Mapping of psoralen cross-linked nucleotides in RNA

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ABSTRACT

A method is described for using the cross-linking reagent 4'-(hydroxymethyl)-4,5',8-trimethylpsoralen (HMT) to map base paired regions and higher-order structure within RNA molecules. Applying this method to yeast tRNA^{Phe}, we have specifically identified cross-links within the acceptor stem between U₆ X U₆₈, in the D-stem between C₁₁ X C₂₅, and in the TΨ-stem between U₅₀ X C₆₃ and U₅₂ X C₆₃. We have also identified a unique cross-link between U₈ X C₄₈ which are trans pyrimidines in the core region due to tertiary interactions between U₈:A₁₄ and C₄₈:G₁₅. The precise point of cross-linking was deduced in every case by using purine-specific U₂ ribonuclease along with cytidine-specific CL3 ribonuclease which will anomalously cleave after photoreversed pyrimidines. The ability to map the precise point of cross-linking should prove invaluable in identifying nucleotides in close proximity within the tertiary structure of other RNA molecules.

INTRODUCTION

Determination of the structure of RNA molecules is crucial to our understanding of their biological function within a cell. Though detailed structural information can be obtained from X-ray crystallographic studies (1), the elucidation of conformational changes which RNA molecules might undergo while functioning requires a different approach. Recently, structure-specific enzymes and chemicals have proven very useful for mapping both single- and double-stranded regions within a renatured ³²P-end-labeled RNA molecule in solution (2-7). However, definitive evidence for which nucleotides are base paired, as well as, information regarding the proximity of specific nucleotides within the tertiary structure is unobtainable by this method. One method that may be useful in obtaining such information is psoralen cross-linking.

The family of furocoumarin derivatives known as psoralens have been used increasingly in recent years to study nucleic acid structure (8-12). The psoralen derivative 4'-(hydroxymethyl)-4,5',8-trimethylpsoralen (HMT) is more water soluble and more reactive than the parent compound (9,13,14), and has been used quite successfully in studies on RNA structure in solution

(11-14). HMT is a planar heterocyclic molecule that can intercalate between the stacked bases. Upon irradiation at 365 nm, the intercalated molecule will undergo cyclophotoaddition to the 5,6 double bond of a pyrimidine to form a monoadduct. A small conformational change results with some partial unstacking, thus allowing for a second photoaddition reaction to occur with a pyrimidine in the opposite strand in an adjacent base pair. This diadduct between trans pyrimidines can be reversed by irradiation at 254 nm (11-15). The skillful use of HMT cyclophotoaddition in combination with photoreversal and subsequent fractionation of reversal products by polyacrylamide gel electrophoresis, has recently permitted mapping of base paired regions and higher-order interactions in *Drosophila* 5S RNA (11) and *E. coli* 16S rRNA (12,15,16). The assignment of which nucleotides are actually involved in the cross-link, however, relies in part on the assumption that cross-links occur predominately between uridines and requires the use of phylogenetic conservation data to rule out unlikely base paired regions (12).

In this paper we report a method using HMT in conjunction with partial ribonuclease digestion, *in vitro* 32 P-end-labeling, and two-dimensional polyacrylamide gel electrophoresis to map base paired regions and higher-order interactions in RNA. Applying the method to yeast tRNA^{Phe}, we have specifically identified cross-links within the acceptor stem between U₆ X U₆₈, in the D-stem between C₁₁ X C₂₅, and in the TΨ-stem between U₅₀ X C₆₃ and U₅₂ X C₆₃. We have also identified a unique cross-link between U₈ X C₄₈ which are trans pyrimidines in the core region due to tertiary interactions between U₈:A₁₄ and C₄₈:G₁₅. The precise point of cross-linking can be deduced in every case using purine-specific U₂ ribonuclease and in some cases, cytidine-specific CL3 RNase which will cleave after photoreversed pyrimidines. The ability to map the precise point of cross-linking should prove useful in identifying nucleotides in close proximity within the tertiary structure of other RNA molecules.

