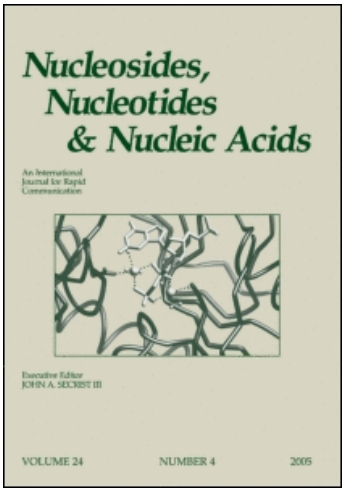


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# Metal-Catalyzed RNA Strand Scission

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# METAL-CATALYZED RNA STRAND SCISSION

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Abstract. Two novel approaches have been employed for mediating RNA strand scission under physiological conditions. One of these involved strand scission by an $Fe^{2+}$ chelate of the antitumor antibiotic bleomycin; the other involved a novel $Mn^{2+}$-dependent cleavage of an RNA hairpin at a specific site. Bleomycin-mediated RNA cleavage was oxidative in nature, while the $Mn^{2+}$-promoted cleavage of the hairpin occurred by a solvolytic mechanism.

## INTRODUCTION

The cleavage of DNA and RNA has been of considerable interest in recent years.[1-4] Reagents of utility for site-selective DNA cleavage include a number of natural and synthetic agents, as well as single-stranded oligonucleotide probes of specific sequence equipped with prosthetic groups capable of mediating sequence-neutral cleavage.[1] Fewer studies have addressed the issue of RNA cleavage, especially in a sequence-selective fashion, although cleavage has been achieved via the agency of small molecules,[5-7] through the use of RNA self-processing motifs,[3,4,8] and by proteins normally responsible for RNA processing that can recognize certain duplexes potentially accessible by exogenous addition of a synthetic oligonucleotide probe to a single-stranded RNA substrate.[9]

Presently, we describe two novel approaches for the highly selective cleavage of RNA under physiological conditions. One of these involves the use of bleomycin (BLM), an antitumor agent long studied for its DNA cleavage properties. Bleomycin-mediated RNA cleavage was found to be highly selective, metal dependent and oxidative in nature. The second reagent employed for RNA cleavage was $Mn^{2+}$, which was shown to catalyze the cleavage of an RNA hairpin at a specific position. Cleavage occurred by a solvolytic mechanism under physiological conditions, and also when the hairpin motif was present within a larger RNA.

## RESULTS AND DISCUSSION

### Bleomycin-Mediated RNA Cleavage

Although the cleavage of DNA by metallobleomycins has been studied in detail,[10,11] the available evidence has suggested strongly that RNA was refractory to BLM-mediated degradation.[12] Recently, however, Magliozzo et al[13] have shown that yeast $tRNA^{Phe}$ could be degraded to a limited extent by very high concentrations of Fe(II)·BLM. To assess whether this limited cleavage might actually have been due to highly efficient cleavage at a limited number of sites, we studied a number of *in vitro* RNA transcripts as potential substrates for Fe·BLM. As illustrated in Figure 1 for a *Bacillus subtilis* $tRNA^{His}$ precursor transcript, some of the substrates were cleaved by Fe(II)·BLM in a highly selective fashion. Cleavage proceeded readily at 22, 37 and 55°C, but not at 0°C. While this experiment was carried out using 300 μM Fe(II)·BLM $A_2$, in other experiments Fe(II)·BLM $A_2$ gave efficient cleavage of the same substrate at concentrations as low as 3 μM.[12] In comparison with BLM-mediated DNA cleavage,[10,11] degradation of the RNA transcript proceeded at comparable concentrations of added bleomycin, but with much greater site selectivity.

