Preparation of $^{13}$C and $^{15}$N labelled RNAs for heteronuclear multi-dimensional NMR studies

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ABSTRACT

A procedure is described for the efficient preparation of isotopically enriched RNAs of defined sequence. Uniformly labelled nucleotide 5' triphosphates (NTPs) were prepared from E.coli grown on $^{13}$C and/or $^{15}$N isotopically enriched media. These procedures routinely yield 180 μmoles of labelled NTPs per gram of $^{13}$C enriched glucose. The labelled NTPs were then used to synthesize RNA oligomers by in vitro transcription. Several $^{13}$C and/or $^{15}$N labelled RNAs have been synthesized for the sequence r(GGCGCUUGCGUC). Under conditions of high salt or low salt, this RNA forms either a symmetrical duplex with two U • U base pairs or a hairpin containing a CUUG loop respectively. These procedures were used to synthesize uniformly labelled RNAs and a RNA labelled only on the G and C residues. The ability to generate milligram quantities of isotopically labelled RNAs allows application of multi-dimensional heteronuclear magnetic resonance experiments that enormously simplify the resonance assignment and solution structure determination of RNAs. Examples of several such heteronuclear NMR experiments are shown.

INTRODUCTION

The discovery that RNAs perform catalytic functions (1, 2) has led to searches for additional roles of RNA in biological systems. A wealth of chemical and biological studies have enormously increased our knowledge of the functional variations of RNAs in cells (3). Many of these studies indicate that the biological activity of an RNA requires that it fold into a precise three-dimensional structure (4). However, very little direct structural data presently exists for RNAs.

X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are currently the only methods available for determining high resolution structures of biomacromolecules (4, 5). Whereas a large number of protein structures have been solved by crystallography, there are only a few RNA crystal structures. The primary reasons for this have been difficulties in obtaining large quantities of pure RNAs of defined sequence and difficulties in generating RNA crystals that diffract to high resolution.

Improved methods for chemical synthesis (6) or in vitro transcription (7) of RNAs have overcome the problem of generating sufficient material, however crystallization of RNAs is still a significant challenge. Thus NMR spectroscopy presently represents the most promising approach for RNA structure determinations.

In recent years, an increasing number of solution structures of small RNAs have been determined by NMR spectroscopy (8). These structure determinations have relied primarily on homonuclear two-dimensional (2D) NMR methods (5, 8), however the very limited resolution of the sugar and aromatic resonances in the $^1$H spectra of RNAs restricts detailed structural studies to relatively small RNAs (<10kDa). The larger dispersion of the $^{13}$C and $^{15}$N chemical shifts in nucleic acids (8) make these nuclei attractive candidates for heteronuclear NMR investigations but such studies are rather impractical due to the low natural abundance of these nuclei. Thus heteronuclear NMR studies of RNA have been generally limited to selectively labelled, naturally occurring RNAs such as 5S rRNAs or tRNAs (9–12).

Here we present a general method for preparation of $^{15}$N and/or $^{13}$C enriched RNAs of defined sequence that involves coupling in vitro transcription (7) of RNAs have overcome the problem of generating sufficient material, however crystallization of RNAs is still a significant challenge. Thus NMR spectroscopy presently represents the most promising approach for RNA structure determinations. Improved methods for chemical synthesis (6) or in vitro transcription (7) of RNAs have overcome the problem of generating sufficient material, however crystallization of RNAs is still a significant challenge. Thus NMR spectroscopy presently represents the most promising approach for RNA structure determinations.

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MATERIALS AND METHODS

All enzymes were purchased from Sigma chemical (St Louis), with the exception of the T7 RNA polymerase, Deoxyribo-nuclease I (DNase I) Type II, Pyruvate Kinase (PK), Adenylate Kinase (AK), and the Nucleotide Monophosphate Kinase (NMPK) were obtained as powders and were dissolved in solutions of 15% glycerol, 1 mM dithiothreitol (DTT) and 10 mM Tris-HCl, pH 7.4 and stored at −20°C. Guanylate Kinase (GK) and Nuclease P₁ were obtained as solutions and stored at −20°C. Phosphoenolpyruvate (PEP, potassium salt) was also obtained from Sigma. The T7 RNA polymerase was prepared according to standard procedures (19). The AG-1X2 200-400 mesh anion exchange resin and the Affi-Gel 601 affinity chromatography resin were purchased from Bio-Rad. Thin layer chromatography was performed with polyethylenimine cellulose coated plates (Aldrich Chemical Co.) using a stepped gradient of 0.3 M, 1.0 M and 1.6 M LiCl. Analytical HPLC separations were performed on a Vydac 303NT405 analytical anion exchange column (Vydac Separations Group) using a gradient of 100% solvent A (0.045M NH₄COOH, pH 4.5 with H₂PO₄) to 100% solvent B (0.5M Na₂HPO₄, pH 2.7 with HCOOH) in 10 minutes.

