

Selective detection of ribose-methylated nucleotides in RNA by a mass spectrometry-based method

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Received April 12, 1999; Revised May 20, 1999; Accepted June 14, 1999

ABSTRACT

Post-transcriptional methylation of ribose at position O-2' is one of the most common and conserved types of RNA modification. Details of the functional roles of these methylations are far from clear, although in tRNA they are involved at position 34 in regulation of codon recognition and in eukaryotic rRNAs they are required for subunit assembly. Experimental difficulties in the mapping of ribose methylations increase with RNA molecular size and the complexity of mixtures resulting from nuclease digestion. A new and relatively rapid approach based on tandem mass spectrometry is described in which any of four ion reaction pathways occurring in the mass spectrometer can be monitored which are highly specific for the presence of 2'-*O*-methylribose residues. These pathways emanate from further dissociation of ribose-methylated mononucleotide (Nmp) ions formed in the electrospray ionization region of the mass spectrometer to then form the base, methylribose phosphate or PO_3^- anions. The mass spectrometer can be set for detection of generic ribose methylation (Nm) in oligonucleotides, selectively for each of the common methylated nucleosides Cm, Gm, Am or Um or for specific cases in which the base or sugar is further modified. By direct combination of mass spectrometry with liquid chromatography the method can be applied to analysis of complex mixtures of oligonucleotides, as for instance from synthetic or *in vitro* reaction mixtures or from nuclease digests of RNA. An example is given in which the single ribose-methylated nucleoside in *Escherichia coli* 16S rRNA (1542 nt), N^4,*O*-2'-dimethylcytidine, is detected in 25 pmol of a RNase T1 digest and localized to the fragment 1402-CCCGp-1405 in a single 45 min analysis.

INTRODUCTION

Ribose methylation at position O-2' is one of the most common and highly conserved forms of post-transcriptional modification in RNA (1–3). Interestingly, this relatively simple modification appears to serve a range of functional roles, which are ultimately based on combinations of factors, including structural stabilization of ribose resulting from thermodynamic preference for the C3'-*endo* ribose conformer (4), resistance to nuclease hydrolysis and influence on sugar hydrogen bonding interactions. One of the clearest examples has been the influence of ribose methylation (often in conjunction with base modification) at position 34 in tRNA on codon recognition (5,6). By contrast, the roles of ribose methylation in rRNA are poorly understood, although they are strongly implicated in ribosomal subunit assembly and processing (see for example 7–13). These modifications in eukaryotes occur in large numbers, for instance 63–65 residues in human 28S rRNA, 11–13 of which are as yet unlocated (2), as well as in rRNAs from the archaeal thermophiles (14). Further interest in rRNA methylation in eukaryotes has been recently heightened by the identification of a system of small nucleolar RNAs that serve as guides, through hybridization to pre-rRNA and rRNA, to precisely target the sites of ribose methylation (for a review and leading citations see 15).

Classical approaches to the problem of mapping sites of ribose methylation include nuclease fingerprinting systems with *in vivo* ^{14}C or ^{32}P labeling (e.g. using RNases T1 or A), as summarized by Maden (2), and reverse transcription (16) and primer extension in conjunction with partial alkaline hydrolysis (16–18). Recently a novel and sensitive method was introduced for detection of 2'-*O*-methylated residues in regions where methylation is predicted, based on RNase H cleavage patterns in RNA and RNA/DNA chimeric duplexes (19). Experimental difficulties in the accurate mapping of ribose methylations increase with RNA molecular size (15) and are dependent on the extent of primary sequence information available, e.g. as inferred from the corresponding gene sequence. In the case of tRNA further uncertainties may derive from the occurrence of ribose-methylated nucleotides that are additionally modified in the base, of which 12 are presently known (3). Limitations of the different methods vary with the problem at hand, but in the case of rRNA they are overall considered to be sufficiently problematic to pose a hindrance to structure–function studies of rRNA methylation (15).

The presence of ribose-methylated residues in intact oligonucleotides can be inferred in some circumstances by using mass spectrometry, from shifts in mass due to methylation (20) (e.g. 14 Da for one methyl group), but the measurements generally require subfractionation of oligonucleotides in order to provide simple mixtures for introduction into the mass spectrometer.

