REFERENCES AND NOTES

1. D. Biniszkiewicz, E. Cesnaviciene, D. A. Shub, *EMBO J.* **13**, 4629 (1994); B. Reinholdhurek and D. A. Shub, *Nature* **357**, 173 (1992).
2. S. K. Westway and J. Abelson, in *tRNA: Structure, Biosynthesis, and Function*, D. Söll and U. Raj Bhandary, Eds. (American Society for Microbiology, Washington, DC, 1995), pp. 79–92; M. R. Culbertson and M. Winey, *Yeast* **5**, 405 (1989); E. M. Phizicky and C. L. Greer, *Trends Biochem. Sci.* **18**, 31 (1993).
3. J. R. Palmer, T. Baltrus, J. N. Reeve, C. J. Daniels, *Biochim. Biophys. Acta* **1132**, 315 (1992); J. Kjems, J. Jensen, T. Olesen, R. A. Garrett, *Can. J. Microbiol.* **35**, 210 (1989).
4. C. R. Trotta, unpublished results.
5. V. M. Reyes and J. N. Abelson, *Cell* **55**, 719 (1988).
6. L. D. Thompson and C. J. Daniels, *J. Biol. Chem.* **265**, 18104 (1990).
7. C. R. Trotta *et al.*, *Cell* **89**, 849 (1997).
8. K. Kleman-Leyer, D. A. Armbruster, C. J. Daniels, *ibid.*, p. 839.
9. C. K. Ho, R. Rauhut, U. Vijayraghavan, J. N. Abelson, *EMBO J.* **9**, 1245 (1990).
10. J. Lykke-Andersen and R. A. Garrett, *ibid.* **16**, 6290 (1997).
11. Recombinant endonuclease was purified from *E. coli* by a Ni-NTA (Qiagen) affinity chromatography step after heat denaturation. Crystals were grown at 22°C by vapor diffusion using KCl as a salting-in precipitant with a starting concentration of 1 M. The well solutions were at pH 5.5 to 6.0 and contained 20 to 80 mM $(NH_4)_2SO_4$. Crystals were soaked for 3 days in 20 mM sodium cacodylate (pH 5.5 to 6.0), 20 mM $(NH_4)_2SO_4$, 25% glucose, 2 mM $Au(CN)_2$ before data collection.
12. B. Lee and F. M. Richards, *J. Mol. Biol.* **55**, 379 (1971).
13. C. C. F. Blake *et al.*, *ibid.* **88**, 1 (1974); T. K. Sixma *et al.*, *Nature* **351**, 371 (1991).
14. C. Walsh, *Enzymatic Reaction Mechanisms* (Freeman, San Francisco, 1979).
15. C. J. Daniels, personal communication.
16. The catalytic triad (residues His^{12}, His^{119}, and Lys^{41}) of RNase A was brought manually to superimpose with Tyr^{115}, His^{125}, Lys^{156} of *M. jannaschii* endonuclease. The best superimposition was obtained when Tyr^{115} aligns with His^{119}, His^{125} with His^{12}, and Lys^{156} with Lys^{41}.
17. H. Li, C. R. Trotta, J. N. Abelson, data not shown.
18. J. D. Puglisi, L. Chen, A. D. Frankel, J. R. Williamson, *Proc. Nat. Acad. Sci. U.S.A.* **90**, 3680 (1993); F. Aboul-ela, J. Karn, G. Varani, *Nucleic Acids Res.* **24**, 3974 (1996).
19. S. Fabbri *et al.*, (1998) *Science*, in press.
20. M. Carson, *J. Mol. Graphics* **5**, 103 (1987).
21. A. G. W. Leslie, *CCP4 AND ESF-EACMB Newsl. Protein Crystallogr.* **32**, 2 (1996).
22. Z. Otwinowski, in *Proceedings of the CCP4 Study Weekend: Data Collection and Processing, 29–30 January 1993*, L. I. Sawyer, N. Isaac, S. Bailey, Eds. (Science and Engineering Research Council, Daresbury Laboratory, Warrington, UK, 1993), pp. 56–62; W. Minor, *XDISPLAYF Program* (Purdue University, West Lafayette, IN, 1993).
23. W. Kabsch, *J. Appl. Crystallogr.* **21**, 916 (1988).
24. Collaborative Computational Project, Number 4, "The CCP4 Suite: Programs for Protein Crystallography," *Acta Crystallogr. D* **50**, 760 (1994).
25. J. Kraut *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **48**, 1417 (1962).
26. V. Ramakrishnan and V. Biou, *Methods Enzymol.* **276**, 538 (1997).
27. T. C. Terwilliger, S. H. Kim, D. E. Eisenberg, *Acta Crystallogr. A* **43**, 34 (1987).
28. Z. Otwinowski, in *Isomorphous Replacement and Anomalous Scattering*, W. E. Wolf, P. R. Leslie, A. G. W. Leslie, Eds. (Science and Engineering Research Council, Daresbury Laboratory, Warrington, UK, 1991), p. 80.
29. K. Cowtan, *Joint CCP4 ESF-EACBM Newsl. Protein Crystallogr.* **31**, 34 (1994).
30. T. A. Jones, J. Y. Zou, S. W. Cowan, M. Kjeldgaard, *Acta Crystallogr. A* **47**, 110 (1991).
31. A. T. Brünger, *X-PLOR Version 3.1, A System for Crystallography and NMR* (Yale Univ. Press, Department of Molecular Biophysics and Biochemistry, New Haven, CT, 1996).
32. R. A. Laskowski, M. W. MacArthur, D. S. Moss, J. M. Thornton, *J. Appl. Crystallogr.* **26**, 283 (1993).
33. R. Luthy, J. U. Bowie, D. Eisenberg, *Nature* **356**, 83 (1992).
34. R. Engh and R. Huber, *Acta Crystallogr. A* **47**, 392 (1991).

