL per liter growth) of STE buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, pH 8.0) and 0.5% SDS. This whole-cell slurry was then sonicated in a Branson Sonifier 450 sonicator at its highest power setting for 4 min, allowed to cool on ice for 5 min, then the procedure was repeated three times. This slurry was then extracted once with 25:24:1 equilibrated phenol(pH 8.0):chloroform:isoamyl alcohol at 60 °C for 30 min with constant stirring. The mixture was centrifuged, and the aqueous phase removed and saved. The phenol layer was back extracted once with 1/2× volume STE buffer, the aqueous phase removed, and pooled with that from the first extraction. The pooled aqueous phase was extracted three times with 1/2× volume chloroform, leaving an aqueous phase essentially free of phenol contamination. The total cellular nucleic acids were precipitated by adding 1/10 volume 3 M sodium acetate and 1× volume isopropyl alcohol and centrifuging.

The pellet was dried and resuspended in P1 nuclease digestion buffer (15 mM sodium acetate, pH 5.2, and 0.1 mM $ZnSO_4$). The nucleic acids were denatured by heating to 95 °C for 1 min and snap cooled in ice. Ten units of P1 nuclease and 100 units of DNase I were added per liter of cell growth and incubation was continued at 37 °C until there were no polymers of nucleic acid left by PAGE analysis, typically 12 h. The desalting procedures and conversions to ribonucleotide triphosphates were identical to those published previously (Batey et al., 1992; Nikonowicz et al., 1992). After complete conversion of the ribonucleotides from the monophosphate to the triphosphate, no further purification was necessary, and the nucleotide triphosphates could be used immediately in transcription reactions.

### Cleavage of RNA with the 2′-O-methyl RNA/DNA chimeras in solution

All RNase H cleavage reactions contain 20 mM HEPES-KOH, pH 9.0, 50 mM KCl, and 10 mM $MgCl_2$. The chimera was annealed to RNA by heating to 90 °C and cooling slowly to room temperature at high concentration; reaction concentrations for chimera–RNA complex were typically between 0.5 and 2.0 mM. The chimera was kept at 1.2× the RNA concentration to insure complete hybridization of the RNA. RNase H was added to a final 20 units per 100 $\mu$L reaction. Hoefer Scientific Mini Gels were used to follow the large-scale reactions to completion, as shown in Figure 3. The reaction typically takes between 30 min and 3 h, and denaturing PAGE was utilized to purify the products.

### Cleavage of RNA with an immobilized biotin labeled 2′-O-methyl chimera

B2′LDR was bound to streptavidin beads (Pierce, Immuno-Pure immobilized streptavidin, crosslinked, on 6% beaded agarose) using the following procedure. The buffers used are 50 mM wash buffer (20 mM Tris-HCl, pH 7.6, 0.01% NP-40, 50 mM NaCl), 250 mM wash buffer (20 mM Tris-HCl, pH 7.6, 0.1% NP-40, 250 mM NaCl), and preblock mix (100 $\mu$g/mL glycogen, 1 mg/mL BSA, 100 $\mu$g/mL tRNA, 33% 50-mM wash buffer). Two milliliters of the 50% bead slurry solution supplied by Pierce was centrifuged to remove the storage solution and washed twice with sterile double-distilled (dd) $H_2O$. Five-hundred microliters of preblock mix was added and mixed slowly with the beads for 20 min at 4 °C. The preblock mix was removed and the beads were rinsed three times with 500 $\mu$L of the 50 mM wash buffer. Forty-five nanomoles of the biotinylated chimera B2′LDR (50 $\mu$L at 0.9 mM) were added to the beads together with 500 $\mu$L of the 250 mM wash buffer for 90 min at 4 °C. The supernatant was removed from the beads and washed three times with the 250 mM wash buffer. There was no UV signal at 260 nM for the supernatant or the washings, indicating that all 45 nmol of B2′LDR was bound completely to the streptavidin beads.

