Characterization of Iron(II)·Bleomycin-Mediated RNA Strand Scission†

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ABSTRACT: The ability of iron(II)·bleomycin to mediate RNA degradation was further characterized. At micromolar concentrations, Fe^{II}·BLM was shown to effect cleavage of *Escherichia coli* $tRNA_1^{His}$ and a *Schizosaccharomyces pombe* amber suppressor tRNA construct in an efficient fashion. In contrast, *E. coli* $tRNA^{Cys}$ and yeast mitochondrial $tRNA^{Asp}$ and $tRNA_f^{Met}$ precursors were not substrates for Fe^{II}·BLM. Also shown to be a good substrate for cleavage by Fe^{II}·BLM was yeast 5S ribosomal RNA. Since HIV-1 reverse transcriptase mRNA has previously been shown to be degraded by Fe·BLM (Carter et al., 1990a), members of the three major classes of RNA have now been shown to undergo Fe·BLM-mediated strand scission. For each of the substrate RNAs, cleavage occurred at sites unique to that substrate. Although RNA cleavage occurred at numerous sequences, 5′-G-pyr-3′ sites were prominent. Likewise, while cleavage was noted in regions anticipated to be double-stranded, as well as in single-stranded regions, a disproportionate number of cleavages were noted at the junction between single- and double-stranded regions. As found in earlier studies, RNA cleavage was much more selective than DNA cleavage. Further, when RNA cleavage was carried out in the presence of reagents such as Mg^{2+}, spermidine, and NaCl, the selectivity of cleavage was further enhanced. The highly selective and efficient cleavage of a number of RNA molecules reinforces our earlier suggestion that RNA may constitute a therapeutically relevant target for bleomycin.

The antitumor antibiotic bleomycin (BLM[1]) is employed widely in clinics for the treatment of several neoplastic diseases, including non-Hodgkins lymphoma, squamous cell carcinomas, and testicular tumors (Blum et al., 1973; Sikic et al., 1985). Originally isolated as a natural product from *Streptomyces verticillus* (Figure 1) (Umezawa et al., 1966), this family of agents has been studied intensively in an effort to define the molecular basis for its antitumor activity (Hecht, 1986; Stubbe & Kozarich, 1987).

Most of the published work on the mechanism of action of bleomycin has focused on its ability to degrade cellular DNA and on the relationship between DNA damage and the cytotoxic effects of BLM (Twentyman, 1984; Berry et al., 1985a). In cell-free experiments, BLM degrades DNA as a 1:1 complex with any of several redox-active metal ions, including Mn (Burger et al., 1984; Ehrenfeld et al., 1984), Cu (Ehrenfeld et al., 1987), and Fe (Ishida & Takahashi, 1975; Sausville et al., 1978a); degradation requires O_2 and is thought to involve reductive activation of oxygen as part of a ternary complex with the metallobleomycin (Burger et al., 1981; Hecht, 1986; Stubbe & Kozarich, 1987; Natrajan et al., 1990a). Two sets of products have been identified, both of which involve oxidative destruction of the deoxyribose moiety in DNA. One of the intriguing observations for bleomycin is that DNA damage is mediated in a sequence-selective fashion, involving a subset of the 5′-GC-3′ and 5′-GT-3′ sequences.

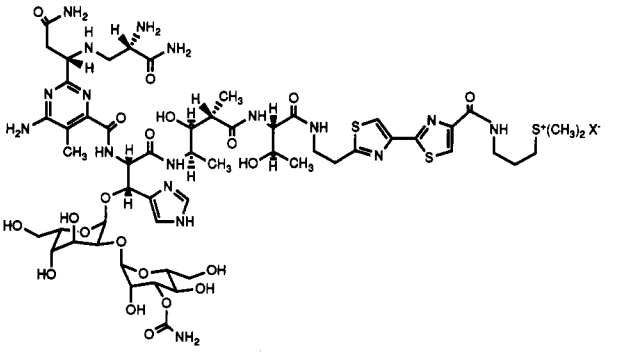

FIGURE 1: Structure of bleomycin A_2.

Systematic variation of the structure of bleomycin could undoubtedly provide important insights into the biochemical loci at which BLM mediates its therapeutic effects. While the structural complexity of BLM has tended to limit the variety of species available, some structural analogs have been prepared by synthesis (Saito et al., 1982; Aoyagi et al., 1982; Otsuka et al., 1986; 1990; Kitakka et al., 1988; Ohno et al., 1988; Carter et al., 1990a; Hamamichi et al., 1992; Boger et al., 1992) and semisynthesis (Takita et al., 1973; Nakayama et al., 1973; Muraoka et al., 1976, 1977; Tanaka, 1977; Naganawa et al., 1977; Umezawa, 1979; Tanaka & Takita, 1979; Takahashi et al., 1979). By the use of these analogs, it has been possible, for example, to better define metal binding and oxygen activation (Kilkuskie et al., 1985; Otsuka et al., 1986; Kittaka et al., 1988; Ohno et al., 1988), DNA binding (Kilkuskie et al., 1985; Morii et al., 1986, 1987), unwinding (Povirk et al., 1981; Fisher et al., 1985; Levy & Hecht, 1988), and cleavage (Sugiyama et al., 1985a; Shipley & Hecht, 1988; Carter et al., 1990c), and the possible existence of additional loci at which the cytotoxic effects of BLM are mediated (Berry et al., 1985b).

One obvious potential therapeutic locus for BLM is RNA. In spite of early reports that RNA was not degraded by BLM (Suzuki et al., 1970; Müller et al., 1972; Haidle et al., 1972; Haidle & Bearden, 1975; Hori, 1979; Krishnamoorthy et al.,

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[1] Abbreviations: BLM, bleomycin; nt, nucleotide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 3-(*N*-morpholino)-propanesulfonic acid; mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA.

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1988), recent studies have provided unequivocal evidence for Fe·BLM-mediated degradation of certain RNA substrates, notably transfer RNAs and tRNA precursor transcripts (Magliozzo et al., 1989; Carter et al., 1990a, 1991a,b). HIV-1 reverse transcriptase mRNA was also shown to be a substrate for Fe·BLM (Carter et al., 1990a). RNA degradation was shown to be more highly selective than BLM-mediated DNA degradation and to afford products whose formation could be envisioned to parallel the oxidative mechanism(s) employed for the degradation of DNA. While the sites of cleavage seemed to reflect RNA tertiary structure rather than recognition of specific sequences, only a limited number of examples have been reported thus far.

To permit a more detailed characterization of BLM-mediated RNA cleavage, several additional RNAs have been studied as potential BLM substrates. These included five additional tRNAs and tRNA precursor transcripts, as well as yeast 5S ribosomal RNA. To facilitate an assessment of RNAs as possible therapeutic targets for BLM, we have also studied the effects of reagents such as Mg^{2+}, spermidine, and salt on Fe·BLM-mediated RNA degradation. Presently, we provide further examples of the highly selective nature of RNA cleavage by Fe·BLM and demonstrate that RNA tertiary structure is a key determinant of the observed selectivity. Additionally, we show that NaCl and Mg^{2+} can further enhance the selectivity of RNA cleavage at physiologically relevant concentrations. One of the RNA substrates was also ^{32}P end labeled at the 5′- and 3′-ends in parallel experiments in order to provide data relevant to the actual chemistry of Fe·BLM-mediated strand scission and to permit evaluation of the possibility that not all of the cleavage sites were primary sites of BLM-induced damage.

EXPERIMENTAL PROCEDURES

Materials. Blenoxane was obtained from Bristol Laboratories; fractionation (Chien et al., 1977; Oppenheimer et al., 1979b) afforded bleomycin A_2, which was employed for all experiments described here. Restriction enzymes *Pvu*II and *Taq*I were purchased from Bethesda Research Laboratories, as were T4 RNA ligase, T4 polynucleotide kinase, T7 RNA polymerase, and the RNA sequencing system. *Eco*RI, *Bst*N1, calf intestinal alkaline phosphatase, and SP6 RNA polymerase were obtained from Boehringer Mannheim. RNasin RNase inhibitor was from Promega; [γ-^{32}P]ATP (>7000 Ci/mmol) was from ICN. QIAGEN columns used for RNA purification were purchased from QIAGEN, Inc.; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Alfa Products) was used to prepare aqueous solutions for combination with bleomycin immediately prior to each reaction. Spermidine and dithiothreitol were purchased from Sigma Chemicals.

A pSP64 plasmid encoding *Bacillus subtilis* $tRNA^{His}$ precursor under the control of an SP6 RNA polymerase promoter and adjacent to an *Eco*RI restriction site was provided by Dr. Barbara Vold (SRI International, Menlo Park, CA). Dr. Dieter Söll (Yale University) provided a plasmid encoding a *Schizosaccharomyces pombe* amber suppressor $tRNA^{Ser}$ construct (SP6 RNA polymerase promoter, *Taq*I restriction site). Plasmids encoding *Saccharomyces cerevisiae* mitochondrial $tRNA^{Asp}$ and $tRNA_f^{Met}$ precursor constructs (each containing a T7 RNA polymerase promoter; *Bst*N1 restriction site) were from Dr. Nancy Martin (University of Louisville).

The dodecamer d($CGCT_3A_3GCG$) was provided by Dr. Jacques van Boom (University of Leiden). Yeast 5S rRNA was purchased from Bethesda Research Laboratories; *Escherichia coli* $tRNA_1^{His}$ and $tRNA^{Cys}$ were from Plenum Scientific. A 230-nucleotide RNA was obtained by transcription from a *Pvu*II-linearized pSP64 plasmid (Carter et al., 1990a).

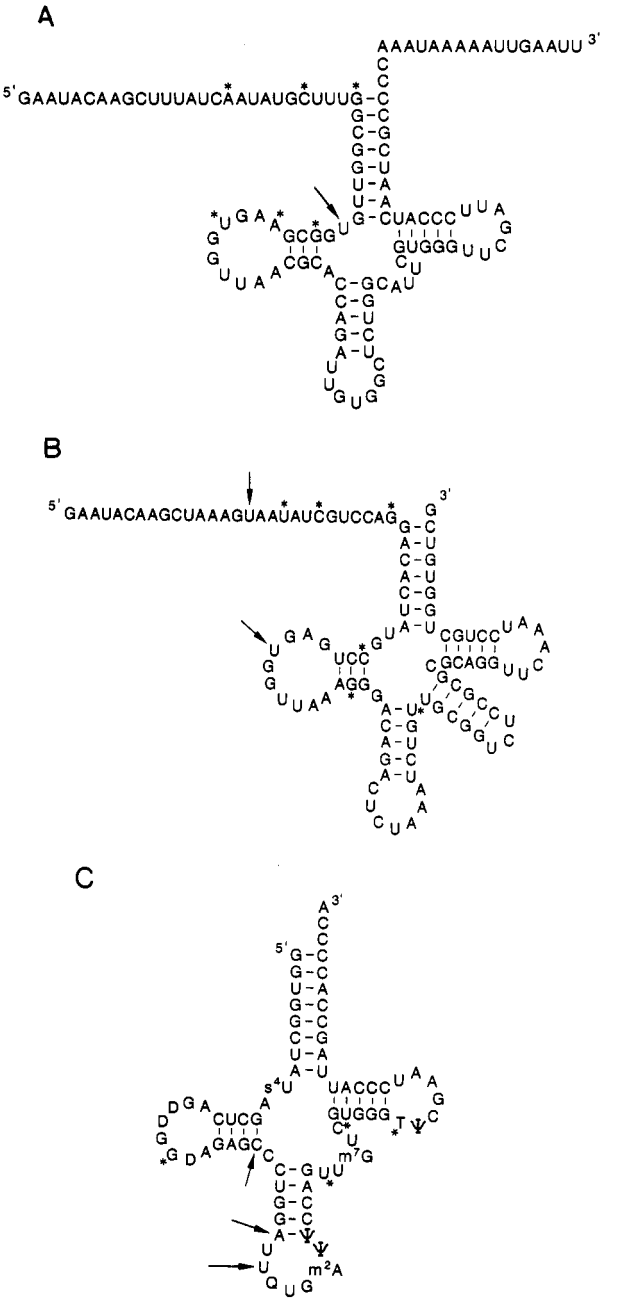


FIGURE 2: Structures of three RNA substrates for Fe^{II}·BLM A_2. The arrows denote the major sites of cleavage of *B. subtilis* $tRNA^{His}$ precursor (A), *S. pombe* amber suppressor $tRNA^{Ser}$ construct (B), and mature *E. coli* $tRNA_1^{His}$ (C). The asterisks denote the minor cleavage sites.