The predominant site of $tRNA^{His}$ precursor cleavage by Fe(II)·BLM was determined by RNA sequence analysis[14] to be at $uridine_{35}$ (Fig 1); in the cloverleaf representation of

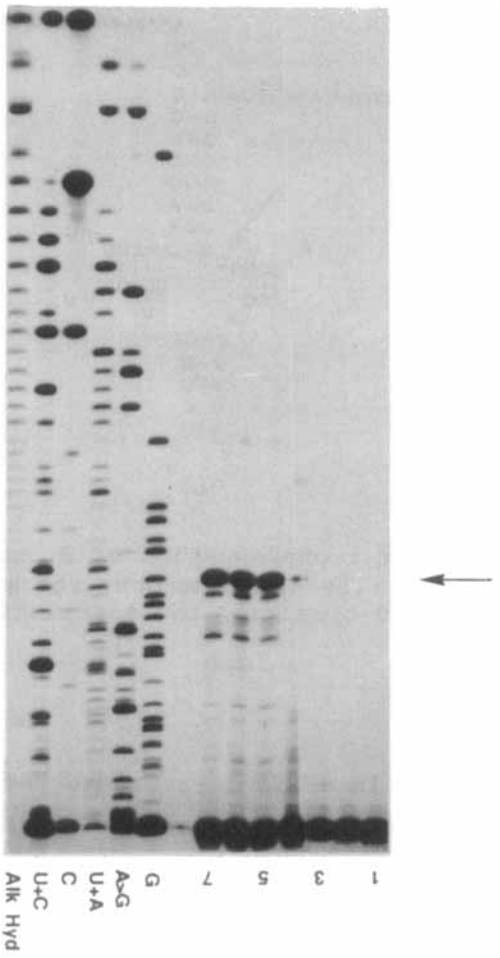


FIG. 1. Fe(II)·BLM-mediated cleavage of 5'-$^{32}$P-labeled $tRNA^{His}$ precursor at each of four temperatures, and enzymatic sequencing reactions identifying the cleavage site(s). Reactions (5 μL total volume) contained radiolabeled $tRNA^{His}$ precursor, 5 mM $NaH_2PO_4$, pH 7.0, and Fe(II)·BLM $A_2$. Reactions were incubated at the indicated temperature for 1 hr and then quenched by the addition of loading buffer and analyzed on a 20% polyacrylamide gel. Lanes 1-7 contained ~ 2.5 μM (final base concentration) 5'-end labeled $tRNA^{His}$ precursor. Lane 1, $tRNA^{His}$ precursor alone; lane 2, 300 μM BLM; lane 3, 300 μM Fe(II); lanes 4-7, 300 μM Fe(II)·BLM at 0, 22, 37, or 55° C, respectively. The remaining lanes were enzymatic sequencing reactions, as indicated.

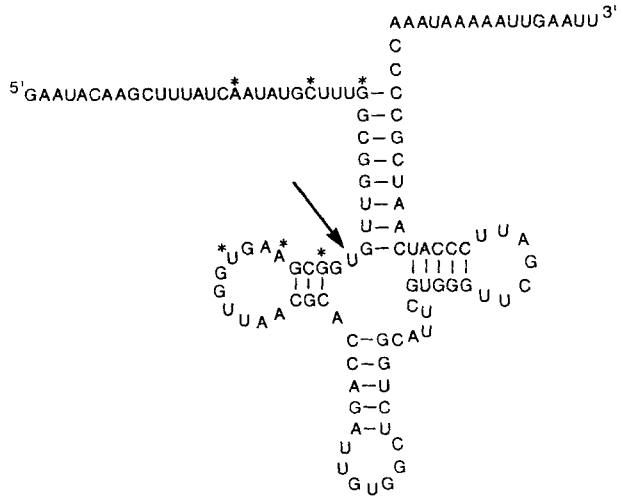


FIG. 2. Cloverleaf representation of *B. subtilis* $tRNA^{His}$ precursor. The arrow denotes the major site of Fe(II)·BLM-mediated cleavage; the asterisks denote the minor sites.