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Additional experiments, such as gas phase dissociation (21) or separate analysis of nucleosides following total hydrolysis (22,23), would be required to distinguish base versus ribose methylation. These problems are greatly reduced as a result of the recent introduction of a new solvent system for liquid chromatography (LC)/electrospray mass spectrometry (MS) analysis of oligodeoxynucleotides (24), which is also applicable to RNA (25). This results in the capability to mass measure individual components of complex oligonucleotide mixtures in a single experiment, which in turn provides the foundation for the rapid detection of ribose-methylated residues in nuclease digests of large RNAs (as described below), of *in vitro* methylation products of T7 transcripts or of chemical synthesis reaction mixtures. The procedure we report is based on detection of reaction pathways which occur in the mass spectrometer that are highly specific for the presence of 2'-*O*-methyl nucleotides. The mass spectrometer can be set for detection of generic ribose methylation (Nm) in oligonucleotides, for each of the common methylated nucleosides (Cm, Gm, Am or Um) or for cases in which the base is also modified (e.g. N^4, 2'-*O*-dimethylcytidine, m^4Cm). The method can be readily extended to other ribose-modified residues in RNA, as well as synthetic 2'-*O*-alkyl derivatives or to any other substitutions or modifications in the sugar which result in a mass different from that of ribose.

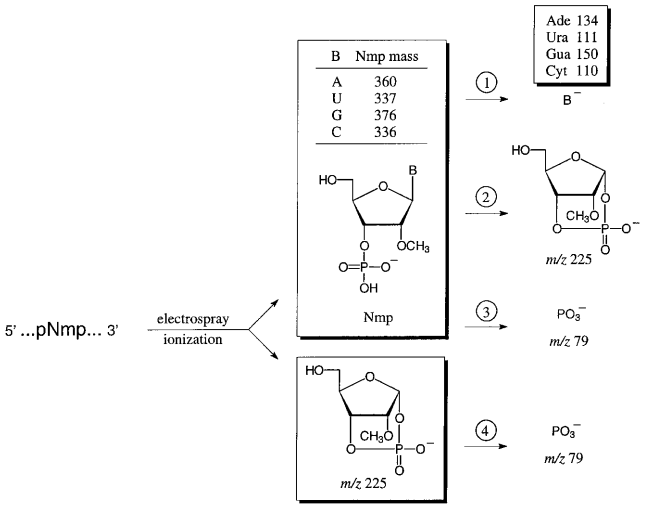

Figure 1. Ion dissociation pathways which can be used for detection of 2'-*O* methylation (or other C-2' modifications) by tandem mass spectrometry. Electrospray ionization (ESI) of oligonucleotides which contain Nm produces Nmp and *m/z* 225 fragment ions in the ionization region. Recognition of subsequent dissociation paths 1, 2 or 3 allows detection of any mass-specified Nm species (such as Gm or Um) or from path 4 the generic detection of O-2' methylation.

MATERIALS AND METHODS

Materials

Synthetic oligonucleotides were prepared at the University of Utah Protein/DNA Synthesis Facility on an Applied Biosystems model 394 synthesizer (Perkin Elmer, Foster City, CA) using standard phosphoramidite technology. Procedures for final 2'-OH deblocking and sample purification have been described (21). 16S rRNA was isolated from *Escherichia coli* MRE 600 cells by E. Bruenger in this laboratory as earlier reported (20). Digestion of 16S rRNA by RNase T1 (Ambion, Austin, TX) was carried out for 30 min at 37°C (20). Approximately 100 pmol were hydrolyzed, from which a 25 pmol aliquot was used for the experiment represented in Figure 4.

Liquid chromatography/mass spectrometry

Analysis of mixtures of either synthetic oligonucleotides (data in Figs 2 and 3) or RNase T1 hydrolysates of 16S rRNA (data in Fig. 4) was carried out using a Quattro II triple quadrupole mass spectrometer (Micromass, Beverly, MA) equipped with a standard electrospray ion source and interfaced to a Hewlett-Packard 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA) with a UV diode array detector, used over the range 190–320 nm.

