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# Positions in the 30S ribosomal subunit proximal to the 790 loop as determined by phenanthroline cleavage

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**ABSTRACT**

**Positioning rRNA within the ribosome remains a challenging problem. Such positioning is critical to understanding ribosome function, as various rRNA regions interact to form suitable binding sites for ligands, such as tRNA and mRNA. We have used phenanthroline, a chemical nuclease, as a proximity probe, to help elucidate the regions of rRNA that are near neighbors of the stem-loop structure centering at nt 790 in the 16S rRNA of the *Escherichia coli* 30S ribosomal subunit. Using phenanthroline covalently attached to a DNA oligomer complementary to nt 787–795, we found that nt 582–584, 693–694, 787–790, and 795–797 were cleaved robustly and must lie within about 15 Å of the tethered site at the 5′ end of the DNA oligomer, which is adjacent to nt 795 of 16S rRNA.**

**Keywords: 582; 693; 790; cleavage; phenanthroline; ribosomes; structure**

## INTRODUCTION

Unraveling the tertiary structure of rRNA within the ribosome remains a perplexing problem. Recent efforts have utilized a number of techniques to ascertain accessible regions of rRNA (Noller & Herr, 1974; Stern et al., 1989; Teare & Wollenzien, 1989; Hill et al., 1990a; Noller et al., 1990), relative positions of specific regions of rRNA (Stiege et al., 1982, 1986; Brimacombe et al., 1988), and detailed interactions between regions of rRNA and various ligands, such as mRNA (Lim et al., 1992; Bogdanov et al., 1993; Bhangu et al., 1994; Huttenhofer & Noller, 1994; Bucklin et al., 1997; Sergiev et al., 1997), tRNA (Moazed & Noller, 1989, 1990; Odom et al., 1990; Dontsova et al., 1992; Bullard et al., 1995, 1998), nascent protein and the ribosome (Picking et al., 1991; Hendrick et al., 1993; Stade et al., 1994, 1995; Choi & Brimacombe, 1998; Choi et al., 1998), and antibiotics (Moazed & Noller, 1987; De Stasio et al., 1989). Even though much information has been accumulated, details of the way in which the rRNA is folded within the ribosomal subunits remains obscure. Using a chemical nuclease, iodoacetamido-1,10-phenanthroline (IoP; Fig. 1), covalently attached to a short DNA oligomer complementary to the loop around nt 790, (787–795), we have harnessed the power of a chemical cleavage to help elucidate regions of rRNA proximal to a target site to a resolution of about 15 Å.

It would be ideal to attach IoP directly to a modified nucleotide within the rRNA itself, and, following cleavage, identify sites proximal to the modified nucleotide. However, such site-specific modification of rRNA is not presently feasible. In lieu of this, delivery of the IoP to a defined site within the ribosome can be accomplished by hybridizing a short, complementary DNA oligomer to a specific region within the rRNA. Upon cleavage, other rRNA sites proximal to the bound phenanthroline can be identified. There is ample evidence that IoP, covalently linked through a single 4-thiouracil residue to tRNA or mRNA in the presence of Cu(II) and a thiol reducing agent, creates a unique cleavage pattern in rRNA (Bullard et al., 1995, 1998; Bucklin et al., 1997). Based on these results, covalent attachment of 5-iodoacetamido-1,10-phenanthroline (IoP) to the 5′ end of a thiophosphate oligodeoxynucleotide (François et al., 1989) should efficiently deliver a nucleolytic reagent to those regions within the ribosome close to position 790 and produce cleavage patterns to map those interactions. A similar approach has been used by Chen & Sigman (1988), wherein they used a DNA oligomer to deliver orthophenanthroline to a specific site on a sequence of *lac* RNA.

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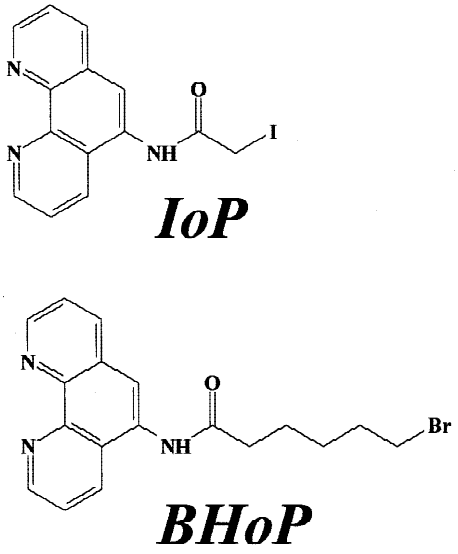


