Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1113–1116, February 1995
Biochemistry

Directed hydroxyl radical probing of 16S rRNA using Fe(II) tethered to ribosomal protein S4

(ribosomal RNA/chemical probing/ribosomes)

GABRIELE M. HEILEK*, ROSEMARY MARUSAK†‡, CLAUDE F. MEARES†, AND HARRY F. NOLLER*

*Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064; and †Department of Chemistry, University of California, Davis, CA 95616

Contributed by Harry F. Noller, October 21, 1994

ABSTRACT **Localized hydroxyl radical probing has been used to explore the rRNA neighborhood around a unique position in the structure of the *Escherichia coli* 30S ribosomal subunit. Fe(II) was attached to ribosomal protein S4 at Cys-31 via the reagent 1-(*p*-bromoacetamidobenzyl)-EDTA. [Fe-Cys^{31}]S4 was then complexed with 16S rRNA or incorporated into active 30S ribosomal subunits by *in vitro* reconstitution with 16S rRNA and a mixture of the remaining 30S subunit proteins. Hydroxyl radicals generated from the tethered Fe resulted in cleavage of the 16S rRNA chain in two localized regions of its 5′ domain. One region spans positions 419–432 and is close to the multihelix junction previously placed at the RNA binding site of S4 by chemical and enzymatic protection (footprinting) and crosslinking studies. A second site of directed cleavage includes nucleotides 297–303, which overlap a site that is protected from chemical modification by protein S16, a near neighbor of S4 in the ribosome. These results provide useful information about the three-dimensional organization of 16S rRNA and indicate that these two regions of its 5′ domain are in close spatial proximity to Cys-31 of protein S4.**

Understanding the molecular mechanism of translation depends on detailed knowledge of the three-dimensional structure of the ribosome. In the absence of an x-ray crystal structure, a wide variety of alternative biochemical and physical approaches have been devised to obtain information concerning the relative locations of ribosomal proteins and rRNA and other macromolecular components of translation in the ribosome (1). Indeed, even with a well-resolved electron density map in hand, information of this kind will likely be essential for its interpretation.

In the studies presented here, we describe a biochemical method for obtaining information about the three-dimensional structure of RNA–protein complexes such as the ribosome. It consists of generating hydroxyl radicals locally from Fe(II) tethered to a single position in the ribosome, which results in cleavage of the rRNA backbone at positions that are in close proximity to the Fe(II) ion. Because of the short lifetime of hydroxyl radicals in aqueous solution, cleavage is usually restricted to positions in the RNA that are within about 10 Å of the Fe(II) ion (2, 3). We use ribosomal protein S4 as a model system for these studies. The binding of S4 to 16S rRNA has been extensively characterized (4–8); it is one of six small-subunit ribosomal proteins that bind specifically to the RNA in the absence of the other proteins (9). Fe(II) is tethered to the unique cysteine residue at position 31 of protein S4 via 1-(*p*-bromoacetamidobenzyl)-EDTA (BABE), a reagent that has successfully been used to map intramolecular proximities in proteins (10, 11). Iron-derivatized protein S4 ([Fe-Cys^{31}]S4) is then bound to 16S rRNA, either alone, or in a fully assembled 30S ribosomal subunit. Hydroxyl radicals are generated after assembly of the ribonucleoprotein complex by using the Fenton reaction (12), and the positions of cleavage of the 16S rRNA chain are identified by primer extension (13).

This approach provides certain advantages over previous methods for studying the RNA neighborhoods of ribosomal proteins. One shortcoming of chemical and enzymatic protection methods is that protection can be caused by protein-induced conformational rearrangements rather than direct protein–RNA contact. As a result, one cannot easily rule out the possibility that protected regions of the RNA might be remote from the site of protein–RNA contact. Crosslinking methods avoid this ambiguity but rely on favorable chemistry at the sites of crosslinking, limiting the scope of such approaches. Moreover, identification of crosslinked moieties often involve difficult and laborious procedures. Directed hydroxyl radical probing has the advantage that ribose moieties in virtually any region of the RNA backbone are susceptible to attack (14) and that the positions of cleavage can be located readily and assigned unambiguously to the proximity of the tethered Fe(II) ion. This general approach has been employed successfully to study DNA–protein interactions (15–18).

