Analysis of RNA secondary structure by photochemical reversal of psoralen crosslinks

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ABSTRACT

Aminomethyltrioxsalen (AMT), a psoralen, is known to cause interstrand crosslinks in double stranded nucleic acids. We have demonstrated the photochemical reversal of this reaction, and have used this result to develop a method for identification of specific sequences which are adjacent because of RNA secondary structure formation. *E. coli* 5S rRNA is used as a model system. We isolated and characterized a product that is derived from the stem region of 5S RNA.

INTRODUCTION

RNA primary structure can be definitively determined by fingerprinting1 or rapid sequencing methods2,3,4,5, but the next level of organization, secondary structure, has resisted systematic analysis. Often the primary structure reveals regions of complementary base sequence; the stability of these local helices can be estimated using the quantitative parameters determined from the study of oligonucleotide stability6,7,8,9. However, the reality of the hypothetical RNA secondary structures which result from this analysis clearly needs direct experimental verification.

More confidence can be placed in secondary structures inferred from base sequences when comparative data are available on related RNAs from a variety of sources. Assuming that a common function derives from a common secondary structure10, the model is adjusted to accommodate all known sequences in the same structural pattern. This approach was successful in predicting the cloverleaf structure of tRNA, and has been applied to 5S rRNA.10,11 Unfortunately, extensive comparative data are not often available for species such as mRNA.

Among the direct methods for RNA secondary structure determination, x-ray crystallography12,13,14,15,16 is the most detailed and exact. How-
ever, tRNA is the only natural RNA crystallized to date, and crystal analysis may in addition be intractable for rare species such as mRNA because of the relatively large amounts of material required by the method. The gross structural features of large RNAs can be examined by electron microscopy but EM is a low resolution technique, and small helices or loops such as those in tRNA or 5S RNA are invisible.

Enzymes and chemical modifying agents have been successfully used to probe RNA structure. This method is particularly appropriate for demonstrating which regions of the molecule are accessible to the solvent, and for identification of sequences which interact with proteins. However, the question of which bases are joined by specific base pairing interactions is not addressed directly by such experiments.

RNA-RNA crosslinking reagents can in principle provide definitive evidence of proximity for specific nucleotides in an ordered RNA structure. In many cases only a few key crosslinks would be necessary to increase greatly the confidence in a particular hypothetical RNA secondary structure deduced from the nucleotide sequence. Wagner and Garrett have reported the use of a bifunctional, reversible RNA crosslinking reagent, 1,4-phenyl-diglyoxal, which reacts with guanosine residues. They found a crosslink in the stem region of E. coli 5S RNA. The structural specificity of their reagent is uncertain: dicarbonyl reagents generally react only with non-base paired guanines, but the phenyl ring may confer intercalating properties.

We have developed a method based on dissociation of RNA-RNA crosslinks, utilizing the reagent aminomethyltrioxsalen (AMT), a psoralen derivative which is expected to intercalate into double helices and crosslink the component strands. AMT putatively reacts with pyrimidines on opposite strands that are in adjacent base pairs. In addition to the general psoralen specificity for double helices, AMT has the desirable property of high solubility in aqueous buffer. Crosslinking can be carried out under a wide variety of conditions on isolated or in situ samples.

Crosslinked oligonucleotides are much simpler to identify if the crosslink can be dissociated. Visible light has been used to reactivate UV-treated DNA but this process is enzyme mediated in vivo and is not effective in reversing psoralen-DNA reactions. On the other hand, irradiation at 240-290 nm decomposes psoralen-thymine adducts. By analogy, it seemed possible that UV treatment might break the RNA-RNA crosslink, releasing the oligonucleotide fragments. Our results show that
photoreversal of AMT crosslinks can be accomplished without significant damage to the RNA chain.

Our method involves the isolation of an RNase digestion fragment containing a crosslink and analysis of its structure. AMT-treated RNA, photocrosslinked at 300-360 nm, is digested with RNase $T_1$. A two dimensional gel system reveals $T_1$ resistant fragments not found in untreated samples. The putative crosslinks are then cleaved with UV (~250 nm) light. The photoreversal products are isolated and characterized by conventional RNA sequence determination methods.

