Preparation of Specifically Deuterated and ^{13}C-Labeled RNA for NMR Studies Using Enzymatic Synthesis†

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Abstract: The enzymatic conversion of glucose into ATP, GTP, UTP, and CTP with several different isotopic labeling patterns is described. Enzymes of the pentose phosphate pathway and enzyme-catalyzed hydrogen exchange were used to convert three types of isotopically labeled glucose into [1′,2′,3′,4′,5′,5′-^{2}H$_6$]NTPs (**1−4**), [3′,4′,5′,5′-^{2}H$_4$]-UTP (**5**), [1′,2′,3′,4′,5′-^{13}C$_5$]NTPs (**6−9**), and [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]NTPs (**10−13**), which were then used to synthesize a 30 nucleotide HIV TAR RNA. Representative NOESY and HSQC spectra were acquired to demonstrate the utility of the new labeling patterns. The spectral editing afforded by ^{2}H and ^{13}C labeling dramatically simplifies the crowded NOESY and HSQC spectra of RNA molecules. The synthetic methods described here will permit the preparation of several specifically deuterated and/or ^{13}C-labeled forms of RNA which should be useful in NMR structural studies of large RNAs.

Introduction

Application of ^{13}C and ^{15}N labeling to protein and RNA NMR spectroscopy has made the study of proteins of up to 20−25 kDa and RNAs of up to 30−40 nucleotides[1] routine, but beyond these molecular weight limits, rapid decay of NMR signals makes heteronuclear experiments very inefficient.[2−7] Both deuteration and heteronuclear labeling have been used to combat the spectral crowding and relaxation problems which interfere with NMR structural studies of large macromolecules. Substitution of deuterium for hydrogen can both remove specific resonances from NMR spectra and greatly reduce dipole−dipole relaxation.[8−17] Random fractional deuteration of proteins and RNA molecules can greatly alter the relaxation properties of

the remaining protons, resulting in sharper lines and more efficient magnetization transfer, but suffers from the drawback of decreased sensitivity. In contrast, specific deuteration reduces spectral crowding and the relaxation rate of the remaining protons, but the sensitivity of the remaining protons is not compromised. Recently, heteronuclear labeling and deuteration have been combined to successfully study proteins on the order of 40−60 kDa.[18] This approach has also been applied to RNA in the form of random fractional deuteration of RNA in combination with heteronuclear labeling,[19,20] although the benefit of this strategy is probably less than that obtained for proteins. In an attempt to ameliorate the problems of large RNA NMR spectroscopy, we have developed isotopic labeling strategies that will allow a high degree of spectral editing and alteration of relaxation properties while still retaining important sequential connectivity information.

In general large isotopically labeled RNAs are prepared by T7 RNA polymerase catalyzed transcription reactions directed by a DNA template using labeled nucleoside triphosphates (NTPs)[21,22] that are produced from bacterial growth on inexpensive isotopically labeled starting materials such as [^{13}C]glucose, [^{13}C]methanol, or [^{15}N]ammonium sulfate.[23,24] Although some specific isotopic labeling patterns can be produced using

† Abbreviations used: NTP, nucleoside triphosphate; NOE, nuclear Overhauser effect; PRPP, 5-phospho-D-ribosyl α-1-pyrophosphate; PEP, phosphoenolpyruvate; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; calcd, calculated; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; LB medium, Luria−Bertani medium; OD, optical density; UPRT, uracil phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; XGPRT, xanthine−guanine phosphoribosyltransferase; TEABC, triethylammonium bicarbonate; 3PGA, 3-phosphoglycerate; PP$_i$, inorganic pyrophosphate; DTT, dithiothreitol; Glu, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; d_6-NTPs, [1′,2′,3′,4′,5′,5′-^{2}H$_4$]NTPs; d_4-NTPs, [3′,4′,5′,5′-^{2}H$_4$]NTPs; ^{13}C$_5$-ribose-NTPs, [1′,2′,3′,4′,5′-^{13}C$_5$]NTPs; d_4-^{13}C$_5$-ribose-NTPs, [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]NTPs; d_6-RNA, RNA prepared from d_6-NTPs (**1−4**); d_4/d_6-RNA, RNA prepared from d_4-UTP (**5**) and d_6-NTPs (**1, 2, 4**); ^{13}C$_5$-ribose-RNA, RNA prepared from ^{13}C$_5$-ribose-NTPs (**6−9**); d_4-^{13}C$_5$-ribose-RNA, RNA prepared from d_4-^{13}C$_5$-ribose-NTPs (**10−13**).

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this method,[25] they are limited to certain types of labeling and difficult to control. Alternatively, chemical synthesis of isotopic labels in ribose moieties and subsequent conversion into nucleosides or nucleotides permits a great deal of control in the placement of isotopic labels,[17,26,27] but with the offsetting additional work and expense of a multistep synthesis. To efficiently and economically incorporate a wide variety of specific deuterium and ^{13}C labeling patterns into RNA, enzymes from the pentose phosphate pathway in combination with enzymes from nucleotide biosynthesis and salvage pathways can be utilized to convert isotopically labeled glucose into nucleotides in a single coupled enzymatic reaction. This approach has been used by Schramm and co-workers to produce several isotopically labeled forms of AMP and NAD$^+$ including [1'-^{2}H-, 5'-^{3}H]AMP, [5'-^{14}C]AMP, and [H$_N$1'-^{3}H]NAD$^+$,[28–30] and it has also been used to incorporate ^{13}C-labeled glucose into UTP.[31] Here we report the combination of these routes with our recently reported procedures to convert ribose into the four NTPs on a preparative scale[17] to produce isotopically labeled RNA from glucose.

Glucose is an attractive starting material for incorporation of isotopic labels into the ribose moieties of RNA for NMR studies because it is commercially available in a variety of ^{13}C- and/or deuterium-labeled forms, including uniformly deuterated, uniformly ^{13}C-labeled, and uniformly ^{13}C- and ^{2}H-labeled. In addition, it has been shown that glucose-6-phosphate isomerase, 6-phosphogluconic dehydrogenase, and ribose-5-phosphate isomerase can be used to exchange the protons on glucose which eventually become the H1' and H2' protons on nucleotides.[29,30,32] Many isotopic labeling patterns that will be useful for RNA NMR studies can be created by combining the wide range of commercially available isotopically labeled glucose with the ability of exchanging hydrogen or deuterium into the 1' or 2' position of nucleotides. We have synthesized nucleotides and RNAs with several isotopic labeling patterns and conducted a few basic NMR experiments on them to demonstrate the flexibility and utility of these methods.

Results and Discussion

(A) Enzymatic Synthesis. Our strategy for the enzymatic synthesis of isotopically labeled RNAs was to utilize enzymes of the pentose phosphate pathway, shown in Figure 1, to convert glucose into 5-phospho-D-ribosyl α-1-pyrophosphate (PRPP), and also to exchange the H1' and H2' protons with solvent,[28–32] and then to convert PRPP enzymatically into the four nucleoside triphosphates required for RNA.[17] The enzymes of the pentose phosphate pathway required to convert glucose into PRPP are all commercially available. Of the remaining enzymes required to convert PRPP into the four NTPs, only adenine phospho-

ribosyltransferase (APRT), xanthine–guanine phosphoribosyltransferase (XGPRT), CTP synthetase, and uracil phosphoribosyltransferase (UPRT) are not commercially available in high activity forms. The purifications of the first three enzymes have been described previously,[17] and the cloning and purification of UPRT is described in the Experimental Section of this paper.[33,34]

To reduce the number of steps required to produce labeled RNA from glucose, we attempted to produce all four nucleotides from glucose in one reaction, rather than synthesize each nucleotide separately as we have reported previously. Since all four nucleotides are mixed together during transcription reactions, simultaneous synthesis of the nucleotides together would reduce the number of reactions and purifications necessary to produce uniformly labeled RNA. Unfortunately, CTP synthetase, which is required to convert UTP into CTP, is inhibited by some component or combination of components in the multienzyme reactions that form NTPs from glucose,[17,35,36] so it was not possible to produce CTP from glucose directly. Instead, it was necessary to convert glucose into ATP, GTP, and UTP in a single coupled enzymatic reaction, and then half of that purified reaction mixture could be used to convert UTP into CTP in a separate reaction. In this way, all four NTPs can be produced from glucose in only two reactions.