Our results show that Cys-31 of ribosomal protein S4 is in three-dimensional proximity to two discrete regions of the 16S rRNA chain, both of which are located within its 5′ domain and one of which has previously been localized by footprinting and crosslinking experiments to the S4 binding site itself. This approach should be generally useful for probing ribosome topography as well as that of other ribonucleoprotein complexes.

MATERIALS AND METHODS

Ribosomes, 30S ribosomal subunits, 16S rRNA, and ribosomal protein S4 were isolated as described (19, 20). Preparation of BABE was done as described (10). Formation of the Fe–BABE chelation complex was done by mixing 2 μl of freshly prepared 50 mM $FeSO_4$ with 18 μl of 11 mM BABE in 100 mM NaOAc (pH 6.0), followed by incubation at room temperature for 30 min. Conjugation of S4 with Fe–BABE or with iodoacetate was carried out by mixing 15 μl of a 2.98 mg/ml solution of S4 (in 80 mM potassium Hepes, pH 7.7/1 M KCl/6 mM 2-mercaptoethanol) with 10 μl of 10 mM Fe–BABE or 10 μl of 10 mM iodo[^{14}C]acetate (Amersham; 5.93 mCi/mmol; 1 Ci = 37 GBq) in 1 M KCl/80 mM Hepes, pH 7.7/0.01% Nikkol (total volume, 100 μl), followed by incubation at 37°C for 15 min. Separation of the modified S4 from excess reagent was done by loading the reaction mixture on a 1-ml Sephadex G-50 spin-column (Pharmacia) preequilibrated with 1 M KCl/80 mM Hepes, pH 7.7/0.01% Nikkol and centrifuging for 4 min at 1700 × *g* on a clinical centrifuge. Derivatized S4 could be stored at −80°C for at least 4 weeks without loss of binding

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: BABE, 1-(*p*-bromoacetamidobenzyl)-EDTA.

‡Present address: Department of Chemistry, Kenyon College, Gambier, OH 43022.

activity. Electrospray ionization mass spectrometry of S4 and [(BABE)-Cys^{31}]S4, was performed on a Finnigan-MAT (San Jose, CA) model TSQ700 triple quadrupole mass spectrometer by Bill Lane at the Harvard Microchemistry Facility.

In control experiments, assays for free and chelated Fe(II) ion using 1,10-phenanthroline (GFS Chemicals, Powell, OH) and 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ; Aldrich), respectively (21), showed that, after G-50 spin-column filtration or incubation under reconstitution conditions at 40°C for 1 hr, Fe remains chelated to BABE-derivatized bovine serum albumin.

Complexes between S4 or [Fe-Cys^{31}]S4 and 16S rRNA were formed, in a typical experiment, by adding 2 μl of a 5 mg/ml solution of *Escherichia coli* 16S rRNA (in 50 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/300 mM KCl) to 41.3 μl of buffer A (80 mM potassium Hepes, pH 7.7/20 mM $MgCl_2$/0.01% Nikkol) containing 200 mM KCl, followed by incubation for 15 min at 40°C. After addition of 6.7 μl of a 0.28 mg/ml solution of S4 or [Fe-Cys^{31}]S4 in buffer A containing 1 M KCl, incubation at 40°C was continued for 1 hr, followed by 10 min on ice. Reconstitution of 30S ribosomal subunits containing [Fe-Cys^{31}]S4 or unmodified S4 was done similarly, except that additionally 10 μl of 8 μM Σ − S4 (a mixture containing all 30S ribosomal proteins except S4) was added to allow formation of complete subunits.

S4–16S rRNA complexes were separated from unbound protein by using a 1-ml Sephacryl S-200 spin-column preequilibrated with buffer A containing 330 mM KCl. Reconstituted 30S subunits were purified by sedimentation in a SW-41 rotor for 18 hr at 35,000 rpm at 4°C with a 10–40% sucrose gradient in 50 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/100 mM KCl.

Hydroxyl radicals were generated by addition of 1 μl of 250 mM ascorbic acid and 1 μl of 2.5% H_2O_2 to 25 μl of a 0.2 mg/ml solution of [Fe-Cys^{31}]S4 complex in buffer A containing 330 mM KCl or to 25 μl of a 0.32 mg/ml solution of [Fe-Cys^{31}]S4 containing 30S subunits, followed by incubation for 10 min at 4°C. The reaction was terminated by addition of a 1/10th volume of 3 M NaOAc (pH 5.2), 1 μl of a 10 mg/ml solution of glycogen, and 2.5 volume of ethanol. The modified RNA was extracted and analyzed by primer extension as described (13). Kethoxal probing was used to control for assembly of S4 as described (7), and poly(U)-dependent binding of tRNA^{Phe}, performed as described (22), was used as an assay for the activity of reconstituted 30S subunits.

