CHAPTER 2 "AN RNA ENDONUCLEASE"

2.1 Summary

This chapter presents a method for site-specifically cleaving RNA of any sequence, with high reaction yield. The reaction has been adapted to the cleavage of milligram quantities of RNA, suitable for the sample preparation needs of nuclear magnetic resonance and X-ray crystallographic studies.

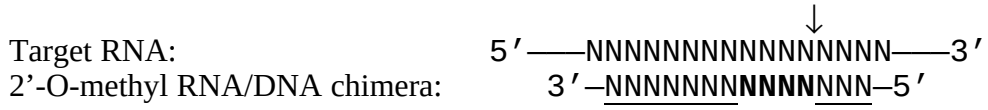
2.2 Introduction

Site-specific endonuclease cleavage of DNA is a powerful tool for molecular biologists, making possible procedures such as gene cloning. This reaction is accomplished by means of restriction enzymes that recognize, bind and cleave specific DNA sequences and are usually high yielding. Site-specific restriction enzymes, however, do not exist for RNA. Rather, the world is replete with the bane of the RNA biologist, the non-specific nuclease. One such nuclease, Ribonuclease H (RNase H), has the interesting property in that it only binds to RNA that is base paired with DNA and catalyzes the hydrolysis of the phosphodiester backbone between the nucleotides of the RNA strand. Thus, RNase H'es are biologically important for "cleaning up" during processes which would generate long stretches of RNA/DNA strands, such as during reverse transcription (reverse transcriptase has a built-in RNaseH functionality).

RNaseH is not, however, very site-specific by nature. If one binds a long sequence of complementary DNA to RNA, the cleavage can occur in any position shared by the RNA/DNA duplex. In 1987, Inoue *et al.*, recognized that this could be exploited to cleave "specifically" if one could reduce the number of DNA nucleotides bound to the RNA. In fact, they showed that exactly four DNA nucleotides gave a single specific

RNA cleavage, presumable due to the RNaseH requiring a four base pair binding site. In order to increase the thermodynamic stability of this complex, they surrounded the four DNA nucleotides with stretches of 2'-O-methyl RNA (Fig. 2.1). The 2'-O-methyl RNA is not recognized by the RNase H as a suitable substrate for binding, and thus does not interfere in the reaction, serving to "hold" the DNA in place. A few more papers were published by the Japanese group (Inoue, *et al*., 1988; Hayase, *et al*, 1990), the later paper demonstrated that a tRNA could be cleaved in different positions using this technique.

Figure 2. 1 RNase H cleavage position



Underlined characters, N, represent 2'-O-methylated RNA. Bold characters, **N**, represent DNA. Regular characters, N, represent RNA. The arrow, ↓, indicates the position of cleavage.

This is an important reaction for the RNA biologist that may not have been fully realized. There are a number of very interesting properties of this reaction, the reaction time is short, the efficiency of cleavage is high and the cleavage products of RNase H reactions have a 5'-phosphate and a 3'-hydroxyl (Berkower *et al*., 1973; Zawadzki & Gross, 1991). These properties can be used to accomplish a number of highly desirable tasks for someone working with RNA. First, this allows for a procedure to synthesize RNAs that have no sequence requirements at their 5' end. The synthesis of large quantities of RNA for biophysical study is often accomplished using the bacteriophage T7 RNA polymerase and a DNA template. One of the unfortunate problems of using this enyzme is that the transcription yield of the target RNA is often highly dependent on the nucleotide sequence of the 5' end. Synthesizing a target RNA with a "high yield" 5' end

sequence, followed by a cleavage reaction to produce the final target RNA product can circumvent this problem. The second use of this RNA endonuclease technique is that the RNA products of the cleavage reaction can be used directly in a subsequent religation reaction. This makes it possible, for example, to synthesize "segmentally isotope labeled" RNA for use in NMR (Xu, *et al*., 1996).

The bacteriophage protein T7 RNA polymerase has been used in *in vitro* transcription reactions to generate large quantities of RNA (Milligan *et al*., 1987; Milligan & Uhlenbeck, 1989). While 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 readily obtained in large quantities by over expression and purification techniques (Grodberg & Dunn, 1988; Davanloo *et al*., 1984; 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 biophysical studies produced by T7 RNA polymerase often contain modifications at their 5' end sequence to maximize transcription yield, a compromise that sometimes must be avoided.

Under the optimum conditions this reaction occurs rapidly, can be scaled up to milligram quantities of RNA, is highly efficient and is absolutely site specific. We have exploited this cleavage reaction to circumvent the problems the T7 RNA polymerase has with transcribing low yielding RNA sequences. We demonstrate that the 2'-O-methyl RNA on the 5' side of the DNA is not a necessary component for the reaction to proceed.

This allows for the same chimeric construct to be used in the production of any RNA sequence, since the base pairing between the chimera and the target RNA occurs only along the 5' side of the cleavage site, as shown below in figure 2.2.