MATERIALS

4'-(hydroxymethyl)-4,5',8-trimethylpsoralen (HMT) was obtained from HRI Associates, Inc., Emeryville, CA. Yeast phenylalanine tRNA^{Phe}, calf intestinal alkaline phosphatase (CIAP), and T₄ polynucleotide kinase were obtained from Boehringer-Mannheim. Ribonucleases T₁ and U₂ were prepared by Sankyo and obtained through Calbiochem, La Jolla, CA. *Physarum* RNase I and *B. cereus* RNase were purchased from P&L Biochem, Inc. Cytidine-specif-

ic chicken liver enzyme (CL3) was obtained from Bethesda Research Labs. γ -[^{32}P]-ATP (7000 Ci/mmol) was prepared from carrier-free ^{32}Pi (ICN 64014) by the procedure of Johnson and Walseth (17). Spectrapor dialysis tubing with a 3,500 dalton molecular weight cut off was purchased from Arthur H. Thomas Co. UV irradiations were performed with Chromato-vue transilluminators (model C-16 shortwave, model C-62 longwave) obtained from Fisher Scientific Company. Irradiation doses were measured with Blak-Ray UV meters (model J-225 shortwave, J-221 longwave), also from Fisher Scientific Company.

METHODS

Crosslinking Reactions

Yeast tRNA^{Phe} was dephosphorylated with calf alkaline phosphatase (CIAP) and subsequently purified by polyacrylamide gel electrophoresis (5,18). 0.5 A₂₆₀ units of dephosphorylated tRNA^{Phe} was placed in 20 μl of reaction buffer (5 mM Tris-HCl, pH 7.2, 0.1 mM EDTA) and incubated with HMT (5 $\mu\text{g}/\text{ml}$) for 15 min at 4°C to allow intercalation. The reaction mixture was then irradiated at 365 nm (122.5 mW/cm²) for 30 min. Both the intercalation and irradiation procedures were repeated twice to give a final concentration of 15 $\mu\text{g}/\text{ml}$ for HMT. Unreacted psoralen was removed by extracting twice with phenol, once with ether and precipitating the RNA with ethanol (11).

Partial Digestion with T₁ Ribonuclease

The psoralen-reacted tRNA^{Phe} was pelleted by high speed centrifugation according to the method of Shapiro (19). The RNA was dissolved in 20 μl of structure buffer (10 mM Citrate, pH 5.2, 200 mM NaCl, 10 mM MgCl₂) and digested with T₁ RNase (2.5 x 10⁻²) units/ μg RNA) for 10 min at 4°C. The digestion was terminated by the addition of proteinase K to a final concentration of 200 $\mu\text{g}/\text{ml}$ and incubated for 25 min at 25°C. The reaction mixture was then phenol extracted and the RNA ethanol precipitated (20).

5'-[^{32}P]-End-Labeling of RNA Fragments

The partially digested yeast tRNA^{Phe} was recovered by high speed centrifugation (19). The RNA was dissolved in 5 μl of 2 mM EDTA, heated at 65°C for 5 min and quick-cooled on ice. The RNA was brought to a final volume of 20 μl in kinase buffer (40 mM Tris-HCl, pH 8.5, 15 mM DTT, 9 mM MgCl₂, 15% DMSO). This mixture was transferred to a tube containing 4 mCi (400 pmol) of freshly lyophilized γ -[^{32}P]-ATP (7000 Ci/mmol) to which 7 units of T₄ polynucleotide kinase was added. The reaction mixture was incubated for 30 min at 37°C and terminated by the addition of 0.20 M EDTA

to a final concentration of 20 mM and 40 μ l of loading solution (99% formamide 0.1% w/v xylene cyanol and bromophenol blue dyes).