**FIGURE 1.** Structures of phenanthroline derivatives used to cleave ribosomal RNA.

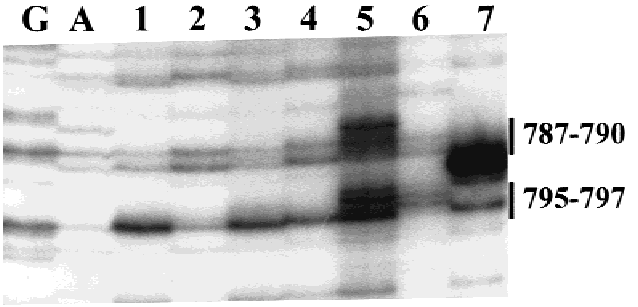


**FIGURE 2.** Cleavage of 30S ribosomal subunits with 790-IoP. Lanes G,A: dideoxy sequencing lanes; lane 1: free IoP; lane 2: 16S rRNA; lane 3: free IoP + 790 probe control; lane 4: 790 probe control; lane 5: cleavage with IoP covalently attached to 790 probe; lane 6: IoP covalently attached to mismatch probe; lane 7: RNase H cleavage. All cleavages were for 1 h.

Under the experimental conditions used in the present study, phenanthroline-Cu(II) does not produce a diffusible hydroxyl radical. Elegant mechanistic studies have shown that the hydroxy radical produced under these mild conditions remains coordinated to the phenanthroline-copper complex (Johnson & Nazhat, 1987). This critical difference in mechanism allows us to confidently report distance proximities to the 5′ end of the probe with much greater certainty than possible if the hydroxyl radical were to diffuse. Therefore we can report that the distance between the 5′ end of the 787–795 DNA oligomer to the cleaved nucleotides found at positions 582–584, 693–694, 787–790, and 795–797 in 16S rRNA must be approximately 15 Å or less in 30S ribosomal subunits that have not been reconstituted.

## RESULTS

The DNA oligomers for this study were prepared and modified at the 5′ end as outlined in Materials and Methods. To prevent reverse transcriptase (RT) from initiating transcription on the DNA probe in the 790 region, all DNA probes were synthesized with a 3′ deoxyribose. The DNA oligomers containing the 5′-thiophosphate group were reacted with IoP or 5-bromohexnanoyl-1,10-phenanthroline (BHoP; Fig. 1). A mock IoP-probe control was created by reacting IoP with an unmodified 5′-phosphate probe to determine if the observed cleavages could be attributed to modifications to the probe other than the intended reaction. We observed no cleavages using this mock probe (data not shown).

To establish the specificity of probe binding, the ribosome-DNA conjugates were incubated in the presence of RNaseH. Figure 2, lane 7, shows cleavage of nt 791–794, indicating the probe was bound in the desired position. No additional RNaseH cleavages were observed on sequencing the entire 16S rRNA (data not shown). The use of free IoP (Fig. 2, lane 2), free IoP + probe (Fig. 2, lane 3), and probe alone (Fig. 2, lane 4) provided controls for nonspecific phenanthroline cleavage and possible artifacts created in primer extension analysis. No cleavages from these controls were seen in the 790 region. All cleavages attributed to IoP-DNA were attenuated completely in competition reactions where an excess of 5′-phosphate probe was annealed simultaneously with IoP-DNA probe (data not shown). The 790 probe with IoP covalently attached to the 5′ end (790-IoP) produced dramatic cleavages at nt 787–790 and 795–797 (Fig. 2, lane 5), further corroborating that the modified probe was bound at the desired position within the 30S ribosomal subunit. A probe with a sequence not complementary to any sequence of nucleotides in the 16S rRNA, modified to carry phenanthroline at the 5′ end (mismatch-IoP), did not show cleavage in the 790 region (Fig. 2, lane 6).

Upon sequencing the entire 16S rRNA, additional cleavages attributed to 790-IoP were observed at nt 582–584 (Fig. 3, lane 3), and 693–694 (Fig. 4, lane 3). The cleavages at 582–584 and 693–694 were obtained with a 5-min cleavage reaction, using a tenfold molar excess of probe. Strikingly, the cleavages in the 790 loop itself were very faint with a 5-min cleavage reaction, although very evident after 1 h, also at a 10:1 probe/ribosome ratio. All cleavages emanating from the 790 loop were independent of buffer conditions, provided the minimal requirements were present to maintain ribosomal integrity.