Figure 2. 2 RNase H cleavage occurs without the 5' 2'-O-methyl RNA

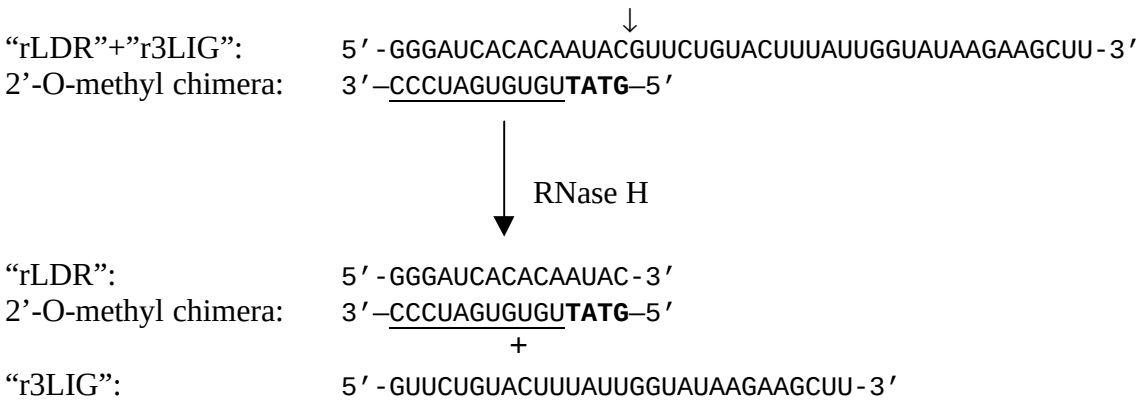
Target RNA: 5′——NNNNNNNNNNNN↓NNNN——3′
2'-O-methyl RNA/DNA chimera: 3′—NNNNNNN**NNNN**—5′

Underlined characters, N, represent 2'-O-methylated RNA. Bold characters, **N**, represent DNA. Regular characters, N, represent RNA. The arrow, ↓, indicates the position of cleavage.

We also demonstrate that this reaction can also be executed off a solid phase media. The chimera can be produced with a 3'-biotin label and adhered to a streptavidin-agarose bead, and the RNase H cleavage reaction can then be performed off this solid phase matrix. The reaction occurs with similar results to those obtained in the solution phase reaction, with the added benefit of an easy route for reusing the 2'-O-methyl RNA/DNA chimera for future reactions.

We demonstrate the practical applicability of this RNA endonuclease reaction by synthesizing an ^{15}N isotope labeled 30 nucleotide RNA hairpin "r3LIG", which contains an inherent poorly transcribing 5' end sequence. The 5' end of this RNA could not be modified since future experiments with the RNA involve ligation of an unlabeled piece of RNA to the 5' end. Any modifications to the sequence would then become internal sequence modifications, which may change the physical properties of the RNA. The RNA was produced by adding 15 nucleotide high-yielding leader sequence "rLDR", as shown below in figure 2.3, and subsequently cleaving rLDR away from the r3LIG RNA.

Figure 2. 3 Synthesis of r3LIG RNA



Underlined characters, N, represent 2'-O-methylated RNA. Bold characters, **N**, represent DNA. Regular characters, N, represent RNA. The arrow, ↓, indicates the position of cleavage.

A two-dimensional ^{1}H-^{15}N HMQC NMR spectrum is shown from the final product to demonstrate that these reactions can indeed be accomplished on large quantities of RNA.

2.3 Results

2.3.1 Enhancement of transcription yield with a leader sequence

Transcription of the 30 nucleotide hairpin of *C. fasciculata* r3lig with its wild type sequence (5'-rGUUUCUGUACUUUAUUGGUAUAAGAAGCUU-3') using T7 RNA polymerase at best gave a yield of 0.32 nmoles of RNA per 1 ml of reaction after gel purification. Synthesis of an NMR sample of this RNA would require greater then 200 mL of transcription. However, addition of the 15 nt leader sequence rLDR (5' GGGAUCACACAAUAC 3') to the 5' end of the r3lig sequence increased the yield to an average of 10 nmoles 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 á-^{32}P UTP and using a phosphorimager to analyze the purification gel (Fig. 2.4). These data show an approximate 13 fold increase in the molar yield of the RNA product of the rLDRr3lig over the r3lig RNA, after taking into account the difference in the number of uridines in the two RNAs.

2.3.2 Yield and site specificity of the cleavage reaction

The rLDRr3lig RNA must be post transcriptionally processed by RNase H to generate the final r3lig RNA. Two chimeras were constructed, 2'SURROUND and 2'LDR, to test for the necessity of having the 2'-O-methyl RNA flanking both sides of the four DNA nucleotides. The RNase H cleavage reaction was attempted with both chimeras in solution (see Fig. 2.5a), and it is clear that both reactions were successful. Scaling the reaction up to NMR quantities of RNA (Fig. 2.5b) shows the typical large scale cleavage yield obtained. More than 90 % of the input target RNA is converted to

