Directed hydroxyl radical probing of 16S rRNA in the ribosome: Spatial proximity of RNA elements of the 3' and 5' domains

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ABSTRACT

We have shown previously that directed hydroxyl radical probing of 16S rRNA from Fe(II) tethered to specific sites within the RNA gives valuable information about RNA–RNA proximities in 70S ribosomes. Here, we extend that study and present probing data from nt 424 in 16S rRNA. To tether an Fe(II) to position 424 in the rRNA we created a specific discontinuity in the RNA by *in vitro* transcription of the RNA as two separate fragments corresponding to nt 1–423 and 424–1542. An Fe(II)-BABE was covalently attached to a 5'-guanosine- α -phosphorothioate at position 424 and 30S subunits were reconstituted from the two pieces of rRNA and the small subunit proteins. Reconstituted 30S subunits capable of associating with 50S subunits were selected by isolation of 70S ribosomes. Hydroxyl radicals, generated *in situ* from the tethered Fe(II), cleaved positions in the RNA backbone that were close in three-dimensional space to the Fe(II), and the sites of cleavage were identified using primer extension. Fe(II) tethered to position 424 induces cleavage around nt 424, 513, and 531 in the 5'-domain of 16S rRNA and around nt 1008, 1029, 1044, and 1208 in the 3'-domain of 16S ribosomal RNA. These data constrain the positions of the 420, 1015, 1030 and 1000/1040 helices, for which there is little structural information. Since the 5'- and 3'-domains of 16S rRNA constitute the body and head, respectively, of 30S subunits, these findings provide direct evidence for proximity of RNA elements in the body and head of 30S.

Keywords: 30S ribosomal subunit; BABE; chemical probing; hydroxyl radicals; rRNA folding

INTRODUCTION

The 16S ribosomal RNA (rRNA) plays a critical role in the structure and function of 30S ribosomal subunits (reviewed in Noller, 1991). Thus, to understand how 30S subunits function in translation, elucidation of the three-dimensional folding of the 1,542-nt 16S rRNA within the ribosome is a necessary step. An extensive body of biochemical data has been used to construct low-resolution models of the three-dimensional folding of 16S rRNA in the 30S subunit by several groups of investigators (Stern et al., 1988b; Hubbard & Hearst, 1991; Malhotra & Harvey, 1994; Fink et al., 1996; Mueller & Brimacombe, 1997). In all of these models the three major secondary structure domains of 16S rRNA can be identified with distinct morphological features that are seen in electron micrographs of 30S subunits

(for example, Lata et al., 1996); the 5', central, and 3' major domains correspond to the body, platform, and head, respectively, of the subunit. There remain, however, several regions of rRNA in the 5' and 3' domains that have few structural constraints and are thus either not included in models or are placed with a high degree of uncertainty. Our goal has been to obtain structural information about these poorly constrained regions of 16S rRNA.

In a previous article, we described a method of probing RNA–RNA proximities directly from internal positions of 16S rRNA using hydroxyl radicals generated *in situ* from Fe(II) tethered to specific sites within 16S rRNA (Newcomb & Noller, 1999). To covalently attach Fe(II) to the rRNA, we transcribed the RNA *in vitro* as two separate fragments and tethered Fe(II) to a guanosine- α -phosphorothioate (GMPS) at the 5' terminus of the 3' fragment via a 1(*p*-bromoacetamidobenzyl)-EDTA (BABE) linker (DeRiemer et al., 1981). Ribosomal 30S subunits were then reconstituted from the two fragments of rRNA and a total mixture of the 30S ribosomal proteins (TP30). Initiation of hydroxyl radical

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formation (Joseph & Noller, 1996) then allowed us to probe the RNA neighborhood around the tethered Fe(II).

