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Mapping the rRNA neighborhood of the acceptor end of tRNA in the ribosome

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In order to map the rRNA environment of the acceptor end of tRNA in the ribosome, hydroxyl radicals were generated *in situ* from Fe(II) attached via an EDTA linker to the 5′ end of tRNA. Nucleotides in rRNA cleaved by the radicals were identified by primer extension, and assigned to the ribosomal A, P and E sites by standard criteria. In the A site, cleavages were found in the 2555–2573 region of 23S rRNA, around bases previously shown to be protected by A site tRNA, and in the α-sarcin loop, the site of interaction of elongation factors EF-Tu and EF-G. P site cleavages occurred in the 2250 loop, where a base pair is made with C74 of tRNA; and around the 2493 region in domain V. Interestingly, two clusters of nucleotides in 23S rRNA are accessible to both A site and P site tRNA probes. The first cluster is in the 1940–1965 region of domain IV, around the site of affinity labeling by the 3′ end of tRNA, and the second cluster is around the bulged adenosine A2602, whose accessibility to chemical probes is enhanced by P site tRNA and decreased by A site tRNA. From the E site, cleavages occur in the 2390–2440 region, surrounding C2394, a base protected from dimethyl sulfate by E site tRNA, and in the phylogenetically variable stem at positions 1860/1880 of domain IV. Unexpectedly, no cleavages were detected in the central loop of domain V of 23S rRNA.

Keywords: chemical probing/hydroxyl radical/ribosome/50S subunit/tRNA

Introduction

Many lines of evidence point to a functional role for rRNA in translation (reviewed in Noller, 1991). One of the most crucial ribosomal functions is catalysis of peptide bond formation, the peptidyl transferase reaction, which involves interactions between the acceptor end of tRNA and the large (50S) ribosomal subunit (Maden *et al.*, 1968; Monro, 1971; Noller *et al.*, 1992). Photochemical cross-linking and chemical protection studies have placed the acceptor end of tRNA in proximity to conserved sequences of 23S rRNA (Barta *et al.*, 1984; Steiner *et al.*, 1988; Wower *et al.*, 1989; Moazed and Noller, 1989, 1991; Podkowinski and Gornicki, 1991; Mitchell *et al.*, 1993). Both of these approaches, however, have limitations; efficient cross-linking depends on favorable chemical and stereochemical circumstances, while interpretation of footprinting results is subject to the ambiguity of whether protection of rRNA by tRNA is direct or indirect.

We present an alternative approach utilizing directed probing with hydroxyl radicals to explore the rRNA neighborhood of tRNA in the ribosome. Fe(II)–EDTA complexes, in the presence of a reducing source, are believed to generate neutral hydroxyl radicals, which cleave the nucleic acid backbone via oxidative attack on the ribose moiety, through a process called the Fenton reaction (Hertzberg and Dervan, 1982; Bull *et al.*, 1983; Hertzberg and Dervan, 1984; Tullius and Dombroski, 1985; Celander and Cech, 1990). Cleavage is restricted to a sphere of a few Angstroms around the position of the metal complex, and is relatively sequence and secondary structure independent. Tethered Fe(II)–EDTA complexes have proved useful in the structural analysis of ligand–DNA complexes (Taylor *et al.*, 1984; Dreyer and Dervan, 1985; Moser and Dervan, 1987; Sluka *et al.*, 1990), group I ribozymes (Wang and Cech, 1992) and in probing the rRNA environment of individual ribosomal proteins (Heilek *et al.*, 1995). In the experiments presented here, we have used 1-(*p*-bromoacetamidobenzyl)-EDTA (BABE) (DeRiemer *et al.*, 1981; Rana and Meares, 1990) to tether Fe(II) to the 5′ terminus of tRNA bearing a 5′-phosphorothioate, and have studied the cleavage patterns obtained in rRNA when 5′-Fe-BABE-modified tRNA is bound to the ribosomal A, P and E sites.

Results

5′-Fe-BABE-modified tRNA^{Phe} binds normally to 70S ribosomes

The characteristic 'footprints' that are observed in 16S and 23S rRNA when tRNA is bound to the ribosome serve as a sensitive and detailed assay for tRNA binding. In order to determine whether the 5′-tethered Fe-BABE affects proper binding of tRNA to the ribosome, we carried out chemical probing experiments with dimethyl sulfate (DMS) and kethoxal and compared the characteristic footprints observed in 16S and 23S rRNA (Moazed and Noller, 1986, 1989, 1990) when either unmodified or 5′-Fe-BABE-modified tRNAs were bound to ribosomes in a poly(U)-dependent fashion. Similar protection patterns were observed at G926 of 16S rRNA and G2252/G2253 of 23S rRNA (Figure 1), and other regions (data not shown), whether unmodified or modified tRNAs were bound to the ribosomal A, P and E sites, providing evidence that the 5′-Fe-BABE moiety does not interfere with tRNA–ribosome interactions.