35. We thank D. Graham and C. Woese for *M. jannaschii* genomic DNA, P. Bjorkman and D. Rees for their helpful discussions and generosity in sharing their equipment, C. M. Ogata for x-ray beam time allocation, F. T. Burling and S. Diana for assistance in data collection, R. Story and M. Saks for critical review of the manuscript, and other members of the Abelson laboratory for their advice and support. This work was supported by American Cancer Society grant NP802 and NIH Individual National Research Service Award F32 GM188930-01. PDB code for the coordinates is 1a79.

10 December 1997; accepted 13 February 1998

Conservation of Substrate Recognition Mechanisms by tRNA Splicing Endonucleases

Stefania Fabbri, Paolo Fruscoloni, Emanuela Bufardeci, Elisa Di Nicola Negri, Maria I. Baldi, Domenica Gandini Attardi, Emilio Mattoccia, Glauco P. Tocchini-Valentini*

Accuracy in transfer RNA (tRNA) splicing is essential for the formation of functional tRNAs, and hence for gene expression, in both Eukaryotes and Archaea. The specificity for recognition of the tRNA precursor (pre-tRNA) resides in the endonuclease, which removes the intron by making two independent endonucleolytic cleavages. Although the eukaryal and archaeal enzymes appear to use different features of pre-tRNAs to determine the sites of cleavage, analysis of hybrid pre-tRNA substrates containing eukaryal and archaeal sequences, described here, reveals that the eukaryal enzyme retains the ability to use the archaeal recognition signals. This result indicates that there may be a common ancestral mechanism for recognition of pre-tRNA by proteins.

The endonucleases responsible for cleavage of pre-tRNAs during splicing appear to use fundamentally different mechanisms for determining the sites of cleavage (*1*). The eukaryal enzymes bind to invariant nucleotides in the body of the mature RNA and measure the distance to equivalently positioned intron-exon junctions (*2*, *3*), whereas the archaeal enzymes recognize a structure, the bulge-helix-bulge (BHB) motif, that defines the intron-exon boundaries (*4*).

A curious feature of the eukaryal enzymes is their dependency on base-pairing near the site of 3′ cleavage, the so-called anticodon-intron pair (A-I pair) (*5*). Because the A-I pair generates a three-nucleotide (nt) bulged structure that resembles half of the BHB, we studied whether the eukaryal endonucleases retained elements of an ancestral RNA recognition mechanism that is still used by the archaeal enzymes. In general, archaeal pre-tRNAs are not recognized by the eukaryal endonuclease, and vice versa (*4*).