Control reactions with free IoP at a tenfold molar excess to the ribosome showed only background cleavage of the 582–584 region even with reactions up to 15 h. Free IoP did cleave the 693–694 region, but only under extreme conditions of 10:1 probe/ribosome ratio, for 3 h with 4 mM ascorbic acid as the reductant. The

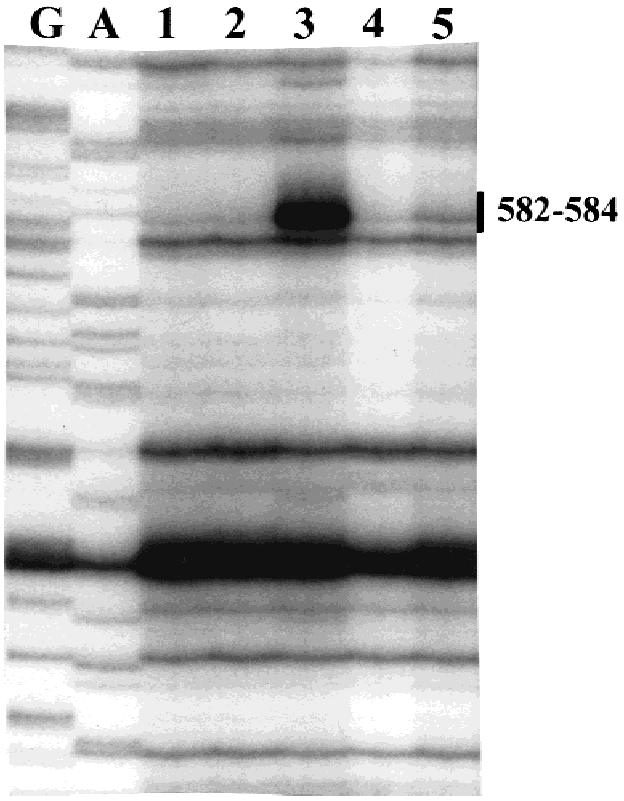


**FIGURE 3.** Cleavage of 30S ribosomal subunits with DNA-IoP probes. Lanes G,A: dideoxy sequencing lanes; lane 1: 16S rRNA; lane 2: 530-IoP probe cleavage; lane 3: 790-IoP probe cleavage; lane 4: 1400-IoP cleavage; lane 5: mismatch-IoP probe cleavage. Cleavages were for 5 min.

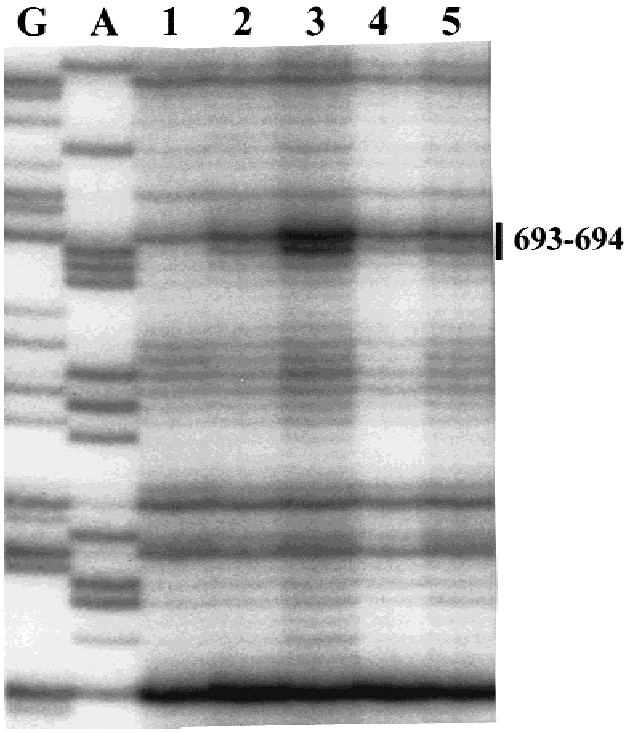


**FIGURE 4.** Cleavage of 30S ribosomal subunits with DNA-IoP probes. Lanes G,A: dideoxy sequencing lanes; lane 1: 16S rRNA; lane 2: 530-IoP probe cleavage; lane 3: 790-IoP probe cleavage; lane 4: 1400-IoP cleavage; lane 5: mismatch-IoP probe cleavage. Cleavages were for 5 min.

mild conditions used to obtain the probe-directed cleavage of 693–694 did not show cleavage of the 693 region with free IoP. Additionally, nt 845, which is ordinarily cleaved robustly by free IoP, was not cleaved at all in reactions containing IoP covalently attached to the 790 probe or mismatch probe (G.W. Muth & W.E. Hill, unpubl. observations). This negative result indicated a minimal amount of free IoP present in the probe preparation.

To confirm the specificity of the cleavage events, additional IoP-DNA oligomers, complementary to other regions of the 30S ribosomal subunit, were prepared. DNA oligomers complementary to nt 1396–1403 (1400-IoP) and nt 518–526 (530-IoP) did not cleave the 790 loop, nor the 582–584 (Fig. 3) or 693–694 (Fig. 4) regions. The mismatch-IoP probe also did not cleave either of these regions. The cleavage events of 16S rRNA from phenanthroline anchored at nt 795 are summarized in Figure 5.