Mixtures of synthetic oligonucleotides or hydrolysates of 16S rRNA were injected onto a 300 × 1 mm Supelcosil LC-18-DB column (Supelco, Bellefonte, PA) with a 15 × 1 mm Opti-Guard C-18 precolumn cartridge (Optimize Technologies, Oregon City, OR). The solvent system consisted of 0.8 M 1,1,1,3,3,3-hexafluoro-2-propanol (J. T. Baker; Phillipsburg, NJ), adjusted to pH 7.0 with triethylamine, half of which was diluted 1:1 with HPLC grade water (buffer A) and the other half 1:1 with methanol (buffer B) (24). The column was eluted using a linear gradient of 0–100% B in A + B over 40 and 45 min for synthesized oligomers and digests of 16S rRNA, respectively, at a flow rate of 60 µl/min.

The chromatographic eluate was conducted into the mass spectrometer ion source without prior splitting. The instrument was operated in negative ion mode, with the ion source temperature maintained at 125°C. Capillary and lens voltages were typically in the range –2.8 to –3.3 and 0.4 to 0.6 kV, respectively. Cone voltages were 60 V for the full scan function and 110 V for the multiple reaction monitoring (MRM) scan function. Argon was used as the collision gas in MRM experiments at a pressure of 3×10^{-3} mbar, monitored by the collision cell ion gauge. Collision energies were 15, 20, 23 and 25 eV for reaction channels Nmp→*m/z* 225, *m/z* 225→79, Nmp→79 and Nmp→base, respectively.

RESULTS AND DISCUSSION

The principle of the method presently reported is schematically illustrated in Figure 1. The electrospray ionization of polynucleotides produces, under the conditions described above (cone voltage 110 V), a range of small fragment ions formed in the ionization region of the mass spectrometer (the so-called nozzle-skimmer region). These include mononucleotides Np (not distinguished for present purposes from pN) and Nmp if 2'-*O*-methylated species are present and the ribose phosphate ion (boxed structures in Fig. 1). Upon systematic exploration of the further dissociation paths of these ions using standard tandem MS methods, four pathways were found which may be utilized for rapid recognition of Nm in the oligonucleotide undergoing ionization. These are (Fig. 1): from the Nmp ion formation of the base anion B^- (path 1), the methylribose ion

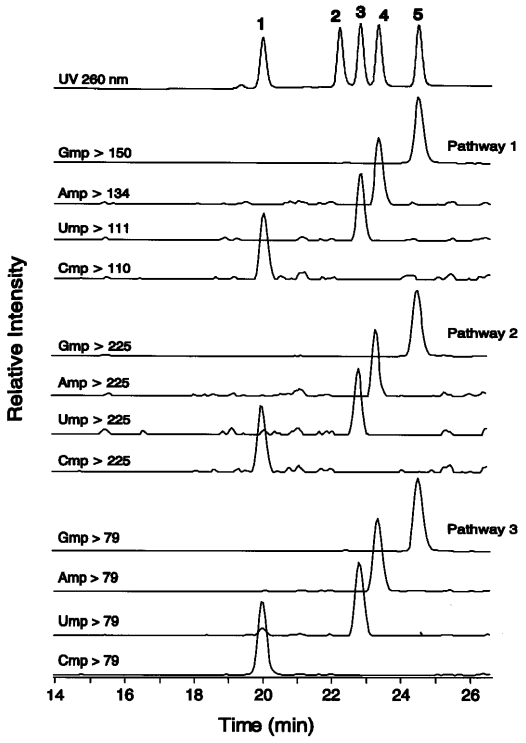

Figure 2. Selective detection of O-2' ribose-methylated residues in a mixture of oligonucleotides using LC/MS/MS. (Top) Chromatographic separation of oligonucleotides with UV absorption detection. Components: 1, GCmUG; 2, GUAUG; 3, GUmAUG; 4, GCAmUG; 5, GmCCAA. (Lower) Mass spectrometric detection for each of the four ribose-methylated nucleosides (Gm, Am, Um and Cm), based on monitoring of reaction pathways 1–3 (see Fig. 1).