We designed and constructed a hybrid pre-tRNA molecule, pre-$tRNA^{Archeuka}$, that is a substrate for both the eukaryal and archaeal endonucleases. This molecule consists of two regions derived from yeast pre-$tRNA^{Phe}$ (nt 1 to 31 and nt 38 to 76) joined by a 25-nt insert that corresponds to the BHB motif of the archaeal pre-tRNAs (Fig. 1). It has a typical eukaryal mature domain with putative cleavage sites located at the prescribed distance from the reference elements as well as a correctly positioned A-I base pair, all of which should ensure correct recognition by the eukaryal endonuclease. In addition, the presence of the BHB motif should confer substrate characteristics that are recognizable by the archaeal enzyme. Indeed, we found that precise excision of the intron was catalyzed by the eukaryal *Xenopus* and yeast endonucleases and by the archaeal *Sulfolobulus sulfataricus* endonuclease (Fig. 1, lanes 3, 7, and 11). Thus, pre-$tRNA^{Archeuka}$ is a universal substrate.

Because the reference sites for eukaryal endonucleases are in the mature domain of the tRNA (*2*, *3*, *5*), insertion of two base pairs into the anticodon stem, to generate pre-$tRNA^{Phe}\left(\begin{smallmatrix}U\cdot A\\ G\cdot C\end{smallmatrix}\right)_V$, increases the size of the

S. Fabbri and E. Bufardeci, EniChem, Istituto Guido Donegani SpA, Laboratori di Biotecnologie, 00015 Monterotondo, Rome, Italy.
P. Fruscoloni, E. Di Nicola Negri, M. I. Baldi, D. Gandini Attardi, E. Mattoccia, Istituto di Biologia Cellulare, CNR, Viale Marx 43, 00137 Rome, Italy.
G. P. Tocchini-Valentini, Istituto di Biologia Cellulare, CNR, Viale Marx 43, 00137 Rome, Italy, and Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, USA.

*To whom correspondence should be addressed. E-mail: glauco@biocell.irmkant.rm.cnr.it

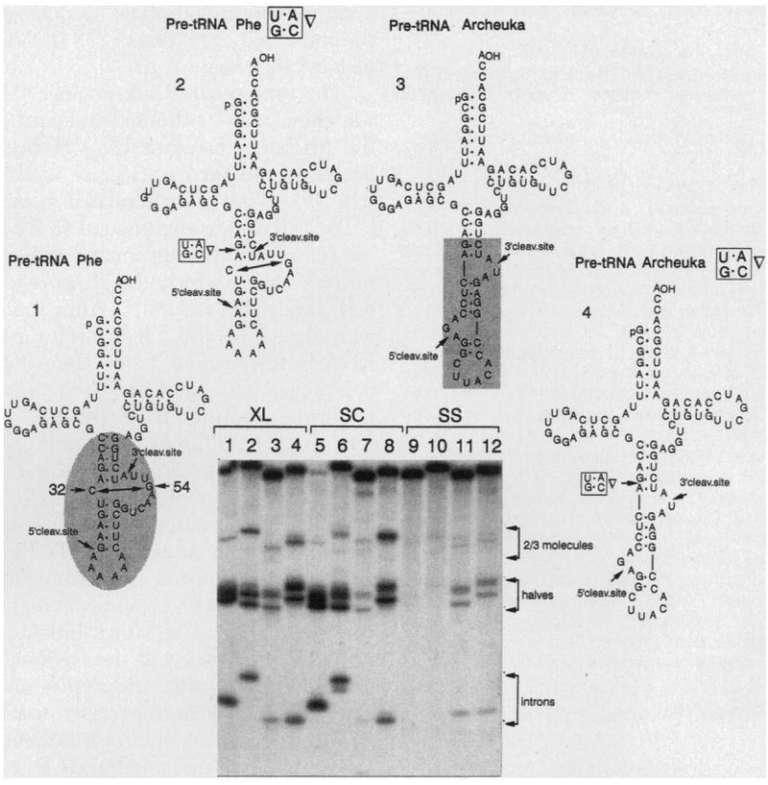

Fig. 1. Cleavage of pre-tRNA$^{Archeuka}_{(G\cdot C)}$ by eukaryal enzymes in the absence of mature domain elements (*12*). The eukaryal tRNA splicing endonucleases normally recognize a tripartite set of RNA elements (*7*). The enzymes bind to the mature domain and use a measurement mechanism to identify the positions of the two cleavage sites (*2*, *3*). Specific features are required at the intron-exon boundaries (*5*, *7*), including a base pair between the pyrimidine at position 32, the first nucleotide in the anticodon loop, and a purine in the intron (the anticodon-intron pair, A-I pair). The purine in the A-I pair is normally located three bases before the cleavage site. The archaeal endonucleases do not require a mature tRNA structure. Instead, they recognize the BHB motif, which is composed of two 3-nt bulge loops. Oval box: Recognition region for eukaryal endonuclease derived from yeast pre tRNA^{Phe}. Rectangular box: Recognition region for archaeal endonuclease corresponding to the BHB motif. XL, SC, and SS indicate products of digestion by the tRNA endonuclease of *X. laevis* (XL), *S. cerevisiae* (SC), and *S. sulfataricus* (SS). The substrates were uniformly labeled pre-tRNA^{Phe} (lanes 1, 5, and 9), pre-tRNA$^{Phe}_{(G\cdot C)}$ (lanes 2, 6, and 10), pre-tRNA^{Archeuka} (lanes 3, 7, and 11), and pre-tRNA$^{Archeuka}_{(G\cdot C)}$ (lanes 4, 8, and 12).