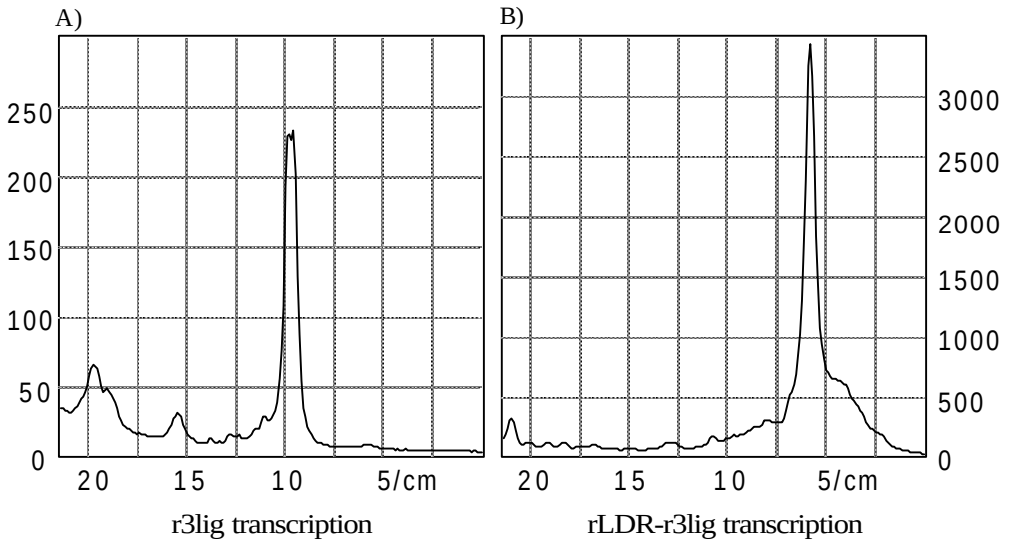


Figure 2. 4 Transcription Comparison: r3lig with rLDRr3lig

Phosphorimage data from 20 uL transcription reactions spiked with á ^{32}P rUTP (40mM Tris-HCl pH 8.3, 20 mM MgCl2, 50 mg/ml PEG 8000, 5 mM DTT, 1 mM Spermidine, 0.01% NP-40, 200 nM DNA template, 4 mM each rNTP, 5 uCi á ^{32}P rUTP and 0.1 mg/ml T7 RNA polymerase at 37 C for 4 hours). Yield comparisons of (A) r3lig and (B) rLDRr3lig demonstrates the poor transcription yield of the r3lig RNA.

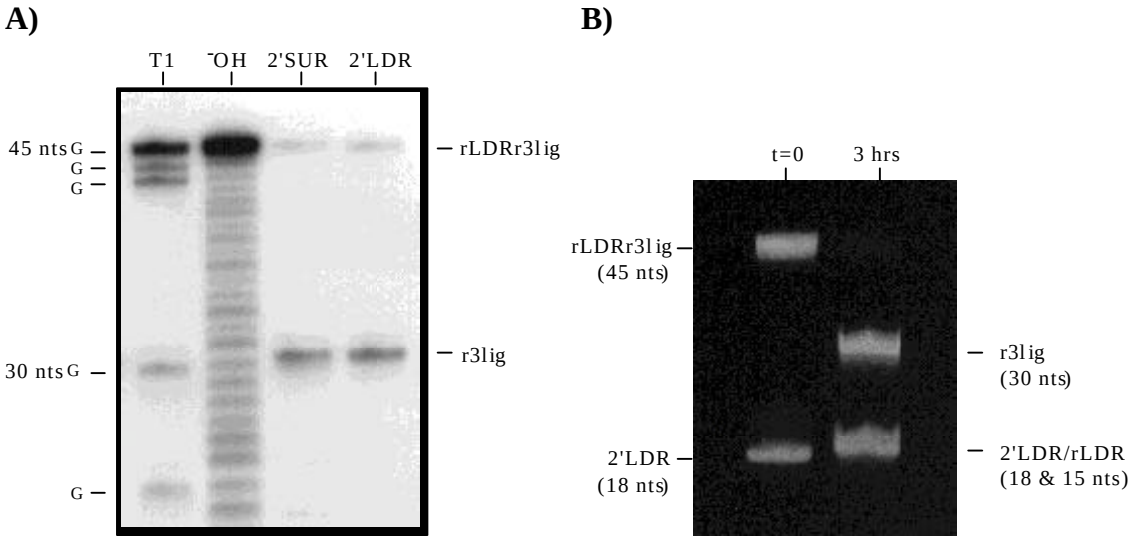


Figure 2. 5 Site specificity of the cleavage and large scale cleavage

A) 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 rLDRr3lig is 3' end labeled 30 nucleotide r3lig. **B)** Ethidium stained 20% PAGE of NMR scale cleavage of rLDRr3lig by 2'LDR.

the desired 30 mer RNA r3LIG, which runs near the expected size of 30 nucleotides on the ethidium stained gel.

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. 2.5a). For both the 2'SURROUND and 2'LDR chimera-directed cleavage, there is a barely detectable level of a 31 nucleotide product, in addition to the dominant 30 nucleotide band. However, comparison with the partial T1 ribonuclease digestion land 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 production of any RNA sequence. All large scale cleavage reactions were consequently performed with this 2'LDR chimera.