MATERIALS AND METHODS

$^{32}$P RNA: $^{32}$P 5S RNA was prepared from *Eschericia coli* MRE 600 by the whole cell phenol extraction method of Jordan. Approximately $1 - 2 \times 10^7$ cpm were recovered from 100 ml of culture labeled with 10 mCi $^{32}$P (New England Nuclear NEX-054). 5S RNA was separated by electrophoresis on a composite preparative gel (Buffer: 0.09 M Tris, 0.112 M borate, 1 mM magnesium acetate, 20 mM sodium chloride). The top 1/3 of the gel was 3% polyacrylamide, 0.5% agarose and the bottom portion was 12% polyacrylamide. After autoradiography and excision of the appropriate band, the 5S RNA was electrophoretically eluted from the gel (extraction buffer: 20 mM Tris-acetate, 1 mM MgCl$_2$, pH 8) for a period of 24-36 hours. For the last hour of elution the bottom (positive) reservoir was made 1 M in NaCl. Carrier 5S RNA was added; the sample was ethanol precipitated, phenol extracted, and precipitated again.

AMT: Aminomethyl trioxsalen (AMT) was synthesized according to Isaacs et al. from trioxsalen (gift of Paul Elder Co., Bryan, Ohio).

Crosslinking: Crosslinking reaction mixtures were exposed to long wavelength UV light in a Rayonet Type RS photochemical reactor (So. New England UV Co.). A combination of bulbs with either $\lambda_{\text{max}} = 300$ nm or $\lambda_{\text{max}} = 350$ nm was used. The Rayonet reactor is surrounded by reflecting surfaces, and the maximum energy flux in the empty reactor is quoted by the manufacturer as 40-50 W/cm$^2$. This value is doubtless strongly reduced when the reactor contains absorbing material. We found by using a $^3$H labelled sample of hydroxymethyltrioxsalen (HMT) (kindly supplied by Dr. John Hearst) that 3-4 hours irradiation was required to yield a plateau value for the extent of HMT-5S RNA attachment.

The sample holder was constructed as follows: four 12 x 100 mm test
tubes were sawed to a length of 3.2 cm and inserted through holes in a 8.5 x 7 x 3.5 cm clear plastic box. Dow Corning Silicone Rubber Sealant was used to make the joints water tight and a 2-hole stopper was inserted through a fifth hole for water circulation. Samples were irradiated in Eppendorf 1.5 ml polypropylene centrifuge tubes in 20 μl of 20 mM Tris-HCl, 10 mM MgCl₂, pH 7.5 (crosslinking buffer) at a temperature of 15°C.

**Photoreversal:** Samples in Eppendorf tubes were placed in an ice bath with the caps of the tubes open. A short wavelength illuminator (Chromatovue Transilluminator, Model C-61, UV Products, San Gabriel, California) with the filter removed was inverted 2 cm over the samples. Irradiation with UV light (7 mW/cm² + 20% at 250 nm) was performed for varying lengths of time.

To concentrate the RNA, the samples were diluted 5-fold with water and loaded onto DE-52 ion exchange resin. This was easily accomplished by preparing 1 ml Rainin Pipetman tips with glass wool and approximately 100 μl of DE-52. The pipet tips were then fastened on the ends of 10 ml plastic pipets, through which the sample could be loaded and washed. After washing with water, the pipet tips were placed in siliconized 10 x 75 mm culture tubes and spun dry in a clinical centrifuge. In this arrangement, 16 samples could be conveniently handled at once.

The RNA was eluted from the DE-52 by two additions of 100 μl 30% triethylammonium carbonate (TEAC), and dried as described below.

**Gel Electrophoresis:** Unless otherwise indicated, gel electrophoresis was performed on 12.5% or 15% polyacrylamide slab gels in 7 M urea-TBE buffer (50 mM Tris-borate, 1 mM EDTA, pH 8.3). Total digests of cross-linked samples were run on 38 cm 15 or 20% gels at 800-1000 V until the Bromophenol Blue was 10 cm from the bottom. 16 cm acid-urea gels 31 were run to separate oligonucleotides by base composition.

Except for the initial 5S preparation, samples were eluted from gels by crushing and soaking in extraction buffer (.5 M NH₄OAc, .01 M MgOAc) for at least 4 hours at room temperature.