Tethered hydroxyl radical probing with 5′-Fe-BABE-modified tRNA^{Phe} bound to 70S ribosomes

Unmodified or 5′-Fe-BABE-modified tRNAs were bound to ribosomes, either in the presence or absence of poly(U)

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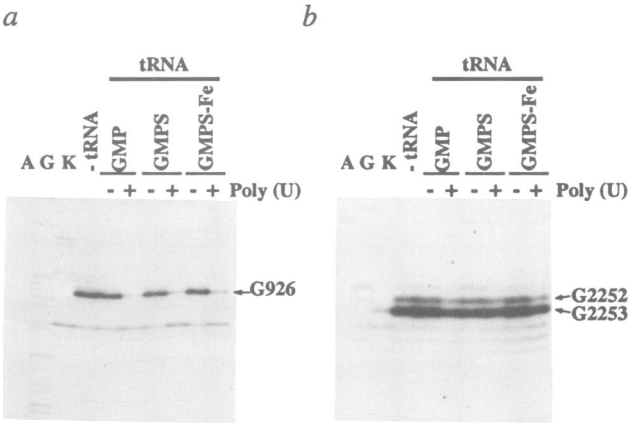

Fig. 1. The 5′-Fe-BABE modification does not interfere with binding of $tRNA^{Phe}$ to 70S ribosomes. Protection of (**a**) G926 of 16S rRNA and (**b**) G2252 and G2253 of 23S rRNA from kethoxal attack by unmodified or 5′-Fe-BABE-modified $tRNA^{Phe}$. A and G, dideoxy sequencing lanes. K, unmodified rRNA; –tRNA, ribosomes probed in the absence of tRNA; GMP, GMPS and GMPS-Fe, ribosomes probed after incubation with an equimolar amount of tRNA transcript containing 5′-guanosine monophosphate, 5′-guanosine-α-phosphorothioate or 5′-guanosine-Fe-BABE-α-phosphorothioate, respectively. + and – indicate the presence or absence, respectively, of poly(U) mRNA.

message, under low [Mg^{2+}] conditions (20 mM) where binding of tRNA to the P and A sites is poly(U) dependent, while binding to the E site is poly(U) independent (Grajevskaja *et al.*, 1982; Kirillov *et al.*, 1983; Lill *et al.*, 1984, 1986; Moazed and Noller, 1990). Hydroxyl radicals were then generated from the tethered Fe(II) by initiation of the Fenton reaction. Positions of cleavage of the rRNA chains were detected by scanning the entire 23S, 16S and 5S rRNA sequences by primer extension with reverse transcriptase using a set of synthetic DNA oligonucleotide primers (Stern *et al.*, 1988). No cleavage of 5S or 16S rRNA was detected, but both poly(U)-dependent and -independent strand scissions were observed in 23S rRNA (Figure 2). Site-specific poly(U)-independent cleavage was detected in 23S rRNA in domain IV (positions 1859–1862 and 1881–1884; Figure 2a) and domain V (positions 2074–2076, 2395–2401, 2421–2424 and 2431–2436; Figure 2c and d). Poly(U)-dependent cleavage was detected mainly in domain IV (positions 1941–1947, 1954–1956 and 1959–1966; Figure 2b) and domain V (positions 2251–2257, 2492–2494, 2555–2561, 2566–2573, 2594–2596 and 2601–2603; Figure 2d, e and f). Interestingly, poly(U)-dependent cleavage was also detected in the α-sarcin loop of domain VI (positions 2659–2664; Figure 2f), indicating the close proximity of this highly conserved functional site to the 5′ end of Fe-BABE-modified tRNA. Since only E site binding is poly(U) independent under these conditions, we assign the poly(U)-independent cleavages of Figure 2 to E site-bound tRNA, and the poly(U)-dependent cleavages to either P or A site-bound tRNAs. The intensity of the E site cleavages decreases markedly in the presence of poly(U) (Figure 2a and d). This can be explained either by competition of the higher affinity A and P sites for the available tRNA, or by a decrease in E site binding upon occupancy of the A site (Rheinberger and Nierhaus, 1986).