Purification of Cross-Linked Oligonucleotides by Two-Dimensional Polyacrylamide Gel Electrophoresis

The kinase mixture in loading solution was layered directly onto a 15% polyacrylamide slab gel (80cm long x 20 cm wide x 0.15cm thick) 32:1 acrylamide to bisacrylamide and 7M urea in TBE buffer (150 mM Tris-Borate, pH 8.3, 3mM EDTA). After electrophoresis for 21,000 volt-hours, the gel was autoradiographed and the lane containing the 5'-[32 P]-end-labeled T_1 fragments was excised into two equal sections (25cm long x 1.5cm wide). These two gel sections shown in Figure 1, the bottom (region I) and the middle (region II), were each placed at the bottom of two separate glass plates (33cm wide x 60 cm long) and dampened with TBE buffer. The psoralen cross-links were then reversed by irradiating the gel sections at 254 nm (1.4 mW/cm^2) for 5 min at 4°C. Each irradiated gel section was covered with another glass plate of equal size (33 cm wide x 60 cm long) and sealed to form a polyacrylamide gel mold. The irradiated gel strips were overlayed with 8% stacker gel (19:1 acrylamide: bisacrylamide, 7M urea, TBE buffer) to a level of 1 cm above the top of each strip. After polymerization of the stacker gel, the molds were filled with 20% polyacrylamide (16:1 acrylamide: bisacrylamide, 7M urea and TBE buffer). These gels were electrophoresed in the second dimension from bottom to top until the bromophenol blue dye was 10cm from the top in the region I gel and the xylene cyanol 15cm from the top of the region II gel. After autoradiography, the 5'-[32 P]-end-labeled photoreversed oligonucleotides migrating above the major diagonal (Figure 2) were excised and recovered by electrophoretic elution into Spectrapor dialysis tubing (3,500 dalton cut off) and ethanol precipitated (19,20). The oligomers were recovered by high speed centrifugation according to Shapiro (19), and dissolved in 15 μ l of H_2O for nucleotide sequence analysis.

Partial Enzymatic Digestion of the 5'-[32 P]-End-Labeled Photoreversed Oligonucleotides for Sequence Analysis

Partial enzymatic digestion of the purified 32 P-end-labeled oligonucleotides with T_1 RNase (4×10^{-3} units/ μ g RNA) and Phy I ribonuclease (0.6 units/ μ g RNA) was performed in buffer containing 50 mM citrate (pH 5.0), 7M urea, 1 mM EDTA, 1.0 μ g/ μ l carrier tRNA, 0.025% xylene cyanol and bromophenol blue, at 50°C for 30 min. U_2 RNase (1×10^{-4} units/ μ g RNA) digestions were performed in 50 mM citrate buffer (pH 3.5) and 7M urea at 50°C for 30 min. *B. cereus* ribonuclease (1.5 units/ μ g RNA) digestions were carried out in the absence

FIGURE 1. Schematic diagram of the method used to map psoralen cross-linked nucleotides in RNA.

of urea as previously described (21) at 50°C for 30 min. Chicken liver ribonuclease (CL3, 0.3 units/ug RNA) digestions were carried out in 20mM Tris-HCL, pH 7.2, and 75% DMSO at 50°C for 30 min. Controlled acid hydrolysis (H⁺) was performed as previously detailed (5,20) except the final acid concentration was adjusted to 0.2 N H₂SO₄ and reactions were incubated at 100°C for 45 seconds. Partial digests were electrophoresed on polyacrylamide sequencing gels.

RESULTS

Figure 1 outlines the method used to map base paired regions and higher-order interactions in RNA. In the studies described here, yeast tRNA^{Phe} molecules are first incubated with HMT to allow intercalative binding, then irradiated at 365 nm to form monoadducts and to cross-link trans pyrimidines. Because the binding constant for HMT is sufficiently low, there is little perturbation of the RNA structure (8). Since the unbound HMT will photo-destruct during the course of the irradiation, the intercalative binding and irradiation steps are repeated twice with fresh additions of HMT. A total of 2 psoralens/tRNA molecule produced maximal cross-linking. The reaction was further optimized by eliminating both Mg⁺⁺ and Na⁺ from the irradiation mixture. Due to the dense conformational packing of yeast tRNA^{Phe} (1), we found that the variety and quantity of cross-links could be increased in the presence of 0.1 mM EDTA. However, these salt conditions may not be optimal for other RNA molecules. For example, maximal cross-linking of mouse 5S RNA required 5 mM Mg⁺⁺. It is necessary therefore, that both the HMT concentration

and buffer conditions be optimized for the specific RNA molecules being studied.