tRNA, this uridine is in a single-stranded region located adjacent to a double-stranded region of RNA (Fig 2). Because the enzymatic RNA sequencing method employed to determine the sites of BLM-mediated RNA cleavage produces strand scission by hydrolysis of phosphodiester bonds with concomitant formation of oligonucleotides having nucleoside cyclic 2',3'-phosphates at their 3'-termini, the bands from individual base-specific reactions actually consist of chemical species different than those which result from agents that produce strand scission by chemical degradation of individual nucleotides. Accordingly, the assignment of the position of BLM-mediated cleavage assumed that Fe(II)·BLM cleaved the RNA transcript in the same (oxidative) fashion in which it has been shown to degrade DNA.[10,11]

The actual nature of Fe·BLM-mediated RNA degradation was studied in a few different ways to test the validity of the foregoing assumption. In common with the BLM-mediated

degradation of DNA,[10,11] the cleavage of $tRNA^{His}$ precursor proceeded readily in the presence of Fe(II)·BLM but not Fe(III)·BLM (Fig 3). The addition of exogenous reductants has been noted to potentiate DNA degradation by Fe·BLM; in fact, added ascorbate also promoted increased cleavage of $tRNA^{His}$ precursor by Fe(II)·BLM (not shown). The products of oxidative DNA damage by Fe·BLM are known to include free bases.[10,11] Since cleavage of $tRNA^{His}$ precursor was believed to occur primarily at $U_{35}$, it was anticipated that oxidative cleavage at that position should result in the release of uracil. In fact, Fe(II)·BLM treatment of a $tRNA^{His}$ precursor generally radiolabeled within the pyrimidine moieties of the uridines produced the expected amounts of a product that co-migrated with authentic uracil (reverse phase HPLC).

Also studied was the ability of Fe(II)·BLM to effect the cleavage of other RNA substrates. Analogous treatment of *E. coli* $tRNA^{Tyr}$ precursor failed to produce significant amounts of strand scission, even when much greater amounts of Fe(II)·BLM were employed. On the other hand, a 231-nucleotide RNA transcript produced from pSP64 plasmid DNA afforded cleavage predominantly at two major sites. The sites of cleavage were determined by RNA sequence analysis; both were located in regions believed to be at the junction between single-and double-stranded regions of the RNA. Likewise, a 5'-$^{32}$P end labeled RNA transcript 270 nucleotides in length, encoding the N-terminus of HIV reverse transcriptase, was found to be susceptible to cleavage by Fe(II)·BLM. At a BLM: RNA nucleotide ratio similar to that used for cleavage of *B. subtilis* $tRNA^{His}$ precursor, the mRNA was cleaved at four sites, two of which were strong cleavage sites.

The selectivity of RNA cleavage was studied further by carrying out the degradation of $^{32}$P-end labeled $tRNA^{His}$


FIG. 3. Cleavage of a 5'-$^{32}$P-labeled $tRNA^{His}$ precursor by Fe(II)·BLM $A_2$ and Fe(III)·BLM $A_2$. The reactions were carried out essentially as described in the legend to Figure 1, at 22° C. The left lane contained 25 μM Fe(III)·BLM $A_2$; the right lane 25 μM Fe(II)·BLM $A_2$.

precursor in the presence of a large excess of unfractionated tRNA's or calf thymus DNA. As described,[12] even in the presence of a large (>$10^3$) molar excess of unfractionated *E. coli* tRNA, the ability of Fe(II)·BLM $A_2$ to effect strand scission of $tRNA^{His}$ precursor at $U_{35}$ was diminished only about 10-fold. On the other hand, while admixture of an equimolar amount of DNA to $tRNA^{His}$ precursor had no effect on Fe(II)·BLM-mediated degradation of $tRNA^{His}$ precursor, no RNA strand scission was observed in the presence of a large excess of DNA. The ability of DNA to diminish BLM-mediated RNA degradation was quantified by treatment of $^{32}P$-end labeled $tRNA^{His}$ precursor with 10 μM Fe(II)·BLM $A_2$ in the presence of several different concentrations of calf thymus DNA. As illustrated in Fig 4, $tRNA^{His}$ precursor cleavage was diminished substantially in the presence of 5 μM calf thymus DNA, and essentially completely at DNA nucleotide concentrations ≥50 μM.