**Sequence Analysis:** RNase T₁ (Sankyo Grade B, Calbiochem) was used at a concentration of 2500 units/ml. RNase A (Worthington) was used at a concentration of 200 μg/ml. Fingerprinting analysis was performed as described by Barrell (1), except that the second dimension, homochromatography, was run on PEI plates (Brinkman Cel 300 PEI) instead of DEAE plates. RNase A products were electrophoresed at pH 3.5 on DEAE paper or on Brinkman 20 x 40 cm DEAE plastic backed plates. Samples were eluted from PEI in the
following manner: small pipet tips (200 μl, Rainin) were plugged with a small amount of glass wool, then attached to an aspirator vacuum hose. The PEI was scraped into the pipet tip and was washed with 0.5-1 ml ethanol. The pipet tip was placed in a holder (easily made from 12 x 75 mm plastic tube caps, Faldon, Oxnard, CA) in 10 x 75 mm siliconized culture tubes. 75 μl of 30% triethyl ammonium carbonate (TEAC), pH 8.5 was added; and the sample was spun briefly in a clinical centrifuge. An additional 50 μl of TEAC was added, and the sample was spun dry. The liquid was poured onto a plastic sheet in a large Petri dish and the salt was removed by drying with a hairdrier attached to a variable voltage source. The spots were washed twice with 4 drops of water and redried to remove traces of TEAC.

RESULTS

Assay for crosslinking and photoreversal: It is known from modification and partial enzymatic cleavage studies that one of the most accessible residues in 5S RNA is G₄₁.²⁰ Jordan and Monier showed that a molecule having identical polyacrylamide gel electrophoresis and fingerprint characteristics to 5S RNA can be formed by reassociation of the two pieces (I = residues 42 → 120, II = residues 1 → 41) which result from partial T₁ digestion. We have used this system to show that the two fragments I and II can be photocrosslinked, and that the covalent linkage can be photodissociated.

Figure 1a shows a partial digest (10⁻³ units of T₁/μg RNA, 0°C for 15 min) of 5S RNA. Bands I and II were eluted from the gel in elution buffer and the solution was made 30 mM in Mg²⁺. Equimolar amounts of I and II (total RNA concentration ~ 1 OD₂₆₀) were mixed and heated at 60°C for 15 min. The sample was cooled slowly and 50 μg carrier RNA was added. After ethanol precipitation the sample was redissolved in crosslinking buffer, AMT was added to an AMT:base pair ratio of 1:3, and the sample was exposed to 300-360 nm light for varying time intervals. Samples were run on a 12.5% denaturing gel until the bromophenol blue was almost at the bottom of the gel. Figure 1b shows that only a combination of psoralen plus near UV light results in covalent binding of fragment I to fragment II. This species, 5Y, has a slightly lower mobility than 5S RNA, probably due to bound AMT molecules.

Photoreversal was demonstrated using the crosslinked 5S RNA fragments. Figure 1c shows the results after several different times of irradiation.
5S RNA
Fragment I
(X)
Fragment II

(a)

1 2 3 4 5 6

5Y
I
II

(b)
Figure 1. Electrophoresis of 5S RNA fragments on 12.5% polyacrylamide TBE-urea gels. X indicates position of xylene cyanol FF, and B indicates position of bromophenol blue. (a) Partial digest of 5S RNA. (b) 1) Isolated fragment I. The shadow below the primary band is a degradation product of Band I, missing 13-15 nucleotides from the 5' end. 2) Isolated fragment II. In slots 3, 4, 5, and 6, fragments I and II were mixed and annealed as described in Results, then treated as indicated: 3) Neither AMT nor light. 4) 365 nm irradiation, 1 h., no AMT. 5) AMT (1 AMT:3 base pairs), no light. 6) AMT plus light results in crosslinked band (5Y). The degradation product of Band I can participate in the crosslinking reaction. Thus the degraded portion of the molecule is not essential for reannealing and crosslinking. (c) Photoreversal of 5Y with far UV light. 1) 0', 2) 7.5', 3) 15', 4) 30', 5) 60'.

Microdensitometry of the different slots of the gel autoradiogram shows that after 15 min the reversal is 90% complete. By 30 min all the radioactivity has moved out of the crosslinked band, with most of the counts appearing in the fragment bands I and II. This constitutes proof that AMT can be used as a reversible RNA-RNA crosslinking agent.

