Identification of a site of psoralen crosslinking in *E. coli* 16S ribosomal RNA

Seán Turner, John F. Thompson*, John E. Hearst* and Harry F. Noller

Thimann Laboratories, University of California, Santa Cruz, CA 95064, and *Department of Chemistry, University of California, Berkeley, CA 94720, USA

Received 1 March 1982; Revised and Accepted 5 April 1982

ABSTRACT

We have developed a 2-dimensional gel method for identification of RNA sequences crosslinked by the intercalative drug 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT). This method is being used to localize such sites in *E. coli* ribosomal RNA. We report here the identification of a site for HMT crosslinking within positions 434 and 497 of 16S rRNA. We suggest a likely site for HMT intercalation, in which residues U₄₅₈ and U₄₇₃ become crosslinked via the drug.

INTRODUCTION

The 16S rRNA of prokaryotic ribosomes is known to be intimately involved in a number of facets of protein synthesis, including messenger RNA selection (1, 2, 3), tRNA binding (4, 5, 6), ribosomal subunit association (7, 8, 9), and antibiotic sensitivity or resistance (10). A knowledge of its structure is indispensable for a complete understanding of its participation in the process of translation. Since the elucidation of the primary structure of 16S rRNA from *Escherichia coli* (11, 12), much effort has been devoted to deducing a sound secondary structure for this important part of the prokaryotic ribosome. 4'-Hydroxymethyl-4,5',8-trimethylpsoralen (HMT) has been used in conjunction with electron microscopy to probe the secondary structure of *E. coli* 16S rRNA (13, 14), and a map of crosslinked sites has been reported (15). Several of these sites correspond to features of the secondary structure models derived independently on the basis of phylogenetic comparison, UV-crosslinking, and chemical and enzymatic probe studies (16, 17, 18). The remaining HMT sites do not appear to have obvious counterparts in the secondary structure models, and so may indicate heretofore undetected secondary or other structural features. We are therefore developing methods for precise localization of the sites of HMT crosslinking of rRNA.

Psoralens (furocoumarins) are known to interact with nucleic acids and form covalent C₄-cycloaddition adducts with pyrimidines when irradiated with

ultraviolet light at 320-400 nm (reviewed in 19, 20). These drugs are particularly well-suited for conformational studies of nucleic acids due to the non-damaging photoreversibility of the cycloaddition adducts when irradiated with light of 240-260 nm (21). The covalent crosslinking of double-helical nucleic acids by psoralens has been demonstrated for both DNA (22, 23, 24) and RNA (24, 25). Monoadduct sites have been determined to nucleotide specificity for *E. coli* tRNA (26, 27) and to a lesser extent in *E. coli* 16S rRNA (28). Sites of covalent crosslinking have been determined to sequence specificity for 5S rRNA from *E. coli* (21) and from *Drosophila melanogaster* (29). In this paper we report a method for isolating such regions from large rRNAs and give examples of sequences that are found.

MATERIALS AND METHODS

Ribosomal RNA Preparation

70S ribosomes uniformly labeled with ^{32}P were prepared from *E. coli* MRE-600 cells as described by Chapman and Noller (8). Ribosomes (4×10^6 cpm/ μg) were suspended at a concentration of 75 $\mu\text{g}/\text{ml}$ in buffer containing 10 mM Tris·HCl (pH 7.5), 0.1 M LiCl, 1 mM EDTA, $\text{Na}_2\text{S}_2\text{O}_4$, 0.5% SDS, and twice extracted with H_2O -saturated phenol. The aqueous phase was then loaded on 15%-30% linear sucrose gradients of the same buffer and centrifuged at 25,000 rpm for 22 hours, 10°C in a Beckman SW-27 rotor (30). Fractions containing 16S rRNA were pooled and the RNA twice precipitated with three volumes of ethanol at -20°C overnight.