We now extend this approach by probing from Fe(II) attached to nt 424, which is at the end of a helix with phylogenetically variable sequence. The 420 helix is known to be near ribosomal protein S4, which has been cross-linked to nt 413 using nitrogen mustard (Greuer et al., 1987). Nucleotides in this helix are also protected from solution hydroxyl radical cleavage by protein S4 (Powers & Noller, 1995b), and directed hydroxyl radical probing from Fe(II) tethered to position 31 of protein S4 results in cleavage of the RNA backbone at the end of the 420 helix (Heilek et al., 1995). Protein S4 has been shown by neutron diffraction and immunoelectron microscopy studies to be in the upper part of the body of the 30S subunit (Winkelmann et al., 1982; Stöffler-Meilicke et al., 1984; Capel et al., 1987). Although the 420 helix is believed to be located in the upper region of the body of the 30S subunit, its exact location has not been determined. The results of this study constrain the 420 helix relative to 16S rRNA elements in the body of the subunit, and, most interestingly, relative to specific RNA features in the head of the 30S subunit.

RESULTS

Reconstitution of functional 30S subunits from fragmented 16S rRNA

Escherichia coli 30S subunits were reconstituted from unmodified or Fe(II)-BABE-modified in vitro transcribed 16S rRNA fragments 1–423 and 424–1542 following a procedure described in Newcomb & Noller (1999) with the modification that Fe(II)-BABE was attached to the rRNA before, rather than after, heating and snap-cooling the rRNA. This change reproducibly resulted in higher yields of reconstituted 30S subunits (see Materials and Methods). Functional subunits were selected by associating reconstituted 30S subunits with natural 50S subunits and isolating the resulting 70S ribosomes by sucrose gradient sedimentation (Fig. 1). There was no detectable difference in sedimentation behavior between ribosomes reconstituted from unmodified or Fe(II)-BABE-modified rRNA (data not shown). Furthermore, there was no detectable difference in sedimentation between 70S ribosomes formed from 30S subunits reconstituted from natural 16S rRNA (Fig. 1A), 5'-Fe(II)-modified full-length in vitro-transcribed 16S rRNA (Fig. 1B), and rRNA fragments 1–423 and Fe(II)-modified 424–1542 (Fig. 1C).

In addition to assaying the ability of the reconstituted 30S subunits to associate with 50S subunits, we tested the tRNA binding activity of 70S ribosomes containing rRNA fragments 1–423 and 5'-Fe(II) 424–1542. These modified ribosomes containing a discontinuity at position 423/424 bound tRNA 0.85 times as efficiently as ribosomes containing natural 30S subunits (Table 1).

FIGURE 1. Sucrose gradient analysis of 70S ribosomes associated from natural 50S subunits and 30S subunits reconstituted in vitro with different constructs of control and modified 16S rRNA. 30S subunits reconstituted with (A) 60 pmol natural 16S rRNA plus 38 pmol 50S subunits, (B) 210 pmol in vitro transcribed 5'-Fe(II) 16S rRNA plus 30 pmol 50S subunits, (C) 210 pmol 16S rRNA fragments 1–423 and Fe(II) 424–1542 plus 23 pmol 50S subunits. D: 23 pmol of 50S subunits. Sedimentation is from left to right and absorbance was monitored at 254 nm.

At 20 mM $MgCl_2$, poly(U)-independent tRNA binding by all particles was about one-third that observed in the presence of poly(U).

Probing 70S ribosomes

Isolated 70S ribosomes were probed with hydroxyl radicals generated *in situ* and the rRNA was isolated and analyzed by primer extension to identify the cleavage targets (Stern et al., 1988a). For each probing experiment, three versions of rRNA were reconstituted. As a first control, 30S subunits were reconstituted from 16S rRNA in which guanosine monophosphate (GMP), rather than GMPS, was incorporated at the 5' end of the 3'

TABLE 1. Yield and tRNA binding activity of 70S ribosomes formed from reconstituted 30S subunits

30S subunits	Percent yield of 70S ribosomes ^a	Relative tRNA binding with poly(U) ^b	Relative poly(U)-independent tRNA binding ^b
Natural 30S	44	1.00	0.48
Reconstituted from:			
Natural 16S rRNA	28	1.10	0.48
5'-Fe(II) in vitro transcribed 16S rRNA	5.4	0.88	0.22
1-423/Fe(II)-424-1542	3.9	0.85	0.17
Natural 50S subunits ^c	—	0.13	0.11

Sucrose gradient purification and tRNA binding of ribosomes were performed as described in Materials and Methods.

^aYield of ribosomes isolated after sucrose gradient sedimentation is based on input rRNA.

^bValues are relative to binding of 1.20 pmol tRNA bound per pmol 70S ribosomes formed from natural 30S subunits.