In order to isolate discrete regions of the RNA containing cross-links, the tRNA^{Phe} was partially digested with T₁ RNase in structure buffer. This digestion generates a set of overlapping fragments containing cross-linked helices, cross-linked hairpin loops, and unreacted oligomers. These fragments were then labeled at their 5' termini with ³²P and fractionated on an 80 cm long denaturing polyacrylamide gel (Figure 1). After autoradiography, a reproducible pattern of bands containing both cross-linked and uncross-linked oligomers is discernible. This gel lane was then divided into two separate sections. The bottom region (I), contained fragments less than 26 nucleotides in length and the middle region (II) contained the remaining fragments. Both regions I and II were individually excised and each placed at the bottom of two separate glass plates. The strips were then irradiated at 254 nm to reverse the cross-links. Both the intensity and the time of irradiation, however, were substantially reduced compared with conditions previously reported (10, 12). Photoreversal times longer than five minutes at 1400 $\mu\text{W}/\text{cm}^2$ generally caused RNA to irreversibly bind to the acrylamide and removed the majority of the HMT monadducts. These monadducts (psoralen modified pyrimidines) assist in mapping the precise nucleotides involved in the cross-link.

To identify which regions of the RNA molecule are cross-linked, the photoreversed oligonucleotides must first be purified from unreacted fragments. This can be accomplished by polymerizing the irradiated gel strip within the bottom of a second denaturing polyacrylamide gel of a higher percentage and electrophoresing in the second dimension perpendicular to the strip. Cross-linked fragments (dimers, trimers, cross-linked hairpin loops) migrating within the first dimension will resolve into their component oligomers in the second dimension. After autoradiography, the 5'-[³²P]-end-labeled structural counterparts migrating in a line, one above the other, can be excised, the radioactive fragments recovered, and their nucleotide sequences determined enzymatically (19,21).

Figure 2 shows autoradiograms of two second dimension gels for yeast tRNA^{Phe}. In Figure 2A, photoreversed oligonucleotides from region I of the first dimension gel are resolved, while Figure 2B resolves counterparts from region II. The migration of fragments above the major diagonal was entirely dependent on both HMT cross-linking and photoreversal at 254 nm. Figure 3A shows a 2-D gel analysis of yeast tRNA^{Phe} subjected to the cross-linking

Figure 2. Autoradiograms of the two-dimensional polyacrylamide gels used to purify photoreversed 5'-[^{32}P]-labeled T_1 fragments of yeast tRNA^{Phe} . First-dimension: 15% polyacrylamide-7M urea. Second-dimension: 20% polyacrylamide-7M urea. A. Region I of first-dimension gel B. Region II of first dimension gel.

reactions and photoreversal in the absence of HMT. No photoreversal products are present. When the tRNA is cross-linked with HMT, but photoreversal omitted as shown in Figure 3B, no ^{32}P -end-labeled oligonucleotides are found above the diagonal. This data also demonstrates that photoreversal products are not a result of nonspecific photochemical breakdown.

The 5'-[^{32}P]-end-labeled photoreversal products #1-#32 in Figure 2, were reproducible and of high enough specific activity to sequence enzymatically. The nucleotide sequences of these oligonucleotides are listed in Table I. Most of these fragments were useful in mapping pyrimidines likely involved in HMT cyclophotoaddition reactions. However, the photoreversal products which were structural counterparts and generated the most useful

Figure 3. Autoradiograms of control two-dimensional polyacrylamide gels. A. 1-D: -HMT/ + U.V. 365 nm; 2-D +U.V. 254 nm. B. 1-D: + HMT/ + U.V. 365 nm; 2-D: - U.V. 254 nm.

information about the structure of yeast tRNA^{Phe} are summarized in Table II. The cross-linked nucleotides within these counterparts were assigned based on three criteria:

- i) determination of the most stable base pairing interaction between counterparts.
- ii) location of all possible trans pyrimidines within the putative helix.
- iii) identification of the trans pyrimidines cleaved anomalously with U₂ and CL3 ribonucleases.