In order to distinguish between A and P site cleavages, binding was next carried out under higher [Mg^{2+}] conditions (25 mM) where tRNA binding to the P and E site are both essentially poly(U) independent, but where tRNA binding to the A site remains strictly poly(U) dependent (Figure 3) (Wurmbach and Nierhaus, 1979; Rheinberger *et al.*, 1981; Grajevskaja *et al.*, 1982; Lill *et al.*, 1984, 1986; Moazed and Noller, 1990). Under these conditions, a subset of strand scissions that were poly(U) dependent in the previous experiment became poly(U) independent (nucleotides 1941–1947, 1954–1956, 1959–1966, 2251–2257, 2492–2494, 2594–2596 and 2601–2603; Figure 3b, c, e and f. We infer that these newly poly (U)-independent cleavages originate from P site-bound tRNA, while those that remain strictly poly(U) dependent originate from A site-bound tRNA. A further possibility is that some targets will be accessible from both the A and P sites. Such targets should show poly(U)-independent cleavages that further increase in intensity in the presence of poly(U). This behavior is, in fact, observed for the 1940–1970 and 2600 region nucleotides (Figure 3b, e and f); accordingly, we suggest that these regions are proximal to both A and P site tRNA. In contrast, no such increase in cleavage intensity is observed for nucleotides in the 2250 and 2493 regions, which we ascribe purely to P site tRNA (Figure 3c and e). Since the ribosomal P site has a higher affinity for deacylated tRNA than the A site (Wurmbach and Nierhaus, 1979; Rheinberger *et al.*, 1981; Lill *et al.*, 1986; Rheinberger and Nierhaus, 1986), we further tested our assignments by tRNA titration studies. At low tRNA:ribosome ratios (1:1 or 0.5:1), the cleavages assigned to the A site diminish, while those assigned to the P site persist, confirming our assignments (data not shown). Finally, chemical footprinting experiments carried out under the same conditions of directed hydroxyl radical cleavage (25 mM Mg^{2+}) confirm binding of 5′-Fe-BABE-tRNA to the A, P and E sites (Figure 1 and data not shown).

To exclude the possibility that some of the observed cleavages might originate from 5′-Fe-BABE-tRNAs bound to spurious sites, we asked whether excess unmodified tRNA can compete for binding of the modified tRNA to eliminate the observed strand scissions. Indeed, all of the observed cleavages were abolished by titration with excess unmodified tRNA (Figure 4 and data not shown), providing further evidence that they originate from 5′-Fe-BABE-modified tRNAs bound to the ribosomal tRNA binding sites.

Discussion

Primer extension scanning of all three rRNAs from ribosomes subjected to directed hydroxyl radical cleavage with 5′-Fe-BABE-tRNA shows that the 5′ end of tRNA lies in proximity to 23S, but not 5S or 16S rRNA (Figure 5, upper panel). Autocleavage experiments with 5′-Fe-BABE-tRNA in solution and bound to the ribosome show that the Fe(II) probe is proximal to tRNA positions C74, C75 and A76 (data not shown), a result that is not surprising in view of the pairing of the 5′- and 3′-proximal sequences in the secondary structure of tRNA. According to our assignments, cleavages generated exclusively from A site tRNA were found in the stem–loop region around position 2560 in domain V (2043–2625), close to G2553, a base whose protection from kethoxal attack was shown