2.3.3 Cleavage on a solid state matrix

A 2'-O-methyl RNA/DNA chimera, "B2'LDR", was synthesized with a 3' end biotin label. This chimera was complexed to a streptavidin-agarose bead matrix and was successfully employed 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, 5' ^{32}P end labeled rLDRr3LIG was incubated with the beads and cleaved with RNase H. After 3 hours of the reaction, greater then 90% of the counts remained bound to the beads (Fig. 2.6), demonstrating that the rLDR remains bound to

the beads after cleavage. In the second reaction, 3' ^{32}P end labeled rLDRr3LIG was produced, bound to the B2'LDR beads and cleaved. After 3 hours of reaction, 70-80% of the counts could be found in the supernatant (Fig. 2.6). As the reaction proceeds, the 3' end of the rLDRr3lig is released into the supernatant. This radiolabeled piece of RNA was analyzed by sequencing and found to indeed be the 30 nucleotide r3LIG RNA (data not shown).

This "cleavage column" was not tested for the ability to scale up to NMR quantities of RNA, because it would require large quantities of bead matrix. However, the method worked quite well in the small-scale reactions, especially when working with radiolabeled RNA. The reaction is followed easily when working with 3' ^{32}P end labeled RNA by observing the increase in counts in the supernatant as the RNA is cleaved from the beads, 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.

2.3.4 NMR sample preparation

Preparation of a NMR sample of r3LIG required 30 mL of rLDRr3LIG transcription, at an average yield of 5.4 nmoles of RNA per ml of reaction after RNase H cleavage and PAGE purification. The 2'-O-methyl RNA/DNA chimera 2'LDR was used to direct the cleavage the RNA in solution by RNase H and the reaction was followed by

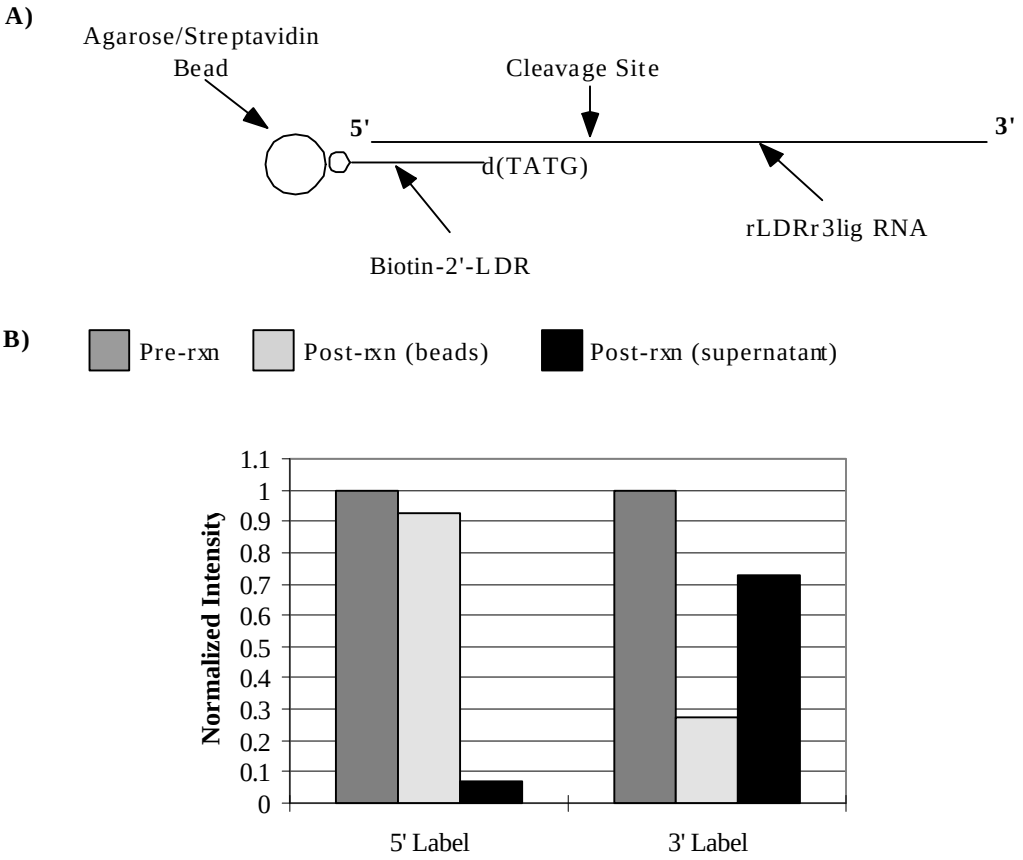


Figure 2. 6 Solid State RNase H Cleavage

A) Diagram of rLDRr3lig RNA bound to B2'LDR column. **B)** Results of RNase H cleavage of rLDRr3lig RNA bound to B2'LDR column. After 3 hours of reaction, the supernatant was removed from the beads by centrifugation and the beads were rinsed. The 5' end labeled RNA remained bound to the beads, while the 3' end labeled RNA came off with the supernatant.

denaturing mini-gel until completion, taking an average of 3 hours. After the final gel purification a final yield of 75 nmoles of r3lig RNA was obtained.