To follow the cleavage of the rLDR3lig RNA on the B2′LDR column, the RNA was prepared 3′ end-labeled and 5′ end-labeled in two separate reactions. The 3′ end-label cleavage reaction (100 μL B2′LDR beads, 40 μL 5× RNase H buffer, 10 μL 20-mM DTT, 70K cpm pCp 3′ end-labeled rLDRr3lig, and 3 μL RNase H at 1.9 U/μL) and the 5′ end-label cleavage reaction (100 μL B2′LDR beads, 40 μL 5× RNase H buffer, 10 μL 20-mM DTT, 70K cpm 5′ end-labeled rLDR3lig, and 3 μL RNase H at 1.9 U/μL) were heated to 70 °C for 1 min and slow cooled before adding enzyme. Reactions ran for 3 h at room temperature while mixing slowly to keep the beads in solution. Reactions were harvested by centrifugation and removal of the supernatant.

For the 5′ end-labeled reaction, greater than 95% of the counts remained on the column beads after removal of the supernatant and repeated washings, as shown in Figure 5. For the 3′ end-labeled reaction, greater than 70% of the counts came off in the supernatant and the PAGE analysis confirmed production of the correct product, r3lig.

### Recycling the B2′LDR column

After an RNase H cleavage of an RNA with the rLDR sequence at its 5′ end, the B2′LDR column may be regenerated. The rLDR sequence is bound to the column via base pairing to B2′LDR and must be removed before the column may be used again. Two or three washings of an equal volume of denaturing buffer (6 M urea, 1 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, and 20% acetonitrile) to bead material for 30 min at 60 °C removes the rLDR. The column must then be rinsed several times with sterilized $ddH_2O$ to prepare it for the next reaction. This procedure removes 95% of the counts from the 5′ end-labeled reaction, and the column was able to cleave another batch of RNA successfully.

### Analysis of RNA after RNase H cleavage

To analyze cleavage products, the RNA was 5′ end-labeled by sequential dephosphorylation with calf intestine phosphatase and kinased with polynucleotide kinase and $\gamma$-$^{32}$P-ATP. The radiolabeled products were run on denaturing gels next to RNA sequencing lanes. In addition, a 3′-labeled sample prepared as described above was purified on a denaturing polyacrylamide gel to separate polymerization products $n$ and $n + 1$, subjected to the RNase H cleavage reaction, and the product was analyzed on an RNA sequencing gel (see Fig. 4). To provide additional proof that the cleavage reaction proceeds site specifically (data not shown), the 3′ cleavage product was ligated to another RNA at its 5′ end (the site of the cleavage). The ligation reactions were conducted using a buffer of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 50 μg/mL BSA. The two pieces of RNA to be ligated are annealed to a complementary strand of DNA, which is of a different size than the RNAs or the RNA ligation product (17 nt longer than the product in this case) to facilitate purification of the products. The complex formation can be followed by native PAGE. Typical annealing conditions are to heat to 90 °C and slow cool to room temperature over 30 min time. All reactions were performed at room temperature and used 1/10 of the total reaction volume as ligase (at 400 U/μL). Yields of the ligations varied from 50% to 80% and are consistent with typical RNA ligation yields.

### NMR procedures

NMR samples were dialyzed repeatedly against 20 mM phosphate buffer, pH 6.5, 10% $D_2O$ was added for the lock carrier signal, and the final volume of the sample was 200 μL in a Shigemi NMR tube. NMR spectra shown (see Fig. 6) were taken on a General Electrics Omega 500 spectrometer using a Bruker Instruments $^1H$, $^{13}C$, $^{15}N$ triple resonance probe with X, Y, Z pulsed-field gradient coils. The $^1H$-$^{15}N$ HMQC experiment was adapted from Szewczak et al. (1993), utilizing GARP decoupling of the nitrogen heteronucleus (Shaka et al., 1985). The 0.5-mM r3lig sample required 3 h of spectrometer time to collect 128 experiments of 64 scans. All NMR data were processed on a Silicon Graphics workstation using Biosym Technologies Felix v2.3 NMR processing software.

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