RESULTS

Our goal was to anchor a single Fe(II) ion to a unique position in the 30S ribosomal subunit, from which hydroxyl radicals could be generated locally via the Fenton reaction (12) to induce site-specific cleavage of the 16S rRNA chain. The first step was to derivatize ribosomal protein S4 with Fe–BABE at its single cysteine residue at position 31. Derivatization of S4 was monitored by competition of Fe–BABE with iodo[^{14}C]acetate, which alkylates cysteine via a similar nucleophilic displacement mechanism. According to this assay, reaction of S4 with iodoacetate was fully blocked following derivatization with Fe–BABE under our conditions, indicating that the available cysteine (amounting to 0.45 ± 0.05 mol per mol of S4) was fully derivatized with Fe–BABE. This was supported by mass spectrometric analysis of BABE-Cys^{31}-S4, which showed that about half of the protein sample had an increase in mass corresponding to that of the covalently attached acetamidobenzyl-EDTA moiety (data not shown).

The ability of [Fe-Cys^{31}]S4 to bind to 16S rRNA was tested by chemical footprinting of a complex formed between the single modified protein and the RNA under *in vitro* reconstitution conditions. The results of chemical probing experiments show that the protection pattern for Fe-C31-S4 is similar to that seen for unmodified S4. Lanes 2 and 3 in Fig. 1*b* show protection of guanosine at position 497 from kethoxal attack by bound [Fe-Cys^{31}]S4, diagnostic for proper binding of S4 (7).

Hydroxyl radicals were then generated locally from the Cys-31-tethered Fe(II) in the [Fe-Cys^{31}]S4 complex via the Fenton reaction, initiated by addition of ascorbate and H_2O_2. The sites of cleavage of the RNA backbone were identified by primer extension by using a series of synthetic DNA oligonucleotide primers that allow scanning of the entire 16S rRNA chain (except for the very 3′ terminus) (13). Authentic [Fe-Cys^{31}]S4-dependent cleavage events were distinguished from spontaneous reverse transcriptase stops and other primer extension artifacts by a series of control experiments. Generation of hydroxyl radicals from possible contaminating metal ions bound to 16S rRNA was excluded by experiments in which unmodified S4 was complexed with RNA and subjected to the same chemical treatments as for the [Fe-Cys^{31}]S4 complex. Cleavage due to nuclease contamination was excluded by primer extension analysis of complexes that had been subjected to all of the same treatments, except for initiation of the Fenton reaction.

The results of experiments using the [Fe-Cys^{31}]S4–16S rRNA complex show that cleavage of the RNA chain is localized to two regions of the 5′ domain (Fig. 2). One region, spanning positions 419–432, is within a part of 16S rRNA where the binding site for protein S4 was previously localized by chemical footprinting experiments (7). A second region, including positions 292–310, overlaps the site that was previously shown to be protected from chemical modification by protein S16 (23), a near neighbor of S4 in the 30S subunit (24). These hydroxyl radical-dependent cleavages disappear in competition experiments, where excess unmodified S4 is present during complex formation (Fig. 1*a*, lanes 3 and 4), providing evidence that the observed cleavages are due to [Fe-Cys^{31}]S4 that is bound to 16S rRNA at the normal S4 binding site.

We next constructed complete 30S subunits containing Fe tethered to a unique position by *in vitro* reconstitution using [Fe-Cys^{31}]S4, 16S rRNA, and a full-complement mixture of ribosomal proteins made up of stoichiometric amounts of individually purified 30S proteins lacking S4 (Σ − S4). Reconstitution into 30S particles was monitored by cosedimentation with radioactively labeled 30S subunits in sucrose gradient centrifugation. All native 30S subunits and particles reconstituted with modified or unmodified S4 showed similar