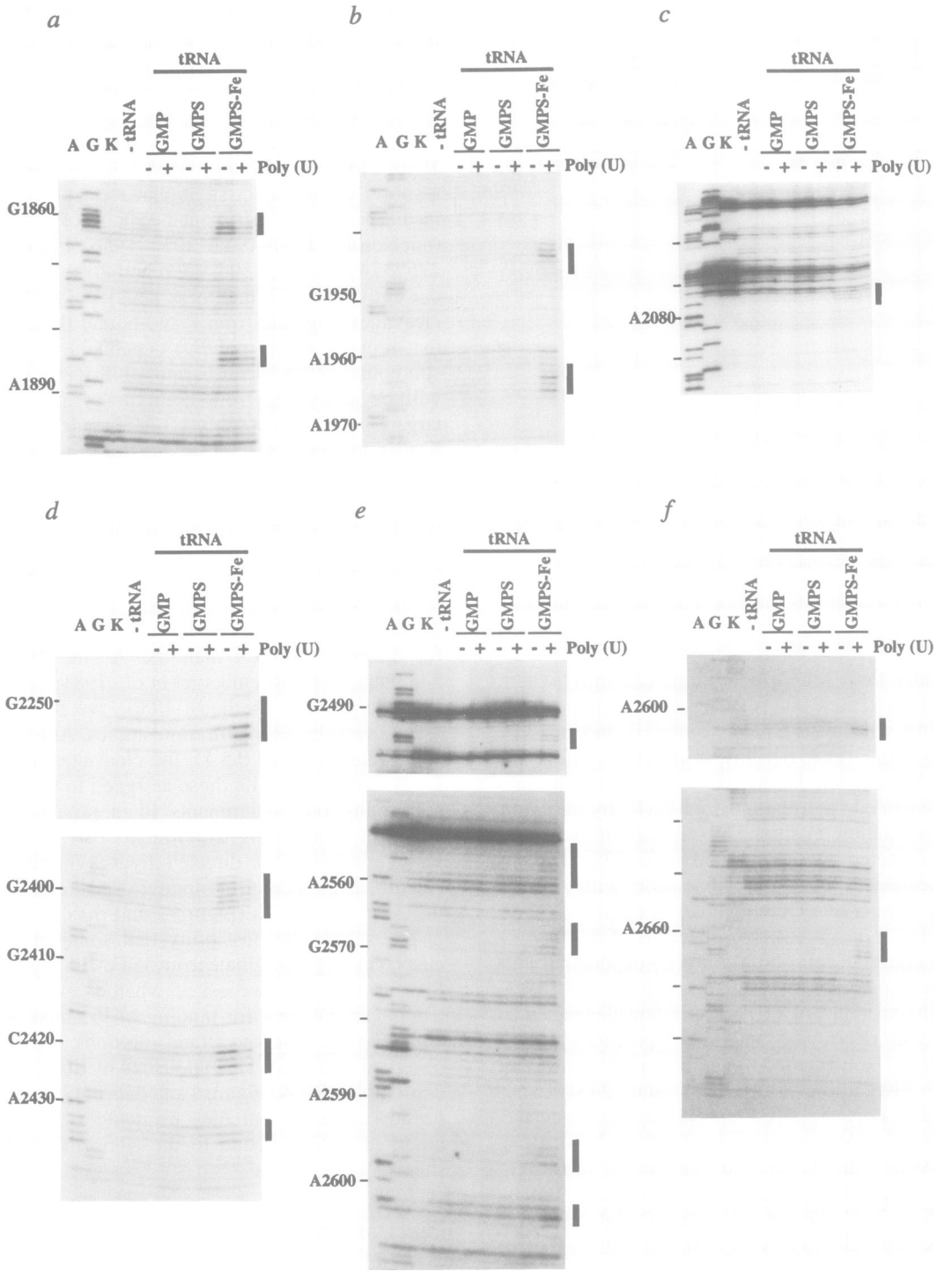

Fig. 2. Strand scission of 23S rRNA by hydroxyl radicals generated from 5′-Fe-BABE-modified $tRNA^{Phe}$ bound to *E.coli* 70S ribosomes at 20 mM $[Mg^{2+}]$. Ribosomes were incubated with a 5-fold excess of tRNA or modified tRNA, and the complexes were treated as described in Materials and methods. Lane designations are as described in Figure 1, except that all but the first three lanes were treated with ascorbate and H_2O_2. Positions of strand scission are indicated by the bars.

to be dependent on the 3′-adenosine of A site tRNA (Figure 5, lower panel; Moazed and Noller, 1989). In addition, cleavage at several nucleotides around position 2660 places the acceptor end of A site tRNA near the α-sarcin loop (2650–2670), which has been shown to interact with elongation factors EF-Tu and EF-G (Moazed *et al.*, 1988). Thus EF-Tu, which is responsible for delivering aminoacyl-tRNA to the ribosome, interacts with 23S rRNA at a location that is not far from the position of the acceptor end of tRNA when it is bound in the ribosomal A site.

Cleavages generated exclusively from P site-bound tRNA were centered on the 2250 stem–loop and around position 2493 (Figure 5, upper panel). Recently, it has