2.3.5 NMR spectroscopy

The NMR spectroscopy demonstrates that the isotopically labeled nucleotides were incorporated into the sample and that the sample is adequately concentrated. The 2 dimensional ^{1}H - ^{15}N HMQC (Fig. 2.7) clearly shows 4 A-U base pairs and 2 G-C base pairs. We do not detect the G7-U21 and G27-U2 base pairs at the temperature and buffer used in this experiment, but we have been able to observe corresponding resonances at colder temperatures and higher ionic strength buffers. We have not been able, however, to observe the U11-G17 or G1-C26 base pairs at any condition, probably because of fast solvent exchange due the hairpin loop opening and helical end fraying respectively.

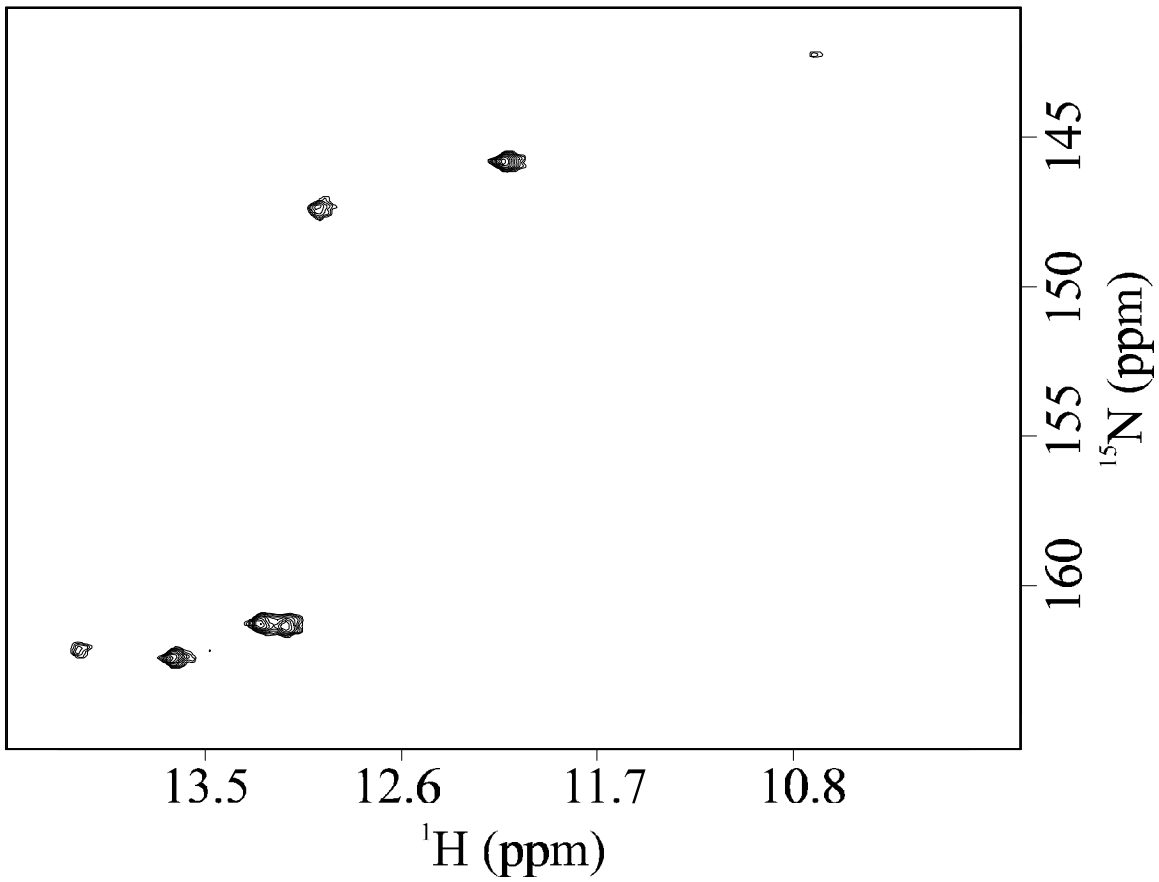


Figure 2. 7 2D ^{1}H-^{15}N HMQC of r3lig product from the RNase H cleavage

2 dimensional ^{15}N-^{1}H HMQC spectra of r3lig obtained from the RNase H cleavage reaction.

2.4 Discussion

In summary, we describe a method that permits the synthesis of large quantities of RNA without regard to the final 5' end sequence. This is accomplished by means of adding a 15 nucleotide leader sequence to the 5' end of the desired RNA, which is subsequently cleaved away from the final product via site-directed RNase H cleavage. The use of the removable 5' leader RNA sequence greatly enhances transcription yield because it can be constructed out of any high transcription yield sequence. Cleavage of the leader sequence from the desired RNA occurs in high yield (>90%), and can be scaled up to large quantities of RNA (milligram).