To enzymatically convert glucose into nucleoside triphosphates, five phosphate equivalents are supplied by ATP and phosphoenolpyruvate (PEP), and two oxidizing equivalents are supplied by NADP$^+$. To prevent dilution of the isotopic label and keep the cost of the reactions down, the cofactors ATP and NADP$^+$ were added in catalytic amounts and regenerated during the nucleotide forming reactions. The ATP that was consumed during the enzymatic reactions was regenerated by a PEP/pyruvate kinase/myokinase system where the phosphoenolpyruvate (PEP) used to drive the reaction was generated *in situ* from an excess of 3-phosphoglycerate (3PGA).[37,38] The NADP$^+$ required for oxidation of glucose-6-phosphate (G6P) and 6-phosphogluconate (6PG) was regenerated by reductive amination of α-ketoglutarate with NADPH and ammonia catalyzed by glutamic dehydrogenase.[30]

Enzyme-Catalyzed Hydrogen Exchange. The H1' proton of NTPs can be exchanged with solvent during enzymatic synthesis by using glucose-6-phosphate isomerase, as shown in Figure 1.[29] G6P isomerase catalyzes the isomerization of a C1 aldose (G6P) into a C2 ketose (F6P) through an enediolate intermediate (Figure 2a). The mechanism of the isomerization involves a protein base abstracting a C2 proton and then replacing it on the C1 for the conversion of the aldose into the ketose. While the abstracted proton resides on the protein there is the possibility of exchange with solvent, and if the isomerization is carried out for a long enough time, complete exchange of the C2 proton can be achieved. The proton that will become the H1' of NTPs is exchanged with solvent when the proton on the C2 of G6P is exchanged. This is because the pentose phosphate pathway oxidatively removes the C1 of glucose, making what was the C2 of glucose into the C1 of ribose-5-phosphate (Figure 1). The H1' exchange can be accomplished by phosphorylation of glucose with hexokinase in the presence

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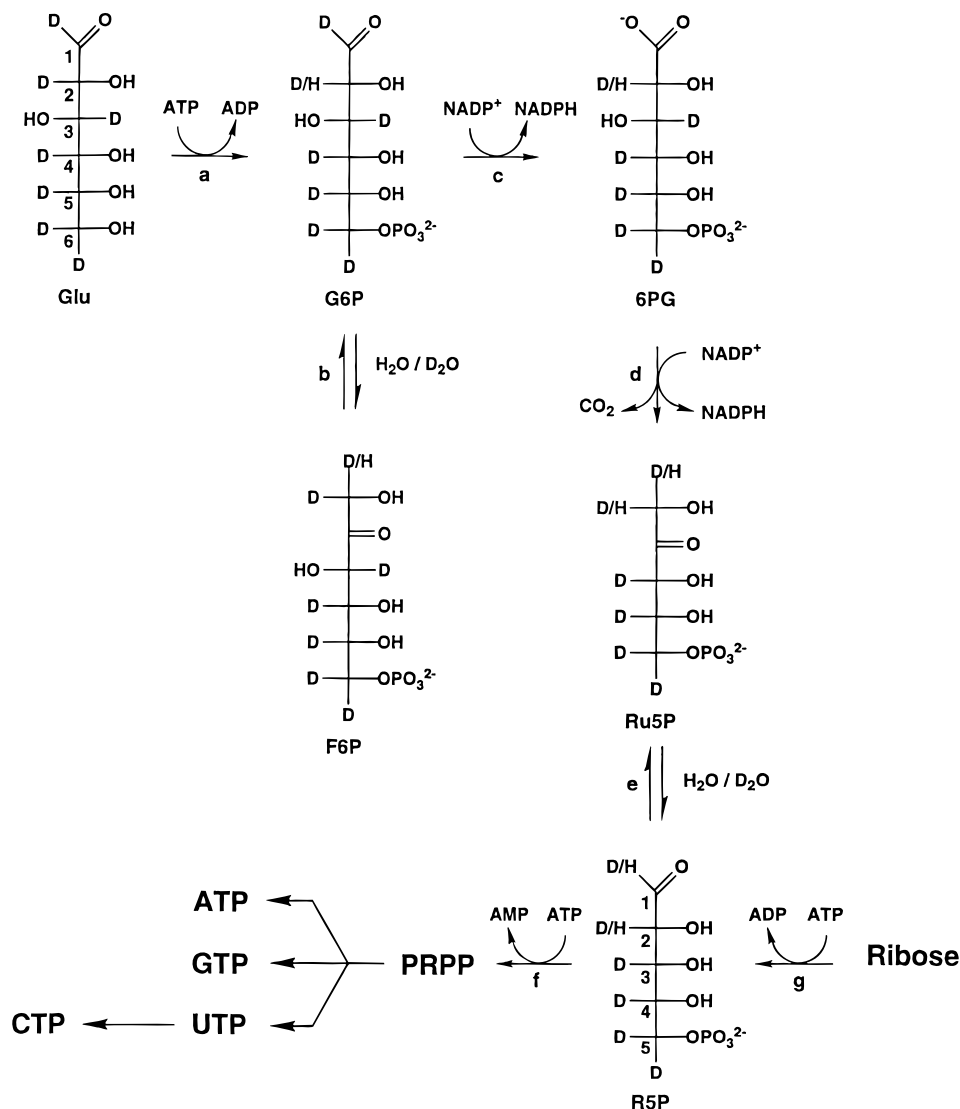
Figure 1. Enzymatic conversion of fully deuterated glucose into PRPP showing possible hydrogen exchange of what will become the H1′ and H2′ of NTPs and subsequent conversion of PRPP into the four NTPs of RNA: (a) hexokinase, (b) glucose-6-phosphate isomerase, (c) glucose-6-phosphate dehydrogenase, (d) 6-phosphogluconate dehydrogenase, (e) ribose-5-phosphate isomerase, (f) PRPP synthetase, and (g) ribokinase.

of glucose-6-phosphate isomerase, allowing sufficient time to pass before beginning the conversion of glucose-6-phosphate into NTPs. In addition, elevating the temperature of the reaction to 34 °C facilitates complete exchange with solvent. Approximately 60% H1′ exchange with solvent was observed in the reaction that was used to produce nucleotides **10**−**12** when the exchange reaction was carried out at room temperature for 2 days. In contrast, 100% exchange of the H1′ was observed in the [3′,4′,5′,5′-^{2}H$_4$]UTP (**5**) forming reaction when it was heated to 34 °C for 20 h while exchanging the H1′ for a total of approximately 2 days.

Exchange of the proton destined to become the H2′ proton of NTPs with solvent is unavoidable when using the pentose phosphate pathway to convert glucose into NTPs. This is because 6-phosphogluconate dehydrogenase removes the only proton on the C3 of 6-phosphogluconate during the oxidation of the 3 hydroxyl group by NADP$^+$ (Figure 2b). During the decarboxylation of the resulting β-keto carboxylic acid, a solvent proton is stereospecifically placed on the C1 of ribulose-5-phosphate (Ru5P).[32] This former solvent proton is stereospecifically removed from the C1 of Ru5P by ribose-5-phosphate isomerase and replaced onto the C2 to produce ribose-5-phosphate (reverse of the general reaction in Figure 2a). This results in the H2 of R5P being equilibrated with solvent as a

side effect of the enzymatic conversion of 6-phosphogluconate to R5P. Because of this, the solvent that the oxidation and isomerization are conducted in must be the same hydrogen or deuterium composition that the H2′ is to be labeled with. When ^{1}H labeling of the H2′ is desired, the reactions simply need be conducted in H$_2$O, but when deuterium labeling is desired, the chemicals and enzymes must be exchanged with D$_2$O and the reactions must be conducted in D$_2$O to achieve a high level of deuteration.

(B) Preparation of Specific Isotopic Labeling Patterns from Glucose. The use of glucose as a starting material for isotopic labeling of RNA allows several different isotopic labeling patterns to be created from glucose with only slight variations in the same general reaction conditions. By simply changing the type of isotopically labeled glucose that is used as a starting material, whether glucose-6-phosphate isomerase is added to the reaction, and whether the reaction is conducted in D$_2$O or H$_2$O, many different labeling patterns can be derived, as outlined in Figure 3. We have used this general strategy to produce three new isotopic labeling patterns and a more efficient synthesis of a previously reported labeling pattern.[17]