Methods. All plasmids were isolated from *E. coli* essentially as described (Sambrook et al., 1989) but using QIAGEN columns. The isolated DNAs were linearized with the appropriate restriction endonucleases; phenol extraction and ethanol precipitation afforded the linearized DNA duplexes, which were shown to have the anticipated mobilities when analyzed by agarose gel electrophoresis.

In vitro RNA run-off transcripts were prepared from the linearized plasmids in reaction mixtures that contained 2.5–5 μg of DNA in 100 μL (total volume) of 40 mM Tris-HCl, pH 7.5, with 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10

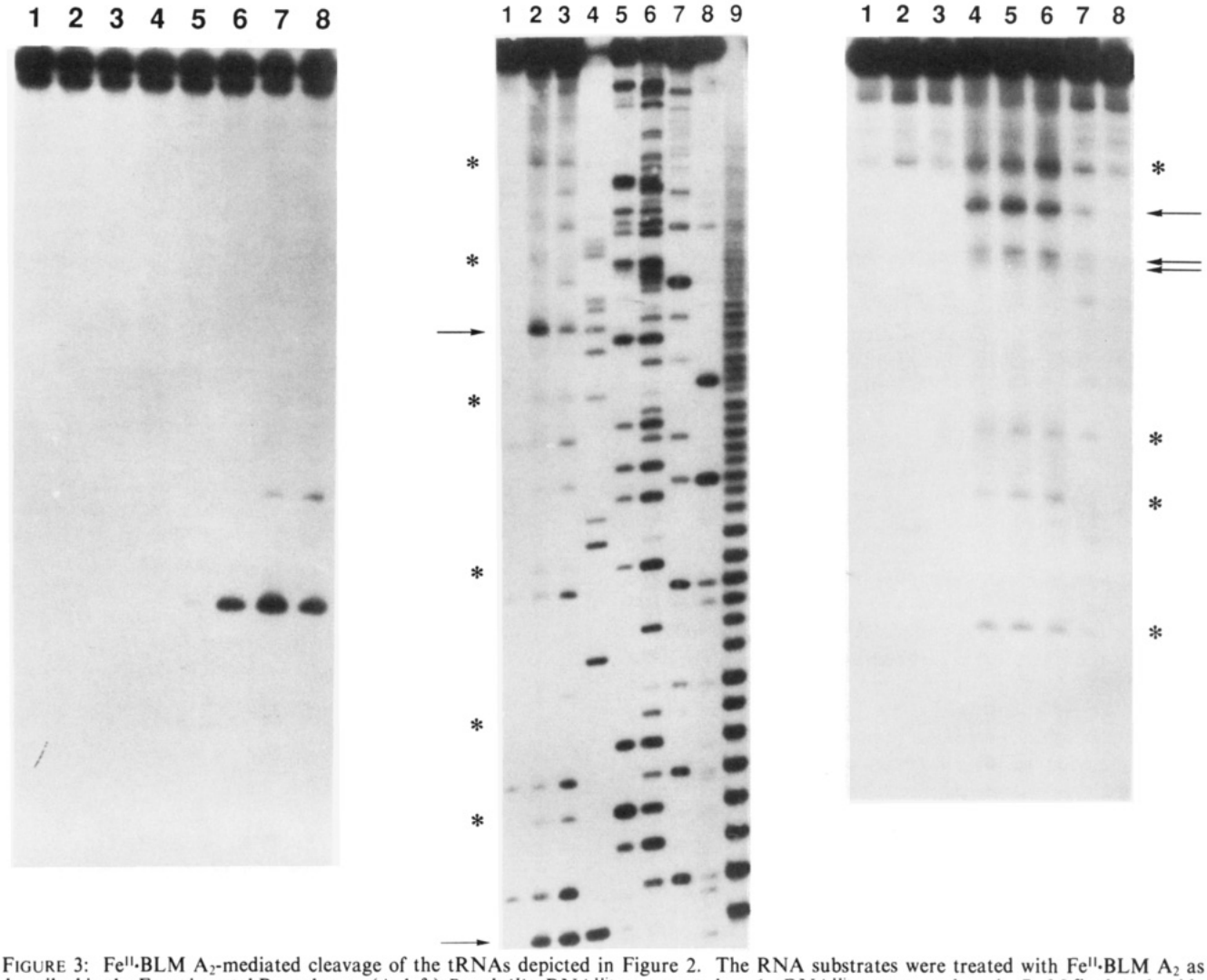


FIGURE 3: Fe^{II}·BLM A_2-mediated cleavage of the tRNAs depicted in Figure 2. The RNA substrates were treated with Fe^{II}·BLM A_2 as described in the Experimental Procedures. (A, left) *B. subtilis* $tRNA^{His}$ precursor: lane 1, $tRNA^{His}$ precursor alone (~7 μM final nucleotide concentration); lane 2, 1.25 μM BLM A_2; lanes 3–8, 0.25, 0.5, 1.25, 2.5, 25, and 250 μM Fe^{II}·BLM A_2, respectively. (B, center) *S. pombe* amber suppressor $tRNA^{Ser}$ construct at ~1 μM final nucleotide concentration: lane 1, 250 μM Fe^{II}; lane 2, 250 μM Fe^{II}·BLM A_2; lane 3, 100 μM Fe^{II}·BLM A_2; lanes 4–8, enzymatic sequencing lanes G, A > G, U + A, U + C, and C, respectively; lane 9, alkali-treated RNA. Control reactions containing RNA alone or RNA + 250 μM BLM A_2 gave essentially the same result as the Fe^{II} control reaction. (C, right) *E. coli* $tRNA_1^{His}$: lane 1, $tRNA_1^{His}$ alone (~2 μM final nucleotide concentration); lane 2, 250 μM BLM A_2; lane 3, 250 μM Fe^{2+}; lanes 4–8, 500, 250, 125, 50, and 5 μM Fe^{II}·BLM A_2, respectively. The uppermost site, denoted by an asterisk, was actually two bands; only the fainter (upper) band was Fe^{II}·BLM-dependent.

mM dithiothreitol, 30 units of RNasin, 0.5 mM ATP, GTP, UTP, and CTP, and 75–150 units of SP6 or T7 RNA polymerase. After incubation at 37 °C for ≥12 h, the reaction mixture was diluted with approximately 200 μL of 50 mM Mops buffer, pH 7.0, containing 400 mM NaCl. The sample was then adsorbed onto a QIAGEN tip-20 column (preequilibrated with the same buffer containing 15% ethanol) and washed with 6 mL of the preequilibration buffer. The column was then washed with 600 μL of freshly prepared 50 mM Mops, pH 7.0, containing 1.05 M NaCl, 15% ethanol, and 2 M urea. The RNA eluate was precipitated with isopropyl alcohol. The purified transcripts were dephosphorylated using calf intestinal alkaline phosphatase (3 units in 50 mM Tris-HCl, pH 9.0, containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine, 37 °C, 1 h) and then 5′-^{32}P end labeled (20–30 units of T4 polynucleotide kinase in 50 mM Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$ and 0.3 mCi of [γ-^{32}P]-ATP, 2–3 h, 37 °C), essentially as described (Carter et al., 1990b). *S. cerevisiae* 5S rRNA was 5′-^{32}P end labeled in the same fashion.

S. cerevisiae 5S rRNA, *E. coli* $tRNA_1^{His}$, and $tRNA^{Cys}$ were 3′-^{32}P end labeled using T4 RNA ligase and [5′-^{32}P]-pCp, essentially as described (Uhlenbeck & Gumport, 1982).

Iron(II)·Bleomycin-Mediated RNA Cleavage. Reaction mixtures (5 μL total volume) contained 0.5–5 μM labeled RNA in 5 mM sodium phosphate buffer, pH 7.5. The reactions were initiated by the simultaneous addition of equal amounts of Fe^{2+} and BLM A_2 to the concentrations indicated in the figure legends. Where indicated, exogenous agents (e.g., glycerol, NaCl, spermidine, $MgCl_2$, or DNA) were added in the appropriate amounts prior to the addition of Fe + BLM. Reaction mixtures were incubated at 22 °C for 15 min and then analyzed by denaturing polyacrylamide gel electrophoresis.

RESULTS

Characterization of tRNA Substrate Cleavage. The ability of Fe^{II}·BLM to cleave RNA was investigated initially using *B. subtilis* $tRNA^{His}$ precursor as a substrate (Figure 2A) (Carter et al., 1990a). When treated with Fe^{II}·BLM A_2, this

118-nt, 5′-^{32}P end labeled RNA was cleaved at a single major site (uridine$_{35}$) and several minor sites (Figure 3A). As shown in the figure, when the substrate RNA was used at ~7 μM nucleotide concentration, the major product band was evident at 1.25–250 μM Fe^{II}·BLM concentrations. The minor cleavage sites, in comparison, were evident only when relatively high concentrations of Fe^{II}·BLM were used.

Sequencing of the 5′-end-labeled RNA substrate was carried out using partial alkaline hydrolysis and base-specific nucleases; the reported sequence (Green & Vold, 1988) was verified. The positions of Fe·BLM-mediated RNA cleavage were determined by comparison of the bands with those produced by the RNA sequencing reagents. The assignments rest on the assumption that the chemistry of Fe·BLM-mediated RNA cleavage, like that of DNA cleavage, involves oxidative destruction of the ribose moiety. This assumption is supported by analysis of the chemical products resulting from RNA cleavage (Magliozzo et al., 1989; Carter et al., 1990a) and also by sequence analysis of the cleavage products resulting from Fe·BLM treatment of an RNA that was 5′- and 3′-^{32}P end labeled in parallel experiments (vide infra).

As shown in Figure 2A (arrow), the major site of cleavage was uridine$_{35}$. While this was located within a 5′-GU-3′ sequence, analogous to the preferred 5′-G·pyr-3′ sites in DNA (D'Andrea & Haseltine, 1978; Takeshita et al., 1981; Mirabelli et al., 1983), four of the six minor cleavage sites involved sequences other than 5′-G·pyr-3′. Further, while Fe·BLM-mediated DNA cleavage has been found to be limited to double-stranded substrates, most of the RNA cleavage occurred at sites believed to represent junctions between single- and double-stranded regions.