The same 2'-O-methyl RNA/DNA chimera can be used for any cleavage reaction. The requirement that the RNA portion of the site-directing chimera exist on both sides of the 4 deoxyribonucleotides is not necessary for efficient, site-specific cleavage of RNA. If the chimera is constructed like the 2'LDR sequence, it can be reused for many RNA molecules, since there is no base pairing overhangs between the chimera and the unknown final RNA target sequence. This affords a great advantage in that the chimera can be produced before knowing what RNA sequence is desired. It is also shown that these reactions can be carried out on a solid phase via a biotin-streptavidin linkage between an agarose bead and the chimera. This has interesting possibilities for the construction of a "RNA cleavage column" which could be reused.

2.4.1 The religation of RNA cleavage products

The RNA products from the reaction terminate with a 3' hydroxyl and a 5' phosphate for the 5' and 3' piece respectively. This is intriguing in that these are the

required end chemistries for further biochemistry, such as in use with DNA ligase. In fact, this fact has been taken advantage of in a variety of ways. Xu et al. (1996) used this idea to construct a "segmentally" labeled *Caenorhabditis elegens* spliced leader RNA for NMR studies in which sections of the RNA was isotopically labeled. This was accomplished by means of synthesizing of a fully labeled and unlabeled version of the RNA, cleaving them at the same position using this technique, and finally religating a labeled section onto an unlabeled section (and vice versa). In this manner, the secondary structure of the RNA could be unambiguously assigned.

Yu and Steitz (1997b) used this technique to introduce a 4-thiouridine (^{4S}U) nucleotide into a pre-mRNA substrate. The ^{4S}U nucleotide is then used as a structural probe by its propensity to crosslink when exposed to UV light. The pre-mRNA molecule is first cleaved site specificially using this method, then the ^{4S}U nucleotide is added to the 5' half of the RNA with T4 RNA ligase (a template free reaction). The 5' and 3' half RNA are then ligated using a DNA guide template and T7 DNA ligase. The final product contains a single ^{4S}U nucleotide at any desired position within the molecule.

2.4.2 Detection of 2'-O-methyl sites in RNA

Yu *et al*. (1997a) also used this technique as a method of detecting sites of 2'-O-methylation in RNA molecules. Since the cleavage of RNA by RNase H is presumed to go through a 2'-O-P-O-3' intermediate, they assumed that if the 2' hydroxy of the target RNA were blocked with a methyl group, the reaction would not occur. They were indeed able to detect sites of 2'-O-methylation, in both chemically synthesized RNAs with known sites of methylation and in biologically interesting RNA with unknown sites.

2.4.3 The RNase H enzyme source affects the cleavage position

The work of Yu and Steitz (1997a,b), however, did raise one question about the technique. They found that the cleavage position for their chimeric constructs composed of four deoxyribonucleotides was at a position one nucleotide in the 5' direction on the target RNA, as shown below in figure 2.8B.

Figure 2. 8 RNase H cleavage positions for different enzyme sources

A) Pharmacia (cat. # 27-0894), Sigma (cat. # R-6501) or Takarashuzo RNase H

RNA: 5′——NNNNNNNNNNNN↓NNNN——3′
2'-O-methyl RNA/DNA chimera: 3′—NNNNNNN**NNNN**NNN—5′

B) Boehringer Mannheim (cat. # 786 349) RNase H four deoxyribonucleotides

RNA: 5′——NNNNNNNNNNNN↓NNNN——3′
2'-O-methyl RNA/DNA chimera: 3′—NNNNNNN**NNNN**NNN—5′

C) Boehringer Mannheim (cat. # 786 349) RNase H three deoxyribonucleotides

RNA: 5′——NNNNNNNNNNNNN↓NNN——3′
2'-O-methyl RNA/DNA chimera: 3′—NNNNNNNN**NNN**NNN—5′

Underlined characters, N, represent 2'-O-methylated RNA. Bold characters, **N**, represent DNA. Regular characters, N, represent RNA. The arrow, ↓, indicates the position of cleavage.

While the gel data (Fig. 2.5) clearly shows that the cleavage position is as demonstrated above in figure 2.8A, one of Jing Xu's NMR experiments on her segmentally labeled RNAs unequivocally demonstrates that we had correctly assigned the cleavage position (Fig. 2.9). The difference in positioning was finally understood when the source of the enzymes used in each study was examined. In all the studies previous to the Steitz experiments, the RNase H enzyme source was from either Pharmacia, Sigma or the Takarashuzo companies. The Steitz lab had used Boehringer Mannheim enyzme. An