In the aggregate, the results obtained for Fe(II)·BLM-mediated RNA degradation suggest that strand scission proceeds in a highly selective fashion that reflects a preference for certain RNA conformations rather than specific sequences, as noted for BLM-mediated DNA degradation.[12,15,16] In addition to the obvious implications of our findings for the molecular mechanism(s) by which bleomycin exerts its therapeutic effects, the apparent ability of activated Fe(II)·BLM to discriminate among conformationally distinct sites in RNA suggests that BLM and its structural congeners[17] could be of substantial utility in analyzing RNA tertiary structure.

$Mn^{2+}$-Dependent Cleavage of an RNA Hairpin

In recent years, a number of laboratories have described chemical transformations of RNA structures mediated by the RNA's themselves.[3,4,8] Several RNA self-processing motifs believed to be of importance in the intact

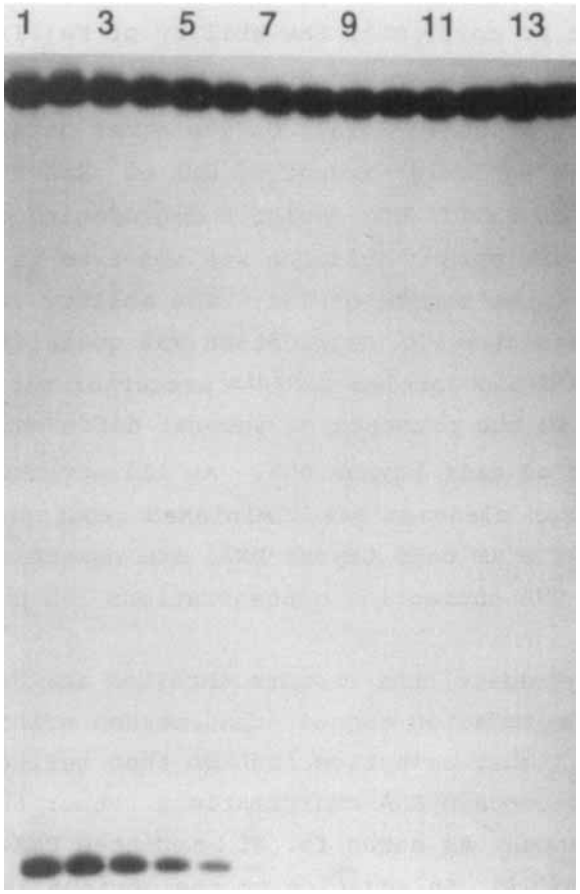


FIG. 4. Cleavage of a 5'-$^{32}$P-labeled $tRNA^{His}$ precursor by Fe(II)·BLM $A_2$ in the presence of calf thymus DNA. The reactions were run essentially as described in the legend to Figure 1, using 270 μM (nucleotide concentration) $tRNA^{His}$ precursor and the indicated concentrations of calf thymus DNA. The reactions were incubated at 22°C for 15 min. Lanes 1-11 contained 10 μM Fe(II)·BLM $A_2$ and 0, 0.05, 0.1, 5, 10, 50, 100, 150, 200, 300 and 400 μM calf thymus DNA, respectively. Lane 12, 10 μM BLM + 200 μM calf thymus DNA. Lane 13, 10 μM $Fe^{2+}$ + 200 μM calf thymus DNA. Lane 14, 200 μM calf thymus DNA.