Preparation of the isotopically enriched nucleotide 5′ triphosphates

E. coli cells (strain MY285) were grown in 1.5 l of minimal media (20) containing 3.0 g (0.2% wt/vol) 99% ¹³C enriched glucose (Isotec) and/or 0.1 g (0.005% wt/vol) 99% ¹⁵N enriched ammonium sulphate (Isotec) as the sole carbon or nitrogen sources, respectively. The cells were harvested at late log phase and frozen overnight at −80°C. The 4.0 g of wet cells were thawed and resuspended in 30 ml of buffer C (10 mM sodium acetate, 10 mM Tris-HCl, 50 μmoles PEP, 20 mM MgCl₂, 1 mM DTT and 0.2 mM 2-mercaptoethanol) (21). The cells were ruptured by passing through a French Press, 0.2 mg of DNase I was added and the cell paste was passed through the French Press two more times. The cell paste was incubated on ice for 10 minutes and then centrifuged for 20 minutes at 20,000 g in a JA-20 rotor (Beckman). The supernatant was decanted and the cell pellet was washed with 5 ml of buffer C. The combined supernatants were centrifuged at 120,000 g for 14 hours at 4°C using a Ti70.1 rotor (Beckman). This high speed supernatant was decanted and the ribosomal pellet resuspended in 3 volumes of 0.1 M sodium acetate (pH=8.0), 10 mM EDTA and 0.1% SDS. The rRNA was separated from protein by serial extractions with phenol/chloroform/isooamyl alcohol (25/24/1) and the rRNA was precipitated in 3 volumes of ethanol overnight at −20°C. The polymeric rRNA was dissolved in 8.0 ml of 50 mM sodium acetate (pH=5.5) and 0.1 mM ZnCl₂. The reaction mixture was heated to 45°C and Nuclease P₁ was added (0.3 units per mg of RNA). The degradation reaction was monitored using TLC and when complete (1-1.5 hours) the mixture was brought to 0.5 mM EDTA, heated to 90°C for 2 minutes and stored at −20°C.

At this point, the nucleotide 5′ monophosphates were either separated over an anion exchange column or used directly in the triphosphate synthesis reaction. Ion exchange separation was performed at 4°C with a mixed eluent of H₂O:ethanol (8:2) that was acidified to specific pH values using 5 M HCl. The NMP mixture was applied to an AG-1X2 anion exchange column (6 cm × 2.5 cm) equilibrated at pH=5.0, then washed with 5 column volumes of eluent (pH=2.8 at 20°C, uncorrected for ethanol). The pH of the column was then decreased 1.0 pH unit in steps of 0.1 pH units with approximately 200 ml passed through the column at each pH step. The NMPs eluted in the following order: C, A, G and U at pH values of 2.7, 2.5, 2.0 and 1.9, respectively. The pH of each fraction was set to 7.0 with 5 M KOH and the volume was reduced by rotary evaporation. Each NMP was ethanol precipitated overnight at −20°C and dried under vacuum. Both 99% ¹³N and 99% ¹⁵C/¹⁵N NMPs were generated by these methods.