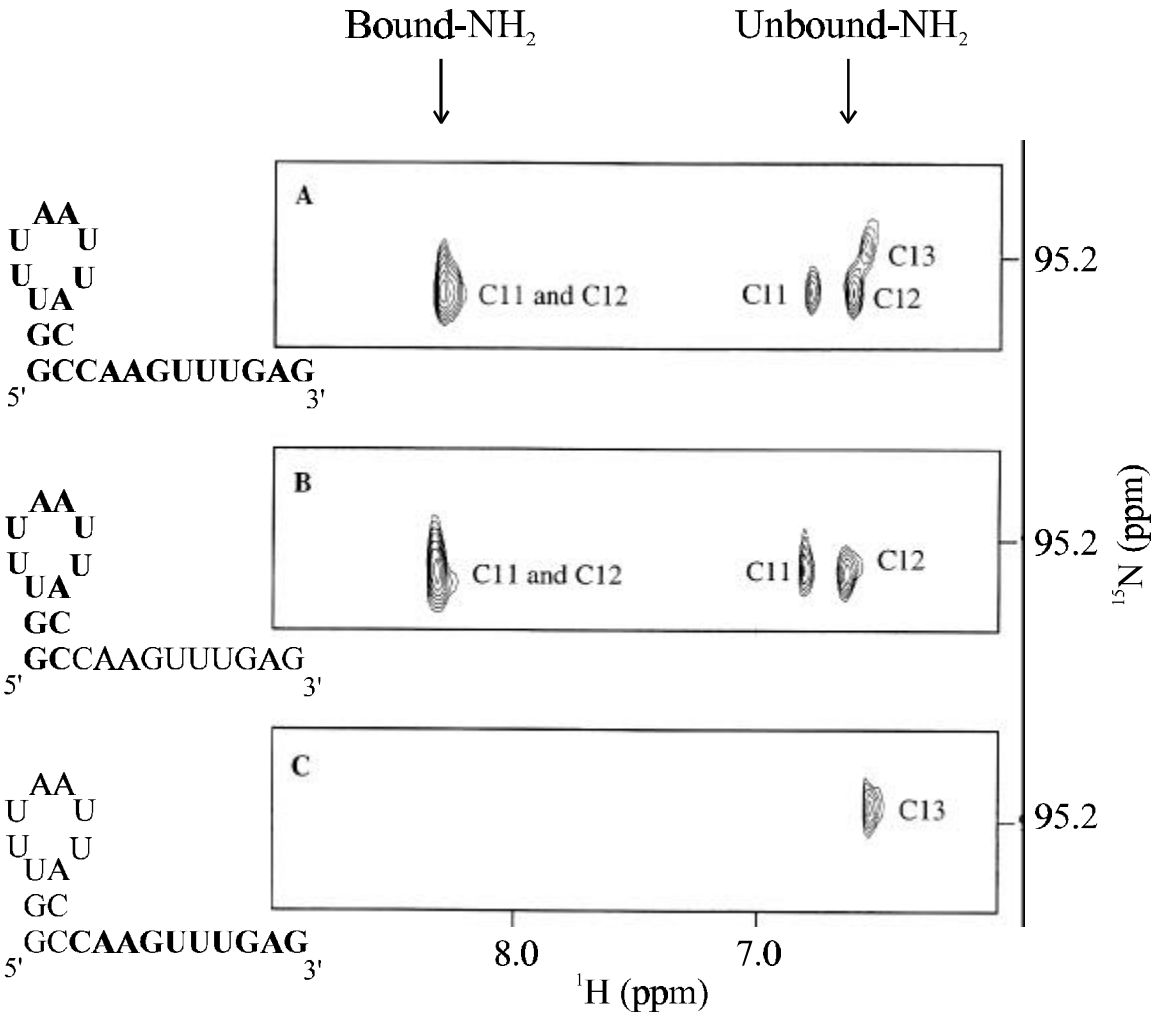


Figure 2. 9 Site specificity of the RNase H cleavage as seen by NMR

Figure kindly provided by Jing Xu (1997). The amino region of ^{1}H-^{15}N HMQC from the 22 nt RNA "CEDONOR" which was synthesized segmentally labeled with ^{15}N (Xu, 1996). **A)** Fully ^{15}N labeled CEDONOR, **B)** the 5' half labeled RNA and **C)** the 3' half labeled RNA. The three amino assignments, C11, C12 and C13 are shown in the figure. It is clear that the two base paired aminos from C11 and C12 are present in the 5' half labeled sample and that the unbase paired C13 is present in the 3' half labeled sample.

experiment in which the same RNase H cleavage reaction was performed side-by-side, except one reaction used Pharmacia RNase H and one used Boehringer Mannheim RNase H. The results (Lapham, *et al*., 1997) (data not shown) were that indeed the position of the cleavage was different by one nucleotide. While it is unknown what the exact reason is for the differences, we do note that the storage buffer for the Boehringer Mannheim RNase H does not contain EDTA and is relatively low in salt concentration compared to the other enzymes. Therefore, when precise cleavage using chimeric oligonucleotides is required, we recommend caution in the construction of the oligonucleotides and in the choice of supplier of enzyme.

2.5 Materials and methods

2.5.1 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 μmole quantities. The three 2'-O-methyl RNA/DNA chimeras were synthesized by the Keck Foundation Oligonucleotide Synthesis Facility at Yale University in 1 μmole quantities. All oligonucleotides were purified by electrophoresis on denaturing 15% polyacrylamide gels. The sequences and names of these chimeras are as follows:

Abbreviation	Full Name	Sequence (5' - 3') (**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)

2.5.2 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 nanomole acid-soluble RNA nucleotide in 20 minutes 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 (Grodberg & Dunn, 1988; Davanloo *et al.*, 1984; Zawadzki & Gross, 1991).