The synthesis of [1′,2′,3′,4′,5′,5′-^{2}H$_6$]-[ATP (**1**), GTP (**2**), UTP(**3**)] (d_6-NTPs) was achieved in a single reaction beginning with [1,2,3,4,5,6,6-^{2}H$_7$]-D-glucose. No glucose-6-phosphate
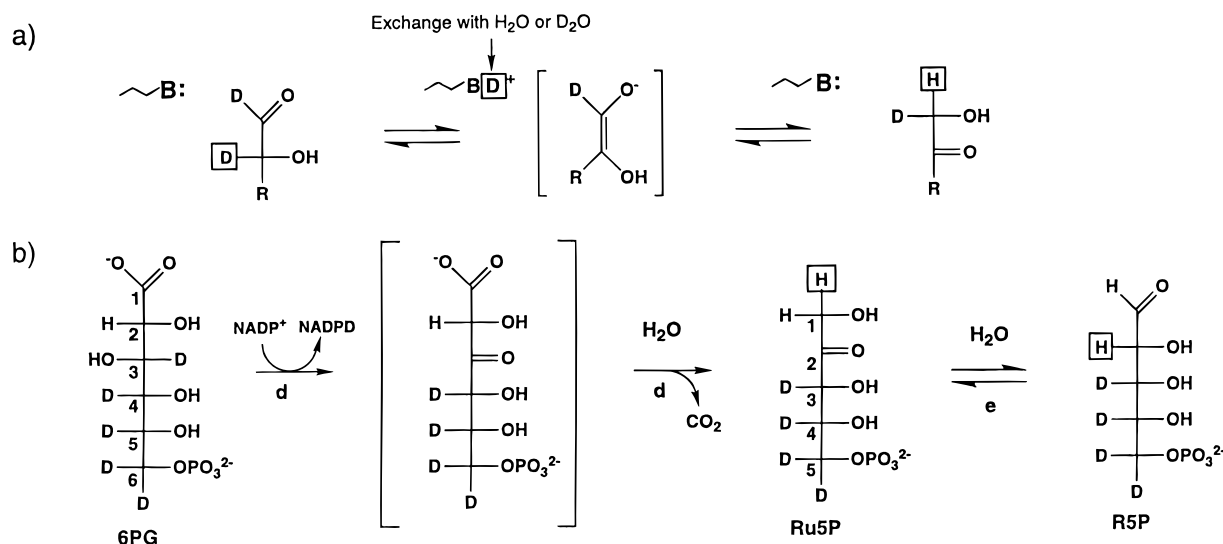
Figure 2. (a) General mechanism for glucose-6-phosphate and ribose-5-phosphate isomerases showing exchange of sugar proton with solvent while it resides on a protein base. (b) Enzymatic conversion of 6-phosphogluconate into ribose-5-phosphate catalyzed by 6-phosphogluconate dehydrogenase and ribose-5-phosphate isomerase (enzymes d and e in Figure 1). The boxed hydrogen arises from solvent.
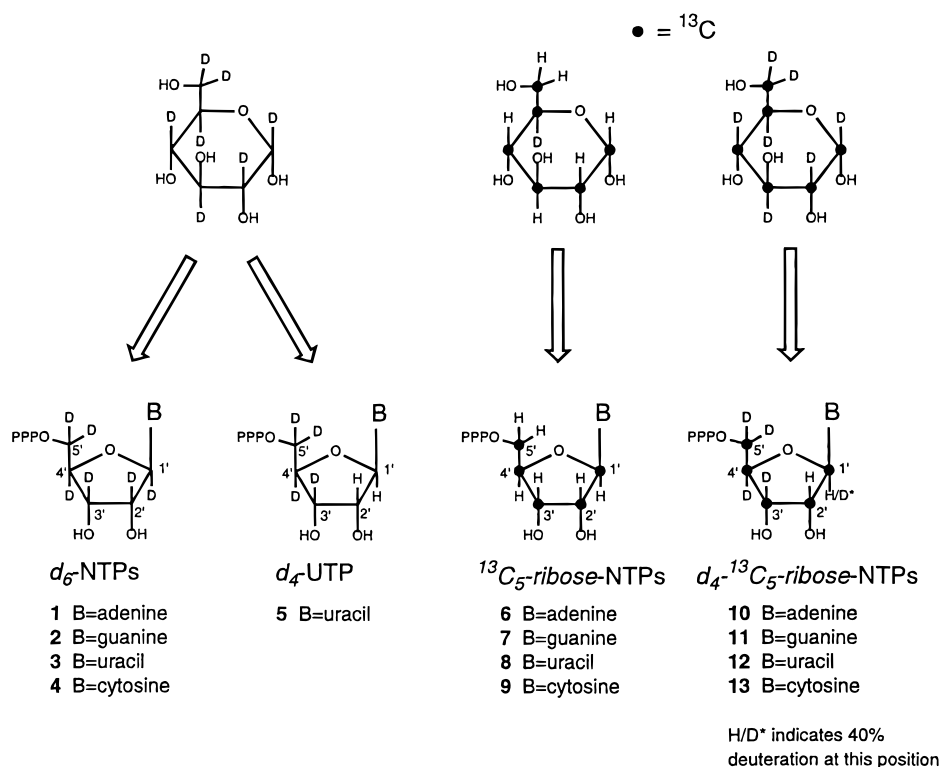
Figure 3. Different isotopically labeled NTPs synthesized from labeled glucose in this paper.

isomerase was added to this reaction since no exchange of the H1′ of the NTPs was desired. The reaction was conducted in D_2O, and the enzymes and chemicals used in the reaction were exchanged into D_2O to insure a high level of deuteration at the H2′ of NTPs. In this reaction, 84% of the starting $[^2H_7]$glucose was converted into d_6-ATP (**1**), d_6-GTP (**2**), and d_6-UTP (**3**). d_6-CTP (**4**) was prepared from d_6-UTP (**3**) in a separate reaction catalyzed by CTP synthetase.

The synthesis of $[3′,4′,5′,5′-^2H_4]$UTP (**5**) (d_4-UTP) from $[1,2,3,4,5,6,6-^2H_7]$-D-glucose was carried out in H_2O so that the protons that would become the H1′ and H2′ of ribose were exchanged with solvent by the action of glucose-6-phosphate isomerase, 6-phosphogluconic dehydrogenase, and ribose-5-phosphate isomerase. First, glucose was phosphorylated by hexokinase, and once glucose-6-phosphate had been formed,

the reaction was heated to 34 °C in the presence of glucose-6-phosphate isomerase to speed the exchange of the H2 of glucose-6-phosphate. The reaction was then cooled, and the remaining enzymes and chemicals required for UTP formation were added. This reaction converted 67% of the starting $[^2H_7]$glucose into d_4-UTP (**5**).