Irradiation Technique

Precipitated RNA was taken up to a concentration of 35 $\mu\text{g}/\text{ml}$ in the 30S reconstitution buffer of Traub and Nomura (31) containing 30 mM Tris·HCl (pH 7.8), 20 mM MgCl_2 , 0.3 M KCl and incubated at 37°C for 30 minutes. Hydroxymethyltrimethylpsoralen (HMT) was taken from a concentrated stock solution and added to the RNA so that the final concentration of HMT was 43 $\mu\text{g}/\text{ml}$. The solution was then irradiated for 5 minutes at 17°C with UV light of 365 nm exactly as described by Bachellerie et al. (32). A second aliquot of HMT half as large as the first was added and the irradiation repeated as before. The RNA was then precipitated overnight at -20°C with ethanol.

Partial Digestion of RNA

Residual unreacted HMT and HMT photobreakdown products not removed by ethanol were extracted by resuspending the RNA to a concentration of 0.35 mg/ml in digestion buffer containing 10 mM Tris·HCl (pH 7.5), 10 mM MgCl_2 , 0.1 M NH_4Cl and extracting once with H_2O -saturated phenol and twice with H_2O -

saturated diethyl ether. Partial enzymatic digestion of the RNA was effected by incubating the extracted aqueous phase at 37°C for 30 minutes, adding RNase T₁ (1:200 w/w, enzyme:RNA), and incubating the solution for one hour at 0°C. Digestion was halted by the addition of 1/10 volume diethylpyrocarbonate (10% in ethanol), 1/5 volume 10% SDS, and diluting 1:4 with digestion buffer. RNase T₁ was then removed by thrice extracting with phenol and twice with ether as described above. RNA fragments in the aqueous phase were precipitated with three volumes of ethanol at -20°C overnight.

First Dimension Electrophoresis

Precipitated RNA was taken up in 25 µl of gel-loading solution containing 90% deionized formamide, 1 mM EDTA, Na₂, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol FF and incubated at 40°C for 20 minutes. The solution was then loaded on a 12% (30:1 monomer:bis) polyacrylamide gel (33 × 14 × 0.15 cm) containing 7 M urea, 90 mM Tris·borate (pH 8.3), 2.5 mM EDTA, Na₂, with the gel reservoirs containing the same buffer except without urea. Electrophoresis was carried out at 350 V at 6°C until the Bromophenol Blue was approximately 8 cm from the bottom of the gel.

Reversal of HMT Crosslinks

Following autoradiography, the lane containing the RNA was cut out from the gel in three pieces of equal length, covered with "Saran Wrap," and irradiated for 45 min at 6°C with a UV illuminator (Chromatovue Transilluminator, Model C-61, UV Products) inverted 3 cm above the gel pieces, as described by Rabin and Crothers (21).

Second Dimension Electrophoresis

Gel strips were each soaked for a total of one hour at room temperature in two changes of 50 ml of buffer containing 7 M urea, 4.5 mM Tris·borate (pH 8.3), 0.125 mM EDTA, Na₂, 0.01% Bromophenol Blue, 0.01% Xylene Cyanol FF, followed by polymerization into second dimension gels of identical composition as the first dimension. Soaking the gel strips in low ionic strength buffer between dimensions allows them to act effectively as stacking gels, thus causing the diagonal to run as a narrow band and facilitating the concentration of individual off-diagonal spots into smaller areas of the gel. Electrophoresis was carried out as before until the Bromophenol Blue had reached the bottom of the gel.

Sequence Analysis of HMT-Crosslinked Fragments

Following autoradiography, off-diagonal spots were cut from the gels and each extracted overnight on a rotary shaker at 2°C with 0.2 ml of extraction

solution containing 0.5 M ammonium acetate, 0.1 mM EDTA, Na_2 , 0.5% SDS. A second extraction with 0.1 ml extraction solution was carried out under identical conditions. Extracts for each spot were pooled and precipitated overnight at -20°C with three volumes of ethanol in the presence of 20 μg unlabeled yeast RNA as carrier. Enzymatic digestions and analyses of the RNA fragments thus isolated were done as described by Barrell (33).