The NMPs were enzymatically converted to NTPs using modifications of procedures developed by Whitesides and coworkers (15). For the separate NTP syntheses, 20 μmoles of AMP, UMP or CMP or 12 μmoles of GMP were dissolved into a 5.0 ml solution containing the following: 20 mM KCl, 80 mM Tris-HCl, 50 μmoles PEP, 20 mM MgCl₂, 1 mM DTT and 0.2 μmoles ATP (except for the AMP reaction which contained 0.1 μmoles ATP). The pH of the solution was adjusted to 6.9 using 3 M KOH. The reactions were degassed by passing nitrogen over the stirred mixtures for 30 minutes and then 80 units of PK were added to each reaction. 0.2 units and 0.4 units of GK and NMPK were added to the GTP and UTP reactions respectively. 40 units and 25 units of AK were added to the CTP and ATP reactions respectively. Each reaction was then brought to 37°C. The conversion of NMPs to NTPs was monitored by TLC. Completion time for the four reactions was 4 hr., 10 hr., and 30 hr. for ATP, CTP, GTP and UTP, respectively, and the conversion from NMPs to NTPs was confirmed by HPLC. For the NTP synthesis of the mixed NMPs, a ‘one pot’ reaction was carried out under the conditions listed above with the following modifications: A 7.5 ml reaction contained ~10 μmoles of each NMP, 100 μmoles of PEP and 0.05 μmoles of ATP. The enzyme concentrations were also modified as follows: 0.08 units GK, 18 units AK, 0.06 units NMPK and 60 units PK. The progress of the reaction was monitored by TLC and confirmed by HPLC. The NTPs from all reactions were precipitated from ethanol and in most cases could be used without further purification. In some cases the NTPs were desalted by adsorbing the NTPs to a cis-diol specific affinity column (Bio-Rad Affi-gel 601) at 4°C equilibrated with 1 M triethylamine (the pH was set to 9.2 by bubbling CO₂ through the solution) (22). The column was washed with 3 column volumes of this buffer, and the NTPs were eluted with a solution of saturated carbonic acid (pH=-4.5). The NTPs were then rotary evaporated and lyophilized. The NTPs were redissolved in H₂O and ethanol precipitated.

Preparation of the ¹⁵N or ¹³C/¹⁵N labelled RNA

RNA oligomers were prepared by in vitro transcription with T7 RNA polymerase using synthetic DNA templates as previously described (7, 13). The labelled molecules were prepared as separate 12.0 ml transcriptions using 200 nM DNA template, 1 mg T7 RNA polymerase, 40 mM Tris-HCl (pH=7.6), 30 mM MgCl₂, 5 mM DTT, 1 mM spermidine, 0.1% Triton X-100 (v/v), 2 mM GTP, 2 mM CTP and 1 mM UTP, where the NTPs were either ¹⁵N or ¹³C/¹⁵N labelled, or in the case of the partially ¹⁵N labelled RNA duplex commercial (Sigma) unlabelled UTP was used. The reaction mixture was incubated at 37°C for 5.5 hours. The RNA transcripts were precipitated from ethanol and applied to 20% polyacrylamide gels (40×60×0.3 cm) containing 7 M urea were run until the bromophenol blue dye maker reached the bottom of the gel. The bands on the gels were visualized by UV shadowing and the band

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corresponding to the full length transcript was excised and electro-eluted from the gel (13). The RNA transcript was then ethanol precipitated from the eluate overnight at −20 °C and dried. The RNA samples from the transcription reactions were dissolved in 2.0 ml of 1.0 M NaCl, 20 mM potassium phosphate (pH = 6.8), 2 mM EDTA and dialyzed against a buffer of 150 mM NaCl, 10 mM potassium phosphate (pH = 6.8) and 0.1 mM EDTA using a Centricron-3 concentrator (Amicon, Inc.). The 13C/15N labelled RNA hairpin was further dialyzed against a buffer of 2.5 mM sodium phosphate (pH = 6.8) and 0.03 mM EDTA.

NMR sample preparation

After dialysis of the 15N labelled RNAs the volume of each sample was brought to 550 μl with 90% H2O/10% D2O in 150 mM NaCl, 10 mM potassium phosphate pH = 6.8, and 0.1 mM EDTA. Immediately before each NMR experiment the NMR sample was heated to 90 °C for 2 minutes and allowed to cool slowly. The final concentrations for the 15N G/C labelled and the uniformly 15N labelled RNA duplexes were 0.45 mM (0.9 mM single strand) and 0.7 mM (1.4 mM single strand) respectively. For the 13C/15N labelled RNA samples the volume of each was brought to 550 μl with appropriate buffer, lyopholized to dryness, redissolved in 550 μl of 99.96% D2O, lyopholized and resuspended in 550 μl of 99.996% D2O to give final concentrations of 0.9 mM (1.8 mM single strand) for the duplex and 0.8 mM for the hairpin.

NMR Spectroscopy

All experiments were performed on a Varian VXR-500S spectrometer equipped with an inverse detection (1H observe/X decouple) probe (Nalorac). All the NMR data were processed on Sun or Silicon Graphics computers using the FELIX program (Hare Research Inc.).