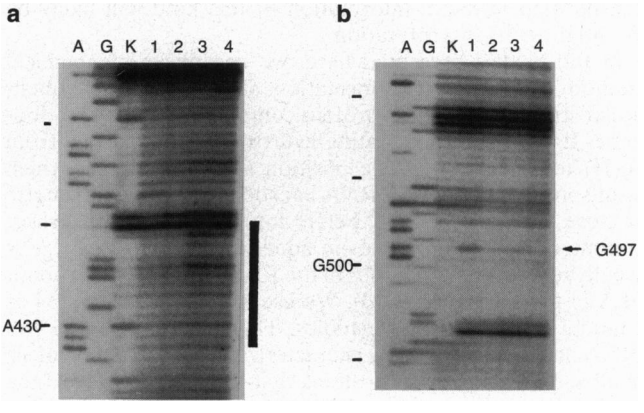

FIG. 1. Binding of [Fe-Cys^{31}]S4 to 16S rRNA and direct hydroxyl radical cleavage. (*a*) Hydroxyl radical cleavage of 16S rRNA by [Fe-Cys^{31}]S4. The bar indicates the extent of cleavage. (*b*) Protection of guanosine at position 497 from kethoxal attack, showing binding of [Fe-Cys^{31}]S4 to the S4 binding site of 16S rRNA. All lanes show results of primer extension using the 480 primer (13). Lanes: A and G, sequencing lanes; K, unprobed naked 16S rRNA; 1–4, probing lanes (1, 16S rRNA; 2, S4–16S rRNA complex; 3, [Fe-Cys^{31}]S4–16S rRNA complex; 4, [Fe-Cys^{31}]S4–16S rRNA complex formed in the presence of a 2-fold excess of unmodified S4). Positions of adenosine at 430 and guanosine at 500 are indicated.

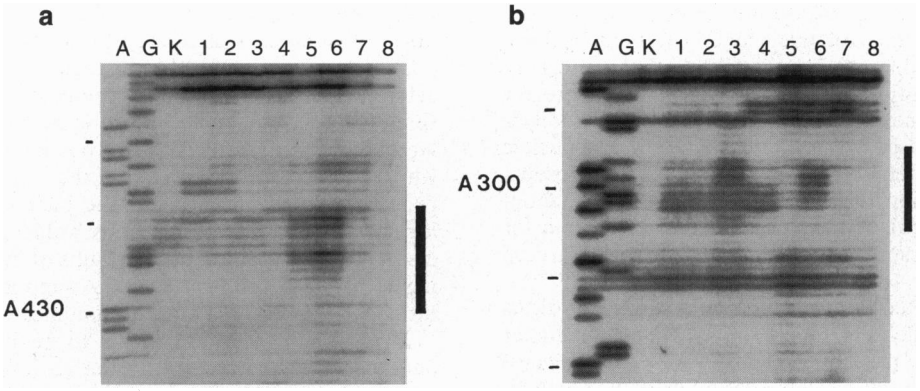

FIG. 2. Directed probing in the [Fe-Cys[31]]S4–16S rRNA complex and in [Fe-Cys[31]]S4-containing 30S subunits. Cleavages in the 420 region were detected with the 506 primer (*a*); cleavages in the 300 region were detected with the 377 primer (*b*). Lanes A, G, and K are as in Fig. 1. Lanes in *a*: 1, 16S rRNA; 2, S4-deficient 30S subunits; 3, 16S rRNA–S4 complex; 4, 30S subunits reconstituted with Σ mix + S4; 5, [Fe-Cys[31]]S4–16S rRNA complex; 6, [Fe-Cys[31]]S4 30S subunits; 7, 30S subunits reconstituted with total ribosomal proteins extracted from 30S subunits; 8, isolated 30S subunits. Lanes in *b* are the same as in *a* except for the following: 2, 16S rRNA–S4 complex; 3, [Fe-Cys[31]]S4–16S rRNA complex; 4, S4-deficient 30S subunits; 5, 30S subunits reconstituted with Σ mix + S4. Positions of adenosine at positions 300 and 430 are indicated.

sedimentation values (Fig. 3). A further test for correct assembly is biological activity, which was monitored by poly(U)-dependent binding of $tRNA^{Phe}$ to the reconstituted 30S particles. The activity of subunits reconstituted with [Fe-Cys[31]]S4 was indistinguishable from that of subunits reconstituted with unmodified S4 (Table 1).