Crosslinking and photoreversal of 5S RNA: Crosslinking of intact 5S RNA and photoreversal of the digestion products followed closely the protocol established for the mixture of fragments I and II. A typical crosslinking mixture contained 20 μg (10^6 cpm) 32P 5S RNA and 1.5 μg AMT in 20 μl of 20 mM Tris-HCl, 10 mM MgCl2, pH 7.5. After 1 hour in the Rayonet illuminator, the sample was alcohol precipitated, redissolved in the same buffer, and another 1.5 μg of AMT was added. After another 1 hour irradiation, it was precipitated again and washed twice with 70% ethanol. 5 μl T1 stock was added to the dried pellet and it was incubated at 37° for 30 min.
It was then run on a 38 cm 15% TBE gel. Figure 2 shows the evolution of crosslinked bands as a function of increasing concentration of AMT. Figure 3 shows the digestion pattern of a crosslinked sample as a function of RNase concentration. For further analysis, the high molecular weight bands were excised and soaked in 6 M urea for 30 min.

A second dimension of separation was achieved by running the samples on a 10% gel at pH 3.5. Under this condition the mobilities of the fragments depend on their base composition. The gel pieces from the 15% sizing

![Gel Image](image_url)

**Figure 2.** Crosslinking as a function of AMT:base pair ratio. The 5S RNA has been crosslinked at various ratios, digested with RNase T1, then run on a 15% TBE-urea gel. (a) no AMT, (b) 1:30, (c) 1:10, (d) 1:3, (e) 1:1.
Figure 3. T₁ RNase digestion of crosslinked 5S RNA at varying enzyme:substrate ratios, run on a 20% TBE-urea gel. (a) 1:200, (b) 1:100, (c) 1:40, (d) 1:20, (e) 1:5, (f) 1:1, (g) control 5S, 1:20.

gel were applied to the top of a base composition gel and a mixture of Xylene Cyanol FF, Acid Fuchsin, and Orange G in glycerol was layered on top. Electrophoresis was performed until the Orange G reached the bottom of the gel. This second gel gave a significant purification; some bands split into 4 or 5 distinct species. Band 1 (Figure 2) was homogeneous in both size and base composition dimensions (data not shown). The bands were crushed and soaked in 900 µl extraction buffer in 1.5 ml Eppendorf centrifuge tubes. After removal of 100 µl for a nonreversed control, 200 µl glacial acetic acid was added. The samples were photoreversed for 30 minutes.
To separate photoreversed products the samples were developed by homochromatography by using homomix C-15 (1) on PEI plates. Band 1 gave the most clear cut photoreversal pattern, decomposing into two spots (Figure 4). The spots were eluted with 30% TEAC (75 μl then 50 μl as above), redigested with RNase T₁, and re-run on PEI plates. Figure 5 shows the redigestions of band 1 and the photoreversed products, 1b and 1c. Although band 1 can be further degraded by RNase T₁ (Figures 3 and 5), 1b and 1c are far more susceptible to digestion, leaving little trace of the starting material (Figure 5).

The T₁ digestion spots shown in Figure 5 were eluted and analyzed by digestion with RNAse A. Table I gives these results. Spot 1b6 is curious because there is no T₁ fragment in 5S RNA such as (AC)ₙG. There are two possible structures for this fragment. It could either be an AMT monoaddition product or it could be the 5' terminal pUGp. In the T₁ fingerprint the 5'-end runs approximately as a 6 or 7 base oligomer, and the RNAse A analysis of this fragment gives bands of identical mobility to 1b6. Hence we conclude that 1b6 is pUGp.

The oligomers that constitute 1b correspond to the 5'-terminal region of 5S RNA, positions 1 - 9. 1c comprises nucleotides 108 - 116. In current models of 5S RNA¹⁰,²⁰, the complementary regions 1-10 and 110-119 form an

![Figure 4. Homochromatograph of photoreversal of band 1 on PEI plate. (Y) indicates position of Orange G. (1) Non-reversed control, (2) Photoreversal products.](image-url)
Figure 5. RNAse T₁ redigestion of photoreversed products, (a) size markers, (b) band 1 that has not been reversed, (c) lb, (d) lc. Numbers at left refer to size of nucleotides. (Y) indicates position of Orange G.

uninterrupted 10 base pair helix, the longest and most stable helix in 5S RNA (Figure 6). Crosslinking of oligomers lb and lc clearly reflects the existence of this helix. The identity of the other 5S RNA crosslinked products will be the subject of a later paper.