One-dimensional 1H experiments in H2O were acquired at 15°C with a binomial 1331 pulse sequence (23). The two-dimensional 15N-HMQC experiments (24, 25) were acquired at 15°C with sweep widths of 10000 Hz in the 1H acquisition period (t1) and 1500 Hz in the 15N evolution period (t2). 16 scans and 2048 complex points were collected in t2 and 128 complex points were collected in t1 using the TPPI-States method for quadrature detection (26). Jump-return pulses were used to create the 90 and 180 degree 1H pulses in the 15N-HMQC experiments (25). 15N-WALTZ decoupling was applied during the acquisition period. A delay of 5.2 ms (optimized for 1JHN=92 Hz) was used to allow anti-phase magnetization to develop and refocus before and after the t1 delay. The time domain data was zero filled in t1 and t2 before Fourier transformation to give a final real matrix size of 512×2048 points. The proton spectra at 20°C were referenced by setting the internal water resonance to 4.76 ppm.

The 2D DQF-COSY(5) spectrum was acquired at 20°C with sweep widths of 4500 Hz in t2 and t1. 32 scans and 2048 complex points were collected in t2 and 380 complex points were collected in t1, using the TPPI-States method for quadrature detection (26). The HCC-H-COSY (27, 28) spectrum was acquired using the same conditions except that a sweep width of only 1500 Hz and 200 complex points were used in t1, 48 scans and 2048 complex points were used in t2, and 13C-WALTZ decoupling was applied during t2. The residual water signal was irradiated during the recycle delay of 1.5 s in both spectra. These time domain data were zero filled in t1 before Fourier transformation to give a final real matrix size of 1024×2048 points. The proton spectra at 20°C were referenced by setting the internal water resonance 4.76 ppm.

RESULTS AND DISCUSSION

As elegantly illustrated in protein structure determinations, a very powerful method for simplifying a complex 1H spectrum of a biopolymer is the application of heteronuclear multi-dimensional NMR techniques (29, 30). These methods have extended the size limit for which NMR solution structures of proteins can be determined to beyond 20 kDa. However, most of these NMR techniques require molecules with a high level (>95%) of 13C and/or 15N isotopic enrichment. Although in vivo biosynthetic techniques are routinely used to generate milligram quantities of 13C and/or 15N labelled proteins (31), such methods appear to have limited applications for production of RNAs since only naturally occurring and/or nuclease stable RNAs can be obtained in large quantities. The method presented here allows efficient generation of milligram quantities of isotopically labelled RNAs of defined sequence.
Preparation of isotopically labelled RNAs

The procedure used for generating the isotopically labelled RNAs is outlined in Figure 1. Polymeric rRNA was isolated from *E. coli* grown in media having $^{13}$C or $^{15}$N as the only carbon or nitrogen source, respectively. We chose to use only the rRNA in this procedure since it has a very low level of modified nucleotides (~0.5%) (32) but still makes up ~80% of the cellular RNA. Thus phenol extraction of the ribosomes provides a pool of isotopically enriched polymeric RNA that is relatively free of modifications. The rRNA is then quantitatively degraded to 5' nucleotide monophosphates by Nuclease P1 digestion. Anion exchange chromatography was used to separate the four NMPs for generating RNA oligomers labelled on only one or several nucleotide types. At this point $^1$H NMR and HPLC showed the NMPs to be pure and free of organic contaminants including dNMPs. The four NMPs were then enzymatically converted to their respective NTPs with greater than 95% efficiency using the procedures outlined in the Methods. The yield of NTPs obtained from 3 grams of glucose (4 grams of wet *E. coli* cells) was approximately 120 μmoles of UTP, 130 μmoles of ATP and CTP and 160 μmoles of GTP. Chromatographic separation of the four NMPs is not necessary if all base types in the RNA are to be uniformly labelled. Instead a one pot enzymatic synthesis can be performed with no loss of NMP to NTP conversion efficiency and this reaction is complete in ~24 hours. Normally, ethanol precipitation of the NTPs from the reaction mixture yields NTPs of sufficient purity for *in vitro* transcription reactions. However, occasionally we have found that NTPs prepared in this manner gave lower yields in test transcription reactions than control reactions with commercial (unlabelled) NTPs. We hypothesize that the lower yields are due to the presence of excess salts in the NTPs and therefore these NTPs can be further purified by desalting over a cis-diol specific affinity column as described in Methods.