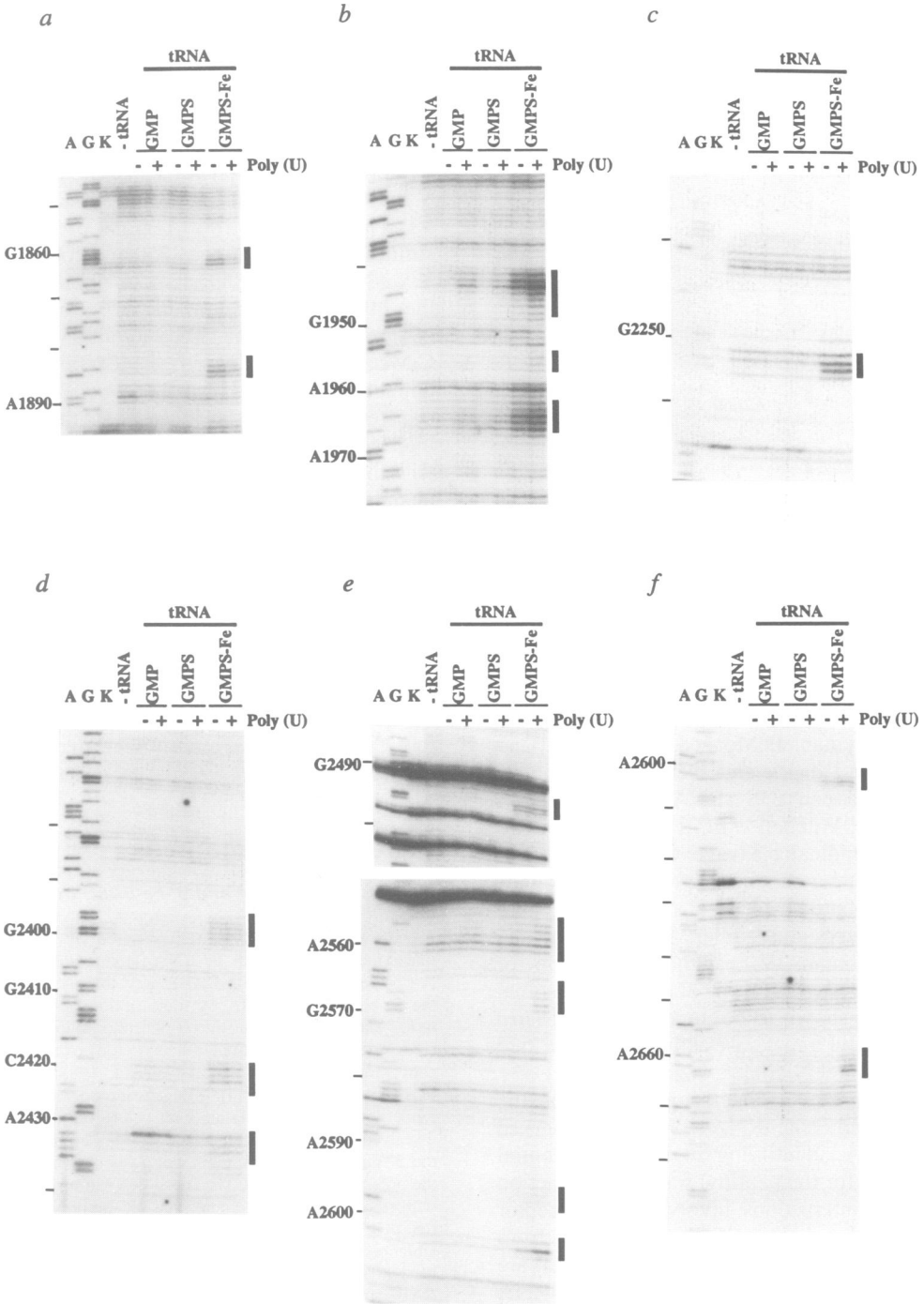

Fig. 3. Strand scission of 23S rRNA by hydroxyl radicals generated from 5′-Fe-BABE-modified $tRNA^{Phe}$ bound to *E.coli* 70S ribosomes at 25 mM [Mg^{2+}]. Ribosomes were incubated with a 15-fold excess of tRNA or modified tRNA, and the complexes were treated as described in Materials and methods. Lane designations are as described in Figure 2. Positions of strand scission are indicated by the bars.

been shown by *in vitro* genetics that C74 of tRNA forms a Watson–Crick base pair with G2252 of 23S rRNA (Samaha *et al.*, 1995), in close agreement with our probing results. The remaining P site cleavages have a somewhat different behavior, becoming enhanced in the presence of poly(U) at high Mg^{2+} concentration (see above). We infer that these nucleotides, in the 1940–1970 and 2600 regions, are accessible to both the A and P site probes. Interestingly, the bulged nucleotide A2602 was shown to gain enhanced reactivity toward DMS in the presence of P site tRNA, and to become protected when tRNA is then bound to the A site (Figure 5, lower panel; Moazed and Noller, 1989). The 1940–1970 region cleavages surround the site of photo-cross-linking of 3′-azidoadenosine-derivatized tRNA (A76), at position 1945 (Figure 5, lower panel; Wower *et al.*, 1989). Also, nearby in domain IV (1648–2009) are bases that are protected by both P site tRNA (Figure 5, lower panel; Moazed and Noller, 1989) and