Materials

Unless otherwise stated, all chemicals were of Reagent Grade purity and were used without further purification. ^{32}P (orthophosphate) was from New England Nuclear. HMT was synthesized by the method of Isaacs et al. (24). Sucrose and urea were of ultrapure grade from Schwarz-Mann. Phenol was from Mallinckrodt and was redistilled prior to use. Diethylpyrocarbonate (diethylloxydiformate) was from Eastman. Acrylamide and bisacrylamide were of "electrophoresis purity" grade from Bio-Rad. Triethylamine was from Aldrich and was redistilled prior to use. Ribonuclease T_1 (Sankyo Grade B) was from Calbiochem. Ribonuclease A was from Worthington. Yeast RNA (Type VI) was from Sigma and was extensively extracted with phenol prior to use. DEAE-cellulose was from Toyo Roshi, Nuclepore. In subsequent experiments, we have found that the best conditions for analysis of the products of enzymatic digests are the use of DEAE-cellulose DE-81, provided by Whatman as rolls, for the separation of complete RNase T_1 digestion products, and the use of DEAE-cellulose provided by Toyo Roshi as 50 x 40 cm sheets for the analysis of complete RNase A digestion products. Both Whatman DE-81 and Toyo Roshi DEAE cellulose provided as rolls are unsuitable for the analysis of RNase A digestion products, as Cp comigrates exactly with Gp under the above conditions. In addition, the latter is barely suitable for the separation of RNase T_1 digestion products in our hands, not only because of the severe streaking these undergo during separation under standard conditions, but also because it is extremely fragile under conditions of high-voltage electrophoresis.

RESULTS

Although other studies have shown that the incorporation of psoralens into both natural and synthetic RNAs is optimized by the use of buffers of low ionic strength and the absence of Mg^{2+} (13, 29, 34), we have chosen to use the optimal reconstitution buffer of Traub and Nomura containing high levels of salt and Mg^{2+} (31), since under these conditions the 16S rRNA can be incorporated into a 30S ribosomal subunit capable of participating in *in vitro* protein synthesis. This may be construed as indicative of biologically signifi-

cant secondary and tertiary structure, particularly in light of results indicting the structure of 16S rRNA under these conditions to be markedly similar to its apparent structure in the 30S subunit, based both on spectroscopic and hydrodynamic data (35), and electron microscopy studies (36). Low ionic strength and the absence of Mg^{2+} are known to alter the structure of E. coli rRNAs (37, 38), and secondary and tertiary structures produced under these conditions, although chemically interesting in themselves, are of uncertain biological significance. Although it is known that 16S rRNA undergoes necessary conformational changes during the reconstitution process (39, 40, 41), it is not unreasonable to assume that reaction of 16S rRNA with HMT in reconstitution buffer may lock the RNA in the conformation it must initially have in order to begin reconstitution with 30S ribosomal proteins.

HMT can be expected to crosslink base paired segments of 16S rRNA be they long-range interactions or short-range hairpin-type structures. The two-dimensional gel system used in this project was designed to separate HMT-crosslinked oligonucleotides, which may be present in low yields, from the main body of unreacted oligonucleotides. The rationale employed was that under the denaturing conditions of the gel, RNA fragments will be unfolded. A crosslinked oligomer complex would migrate more slowly than each of its component strands, due both to its greater molecular weight, and possibly also to the potentially retarding effect of its multi-armed, "octopus-like" nature as suggested by Zwieb and Brimacombe (42). Upon photoreversal of the crosslink, the strands separate and in the second-dimension would migrate as discrete oligonucleotides, each smaller than the crosslinked complex, and would appear as individual spots aligned in the direction of the second dimension and below the diagonal composed of unreacted oligonucleotides. Additionally, an oligonucleotide connected by an intrastrand HMT-crosslink, such as a hairpin loop might be, would be constrained to a smaller effective hydrodynamic radius than in the absence of such a crosslink due to "snapback" and basepairing of the arms of the hairpin promoted by the short-range crosslink. Following the first dimension, photoreversal of the crosslink allows the oligomer to unfold and assume a conformation of greater effective hydrodynamic radius, thus retarding its migration in the second dimension. It would then be seen as a single spot above the diagonal.

Figure 1 shows the electrophoretic pattern obtained with E. coli 16S rRNA crosslinked with HMT in reconstitution buffer. It is important to confirm that the observed off-diagonal spots are indeed the products of photoreversed HMT crosslinks and not artifacts produced by the experimental procedures.