The second RNA substrate studied was a *S. pombe* $tRNA^{Ser}$ amber suppressor construct, prepared by primer-directed mutagenesis of a $tRNA^{Ser}_{AGA}$ gene (Krupp et al., 1985) and shown to function as an amber suppressor tRNA in vivo. In common with the $tRNA^{His}$ precursor, the $tRNA^{Ser}$ construct contained a 5′-leader sequence and lacked any post-transcriptional modification. Unlike $tRNA^{His}$, however, the $tRNA^{Ser}$ construct contained the extended variable arm that can be represented as a stem–loop structure, characteristic of class II tRNA molecules (Figure 2B). Treatment of this 5′-^{32}P-labeled tRNA transcript with Fe·BLM at 100 and 250 μM concentrations produced cleavage predominantly at U_{16} and U_{44} (Figure 3B), both of which were part of 5′-GU-3′ sequences (arrows, Figure 2B). Lesser amounts of cleavage occurred at six other sites, including U_{19}, C_{22}, G_{28}, C_{38}, G_{52}, and U_{71}. It is interesting that both of the strong cleavage sites were in regions of the tRNA that were ostensibly single-stranded. It may be noted, however, that U_{44} is part of the dihydrouridine loop; structural studies of other tRNAs have demonstrated that this region is involved in tertiary structure interactions with other regions of those tRNAs. Another class II tRNA molecule studied as a substrate for Fe^{II}·BLM was an *E. coli* $tRNA^{SeCys}$ precursor construct (Burkard & Söll, 1988). It had a single efficient cleavage site within loop IV and a weak cleavage site at the base of the acceptor stem (data not shown).

Also studied was the ability of Fe^{II}·BLM to effect the cleavage of mature tRNAs. As shown in Figure 3C, treatment of *E. coli* $tRNA_1^{His}$ with Fe^{II}·BLM A_2 resulted in cleavage at several sites. RNA sequence analysis indicated that the strong cleavage sites were at C_{26}, A_{32}, and U_{34}; the first of these was part of a 5′-GC-3′ sequence. Also noted was less efficient cleavage at four sites, including G_{20}, U_{45}, C_{49}, and T_{55}. The last of these is unique, as it constitutes the first

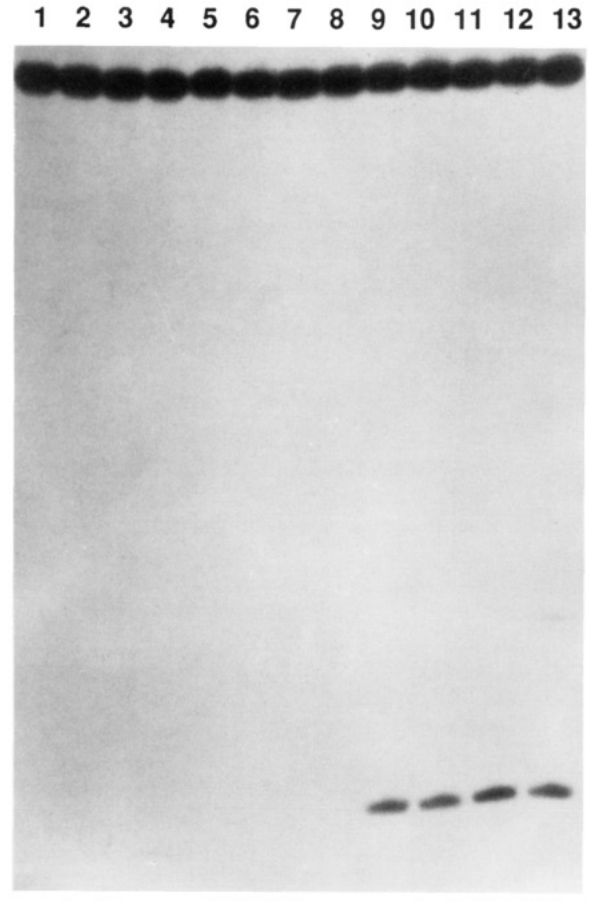


FIGURE 4: Cleavage of $tRNA^{His}$ precursor by Fe^{II}·BLM in the presence of a DNA oligonucleotide. *B. subtilis* $tRNA^{His}$ precursor was treated with Fe^{II}·BLM A_2 in the presence of 5′-d($CGCT_3A_3$-GCG)-3′ as described in the Experimental Procedures: lane 1, $tRNA^{His}$ precursor alone (270 μM final nucleotide concentration); lane 2, 10 μM Fe^{2+}; lane 3, 10 μM BLM A_2; lanes 4–13, 10 μM Fe^{II}·BLM A_2 + 400, 300, 200, 150, 100, 50, 10, 5, 0.5, and 0 μM dodecamer, respectively.

observation of cleavage of a modified RNA nucleotide. As the summary of cleavage sites illustrates (Figure 2C), while the distribution of lesions differed substantially from those of the other tRNAs studied, most of the cleavage sites in this case were also localized at positions believed to be junctions between single- and double-stranded regions of the tRNA.

One interesting facet of the foregoing tRNA cleavage experiments was the difference in the facility with which the individual substrates were degraded by Fe^{II}·BLM A_2. *B. subtilis* $tRNA^{His}$ precursor was degraded with the greatest efficiency. Substantial cleavage at the major site was obtained at 2.5 μM concentration and was readily detectable at 1.25 μM; comparable results have been obtained on numerous occasions. In comparison, cleavage of the $tRNA^{Ser}$ construct was never observed at concentrations of Fe^{II}·BLM of less than 10 μM (data not shown). Likewise, cleavage of *E. coli* $tRNA_1^{His}$ was evident at 50 μM Fe^{II}·BLM, but not at 5 μM. The greater efficiency of cleavage of the $tRNA^{His}$ precursor was obtained in spite of the fact that the sample employed was 5′-^{32}P end labeled at somewhat lower specific activity than the other tRNA samples. It may be noted that the present experiments were carried out successfully in phosphate buffer, which supported RNA degradation optimally, as has also been noted for Fe·BLM-mediated cleavage of DNA (Sausville et al., 1978b). Cleavage of $tRNA^{His}$ precursor has also been carried out successfully in cacodylate, Hepes, and Mops buffers. For $tRNA^{His}$ precursor, as well as the other RNA

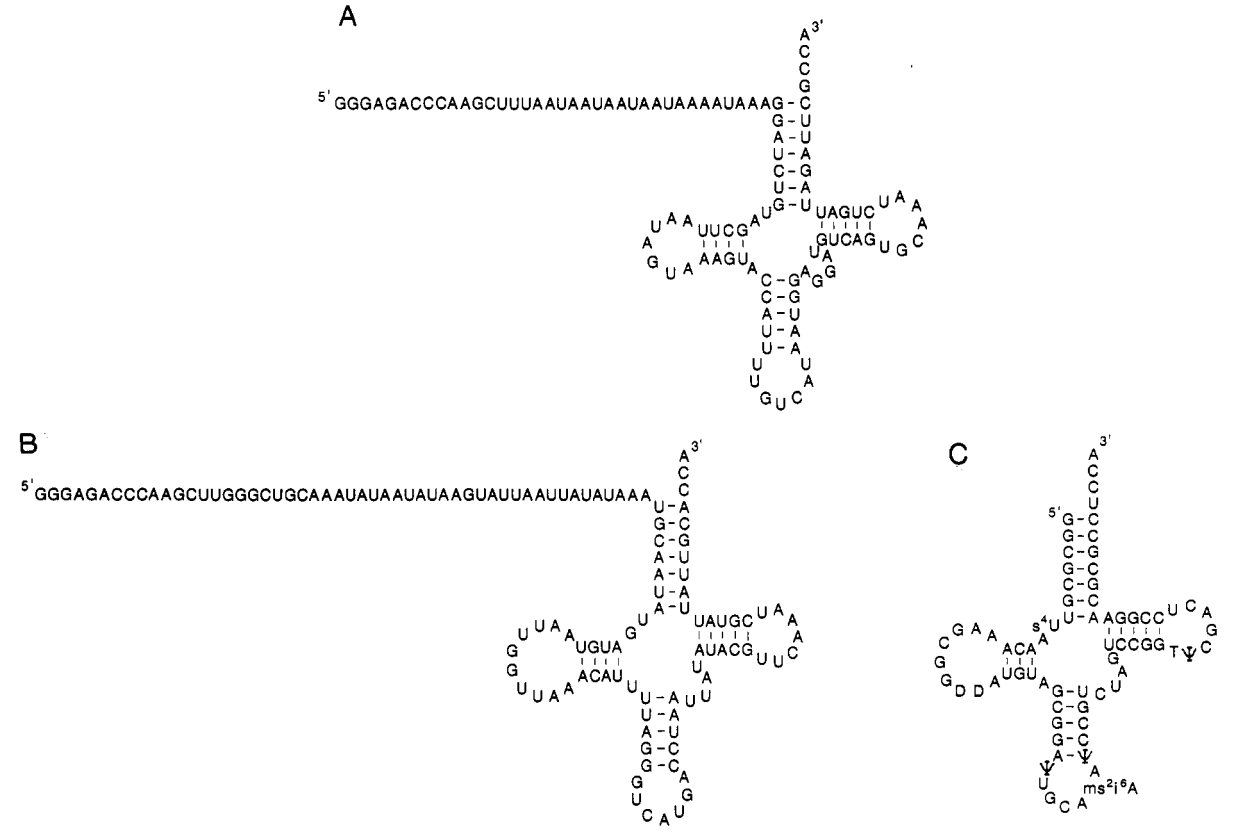


FIGURE 5: Structures of three RNAs that were not substrates for Fe^{II}·BLM A_2-mediated cleavage. Samples of yeast mitochondrial (*S. cerevisiae*) $tRNA^{Asp}$ precursor construct (A), yeast mitochondrial (*S. cerevisiae*) $tRNA_f^{Met}$ precursor construct (B), and *E. coli* $tRNA^{Cys}$ (C) were treated with Fe^{II}·BLM at concentrations ranging up to 500 μM. No significant strand scission was observed.

substrates studied, treatment with much higher concentrations of Fe·BLM caused occasional precipitation of the RNA substrate but no alteration of the sequence selectivity of cleavage.

The modest concentrations of Fe^{II}·BLM required to promote RNA cleavage of the foregoing tRNAs suggested that RNA degradation can be obtained with a facility comparable to that of DNA. In fact, Fe·BLM-mediated cleavage of *B. subtilis* $tRNA^{His}$ precursor has been shown to proceed in the presence of comparable concentrations of calf thymus DNA (Carter et al., 1991a). To further assess the efficiency of RNA cleavage, we employed a sample of *B. subtilis* $tRNA^{His}$ precursor that was 5′-^{32}P end labeled at relatively low specific activity (i.e., which required many strand scission events to produce a detectable product band on a polyacrylamide gel). Cleavage of this RNA was carried out with 10 μM Fe^{II}·BLM A_2 and also with the same amount of Fe^{II}·BLM A_2 in the presence of 5′-d($CGCT_3A_3GCG$)-3′, a DNA oligonucleotide known to be a particularly good substrate for binding and cleavage by Fe^{II}·BLM A_2 (Sugiyama et al., 1985b). As shown in Figure 4, efficient cleavage of the tRNA substrate was obtained even in the presence of 10 μM 5′-d($CGCT_3A_3GCG$)-3′.

Nonsubstrate tRNA Molecules. As noted previously for *E. coli* $tRNA^{Tyr}$ precursor (Carter et al., 1990a), some RNAs do not act as substrates for cleavage by Fe^{II}·BLM. In the present study, three additional tRNAs were found to be refractory to cleavage by Fe^{II}·BLM A_2 (Figure 5). These included a yeast mitochondrial $tRNA^{Asp}$ precursor construct, which afforded no detectable degradation product even when treated with Fe^{II}·BLM at concentrations as high as 500 μM. To ensure that the lack of cleavage was not due to a contaminant or some other experimental artifact, the attempted cleavage of this $tRNA^{Asp}$ precursor construct was repeated in a solution also containing $tRNA^{His}$ precursor. As shown in Figure 6, the $tRNA^{Asp}$ precursor was not cleaved by Fe^{II}·BLM A_2 at any tested concentration in the presence or absence of equimolar *B. subtilis* $tRNA^{His}$ precursor. However, $tRNA^{His}$ precursor was cleaved at all tested concentrations of Fe^{II}·BLM A_2, as anticipated.