$^{15}$N NMR spectroscopy

To demonstrate the applicability of the $^{15}$N heteronucleus to NMR assignment and structure determinations, we prepared two differentially $^{15}$N labelled RNA duplexes. One molecule was fully $^{15}$N labelled and the second was only $^{15}$N labelled on the

![Figure 2](image.png)

Figure 2. Imino proton region of the RNA duplex with A) $^{15}$N labelled guanine and cytidine residues and B) with no $^{15}$N labelled residues. Only the guanine imino protons exhibit the characteristic 92 Hz heteronuclear $^1$H-$^1$N J coupling since they are directly attached to a $^{15}$N nucleus. Imino proton assignments are shown above the peaks in 2B.

![Figure 3](image.png)

Figure 3. The imino region of the two-dimensional $^1$H-$^{15}$N HMQC spectrum of the RNA duplex with A) $^{15}$N labelled guanine and cytidine residues and B) with $^{15}$N labelled guanine, cytidine and uridine residues. The two imino proton resonances at 13.2 ppm are resolved in the HMQC spectra because of the different chemical shifts of the directly attached nitrogens. As previously observed (38), the guanine imino nitrogens resonate upfield of the uridine imino nitrogen resonances. The imino nitrogen peaks of the non-canonical U6-U7 base pairs resonate in the region expected for uridine imino nitrogens. The imino resonances of U6 and U7 have been identified but are not yet distinguished from each other.
G and C residues (note that there are no adenosine residues in this RNA). Figure 2A shows the imino region of the 1D $^1$H spectrum of the G and C labelled RNA duplex. Since no $^{15}$N decoupling was performed in this experiment, the imino protons on guanine residues show a distinctive 92 Hz splitting arising from the one-bond $^1$H-$^{15}$N J coupling. It is not possible to assign the imino resonances to nucleotide type in the spectrum of the unlabelled sample shown in Figure 2B, however the imino proton resonances of the guanine and uridine residues are easily distinguished in the 1D spectrum in Figure 2A.

Figure 3 extends these results by comparing the imino regions of 2D $^1$H-$^{15}$N HMQC spectra of the partially (G and C) $^{15}$N labelled and the fully $^{15}$N labelled duplexes. Since a $^1$H-$^{15}$N HMQC spectrum only generates crosspeaks between protons directly attached to $^{15}$N nuclei, the uridine resonances do not appear in the spectrum of the partially $^{15}$N labelled duplex but are easily observed in the fully labelled duplex (Figures 3A and 3B respectively). Using heteronuclear NMR techniques it is possible to select for or to filter out resonances that are directly attached to an $^{15}$N nucleus (33).

Since the structure determination of biomolecules by NMR spectroscopy relies heavily on extracting interproton distance information from $^1$H-$^1$H nuclear Overhauser effect (NOE) experiments, it is critical that as many $^1$H resonances as possible be assigned to specific atoms in the molecule. The 2D $^1$H-$^1$H NOE (NOESY) experiment has traditionally been the vehicle that provides the interproton distance information in RNA studies (5,8). However, spreading the proton spectral information in only two homonuclear frequency dimensions does not always provide adequate resolution to extract important interproton distance information in RNAs. For example, in a 2D $^1$H-$^1$H NOESY spectrum of this RNA duplex it is not possible to distinguish between NOEs involving the imino protons of G8 and G10 because these protons have identical chemical shifts (see Figure 2A). However because these two bases have different imino nitrogen frequencies (see Figure 3A) the information on the G8 and G10 imino protons are easily separated when the $^1$H-$^1$H NOE data are spread out by the $^{15}$N chemical shifts of the imino nitrogens, such as in a 3D NOESY-HMQC experiment (29,30). Thus the incorporation of isotopic labels also provides another frequency dimension that can be used to help resolve the proton spectrum. We have previously reported an application of this method to RNA structure determinations (34).

$^{13}$C NMR Spectroscopy

The ribose 2' to 5'5' protons normally resonate over a narrow spectral width of ~1.0 ppm in RNAs which leads to severe resonance overlap. This problem is dramatically reduced by

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**Figure 4.** The full aromatic and ribose regions of the $^1$H-$^{13}$C HMQC spectrum of the RNA duplex. The aromatic C8 and C6 resonances are shifted far downfield (139—145 ppm) from the C5 and the C1'-C5' resonances (64—106 ppm). Note that although the 2'—5'5' sugar proton resonances are crowded within ~1 ppm, the C1' to C5' resonances are dispersed over ~35 ppm. The multiplet structure observed in the $^{13}$C dimension is due to the 1-bond $^{13}$C-$^{13}$C J couplings.