The base pairing interaction and the cross-link between fragments #17 and #19 (Figure 2B) were determined using the polyacrylamide sequencing data shown in Figure 4. Fragment #17 (G₁-G₁₅) contains the 5'-end of both the acceptor and D-stems, while its counterpart, fragment #19 (m¹A₅₈-A₇₆) contains the 3'-end of the acceptor stem, as well as, the TΨC-stem. The most stable and the most likely association between these two oligonucleotides is indicated in Table II. The two possible trans pyrimidines within the putative helix are U₆ X U₆₈ and C₂ X C₇₀. In Figure 4, purine-specific U₂ ribonuclease cleaves after U₆ and U₇ of fragment #17, as well as, U₆₈ and

TABLE 1: Catalog of Nucleotide Sequences for the 5'-[³²P]-End-Labeled
Photoreversed T₁-Fragments of Yeast tRNA^{Phe}

FRAGMENT NUMBER	5' SEQUENCE 3'
1	N.D.
2	N.D.
3	N.D.
4	N.D.
5	N.D.
6	N.D.
7	N.D.
8	58 ^m 1 [*] AUCCACAGAAUUCG ₇₁
9	N.D.
10	66AA [*] UUCGCACCA ₇₆
11	5 [*] AUUUAm ² GCUCAG ₁₅
12	54TψCGm ¹ AUCCACAGAAUUCGCAC ₇₄
13	N.D.
14	21AGAGCm ² GCCAGACmUGmAAAYAm ⁵ CUG ₄₂
15	54TψCGm ¹ AUCCACAGAAUUCGCACCA ₇₆
16	N.D.
17	1CGGGAUUUAm ² GCUCAG ₁₅
18	N.D.
19	58 ^m 1 [*] AUCCACAGAAUUCGCACCA ₇₆
20	44AGm ⁷ GUCm ⁵ CUGUGTψCG ₅₇
21	1CGGGAUUUAm ² GCUCAGDDG ₁₇
22	58 ^m 1 [*] AUCCACAGAAUUCGCACCA ₇₆
23	44AGm ⁷ GUCm ⁵ CUGUGTψCGm ¹ AUCCACAGAAUUCGCA ₇₃
24	N.D.
25	2CGGAUUUAm ² GCUCAG
26	46 ^m 7 [*] GUCm ⁵ CUGUGTψCGm ¹ AUCCACAG ₆₅
27	21AGAGCm ² GCCAGACmUGmAAAYAm ⁵ CUG ₄₂
28	21AGAGCm ² GCCAGACmUGmAAAYAm ⁵ CUGG ₄₃
29	44AGm ⁷ GUCm ⁵ CUGUGTψCGm ¹ AUCCACAG ₆₅
30	21AGAGCm ² GCCAGACmUGmAAAYAm ⁵ CUG ₄₂
31	N.D.
32	18GGGAGAGCm ² GCCAGACmUGmAAAYAm ⁵ CUG ₄₂
B1	25Cm ² GCCAGACmUGmAAAYAm ⁵ CUGGAGm ⁷ GUCm ⁵ CUG ₅₁
B2	1CGGGAUUUAm ² GCUCAGDDGGGAGAGCm ² GCCAG ₃₀
B3	N.D.
B4	N.D.
B5	25Cm ² GCCAGACmUGmAAAYAm ⁵ CUGGAG ₄₅
N.D. (Not Determined-Impure) * (Psoralen Modified Pyrimidine)

Figure 4. Autoradiograms of sequencing gels for the structural counterparts #17 and #19 from the acceptor stem of yeast tRNA^{Phe}. The circled pyrimidines were anomalously cleaved with either U2 or CL3 RNase.