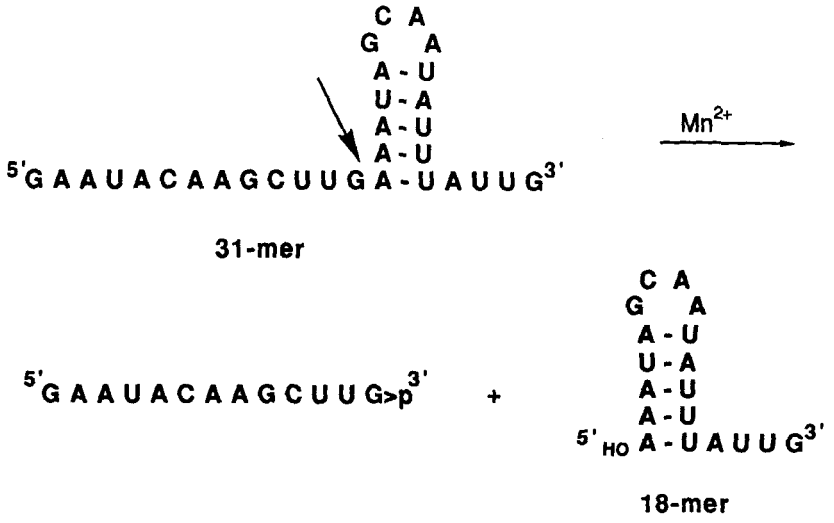


FIG. 5. $Mn^{2+}$-catalyzed cleavage of a 31-nucleotide RNA transcript.



biological systems in which they occur have been described;[3,4] all characterized examples appear to require $Mg^{2+}$ to support the structural transformations noted.

RNA-directed RNA strand scission potentially provides the wherewithal to effect cleavage of a target RNA under physiological conditions; this strategy has been addressed by a few laboratories.[9,18-20] In an effort to identify an RNA-directed cleavage reaction that would proceed with facility in response to some unique exogenous signal, we have investigated the cleavage of an RNA hairpin identical in sequence with the 15-nucleotide hairpin excised from the 5'-end of the 414-nucleotide *Tetrahymena* intron during $Mg^{2+}$-dependent autocyclization.[3] The rather limited stability predicted for this hairpin suggested to us that $Mn^{2+}$ might provide additional stabilization of the hairpin by binding to an RNA base[21] in addition to the phosphate ester binding anticipated for $Mg^{2+}$. As reported previously,[8] $Mn^{2+}$ was

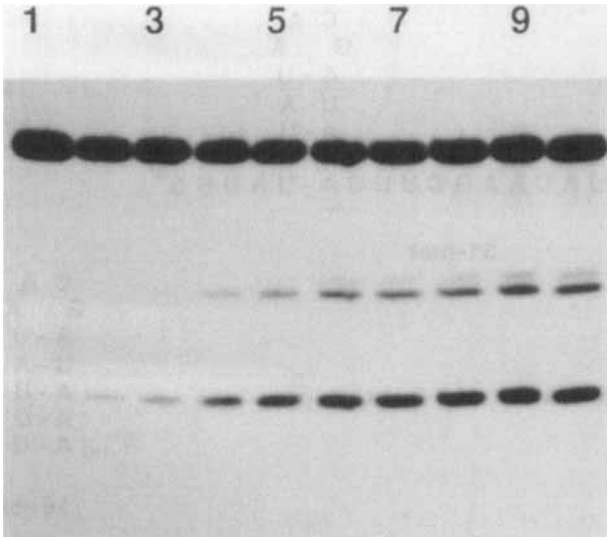


FIG. 6. Time dependent cleavage of a 31-nucleotide RNA transcript in the presence of $Mn^{2+}$. A generally labeled RNA transcript (1.6 x $10^6$ cpm) having the sequence shown in Fig. 5 was incubated in a 120-μL reaction mixture containing 40 mM Tris-OAc, pH 7.5, 3.7 mM spermidine, 100 mM NaCl and 10 mM $MnCl_2$. The reaction mixture was incubated at 45°C and 8-μL aliquots were removed at 0, 10, 20, 30, 40, 55, 70, 85, 100 and 120 min (lanes 1-10, respectively) and analyzed on a denaturing 20% polyacrylamide gel.



found to catalyze the cleavage of 47-and 31- nucleotide RNA's at a specific site on the 5'-end of the hairpin.

