Use of dimethylsulfate to probe RNA structure in vivo

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INTRODUCTION

Understanding how RNA works requires the coordinated use of diverse experimental approaches. Knowledge of RNA structure and its relationship to function is an essential ingredient for interpreting the biological mechanisms of RNA action. Use of genetic, phylogenetic, biophysical, and computational approaches to divining RNA structure and structural dynamics is greatly enhanced by the application of chemical and enzymatic probes of RNA structure in solution. Among the most versatile chemical probes available for studying RNA and ribonucleoprotein structure is dimethylsulfate (DMS), which can directly donate a methyl group to specific hydrogen-bond accepting ring nitrogens on A, C, and G residues in RNA. The efficiency of methylation reports the chemical environment of the sensitive ring nitrogens in each base: hydrogen bonding or poor solvent accessibilty results in protection from methylation, whereas solvent exposure or an unusual chemical environment may enhance methylation[1]. The efficiency of methylation can be estimated at many positions along the RNA chain by evaluating methylation-dependent stops to primer extension by reverse transcriptase[2,3]. This information provides clues about the environment of individual nucleotides that can be compared to RNA structural models and hypotheses about RNA function.

A major experimental convenience of DMS is its rapid penetration into all compartments of the cell. This feature has allowed probing of RNA structure in a wide variety of cells including gram negative[4,5] and gram positive[6] bacteria, yeast[7], protozoa[8,9] and plant[10], including the nucleus[7], nucleolus[11], and chloroplasts[12]. Short incubation times at physiological temperatures allow for a quick snapshot of RNA structure in vivo with a minimum of perturbation or concern that secondary effects lead to the observed structure. The method can be applied to many cultures simultaneously, facilitating direct determination of the effect of different mutations or treatments on folding of the target RNA. Using primers specific for a number of RNAs, the structure of many RNAs can be determined in the same sample. In this paper we present methods for the probing of RNA structure in yeast cells using DMS.

THEORY

If a particular base in RNA is involved in a secondary or tertiary RNA structure, or interacts with a protein or other ligand, it may have altered reactivity with DMS. For example, reactivity toward DMS of N1 of A and N3 of C would be reduced when the base is involved in a Watson-Crick base pair, as they accept hydrogen bonds and are not accessible to DMS. A similar situation may occur during interaction of the base with a protein or other ligand. The N7 of G is also methylated by DMS. Although not involved in Watson-Crick base pairing, N7 of G residues can be evaluated for unusual base-base interactions, ligand interactions or special chemical environments as well.

Because DMS readily penetrates cells without the need for extended permeabilization treatments, modification of RNA occurs under nearly *in vivo* conditions. Stopping DMS modification during RNA extraction can pose a significant challenge, however. To quench the DMS reaction, high concentrations of 2-mercaptoethanol are added to react with excess soluble DMS. Aqueous insoluble DMS that may contaminate the cell pellet can be removed by extraction of the culture with water-saturated isoamyl alcohol prior to centrifugation, a treatment that does not reduce the recovery of RNA.

Mapping of the methylated sites can be achieved in the total RNA population using a labeled primer specific for the target RNA. In the case of A and C, the methylated base directly inhibits reverse transcriptase, because the methyl group alters the Watson-Crick face of the base. In order to map methylation at N7 of G, the RNA must be treated with aniline and borohydride to cleave the RNA chain at the methylated Gs prior to reverse transcription. By comparing the pattern of modification-dependent reverse transcription stops to dideoxynucleoside triphosphate-generated stops using the same labeled primer, the sites of methylation can be mapped to the RNA sequence. Methylation-dependent stops occur one position before the corresponding dideoxynucleotide stop.

PRACTICE

The method has the advantages that it is neither technically demanding nor labor intensive, but care and a few pilot experiments for the empirical determination of certain variables will pay off. In outline, the method involves incubating an aliquot of cells in culture with DMS, quenching the reaction and removing the DMS from the cells, extracting total RNA, and mapping the sites of modified nucleotides using a primer-extension assay.