DISCUSSION

The technique outlined here involves directly isolating covalently linked regions of RNA that are in a base-paired configuration. After
Table I

<table>
<thead>
<tr>
<th>Photoreversed</th>
<th>T₁ Redigestion Fragment Size</th>
<th>RNase A Products</th>
<th>Sequence of T₁ Oligomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>1</td>
<td>G</td>
<td>Gp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gp</td>
<td>pUGCCUGCGG(G)p</td>
</tr>
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<td>2</td>
<td>2</td>
<td>C,G</td>
<td>Cgp</td>
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<tr>
<td>4</td>
<td>4</td>
<td>C,U,G</td>
<td>CC Ugp</td>
</tr>
<tr>
<td>6</td>
<td>&quot;AC&quot;,G</td>
<td></td>
<td>pUgp</td>
</tr>
<tr>
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<td>AG,C</td>
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<tr>
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<td></td>
<td>AACUGCCAGp</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>AAC,U,G</td>
<td>AACUGp</td>
</tr>
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</table>

photodissociation of the products, they are separated and analyzed by standard RNA sequencing methods. Musajo et al. report that the decomposition of psoralen-thymine adducts yields psoralen plus thymine, while decomposition of psoralen-cytosine adducts yields psoralen plus uracil. They state that the deamination of cytosine occurs during the post-irradiation processing. We have no firm evidence that the structure of

Figure 6. Possible secondary structure model of 5S RNA, adopted from Fox and Woese.
crosslinked then photoreversed bases is identical to the original bases, except that the fragments resulting from photoreversal are well behaved when subjected to sequence analysis. However, care must be taken in the analysis of photoreversed products because single strands with monoadducts have anomalous mobilities. The relative rates of mono- and diadduct photorelease have not been measured.

It is likely that the probability of crosslinking a certain helix depends on the accessibility of that region. Proteins or globular RNA configurations could protect helices from psoralen intercalation. This is a disadvantage in that information on inaccessible regions is limited. It is attractive, however, in that psoralen binding is not a strongly disruptive process.

The primary difficulty in our method is purifying enough homogeneous product to permit subsequent analysis. Band 1 (Figures 2 and 3) is well separated on the sizing gel, it is homogeneous on the base composition gel, and it decomposes into two fragments upon photoreversal. There were several bands resulting from irradiation that, unlike band 1, remained a single species after photoreversal. We take this to mean that in some cases monoaddition of AMT can cause incomplete enzyme digestion. Monoaddition could be due to incomplete photoreaction, improper intercalation, or the unavailability of a pyrimidine on the opposite strand in the adjacent base pair.

Other techniques could be used to separate the photoreversal and subsequent T1 products, for example polyacrylamide gels. However, we chose the homochromatography system for two reasons: (1) for long film exposures there is no diffusion problem on PEI because it is dried after chromatography, and (2) elution and recovery of small oligonucleotides is more easily accomplished from PEI than from a gel. Another chromatography system that has been used with 5S RNA fragments21,34 is electrophoresis on DEAE paper. In a small molecule such as 5S RNA, almost all the T1 products are well separated, but in a larger RNA molecule secondary analysis of the products would be necessary.

In principle, one could use psoralen crosslinking to probe larger RNA's such as 16S or 23S rRNA. Crosslinks protect helices from single stranded RNase digestion. This is observed in 5S RNA: after photoreversal spots 1b and 1c are more T1 susceptible than the precursor band 1 (Figure 5). Partial products would be very useful in working with a large molecule. Medium sized T1 fragments normally contaminated by other sequences of the
same chain length would become distinctive if still attached to the sur-
rounding oligonucleotides. If there were several oligonucleotides of the
same length in proximity, a two dimensional fingerprint could be run on the
photoreversed products. Subsequent RNase A and alkaline hydrolysis analysis
would unambiguously identify the products. In a molecule such as *E. coli*
16S RNA where the sequence is known³⁵, an analysis such as outlined here
could pinpoint crosslinks that have been approximately located by electron
microscopy.

We believe that this crosslinking technique will be a powerful addition
to present methods for elucidation of RNA secondary structure.

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REFERENCES

1. Barrell, B.G. (1971) in Proceedings in Nucleic Acid Research, Cantoni,
   York.
   Res. 4, 2527-2538.
4. Axelrod, V.D., Vartikyan, R.M., Aivazashvili, V.A., and Bebealashvili,
   5334-5338.
6. Tinoco, I., Jr., Borer, P.N., Dengler, B., Levine, M.D., Uhlenbeck,
   O.C., Crothers, D.M. and Gralla, J. (1973) Nature New Biol. 246, 40-
   41.
   J. Mol. Biol. 86, 843-853.
   649.