A second RNA of interest as a possible substrate for Fe^{II}·BLM A_2 was a yeast mitochondrial $tRNA_f^{Met}$ precursor construct (Figure 5) having 11 5′-G·pyr-3′ sequences and a dihydrouridine loop differing from *B. subtilis* $tRNA^{His}$ precursor by only one nucleotide (cf. Figure 2A). In spite of these structural elements, treatment with 25–500 μM Fe^{II}·BLM A_2 had no effect on this RNA (data not shown).

Another RNA that proved refractory to cleavage was *E. coli* $tRNA^{Cys}$ (Figure 5); 125–500 μM Fe^{II}·BLM A_2 had no effect on this RNA. Again, a comparison of the sequence of this tRNA with those of known substrates (Figure 2) would predict several cleavage sites if Fe·BLM recognition were based entirely on a 2–3-base sequence recognition. Also studied as a substrate for cleavage by Fe^{II}·BLM A_2 was a 5′-^{32}P end labeled suppressor tRNA construct modeled after *E. coli* $tRNA^{Gln}$ (D. Söll, personal communication). Although the sensitivity of detection in this case was diminished due to the (~100-fold) lower specific activity of the radiolabeled tRNA as compared with the other tRNAs in Figure 5, no cleavage was observed using Fe^{II}·BLM A_2 at concentrations up to 500 μM (data not shown). In all cases, the lack of cleavage of a given RNA was verified by admixture of that species to a solution containing a known substrate, the latter of which was cleaved normally by Fe^{II}·BLM in the presence of the refractory RNA (vide supra).

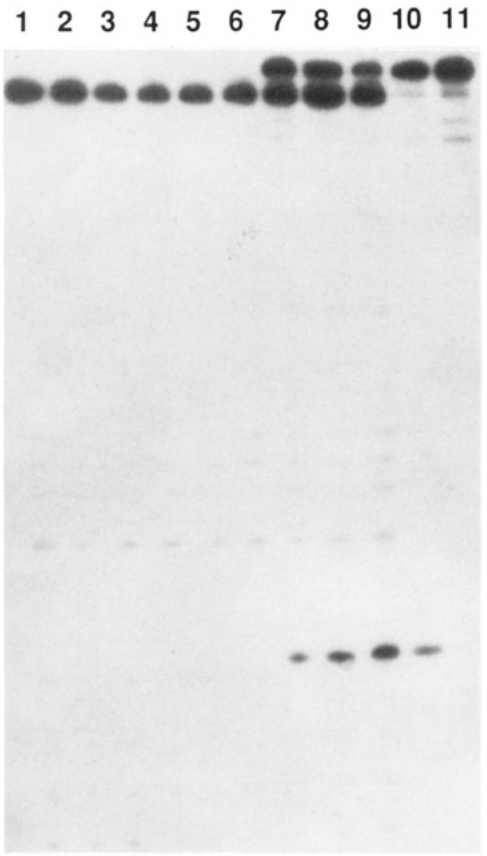
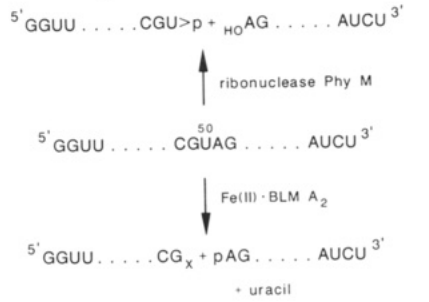


FIGURE 6: Attempted cleavage of yeast mitochondrial $tRNA^{Asp}$ precursor construct with Fe^{II}·BLM A_2. 5′-End-labeled $tRNA^{Asp}$ precursor was treated with Fe^{II}·BLM A_2 alone and also in the presence of *B. subtilis* $tRNA^{His}$ precursor as described in the Experimental Procedures: lane 1, $tRNA^{Asp}$ precursor alone (~0.6 μM final nucleotide concentration); lane 2, $tRNA^{Asp}$ precursor + 250 μM Fe^{2+}; lane 3, $tRNA^{Asp}$ precursor + 250 μM BLM A_2; lanes 4–6, $tRNA^{Asp}$ precursor + 25, 250, and 500 μM Fe^{II}·BLM A_2, respectively; lanes 7–9, $tRNA^{Asp}$ precursor + $tRNA^{His}$ precursor (0.5 μM final nucleotide concentration) + 25, 250, and 500 μM Fe^{II}·BLM A_2, respectively; lane 10, $tRNA^{His}$ precursor + 250 μM Fe^{II}·BLM A_2; lane 11, $tRNA^{His}$ precursor alone.

The lack of cleavage of several RNAs whose structures are not dramatically different than those of authentic substrate RNAs (cf. Figures 2 and 5) further supports the thesis that RNA recognition by Fe^{II}·BLM depends importantly on secondary and tertiary structure elements in the RNAs.

Cleavage of Yeast 5S Ribosomal RNA. 5S ribosomal RNA is an abundant, evolutionarily conserved species essential for ribosome assembly and function. The susceptibility of this RNA to Fe^{II}·BLM-mediated cleavage was assessed using both 3′- and 5′-^{32}P end labeled rRNA substrates. As shown in Figure 7B, treatment of the 3′-end-labeled 122-nt rRNA with 210 or 250 μM Fe^{II}·BLM A_2 resulted in strong cleavage at three sites and cleavage to a lesser extent at a few additional sites. The same result was obtained following treatment of the 5′-end-labeled rRNA with 125 and 250 μM Fe^{II}·BLM A_2; sequence analysis indicated that the major lesions had formed at U_{50}, U_{62}, and U_{83} (cf. Figure 7A,C). Interestingly, each of these uridines was present within a 5′-GUA-3′ sequence. Four such sequences exist in yeast 5S rRNA. The three that were cleaved by Fe^{II}·BLM are all present within helical regions of the RNA; all three of these sites also have a one-nucleotide bulge 1–2 bases to the 3′-side of the site of cleavage. At least two of these three bulges have been conserved (McDougall & Nazar, 1983; Nazar, 1991); two of them have been postulated to serve as protein recognition sites (Nazar, 1979) and all three as stabilizers of tertiary structure (McDougall & Nazar, 1983). The fourth 5′-GUA-3′ site, involving U_{78}, is located within a single-stranded region of the RNA.

Scheme I: Postulated Chemistry of 5S Ribosomal RNA Cleavage by Fe^{II}·BLM A_2, Illustrated for the Lesion Produced at $Uridine_{50}$[a]



[a] Fe^{II}·BLM produces an RNA fragment believed to contain a phosphoroglycolate moiety at the 3′-terminus.

The use of both 5′- and 3′-^{32}P end labeled rRNAs as substrates for Fe^{II}·BLM provided two additional types of data. First, the fact that both substrates afforded the same three cleavage products (cf. Figure 7B,C) indicates that all three sites are primary cleavage sites for Fe^{II}·BLM in the intact 5S rRNA and that no double-stranded RNA cleavage occurred. Any secondary cleavage site, i.e., a site susceptible to cleavage only after strand scission had occurred at a primary site, would fail to yield a (radiolabeled) product band corresponding to that site for one of the two end-labeled RNAs. Likewise, simultaneous double-strand cleavage of the 5S rRNA, analogous to that noted for DNA (Povirk et al., 1977, 1989; Lloyd et al., 1978; Burger et al., 1986), would give a unique end-labeled cleavage product from each of the radiolabeled substrates.

The second type of data accessible by the use of both 5′- and 3′-end-labeled RNAs is related to the chemistry of RNA cleavage. The 5′-end-labeled RNA gave major bands (Figure 7C) believed to have 3′-phosphoroglycolate termini (Carter et al., 1990a). As anticipated, these migrated ahead of the corresponding bands in the sequencing lanes, the latter of which are known to have 2′,3′-cyclic phosphates at their 3′-termini. This was consistent with the belief that Fe·BLM-induced lesions were formed by oxidative destruction of uridine with concomitant formation of free uracil (Magliozzo et al., 1989; Carter et al., 1990a) (Scheme I). Although some of the bands in Figure 7C appeared to be partially resolved into doublets, this effect was not seen reproducibly. In contrast, Fe·BLM-mediated cleavage of 3′-end-labeled RNA gave single bands (Figure 7B). These bands migrated slightly ahead of the corresponding bands in the U-lane of the RNA sequencing gel (not shown). The ribonucleases that produced the sequencing bands are known to afford oligonucleotides having 5′-OH termini; the slightly greater mobility of the oligonucleotides produced by Fe·BLM is consistent with the presence of 5′-phosphate termini, as demonstrated previously for Fe·BLM-mediated DNA cleavage (Hecht, 1986; Stubbe & Kozarich, 1987). The inferred chemical transformations of the RNA substrate are summarized in Scheme I.

The facility of cleavage of yeast 5S rRNA was influenced by Na^+ and Mg^{2+}, each of which can play a role in stabilizing RNA tertiary structure (Reeves et al., 1970; Cole et al., 1972; Cole & Crothers, 1972; Reid & Cowan, 1990). As shown in Figure 7B, Fe^{II}·BLM-mediated cleavage of the 5S rRNA was diminished in the presence of 85 mM NaCl and abolished in the presence of 85 mM NaCl + 17 mM Mg^{2+} (cf. lanes 5–7).