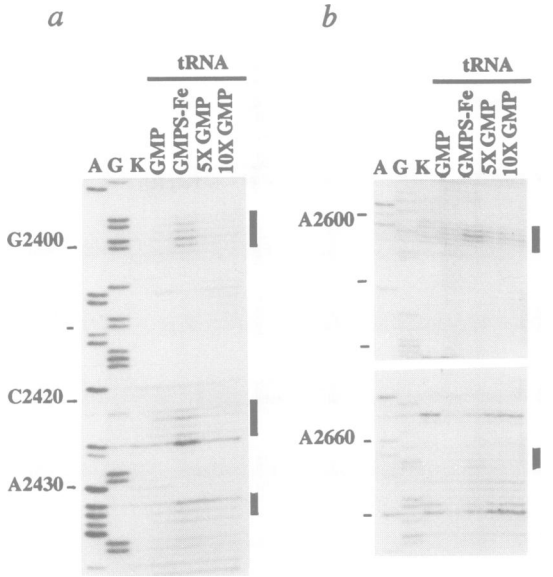

Fig. 4. Competition of 5′-Fe-BABE-modified tRNA^{Phe} by unmodified tRNA^{Phe}. Lane designations are as described in Figure 2. 5× and 10× indicate the presence of 5-fold or 10-fold excess of unmodified 5′-GMP tRNA over 5′-Fe-BABE-tRNA. Positions of strand scission are indicated by the bars.

30S subunits (C.Merryman, D.Moazed, J.McWhiter and H.F.Noller, unpublished data), and the site of an inter-subunit cross-link between 23S rRNA and 16S rRNA (Figure 5, lower panel; Mitchell *et al.*, 1992). Finally, two clusters of hydroxyl radical cleavage were found for E site tRNA (Figure 5, upper panel). One of these surrounds C2394, a site strongly protected by E site tRNA (Figure 5, lower panel; Moazed and Noller, 1989), while the second cluster is in domain IV near the binding site for protein L2 (Egebjerg *et al.*, 1991), one of the few ribosomal proteins that has been shown to be important for *in vitro* reconstitution of peptidyl transferase activity from purified components (Schulze and Nierhaus, 1982).

Our findings demonstrate directly proximity of the acceptor end of A, P and E site tRNA to specific conserved features of 23S rRNA, identifying regions of potential functional importance for the peptidyl transferase reaction and other ribosomal interactions involving the acceptor end of tRNA. These data help to distinguish direct from indirect protections of 23S rRNA by tRNA; sites that are protected by tRNA and cleaved by 5′-Fe-BABE-tRNA are strong candidates for tRNA–rRNA contacts. One such site is the 2250 loop, which is cleaved by P site-bound 5′-Fe-BABE-tRNA, and protected by P site tRNA (Moazed and Noller, 1989). Since C74 of tRNA interacts with G2252 of 23S rRNA in a Watson–Crick fashion (Samaha *et al.*, 1995), we can infer that our 5′-tethered Fe(II) probe is in close proximity to C74 of tRNA when it is bound to the 50S P site. Unexpectedly, we do not detect any cleavage in the central loop of domain V, the 'peptidyl transferase loop' (the multibranch loop in the lower half of domain V), suggesting either that the loop itself is not in close proximity to the 5′ end of tRNA, or that the loop is somehow shielded from attack by hydroxyl radicals.

Additionally, our data demonstrate proximity between certain features of 23S rRNA, providing constraints for its three-dimensional folding. Given that the probe length itself is ~12 Å (Rana and Meares, 1991), and that hydroxyl radicals have a path of ~10 Å (Dreyer and Dervan, 1985; Moser and Dervan, 1987), cleavages that are generated from a uniquely positioned probe are expected to lie within a 40–50 Å sphere. Thus, the 2550 loop of domain V must be within 40–50 Å of the α-sarcin loop, in domain VI. The 1940 and 1960 regions of domain IV must be similarly proximal to the 2250 and 2600 regions of domain V, and the 1860/1880 stem of domain IV must be near the 2400 region of domain V. Because of the proximity of domain IV to the 30S subunit (Mitchell *et al.*, 1992; C.Merryman, D.Moazed, J.McWhirter and H.F.Noller, unpublished data), it can be concluded that the peptidyl transferase center is located at the 30S–50S subunit interface.

Tethered hydroxyl radical probing, like cross-linking, unambiguously identifies regions of rRNA that are in proximity to the Fe(II) probe. However, the tethered approach has two important advantages. First, the sites of cleavage are identified readily by primer extension, and second, the relative indifference of hydroxyl radicals to the structure of their RNA targets provides a more comprehensive survey of the RNA neighborhood of the probe. Clearly, this approach can be extended by tethering Fe(II) to other positions of tRNA (Han and Dervan, 1994) to localize its rRNA environment within the ribosome in even greater detail.