The synthesis of $[1′,2′,3′,4′,5′-^{13}C_5]$-[ATP (**6**), GTP (**7**), UTP (**8**)] ($^{13}C_5$-ribose-NTPs) was effected in a single reaction from $[1,2,3,4,5,6-^{13}C_6]$-D-glucose. No glucose-6-phosphate isomerase was added to this reaction since no exchange of the H1′ of the NTPs was desired. The reaction was conducted in H_2O since the desired labeling of the H2′ of the NTPs was hydrogen. This reaction converted 88% of the starting $[^{13}C_6]$glucose into $^{13}C_5$-ribose-[ATP (**6**), GTP (**7**), UTP (**8**)]. $^{13}C_5$-ribose-CTP (**9**) was

```
          G  G
      U        G
      C        A
          C • G
          G • C
          A • U
          G • C
      U         |
      U
          A • U
          G • C
          A • U
          C • G
          C • G
          G • C
          G • C
         5'    3'
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Figure 4. Secondary structure of the 30 nt HIV-2 TAR RNA.

prepared from $^{13}C_5$-*ribose*-UTP (**8**) in a separate reaction catalyzed by CTP synthetase.

The synthesis of $[3',4',5',5'-^2H_4-1',2',3',4',5'-^{13}C_5]$-[ATP (**10**), GTP (**11**), UTP (**12**)][39] (d_4-$^{13}C_5$-*ribose*-NTPs) was achieved from $[1,2,3,4,5,6,6-^2H_7-1,2,3,4,5,6-^{13}C_6]$-D-glucose in H_2O so that the protons that become the H1′ and H2′ of ribose are exchanged with solvent by the action of glucose-6-phosphate isomerase, 6-phosphogluconic dehydrogenase, and ribose-5-phosphate isomerase. This reaction was conducted in a manner similar to the reaction that produced d_4-UTP (**5**), except for that it was not heated above room temperature during the glucose-6-phosphate isomerase step, resulting in only approximately 60% exchange of the H1′ position with H_2O. The glucose-6-phosphate exchange was carried out at room temperature for 2 days, and then the remaining enzymes and chemicals required for ATP, GTP, and UTP formation were added to the reaction. In this reaction, 92% of the starting $[^2H_7-^{13}C_6]$glucose was converted into d_4-$^{13}C_5$-*ribose*-[ATP (**10**), GTP (**11**), UTP (**12**)]. d_4-$^{13}C_5$-*ribose*-CTP (**13**) was produced in a separate reaction from d_4-$^{13}C_5$-*ribose*-UTP (**12**) with CTP synthetase.

(C) NMR of Isotopically Labeled RNA. To illustrate the utility of these isotopic labeling patterns, we have prepared a 30 nucleotide RNA derived from the HIV-2 TAR bulged loop (Brodsky, A. S.; Williamson, J. R. *J. Mol. Biol.* **1997**, *267*, 624–639), shown in Figure 4, by in vitro transcription with different combinations of the isotopically labeled NTPs prepared in this paper from glucose. NOESY and HSQC spectra of the deuterated and ^{13}C-labeled RNAs are shown to demonstrate the spectral editing effects of different deuteration patterns.

Simplification of NOESY Spectra by Specific Deuteration. d_6-TAR RNA, where all of the ribose protons are deuterated was prepared using d_6-NTPs (**1**–**4**). Since most of the strong NOEs in a NOESY spectrum of RNA (Figure 5a) arise from ribose–ribose and ribose–base NOEs, removal of the ribose protons results in a dramatic simplification of the NOESY spectrum (Figure 5b). The only remaining strong NOEs in the d_6-RNA NOESY are the intrabase pyrimidine H5–H6 cross-peaks. While this type of labeling pattern does not yield very much structural information about the RNA, it does offer the possibility of spectral simplification, either by using this labeling pattern in combination with another labeling pattern, or when studying an RNA–protein complex.

By transcribing the TAR RNA using d_6-ATP (**1**), d_6-GTP (**2**), d_6-CTP (**4**), and d_4-UTP (**5**), it is possible to create d_4/d_6-TAR RNA where the only remaining ribose protons are the H1′ and H2′ of uridine nucleotides. Since only the ribose protons have been deuterated in the d_4/d_6-RNA, and all of the base protons for A, G, C, and U are still present, all of the base to

H1′ and H2′ connectivities are still present for uridine nucleotides in the d_4/d_6-RNA NOESY. Figure 5c,d shows a comparison of uniformly labeled d_4-RNA to the d_4/d_6-RNA. There are six uridines in TAR RNA, and it is easy to locate the six strong H1′-H2′ cross-peaks and the six strong H2′-base cross-peaks in Figure 5d. While that information is also present in the d_4-RNA spectra in Figure 5c, the presence of the NOEs arising from A, G, and C ribose protons makes assignment of the uridine NOEs more difficult, although it is still a much simpler case than assigning uridine H1′ and H2′ NOEs in the unlabeled RNA (Figure 5a). The selective spectral editing shown here will be useful for simplifying the very complicated spectra of larger RNAs.

Simplification of Heteronuclear Spectra by Specific Deuteration. The TAR RNA was transcribed with $^{13}C_5$-*ribose*-NTPs (**6**–**9**) and also with d_4-$^{13}C_5$-*ribose*-NTPs (**10**–**13**) to produce ^{13}C-*ribose*-RNA and d_4-^{13}C-*ribose*-RNA. HSQC spectra of the two different types of RNA were acquired and are shown in Figure 6. As can be seen in Figure 6a, the ^{13}C NMR chemical shifts of ribose are fairly well dispersed by type of carbon, but the C2′ and C3′ carbon NMR chemical shifts overlap. The difficulty of assigning the C2′ and C3′ carbons is accentuated by the fact that the proton H2′ and H3′ NMR chemical shifts also overlap, making the identification of some of the cross-peaks difficult. The cross-peaks within the boxes in Figure 6a are some of the H3′–C3′ cross-peaks of TAR that overlap the H2′–C2′ cross-peak region. Deuteration of the C3′ of ribose in the d_4-^{13}C-*ribose*-RNA (Figure 6b) makes the identification of the H2′–C2′ cross-peaks much easier. The absence of the boxed cross-peaks in Figure 6b, which were present in Figure 6a, make it clear that they are H3′–C3′ cross-peaks. Carbon-13 labeling of RNA from glucose offers the possibility of combining the advantages of multidimensional heteronuclear NMR with the spectral editing advantages of specific deuteration. The ability of deuteration in this context allows alteration of the relaxation properties of RNA and perhaps development of new types of triple-resonance NMR experiments.[40] The ^{13}C-*ribose*-RNA offers an inexpensive and easy way to incorporate ^{13}C labels into the ribose moieties of RNA.

Conclusion

The enzymatic RNA labeling strategy described in this paper is very efficient and flexible. This method requires only two reactions to convert glucose into all four NTPs required for RNA synthesis, with yields ranging from 67 to 92% conversion. The amount of labeled glucose required to make an NMR sample is low when compared to other methods of isotopically labeling macromolecules with glucose. For instance, it has been reported that harvesting nucleoside monophosphates from *Escherichia coli* grown on isotopically labeled glucose medium results in 58 mg of NMPs per gram of glucose added.[24] In contrast, using these enzymatic methods, over a gram of isotopically labeled NMPs can be prepared from 1 g of glucose. Granted, the isotopically labeled NMPs prepared by the enzymatic synthesis will only be labeled in the ribose moieties, but this difference in yield emphasizes the gain in yield that can be obtained when isotopically labeled starting material is directly converted into molecules of interest rather than relying on cellular biosynthesis to make them as a byproduct of metabolism. By combining enzymatic conversion of labeled glucose into NTPs with enzyme catalyzed hydrogen or deuterium exchange, many diverse RNA isotopic labeling patterns that will be useful in NMR structural