To determine some limits of the effective cleavage distance from the 790 loop, we compared the cleavages in the 582–584 region between 790-IoP probe and a 790 probe covalently modified with BHoP, which has a six-carbon tether. The 582–584 cleavages were not only specific to phenanthroline anchored at the 790 loop, but also specific to IoP. When cleavage was repeated with a 790 probe modified with BHoP (Fig. 1), no cleavage above background was noted in either the 582 region (Fig. 6) or the 693 region (data not shown). These data indicate that the cleavages induced by the 790-IoP probe in the 582 and 693 regions were length dependent.

## DISCUSSION

The results reported in this study provide definitive evidence that nt 582–584 and 693–694 are within 15 Å of the tethered site of phenanthroline at the 5′ end of the DNA oligomer complementary to nt 787–795 of 16S rRNA in 30S ribosomal subunits that have not been reconstituted. The cleavages in the 693 region do corroborate evidence from other studies. Brimacombe's group (Atmadja et al., 1986) found crosslinking between fragments 693–696 and 794 (or 799) using a nitrogen mustard. This crosslink was placed into the Harvey model using a distance of 11 $\pm$ 4 Å (Malhotra & Harvey, 1994). More recently Mundus & Wollenzien (1998) have reported a distance of $<$25 Å between nt 788–789 and 693 using site-specific photocrosslinking. Our results confirm the results reported by Brimacombe and refine the findings of Mundus and Wollenzien, limiting the 693 region to be within