^ctRNA binding was measured after sucrose gradient sedimentation of 50S subunits isolated along with 70S ribosomes resulting from approximately 5% contamination of the 50S subunits by 30S subunits.

fragment (5'-GMP 424-1542). This rRNA was treated with Fe(II)-BABE to control for any cleavage induced independent of GMPS-tethered Fe(II). As a second control, subunits were reconstituted using rRNA containing an underivatized GMPS at the 5' end of the 3' fragment (5'-GMPS 424-1542). Lastly, 30S subunits were reconstituted from rRNA in which Fe(II) was tethered via BABE to a 5'-GMPS on the 3' fragment (5'-Fe(II) 424-1542). The Fe(II)-modified rRNA will be referred to as Fe(II)-424.

Figure 2 shows autoradiographs of primer extension analyses of 16S rRNA extracted from ribosomes containing 16S rRNA fragments 1-423 and 424-1542, with Fe(II) tethered to position 424 of the rRNA, that were subjected to directed hydroxyl radical probing. Sites of cleavage in the rRNA backbone appear as additional bands in lane 3 of the gels. Cleavage occurred in the rRNA backbone near the point of attachment of the probe, from nt 424-435 (Fig. 2A). Probing from nt 424 also resulted in cleavage in the 530 stem and loop at

FIGURE 2. Cleavage of 16S rRNA in 70S ribosomes assembled from 30S subunits reconstituted with 16S rRNA fragments 1-423 and Fe(II)-derivatized 424-1542 detected by primer extension. A and G are dideoxy sequencing lanes. Lanes 1-3: directed hydroxyl radical cleavage of 70S ribosomes containing 16S rRNA fragments 5'-GMP 1-423 and (lane 1) Fe(II)-BABE treated 5'-GMP 424-1542; (lane 2) mock-treated 5'-GMPS 424-1542; (lane 3) 5'-Fe(II) 424-1542. Hydroxyl radical cleavages are seen as additional bands in lane 3 and are indicated by bars.

positions 510–516, 530–532 and 537–538 (Fig. 2B). Finally, cleavage was observed in the 3'-domain of 16S rRNA at positions 996–1002, 1027–1030, 1032–1035, 1040–1045, 1071–1073 (very weak) (Fig. 2D), and 1203–1215 (Fig. 2C).

The cleavages observed when probing from Fe(II) at position 424 of 16S rRNA are summarized on the secondary structure of 16S rRNA in Figure 3. Relative cleavage intensities were estimated by comparison of band intensity with the intensity of bands in the sequencing lanes (Fig. 3). Distances between the tethered probe and rRNA targets can be estimated, based on previous calibration experiments (Joseph et al., 1997), to lie within the range of 0 to 22 Å for strong, 12 to 36 Å for medium, and 20 to 44 Å for weak cleavages.

DISCUSSION

In a continuation of our studies in which 16S rRNA is probed from internal positions of the RNA (Newcomb & Noller, 1999; Samaha et al., 1999), we have shown that functional 30S subunits can be reconstituted from *in vitro* transcribed 16S rRNA fragments 1–423 and 424–1542 together with the small subunit proteins. These subunits, in which there is a discontinuity in the rRNA chain between positions 423 and 424, associate with

natural 50S subunits and the resulting 70S ribosomes bind tRNA in a message-dependent manner nearly as efficiently as ribosomes containing natural 30S subunits (Table 1). These functional tests provide strong evidence for the correct assembly of the reconstituted 30S subunits.

Further evidence for correct folding of the 424 region of 16S rRNA in our Fe(II)-BABE-derivatized 30S subunits comes from the fact that the 420 helix is part of the binding site for protein S4 (Powers & Noller, 1995a, 1995b). S4 is a primary RNA-binding protein whose incorporation is essential for the correct assembly of 30S subunits (Held et al., 1974). The fact that our Fe(II)-BABE-derivatized particles cosediment with 30S subunits and are active in both tRNA binding and subunit association suggests that S4 is properly assembled, and that the 420-region helical elements are properly folded. Although we cannot exclude some mobility of the nucleotide itself, this level of uncertainty would not severely compromise the relatively low-resolution information obtained by our method.