Figure 1. Two-dimensional gel electrophoresis of 16S rRNA partially digested with RNase T₁ and crosslinked with HMT in the first dimension (left to right), followed by photoreversal of HMT crosslinks and electrophoresis in the second dimension (top to bottom). Off-diagonal spots 1-4 were excised and analyzed as discussed in the text.

Although it is known that irradiation of rRNA with short-wavelength UV light, such as that used to reverse HMT-RNA covalent bonds in this study, can itself crosslink rRNA (43), we have found that control samples treated in the same manner as test samples, but in the absence of HMT, show no significant off-diagonal spots after two-dimensional electrophoresis (data not shown). Bachellerie and Hearst (27) and Thompson et al. (29) have reported a retarding effect of HMT monoadducts to oligonucleotides when run in high percentage polyacrylamide gels. In the system employed here, this would be expected to manifest itself by the appearance of individual spots below the diagonal, since oligonucleotides so affected would migrate slightly faster in the second dimension following removal of the HMT moiety. No such pattern is seen. Thus, potentially aberrant electrophoretic behavior attributable to irradiation

tion or the presence of HMT monoadducts does not appear to be a problem under the conditions used here.

Off-diagonal spots were excised from the gel and their oligonucleotide sequence analyzed. As an example of the results obtained, the sequence analyses of spots numbered 1-4 in Fig. 1 are presented in Table I. Results of the analysis of the remaining spots will be presented elsewhere.

DISCUSSION

The electron microscopy studies of Wollenzien et al. (13) and Thammana et al. (14) clearly demonstrated the existence of long-range crosslinks in 16S rRNA reacted with HMT. In addition, the existence of short-range RNA-RNA contacts crosslinked via HMT was implicated by the shorter contour length of 16S rRNA molecules reacted with the drug compared to that of untreated control molecules. Direct sequencing of HMT-crosslinked regions would be of great value in elucidating the secondary structure taken by 16S rRNA under various reaction conditions, and would help to dispel some of the differences between

TABLE I
Sequence Analysis of Spots 1-4 from Fig. 1

RNase T ₁ Digestion Product	RNase A Analysis*	Proposed Sequence	Presence in 2-D gel spot [†]			
			1	2	3	4
1	G	G	5-6	5-6	5-6	1-2
2	C,G,AG	CG +AG	1 2	1 2	1 2	- -
3	C,G,AC,AG	CAG +ACG	1 1	1 1	- 1	- -
4	AAG	AAG	2	1	1	1
5	U,AAAG	UAAAG	1	1	1	1
6	<u>U</u> , <u>C</u> ,G,AC	UUACCCG	1	1	1	-
7	<u>U</u> , <u>C</u> ,G,AU	CUCAUUG	1	1	1	1
8	<u>U</u> , <u>C</u> ,AC,AG	UACUUCAG	1	1	1	-
9	<u>U</u> , <u>C</u> ,G,AC,AAU	UUAUACCUUG	1	1	1	1

*Following the convention of (44), each underline represents an additional residue of the underlined species. †Numbers represent estimated stoichiometric amounts present in each gel spot as determined by visual examination of autoradiographs of both RNase T₁ and RNase A digestion products.

authors on the basis of independent evidence (16-18). The precise site of HMT crosslinking is putative, since regions that have been crosslinked will not migrate off the diagonal unless the crosslink has been reversed, necessitating removal of the HMT moiety. However, there is much evidence supporting the hypothesis that the crosslink is between U₄₅₈ and U₄₇₃, and that HMT intercalates into the helix as shown in Fig. 3. First, it is well established that the base in RNA most reactive with psoralens is uracil (19, 26, 32). Second, it has been reported that many reactive sites occur at G·U base pairs in helices (20), which would be of lower stability than that of standard Watson-Crick base pairs (47). Finally, runs of adjacent uracils within weak helices of natural RNAs seem to be particularly susceptible to reaction with these drugs (27-29). All three conditions are satisfied by the proposed site of the intercalation; a G·U base pair exists between U₄₅₈ and G₄₇₄ in direct proximity to three adjacent uracils in the hairpin structure of Fig. 3 (U₄₇₁-U₄₇₃).