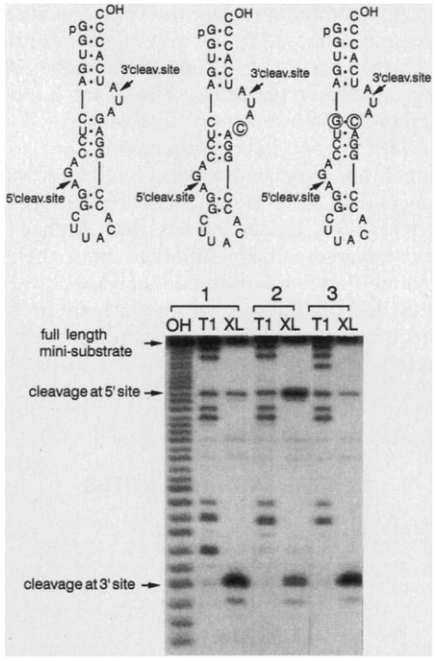

Fig. 2. Products of cleavage of 3′ end–labeled mini-BHB substrates by the *X. laevis* tRNA endonuclease (XL). The two labeled products derived by cleavage at sites corresponding to the 5′ and 3′ ends of the intron are indicated by arrows. Molecular size markers of a partial alkaline digest (OH) and partial ribonuclease T1 digest (T1) were included on the gel.

excised intron by four bases, two at each end (Fig. 1, lanes 2 and 6). Neither pre-tRNA^{Phe} nor pre-tRNA$^{Phe}_{(G\cdot C)}$, which lack the BHB motif, is cleaved by the archaeal endonuclease (Fig. 1, lanes 9 and 10).

Remarkably, the intron fragments excised from the cleavage of pre-tRNA$^{Archeuka}_{(G\cdot C)}$ by the eukaryal and the archaeal endonucleases were identical (Fig. 1, lanes 4, 8, and 12). This result shows that the eukaryal enzymes fix the sites of cleavage of this particular substrate not by reference to the mature domain but by recognition of local structures at the intron-exon boundaries, in the same manner as the archaeal enzyme. Presumably the mature domain of pre-tRNA^{Archeuka} interferes with the interaction between the eukaryal enzymes and the symmetrically disposed cleavage sites, because the RNA with a longer anticodon stem is a better substrate (Fig. 1, lanes 3, 4, 7, and 8). In contrast, the archaeal enzyme, which does not normally interact with the mature domain, is unaffected (lanes 11 and 12).

The eukaryal enzyme can operate independently of the mature domain on a minisubstrate (mini-BHB) that corresponds to the BHB motif of the archaeal pre-tRNAs (Fig. 1, rectangular box). Such a substrate, labeled at the 3′ end, was cleaved at both cleavage sites by the *Xenopus* endonuclease (Fig. 2, substrate 1). When mini-BHB was labeled at the 5′ end, the labeled products were the 5′ half and the 5′ half plus intron (*6*). When one of the bulges was disrupted by substitution of one of the flanking nucleotides (resulting in disruption of the A-I pair), the *Xenopus* enzyme cleaved at the opposite bulge, at a site corresponding to the 5′ site, and only very inefficiently at the 3′ site (Fig. 2, substrate 2). The compensatory mutation that allowed the formation of a new A-I pair in the double mutant produced a substrate that was cleaved efficiently at both sites by the eukaryal enzyme (Fig. 2, substrate 3). We conclude that the local structural information present at the intron-exon boundaries of the mini-BHB is sufficient to direct the *Xenopus* enzyme to the correct cleavage sites.