DMS is corrosive and toxic, and is a suspected carcinogen. It is volatile and inhalation is hazardous. It is readily absorbed through the skin. For these reasons, DMS should only be handled in the hood or in tightly closed containers. Non-latex gloves that resist penetration by organic chemicals should be worn and care should be taken to ensure that clothing does not become contaminated. The isoamyl alcohol and 2-mercaptoethanol used in the protocol are also volatile and noxious. Thus, closed containers and proper hood ventilation are necessary for the safe practice of this protocol. Information about DMS is available at http://www.state.nj.us/health/eoh/rtkweb/0768.pdf.

DMS treatment of yeast cultures

Yeast can be grown in a variety of media including YEPD or synthetic complete medium[13] at 18-37° C depending on the design of the experiment.

1. Take 10 ml aliquots of yeast culture at A_{600}= 0.75-1.5 and place in a 50 ml polypropylene tube in the hood. In order to maintain temperatures, tubes should be pre-warmed. Set up a "stop control" tube to evaluate the effectiveness of the DMS quench treatment.

2. Add 200 ul of a fresh (same day) dilution of DMS (Aldrich, 99%+ grade) in 95% ethanol (1:4, v/v). Mix well, but without cavitation. Do not add DMS to the "stop control" sample yet. Cap the tubes tightly.

3. Incubate with shaking for about 2 minutes if incubation temperature is 30°C or higher. At 18°C it may take 10 minutes to achieve a good reaction.

4. Stop the reaction by placing the tube on ice and adding 5 ml of 0.6 M 2-mercaptoethanol and 5 ml of water-saturated isoamyl alcohol. Cap tightly and shake well. After addition of stop solutions to the "stop control" tube, add an equivalent amount of DMS as the experimental tubes received in step 2 and mix again.

5. Centrifuge the cells at 3000x*g* in the cold for 5-10 minutes in order to pellet the cells and float the DMS-containing isoamyl alcohol phase away from the pellet. Carefully remove the upper isoamyl alcohol phase and the lower aqueous phase from the pellet. The isoamyl alcohol solubilizes any DMS micelles that may pellet with the yeast cells and react with RNA during subsequent manipulations.

6. Suspend the cell pellet in another 5 ml of 0.6 M 2-mercaptoethanol and centrifuge again.

7. Extract RNA from the cell pellet.

We usually do at least one stop control reaction during each experiment and have found that confidence in the data is greatly enhanced by these controls. Significant DMS reactivity can occur during RNA extraction and generally shows that all A and C residues are accessible. The stop control provides an assurance that the modification pattern observed was generated during the incubation period instead of during the extraction procedure.

RNA extraction

It may be important to begin RNA extraction immediately after stopping the modification reaction. Although frozen cell pellets from untreated yeast cultures can be stored and extracted by the following protocol with satisfactory results, we have not tried to store and extract RNA from frozen, DMS-treated yeast cultures. As with any RNA extraction protocol, use of DEPC-treated water for making buffers, baked glassware or RNAse-free plasticware, gloves, and other precautions are necessary.

1. Suspend cell pellets in 250 ul of AK buffer [AK buffer is 1 g triisopropylnapthalene sulfonic acid (ACROS Organics, NJ), 6 g sodium *p*-amino salicylate (Sigma), 1.17 g sodium chloride and 6 ml of water-saturated phenol, dissolved in water and brought to a final volume of 100 ml, can be stored frozen for several months] and rapidly transfer them to microcentrifuge tubes containing 0.5 mls of water-saturated phenol pre-incubated at 65° C, followed by vortexing at high speed for 30 seconds.

2. Continue incubation of the emulsified phenol-AK buffer mixture at 65° C for 30 minutes, vortexing for 30 seconds at 10 minute intervals.

3. Cool the mixture on ice for 2 minutes and centrifuge at 12,000xg for 10 minutes in a microcentrifuge. Transfer the aqueous phase to a fresh tube and re-extract the organic phase with 200 ul AK buffer.

4. Pool the aqueous phases and extract once with phenol/chloroform/isoamyl alcohol, 25:24:1, and once with chloroform/isoamyl alcohol, 24:1.