2.5.3 T7 RNA Polymerase Transcriptions

All RNA transcriptions utilized a bottom strand DNA template coding for the RNA plus a 5' 17 nucleotide T7 RNA polymerase promoter sequence. The top strand DNA template was complementary to the 17 nucleotide 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 (Nikonowicz *et al*., 1992; Batey *et al*., 1992), modified as described below. The reaction conditions for the transcriptions were 40 mM Tris HCl (pH 8.3 @ 20° C), 5mM DTT, 1mM spermidine, 20 mM $MgCl_2$, 0.01% NP-40, 50 mg/ml PEG 8000, 2mM in each rNTP, 200nM DNA template, and 0.1 mg/ml T7 RNA polymerase. All reactions were carried out at 37° C for 4-8 hours. Products of the transcriptions were purified by 15% denaturing PAGE.

Comparisons of transcription yields between r3lig and rLDRr3lig, shown in fig. 2.2, were carried out by analyzing 20 µl transcriptions spiked with 5 µCi of α-^{32}P-UTP, run on 15% denaturing gels, and quantitated by phosphorimager (Fuji Inc., Fujix 2000) analysis. Calculations of transcription yields for the body α-^{32}P-UTP labeled RNAs included a correction factor for the number of uridines in the sequence.

2.5.4 ^{15}N NTP isolation and purification

We used the methods of Batey *et al*. and Nikonowicz *et al*. (1992; 1992), with modification of the method of isolation of nucleic acids from the cell extract. *E. 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 brand sonicator at its highest power setting for 4 minutes, allowed to cool on ice for 5 minutes, then the procedure was repeated 3 times. This slurry was then extracted once with 25:24:1 equilibrated phenol (pH 8.0) : chloroform : isoamyl alcohol at 60° C for 30 minutes with constant stirring. The mixture was centrifuged, and the aqueous phase removed and saved. The phenol layer was back extracted once with 1/2x volume STE buffer, the aqueous phase removed, and pooled with that from the first extraction. The pooled aqueous phase was extracted 3 times with 1/2x 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 1x 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 minute and snap cooled in ice**.** 10 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 hrs. The desalting procedures and conversions to ribonucleotide triphosphates were identical to those published previously (Nikonowicz *et al*., 1992; Batey *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.

2.5.5 Cleavage of RNA with the 2'-O-methyl RNA/DNA chimeras in solution

All RNase H cleavage reactions contain 20 mM HEPES-KOH pH 8.0, 50 mM KCl, and 10 mM $MgCl_2$. The chimera was annealed to RNA by heating to 90° C and slowly cooling to room temperature at high concentration, typically in the millimolar range. The chimera was kept at 1.2 times the RNA concentration to insure complete hybridization of the RNA. RNase H was added to a final 20 units per 100 µl reaction. Hoefer Scientific Mini Gels were used to follow the large scale reactions to completion, as shown in fig. 3. The reaction typically takes between 30 minutes to 3 hours and denaturing PAGE was utilized to purify the products.

2.5.6 Cleavage of RNA with an immobilized biotin labeled 2'-O-methyl chimera

B2'LDR was bound to streptavidin beads (Pierce, ImmunoPure 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 µg/ml glycogen, 1 mg/ml BSA, 100 µg/ml tRNA, 33% 50 mM Wash Buffer). 2.0 mL 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. 500 µl of preblock mix was added and mixed slowly with the beads for 20 minutes at 4 °C. The preblock mix was removed and the beads were rinsed 3 times with 500 µl of the 50 mM wash buffer. 45 nmoles of the biotinylated chimera B2'LDR (50 µl at 0.9 mM) were added to the beads with 500 µl of the 250 mM wash buffer for 90 minutes at 4° C. The supernatant was removed from the beads and washed 3 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 nmoles 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 5x 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 5x 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 minute and slow cooled before adding enzyme. Reactions ran for 3 hours 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 fig. 2.4. 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.

2.5.7 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 (6M urea, 1mM Tris-HCl pH 7.6, 0.1 mM EDTA, and 20% acetonitrile) to bead material for 30 minutes 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.

2.5.8 Analysis of RNA After RNase H Cleavage

To analyze cleavage products, the RNA was 5' end labeled by sequential dephosphorylation with calf intestine phophatase and kinased with polynucleotide kinase and γ-^{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. 2.3a). 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 carried out 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 nucleotides 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 minutes 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 percent and are consistent with typical RNA ligation yields.

2.5.9 NMR Procedures

NMR samples were dialyzed repeatedly against 20 mM phosphate buffer at 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 spectrum shown (see Fig. 2.5) was collected on a General Electrics Omega 500 spectrometer using a Bruker Instruments ^{1}H, ^{13}C, ^{15}N triple resonance probe with X, Y, Z pulsed field gradient coils. The ^{1}H - ^{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 hours of spectrometer time to collect 128 experiments of 64 scans. All NMR data was processed on a Silicon Graphics workstation using Biosym Technologies' Felix v2.3 NMR processing software.

2.6 References

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