C₆₃ in fragment #19. These anomalous scissions after certain pyrimidines occur within photoreversed oligonucleotides only and could indicate that these pyrimidines have been modified by HMT cyclophotoaddition. Cytidine-specific chicken liver ribonuclease (CL3) also strongly cleaves after U₈ of fragment #17. U₆ and U₆₈ are the only trans pyrimidines within the proposed helix which undergo anomalous cutting. Together, this data suggests that the acceptor stem within yeast tRNA^{Phe} can be cross-linked with HMT via the formation of a diadduct between the trans pyrimidines U₆ and U₆₈.

TABLE II. NUCLEOTIDE SEQUENCES FOR THE 5'-³²P-OLIGONUCLEOTIDES OF YEAST tRNA^{PHE} MIGRATING AS COUNTERPARTS ABOVE THE DIAGONAL WITHIN THE 2-D POLYACRYLAMIDE GEL. (*) INDICATES P³²-ORALEN MODIFIED PYRIMIDINES.

LOCATION	SEQUENCE
ACCEPTOR STEM	
#11	5'...C A C G C U ³² pA U U U A m ² G C U C A G ₁₅ 3'
#12	3'...A C C A C G C U ³² pA U U U A m ² G C U C A G ₁₅ C C U m ¹ A G C C U T ₅₄ 5'
#17	5'...pG C G G A U U U A m ² G C U C A G ₁₅ 3'
#19	3'...A C C A C G C U ³² pG C G G A U U U A m ² G C U C A G ₁₅ C C U m ¹ Ap ₂₂ 5'
D-STEM	
#25	5'...pC G G A U U U A m ² G C U C A G ₁₅ 3'
#27	3'...G U m ¹ C U A Y A A G m U C m A G A C C m ² G C G A C Ap ₂₂ 5'
#25	5'...pC G G A U U U A m ² G C U C A G ₁₅ 3'
#28	3'...G U m ¹ C U A Y A A G m U C m A G A C C m ² G C G A C Ap ₂₂ 5'
T-Ψ STEM	
#23	5'...pA G m ² G U C m ¹ C U G U G T C G m ¹ A U C C A C A G A A U U C G C A ₇₃ 3'
#22	3'...A C C A C G C U U A A G A C C U m ¹ Ap ₂₂ 5'
CORE REGION	
#21	5'...pG C G G A U U U A m ² G C U C A D D G ₁₅ 3'
#20	3'...G C C T G U G U m ¹ C U m ² G G Ap ₂₂ 5'

To confirm the U₆ and U₆₈ cross-link within the acceptor stem, another pair of photoreversal products from the same region of the RNA was identified. Fragments #11 (A₅-G₁₅) and #12 (T₅₄-C₇₄) exist as counterparts in Figure 2A and, as shown in Table II, are subfragments of #17 and #19, respectively. Fragment #11, like fragment #17, contains the anomalously cleaved pyrimidines U₆ and U₈. Fragment #12 shows the anomalous cuts with U₂ RNase after U₆₈, C₆₃, as well as, U₅₉. Both U₆ and U₆₈ in this instance are the only trans pyrimidines within the proposed helix. This data supports the notion that the cross-link within the acceptor stem occurs between U₆ X U₆₈. These data illustrate that the generation of overlapping fragments with T₁ ribonuclease is useful in identifying both the cross-linked regions, as well as, verifying the nucleotides most likely involved in the cross-link. It is still not clear whether the anomalous cleavages occurring after the other pyrimidines are due to the presence of psoralen monoadducts, or are a result of photoreversal from a yet unidentified fragment. Both fragments #13 and #18 which are likely counterparts to #11/#12 and #17/#19, respectively, were too impure for sequence analysis (Table I).

The region encompassed by fragments #11 and #17 is also present in another fragment #25 (C₂-G₁₅) of Figure 2B. Fragments #27 (A₂₁-G₄₂) and

Figure 5. Autoradiograms of sequencing gels for the structural counterparts #25 and #28 from the D-stem of yeast tRNA^{Phe}. The circled pyrimidines were anomalously cleaved with either U₂ or CL3 RNase.