5. Bring the aqueous phases to a final concentration of 0.3 M sodium acetate (pH 5.2), and precipitate the RNA by the addition of two volumes of ethanol.

6. Collect the precipitate, rinse with 70% ethanol, dry, and redissolve in water at a concentration of 1 mg RNA per ml (determined by absorption at 260 nm). From 10 ml of yeast cells at A_{600}=1, the usual yield is about 200 ug of RNA. Take care to observe that the RNA pellet is completely redissolved.

To map the sites of A and C methylation, proceed directly to primer extension (below). To map the methylation sites of N7 of G, it is necessary to chemically cleave the RNA at 7-methylguanosine (7mG) residues before primer extension. The chemistry of the cleavage of RNA at 7mG was first reported by Wintermeyer and Zachau[14] who showed that yeast $tRNA^{phe}$ could be cleaved specifically at a naturally occurring methylated guanosine. The methylated base is reduced with sodium borohydride followed by strand scission of the polynucleotide chain at the reduced residue with aniline. The method described below is an adaptation of the guanine specific cleavage reaction used in chemical sequencing of RNA[15].

Borohydride reduction and aniline cleavage at 7-methylguanosine

1. To 5 ug of modified RNA, add 1 ug carrier *E. coli* tRNA. Ethanol precipitate by the addition of 0.1 volume of 3M NaOAc pH 5.2 and 2.5 volumes of 95% ethanol. The addition of carrier 7mG-containing RNA such as tRNA has been shown to be critical for the site specific cleavage of some RNAs[16].

2. Resuspend the RNA in 10 ul 1M Tris-HCl pH 8.2. Add 10 ul freshly prepared 0.2 M $NaBH_4$ (Aldrich) and incubate 30 min at 0° in the dark. Stop the reaction by the adding 1 ul 20% 2-

mercaptoethanol, 70 ul 95% ethanol, and 10 ul 3M NaOAc pH 5.2. Freeze on dry ice and collect the precipitate by centrifugation at 12,000x*g* for 10 minutes.

3. Rinse the precipitate with 70% ethanol, dry, and resuspend the pellet in 10 ul of freshly prepared 1.0 M aniline/acetate buffer pH 4.5 [378 ul ddH_2O, 136.5 ul glacial acetic acid, 45.5 ul aniline (Aldrich 99.5+%)] and incubate 20 min at 55° in the dark.

4. Add 100 ul 0.2M NaOAc (pH not adjusted) and extract with 120 ul water-saturated phenol followed by extraction with 120 ul chloroform. Precipitate the RNA from the aqueous phase with 95% ethanol, rinse with 70% ethanol, dry and resuspend in 5-10 ul ddH_2O.

Primer extension to map modification sites

To map primer extension stops induced by DMS methylation, it is important to have a primer specific for the target RNA. Reaction of the primer with other RNAs in the cell can obscure signals from the target and complicate interpretation. Detection of signals from low abundance targets may be enhanced by optimizing primer annealing and reverse transcriptase parameters.

1. Kinase the oligonucleotide primer. Mix 2 pmoles primer with 1 ul 10X kinase buffer [0.5 M Tris-Cl, pH 7.5, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 10 mM EDTA], 4 ul (40 uCi, 3000 Ci/mmole) □-[^{32}P]-rATP, and enough water to bring the volume to 9 ul. Add 10 units (1 ul) polynucleotide kinase (New England Biolabs) and incubate at 37°C for 30-45 minutes. Stop by adding 90 ul of 10 mM Tris-Cl pH 8.0, 1 mM EDTA and heating to 65°C for 5-10 minutes. To check that the reaction was successful, spot 1 ul on a rectangular piece of Whatmann 3MM paper about 1.5 cm from the bottom. Place in a beaker filled to a depth of 1 cm with 0.75 M K_2PO_4 pH 3.4 and allow the liquid front to move 5-6 cm. Wrap in plastic wrap and autoradiograph for 2 minutes. Labeled oligo remains at the origin, and unincorporated rATP migrates at the front.