Materials and methods

In vitro transcription and 5′-Fe-BABE modification of E.coli tRNA^{Phe}

Escherichia coli tRNA^{Phe} was prepared by *in vitro* transcription of linearized p67CF10 with T7 RNA polymerase (Sampson *et al.*, 1989). For 5′-derivatization with BABE, a 5′-thiophosphate was introduced at the 5′ terminus of tRNA by transcription in the presence of a 5-fold molar excess of 5′-guanosine-α-phosphorothioate (GMPS; or GMP, for control tRNA) (5 mM final concentration) over each NTP (1 mM final concentration) (Sampson and Uhlenbeck, 1988; Burgin and Pace, 1990). tRNAs were isolated on 10% denaturing polyacrylamide gels, extracted twice with phenol and twice with chloroform, ethanol precipitated and dissolved in water. The level of incorporation of GMPS (or GMP) at the 5′ end of the tRNA transcripts was determined by transcription in the presence of [α-^{32}P]CTP. These tRNA transcripts were then digested with ribonuclease T2 and analyzed for the presence of 5′-thio-pG[^{32}P]p (or pG[^{32}P]p), by TLC and by paper ionophoresis (Sampson and Uhlenbeck, 1988; Burgin and Pace, 1990). The analysis showed that at least 80% of the transcripts initiated with GMPS (or GMP). For Fe-BABE modification of 5′-GMPS-tRNA, BABE was first loaded with Fe(II) as described (Heilek *et al.*, 1995), followed by addition of 400 pmol of 5′-GMPS-tRNA^{Phe} and 1 µl of 400 mM potassium phosphate buffer pH 8.5 (final reaction volume 10 µl) and incubation for 60 min at 37°C. The reaction was stopped by extracting twice with phenol to remove excess unreacted Fe-BABE and ethanol precipitated to recover 5′-Fe-BABE-tRNA^{Phe}. 5′-GMPS-tRNA^{Phe} reacts essentially to completion with Fe-BABE, as shown by TLC and paper ionophoresis as described above.

Autocleavage of 5′-Fe-BABE-modified E.coli tRNA^{Phe}

5′-Fe-BABE-modified, 3′-[α-^{32}P]pCp end-labeled tRNAs were incubated in 80 mM K cacodylate pH 7.2, 20 mM Mg acetate, 150 mM ammonium chloride at 37°C for 20 min, followed by addition of ascorbate (5 mM final concentration) and hydrogen peroxide (0.05% final concentration) and incubation for an additional 10 min at room temperature. As a control, 5′-GMP tRNA was treated with Fe-BABE and subjected to identical treatment. Reactions were stopped by addition of an equal volume of gel-loading buffer (95% formamide, 20 mM