Directed hydroxyl radical probing of the singly Fe–BABE-substituted 30S subunits resulted in cleavage of the 16S rRNA chain in the same two regions as those observed for the S4–16S rRNA complex (Fig. 2). The only detectable differences between the two probing patterns is that, whereas the cleavages in the 300 region extend from around position 292 to 310 for the S4–RNA complex, in fully assembled 30S subunits cleavage is restricted to nucleotides 299–303 (Fig. 2*b*, lanes 3 and 6). Possible explanations for these differences are discussed below.

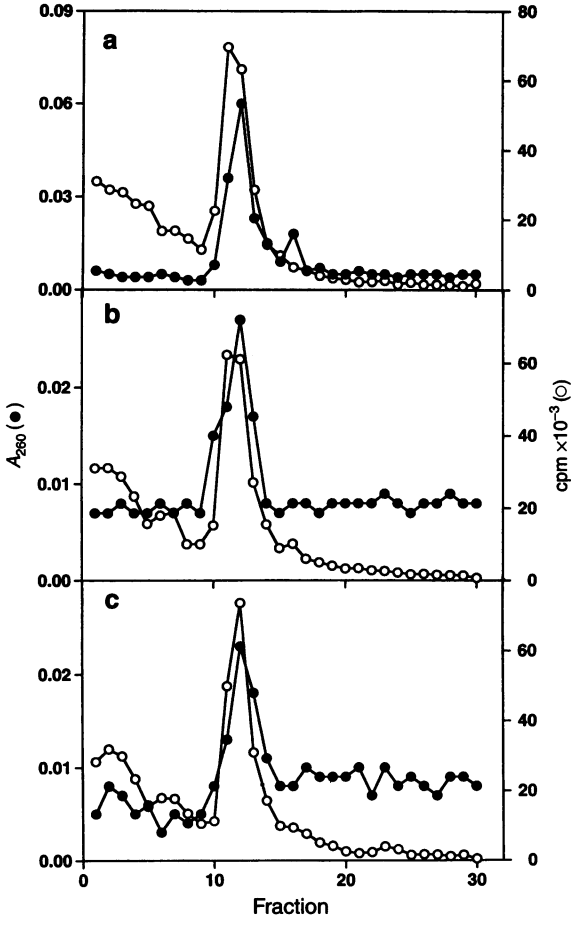

FIG. 3. Sedimentation analysis of 30S subunits reconstituted with [Fe-Cys[31]]S4. ○, ^{32}P-labeled reconstituted marker 30S subunits; ●, unlabeled control or reconstituted subunits. (*a*) Isolated 30S subunits. (*b*) Subunits reconstituted with unmodified S4. (*c*) Subunits reconstituted by using [Fe-Cys[31]]S4. Sedimentation is from left to right.

DISCUSSION

Directed probing with hydroxyl radicals is a potentially powerful method for exploring structural neighborhoods in nucleic acid–protein complexes. This approach has been employed previously with success in studies of protein–DNA complexes. One application has been to map chromosomal DNA using site-directed cleavage by Fe(II) bound to DNA-binding proteins (15, 16). Other studies have used binding geometry of proteins relative to their DNA binding sites (17, 18). Applications of tethered Fe(II) to the study of RNA structure include its use for locating small ligands bound to an RNA (25) and for probing structure within a single RNA molecule (26). Hydroxyl radicals attack the ribose, presumably at its C1′ and C4′ hydrogens (27) in a reaction that leads to scission of the RNA backbone. In contrast to RNA probing chemistries that rely on attack at nucleophilic positions of base moieties (7), the hydroxyl radical reaction is generally insensitive to secondary structure and base–base interactions (14). A second difference

Table 1. tRNA binding activity of 30S subunits reconstituted with BABE-derivatized protein S4

30S Subunits	$tRNA^{Phe}$ bound, pmol	Activity, %
Native	2.29	100
Reconstituted from 16S rRNA		
+ TP30*	0.70	29
+ Σ mix†		
+ untreated S4	0.94	41
+ mock control S4	1.04	46
+ [Fe-Cys[31]]S4	1.08	47

Native or reconstituted particles (5 pmol) were incubated with 5 pmol of [^{32}P]$tRNA^{Phe}$ and 5 μg of poly(U) in 75 μl of 20 mM $MgCl_2$/100 mM KCl/80 mM Hepes, pH 7.7, for 15 min at 37°C and for 10 min on ice. Bound tRNA was measured by filter binding (22).

*TP30, mixture of total ribosomal proteins extracted from 30S subunits.