2. Set up the primer annealing reactions in a total volume of 7 ul. To up to 5 ug RNA, add 0.5 ul 10x reverse transcriptase buffer [1.25 M Tris-Cl pH 8.3, 175 mM KCl], and 0.5-1 ul of the kinased oligonucleotide. This is ~20 nM stock, final oligonucleotide concentration in the annealing will be 1.5-3 nM. Usually this concentration is sufficient for oligonucleotides with good specificity and targets present at about 10^3 molecules per cell or less, with the lower limit of detection depending on the specific activity of the probe. It is possible to use less RNA and more primer if the target is more abundant and the oligonucleotide is sufficiently specific. Set up annealing reactions on the stop control and on unmodified RNA as well. To generate a sequence ladder, set up four additional annealing reactions with unmodified RNA.

3. Anneal the primer by heating to 95°C for 2 minutes, bring the reaction to 65°C for 5 minutes and then to the annealing temperature of the oligo for 30-45 minutes. The annealing temperature is best determined empirically but can be approximated by adding four times the number of GC base pairs and 2 times the number of AT and AU base pairs in the hybrid, and subtracting 5. Fancier formulas can also be used. Bringing the mixture to the annealing temperature and allowing it to slow cool at least 15 degrees over the course of 45 minutes usually ensures that efficient and specific annealing will occur. Allowing the temperature to go too low (e.g. placing the annealing reactions on ice) may encourage the formation of hybrids between the oligonucleotide and non-target RNAs.

4. For the reverse transcriptase sequencing reactions, add 0.5 ul of one of each of the ddNTP (Pharmacia) stocks to each of the four annealing reactions containing unmodified RNA. The concentration of the ddNTP stock necessary to obtain aesthetically pleasing and informative sequence ladders must be determined empirically and will be influenced by the base composition of the region being sequenced, the particular reverse transcriptase being used, and the quality (effective concentration) of the ddNTP and dNTPs in the reaction. Good starting points for the reaction

below using AMV reverse transcriptase would employ stocks of about 0.75 mM of ddATP or ddGTP and 1-1.5 mM of ddCTP and ddTTP.

5. Assemble a "premix" on ice. Per annealing reaction use 0.5 ul reverse transcriptase buffer (as defined above), 0.5 ul 0.1 M DTT, 1 ul 0.1 M $MgCl_2$, 0.5 ul of a mixture of 2.5 mM each dNTP (Pharmacia), 0.25 ul of 1 mg/ml Actinomycin D (Sigma, catalog number A1410), 0.25 ul of 20 units/ul AMV reverse transcriptase (Life Sciences, Inc.). To set up *n* reactions, multiply the above volumes by (*n*+1), mix and distribute 3 ul to each annealing reaction. Incubate at 42°C for 30-45 minutes.

6. Stop the reaction by adding 5 ul of a solution of 10 ug/ml RNase A, 30 mM EDTA, 0.6 M NaOAc pH 5.4. Incubate 5 minutes at 42°C.

7. Remove the RNase by adding 5 ul of a solution of 0.2% SDS, 20 ug/ml proteinase K, 0.6 M NaOAc pH 5.4. Incubate 10 minutes at 42°C.

8. Add 50 ul 95% ethanol and freeze on dry ice 10 min. Add 1 ml 70% ethanol, mix by inversion and centrifuge at 12,000x*g* for 10 minutes. Remove ethanol and dry. Resuspend pellet in 1 ul of 20 ug/ml proteinaseK, 25 mM EDTA. Incubate 2-5 minutes at 65°C.

9. Add 2 ul 98% formamide with dyes. Heat 5 minutes at 65°C. Load on 6-8% acrylamide, 7.5 M urea sequencing gel.

EXAMPLES AND INTERPRETATION

Probing of U2 small nuclear RNA in vivo

Structure-probing data for RNA in solution is often found to be in good agreement with RNA structures determined by other methods. Data obtained by probing *in vivo* can provide support for the biological relevance of structural models based on other data. Probing mutant derivatives of the RNA of interest provides a way to understand the impact of the mutation on RNA structure. As an example, we compare the results of *in vivo* structure probing of wild type and mutant U2 RNAs in the stem loop IIa region with the high resolution NMR structure of a model oligoribonucleotide of U2 stem loop IIa obtained by Stallings and Moore[17] (Figure 1, PDB Entry 1U2A).