A eukaryal mini-substrate containing only the extended anticodon stem of tRNA^{Phe} (Fig. 1, oval box) is cleaved exclusively at the 3′ site, although this RNA contains a bona fide 5′ site (*7*). Taken with the results in Fig. 2, this shows that the eukaryal endonuclease uses the mature domain as a reference point for cleavage at the 5′ splice site, when the corresponding bulge is missing from the substrate. However, when a substrate contains the whole BHB motif, the conserved spatial positioning of the two active sites of the eukaryal and archaeal endonucleases allows for cleavage at both ends of the intron. This remarkable finding demonstrates a common ancestral mechanism for recognition of tRNAs and perhaps other RNA substrates.

A structural model for the yeast endonuclease is proposed in the accompanying report (8). A central feature of the model is represented by the strong Sen2-Sen54 and Sen34-Sen15 interactions that were detected with the two-hybrid system (9). The two dimers are associated to form a tetramer by way of the L10 loop sequences of Sen15 and Sen54. This model implies that Archaea and Eukaryotes have inherited from their common ancestor the endonuclease active sites and the means to array them in a precise and conserved spatial orientation. Our results with the artificial pre-tRNA$^{\text{Archeuka}}\left(\begin{smallmatrix}\text{U·A}\\ \text{G·C}\end{smallmatrix}\right)_{\nabla}$ and mini-BHB substrates strongly support that model.

REFERENCES AND NOTES

1. M. Belfort and A. Weiner, *Cell* **89**, 1003 (1997).
2. V. M. Reyes and J. Abelson, *Ibid.* **55**, 719 (1988).
3. E. Mattoccia, M. I. Baldi, D. Gandini Attardi, S. Ciafrè, G. P. Tocchini-Valentini, *ibid.*, p. 731.
4. L. D. Thompson and C. J. Daniels, *J. Biol. Chem.* **265**, 18104 (1990).
5. M. I. Baldi, E. Mattoccia, E. Bufardeci, S. Fabbri, G. P. Tocchini-Valentini, *Science* **255**, 1404 (1992).
6. S. Fabbri *et al.*, data not shown.
7. E. Di Nicola Negri *et al.*, *Cell* **89**, 859 (1997).
8. H. Li, C. R. Trotta, J. Abelson, *Science* **280**, 279 (1998).
9. C. R. Trotta *et al.*, *Cell* **89**, 849 (1997).
10. D. Gandini Attardi, M. I. Baldi, E. Mattoccia, G. P. Tocchini-Valentini, *Methods Enzymol.* **181**, 510 (1989).
11. L. D. Thompson and C. J. Daniels, *J. Biol. Chem.* **263**, 1751 (1988).
12. Pre-tRNA$^{\text{Phe}}$ and pre-tRNA$^{\text{Phe}}\left(\begin{smallmatrix}\text{U·A}\\ \text{G·C}\end{smallmatrix}\right)_{\nabla}$ were synthesized as described (*3*, *5*, *7*). Templates for the synthesis of the Archaeuka precursors were constructed by polymerase chain reaction (PCR). The PCR templates were the full-length pre-tRNAs. One primer contained the T7 promoter and part of the 5′ exon. The other was composed of the desired sequence of the 3′ exon. Conditions for PCR, transcription by T7 RNA polymerase, and endonuclease assays were as described (*3*, *5*, *7*). *Xenopus laevis* endonuclease was purified as in (*10*), *Saccharomices cerevisiae* endonuclease as in (*9*), and *S. sulfataricus* endonuclease as in (*11*).

13. We thank J. Dahlberg for help with the manuscript; J. Abelson for helpful comments, for communicating results before publication, and for yeast endonuclease; R. H. Haselkorn for critical reading of the manuscript; M. Rossi for *S. sulfataricus* cells; G. Di Franco for technical assistance; and A. Sebastiano for typesetting help with the manuscript. Supported in part by Progetto Finalizzato (CNR) Ingegneria Genetica, Progetto Finalizzato (CNR) Biotecnologie, Human Frontiers Science Program Organization (HFSPO), and Fondo Cinquepercento: Biotecnologie.

15 December 1997; accepted 9 February 1998

Ribosome-Catalyzed Peptide-Bond Formation with an A-Site Substrate Covalently Linked to 23S Ribosomal RNA

Rachel Green,* Christopher Switzer, Harry F. Noller†

In the ribosome, the aminoacyl–transfer RNA (tRNA) analog 4-thio-dT-p-C-p-puromycin crosslinks photochemically with G2553 of 23S ribosomal RNA (rRNA). This covalently linked substrate reacts with a peptidyl-tRNA analog to form a peptide bond in a peptidyl transferase–catalyzed reaction. This result places the conserved 2555 loop of 23S rRNA at the peptidyl transferase A site and suggests that peptide bond formation can occur uncoupled from movement of the A-site tRNA. Crosslink formation depends on occupancy of the P site by a tRNA carrying an intact CCA acceptor end, indicating that peptidyl-tRNA, directly or indirectly, helps to create the peptidyl transferase A site.