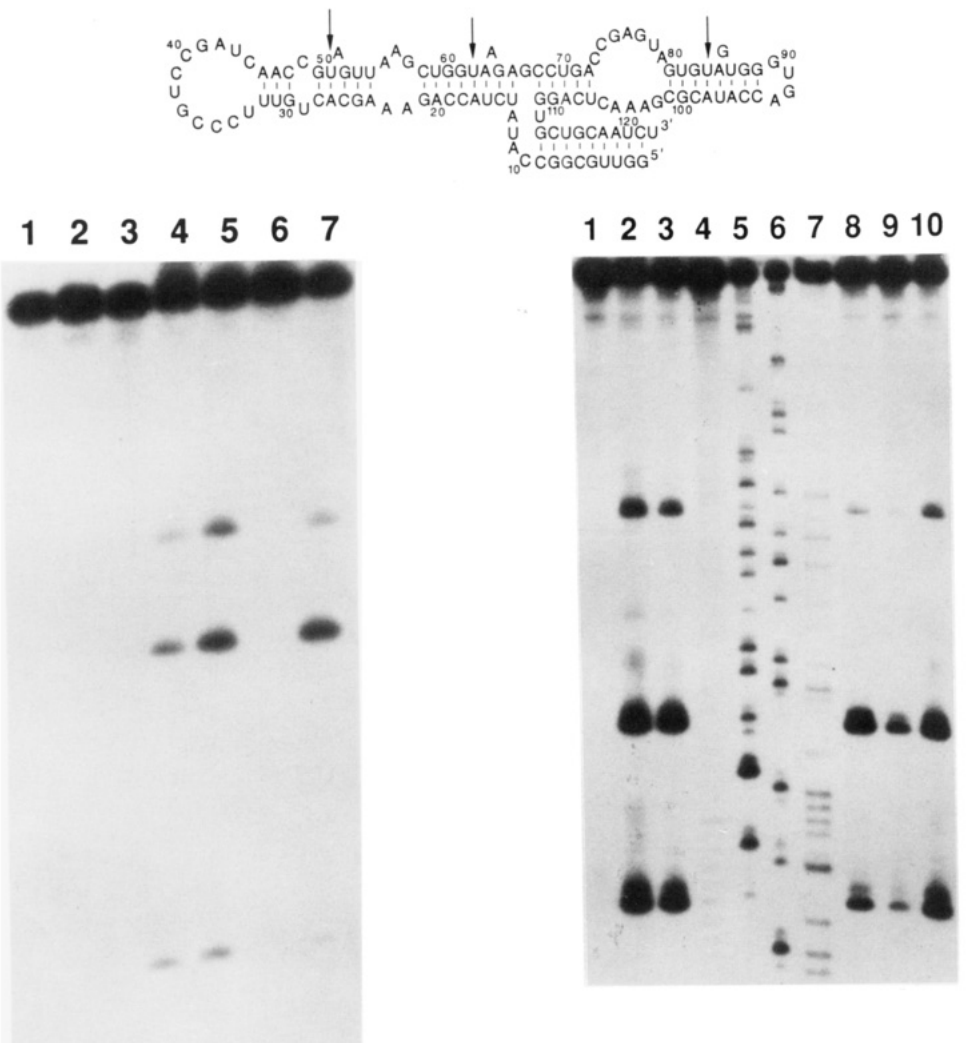


FIGURE 7: Fe^{II}·BLM A_2-mediated cleavage of yeast 5S rRNA. (A, top) Proposed secondary structure of yeast 5S rRNA. The three sites of Fe^{II}·BLM A_2-mediated cleavage are indicated by arrows. (B, bottom left) Fe^{II}·BLM A_2-mediated cleavage of 3′-^{32}P end labeled 5S rRNA: lane 1, rRNA alone (~1 μM final nucleotide concentration); lane 2, 250 μM BLM A_2; lane 3, 250 μM Fe^{2+}; lane 4, 250 μM Fe^{II}·BLM A_2; lane 5, 210 μM Fe^{II}·BLM A_2; lane 6, 210 μM Fe^{II}·BLM + 85 mM NaCl + 17 mM Mg^{2+}; lane 7, 210 μM Fe^{II}·BLM + 85 mM NaCl. Mg^{2+} and NaCl were added directly to the RNA solution prior to Fe^{II}·BLM A_2 addition; the RNA was not renatured experimentally prior to Fe·BLM addition. (C, bottom right) Fe^{II}·BLM A_2-mediated cleavage of 5′-^{32}P end labeled 5S rRNA: lane 1, rRNA alone (~1 μM final nucleotide concentration); lane 2, 250 μM Fe^{II}·BLM A_2; lane 3, 125 μM Fe^{II}·BLM A_2; lane 4, alkali-treated RNA; lane 5, G-lane; lane 6, A > G lane; lane 7, U + A lane; lane 8, 250 μM Fe^{II}·BLM A_2 + 100 mM NaCl; lane 9, 250 μM Fe^{II}·BLM A_2 + 5 mM Mg^{2+}; lane 10, 250 μM Fe^{II}·BLM A_2 + 1 mM Mg^{2+}.

It is interesting that cleavage at U_{62} was essentially unaffected by NaCl, while the lesions at U_{50} and U_{83} diminished substantially. In contrast, the diminution of cleavage noted in the presence of 1 and 5 mM Mg^{2+} occurred roughly in the same proportions at each of the three cleavage sites (Figure 7C).

Fe·BLM Cleavage of a 230-nt RNA Transcript. In vitro transcription from a *Pvu*II-linearized pSP64 plasmid afforded a 230-nt RNA transcript (Figure 8A) that was also studied as a potential substrate for cleavage by Fe^{II}·BLM A_2, affording cleavage predominantly at three sites (Figure 8B). RNA sequence analysis (not shown) indicated the sites of cleavage to be U_{75}, U_{92}, and G_{121} (cf. Figure 8A). The former two were present within 5′-GU-3′ sequences; a folding analysis (Zuker & Stiegler, 1981) of the RNA suggested that both sites may be in helical regions close to junctions with single-stranded regions of the RNA. The folding analysis suggested that G_{121} is also at the junction of single- and double-stranded regions of the RNA. Also noted among four minor cleavage sites were U_{82}, U_{84}, and A_{86}, which were assigned to one double-helical region by the folding analysis. According to this analysis, A_{86} was a mismatched base.

Effect of RNA Conformational Change on the Facility of Cleavage. The effect on Fe·BLM-mediated RNA cleavage of agents known to alter RNA conformation was studied in more detail using *B. subtilis* $tRNA^{His}$ precursor. The 5′-^{32}P end labeled RNA was treated with 25 μM Fe^{II}·BLM A_2 in the presence of spermidine, glycerol, NaCl, and $MgCl_2$. As indicated in Table I, 5 mM or 500 μM spermidine completely inhibited BLM-induced cleavage. The same result was obtained in the presence of 50 mM Mg^{2+}, consistent with observations made for cleavage of yeast 5S rRNA (Figure 7). In contrast, slight enhancement of cleavage by 25 μM Fe^{II}·BLM A_2 was observed in the presence of 5% glycerol or 50 mM NaCl. No change in the site of cleavage was observed with any of the added agents.

Polyamines have been shown to bind in the minor groove of tRNA helices and induce distance changes between structural domains of tRNAs (Rich et al., 1979). Since the major site of cleavage of $tRNA^{His}$ precursor by Fe·BLM does

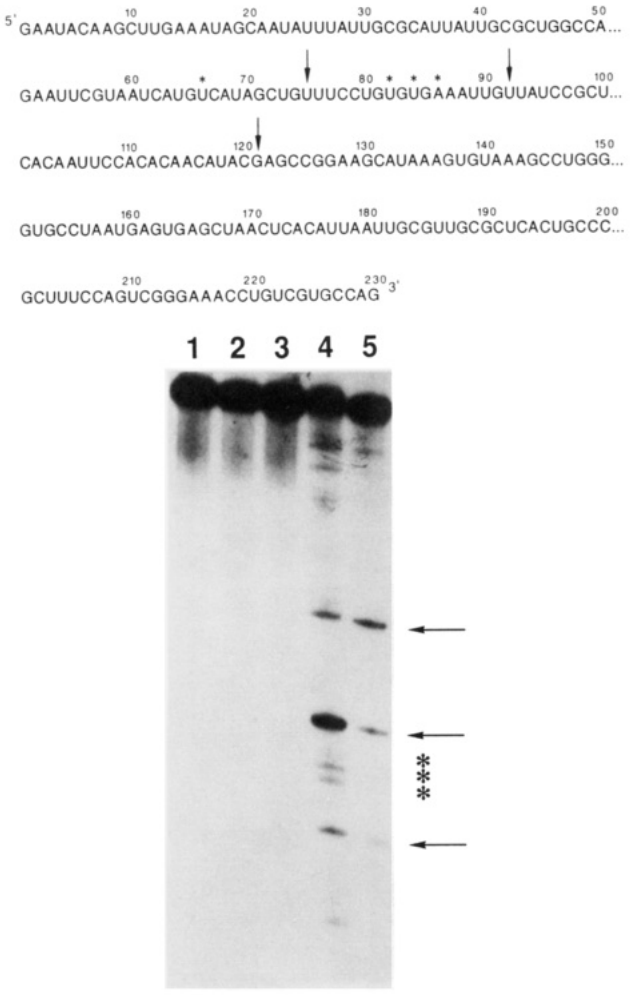


FIGURE 8: Fe^{II}·BLM-mediated cleavage of a 230-nt *E. coli* RNA transcript. An RNA 230 nt in length was transcribed from a *Pvu*II-linearized pSP64 plasmid, 5′-^{32}P end labeled, and treated with Fe^{II}·BLM A_2 as described in the Experimental Procedures. (A, top) Sequence of the transcribed RNA. The major Fe·BLM cleavage sites, determined by RNA sequencing reactions, are indicated by arrows; minor cleavage sites are indicated by asterisks. (B, bottom) Fe^{II}·BLM A_2-mediated cleavage of the RNA substrate: lane 1, RNA alone (2×10^4 cpm); lane 2, 100 μM BLM A_2; lane 3, 100 μM Fe^{II}; lane 4, 100 μM Fe^{II}·BLM A_2; lane 5, 25 μM Fe^{II}·BLM A_2.

not occur within a helical region, it seems likely that the observed inhibition was due to an induced conformational change, rather than direct binding competition for the site at which cleavage occurs. Likewise, Mg^{2+} has been shown to tighten and stabilize overall RNA tertiary structure, and inhibition of BLM cleavage by this species could well be due to an overall change in RNA conformation.

The effect of Mg^{2+} was characterized further using several different substrate molecules. As shown in Figure 9 for *B. subtilis* $tRNA^{His}$ precursor (17 μM final nucleotide concentration), cleavage mediated by 250 μM Fe^{II}·BLM A_2 at U_{35} was essentially unaffected by concentrations of Mg^{2+} up to 125 μM. In contrast, under the same conditions BLM-induced cleavage was eliminated at all of the minor cleavage sites except for C_{23}. At Mg^{2+} concentrations from 250 to 500 μM, cleavage at all of the minor sites was completely abolished. Cleavage of the same substrate RNA in the presence of Mg^{2+} concentrations up to 5 mM significantly reduced, but did not eliminate, cleavage at U_{35} by 250 μM Fe^{II}·BLM (data not shown). However, when mature *E. coli* $tRNA^{His}$ was used as a substrate, concentrations of Mg^{2+} greater than 1 mM completely inhibited cleavage by 500 μM Fe^{II}·BLM. Thus, the susceptibility of the $tRNA^{His}$ precursor to cleavage by BLM in the presence of Mg^{2+} varied from site to site, consistent with the interpretation that inhibition of cleavage was caused by local conformational changes in the RNA substrates.

Table I: Effect of Exogenous Agents on Iron(II)·Bleomycin-Mediated Cleavage of $tRNA^{His}$ Precursor[a]

Fe^{II}·BLM A_2 (μM)	additions	% cleavage of substrate[b]
0		0[c]
250		25
25		7
0	5 mM spermidine	0[c]
250	5 mM spermidine	0[c]
25	500 μM spermidine	0[c]
0	5% glycerol (v/v)	0[c]
250	5% glycerol (v/v)	18
25	5% glycerol (v/v)	20
0	50 mM NaCl	0[c]
250	50 mM NaCl	21
25	50 mM NaCl	18
0	50 mM $MgCl_2$	0[c]
250	50 mM $MgCl_2$	0[c]
25	50 mM $MgCl_2$	0[c]

[a] Reaction conditions were as described in the Experimental Procedures. Exogenous agents were added to the reaction prior to the addition of Fe^{2+} + BLM. [b] Represents the percent conversion of substrate into the major cleavage product (corresponding to cleavage at U_{35}). [c] Less than 2%.