These mutations were constructed and described in previous studies[7,18,19]. In this experiment, cells expressing only the indicated U2 gene were incubated with DMS as described above for two minutes at 30°C, the reaction was stopped, RNA was extracted from the cells and used for primer extension. Wild type U2 stem IIa loop nucleotides show a characteristic reactivity in vivo (Figure 1A) that agrees well with the NMR structure of the model stem IIa. The hyperreactivity of A60 toward DMS is consistent with its participation in an interaction through its N6 and N7 with the N3 and N2 of G55 (Figure 1B), thus holding the DMS reactive N1 of A60 into solvent. Residues 57-59 are stacked above A60 and project into solvent[17]. A61 does not quite pair with U54 in the NMR structure[17], and this is consistent with its poor protection. Paired residues 62, 52, and 51 are more protected, but not entirely due to alternative conformations of the RNA in vivo[19].

The problem of dynamic RNA structure is not well addressed by the solution structure probing technique, because the reactivity observed at any position is the sum of reactivities at that position of all structures found in the population during the incubation time. This means that the most abundant structure will contribute most to the reactivity pattern. The more complex the structural heterogeneity, the more difficult it will be to interpret the results. In this case, a conserved

U2 sequence downstream of the loop of stem loop IIa has the ability to pair with the loop, forming a competing structure that disrupts the stem[19]. Thus signals from the stem loop structure and the competing structure are superimposed. The existence of this alternatively folded form in the pool of U2 molecules in the cell likely explains the incomplete protection of bases in stem IIa.

Mutations in the loop that destabilize stem IIa enhance the formation of the competing structure, as seen by increased reactivity of C51 and A52 in the U56G and tmB mutants (Figure 1A). The NMR structure places the base moiety of U56 in a space beneath the loop that will not easily accommodate the bulky G residue[17], and the destabilizing effect on the loop may account for the increased reactivity observed. The increased reactivity of stem bases in the tmB mutant, which replaces residues 58-60 with GUU, may be caused by the loss of the G55-A60 base pair, as well as the stacking interactions above it. Another possibility is that the loop mutations could inhibit the binding of a protein to the loop, destabilizing the stem. This seems less likely considering the effect of tmB' (which destroys the complementary sequence needed to form the alternative structure) and the A57C mutation (which also reduces complementarity between the loop and the downstream sequence). Both of these mutants show increased protection of the stem bases, especially C62. Thus in vivo, the structure of wild type U2 stem loop IIa in the context of the full length U2 snRNA is very similar to the model oligonucleotide studied by Stallings and Moore[17]. Furthermore, the effect of mutations on the DMS accessibility of the RNA in vivo corresponds to the expected effect of the mutations on the structure.

Probing DMS reactivity at N7 of G residues in RNA

To demonstrate the usefulness of this method for identifying reactivities at N7 of G we probed 18S ribosomal RNA. Primer-extensions were performed using a primer complementary to nucleotides 1629-1645, just downstream of a conserved region of 18S rRNA (Figure 2). The reactivity pattern obtained is largely consistent with the assignment of helical regions proposed by phylogenetic analysis for this portion of yeast 18S rRNA[20]. Analysis of the pattern of A and C

accessibility (Figure 2A, left panel) observed for the regions represented as being single-stranded in the model[20] (Figure 2B), however, requires more information than sequence comparison can provide. Protections and enhancements in these areas may be due to protein, higher order structure or other unique environments. Reactivities of nucleotides in the homologous region of *E. coli* rRNA have been shown to be enhanced by binding of ribosomal proteins S7, S9 and S19[21].