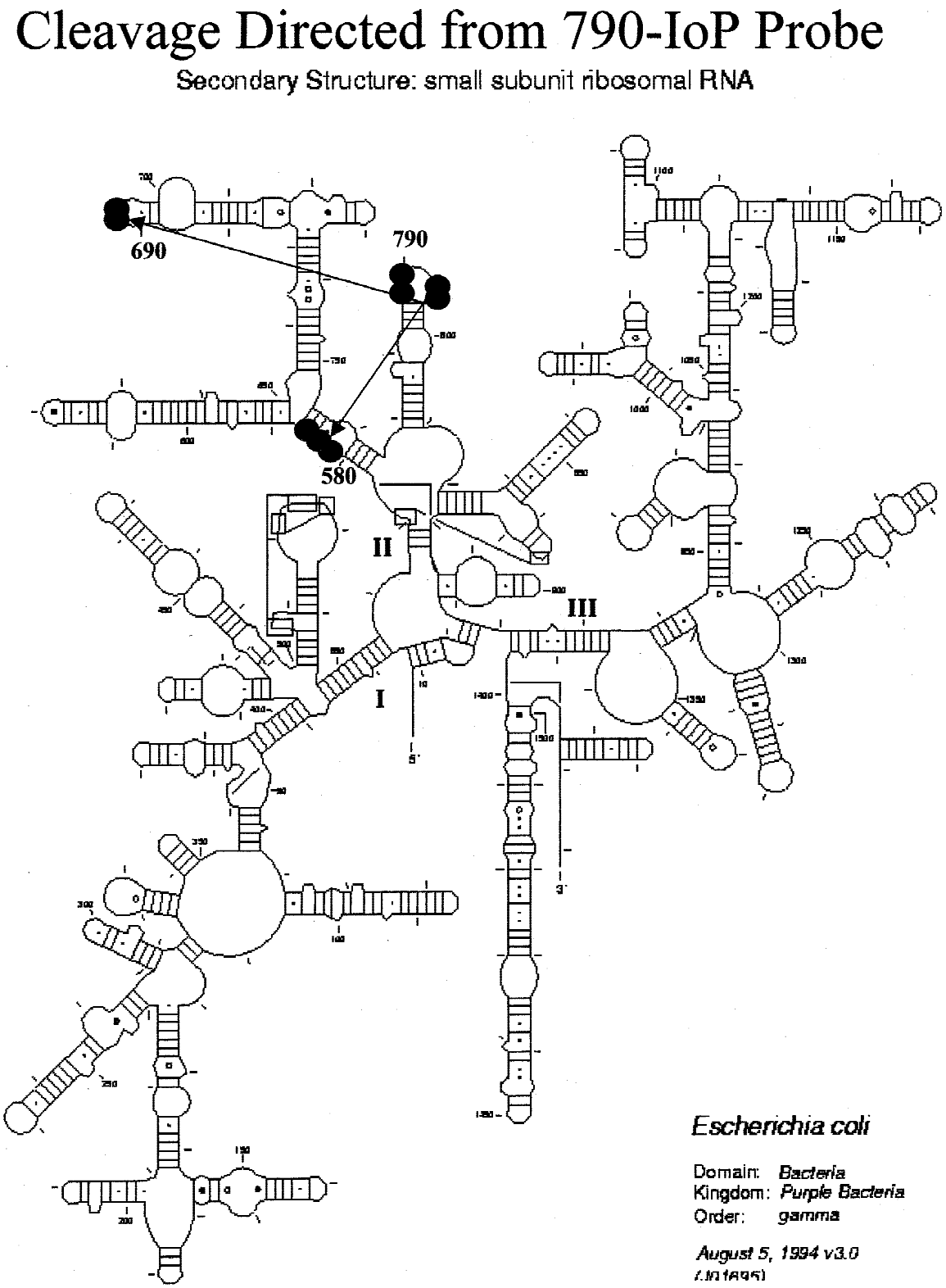


**FIGURE 5.** Secondary structure map of 16S ribosomal RNA from *E. coli*. Arrows indicate potential proximity of 690 region and 580 region from the 790 region as elucidated by phenanthroline cleavage.

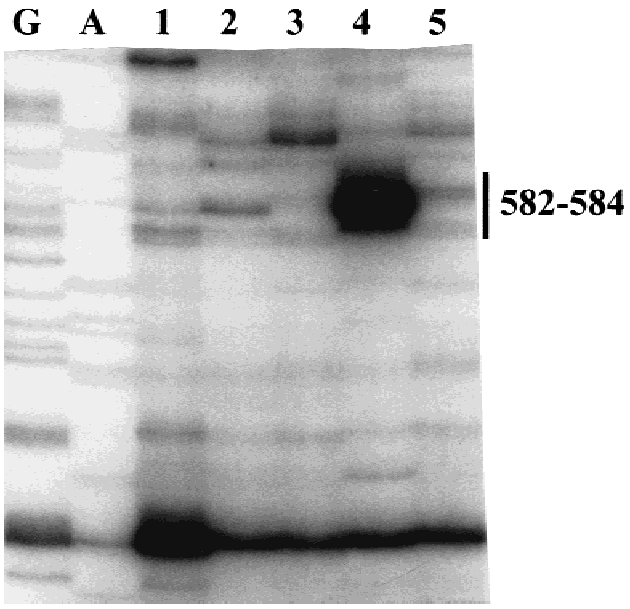


**FIGURE 6.** Cleavage of 30S ribosomal subunits with DNA-IoP probes and DNA-BHoP probes. Lanes G,A: dideoxy sequencing lanes; lane 1: 16S rRNA; lane 2: free IoP cleavage; lane 3: free BHoP cleavage; lane 4: 790-IoP probe cleavage; lane 5: 790-BHoP cleavage. Cleavages were for 5 min.

15 Å of the tethered site at nt 795. Placing nt 693 within 15 Å of nt 795 also fits the reconstruction reported by Mueller & Brimacombe (1997), wherein they showed helixes 24.3 (790 region) and 23.2 (690 region) to be both proximal and accessible to subunit association.

The proximity of the 582–584 region to the 790 region has not been previously reported. Although the central domain of 16S rRNA has been well localized in the model published by Malhotra & Harvey (1994), parts of helix 20 (nt 581–587) have a reported standard deviation of 15 Å due to the lack of structural data available. The results presented here will allow the placement of helix 20 with greater certainty than previously reported.

Two conditions are needed for effective directed phenanthroline cleavage of ribosomal subunits. The first condition involves using a suitable oligomeric probe to direct phenanthroline to regions within the ribosome. DNA oligonucleotides have been extensively used to probe the availability of certain sites within the ribosome (Tapprich & Hill, 1986; Hill et al., 1988, 1990b; Weller & Hill, 1991). Probes complementary to the 790 region, specifically complementary to nt 787–795, previously showed binding at 38% efficiency when used at a 10:1 ratio of probe to 30S ribosomal subunits (Tapprich & Hill, 1986). A short DNA oligomer complementary to this region, complexed with two phenanthroline reagents IoP and BHoP (ee Fig. 1), was used in this study.

The second condition involves need for partial intercalation or "bookmarking" of phenanthroline itself between stacked or bulged single-stranded bases, as described by Hermann & Heumann (1995). In this study, they showed that phenanthroline (oP)-copper complexes exhibit remarkable specificity for certain regions of tRNA, most easily explained by a model in which the phenanthroline was partially intercalated between adjacent bases. Only in those regions of RNA where such partial intercalation can take place will cleavage occur. The chemical mechanism by which the cleavage of the phosphodiester bond by phenanthroline occurs does not involve base recognition, nor is it base specific. Rather, phenanthroline shows a dramatic preference for single-stranded regions of RNA, especially those that are in a strained conformation (Murakawa et al., 1989; Mazumder et al., 1992; Hermann & Heumann, 1995; Muth & Hill, 1999).