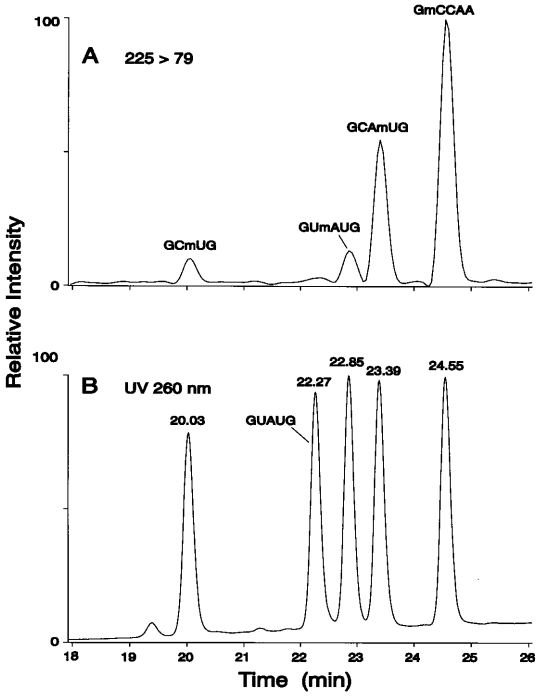

Figure 3. Detection of all O-2' ribose-methylated residues using LC/MS/MS. (**A**) MS-based detection using pathway 4. (**B**) UV absorbance detection (see Fig. 2).

species, *m*/*z* 225 (26) (path 2) and PO_3^-, *m*/*z* 79 (path 3); from the *m*/*z* 225 ion the PO_3^- ion (path 4). The characteristic fragment ion *m*/*z* 225 is formed both by initial dissociation in MS-1 as well as by subsequent dissociation of Nmp. This ion contains the C-2' substituent and so appears at other mass values when modifications occur at this position. Thus the analogous ion occurs at *m*/*z* 211 in mass spectra of normal ribonucleotides and at *m*/*z* 195 in the case of DNA (26).

Selectivity in the detection of O-2' methylation is gained by setting the two mass analyzers MS-1 and MS-2 to transmit ions selective for any of the four reaction paths. Using pathway 1 for instance, if MS-1 is set to transmit the 2'-*O*-methylguanosine monophosphate (Gmp) ion (*m*/*z* 376) and MS-2 to detect the guanine ion (*m*/*z* 150), then a detector signal indicating the presence of 2'-*O*-methylguanosine in the oligonucleotide will be obtained only when the reaction represented by pathway 1 ($376^- \rightarrow 150^-$) occurs in the gas collision cell located between MS-1 and MS-2. Note that detection of B^- or PO_3^- in a single mass analyzer experiment would be of little use because virtually all oligonucleotides produce these ions. Using one mass analyzer, detection of any of the allowed mass values for Nmp or the generic methylribose indicator *m*/*z* 225 would have greater value in terms of selectivity. However, maximum selectivity is achieved through the use of tandem mass analyzers to define the presence of specific Nm species in which the mass value of B is predefined (paths 1, 2 or 3) or of any 2'-*O*-methylated species (path 4). Because the mass values of Nmp precursor ions in paths 1, 2 and 3 also specify the mass of the base, the technique can in principle test for the presence of residues modified simultaneously in the ribose and the base. Any of the four pathways can be used to detect residues modified in other ways at C-2', either in RNA or DNA, by appropriate adjustment of mass settings for the values shown for Nmp and for *m*/*z* 225 ion.

These measurements can be readily carried out by simple infusion of samples into the mass spectrometer, but the materials should be reasonably pure in order to avoid ambiguity in interpretation of the data. However, the ability of the instrument to rapidly switch among mass channels representing any of the four pathways permits introduction of the sample by liquid chromatograph (27) so that chromatographic eluants can be measured sequentially as they pass from the UV detector into the mass spectrometer, thus extending the method to mixtures of oligonucleotides.

An example demonstrating the selectivity of the detection for specific ribose-methylated nucleosides is given in Figure 2. Four of the five components contain one of the common ribose-methylated nucleotides (Gm, Am, Um or Cm), while the fifth, GUAUG, contains only normal ribose. The overall chromatographic separation is shown in the top panel of Figure 2 using UV absorbance detection at 260 nm from a detector