As illustrated in Figure 5 for the 31-nucleotide RNA construct, $Mn^{2+}$ promoted cleavage on the 3'-side of $G_{13}$. The products consisted of a 13-nucleotide fragment having a 2',3'-cyclic phosphate at the 3'-terminus, and an 18-nucleotide fragment having a free 5'-OH at the 5'-terminus. Shown in Figure 6 is the time dependence of cleavage of the (generally radiolabeled) 31-nucleotide RNA transcript at 45°C and pH 7.5. The cleavage reaction proceeded steadily over the 2 hr time course of the incubation; in separate

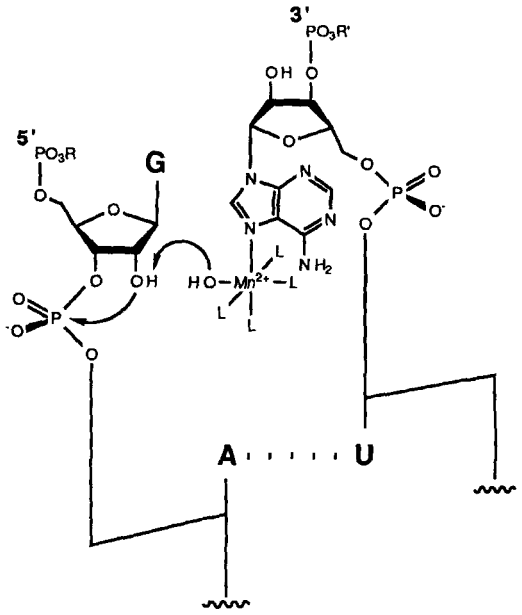


FIG. 7. A possible molecular mechanism for $Mn^{2+}$-catalyzed RNA hairpin cleavage.



experiments it was shown that under similar conditions the same cleavage reaction proceeded readily between pH 6.9 and 7.8, and at temperatures between 23 and 45°C. The cleavage reaction occurred with greatest facility at $Mn^{2+}$ concentrations from 5-10 mM $Mn^{2+}$ and was unaffected by adding $Mg^{2+}$ at concentrations up to 25 mM. The addition of NaCl actually resulted in somewhat more $Mn^{2+}$ dependent strand scission under the conditions utilized, presumably due to a lessening of charge repulsion between the RNA phosphodiester moieties; as anticipated, 25 mM EDTA completed inhibited the cleavage reaction.[8]

Although the data presently available do not permit the chemical mechanism of $Mn^{2+}$-dependent cleavage to be understood with certainty, a plausible mechanism is provided in Figure 7. As suggested in the figure, the observed strand scission could well result from $Mn^{2+}$-hydroxide

promoted deprotonation of the 2'-OH group of $G_{13}$; this would lead to nucleophilic attack of the 2'-alkoxide on the adjacent phosphate ester, with eventual strand scission and formation of the observed products (Fig 5). The postulated involvement of $A_{28}$ in the mechanism provides a rationale for the involvement of $Mn^{2+}$, since this metal ion is known[21] to bind to N-atoms in the nucleic acid bases. In fact, in preliminary experiments it was found that replacement of $A_{28}$ with uridine eliminated $Mn^{2+}$-dependent RNA cleavage, while replacement with cytidine or guanosine afforded constructs whose cleavage in the presence of $Mn^{2+}$ was less facile.

Although identification of the RNA hairpin structures optimal for $Mn^{2+}$-dependent cleavage is ongoing, the ability of $Mn^{2+}$ to effect cleavage of the present hairpin contained within a larger RNA transcript has been demonstrated by the use of a 231-nucleotide RNA transcript that includes the present hairpin structure. It is anticipated that the identification of hairpin motifs cleaved with even greater facility would permit the use of $Mn^{2+}$ as an exogenous chemical switch to regulate the levels of specific RNA transcripts in systems that model transcriptional regulation of gene expression.



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