To assay the accessibility of the N7 position of G, the RNA was subjected to reduction with sodium borohydride and aniline-induced cleavage. Primer extensions were performed with the same primer (Figure 2A, right panel). A nearly quantitative stop results from the natural methylation of the G at position 1577 in yeast 18S rRNA[22] in all samples subjected to aniline cleavage (arrow, 7mG1577). A stop observed only in the modified and aniline cleaved RNA was observed at G1592 (asterisk). The stop at G1592 represents a G residue that is especially available for methylation by DMS at N7. The reason for the accessibility of this residue is unclear but it may represent a disruption of the helical structure due to protein binding. Corresponding residues in *E. coli* 16S rRNA are also sensitive to N7 methylation[23]. We have been able to observe hyperreactive G residues using this method, but the reactivity of the typical G residue in RNA seems quite low. Aniline-induced strand scission can produce a background ladder due to a low rate of cleavage at many sites, obscuring subtle protections. It has not been easy to monitor A, C and G reactivities in the same sample, so we routinely run the aniline treated samples only to determine G accessibility. A recent report[24] used this approach to detect a hyperreactive G in the HIV Rev-response element RNA expressed in the context of a yeast mRNA.

Occasional U residues are accessible to DMS in vivo

U3 RNA is located in the nucleolus and is also accessible to modification by DMS *in vivo*[11], as shown in Figure 3. The region shown includes the most 3' stem loop of the U3A structure (A263-U308), and the adjacent short loop of conserved nucleotides (G251-G262),

referred to as "box C", which is required in mammalian U3 for protein binding[25]. The sites of strong DMS modification (Figure 3A, lane 1, labeled on the right) are indicated in Figure 3B in bold case. The pattern of modifications and protections corresponds well to the proposed structure of the stem loop. U282, which occurs within a run of six U residues in the terminal loop of the stem, is also specifically modified by DMS. This modification has been observed by others in vivo, but not in naked RNA or partially purified U3 snRNPs[11]. Methylation of U and G by DMS is rarely but reproducibly observed, and its chemical basis is not understood[26]. We suggest that the special environment of this residue in vivo may stabilize an enol tautomer of certain U residues, rendering them reactive to DMS at the N3 position. Stabilization of the enol tautomer of G (and methylation at N1 of G) would also explain the occasional G residue that reacts with DMS and leads to a reverse transcription stop in the absence of aniline treatment[26].

TROUBLE SHOOTING

Difficulties encountered in the use of DMS in vivo have usually involved either poor modification, or inability to stop the modification (e. g. "stop control" reactions that show that modification occurs during RNA extraction). In the case of poor modification, more of a 1:4 dilution of DMS in ethanol should be used per 10 ml of culture, rather than the same amount of a less dilute stock of DMS. Pure DMS has a limited shelf life and it may simply be worth ordering a fresh bottle if modification is poor. A small bottle of fresh DMS every few months, rather than a single lifetime supply is recommended.

In the case of modification in the stop control samples, too much DMS is being used, and more dilute stocks (e. g. 1:6, 1:8 of the DMS in ethanol) should be tried until the modification reaction is suppressed in the stop control samples, but is readily detected in the experimental samples.

One problem encountered with primer extension is poor specificity of the oligonucleotide primer. This causes multiple stops that are not interpretable. A specific primer used under optimal reverse transcription conditions for the unmodified RNA target will generate a very strong stop corresponding to the 5' end of the RNA, as well as several structure-induced stops characteristic of the target. Non-target RNAs may also produce such stops, but these may be longer than the longest product expected from the target. It is advisable to determine empirically the optimum annealing conditions for each primer.

A second problem is primers that do not label efficiently or do not prime efficiently due to formation of competing structure within the primer or the target. These problems may require using alternative primers. If several primers on the same target do not produce satisfactory signals, the target may not be abundant enough. Enriching the RNA sample for the target, or optimizing the KCl concentration in the reverse transcriptase reaction may help.

A third problem that is usually diagnosed during evaluation of primer extensions is that of ribonuclease contamination of samples. This is most often seen when a strong stop is observed at a position (or several positions) equivalent to a dideoxyTTP-induced stop at an A residue in the target RNA. The A will almost always be preceded by a U in the sequence. This usually indicates that the target RNA has been hit by a pancreatic-type (RNaseA) nuclease which prefers UpA sites for cleavage, producing a strong stop at the cleavage site.