**Figure 5.** The H1' to H2' region for the CUUG hairpin is shown. A) A DQF-COSY spectrum of the unlabelled CUUG hairpin (this molecule was made with unlabelled NTPs by standard procedures) (7,14). Only 3 H1'/H2' connectivities are observed in the spectrum due to the small H1'-H2' J coupling (see text). Both positive and negative contours are plotted. B) Identical region of the 2D HCCH-COSY spectrum of the $^{13}$C/T/$^{15}$N labelled CUUG hairpin. All H1'/H2' connectivities are easily observed in this spectrum but the H1'/H2' connectivity for U7 is in a different region of the spectrum and therefore is not seen in this plot.
application of $^{13}$C NMR techniques analogous to those employed for $^{15}$N. Thus the $^{13}$C nucleus provides another frequency dimension for resolving the $^1$H resonances. Figure 4 shows a $^1$H-$^{13}$C HMOC spectrum of the $^{13}$C/$^{15}$N labelled RNA duplex and illustrates that although the 2' to 5'/5' protons resonate in a very narrow region of the $^1$H spectrum, the 2' to 5' carbons resonate over a much larger region (~25 ppm) of the $^{13}$C spectrum (8). This $^{13}$C/$^{13}$C HMOC pulse sequence can be combined with a $^1$H-$^1$H NOE$^*$ pulse sequence to form a 3D NOE$^*$-HMOC experiment (30) or a 4D HMOC-NOE$^*$-HMOC experiment (35). We have recently described the advantages these 3D and 4D techniques for structure determinations of RNAs (34, 36).

Uniform $^{13}$C enrichment also allows very efficient through-bond correlation of proton resonances in RNAs. For example, the proton resonances of the ribose ring are easily identified via HCCH type experiments (27, 28). The techniques take advantage of the large 1-bond $^{13}$C-$^1$C (33-45 Hz) and $^1$H-$^{13}$C (~165 Hz) J couplings and have proved to be invaluable techniques for determining scalar connectivities in labelled proteins (30) and will also have important applications in assignment and structure determinations of nucleic acids. Figure 5 shows a comparison of the H1' to H2' connectivities observed in a standard $^1$H-$^1$H 2D DQF-COSY spectrum and in a HCCH-COSY spectrum. The very small (~1Hz) J coupling for the 1' to 2' protons in a RNA A-form helix (8) leads to missing or very weak crosspeaks for the nonterminal sugars in the stem of this RNA hairpin. However all the 1' to 2' connectivities are readily observed in the HCCH-COSY spectrum as illustrated in Figure 5b (the H1' to H2' connectivity for U7 occurs in a different part of the spectrum and therefore is not seen in this figure). Thus the HCCH-COSY spectrum contains much more information than the conventional DQF-COSY spectrum. We have previously demonstrated (34) that this HCCH-COSY experiment can be extended into three-dimensions to improve the resolution of NMR spectra and thus even further simplify the NMR data analysis of RNAs.

CONCLUSION

Uniformly isotopically enriched RNAs can be efficiently prepared from enriched NTP building blocks using in vitro transcription with T7 RNA polymerase. The uniformly labelled NTPs are prepared in high yields by enzymatic conversion of NMPs to NTPs, with the labelled NMPs being biosynthetically prepared from E.coli. The incorporation of $^{13}$N helps resolve the imino proton spectrum of RNAs and thus should aid in the identification and assignment of both Watson-Crick and non-standard base pairs (4). We have also shown that selective labelling by nucleotide type helps simplify NMR spectra of RNA. Tinoco and coworkers (37) have recently shown how $^{13}$C heteronuclear experiments aid in the assignment and structure determination of RNAs, however these studies were performed at natural abundance and therefore require large amounts of NMR time (>24 hours for a $^{13}$C-HMOC spectrum). For 99% $^{13}$C labelled RNAs the acquisition time of this experiment can be reduced to less than 15 minutes. Thus the high sensitivity afforded by the isotopic labelling techniques allows application of a wide range of extremely powerful heteronuclear magnetic resonance experiments that are impossible at natural abundance. For example, incorporation of $^{13}$C dramatically simplifies the aromatic and sugar proton spectral regions in RNAs by application of 3D (34) and 4D NMR experiments (36). These NMR experiments enormously facilitate resonance assignment and extraction of NMR structural data on RNAs and therefore will make it possible to determine high resolution structures of larger RNAs.

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