(39) The $[3',4',5',5'-^2H_4-1',2',3',4',5'-^{13}C_5]$NTPs prepared in this paper are present as a 3:2 mixture of $[3',4',5',5'-^2H_4-1',2',3',4',5'-^{13}C_5]$NTPs and $[1',3',4',5',5'-^2H_5-1',2',3',4',5'-^{13}C_5]$NTPs due to partial deuteration of the H1′. The entire mixture is characterized in the experimental, but since the $[3',4',5',5'-^2H_4-1',2',3',4',5'-^{13}C_5]$NTPs give rise to the NMR spectra of interest, this mixture is referred to as d_4-$^{13}C_5$-*ribose*-NTPs.

(40) Dayie, K. T.; Tolbert, T. J.; Williamson, J. R. *J. Magn. Reson.* In press.
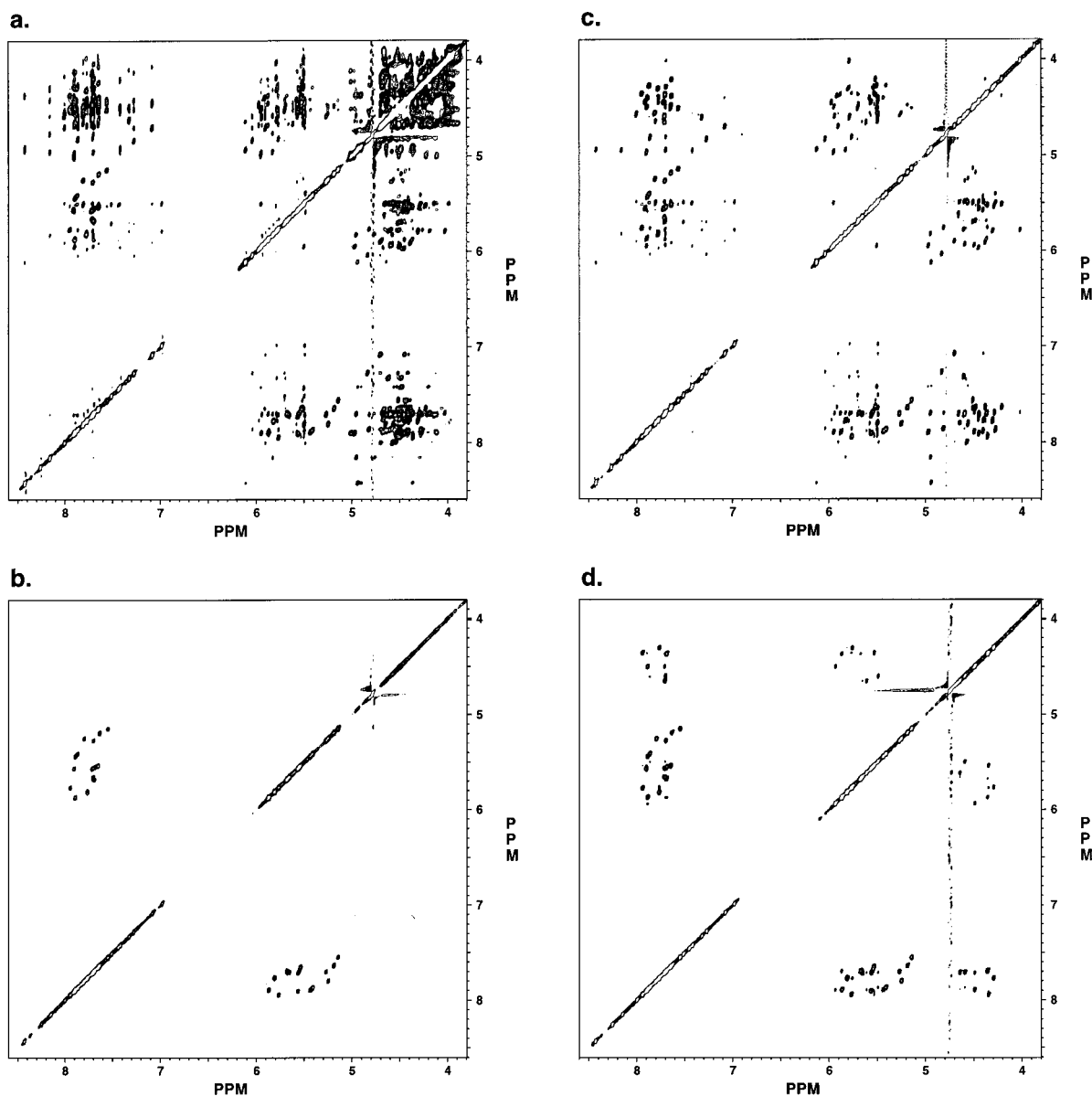
a.

c.

b.

d.

Figure 5. Spectral simplification by deuteration in proton–proton NOESY spectra of TAR RNA: (a) unlabeled TAR RNA, (b) d_6-TAR RNA, (c) d_4-TAR RNA, and (d) d_4/d_6-TAR RNA.

studies can be produced with a minimum amount of effort. Several specifically deuterated forms of RNA can be produced by exchanging either hydrogen or deuterium into the H1′ or H2′ positions, and the same specifically deuterated forms of RNA can be made with ^{13}C labeling by simply starting with the ^{13}C-labeled forms of glucose. Base labeling[41] can also be incorporated into this labeling strategy by simply using isotopically labeled bases as starting materials, and several isotopically labeled bases (^{13}C and ^{15}N) have recently become commercially available. The RNA labeling patterns described here demonstrate how ribose deuteration can be used to simplify RNA NMR spectra while still retaining sequential connectivity information and also demonstrate spectral editing with specific deuteration in combination with ^{13}C labeling. These new isotopic labeling strategies should allow labeling patterns to be specifically designed for good relaxation properties and advantageous spectral editing, extending the size of RNAs that can be studied with NMR spectroscopy.

(41) SantaLucia, J.; Shen, L. X.; Cai, Z.; Lewis, H.; Tinoco, I. *Nucleic Acids Res.* **1995**, *23*, 4913–4921.

Experimental Section

Materials and Methods/General Procedures. Chemicals were purchased from Aldrich and Sigma. [1,2,3,4,5,6-^{13}C$_6$]-D-glucose (>99% ^{13}C) and D$_2$O were purchased from Cambridge Isotopes Laboratories. [1,2,3,4,5,6,6-^{2}H$_7$]-D-Glucose (>97% ^{2}H) and [1,2,3,4,5,6,6-^{2}H$_7$-1,2,3,4,5,6-^{13}C$_6$]-D-glucose (>97% ^{2}H, >98% ^{13}C) were purchased from Martek Corporation. The sodium salt of 3-phosphoglycerate was prepared from the barium salt of 3-phosphoglycerate by exchanging the barium for sodium with amberlite IR-120PLUS strongly acidic cation exchanger, sodium form (16–50 mesh) obtained from Sigma.[24] Phosphoglycerate mutase from rabbit muscle and nucleoside monophosphate kinase from beef liver were purchased from Boehringer Mannheim. Enolase from bakers yeast, myokinase from chicken muscle, pyruvate kinase from rabbit muscle, guanylate kinase from porcine brain, hexokinase from bakers yeast, phosphoglucose isomerase from bakers yeast, glucose-6-phosphate dehydrogenase from bakers yeast, 6-phosphogluconic dehydrogenase from yeast, phosphoribo-isomerase from torula yeast, and L-glutamic dehydrogenase from bovine liver were purchased from Sigma. Adenine phosphoribosyltransferase (APRT), xanthine–guanine phosphoribosyltransferase (XGPRT), and CTP synthetase were purified from overexpressing strains.[17] One unit of enzyme corresponds to 1 μmol/min of activity under assay conditions. Enzymatic reactions were conducted under argon atmosphere. The pH
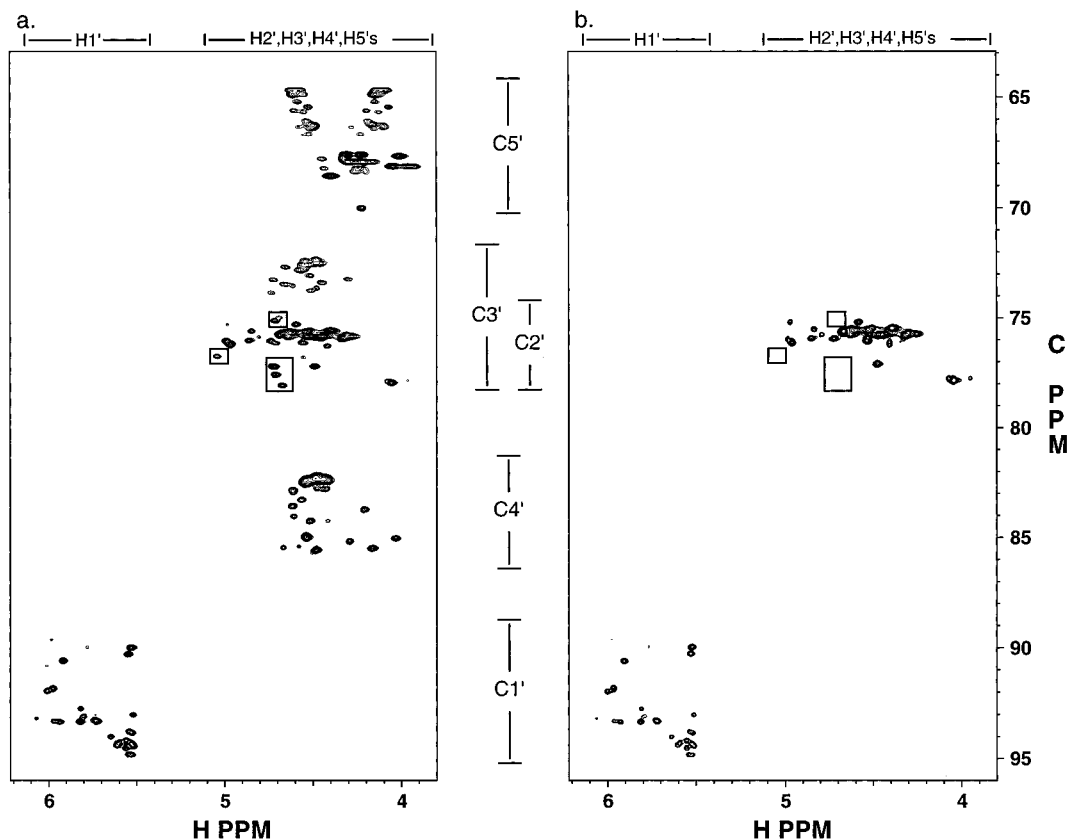
Figure 6. HSQC spectra of (a) $^{13}C_5$-*ribose*-TAR RNA and (b) d_4-$^{13}C_5$-*ribose*-TAR RNA. Chemical shift regions for different types of protons and carbons are indicated. The boxes inside the $^{13}C_5$-*ribose*-TAR RNA spectra outline some of the H3′−C3′ cross-peaks that overlap the H2′−C2′ cross-peak region, and the boxes inside the d_4-$^{13}C_5$-*ribose*-TAR RNA spectra show the absence of these H3′−C3′ cross-peaks in the deuterated RNA spectra.

of aqueous solutions were determined by colorpHast Indicator Strips pH 0−14 (EM Science). NMR spectra were recorded on a Varian Inova-600 MHz spectrometer and ultra violet spectra were recorded on a Hitachi U-2000 UV/Vis spectrophotometer. Mass spectra were obtained on a Hewlett-Packard electrospray mass spectrometer.

Cloning the Gene for Uracil Phosphoribosyltransferase. The gene encoding *E. coli* uracil phosphoribosyltransferase was cloned from the *E. coli* strain JM109 genome on the basis of the reported gene sequence[33] using PCR with the oligonucleotides dCCG CGC GAA TTC TTG AAG ATC GTG GAA GTC AAA CAC and dCCG GCG AAG CTT TTT CGT ACC AAA GAT TTT GTC ACC as primers. The PCR product was digested with EcoR I and Hind III and ligated into expression plasmid pKK223−3 that had been prepared by digestion with EcoR I and Hind III and dephosphorylation with calf intestinal alkaline phosphatase. Transformation of this construct into *E. coli* strain JM109 produced an IPTG inducible uracil phosphoribosyltransferase overproducing strain JM109/pTTU2.

Purification of Uracil Phosphoribosyltransferase.[34] IPTG inducible, uracil phosphoribosyltransferase (UPRT) overproducing strain JM109/pTTU2 was grown on 2 L of LB media containing 50 μg/mL of ampicillin for 12 h at 37 °C after being inoculated with a 5 mL overnight culture, then induced with IPTG (0.23 g/L) for another 6 h. All steps after cell growth were carried out in a 4 °C cold room or on ice. Cells were harvested by centrifugation at 6000 g for 15 min. The cell pellets were resuspended in 65 mM Tris buffer (pH 7.8) with 5 mM 2-mercaptoethanol (buffer A) and disrupted with thirty, 30 s sonication bursts using a Fisher Scientific 550 Sonic Dismembrator on a setting of 7 with a 2.5 min interval between bursts. Cellular debris was removed by centrifugation at 31 000 g for 30 min. A 0.1 volume of a 20% streptomycin sulfate solution was then added to the protein supernatant. After 15 min of stirring, the resulting precipitate was removed by centrifugation at 31 000 g for 30 min. The 0−75% ammonium sulfate fraction was collected from the supernatant of the streptomycin precipitation and then dialyzed against buffer A. The dialysate was subjected to DEAE chromatography with a 500 mL