†Σ mix, stoichiometric mixture of individually purified 30S ribosomal proteins lacking S4.

is that the hydroxyl radicals diffuse out freely from their site of generation and so are relatively unconstrained by local stereochemistry. An important consequence is that many more nucleotides are susceptible to attack, giving a much greater yield of probing information. The lifetime of hydroxyl radicals in aqueous solution, based on their range of attack on a nucleic acid backbone from a tethered position in a duplex (S. Joseph, personal communication), corresponds to a distance of about 10 Å. This is a convenient level of structural resolution for studies on the ribosome, whose maximum dimensions are on the order of 200 Å.

The results presented here show that hydroxyl radicals generated from a single Fe(II) ion tethered via a short linker to Cys-31 of ribosomal protein S4 cause localized cleavage of the 16S rRNA chain in an S4–RNA complex or in reconstituted 30S ribosomal subunits. The cleavage patterns (summarized in Fig. 4) are similar in the two constructs, indicating spatial proximity of the Fe(II) probe to two discrete regions of the 5′ domain, around positions 300 and 425, respectively (Fig. 2). The latter region lies within the previously identified binding site for protein S4, as determined by chemical footprinting experiments in which S4 was localized to the junction of five helical elements in the 400–550 region (7). The other site of directed cleavage, which is centered around position 300, overlaps the footprint observed for protein S16 (23). This result indicates that the section of RNA around position 300 is proximal to Cys-31 of protein S4, although it most likely does not contact S4 directly. Moreover, it is consistent with the evidence from neutron diffraction studies that S16 is a near neighbor of S4 in the 30S subunit (24).

We observe a subtle but reproducible difference in the cleavage patterns in the 300 region between the two kinds of complex (Fig. 2*b*). In the S4–16S rRNA complex, cleavage

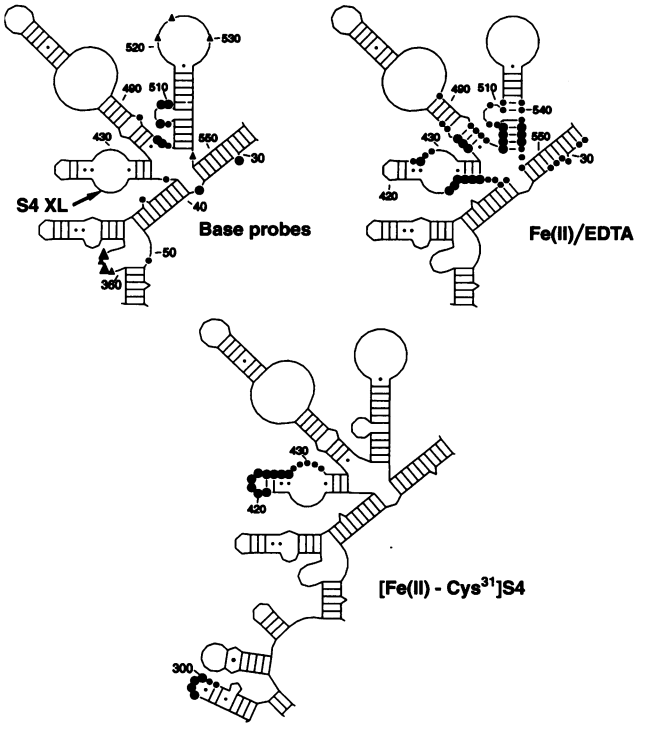

FIG. 4. Summary of sites of cleavage resulting from directed hydroxyl radical probing (*Lower*), compared with S4 footprints obtained by chemical probing (*Upper*) (ref. 7; T. Powers, personal communication) and the site of cross-linking of S4 (S4 XL; ref. 28).

spans approximately positions 292–310, whereas in the assembled 30S subunit, it is limited to positions *ca.* 299–303. One possible explanation for this difference is that in the intact 30S subunit, the particle is more rigidly structured, while in the single protein complex, the probe may be able to explore a larger RNA target size. A second possibility is that protein S16, which protects the 300 region of the RNA from base-specific probes (23), can also shield the backbone from attack by localized hydroxyl radicals, thus reducing the extent of the cleavage pattern. Preliminary results of hydroxyl radical probing with untethered Fe(II)–EDTA support the latter interpretation (T. Powers, personal communication).