Hydroxyl radical probing from Fe(II) tethered to nt 424 results in cleavage of the RNA backbone in both the 5' and 3' major domains of 16S rRNA. In the 5' domain, cleavage is observed in the highly conserved 530 stem and loop, with the strongest cleavage around position 513 in the stem (Fig. 3). Although the location of the 530 loop in relation to other 16S rRNA elements, especially the 1400 decoding region, has been controversial (see, for example, Powers & Noller, 1994; Brimacombe, 1995), the 530 loop has been well constrained to the body of the 30S subunit (Stern et al., 1988b; Malhotra & Harvey, 1994; Fink et al., 1996; Mueller & Brimacombe, 1997).

The cleavages observed in the 3' major domain of 16S rRNA around positions 999, 1029, 1044, and 1206 are especially interesting. While the 5' domain of 16S rRNA is localized to the body of the 30S subunit, the 3' major domain constitutes the head of the subunit. The only covalent connection between the head and the body of the subunit is through the 930 helix, which is believed to be the "neck" of the subunit (Stern et al., 1988b; Malhotra & Harvey, 1994; Fink et al., 1996; Mueller & Brimacombe, 1997). Cleavage of rRNA in the 3' domain from position 424 provides direct evidence for a second point of RNA–RNA proximity between the head and body of the 30S subunit. The cleavage intensities suggest that the closest approach of nt 424 to the head is around position 1044 (Fig. 3).

Previous studies have also suggested proximity between the 420 helix and 530 stem-loop in the 5' domain of 16S rRNA and the 1200 region in the 3' domain. Directed hydroxyl radical probing from a single position on ribosomal protein S5 results in cleavage of 16S rRNA in both of these regions (Heilek & Noller, 1996). Additionally, UV cross-links have been identified between mRNA analogs and 16S rRNA positions 1052,

FIGURE 3. Secondary structure of 16S rRNA summarizing the sites of directed hydroxyl radical cleavage from Fe(II)-424 16S rRNA. The sizes of the circles correspond to the intensities of cleavage seen at each target site.

1196, and 532 (Rinke-Appel et al., 1993; Sergiev et al., 1997). Although these cross-links span 5 nt of the mRNA sequence, they provide further support for proximity between the 530 and 1050/1200 regions of 16S rRNA.

The RNA–RNA proximity that we observe between the 5' and 3' domains of 16S rRNA may correspond to the close approach between the shoulder of the body and the head of the 30S subunit seen in electron micrograph reconstructions of ribosomes (Stark et al., 1995, 1997; Agrawal et al., 1996; Lata et al., 1996). This is perhaps best seen in a comparison between isolated nonactivated 30S subunits, heat-activated 30S subunits, and 30S subunits bound to 50S subunits at 37 Å resolution (Lata et al., 1996). A large gap between the shoulder of the body of the subunit and the head in nonactivated particles narrows in heat-activated subunits, and is closed in 70S ribosomes, the state of the 30S subunits that were probed in our experiments. Formation of this body-head contact appears to occur concomitantly with a twisting and curling up of the platform region of the 30S subunit towards the head, indicating a conformational change in the 30S subunit upon association with 50S subunits (Lata et al., 1996). It has been postulated that this second contact between the body and head of the 30S subunit forms a hole through which the mRNA passes (Lata et al., 1996; Mueller et al., 1997). An intriguing possibility is that this contact between the body and head of the subunit is mediated by interaction of the 530 loop with the 1050/1200 region of the 3' domain. Such an interaction could begin to explain the observation that mutations in the both the 530 and 1050/1200 regions cause perturbations in translational fidelity (reviewed in O'Connor et al., 1995).

Our data provide some of the first structural constraints for positioning of the 997–1003/1037–1044 helix and the associated 1015 and 1030 helices (also known as the bunny ears), which constitute one of the least defined elements in the 3'-major domain of 16S rRNA. In models for the three-dimensional folding of 16S rRNA, the positions of these helices have either not been specified or have been placed with a high degree of uncertainty (Stern et al., 1988b; Malhotra & Harvey, 1994; Fink et al., 1996; Mueller & Brimacombe, 1997). Our results help constrain these three helices to the lower surface of the head of the 30S subunit. Similarly, the data presented here provide strong constraints for the position of the 420 helix in the 30S subunit. Finally, these findings support the possibility that 16S rRNA domain–domain communications in the 30S subunit may play an important role in translation.