gradient of 50−300 mM KCl in buffer A. Column fractions containing UPRT were identified by UPRT assay, pooled, concentrated by ammonium sulfate precipitation, and stored at −20 °C in buffer A containing 50% glycerol. Uracil phosphoribosyltransferase activity was determined by the method described below, and from 2 L of culture, approximately 40 units of UPRT were obtained.

Uracil Phosphoribosyltransferase Assay. Uracil phosphoribosyltransferase activity was determined by a spectrophotometric assay on the basis of the change of absorbance at 271 nm that occurs when uracil is converted into UMP. The assay solution (1 mL) contained 50 mM tris-HCl, 5 mM $MgCl_2$, 0.1 mM uracil, and 1.5 mM PRPP. The assay was started by addition of a small aliquot of UPRT solution, and the absorbance of the solution was monitored at 271 nm as a function of time. The activity of the UPRT solution was determined using a change in extinction coefficient at 271 nm of 2763 cm^{-1} mol^{-1} for the conversion of uracil into UMP.

Preparative Nucleotide Synthesis. Reactions were monitored by HPLC on a 25 × 4.6 mm Vydac 303NT405 nucleotide column, using a linear gradient from 100% buffer A (0.045M ammonium formate brought to pH 4.6 with phosphoric acid) to 100% buffer B (0.5M NaH$_2$PO$_4$ brought to pH 2.7 with formic acid) in 10 min at a flow rate of 1 mL/min, with detection at 260 or 254 nm. Potassium phosphate buffer (50mM) was used in reactions containing PRPP synthetase because the enzyme is inactivated in solutions with low phosphate concentration. The pH of the reactions was monitored using pH paper and adjusted periodically with 1 M NaOH or HCl (or NaOD or DCl for reactions run in D$_2$O) to maintain the pH between 7.0 and 7.9. In reactions that are to be conducted for extended periods of time, it is useful to add some ampicillin (∼50 μg/mL) to prevent bacterial growth in the reactions. Generally, in reactions where ATP was to be formed, a small catalytic amount of ATP was added to start the reaction, and the enzymes required for ATP formation were added first. Once a significant amount of ATP had formed in the reaction, the remaining enzymes for the other NTP formation were added. This was done to increase the rate of the reactions, since the activities of many of the

enzymes in the reactions are dependent on ATP concentration. In reactions where no ATP was to be formed, a greater initial amount of ATP was added to the reaction.

Boronate Affinity Purification of Nucleotides. All of the nucleotides from the enzymatic reactions were purified by boronate affinity chromatography, using a slight modification of previous procedures.[24] The nucleotide-forming reactions were concentrated under vacuum and then dissolved in a minimum amount of 1 M triethylammonium bicarbonate (TEABC) solution pH = 9.5–10 (which is prepared by bubbling carbon dioxide into a solution of 140 mL triethylamine and 860 mL of H_2O). Once the residue was dissolved, the solution was allowed to rest at room temperature for 15–30 min while a white precipitate usually formed (probably precipitated protein); the precipitate was then removed from the solution by filtration or centrifugation. The filtrate was loaded onto an Affigel 601 boronate affinity column that had been pre-equilibrated with 1 M TEABC pH = 9.5–10, and the previously reported procedure was followed for column washing and elution.[24]

Preparation of [1′,2′,3′,4′,5′,5′-²H₆]-[ATP (1), GTP (2), UTP (3)]. Into a Millipore 10 000 MWC centricon were placed 188 units of phosphoglycerate mutase, 100 units of enolase, 75 units of pyruvate kinase, 50 units of myokinase, 75 units of L-glutamic dehydrogenase, 60 units of hexokinase, 15 units of glucose-6-phosphate dehydrogenase, 5 units of 6-phosphogluconic dehydrogenase, 100 units of phosphoriboisomerase, 2.7 units of PRPP synthetase, 1.3 unit of adenine phosphoribosyltransferase, 2 units of xanthine–guanine phosphoribosyltransferase, 2 units of uracil phosphoribosyltransferase, 1 unit of guanylate kinase, and 0.5 units of nucleoside monophosphate kinase. The enzyme solution was centrifuged at 6000 rpm until 90–95% of its original volume had passed through the centricon dialysis membrane, and then 1.5 mL of D_2O were added to the centricon. The centricon was again centrifuged at 6000 rpm until 90–95% of the liquid had passed through the filter. This was repeated two more times, and then the enzymes were considered to have been exchanged into D_2O and ready to be added to the reaction.

Into a 300 mL round bottom flask were placed sodium 3-phosphoglycerate (6.8 mmol), adenine hydrochloride (0.034 g, 0.2 mmol), α-ketoglutaric acid (0.58 g, 4.0 mmol), NH_4Cl (0.30 g, 5.6 mmol), $MgCl_2$ (0.6 mmol), dithiothreitol (0.21 g, 1.4 mmol), 3 mL of 1 M potassium phosphate buffer pH = 7.5, and 60 mL of H_2O. The pH of the resulting solution was adjusted to 7.7 with 1 M NaOH, and then the mixture was concentrated to dryness under vacuum. The residue was dissolved in 10 mL of D_2O and concentrated to dryness under vacuum; this procedure was repeated a total of three times. The D_2O-exchanged residue was dissolved in 60 mL of D_2O, and the pD of the solution was adjusted to 7.5 with DCl and NaOD. The D_2O-exchanged enzyme solution was added to the mixture, and then d_6-ATP (1.7 μmole) from a previous reaction, $NADP^+$ (0.005 g, 7 μmol), and [1′,2′,3′,4′,5′,6′,6-²H₇]glucose (0.150 g, 0.8 mmol) were added to start the reaction. After 40 h, a significant amount of the adenine had been converted into d_6-ATP (1); therefore, guanine (0.30 g, 0.2 mmol) and uracil (0.45 g, 0.4 mmol) were added to the reaction to begin GTP and UTP formation. Additional D_2O-exchanged 3-PGA (1 mmol) was added to the reaction after 6 days, and the reaction was stopped by freezing it after 7 days. The mixture was purified by boronate chromatography as described above, and nucleotides were quantitated by UV absorbance at 260 nm, assuming a 1:1:2 ratio of ATP:GTP: UTP. Using this quantification, 84% of the [²H₇]glucose was converted into nucleotides and recovered. Half of this product was used to make d_6-CTP (4), and half was used directly in transcription reactions. Characterization was conducted on the mixture of products resulting from this reaction; ¹H NMR and mass spectrometry analysis were consistent with a mixture of d_6-ATP (1), d_6-GTP (2), and d_6-UTP (3) in a 1:1:2 ratio. ¹H NMR (600 MHz, D_2O): δ 8.55 (s, 1H), 8.29 (s, 1H), 8.13 (s, 1H), 7.97 (d, 1H, J = 8.4 Hz), 5.98 (d, 1H, J = 8.4 Hz). MS m/z: 512.0, 528.0, 489.0 (512.08, 528.08, and 489.06 calcd for $C_{10}H_9D_6O_{13}N_5P_3$, $C_{10}H_9D_6O_{14}N_5P_3$, and $C_9H_8D_6O_{15}N_2P_3$, respectively).

Preparation of [1′,2′,3′,4′,5′,5′-²H₆]CTP (4) from a mixture of d_6-ATP (1), d_6-GTP (2), and d_6-UTP (3). Into a 500 mL three-neck flask was placed half of the purified nucleotide mixture from the previous reaction containing d_6-ATP (1) (0.084 mmol), d_6-GTP (2) (0.084 mmol), and d_6-UTP (3) (0.168 mmol). To this were added