These findings should prove to be useful constraints in detailed modeling studies on the structure of the 30S ribosomal subunit. In principle, this approach can be extended to probing the RNA neighborhoods of the rest of the ribosomal proteins and should be amenable to the study of other ribonucleoprotein complexes, such as spliceosomes, telomerase, and messenger ribonucleoproteins.

We thank Douglas P. Greiner for synthesis of BABE, Bryn Weiser for Fig. 4, and members of the Noller lab for discussions. We also thank J. Puglisi for critically reading the manuscript. This work was supported by National Institutes of Health Grants GM-17129 (to H.F.N.), CA-16861 and CA-47829 (to C.F.M.), and a National Institutes of Health postdoctoral fellowship to R.M.

1. Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlessinger, D. & Warner, J. R., eds. (1990) *The Ribosome: Structure, Function and Evolution* (Am. Soc. Microbiol., Washington, DC).
2. Tullius, T. D. & Dombrowski, B. (1985) *Science* **230,** 679–681.
3. Dreyer, G. B. & Dervan, P. B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 968–972.
4. Zimmermann, R. A., Muto, A., Fellner, P., Ehresmann, C. & Branlant, C. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1282–1286.
5. Daya-Grosjean, L., Reinbolt, J., Pongs, O. & Garrett, R. A. (1974) *FEBS Lett.* **44,** 253–256.
6. Schaup, H. W., Sogin, M., Woese, C. & Kurland, C. G. (1971) *Mol. Gen. Genet.* **114,** 1–8.
7. Stern, S., Wilson, R. C. & Noller, H. F. (1986) *J. Mol. Biol.* **192,** 101–110.
8. Sapag, A., Vartikar, J. V. & Draper, D. E. (1990) *Biochim. Biophys. Acta* **1050,** 34–37.
9. Held, W. A., Ballou, B., Mizushima, S. & Nomura, M. (1974) *J. Biol. Chem.* **249,** 3103–3111.
10. DeRiemer, L. H., Meares, C. F., Goodwin, D. A. & Diamanti, C. J. (1981) *J. Labelled Compd. Radiopharm.* **18,** 1517–1534.
11. Rana, T. M. & Meares, C. F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10578–10582.
12. Imlay, J. A., Chin, S. M. & Linn, S. (1988) *Science* **240,** 640–642.
13. Stern, S., Moazed, D. & Noller, H. F. (1988) *Methods Enzymol.* **164,** 481–489.
14. Celander, D. W. & Cech, T. R. (1990) *Biochemistry* **29,** 1355–1361.
15. Chen, C. B. & Sigman, D. S. (1987) *Science* **237,** 1197–1201.
16. Pendergrast, S. P., Ebright, Y. W. & Ebright, R. H. (1994) *Science* **265,** 959–962.
17. Sluka, J. P., Horvath, S. J., Bruist, M. J., Simon, M. J. & Dervan, P. B. (1987) *Science* **238,** 1129–1132.
18. Mazzarelli, J. M., Ermácora, M. R., Fox, R. O. & Grindley, N. D. (1993) *Biochemistry* **32,** 2979–2986.
19. Moazed, D., Stern, S. & Noller, H. F. (1986) *J. Mol. Biol.* **187,** 399–416.
20. Hindennach, J., Stoeffler, G. & Wittman, H. G. (1971) *Eur. J. Biochem.* **23,** 7–11.
21. Schilt, A. A. (1969) *Analytical Application of 1,10 Phenanthroline and Related Compounds* (Pergamon, Oxford), pp. 54–59.
22. Moazed, D. & Noller, H. F. (1989) *Cell* **57,** 585–597.
23. Stern, S., Changchien, L. M., Craven, G. R. & Noller, H. F. (1988) *J. Mol. Biol.* **200,** 291–299.
24. Capel, M. S., Engelman, D. M., Freeborn, B. R., Kjeldgaard, M., Langer, J. A., Ramakrishnan, V., Schindler, D. P., Schneider, D. K., Schoenborn, B. P., Sillers, I. Y., Yabuki, S. & Moore, P. B. (1987) *Science* **238,** 1403–1406.
25. Wang, J. F. & Cech, T. R. (1992) *Science* **256,** 526–529.
26. Han, H. & Dervan, P. B. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4955–4959.
27. Stubbe, J. & Kozarich, J. W. (1987) *Chem. Rev.* **87,** 1107–1136.
28. Zwieb, C. & Brimacombe, R. (1978) *Nucleic Acids Res.* **5,** 1189–1206.