MATERIALS AND METHODS

Reagents

Buffer A contains 20 mM NH_4^+ -HEPES (pH 7.5), 20 mM MgCl_2 , and 500 mM NH_4Cl ; buffer B contains 20 mM

NH_4^+ -HEPES (pH 7.5), 10 mM MgCl_2 , and 100 mM NH_4Cl ; buffer C contains 20 mM NH_4^+ -HEPES (pH 7.5), 20 mM MgCl_2 , and 100 mM NH_4Cl . Preparation of 16S rRNA and 30S, 50S, and 70S ribosomes was done as described (Moazed et al., 1986). Sucrose-gradient analysis and gel-electrophoresis analysis showed that there was less than 4.5% contaminating 30S subunits in the 50S subunits. TP30 was prepared from 30S subunits isolated by zonal centrifugation as previously described (Nierhaus, 1990) and stored in buffer A. Synthesis of BABE was done as previously described (DeRiemer et al., 1981).

Preparation of gene constructs

The gene encoding 16S rRNA was amplified, either in its entirety or in fragments, from pSTL102 (Triman et al., 1989) by PCR. Primers for the 5'-end of the 1–423 construct contained the restriction site for *EcoRI* and the T7 promoter; primers for the 5'-end of all other constructs contained the restriction site for *SacI* and the T7 promoter. Primers for the 3' end of all fragments contained the restriction sites for *BsaI* and *BamHI*. Nucleotides A1 and A2 were mutated to Gs to improve the efficiency of in vitro transcription with T7 polymerase. Constructs were ligated into *BamHI*-, *KpnI*-, *EcoRI*-digested or *BamHI*-, *SacI*-digested pUC118 (USB). DNA sequence analysis confirmed the predicted sequences.

Preparation of 16S rRNA transcripts in vitro

In vitro transcripts were generated essentially as described (Newcomb & Noller, 1999) using T7 RNA polymerase (Milligan et al., 1987) from *BsaI* linearized plasmids described above. To introduce a thiophosphate or monophosphate at the 5' terminus of the rRNA, transcriptions were carried out in the presence of a fivefold molar excess of either GMPS or GMP over each NTP (5 mM final concentration). Under these conditions, about 80% of the transcripts initiate with GMPS. Transcriptions were carried out in buffer containing 40 mM Tris-HCl (pH 7.9 at room temperature), 26 mM MgCl_2 , 3 mM spermidine, 0.01% Triton X-100, and 5 mM DTT for 2 h at 37°C. Transcription was stopped by addition of EDTA and SDS to final concentrations of 50 μM and 0.5% respectively. RNA was purified by successive extractions with phenol and chloroform, and chromatography on a G50-Sephadex (Pharmacia) column (1 × 20 cm) eluted with buffer A. Samples were precipitated on ice for 0.5 h with 0.3 M NH_4OAc and 2.5 vol ethanol. Precipitated RNA was recovered by centrifugation for 20 min at 4°C at 10,000 rpm in a JA-20 rotor, washed with 70% ethanol, resuspended in H_2O , and frozen in aliquots.

BABE modification of rRNA

Fe(II)-BABE was prepared by incubating 11 mM final concentration $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ and 15 mM final concentration BABE, brought to pH 3–4 with diisopropylethylamine, for 1 h at room temperature followed by incubation for 10 min with 2.5 mM final concentration EDTA (Newcomb & Noller, 1999). In a standard reaction, 560 pmol of full-length 5'-GMPS-16S rRNA, or an equimolar mixture of 5'-GMP 1–423 and 5'-GMPS-424–1542 16S rRNA fragments, were sus-

pended in 20 mM NH_4^+ -HEPES (pH 8.5), 100 mM NH_4Cl at a final concentration of 9 μM and incubated with 4 mM final concentration Fe(II)-BABE at 37 °C for 45–60 min. Derivatized RNA was removed from free Fe(II)-BABE by two phenol extractions and one chloroform extraction followed by ethanol precipitation as described above. Under these conditions, 5'-GMPS RNA is essentially fully substituted by Fe(II)-BABE. The RNA was resuspended in H_2O at a final concentration of 5.5 μM and heated in a 95 °C water bath for 4 min, followed by rapid addition of ice-cold H_2O to a final concentration of 0.85 μM . Samples were incubated on ice for 15 min and used directly in reconstitution experiments. Control 5'-GMP rRNA that was treated with Fe(II)-BABE, and control 5'-GMPS RNA lacking Fe(II)-BABE were isolated following similar procedures.