NH_4Cl (0.535 g, 10.0 mmol), sodium 3-phosphoglycerate (0.5 mmol), and 250 mL of a solution containing 5 mM $MgCl_2$ and 1 mM dithiothreitol. To start the reaction, 62 units of phosphoglycerate mutase, 50 units of enolase, 50 units of pyruvate kinase, 5 units of myokinase, and 3 units of CTP synthetase were added to the reaction. This was stirred for 48 h, and then the reaction was purified by boronate chromatography. The yield for this reaction was 97% as determined by UV absorbance. Characterization was conducted on the mixture of products resulting from this reaction; ¹H NMR and mass spectrometry analysis were consistent with a mixture of d_6-ATP (1), d_6-GTP (2), d_6-CTP (4) in a 1:1:2 ratio. ¹H NMR (600 MHz, D_2O): δ 8.53 (s, 1H), 8.27 (s, 1H), 8.13 (s, 1H), 7.99 (d, 1H, J = 7.2 Hz), 6.17 (d, 1H, J = 7.2 Hz). MS m/z: 512.0, 528.0, 488.0 (512.08, 528.08, and 488.07 calcd for $C_{10}H_9D_6O_{13}N_5P_3$, $C_{10}H_9D_6O_{14}N_5P_3$, $C_9H_9D_6O_{14}N_3P_3$, respectively).

Preparation of [3′,4′,5′,5′-²H₄]UTP (5). Into a three-neck flask were placed sodium 3-phosphoglycerate (1.3 mmol), α-ketoglutaric acid (0.29 g, 2.0 mmol), and NH_4Cl (0.15 g, 2.8 mmol). This was dissolved in 40 mL of a solution containing 10 mM $MgCl_2$, 20 mM dithiothreitol, and 50 mM potassium phosphate buffer pH 7.5, and the pH of this solution was adjusted to 7.5 with 1 M NaOH. After the solution had been neutralized, d_6-ATP (20 μmol) and [1,2,3,4,5,6,6-²H₇]glucose (0.075 g, 0.4 mmol) were added to the mixture. The phosphorylation of glucose to G6P, and isomerization of G6P, was started by adding 188 units of phosphoglycerate mutase, 50 units of enolase, 75 units of pyruvate kinase, 38 units of myokinase, 60 units of hexokinase, and 75 units of glucose-6-phosphate isomerase. After 30 h, the phosphorylation of glucose appeared to be complete by HPLC analysis of the ATP in the reaction, and the reaction was heated to 34 °C for 20 h to speed up the exchange of the H2 with solvent. The reaction was cooled to room temperature, and 30 units of glutamic dehydrogenase, 6 units of glucose-6-phosphate dehydrogenase, 2.5 units of 6-phosphogluconic dehydrogenase, 100 units of phosphoriboisomerase, 1 unit of PRPP synthetase, 2 units of uracil phosphoribosyltransferase, 0.5 units of nucleoside monophosphate kinase, 125 units of phosphoglycerate mutase, 50 units of enolase, 50 units of pyruvate kinase, 38 units of myokinase, $NADP^+$ (0.009 g, 12 μmol), and sodium 3PGA (2.7 mmol) were added to begin formation of d_4-UTP (5). After 8 days, the reaction was frozen to stop it and purified by boronate chromatography. The yield of this reaction was 67% as determined by UV absorbance. ¹H NMR (600 MHz, D_2O): δ 7.95 (d, 1H, J = 8.4 Hz), 5.99 (d, 1H, J = 6 Hz), 5.98 (d, 1H, J = 8.4 Hz), 4.39 (d, 1H, J = 4.8 Hz); MS m/z: 487.0 (487.05 calcd for $C_9H_{10}D_4O_{15}N_2P_3$).

Preparation of [1′,2′,3′,4′,5′-¹³C₅]-[ATP (6), GTP (7), UTP (8)]. Into a three-neck flask were placed sodium 3-phosphoglycerate (6.0 mmol), adenine hydrochloride (0.034 g, 0.2 mmol), α-ketoglutaric acid (0.58 g, 4.0 mmol), and NH_4Cl (0.30 g, 5.6 mmol). This was dissolved in 80 mL of a solution containing 10 mM $MgCl_2$, 20 mM dithiothreitol, and 50 mM potassium phosphate buffer pH 7.5, and the pH of this solution was adjusted to 7.5 with 1 M NaOH. After the solution had been neutralized, ATP (8 μmol), $NADP^+$ (0.005 g, 7 μmol), and [1,2,3,4,5,6-¹³C₆]-D-glucose (0.150 g, 0.8 mmol) were added to the mixture. The reaction was started by adding 125 units of phosphoglycerate mutase, 50 units of enolase, 50 units of pyruvate kinase, 50 units of myokinase, 50 units of L-glutamic dehydrogenase, 60 units of hexokinase, 10 units of glucose-6-phosphate dehydrogenase, 2.5 units of 6-phosphogluconic dehydrogenase, 40 units of phosphoriboisomerase, 2 units of PRPP synthetase, 1 unit of adenine phosphoribosyltransferase, 2 units of xanthine–guanine phosphoribosyltransferase, 2 units of uracil phosphoribosyltransferase, 1 unit of guanylate kinase, and 0.5 units of nucleoside monophosphate kinase. At 24 h, a significant amount of ATP had formed, and guanine (0.030 g, 0.2 mmol) and uracil (0.045 g, 0.4 mmol) were added to the reaction to begin GTP and UTP formation. After 3 days, an additional 2.0 mmol of sodium 3-phosphoglycerate was added to the reaction, and the reaction was stopped after 5 days. Boronate purification of the reaction and quantification of the purified products by UV absorbance indicated that 88% of the glucose had been converted into isotopically labeled nucleotides and recovered. Characterization was conducted on the mixture of products resulting from this reaction; ¹H NMR, ¹³C HSQC, and mass spectrometry analysis were consistent with a mixture of [1′,2′,3′,4′,5′-¹³C₅]-[ATP (6), GTP (7), UTP (8)] in a 1:1:2 ratio. ¹H

NMR (600 MHz, D$_2$O): δ 8.55 (s, 1H), 8.28 (s, 1H), 8.13 (s, 1H), 7.98 (dd, 1H, J = 8.4, 2.4 Hz), 6.15 (d, 1H, J = 165.5 Hz), 6.00 (d, 1H, J = 170.4 Hz), 5.93 (d, 1H, J = 166.2 Hz), 5.99 (d, 1H, J = 8.4 Hz), 4.95−4.0 (m, 15 H). ^{13}C HSQC cross-peaks (δ proton, δ carbon): (6.18, 89.81), (6.03, 91.23), (5.95, 89.81), (4.85, 76.49), (4.83, 77.38), (4.60, 73.47), (4.45, 72.76), (4.42, 76.85), (4.43, 87.14), (4.38, 86.97), (4.32, 86.43), (4.30, 68.32), (4.28, 68.15), (4.26, 68.32). MS *m/z*: 511.0, 527.0, 488.0 (511.06, 527.06, and 488.04 calcd for (^{12}C$_5$)(^{13}C$_5$)H$_{15}$O$_{13}$N$_5$P$_3$, (^{12}C$_5$)(^{13}C$_5$)H$_{15}$O$_{14}$N$_5$P$_3$, and (^{12}C$_4$)(^{13}C$_5$)H$_{14}$O$_{15}$N$_2$P$_3$, respectively).

Preparation of [1′,2′,3′,4′,5′-^{13}C$_5$]CTP (9) from a Mixture of [1′,2′,3′,4′,5′-^{13}C$_5$]-[ATP (6), GTP (7), UTP (8)]. [1′,2′,3′,4′,5′-^{13}C$_5$]-CTP (**9**) was prepared from the purified mixture of NTPs **6−8** from the previous reaction in a procedure similar to the one used to produce *d$_6$*-CTP (**4**). The yield of this reaction was 80%. Characterization was conducted on the mixture of products resulting from this reaction; ^{1}H NMR, ^{13}C HSQC, and mass spectrometry analysis were consistent with a mixture of [1′,2′,3′,4′,5′-^{13}C$_5$]-[ATP (**6**), GTP (**7**), CTP (**9**)] in a 1:1:2 ratio. ^{1}H NMR (600 MHz, D$_2$O): δ 8.54 (s, 1H), 8.27 (s, 1H), 8.14 (s, 1H), 7.98 (d, 1H, J = 7.2 Hz), 6.16 (d, 1H, J = 7.2 Hz), 6.15 (d, 1H, J = 165.6 Hz), 6.01 (d, 1H, J = 169.2 Hz), 5.94 (d, 1H, J = 165.6 Hz), 4.94−4.00 (m, 15 H). ^{13}C HSQC cross-peaks (δ proton, δ carbon): (6.18, 89.81), (6.04, 92.11), (5.96, 89.81), (4.85, 76.49), (4.83, 77.38), (4.60, 73.47), (4.43, 87.14), (4.42, 72.23), (4.39, 86.97), (4.36, 77.20), (4.31, 85.90), (4.31, 68.32), (4.31, 67.79), (4.26, 68.32). MS *m/z*: 511.0, 527.0, 487.0 (511.06, 527.06, and 487.05 calcd for (^{12}C$_5$)-(^{13}C$_5$)H$_{15}$O$_{13}$N$_5$P$_3$, (^{12}C$_5$)(^{13}C$_5$)H$_{15}$O$_{14}$N$_5$P$_3$, and (^{12}C$_4$)(^{13}C$_5$)H$_{15}$O$_{14}$N$_3$P$_3$, respectively).