#28 (A₂₁-G₄₃) are migrating as counterparts to fragment #25. Figure 5 shows polyacrylamide sequencing gel analyses for the counterparts #25 and #28. The most stable base pairing scheme between these two fragments is shown in Table II. The only trans pyrimidines occurring within the proposed helix are C₁₁ and C₂₅. Both of these pyrimidines are cleaved by U₂ RNase as shown in Figure 5. In fact, C₂₅ is the only psoralen modified pyrimidine present in both fragments #28 and #27. This data indicates that the D-stem within yeast tRNA^{Phe} can also be cross-linked with HMT by forming a diadduct

Figure 6. Autoradiograms of sequencing gels for the structural counterparts #22 and #23 from the TΨ-stem of yeast tRNA^{Phe}. The circled pyrimidines were anomalously cleaved with U₂ RNase.

between the trans pyrimidines C₁₁ X C₂₅. It is noteworthy that both U₆ and U₈ in fragment #25 are also cut with U₂ RNase (Figure 5). These cleavages could be due to retention of psoralen monoadducts or again be a result of photoreversal from an unidentified oligonucleotide. The only other fragment within this set having a high enough specific activity was #26 (U₄₇-G₆₅), which does not appear to be a structural counterpart to #25, #27, or #28. The anomalous cleavages occurring after U₅₀ and U₅₂ in fragment #26 (Table I), may indicate that it was cross-linked to a yet unidentified counterpart.

Figure 7. Autoradiograms of sequencing gels for the structural counterparts #20 and #21 from the core region of yeast tRNA^{Phe}. The circled pyrimidines were anomalously cleaved with either U₂ or CL3 RNase.

The nucleotide sequence of fragment #26 (U₄₇-G₆₅), nevertheless, reappears within fragment #23 (A₄₄-A₇₃). Within this set of oligonucleotides indicated in Figure 2B, fragment #22 is likely its structural counterpart as shown in Figure 6. The best base pairing scheme between these two oligonucleotides is given in Table II. Three cross-links are possible within this putative helix (U₅₀ X C₆₃, U₅₂ X C₆₃, and U₅₂ X C₆₁). U₂ RNase, however, recognizes and cleaves U₅₀, U₅₂, and C₆₃, but not C₆₁. In summary, this data suggests that the Tψ-stem can also be cross-linked with HMT by formation of diadducts between either U₅₀ X C₆₃ or U₅₂ X C₆₃, but less likely between U₅₂ X C₆₁.

Fragments #20 and #21 are also present within the same set of photo-reversal products (Figure 2B). Nucleotide sequence analyses of these oligonucleotides is shown in Figure 7. The only satisfactory duplex that can

Figure 8. Enzymatic sequence analysis of yeast tRNA^{Phe} that has been irradiated at 365nm (1.5 hrs at 122.5mW/cm²), 5' [³²P]-end-labeled, fractionated on a 12% polyacrylamide gel and irradiated at 254nm (5mins at 1.4mW/cm²). From left to right: (-E) minus enzyme; (T₁) T₁ RNase, 2.4x10⁻³ and 2.4x10⁻⁴U/ug RNA; (H⁺) controlled acid hydrolysis; (U₂) U₂ RNase, 1x10⁻³, 1x10⁻⁴, 5x10⁻⁴, 2.5x10⁻⁴U/ug of RNA; (CL3) Chicken liver RNase, 0.3 and 0.1U/ug RNA. Asterisks denote enzyme concentrations comparable to those used for sequence analysis of photoreversed oligonucleotides.

be drawn between these two fragments involves base pairing between G₃-U₈ (#21) and m⁵C₄₉-A₄₄ (#20). The proposed helix, however, contains two G:U base pairs, one of which includes U₆ already designated to be cross-linked with U₆₈. Furthermore, the only trans pyrimidines present within this helix are U₆ and U₄₇. U₂ RNase does cleave after U₆, but not after U₄₇ as is shown in Figure 7, suggesting that this base pairing scheme is unlikely. C₄₈ is the only pyrimidine in fragment #20 cleaved by U₂ RNase. The only other nucleotides in fragment #21 which are likely candidates for forming a cross-link with C₄₈ are the psoralen modified pyrimidines U₇ and U₈. The obvious lack of a base pairing scheme when either of these two pyrimidines are trans to C₄₈ suggests the interesting possibility that these nucleotides are in close proximity within the tertiary structure of yeast tRNA^{Phe}. This is, indeed, the case as shown in Figure 9. Due to tertiary interactions between U₈:A₁₄ and G₁₅:C₄₈ (1) both U₈ and C₄₈ are trans pyrimidines in the core region of the tRNA^{Phe} molecule.