The proposed mechanism of cleavage is initiated via a proton abstraction from the $C_1'$ carbon of the ribose moiety of the targeted nucleotide (Meijler et al., 1997; Chen & Greenberg, 1998). The phenanthroline cleavage reaction proceeds without the generation of a diffusible hydroxyl radical species, which is quite different from the reaction that occurs with Fe(II)-EDTA cleavage reactions (Sigman et al., 1996). With Fe (II)-EDTA cleavage, a diffusible hydroxyl radical is generated, and cleavage occurs in the region of diffusion. Han & Dervan (1994) have defined this region of cleavage in the case of Fe(II)-EDTA cleavage of tRNA when the EDTA was tethered to position 47 of the tRNA. In the case of rRNA, it has been estimated that weak cleavage using Fe(II)-EDTA may occur as far as 44 Å from the tethering position (Joseph et al., 1997).

We would emphasize that although our cleavage patterns often consist of 2–6 bands, these are most likely due to multiple bookmarking sites of the phenanthroline between adjacent bases, and are not the result of cleavage by a diffusible radical.

The cleavage of nt 582–584 and 693–694 appeared after only a 5-min reaction, whereas the cleavages in the 790 loop itself were nearly absent at 5 min, yet were present after a 1-h incubation. These observations suggest that the 582 and 693 sites are proximal and very susceptible to the tethered phenanthroline. The more robust cleavage at position 582 compared to 693 does not necessarily imply a closer proximity; rather it may suggest a site with a more open structure in which the phenanthroline can more readily insert itself or perhaps the relative orientation of the phenanthroline to each site. We can only speculate as to why the regions adjacent to the probe in the 790 loop cleave less rapidly. Perhaps the 790 loop itself is distorted by the bound probe, thus hindering the tethered IoP from efficiently bookmarking near the site of probe binding. Or perhaps the orientation of the phenanthroline relative to the available sites precluded immediate dock-

ing. Interestingly, the RNA near both the 5′ and 3′ end of the probe are cleaved in a similar pattern, a result also observed with the 1400 probe (data not shown). The molecular model (Fig. 7B) shows the 3′ and 5′ end of the RNA hybridized to the DNA to be within the range of the tethered IoP.

The distance of the cleavage sites from the 5′ end of the complementary DNA oligomer can be estimated from molecular modeling of the copper-phenanthroline complex as it is attached to the thiophosphate group on the 5′ end of the DNA oligomer. Molecular modeling using Tripos Sybyl software of the oP–DNA conjugate shows a distance of 6.4–8.2 Å from the 5′ phosphate to the phenanthroline nitrogens based on several energy minimizations and conformations (Fig. 7). The crystal structure of a $(oP)_2Cu(II)$ complex shows the distance between the phenanthroline nitrogens and the copper molecule to be 2 Å (Sigman et al., 1993). The atomic diameter of copper has been calculated to be slightly less than 2 Å. The estimated distance from copper to the labile $C_1'$ ribose hydrogen is ~2 Å. Thus, the distance from the 5′-thiophosphate of the DNA oligomer to the regions being cleaved would maximally be about 12–14 Å, with shorter distances being possible due to rotation about the methylene carbon of the acylamide tether.

Additional molecular modeling of BHoP showed a dramatic flexibility in the carbon tether. Measurement of the distance between the distal methylene carbon (site of thiophosphate attachment) and the phenanthroline nitrogen in several energy minimized structures was as short as 3 Å. This flexibility may account for the lack of cleavage in the 582 and 693 regions with BHoP.

We must also take into account the actual position of the 5′-thiophosphate group relative to the rRNA to which the probe is bound. If we look at the 5′ end of the probe–rRNA complex, we can see that the thiophosphate group (and the attached phenanthroline) may take one of eleven possible positions “around the clock” (see Fig. 7A). It cannot be known, a priori, in which position the thiophosphate group actually resides. We do know that all of the cleavages have emanated from the thiophosphate positioned at only one of the eleven positions.

It has been suggested that hybridizing a complementary DNA oligomer to the ribosomal RNA may create a significant structural deformation in the RNA. This is difficult to test. Although previous work in this lab has shown that poly(U)-directed poly-phe synthesis in the presence of the 790-probe was maintained at 84% of control (Tapprich & Hill, 1986), there is no evidence that the 790 probe remains annealed to the target. This would be unlikely, since the 790 loop has been shown be involved in subunit association (Herr et al., 1979; Moazed & Noller, 1986; Tapprich & Hill, 1986; Merryman et al., 1999). Successful tRNA binding experiments in the presence and absence of probe would prove little, because the protected nucleotides in the 790 loop have been reported to be nonessential for effective tRNA binding (von Ahsen & Noller, 1995). The presence of the probe did not alter poly U directed $tRNA^{Phe}$ binding nor was the probe dissociated from