Wagner et al. have proposed this region of 16S RNA to be part of the mRNA binding site of the 30S subunit based on the reaction of G₄₆₂ and G₄₇₄ with an mRNA analogue (48). They propose a very similar hairpin structure for this region in which the reactive bases are involved in base pairing interactions, suggesting that the upper helix of Figure 3 is disrupted under their conditions. Their affinity label, which is also aromatic in nature, may itself be

Figure 3. Nucleotides 434-497 of *E. coli* 16S rRNA arranged in a hairpin structure as originally proposed by Woese et al. (16). The 5' and 3' termini of spots 1-4 (Fig. 2) are indicated. The heavy black bar between basepairs U₄₅₈-G₄₇₄ and A₄₅₉-U₄₂₃ denotes the putative site of HMT intercalation and subsequent crosslinking as discussed in the text.

capable of intercalation. The preponderance of sequences from this region of 16S rRNA in off-diagonal spots (Fig. 1) indicates its inherently high affinity for HMT intercalation and subsequent photoreaction. Youvan and Hearst have found similar high-affinity sites for HMT monoaddition in 16S rRNA (28).

Further studies on the use of HMT as a crosslinking reagent for the investigation of both short-range and long-range interactions in 16S and 23S rRNA are currently underway in these laboratories.

ACKNOWLEDGEMENTS

This work was supported by NIH grants no. GM-17129 (to H.F.N.) and GM-11180 (to J.E.H.). Support was also received from the Biomedical and Environmental Research Division of the U.S. Department of Energy under contract #W-2405-ENG-48.

These results were reported in part at the 1981 Pacific Slope Biochemical Conference, Davis, California.

REFERENCES

1. Shine, J. and Dalgarno, L. (1974) *Proc. Nat. Acad. Sci. USA* 71, 1342-1346
2. Steitz, J. A. and Jakes, K. (1975) *Proc. Nat. Acad. Sci. USA* 72, 4734-4738
3. Dunn, J. J., Buzash-Pollert, E. and Studier, W. F. (1978) *Proc. Nat. Acad. Sci. USA* 75, 2741-2745
4. Noller, H. F. and Chaires, J. B. (1972) *Proc. Nat. Acad. Sci. USA* 69, 3115-3118
5. Ofengand, J., Liou, R., Kohut, J., Schwartz, I. and Zimmermann, R. A. (1979) *Biochemistry* 18, 4322-4332
6. Prince, J. B., Hixson, S. S. and Zimmermann, R. A. (1979) *J. Biol. Chem.* 254, 4745-4749
7. Santer, M. and Shane, S. (1977) *J. Bacteriol.* 130, 900-910
8. Chapman, N. M. and Noller, H. F. (1977) *J. Mol. Biol.* 109, 131-149
9. Herr, W., Chapman, N. M. and Noller, H. F. (1979) *J. Mol. Biol.* 130, 433-449
10. Helser, T. L., Davies, J. E. and Dahlberg, J. E. (1979) *J. Mol. Biol.* 130, 433-449
11. Brosius, J., Palmer, M. L., Kennedy, P. J. and Noller, H. F. (1978) *Proc. Nat. Acad. Sci. USA* 75, 4801-4805
12. Carbon, P., Ehresmann, C., Ehresmann, B. and Ebel, J.-P. (1978) *FEBS Lett.* 94, 152-156
13. Wollenzien, P. L., Hearst, J. E., Thammana, P. and Cantor, C. R. (1979) *J. Mol. Biol.* 135, 255-269
14. Thammana, P., Cantor, C. R., Wollenzien, P. L. and Hearst, J. E. (1979) *J. Mol. Biol.* 135, 271-283
15. Cantor, C. R., Wollenzien, P. L. and Hearst, J. E. (1980) *Nucl. Acids Res.* 8, 1855-1872
16. Woese, C. R., Magrum, L. J., Gupta, R., Siegel, R. B., Stahl, D. A., Kop, J., Crawford, N., Brosius, J., Gutell, R., Hogan, J. J. and Noller, H. F. (1980) *Nucl. Acids Res.* 8, 2275-2293
17. Brimacombe, R. (1980) *Biochem. Inter.* 1, 162-172
18. Stiegler, P., Carbon, P., Zuher, M., Ebel, J.-P. and Ehresmann, C. (1981) *Nucl. Acids Res.* 9, 2153-2172