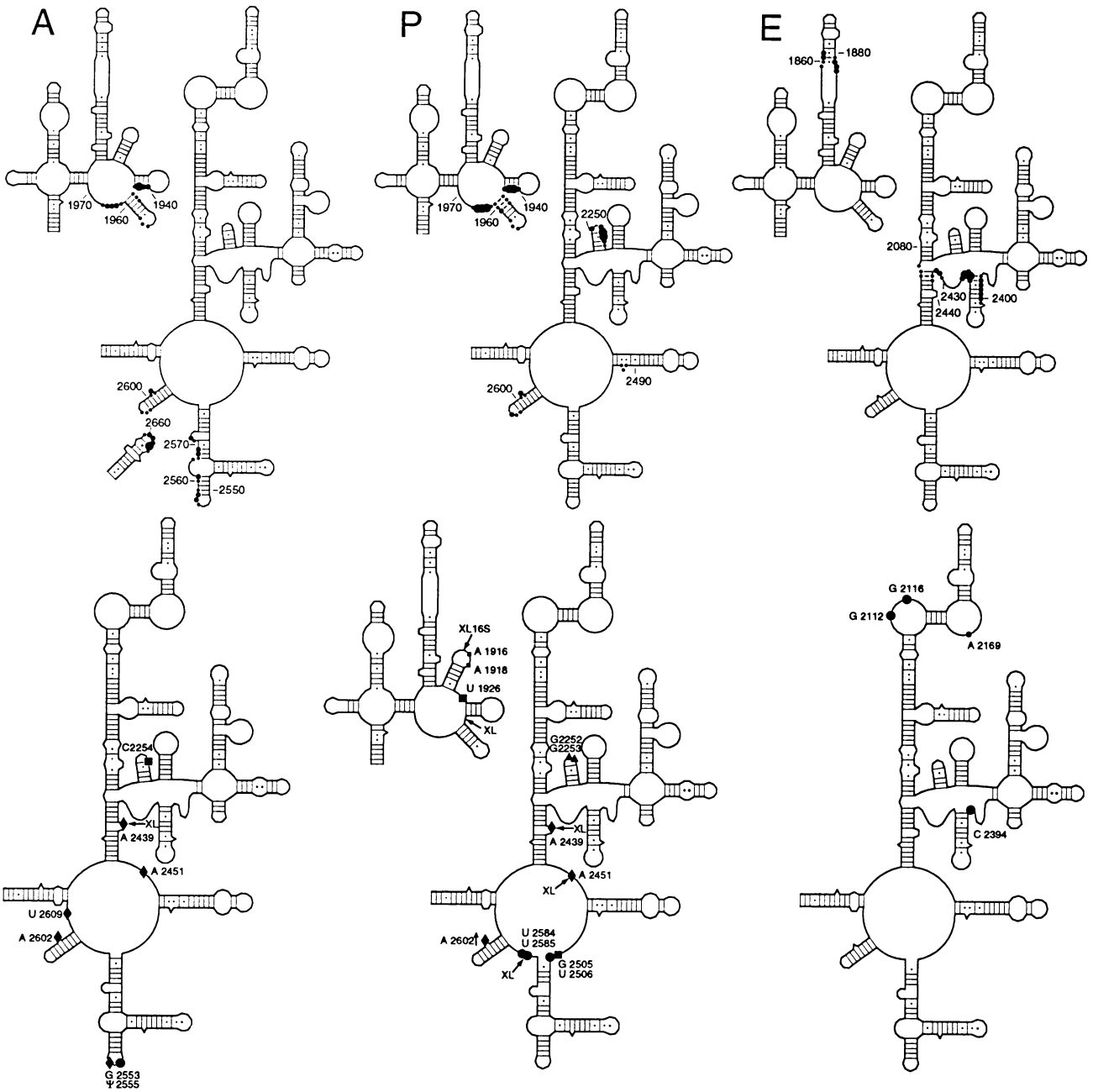

Fig. 5. Summary of 23S rRNA strand scission by hydroxyl radicals originating from 5′-Fe-BABE-modified $tRNA^{Phe}$ bound to *E.coli* 70S ribosomes (upper panel). Assignment to the 50S subunit A, P and E sites is described in the text. Large circles indicate strong cleavages and smaller circles indicate weaker cleavages. Previously identified tRNA chemical protection (Moazed and Noller, 1989), tRNA cross-linking results (Barta *et al.*, 1984; Steiner *et al.*, 1988; Wower *et al.*, 1989; Podkowinski and Gornicki, 1991; Mitchell *et al.*, 1993) and the 16S rRNA cross-linking site (Mitchell *et al.*, 1992) are presented for comparison (lower panel). Symbols in the lower panel indicate dependence of protections on the presence of the 2′, 3′-linked acyl moiety (diamonds) and the 3′-terminal A (circles) or CA (triangles) of tRNA. Squares represent protections that are unaffected by 3′-terminal deletions. Reactivity of A2602 is enhanced by binding of *N*-acetyl-Phe-$tRNA^{Phe}$ to the P site. Small symbols indicate weak effects. XL indicate sites of tRNA–23S rRNA cross-links and XL16S indicates the site of the 16S–23S rRNA cross-link.

EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue; all final concentration) and quick-freezing in a dry-ice–ethanol bath. Reaction products were analyzed on a 25% denaturing gel, and quantified using a Molecular Dynamics PhosphorImager.

Binding of tRNA to 70S ribosomes

Binding of unmodified or 5′-Fe-BABE-modified deacylated tRNAs to ribosomes was carried out essentially as described (Moazed and Noller, 1989). Briefly, 10–50 pmol (0.2–1 μM) of tRNA were incubated with 10 pmol of ribosomes in the presence or absence of 5 μg of poly(U) in 50 μl of 80 mM potassium cacodylate (pH 7.2), 20–25 mM Mg acetate, 150 mM ammonium chloride for 30 min at 37°C and for 20 min on ice. Ribosomes were incubated with or without poly(U) for 10 min at 37°C before addition of tRNA. Hydroxyl radical strand scission was initiated by addition of ascorbate (5 mM final concentration) and hydrogen peroxide (0.05% final concentration) followed by incubation for 10 min at room temperature. Reactions were stopped by addition of 300 μl of cold ethanol and 3 M Na acetate (0.3 M final concentration) and quick-freezing in a dry-ice–ethanol bath. Base-specific chemical modification of tRNA–ribosome complexes were carried out in parallel as previously described (Moazed and Noller, 1989). Extraction of rRNAs from all samples, primer extension and gel electrophoresis were carried out as described previously (Stern *et al.*, 1988). For competition experiments, ribosome binding of 5′-Fe-BABE-modified tRNAs and strand scission

were performed exactly as described above (0.2 μM 70S ribosomes + 1.0 μM of 5′-GMP or 5′-Fe-BABE-GMPS-tRNA^{Phe}), either in the absence or presence of 5-fold or 10-fold excess unmodified 5′-GMP-tRNA. All of the observed Fe-BABE-dependent strand scissions were reproduced qualitatively in two to six experiments using three different ribosome preparations.

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