Difficulty in generating a satisfactory sequencing ladder usually can be remedied by adjusting the concentration of the dideoxyNTP stocks used until an appropriate distribution of stops is obtained for each nucleotide.

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FIGURES

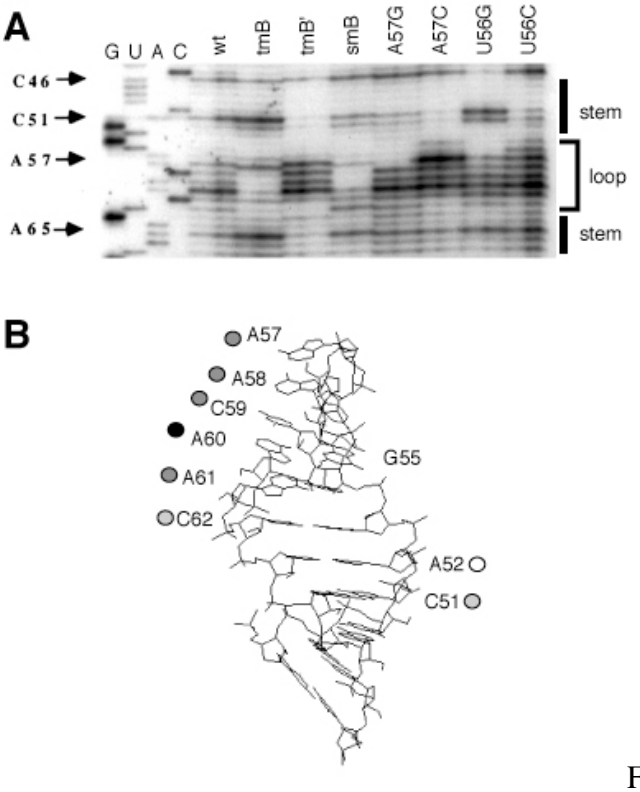

Figure 1.

Figure 1. Comparison of in vivo DMS accessibility with an NMR structure. (A) DMS reactivity of wild type and mutant U2 stem IIa. To determine the structural features associated with specific mutant forms of U2, yeast strains expressing either wild-type or mutant U2 were treated with DMS, total RNA was then extracted and analyzed by primer-extension using an oligonucleotide

complementary to U2. The U2 RNA sequence shown in the first four lanes was generated using unmodified RNA as template by including a different dideoxynucleotide in each of four primer extension reactions. The next lanes show the extension products using modified RNA derived from wild type and seven mutant strains (samples are in duplicate pairs). The mutations include: tmB = 58ACA60 to GUU; tmB' = 99UGU to AAC; and smB contains both tmB and tmB'. The single nucleotide changes are as indicated. Note that some of the changes in the reactivity patterns for these mutants result from sequence changes, for example changing an A to a G results in a loss of a reactive site rather than protection. The samples were separated by electrophoresis in an 8% polyacrylamide gel containing 7 M urea in TBE buffer. Extension products resulting from DMS-induced termination are 1 nucleotide shorter than the corresponding dideoxynucleotide terminations. (B) Comparison of DMS reactivity in vivo with the NMR structure of U2 stem loop IIa of Stallings and Moore[17]. Residues A52, C51 and C62 are relatively protected (white or light gray dots), whereas A57, A58, C59, and A61 are relatively reactive (dark gray dots). A60 is highly reactive (black dot). The N1 of A60 is held in solvent by the formation of a Sanger type II G-A pair with G55[17], explaining its hyperreactivity.