DISCUSSION

In previous studies, Fe^{II}·BLM A_2 has been shown to effect the efficient degradation of *B. subtilis* $tRNA^{His}$ precursor and two in vitro RNA transcripts (Carter et al., 1990a, 1991a,b). While cleavage of these substrates proceeded readily under conditions comparable to those employed for the degradation of DNA, all of the RNA substrates were cleaved with much greater selectivity. The *selectivity* of cleavage was not a function of Fe^{II}·BLM concentration. In addition, at least one potential RNA substrate, *E. coli* $tRNA^{Tyr}$ precursor, was not cleaved readily by Fe^{II}·BLM A_2 at any reasonable concentration, in spite of its obvious structural similarity to *B. subtilis* $tRNA^{His}$ precursor. In the present study, a *S. pombe* amber suppressor $tRNA^{Ser}$ construct was also shown to undergo cleavage by Fe^{II}·BLM, as was post-transcriptionally modified *E. coli* $tRNA_1^{His}$. The $tRNA^{Ser}$ construct was cleaved efficiently at two nucleotides; *E. coli* $tRNA^{His}$ was cleaved at three. In common with *B. subtilis* $tRNA^{His}$ precursor, which is cleaved predominantly at 5′-GU_{35}-3′, three of the five strong cleavage sites in the $tRNA^{Ser}$ construct and *E. coli* $tRNA^{His}$ involved pyrimidine nucleotides within 5′-G-pyr-3′ sequences. At least two of the newly characterized cleavage sites were also at putative junctions between single- and double-stranded regions of the RNA substrates (Figures 2 and 3). However, the remaining strong cleavage sites involved 5′-GA-3′ and 5′-UU-3′ sequences, i.e., sequences different than those shown to be good substrates for Fe^{II}·BLM in B-form DNA duplexes (D'Andrea & Haseltine, 1978; Takeshita et al., 1981; Mirabelli et al., 1983; Shipley & Hecht, 1988). As noted previously for *B. subtilis* $tRNA^{His}$ precursor (Carter et al., 1990a), the minor cleavage sites in the new tRNA substrates also involved sequences not generally found to constitute good substrates in DNA. It is of interest that three of the strong cleavage sites in the newly identified tRNA substrates were located in regions that are nominally single-stranded (Figure 2), although extrapolation from crystallographic studies of other tRNAs suggests that

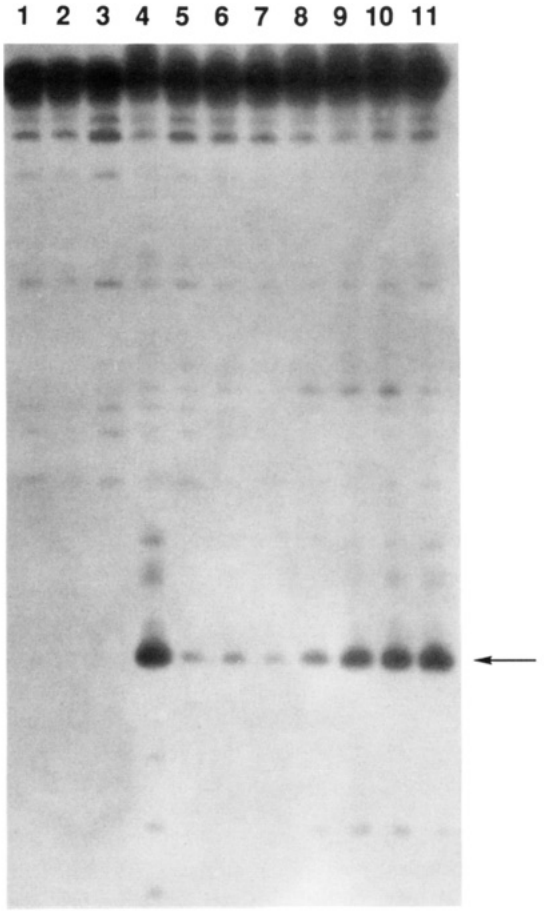


FIGURE 9: Effect of Mg^{2+} on Fe·BLM-mediated cleavage of $tRNA^{His}$ precursor. *B. subtilis* $tRNA^{His}$ precursor was treated with Fe^{II}·BLM A_2 in the presence of varying concentrations of Mg^{2+}, as described in the Experimental Procedures: lane 1, $tRNA^{His}$ precursor (17 μM final nucleotide concentration) + 0.5 mM Mg^{2+}; lane 2, 250 μM Fe^{2+} + 0.5 mM Mg^{2+}; lane 3, 250 μM BLM A_2 + 0.5 mM Mg^{2+}; lane 4, 250 μM Fe^{II}·BLM A_2; lanes 5–11, 250 μM Fe^{II}·BLM A_2 + 0.5, 0.4, 0.3, 0.25, 0.125, 0.1, and 0.05 mM Mg^{2+}, respectively. In each case, Fe^{II}·BLM A_2 was activated aerobically for 15–20 s prior to addition to the individual reaction mixtures.

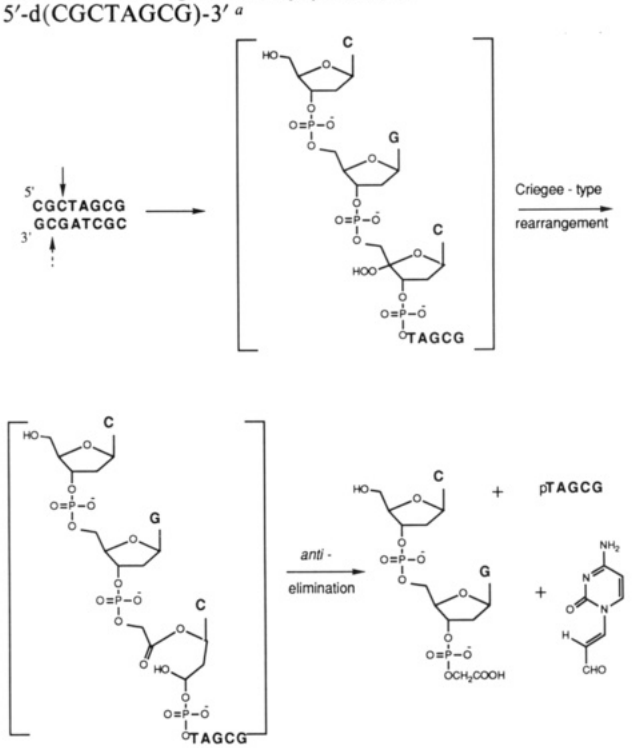
at least some of these regions may actually be involved in tertiary interactions with other regions in the RNAs.

More striking was the finding that several other tRNAs and tRNA constructs *failed* to undergo cleavage by Fe^{II}·BLM A_2 under any of the conditions studied. These included yeast mitochondrial $tRNA^{Asp}$ and $tRNA_f^{Met}$ precursor constructs, *E. coli* $tRNA^{Cys}$, and an *E. coli* suppressor $tRNA^{Gln}$ construct. As noted previously for *E. coli* $tRNA^{Tyr}$ (Carter et al., 1990a), cleavage of these species failed in spite of the presence of numerous structural features ostensibly similar to those in the substrate RNAs (cf. Figures 2 and 5). The fact that cleavage of *B. subtilis* $tRNA^{His}$ precursor also occurred more readily than the other tRNA substrates argues that the susceptibility of RNA to cleavage by BLM is controlled to a considerable extent by RNA conformation (vide infra).

Another RNA found to undergo Fe^{II}·BLM-mediated cleavage with great facility was yeast 5S rRNA; three major cleavage sites were identified and shown to be primary cleavage sites (Figure 7). Each of these involved a uridine within a 5′-GUA-3′ sequence; interestingly, each of these sites had a bulge 1 or 2 bases to the 3′-side of the cleavage site.

The location and efficiency of RNA cleavage were influenced by Na^+ and Mg^{2+}. Low concentrations of Na^+ effected a slight enhancement in the extent of cleavage of *B. subtilis* $tRNA^{His}$ precursor by 25 μM Fe·BLM (Table I). In

Scheme II: Products Resulting from Fe^{II}·BLM-Mediated Oxidative Cleavage of Deoxycytidine$_3$ of 5′-d(CGCTAGCG)-3′ [a]



[a] An analogous set of products was obtained by cleavage at deoxycytidine$_7$ (Sugiyama et al., 1985b).

comparison, as shown in Figure 7B for yeast 5S RNA, 85 mM NaCl diminished cleavage *selectively* at two of three cleavage sites. The addition of Mg^{2+} (at 1 and 5 mM concentrations) diminished cleavage proportionately at the three sites in 5S rRNA; however, the Mg^{2+} concentration dependence of inhibition of Fe·BLM cleavage differed significantly for other RNA substrates tested. For example, BLM-mediated cleavage of mature *E. coli* $tRNA_1^{His}$, as well as yeast $tRNA^{Phe}$ and $tRNA^{Asp}$, was completely inhibited in the presence of 1 mM Mg^{2+} (not shown). Analogous findings have been reported by Hüttenhofer et al. (1992). On the basis of these observations, it would be anticipated that the considerable selectivity of RNA cleavage observed in the present study might well be further enhanced under physiological conditions.

Chemistry of RNA Cleavage. A few lines of evidence suggest that Fe·BLM-mediated RNA degradation, like that of DNA, is oxidative in nature. These include the observations that RNA cleavage was promoted by Fe^{II}·BLM, but not by Fe^{III}·BLM, and that cleavage was potentiated by sodium ascorbate. Bleomycin-mediated DNA strand scission is accompanied by the formation of free bases and base propenals resulting from oxidation of deoxyribose (Hecht, 1986; Stubbe & Kozarich, 1987). Due to differences in the sugar moieties of RNA and DNA, oxidative cleavage of RNA would not be expected to afford base propenals. The analogous RNA products, 2-hydroxylated base propenals, would likely be unstable if formed and undergo ready hydrolysis to liberate free bases. Therefore, the observation that free bases were formed concomitantly with tRNA cleavage by BLM was consistent with a mechanism involving oxidative cleavage. Quantitation of free base release was carried out using *B. subtilis* $tRNA^{His}$ precursor, which is cleaved predominantly at uridine$_{35}$. Treatment of a $tRNA^{His}$ transcript containing [^{3}H]uridine of known specific activity labeled within the pyrimidine moiety afforded a product that coeluted on C_8

reversed-phase HPLC with authentic uracil. The amount of uracil produced was roughly equivalent to the extent of strand breakage at U_{35} (Carter et al., 1990a).

Unequivocal evidence for an oxidative pathway by which Fe^{II}·BLM can degrade RNA nucleotides was obtained by the use of oligonucleotides of defined structure as substrates. The octanucleotide 5′-d(CGCTAGCG)-3′ has been shown to undergo efficient oxidative transformation; cleavage at cytidine$_3$ afforded *trans*-3-(cytosin-1′-yl)propenal and 2′-deoxycytidylyl(3′→5′)[2′-deoxyguanosine-3′-(phosphoro-2″-*O*-glycolate)] in equal amounts (Scheme II) (Sugiyama et al., 1985b). Analogous treatment of an octanucleotide of the same sequence, but containing *ribo*-cytidine at position 3, afforded the same dinucleotide 3′-phosphoroglycolate. The type of transformation depicted in Scheme II has been studied intensively in the case of DNA substrates and has been shown to result from initial oxidation at C-4′ of deoxyribose. That the same product can form from a DNA oligonucleotide containing a ribonucleotide at the site of cleavage strongly supports the thesis that RNA can undergo oxidative cleavage in the presence of Fe^{II}·BLM.

Also consistent with an oxidative cleavage mechanism for RNA were the results obtained from PAGE analysis of ^{32}P-end-labeled RNAs that had been treated with Fe^{II}·BLM. 3′-End-labeled RNAs gave BLM cleavage bands that migrated ahead of authentic standards containing 5′-OH groups. PAGE analysis of 5′-end-labeled RNAs generally gave single bands that migrated ahead of the bands in sequencing lanes, the latter of which are known to have 2′,3′-cyclic phosphates at their 3′-termini. This observation is consistent with the formation of oligonucleotide fragments having 3′-phosphoroglycolate termini. The results of this analysis are summarized in Scheme I for the Fe·BLM-induced lesion at U_{50} in yeast 5S rRNA.