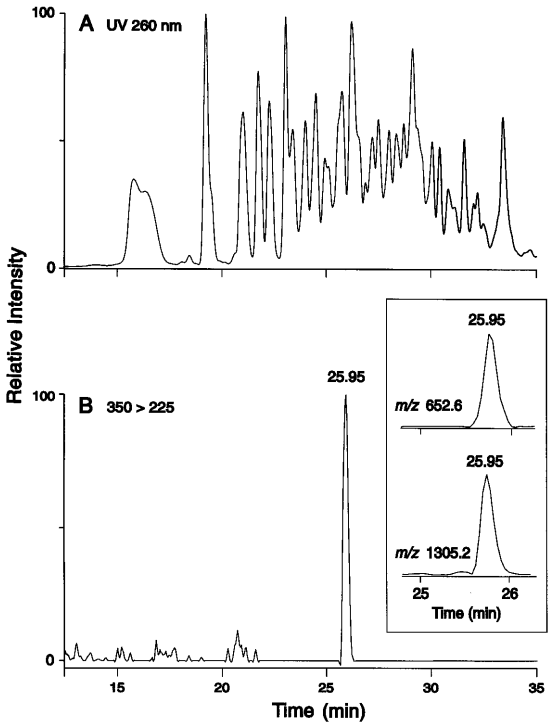

Figure 4. Detection of the single ribose-methylated residue in *E.coli* 16S rRNA (m^4Cm-1402) by LC/MS/MS analysis of a crude RNase T1 digest of 25 pmol of RNA. (**A**) UV absorbance detection. (**B**) MS detection using pathway 2 in Figure 1, showing the m^4Cm-containing oligonucleotide to elute at 25.95 min. (Inset) Chromatographic profiles constructed using ions *m*/*z* 652.6 and *m*/*z* 1305.2, which designate the 25.95 min oligonucleotide eluant to have M_r = 1306.2 and composition {C_3Gp + 2 methyls}.

placed between the liquid chromatograph and mass spectrometer. The mass detection channels (selectively representing each of the Nm residues) are shown in the lower panels, using all three pathways 1, 2 and 3. The ion detection signals in each of the 12 individual chromatograms are normalized to the most abundant signal in each channel. In the first mass channel using pathway 1 the mass analyzers have been set to detect only Gm-containing oligonucleotides by monitoring the dissociation reaction of Gmp to guanine ($376^- \rightarrow 150^-$). The resulting chromatogram shows a signal at 24.55 min elution time, perfectly aligned with the UV detection signal for component 5, GmCCAA. No signal is obtained in this selective mode for any of the other Nm species. Similar selectivity of detection is observed for the other two Gm-specific chromatograms with mass analyzer settings for pathways 2 and 3 and for other channels representing Am, Um and Cm. Component 2 (see UV detection channel) is not represented in any of the mass chromatograms because it contains no 2'-*O*-methylated residues.

Figure 3 shows an example in which the tandem mass analyzers are set for pathway 4, the generic detection of ribose methylation, for LC/MS analysis of the same simple mixture of oligonucleotides. (The data are derived from the same experiment as in Fig. 2 but are shown separately for clarity.) In this case use of the reaction channel $225^- \rightarrow 79^-$ gives good, but not very high, selectivity of detection. Recognition of methylation in the 23.39 and 24.55 min eluants is unambiguous, but due to variability in the ion yields, responses for the 20.03 and 22.85 min eluants would be considered tentative because they differ only 5-fold from the weak signal at 22.27 min (Fig. 3A) from the non-methylated oligonucleotide GUAUG. This mode of detection is useful to survey complex mixtures of oligonucleotides for the presence of O-2' methylated (or otherwise modified) residues.

Several additional points are of note regarding the data in Figure 2. First, in this single experiment, channels representing all three selective detection pathways have been used to demonstrate detection of each of the four Nm species, but in practice only a single type reaction pathway would be required (but for any number of different Nm species). In general we find the order of detection sensitivity to be pathway 3 > 2 > 1. Second, ambiguity in the use of pathway 3 is possible in the event of base methylation because Nmp (ribose methylation) does not differ in mass from mNp (base methylation). Further, the instrumental configuration described is designed for the *detection* of ribose-methylated nucleosides, but not for their sequence placement in the parent oligonucleotide. Because under some circumstances the base composition of an oligonucleotide can be derived directly from its accurately measured molecular mass (28), the location of the ribose-methylated residue in the oligonucleotide might be inferred using the corresponding gene sequence (20). Also, placement of ribose-methylated residues in oligonucleotides (and differentiation from base methylation) can be made from direct mass spectrometric sequencing (21). However, the typical time requirement for such measurements using the triple quadrupole mass spectrometer (one to several minutes data acquisition) is too long when using LC introduction of the sample (peak half-widths of ~20 s for data acquisition) and so an intermediate fractionation step prior to sample infusion into the mass spectrometer would be required.