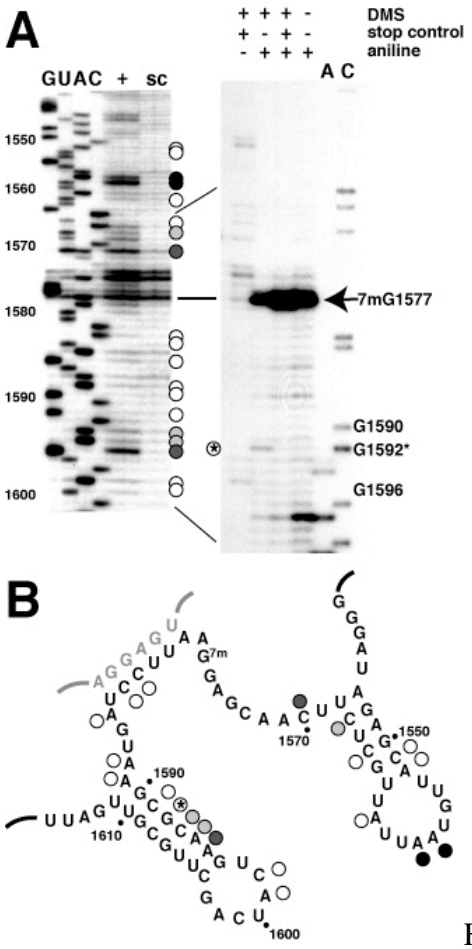

Figure 2.

Figure 2. Mapping reactive 7-methylguanosine residues in yeast rRNA (A) Primer-extension reactions using DMS-modified RNA (lane +) or "stop control" treated RNA (lane sc) either with (right panel) or without (left panel) treatment with aniline. Treatment of the RNA in the right panel is indicated at the top. Sequencing tracks were generated by primer extension on unmodified RNA with ddGTP (lanes C, left and right panels), ddTTP (lanes A, left and right panels), ddATP (lane U) and ddCTP (lane G). The nucleotide positions within 18S rRNA are indicated on the left. The reactions were performed as described above except that the amount of RNA in the primer extension was reduced to 2 ug, and the primer was annealed by heating at 95° C for 2 min followed by slow cooling to 42° C. The G residues marked on the right are positioned to indicate the migration of the expected modification/cleavage induced stop, rather than the dideoxy termination induced stop. (B) Secondary structure model of the region of yeast 18S rRNA[20] displayed in

Figure 2A. The dots indicate reactivity (black and gray), or protection (white). The hyperreactive N7 of G1592 is indicated with an asterisk.

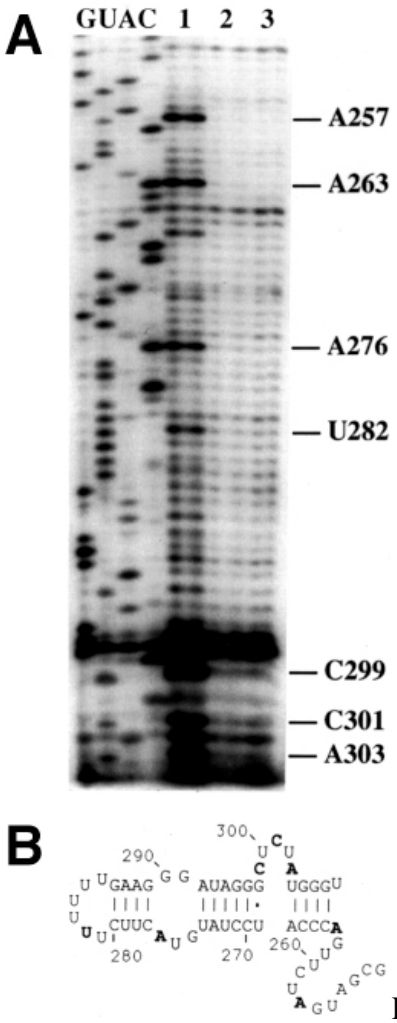

Figure 3.

Figure 3. An unusual U residue in U3 snoRNA is reactive to DMS in vivo. (A) Primer-extension assay to map DMS-reactive bases in U3. DMS-modified RNA, lane 1; "stop control" treated RNA, lane 2; unmodified RNA, lane 3. Duplicate, independent samples are shown for each treatment. Modified nucleotides are indicated on the right. The primer used is complementary to nucleotides 309-329 of yeast U3A. The RNA sequence shown on the left was generated by using the same primer with unmodified RNA and dideoxynucleoside triphosphates as described in the text. (B) Secondary structure model for nucleotides 251-308 of yeast U3A RNA. Modified nucleotides are indicated in bold type.