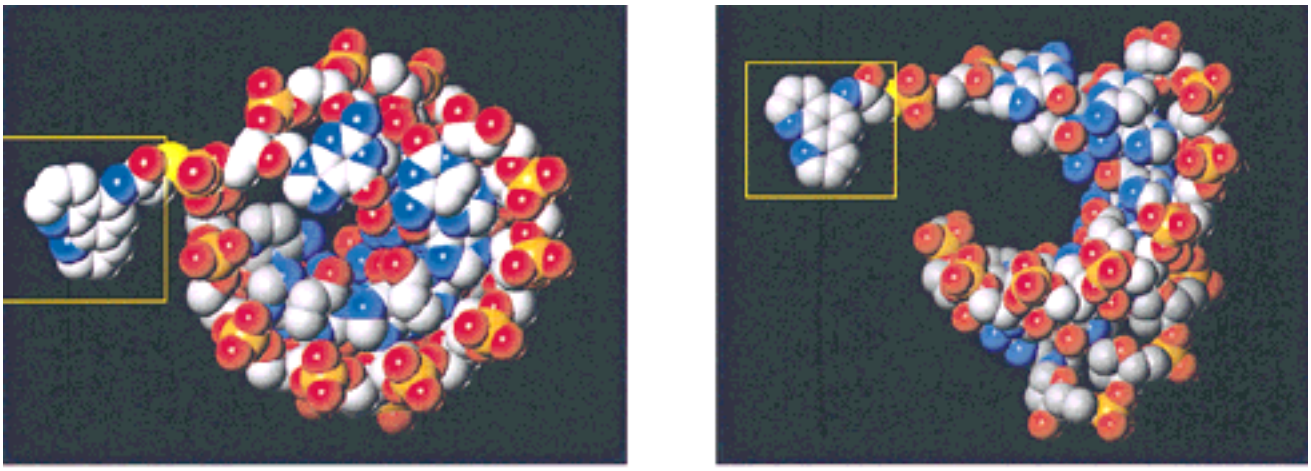


**FIGURE 7.** Molecular model of the possible orientations of the tethered phenanthroline about the 5′ end of the A-form helix composed of the DNA–rRNA hybrid. The phosphate atoms are depicted as orange spheres with attached red oxygen atoms. **A**: 20-Å diameter helix formed by binding the oP–DNA to the rRNA as viewed down the 5′ end of the helical axis. In addition to the phenanthroline (boxed in yellow in both panels) being in one of eleven possible orientations about the helical axis, the phenanthroline can rotate about the methylene carbon to produce a multitude of orientations capable of sweeping out to adjacent structures to produce unique cleavage events. **B**: Helical view with the RNA phosphate backbone in the foreground illustrating the proximity of the tethered phenanthroline to both the 5′ and 3′ ends of the RNA.

the 30S subunit upon $tRNAP^{he}$ binding as determined by a dual-label filter binding assay (data not shown).

Conducting free phenanthroline cleavage in the presence and absence of probe will provide some additional information on possible structural deformation due to the probe. If significant structural deformation were to occur in the presence of the probe, additional phenanthroline cleavage sites should appear, if the deformation is accompanied by the formation of strained regions of RNA. Phenanthroline, which shows a preference for cleaving strained regions of RNA (Muth & Hill, 1999), should cleave the strained RNA in control reactions containing free phenanthroline and the complementary probe. No such probe induced free phenanthroline cleavages were observed when compared to free phenanthroline cleavages (data not shown) throughout the entire 16S rRNA. A final estimation concerning the minimal structural distortion caused by the probe lies in the 693 cleavage itself. An accepted distance between nt 794 and 693 has been reported to be 11 ± 4 Å (Malhotra & Harvey, 1994). Our equivalent distance suggests that if a structural change is occurring because of the probe, it is not affecting this distance.

The 790 loop has been implicated as being on the surface of the 30S subunit and involved in subunit association (Tapprich & Hill, 1986; Tapprich et al., 1989; Santer et al., 1990). In addition, the 790 loop, although protected by P-site-bound tRNA (Moazed & Noller, 1990), along with 693, was found to be nonessential for tRNA binding (von Ahsen & Noller, 1995). These chemical protection studies also implied that A532 and G693, as well as numerous nucleotides in the decoding region, were protected by P-site-bound tRNA. In addition, IF3 both footprints and crosslinks to the 790 loop (Ehresmann et al., 1986; Muralikrishna & Wickstrom, 1989), and a mutation G791C inhibits IF3 binding (Tapprich et al., 1989). These studies suggest the functional importance of the 790 region.