As noted above, Fe^{II}·BLM effected the cleavage of both 5′-d(CGCTAGCG)-3′ and C_3-ribo 5′-CGCTAGCG-3′ at C_3, affording the same oxidative product in each case (vide supra; cf. Scheme II). For DNA, studies in a number of laboratories have indicated that this type of product is formed via the intermediacy of a C-4′ deoxyribose radical (Hecht, 1986; Stubbe & Kozarich, 1987). At least for DNA, this radical can partition to form a second type of product, the alkali labile lesion (Sugiyama et al., 1985c, 1988; Rabow et al., 1986), which predominates at lower oxygen tension (Burger et al., 1982; Wu et al., 1985). The presence of two types of oxidative products formed from DNA is readily apparent, for example, by the appearance of two closely spaced bands on polyacrylamide gels at each cleavage site when a 5′-^{32}P end labeled DNA duplex is treated with Fe^{II}·BLM. Because the alkali labile lesion per se does not result in strand scission, the band derived from this lesion becomes prominent on the polyacrylamide gels only if the DNA is treated with alkali subsequent to Fe·BLM; recently, alkylamines and hydrazine have also been employed for this purpose (Sugiyama et al., 1988). In spite of the occasional appearance of broadened bands on polyacrylamide gels following analogous treatment of 5′-^{32}P end labeled RNA with Fe·BLM (e.g., Figure 7C), there is presently no definitive evidence for the formation of RNA products of the same type as the alkali labile lesions formed in DNA. It may be noted, however, that the detection of such lesions in RNA cannot be accomplished readily using alkali, due to the sensitivity of the RNA to this reagent.

Comparison of Bleomycin-Mediated Cleavage of RNA and DNA. Although several early studies suggested that RNA was not a substrate for cleavage by bleomycin, we have now demonstrated unequivocally that a number of RNA substrates are cleaved by Fe^{II}·BLM. The reaction conditions employed for RNA cleavage were comparable to those employed for DNA, including the (micromolar) concentrations of Fe^{II}·BLM required to produce detectable cleavage of ^{32}P-end-labeled substrates. It has been shown previously that Fe·BLM-mediated cleavage of RNA obtained even in the presence of DNA; this was demonstrated quantitatively in the present study by admixture of a good DNA substrate for Fe·BLM at several concentrations with 5′-^{32}P end labeled $tRNA^{His}$ precursor. As shown in Figure 4, cleavage of $tRNA^{His}$ was obtained even in the presence of 10 μM 5′-d($CGCT_3A_3GCG$)-3′. Further, when we employed an RNA substrate believed to have a secondary structure comparable to that of a DNA of the same sequence, the efficiencies of cleavage of both species were comparable.

Bleomycin-mediated DNA cleavage is characterized by its sequence selectivity; most strand breaks occur at a subset of 5′-G·pyr-3′ sequences. In contrast, while 5′-G·pyr-3′ sequences were also represented to a disproportionate extent for observed RNA cleavage sites, the latter seemed to occur predominantly at sites characterized by their conformational properties (vide infra); many were at the junctions between single- and double-stranded regions of the RNA substrate, while others were in regions anticipated to be nominally single-stranded. Interestingly, many of the single-strand cleavage sites occurred in regions of the tRNAs, such as the dihydrouridine loop, predicted by analogy with structurally characterized tRNAs (Robertus et al., 1974; Kim et al., 1974) to undergo tertiary interactions with other regions of the tRNA molecule. It may be noted that, while DNA cleavage by bleomycin is generally discussed in terms of sequence selectivity, there is some evidence consistent with recognition of DNA conformation by bleomycin. This includes the findings that treatment of DNA plasmids with limited amounts of bleomycin produced lesions localized at a small number of sites (Haidle et al., 1979) and different cleavage patterns in a supercoiled vs a linearized plasmid (Mirabelli et al., 1983). The pattern of DNA cleavage by BLM has been noted to be sensitive both to global variations in DNA structure and to changes in the microenvironment such as those induced by methylation (Hertzberg et al., 1985, 1988; Long et al., 1990) or platination (Mascharak et al., 1983; Gold et al., 1988). Also reported was bleomycin-mediated cleavage within a stem-and-loop structure formed from one strand of DNA (Ueda et al., 1985), analogous to the types of structures present within the tRNA (precursor) substrates studied here.

Perhaps the most interesting observation was the pattern of cleavage obtained using yeast 5S rRNA as a substrate. As indicated in Figure 7, cleavage of this RNA occurred virtually exclusively at three sites, all of which involved uridine nucleotides in the sequence 5′-GUA-3′ and at least two of which were located within evolutionarily conserved duplexes containing a one-nucleotide bulge, 1–2 bases to the 3′-side of the cleavage site. This observation bears at least superficial analogy to the finding of Williams and Goldberg (1988) that Fe·BLM produced nicks in DNA duplexes at the site of 1-base bulges. In this case, however, cleavage was on the strand complementary to the one containing the bulge.

Bleomycin has been shown to produce both single- and double-strand breaks in DNA (Hecht, 1986; Stubbe & Kozarich, 1987). The latter, which may constitute as much as 20% of all breaks (Lloyd et al., 1978, 1979), occur with a frequency well in excess of what would be predicted from the random accumulation of single-strand breaks. In contrast,

thus far there are no examples of double-strand breaks produced in RNA by Fe·BLM. Whether this is due to some intrinsic difference in the mechanism of recognition or cleavage of DNA and RNA by bleomycin or simply reflects the lesser frequency of RNA strand scission is unclear at present.

Fe·BLM-mediated DNA cleavage is believed to be a two-step process (Hecht et al., 1986; Stubbe & Kozarich, 1987; Van Atta et al., 1989). The first step involves binding of Fe·BLM to preferred sites on the DNA, presumably as the activated metallobleomycin (Burger et al., 1981; Natrajan et al., 1990a,b). In a subsequent step, abstraction of C-4′ H from deoxyribose initiates the actual chemical degradation events (vide supra). The selectivity of DNA cleavage presumably results from the existence of preferred binding sites and possibly also from differences in the facility with which activated Fe·BLM can cleave the individual sites to which it has bound (McLean et al., 1989; Kozarich et al., 1989). The association constant for Fe^{II}·BLM binding to duplex DNA has been measured in numerous studies (Dabrowiak, 1982); values on the order of 10^5 M^{-1} are typical. DNA binding is clearly mediated in part via electrostatic interactions (Huang et al., 1980; Kross et al., 1982a; Sakai et al., 1982; Booth et al., 1983) and probably also by intercalation or partial intercalation (Chien et al., 1977; Povirk et al., 1981). Given that the chemical events leading to DNA cleavage take place in the minor groove of DNA, it would not be surprising if DNA association were also dependent on H-bonding interactions between BLM and functional groups on the floor of the minor groove.

The observation of highly selective RNA cleavage by Fe^{II}·BLM is entirely consistent with a binding mechanism for RNA cleavage analogous to that employed for DNA. Further, preliminary experiments indicate that the relative efficiencies of RNA cleavage among key BLM congeners are on the same order as their relative efficiencies of DNA cleavage (not shown), consistent with the interpretation that RNA binding is accomplished by a molecular strategy similar to that employed for DNA binding. If Fe^{II}·BLM-mediated RNA and DNA cleavage both result from similar two-step mechanisms, then there are two logical ways to explain the much greater selectivity of RNA cleavage. The first is that RNA binding per se is much more selective, i.e., that fewer RNA sites are capable of forming complexes with Fe·BLM that can lead to strand scission. The alternative explanation is that strand scission is limited not by the number of sites at which Fe·BLM is bound but by the facility of cleavage at individual sites that contain bound Fe·BLM. Clearly, some combination of these two would also be possible.

RNA Conformation as a Factor in Fe·BLM-Mediated RNA Cleavage. That RNA conformation contributes importantly to the facility of RNA cleavage by Fe^{II}·BLM at a given site can be appreciated from a few different types of data, one of which is the effect of reagents known to be capable of altering RNA conformation. These included a polyamine, Mg^{2+}, and NaCl, whose effects on tRNA conformation have been documented (Reeves et al., 1970; Cole et al., 1972; Cole & Crothers, 1972; Rich et al., 1979; Reid & Cowan, 1990), as well as glycerol. As shown in Table I, 500 μM or 5 mM spermidine inhibited Fe^{II}·BLM A_2-mediated cleavage of *B. subtilis* $tRNA^{His}$ precursor, as did high concentrations of Mg^{2+}. In contrast, 5% glycerol caused slight enhancement of $tRNA^{His}$ precursor cleavage; 50 mM NaCl also enhanced cleavage, but only when a relatively low concentration of Fe^{II}·BLM was employed. In contrast, 85 mM NaCl diminished the extent of cleavage of yeast 5S rRNA by 210 μM Fe^{II}·BLM (Figure 7).

Critically, in several cases these reagents affected RNA cleavage to different extents at individual cleavage sites within the same molecule. Examples included cleavage of yeast 5S rRNA in the presence of NaCl, which inhibited cleavage at U_{50} and U_{83} but not at U_{62}. Cleavage of *B. subtilis* $tRNA^{His}$ precursor by 250 μM Fe^{II}·BLM was readily inhibited at all but one minor cleavage site by 125 μM Mg^{2+}. Cleavage at the major cleavage site, however, was largely unaffected by 125 μM Mg^{2+} and was still readily apparent at 5 mM Mg^{2+}. These observations strongly support the thesis that RNA conformation is an important factor in determining the facility of RNA cleavage by Fe·BLM at a given site. In this context, it may be mentioned that Chow et al. (1992) have made analogous observations for the effects of salt and Mg^{2+} on the cleavage of tRNAs and tRNA precursor constructs by bis(phenanthroline)(phenanthrenequinone diimine)rhodium(III); these workers also attributed variations in cleavage at individual RNA sites to alteration of RNA structure.

Given the importance of electrostatic interactions to metallobleomycin–DNA interaction, and probably to RNA binding as well (vide supra), it seems highly likely that in addition to their effects on RNA conformation Na^+ and Mg^{2+} must also have more direct effects on metallobleomycin–polynucleotide interaction. For example, it was shown that total DNA degradation by BLM decreased at high salt concentrations (Hertzberg et al., 1988), presumably due in part to the association of Na^+ ions with the negatively charged phosphate ester backbone of DNA and concomitant masking of a functionality employed for DNA binding by BLM. In addition, it has been shown that quenching of the fluorescence associated with the bithiazole moiety of BLM upon admixture of DNA, a phenomenon long assumed to reflect DNA binding by BLM (Chien et al., 1977; Strong & Crooke, 1978), could be blocked in part at high ionic strength (Huang et al., 1980). Therefore, while the effects of Na^+, Mg^{2+}, and other reagents on Fe·BLM-mediated RNA cleavage probably did result in part from alteration of RNA conformation, it is likely that some of the inhibitory effects resulted from the ability of these reagents to interfere directly with Fe·BLM binding to RNA.

The exact nature of the structural and conformational parameters in DNA and RNA optimal for binding and cleavage by Fe·BLM is not well defined, in spite of studies that have varied the structures of BLM (Sugiyama et al., 1985a; Ehrenfeld et al., 1987; Shipley & Hecht, 1988; Ohno et al., 1988; Otsuka et al., 1990; Carter et al., 1990c; Hamamichi et al., 1992) and its polynucleotide substrates (Mascharak et al., 1983; Ueda et al., 1985; Hertzberg et al., 1985, 1988; Williams & Goldberg, 1988; Gold et al., 1988; Long et al., 1990; Carter et al., 1990a) and measured the interactions of these species by physicochemical (Povirk et al., 1981; Dabrowiak, 1982; Fisher et al., 1985; Hénichart et al., 1985; Levy & Hecht, 1988; Van Atta et al., 1989) and biochemical methods (Chien et al., 1977; Huang et al., 1980; McLean et al., 1989). The complexity of the structure of BLM and the continuing uncertainty concerning its metal ligands (Takita et al., 1978; Dabrowiak et al., 1978; Dabrowiak, 1980; Oppenheimer et al., 1979a,b, 1980, 1981; Mooberry et al., 1980; Pillai et al., 1980) have contributed to the difficulty in defining the nature of BLM–polynucleotide interaction.