In vitro reconstitution of 30S subunits

In vitro reconstitution was performed essentially as described (Krzyszosiak et al., 1987; Newcomb & Noller, 1999). Briefly, for a 100 μL reaction, 60 pmol of modified rRNA and 1 molar equivalent of TP30 were combined in buffer A containing 0.01% Nikkol and heated sequentially for 15 min at each of the following temperatures: 40, 43, 46, 48, and 50 °C followed by quick cooling to 4 °C. The resulting mixture was used for subunit association.

Subunit association and purification of 70S ribosomes

Reconstituted subunits were incubated with 0.1 to 0.2 molar equivalents (based on input RNA) of natural 50S subunits in buffer containing 50 mM NH_4^+ -HEPES (pH 7.5), 15 mM MgCl_2 , 100 mM NH_4Cl , and 0.002% Nikkol at 37 °C for 30 min. Samples were layered onto 11 mL 10–40% sucrose gradients prepared in buffer B. Gradients were spun in an SW41 rotor at 32,000 rpm for 15.5 h at 4 °C. Peaks sedimenting with 70S ribosomes were isolated and sucrose was removed by centrifugation at 4 °C in a JA-20 rotor at 2,800 rpm in Centricon 100 microconcentrators using 3–4 sequential 2 mL washes with buffer B for probing or with buffer C for tRNA binding.

tRNA binding assays

E. coli tRNA^{Phe} was transcribed from the linearized p67CF10 (Sampson et al., 1989) with α -[³²P] UTP. Isolated 70S ribosomes (2.5 pmol) in 20 μL of buffer C were activated at 42 °C for 20 min. After 15 min on ice, 7.5 pmol *E. coli* tRNA^{Phe} (specific activity 31 Ci/mmol), in 5 μL of buffer C, and either 5 μL of 0.5 $\mu\text{g}/\mu\text{L}$ poly(U) in buffer C or, for message-independent binding, buffer C alone, were added. tRNA binding was performed by incubating this mixture at 37 °C for 10 min. The mixture was placed on ice and 1 mL of cold buffer C was added. Three 300- μL aliquots were filtered on nitrocellulose filters (Millipore). To control for binding that may occur to natural 70S ribosomes resulting from contamination of the 50S subunits, the efficiency of tRNA binding to 2.5 pmol of 50S subunits was tested in an identical manner.

Hydroxyl radical probing and primer extension

In all probing experiments, 70S ribosomes containing each of the two control rRNAs described above as well as BABE-modified rRNA were isolated. Ribosomes were probed by initiating hydroxyl radical formation with 0.01% H_2O_2 and 2 mM ascorbic acid followed by incubating for 10 min on ice (Powers & Noller, 1995a). Reactions were quenched by addition of 0.27 vol of 0.1 M thiourea (20 mM final concentration) and precipitated with 0.3 M NaOAc, 2.5 vol ethanol and 1 μg glycogen (10 mg/mL). RNA was isolated and analyzed by primer extension as previously described (Stern et al., 1988a).

ACKNOWLEDGMENTS

We thank Gloria Culver, Lovisa Holmberg, and Kate Lieberman for critical comments on the manuscript; members of the Noller lab for helpful discussions; J. Moran, D.P. Greiner and S. Joseph for 1-(*p*-bromoacetamidobenzyl)-EDTA, which was synthesized in the laboratory of C. Meares. This work was supported by National Institutes of Health (NIH) grant No. GM-17129 and National Science Foundation grant No. MCB-9406186 (to H.F.N.), NIH postdoctoral fellowship No. GM-17532 (to L.F.N.), and a grant to the Center for Molecular Biology of RNA from the Lucille P. Markey Charitable Trust.

Received January 29, 1999; returned for revision March 8, 1999; revised manuscript received April 8, 1999

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