Numerous results have placed the 530 loop proximal to the 790 region (Dontsova et al., 1992; Rinke-Appel et al., 1993; Alexander et al., 1994; Bucklin et al., 1997; Bullard et al., 1998). The position of the 530 loop has been controversial, as immunoelectron microscopy places the 530 loop on the opposite side of the 30S subunit from the decoding site (Trempe et al., 1982; Scheinman et al., 1992; Malhotra & Harvey, 1994; Mueller & Brimacombe, 1997). We looked extensively for directed phenanthroline cleavages in this region; but none were found. It is possible that the phenanthroline is near the 530 loop, but because of the short tether to the phenanthroline or its orientation, it cannot bookmark the loop, or perhaps the oP is on the distal side of the A-form RNA–DNA probe helix relative to the 530 loop (see Fig. 7). Additional experiments that vary the length of the DNA oligomer, and thereby change the clockwise position around the 790 loop, may provide additional insight into the exact positions of the 790 and the 530 loops with respect to each other in the 30S ribosomal subunit. The sensitivity and specificity provided by directed copper-phenanthroline cleavages should allow these and other delicate interactions within the dynamic 30S ribosomal subunits to be monitored.

## MATERIALS AND METHODS

### Ribosome preparation

Ribosomes were harvested from early log phase *Escherichia coli* strain MRE600 using the protocol outlined by Tam et al. (1981) and Lodmell et al. (1993).

### Synthesis of modified phenanthrolines

5-iodoacetamido-1,10-phenanthroline was obtained from Molecular Probes (Eugene, Oregon) and used without further purification. 5-(ω-bromohexanoamido)-1,10-phenanthroline was prepared according to Thuong & Chassignol (1987) with minor modifications. The product was confirmed by $^1$H NMR (data not shown).

### 5′-thiophosphate-DNA

The complementary DNA probes were prepared in the Murdock Molecular Biology Facility, The University of Montana, using automated phosphoramidite chemistry. The solid support column 3′-dG-CPG (Glen Research, Sterling, Virginia) yielded 2′-OH and 3′-deoxy DNA oligomers. After the addition of the final nucleotide phosphoramidite in the sequence, an additional round of synthesis was conducted using a chemical phosphorylizing reagent (Glen Research) followed by oxidation using a sulfurizing reagent (Glen Research) yielding a 5′-$PO_3S$ group. The sequences of each DNA oligomer were as follows: 530 (GGCTGCTGG), 790 (TATCTAAT), 1400 (GGCGGTGT) and mismatch (GAGAGAGAT), each having a 5′-$PO_3S$ and a 3′ deoxy group to prevent primer extension with reverse transcriptase.

### 5-Iodoacetamido-1,10-phenanthroline-5′-thiophosphate-DNA (IoP-DNA)

A solution of 5-iodoacetamido-1,10-phenanthroline (Molecular Probes) (200 nmol) in dimethylsulfoxide (DMSO) was added to a 1:1 DMSO/water solution containing 20 nmol 5′-thiophosphate-DNA, 0.1 mg $NaBH_4$ and 1% $NaHCO_3$. The reaction was complete after 3 h at room temperature. The solvent was removed *in vacuo* and the residue dissolved in 50 μL $H_2O$. The modified DNA was purified by chromatography (3 × 50 μL $H_2O$ washes, Bio-Spin 6 Chromatography Column (Bio-Rad Laboratories, Hercules, California), 1,100 × *g*, 5 min) yielding 40–57%. Gel electrophoresis (12% acrylamide, 0.1 mg [(*N*-acryloylamino)phenyl]mercuric chloride 10 mA, 30 min) showed a single product and the absence of starting material. The identical procedure was followed to modify the DNA with BHoP.

### Cleavage of ribosomal subunits with oP-DNA

Ribosomal subunits were activated by heating at 37 °C for 20 min in 10 mM Tris-Cl, pH 7.4, 150 mM KCl, and 15 mM $MgCl_2$ (Zamir et al., 1971). Purified ribosomal subunits (25 pmol) were added to a solution of copper sulfate (500 pmol) and IoP-DNA (250 pmol) buffered in 40 mM Tris-Cl, pH 7.4, 100 mM KCl, and 15 mM $MgCl_2$). Incubation up to 3 h at 0 °C insured annealing of the DNA to the complementary region of the rRNA. Addition of 3-mercaptopropionic acid (1 mM) initiated cleavage (0 °C). Cleavage reactions were conduced at 0 °C unless noted. The reaction was quenched by the addition of 100 $\mu$L precipitation buffer (70% ethanol, 8.4 mM NaOAc, pH 5, and 0.8 mM EDTA). Ribosomal proteins were removed by phenol extraction and the purified RNA sequenced by primer extension (Moazed et al., 1986).

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