Catalysis of peptide bond formation requires precise juxtaposition of the acceptor ends of P (peptidyl)- and A (aminoacyl)-site–bound tRNAs in the active site of the ribosome. Accumulating evidence points to a role for the 23S rRNA in the function of peptidyl transferase (*1*–*3*). Identification of a peptidyl transferase–reactive crosslink between a benzophenone-derivatized peptidyl-tRNA and A2451-C2452 localized the central loop of domain V of 23S rRNA to the peptidyl transferase site (*2*). More recently, identification of a base-pairing interaction between C74 of P-site–bound tRNA and G2252 of domain V established a direct role for 23S rRNA in the function of peptidyl transferase (*3*). Here we describe an aminoacyl-tRNA analog, 4-thio-dT-p-C-p-puromycin (s^{4}TCPm), which crosslinks to 23S rRNA with high efficiency and specificity. This substrate remains fully active as an acceptor in the peptidyl transferase reaction while covalently bound to 23S rRNA, imposing constraints on the proposed concerted events of tRNA movement and peptide-bond formation and unambiguously placing the conserved 2555 loop of 23S rRNA at the peptidyl transferase A site.

The aminoacyl-tRNA analog, s^{4}TCPm, was chemically synthesized and purified (*4*); the Michaelis constant (K_m) of this compound for peptidyl transferase is about 10 μM (*5*). The phosphorylated compound, [^{32}P]s^{4}TCPm, was then bound to *Escherichia coli* 70S ribosomes programmed with gene *32* mRNA (*6*) in which the P site was filled with deacylated tRNA$^{\text{Phe}}$. After irradiation with ultraviolet (UV) light (366 nm), total RNA was prepared from the ribosomal complexes and analyzed (Fig. 1A) (*7*). Exclusive labeling of 23S rRNA is consistent with crosslinking to the peptidyl transferase center of the ribosome. Based on incorporation of ^{32}P into 23S rRNA, about 30% of ribosomes were crosslinked in the presence of 20 μM s^{4}TCPm. The absence of substantial crosslinking to ribosomal proteins (*8*) provides evidence for the specificity of the crosslinked A-site ribosomal complex and the RNA-rich nature of the peptidyl transferase site (*9*). This contrasts with the recent proposal of a proteinaceous A-site environment (*10*).

Digestion of the crosslinked *E. coli* 23S rRNA species with ribonuclease (RNase) T1 (*11*) revealed a single crosslinked product (Fig. 1B). The efficiency and specificity of crosslinking was high, which indicates an extremely close juxtaposition of s^{4}TCPm and rRNA, comparable to that between position 34 of tRNA and position C1400 of 16S rRNA (*12*). Typically, even highly efficient crosslinks to the ribosome, such as those obtained with benzophenone-derivatized Phe-tRNA$^{\text{Phe}}$, target multiple rRNA sites (*2*).

Assignment of the site of crosslinking to position G2553 was achieved with RNase H digestion (*13*) followed by primer-extension analysis (Fig. 1C) (*14*). The strong stop induced by crosslinking with s^{4}TCPm is consistent with the estimated crosslinking efficiency of 30%. Crosslinking of s^{4}TCPm to *Bacillus stearothermophilus* ribosomes was similarly efficient and also yielded a single RNase T1 product; primer extension again localized the crosslinked position to nucleotide G2553 (*E. coli* numbering) (*8*).

The crosslink between s^{4}TCPm and 23S rRNA places the conserved 2555 loop at the site of interaction between the ribosome and the conserved CCA end of A-site tRNA. This conclusion is consistent with protection of G2553 from chemical modification by A-site tRNA, which depends on the presence of the terminal adenosine (A76) of tRNA (*15*) and with directed cleavage of this region of the RNA backbone by hydroxyl radicals generated from Fe(II) tethered to the 5′ end of A-site–

R. Green and H. F. Noller, Center for Molecular Biology of RNA, Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064, USA.
C. Switzer, Department of Chemistry, University of California, Riverside, CA 92521, USA.

*Present address: Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.
†To whom correspondence should be addressed. E-mail: harry@nuvolari.ucsc.edu