19. Pathak, M. A., Kramer, D. M. and Fitzpatrick, T. B. (1974) in Sunlight and Man, Fitzpatrick, T. B., Pathak, M. A., Harber, L. C., Seiji, M. and Kukita, A., Eds., pp. 335-368, University of Tokyo Press, Tokyo
20. Hearst, J. E. (1981) *Ann. Rev. Biophys. Bioeng.* 10, 69-86
21. Rabin, D. and Crothers, D. M. (1979) *Nucl. Acids Res.* 7, 689-703
22. Cole, R. S. (1970) *Biochim. Biophys. Acta* 217, 30-39
23. Dall'Acqua, F., Marciani, S. and Rodighiero, G. (1970) *FEBS Lett.* 9, 121-123
24. Isaacs, S. T., Shen, C. J., Hearst, J. E. and Rapoport, H. (1977) *Biochemistry* 16, 1058-1064
25. Wollenzien, P. L., Youvan, D. C. and Hearst, J. E. (1978) *Proc. Natl. Acad. Sci. USA* 75, 1642-1646
26. Ou, C.-N. and Song, P.-S. (1978) *Biochemistry* 17, 1054-1059
27. Bachellerie, J.-P. and Hearst, J. E. (1982) *Biochemistry*, in press.
28. Youvan, D. C. and Hearst, J. E. (1981) *Anal. Biochem.*, in press.
29. Thompson, J. F., Wegnez, M. R. and Hearst, J. E. (1981) *J. Mol. Biol.* 147, 417-436
30. Fellner, P. (1969) *European J. Biochem.* 11, 12-27
31. Traub, P. and Nomura, M. (1969) *J. Mol. Biol.* 40, 391-431
32. Bachellerie, J.-P., Thompson, J. F., Wegnez, M. R. and Hearst, J. E. (1981) *Nucl. Acids Res.* 9, 2207-2222
33. Barrell, B. G. (1971) in Procedures in Nucleic Acid Research, Cantoni, C. L. and Davies, D. R., Eds., Vol. 2, pp. 751-779, Harper and Row, New York.
34. Thompson, J. F., Bachellerie, J.-P., Hall, K. and Hearst, J. E. (1982) *Biochemistry*, in press
35. Allen, S. H. and Wong, K.-P. (1978) *J. Biol. Chem.* 253, 8759-8766
36. Vasiliev, V. D., Selivanova, O. M. and Koteliatsky, V. E. (1978) *FEBS Lett.* 95, 273-276
37. Cox, R. A. and Littauer, U. Z. (1962) *Biochim. Biophys. Acta* 61, 197-208
38. Morris, D. R., Dahlberg, J. E. and Dahlberg, A. E. (1975) *Nucl. Acids Res.* 2, 447-458
39. Sypherd, P. S. (1971) *J. Mol. Biol.* 56, 311-318
40. Held, W. A. and Nomura, M. (1973) *Biochemistry* 12, 3273-3281
41. Dunn, J. W. and Wong, K.-P. (1979) *J. Biol. Chem.* 254, 7705-7711
42. Zwieb, C. and Brimacombe, R. (1980) *Nucl. Acids Res.* 8, 2397-2411
43. Zwieb, C., Ross, A., Rinke, J., Meinke, M. and Brimacombe, R. (1978) *Nucl. Acids Res.* 5, 2705-2720
44. Brownlee, G. G. and Sanger, F. (1967) *J. Mol. Biol.* 13, 373-398
45. Musajo, L., Bordin, Caporale, G., Marciani, S. and Rigatti, G. (1967) *Photochem. Photobiol.* 6, 711-719
46. Straub, K., Kanne, D., Hearst, J. E. and Rapoport, H. (1981) *J. Am. Chem. Soc.* 103, 2347-2355
47. Tinoco, jun., I., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M. and Gralla, J. (1973) *Nature New Biol.* 246, 40-41
48. Wagner, R., Gassen, H. G., Ehresmann, Ch., Stiegler, P. and Ebel, J. P. (1976) *FEBS Lett.* 67, 312-315