Preparation of [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]-[ATP (10), GTP (11), UTP (12)].[39] Into a three-neck flask were placed sodium 3-phosphoglycerate (2.1 mmol), adenine hydrochloride (0.034 g, 0.2 mmol), α-ketoglutaric acid (0.76 g, 4.0 mmol), and NH$_4$Cl (0.30 g, 5.6 mmol). This was dissolved in 80 mL of a solution containing 10 mM MgCl$_2$, 20 mM dithiothreitol, and 50 mM potassium phosphate buffer pH 7.5, and the pH of this solution was adjusted to 7.5 with 1 M NaOH. After the solution had been neutralized, *d$_6$*-ATP (7 μmol) and [1,2,3,4,5,6,6-^{2}H$_7$-1,2,3,4,5,6-^{13}C$_6$]-D-glucose (0.155 g, 0.8 mmol) were added to the mixture. The phosphorylation of the glucose to G6P, and the isomerization of G6P, was started by adding 188 units of phosphoglycerate mutase, 50 units of enolase, 75 units of pyruvate kinase, 38 units of myokinase, 60 units of hexokinase, and 75 units of glucose-6-phosphate isomerase. After 48 h of stirring, 30 units of glutamic dehydrogenase, 7.5 units of glucose-6-phosphate dehydrogenase, 2.5 units of 6-phosphogluconic dehydrogenase, 100 units of phosphoriboisomerase, 1 unit of PRPP synthetase, 1 unit of adenine phosphoribosyltransferase, NADP$^+$ (0.007 g, 10 μmol), and sodium 3PGA (2.7 mmol) were added to begin *d$_4$-^{13}C$_5$*-ATP (**10**) formation. At 72 h, guanine (0.030 g, 0.2 mmol), uracil (0.45 g, 0.4 mmol), 2 units of xanthine−guanine phosphoribosyltransferase, 2 units of uracil phosphoribosyltransferase, 1 unit of guanylate kinase, and 0.5 units of nucleoside monophosphate kinase were added to the reaction to begin GTP and UTP formation. After 5 days, additional 3- phosphoglycerate (1.7 mmol) was added to the reaction, and after 7 days, the reaction was stopped and purified. Boronate purification of the reaction and quantification of the purified products by UV absorbence indicated that 92% of the glucose had been converted into isotopically labeled nucleotides and recovered. Characterization was conducted on the complex mixture of products resulting from this reaction; ^{1}H NMR, ^{13}C HSQC, and mass spectrometry analysis were consistent with a mixture of [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]ATP, [1′,3′,4′,5′,5′-^{2}H$_5$-1′,2′,3′,4′,5′-^{13}C$_5$]ATP, [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]GTP, [1′,3′,4′,5′,5′-^{2}H$_5$-1′,2′,3′,4′,5′-^{13}C$_5$]GTP, [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]UTP, and [1′,3′,4′,5′,5′-^{2}H$_5$-1′,2′,3′,4′,5′-^{13}C$_5$]UTP resulting from complete hydrogen exchange of the H2′ in the nucleotides and approximately 60% hydrogen exchange of the H1′. ^{1}H NMR (600 MHz, D$_2$O): δ 8.54 (s, 1H), 8.28 (s, 1H), 8.13 (s, 1H), 7.97 (d, 1H, J = 8.4 Hz), 6.14 (d, 0.6H, J = 173.4 Hz), 6.00 (d, 0.6H, J = 169.2 Hz), 5.93 (d, 0.6H, J =

167.4 Hz), 5.99 (d, 1H, J = 8.4 Hz), 4.81 (d, 1H, J = 150 Hz), 4.79 (d, 1H, J = 150 Hz), 4.39 (d, 1H, J = 151.2 Hz). ^{13}C HSQC cross-peaks (δ proton, δ carbon): (6.17, 89.81), (6.03, 91.23), (5.95, 89.81), (4.84, 76.49), (4.82, 77.20), (4.42, 76.67). MS *m/z*: 515.1, 516.1, 531.1, 532.1, 492.0, 493.0 (515.09, 516.10, 531.08, 532.09, 492.06, and 493.07 calcd for (^{12}C$_5$)(^{13}C$_5$)H$_{11}$D$_4$O$_{13}$N$_5$P$_3$, (^{12}C$_5$)(^{13}C$_5$)H$_{10}$D$_5$O$_{13}$N$_5$P$_3$, (^{12}C$_5$)-(^{13}C$_5$)H$_{11}$D$_4$O$_{14}$N$_5$P$_3$, (^{12}C$_5$)(^{13}C$_5$)H$_{10}$D$_5$O$_{14}$N$_5$P$_3$, (^{12}C$_4$)(^{13}C$_5$)H$_{10}$D$_4$O$_{15}$N$_2$P$_3$, (^{12}C$_4$)(^{13}C$_5$)H$_9$D$_5$O$_{15}$N$_2$P$_3$, respectively).

Preparation of [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]CTP^{39} (13) from a Mixture of [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]-[ATP (10), GTP (11), UTP (12)].[39] [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]CTP (**13**) was prepared from the mixture of **10**, **11**, and **12** from the previous reaction in a procedure similar to the one used to produce *d$_6$*-CTP (**4**). The yield of this reaction was 89%. Characterization was conducted on the complex mixture of products resulting from this reaction; ^{1}H NMR, ^{13}C HSQC, and mass spectrometry analysis were consistent with a mixture of [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]ATP, [1′,3′,4′,5′,5′-^{2}H$_5$-1′,2′,3′,4′,5′-^{13}C$_5$]ATP, [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]GTP, [1′,2′,3′,4′,5′]^{13}C$_5$-GTP, [3′,4′,5′,5′-^{2}H$_4$-1′,2′,3′,4′,5′-^{13}C$_5$]CTP, and [1′,3′,4′,5′,5′-^{2}H$_5$-1′,2′,3′,4′,5′-^{13}C$_5$]CTP. ^{1}H NMR (600 MHz, D$_2$O): δ 8.54 (s, 1H), 8.27 (s, 1H), 8.13 (s, 1H), 8.00 (d, 1H, J = 6.6 Hz), 6.15 (d, 0.6H, J = 166.2 Hz), 6.17 (d, 0.6H, J = 7.8 Hz), 5.93 (d, 0.6H, J = 165.6 Hz), 6.00 (d, 1H, J = 171.0 Hz), 4.81 (d, 2H, J = 150 Hz), 4.33 (d, 1H, J = 152.4 Hz). ^{13}C HSQC cross-peaks (δ proton, δ carbon): (6.17, 89.81), (6.03, 92.11), (5.96, 89.81), (4.84, 76.49), (4.82, 77.20), (4.36, 77.20). MS *m/z* 515.1, 516.1, 531.1, 532.1, 491.0, 492.0 (515.09, 516.10, 531.08, 532.09, and 491.08, 492.08 calcd for (^{12}C$_5$)(^{13}C$_5$)H$_{11}$D$_4$O$_{13}$N$_5$P$_3$, (^{12}C$_5$)(^{13}C$_5$)H$_{10}$D$_5$O$_{13}$N$_5$P$_3$, (^{12}C$_5$)(^{13}C$_5$)-H$_{11}$D$_4$O$_{14}$N$_5$P$_3$, (^{12}C$_5$)(^{13}C$_5$)H$_{10}$D$_5$O$_{14}$N$_5$P$_3$, (^{12}C$_4$)(^{13}C$_5$)H$_{11}$D$_4$O$_{14}$N$_4$P$_3$, and (^{12}C$_4$)(^{13}C$_5$)H$_{10}$D$_5$O$_{14}$N$_3$P$_3$, respectively).

RNA Synthesis. TAR RNA (5′GGCCAGAUUGAGCCUGGGA-GCUCUCUGGCC3′) was synthesized by *in vitro* transcription[21] with T7 RNA polymerase using unlabeled NTPs from Sigma and the different isotopically labeled NTPs produced in this paper. Transcription conditions were as described in Wyatt and Puglisi,[22] except that nucleotide concentrations were approximately 2 mM at the start of transcription reactions. Unlabeled TAR RNA and *d$_4$*-TAR RNA were prepared as described in our previous paper.[17] *d$_6$*-RNA was produced with *d$_6$*-NTPs (**1−4**). *d$_4$/d$_6$*-RNA was produced with *d$_6$*-[ATP (**1**), GTP (**2**), CTP (**4**)] and *d$_4$*-UTP (**5**). ^{13}C$_5$-*ribose*-RNA was produced with ^{13}C$_5$-*ribose*-NTPs (**6−9**). *d$_4$*-^{13}C$_5$-*ribose*-RNA was produced with *d$_4$*-^{13}C$_5$-*ribose*-NTPs (**10−13**). The RNA was purified by 20% PAGE, electroeluted, and dialyzed against 50 mM NaCl, 0.1 mM EDTA, and 10 mM sodium phosphate pH 6.4.

NMR Experiments. NMR experiments were recorded on a Varian Inova-600 MHz spectrometer. RNA sample conditions were 50 mM NaCl, 0.1 mM EDTA, and 10 mM sodium phosphate pH 6.4, and experiments were conducted at 25 °C. NOESY spectra were acquired with a spectral width of 8000 Hz, acquiring 4096 complex points in t_2 and 1024 in t_1, with 32 scans per FID, a relaxation delay of 2.6 s, and a mixing time of 200 ms. HSQC-CT spectra were acquired with a constant time interval of 25 ms with spectral widths of 6500 and 5000 Hz for proton and carbon dimensions, respectively; 1024 and 224 complex points were acquired for t_1 and t_2, respectively, with 8 scans per FID.