An advantage in the use of on-line liquid chromatography tandem mass spectrometry (LC/MS/MS) for the characterization of ribose-methylation sites lies in its complementarity to other MS-based methods for structure studies of modification in RNA (27). In particular, we envision the method presently described as potentially useful for the mapping of the ribose methylation sites in large RNAs, an area of contemporary interest (17,29,30) which, however, is fraught with experimental difficulties (15). An example of this approach for rRNA is given from the data in Figure 4 showing the selective detection of the single 2'-*O*-methylated residue in *E.coli* 16S rRNA (m^4Cm) located in the decoding region at position 1402 (31). The experiment consists of the LC/MS/MS analysis of 25 pmol of rRNA following digestion by RNase T1, which is expected to produce 138 nt of trimer and larger size. These products, terminating in Gp-3', elute between ~17 and 33 min, producing the complex chromatogram shown in Figure 4A. Mass spectral data (Fig. 4B and inset) are acquired every 1.7 s over the course of the experiment. Each of these 1.7 s acquisition segments is divided into two time periods: one for monitoring of the reaction $350^- \rightarrow 225^-$ (corresponding to pathway 2 in Fig. 1) and the other to record a conventional mass spectrum to determine the mass of the intact oligonucleotide, in which MS-1 scans to detect all ions from *m*/*z* 400 to 1600 and MS-2 is not used.

The MS detection response at 25.95 min in Figure 4B identifies this as the exact elution point of an oligonucleotide which contains a base-methylated cytidine that is also methylated on ribose. Note that the mass values that are used only define methylcytosine; substitution specifically at N^4 cannot from these data be distinguished from N-3 and C-5, which are also known positions of cytosine methylation in RNA. The conventional mass spectra acquired every 1.7 s during elution of the 25.95 min component (data not shown) were then examined to determine the molecular mass values for all RNase T1 fragments eluting in this time window. An oligonucleotide of M_r 1306.2 was detected, as indicated by the ions *m*/*z* 652.6 (corresponding to $\{M_r\text{-}2H\}^{2-}$) and *m*/*z* 1305.2 (corresponding to $\{M_r\text{-}H\}^-$). When plotted chromatographically (Fig. 4B and inset) these two ion profiles track with each other and with the pathway 2 detector signal in Figure 4B, indicating that all three signals are associated with the same eluant of M_r 1306, even though other components elute within this approximate time window. The experimentally measured M_r of 1306.2 defined by the co-tracking *m*/*z* 652.6 and 1305.2 ions corresponds to the composition {C_3Gp + 2 methyls} (calculated 1306.2), which in conjunction with the gene sequence (31) constrains the identity of the mCm-containing oligonucleotide in 16S rRNA uniquely to the RNase T1 fragment 1402-CCCGp-1405, in accord with the reported identity of residue 1402 as N^4,*O*-2'-dimethylcytidine (31,32).

In summary, for the protocol represented in Figure 4, two requirements must generally be met for assignment of 2'-*O*-methylation sites in RNA using LC/MS/MS. (i) Using one or more of the pathways shown in Figure 1, an unambiguous signal with appropriate chromatographic peak shape must be obtained (see for example Fig. 4B). (ii) An accurate molecular mass value should be obtained at the indicated elution time (e.g. at 25.95 min in Fig. 4), which (a) corresponds to an oligonucleotide composition that includes at least one methyl group and (b) also represents a composition allowed by the corresponding gene sequence, if available. LC/MS measurements of this type should result in M_r measurement errors of ±0.010–0.015% using the quadrupole mass analyzer (see for example 25), corresponding to about ±0.13–0.20 Da for the 4mer in the inset of Figure 4B or ±0.33–0.50 Da for oligonucleotides at the 10mer level. For the majority of cases involving RNase T1 products of 16/18S rRNA we find this level of mass accuracy to be sufficient for unique placement of the ribose-methylated oligonucleotide, to the extent that the base composition represented is unique in the RNA.

ACKNOWLEDGEMENTS

The authors are indebted to E. Bruenger and J. Rozenski for technical assistance and to P. F. Crain and J. Rozenski for critical reading of the manuscript. This work was supported by grant GM21584 from the National Institute of General Medical Sciences. The University of Utah DNA and Peptide Core Facility is supported by NIH Cancer Center Support Grant CA42014.

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