To eliminate the possibility that the anomalous cleavages by U₂ and CL3 ribonucleases are due to recognition of U.V. damaged nucleotides, dephosphorylated yeast tRNA^{Phe} was first irradiated at 365nm for 1.5 hr, 5'-³²P-end-labeled, fractionated on a 12% polyacrylamide gel, and the RNA again irradiated at 254nm for 5 min within the gel. Sequence analysis of the double-irradiated tRNA^{Phe} shown in Figure 8 demonstrates the absence of cleavage by U₂ ribonuclease at cytidine and uridine residues designated to be psoralen-modified in Tables I and II. Although weak cleavages after U₆, U₇, and U₈ with CL3 ribonuclease are present (22), the relatively strong cleavage after U₈ in psoralen cross-linked RNA is noticeably absent in the double-irradiated RNA.

The nucleotide sequences for fragments B₁-B₅ migrating below the diagonal in Figure 2 are summarized in Table I. The complete sequences of B₃ and B₄, however, could not be determined due to apparent sequence divergences. These divergences are likely caused by unreversed cross-links still retained within the fragment, possibly explaining their slower migration within the second dimension.

DISCUSSION

The location of each cross-link within the tertiary structure of yeast tRNA^{Phe} is shown in Figure 9. It is clear in every case that the pyrimidines cross-linked by HMT are all trans to one another. Since HMT is more likely to intercalate in helical regions with small bulges and partial unstacking (11), the G₄:U₆₉ wobble base pair within the acceptor stem contiguous to the

Figure 9. Designation of the psoralen cross-linked pyrimidines within the tertiary structure of yeast tRNA^{Phe}. The polynucleotide backbone is reduced to a thin line with the short boardlike structures representing unpaired bases and the longer boards representing base pairs.

U₆ X U₆₈ cross-link may promote HMT intercalation within this region. The variety and quantity of HMT cross-links found attests to the sensitivity of this method. Not only was every major stem, excepting the anticodon arm, cross-linked but cross-links between uracil and cytosine, as well as, between two cytosines were detected. To our knowledge this is the first report of a C-C cross-link (C₁₁ X C₂₅), and the third report of a C-U psoralen cross-

link (11,23).

The cross-linking method detailed in this paper is in principle similar to that used by Turner, et. al (15) for uniformly ^{32}P -labeled 16S rRNA. However, the use of in vitro ^{32}P -end-labeling in conjunction with enzymatic sequence analysis of the ^{32}P -end-labeled photoreversal products allows for specific mapping of the cross-linked nucleotides. The ability of both purine-specific U_2 ribonuclease and cytosine-specific chicken liver RNase (CL3) to recognize and cleave the psoralen modified pyrimidines, is probably due to an unreversed HMT monoadduct. The electrophoretic mobility of these modified pyrimidines is, in most instances, slower than unreacted nucleotides on sequencing gels (Figures 4-7). The ring structure of the psoralen modified pyrimidine somehow may mimic a purine and therefore be cleaved by U_2 RNase. Likewise, an HMT modified uridine may be recognized and cut with CL3 ribonuclease. Though the presence of HMT modified pyrimidines within photoreversed fragments is probably due to both monoaddition (14) as well as, diadduct reversal, these pyrimidines now become candidates for selecting which nucleotides are cross-linked within two structural counterparts. The ability to map the precise point of cross-linking should prove invaluable in identifying nucleotides in close proximity within the tertiary structure of other RNA molecules.

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