Fe·BLM exhibits a strong preference for cleavage of duplex DNA (Umezawa, 1978; Kross et al., 1982b; Ueda et al., 1985); cleavage proceeds optimally under conditions that favor

B-form, rather than Z-form, DNA (Hertzberg et al., 1988). While several modes of BLM–DNA interaction have been proposed (Povirk et al., 1979, 1981; Takeshita et al., 1979; Hénichart et al., 1985; Kuwahara & Sugiura, 1988), it seems virtually certain that the metal-binding domain of BLM resides within the minor groove of B-form DNA, since oxidative damage is initiated by this domain at C-4′ of deoxyribose in the minor groove (Hecht, 1986; Stubbe & Kozarich, 1987). In comparison, RNA duplexes typically adopt an A-form conformation; relative to DNA, these are believed to be tighter structures having a wide, shallow minor groove. As described above, the available evidence suggests that bases lying at the junction between double- and single-stranded RNA regions can be particularly susceptible to cleavage by Fe·BLM. Such junction regions might be anticipated to have more open, distorted conformations, some of which might be particularly conducive to Fe·BLM binding and cleavage.

Several observations have been made which support the thesis that steric accessibility may be important in determining the sites in RNA that can be cleaved by Fe^{II}·BLM and that the tighter structures which are obtained within RNA duplexes tend to limit the facility of BLM-mediated cleavage. It was shown, for example, that Fe·BLM-mediated cleavage of *B. subtilis* $tRNA^{His}$ precursor was temperature dependent (Carter et al., 1991a,b). Cleavage proceeded readily at 22, 37, and 55 °C but was diminished significantly at 0 °C, consistent with the need for a more flexible structure to facilitate BLM binding. The same result was obtained using the *S. pombe* amber suppressor $tRNA^{Ser}$ construct as a substrate for Fe^{II}·BLM (data not shown).

Perret et al. (1990) have studied the structures of yeast $tRNA^{Asp}$ and of an RNA transcript having the same nucleotide sequence. Both were found to have similar tertiary structures, as judged by their recognition by aspartyl-tRNA synthetase and reactivity toward chemical probes, but the unmodified transcript was clearly a more open structure. This finding was entirely consistent with another recent study that has suggested that post-transcriptional modification of tRNA transcripts serves to stabilize tRNA structure and decrease the overall flexibility of the molecule (Sampson & Uhlenbeck, 1988). In this context it is worthy of note that the two RNA substrates cleaved with much greater efficiency than other RNA substrates studied, namely, *B. subtilis* $tRNA^{His}$ precursor and yeast 5S rRNA, were not post-transcriptionally modified. While *E. coli* $tRNA_1^{His}$ (Figures 2 and 3), yeast $tRNA^{Phe}$ (Magliozzo et al., 1989), and yeast $tRNA^{Asp}$ (data not shown) were found to be substrates for Fe^{II}·BLM, they were cleaved less readily; mature *E. coli* $tRNA^{Cys}$ (Figure 5) was not a substrate for Fe^{II}·BLM at all. Further, unfractionated *E. coli* tRNAs had a minimal effect in inhibiting cleavage of *B. subtilis* $tRNA^{His}$ by limiting amounts of Fe^{II}·BLM, suggesting that few of them, if any, bound Fe^{II}·BLM tightly (Carter et al., 1990a).

It is believed that Mg^{2+} and spermidine lead to more stabilized RNA structures. In addition to their effects on RNA conformation and their ability to act as counterions for phosphate ester anions, it may be the case that these cations inhibit BLM-mediated RNA cleavage by leading to tighter structures less sterically accessible to BLM. If more open, flexible structures were an essential element for RNA cleavage by Fe·BLM, the greater sensitivity of this process to inhibition by Mg^{2+} could be readily explained. In this context it is interesting to note that the mature tRNAs cleaved with lesser facility by Fe·BLM (*E. coli* $tRNA_1^{His}$, yeast $tRNA^{Asp}$, and yeast $tRNA^{Phe}$; vide supra) were also more susceptible to inhibition of cleavage by added Mg^{2+}.

As noted previously, Fe^{II}·BLM effected cleavage of yeast 5S rRNA within three of the four 5′-GUA-3′ sequences present in the RNA. While no crystallographic analysis of this RNA has been reported, chemical modification and cross-linking studies have provided much useful information concerning RNA secondary and tertiary structure (Nazar, 1979, 1991; McDougall & Nazar, 1983; Yeh & Lee, 1988). These studies indicated that the 5′-GUA-3′ sequence (at positions 77–79) refractory to Fe·BLM cleavage was not completely accessible to modification by Fe^{II}·EDTA or ethylnitrosourea; the three sites cleaved by Fe·BLM, on the other hand, were readily accessible to these reagents (McDougall & Nazar, 1983; Nazar, 1991).

Protein binding of 5S rRNA by L1a has been found to occur specifically from one side of the RNA molecule between nucleotides 50 and 121; a model for this interaction has been described (Nazar, 1979). It is interesting that Fe·BLM-promoted cleavage of the RNA at U_{50}, U_{62}, and U_{83} involves cleavage of the RNA within the same region of the RNA accessible for binding of ribosomal protein L1a.

The available data are thus consistent with the importance of steric accessibility in determining the position of RNA cleavage by Fe·BLM. The lack of RNA cleavage noted at sites ostensibly similar in structure to authentic cleavage sites (Magliozzo et al., 1989; Carter et al., 1990a) may thus be attributable to lack of steric accessibility caused by the overall conformation adopted by the molecule in solution.

Possible Therapeutic Relevance of Fe·BLM-Mediated RNA Cleavage. Bleomycin exhibits antitumor activity in several experimental animal models and is employed clinically as an anticancer agent. Studies of the mechanism by which bleomycin mediates its antitumor effects have focused on the ability of the drug to degrade DNA; this process has been characterized extensively at chemical, biochemical, and pharmacological levels.

While it is clear that bleomycin mediates DNA degradation and causes loss of cell viability and that both increase in direct proportion to the amount of BLM employed, there are a few observations which suggest that DNA may not be the only cellular locus at which BLM exerts its therapeutic effects. For example, while it has been shown that BLM causes extensive damage to chromatin when employed in even modest amounts (Berry et al., 1985a), the correlation between the extent of DNA damage and KB cell growth inhibition exhibited a wide variation for individual BLM congeners (Berry et al., 1985a). Likewise, it was found that treatment of these cells with the local anesthetic dibucaine rendered them susceptible to inhibition by a BLM congener dysfunctional in DNA strand scission (Berry et al., 1985b). Further complicating the analysis is the compartmentalization of chromosomal DNA within the nucleus of eukaryotic cells. Poddevin et al. (1991) have demonstrated that the toxicity of BLM toward a transformed Chinese hamster lung fibroblast cell line was dramatically enhanced when transport through the cell membrane was facilitated by electropermeabilization. It seems logical to assume that the nuclear membrane would further diminish the expression of cytotoxicity if chromatin were the (sole) target of bleomycin.

As a target for Fe·BLM, RNA differs in several respects from DNA; all of these suggest that RNA may be a relevant therapeutic target for bleomycin. For example, RNA is present within the cytoplasm of eukaryotic cells, obviating the need for delivery of bleomycin across the nuclear

membrane. The paucity of known mechanisms for RNA repair suggests that bleomycin-mediated RNA damage would likely be irreversible; destruction of an RNA critical to cell function could readily produce the therapeutic effects noted for BLM.

In earlier studies, we demonstrated that Fe^{II}·BLM could effect the cleavage of *B. subtilis* $tRNA^{His}$ precursor and HIV-1 reverse transcriptase mRNA. Both of these RNAs were cleaved with much greater selectivity than typical DNA substrates; certain RNAs, such as *E. coli* $tRNA^{Tyr}$ precursor, were not substrates for Fe·BLM. Presently, we extend these findings by demonstrating that Fe^{II}·BLM also mediated the cleavage of a *S. pombe* amber suppressor $tRNA^{Ser}$ construct and of mature (i.e., post-transcriptionally modified) *E. coli* $tRNA_1^{His}$. In common with *B. subtilis* $tRNA^{His}$ precursor, cleavage of the newly tested species occurred at a limited number of sites. The selectivity of RNA cleavage was also further established by the finding that *E. coli* $tRNA^{Gln}$ and yeast mitochondrial $tRNA^{Asp}$ and $tRNA_f^{Met}$ precursor constructs, as well as mature *E. coli* $tRNA^{Cys}$, failed to undergo cleavage at any tested concentration of Fe^{II}·BLM.

The major classes of cellular RNAs include transfer RNAs, messenger RNAs, and ribosomal RNAs. Having demonstrated the susceptibility of certain RNAs from the first two classes to Fe·BLM-mediated degradation, we sought to establish whether ribosomal RNA could also act as a substrate for cleavage. As shown in Figure 7, yeast 5S rRNA was cleaved at three sites, all of which involved the same (5′-GUA-3′) sequence in proximity to a one-nucleotide bulge. The facility of 5S rRNA cleavage was comparable to that of *B. subtilis* $tRNA^{His}$ precursor, demonstrating that the third major type of RNA was also susceptible to cleavage by BLM in a highly selective fashion.

Also investigated as part of the present study were the effects on Fe·BLM-mediated RNA cleavage of reagents (e.g., Mg^{2+}, salt) that would be present under physiological conditions. As illustrated in Figures 7 and 9 and in Table I, several reagents known to bind to RNA or to effect conformational changes in the macromolecule altered the extent (but not the site) of RNA cleavage by Fe·BLM. At concentrations likely to occur physiologically, Mg^{2+} and Na^+ both diminished RNA cleavage by Fe·BLM; remarkably, for some RNA substrates the extent of inhibition varied from site to site within single RNA molecules, suggesting a further potential source of selectivity for RNA cleavage by BLM in intact cells. It may be noted that Hüttenhofer et al. (1992) have also reported inhibition of BLM-mediated yeast $tRNA^{Phe}$ cleavage by Mg^{2+}. These authors concluded that, if this phenomenon proved to be general, it would imply that Fe·BLM-mediated RNA cleavage could not be a therapeutically important process. Our finding that certain RNAs can be cleaved by Fe·BLM under physiologically relevant conditions clearly obviates this concern and underscores the importance of evaluating the action of Fe·BLM using a variety of substrates. Although not investigated at all to date, it may be noted that the BLM cleavage sites in yeast 5S rRNA are known sites of protein binding (Nazar, 1979; Yeh & Lee, 1988); it seems highly likely that the binding of protein to RNA could further enhance the selectivity of RNA cleavage in situ by Fe·BLM.

Whether or not bleomycin mediates its therapeutic effects at the level of RNA degradation, the foregoing discussion underscores the logic for believing that RNA could serve as a viable therapeutic target; it is relatively accessible to exogenous agents, not known to be repaired if damaged, and required as a participant in several essential cell functions. The substantial variety of RNA structures underscores the potential for high selectivity of RNA cleavage, as already demonstrated for Fe·BLM. If we assume that the observed selective toxicity of BLM toward neoplasms results from selective action at the relevant molecular locus, the remarkable selectivity of BLM-mediated RNA strand scission could well provide the molecular basis for this observed therapeutic selectivity. Although RNA cleavage has been explored in less detail than DNA cleavage, several reagents capable of oxidative RNA strand scission have already been characterized (Kean et al., 1985; Chen & Sigman, 1988; Murakawa et al., 1989; Latham & Cech, 1989; Chow & Barton, 1990; Chow et al., 1992). Obvious candidates for further study of RNA cleavage include those agents whose antitumor activity is presently believed to derive from their ability to damage DNA.

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