Iron(II) Bleomycin-Mediated Degradation of a DNA–RNA Heteroduplex†

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ABSTRACT: The effect of iron(II) bleomycin on a DNA–RNA heteroduplex was investigated using a substrate formed by reverse transcription of *Escherichia coli* 5S ribosomal RNA. Both strands of the heteroduplex were cleaved by Fe^{II}·BLM A_2 at comparable concentrations; complete digestion of both strands was observed using 5 μM Fe^{II}·BLM A_2. The DNA strand of the heteroduplex was cleaved predominantly at 5′-G-pyr-3′ sites; the sites of cleavage of the DNA strand were a subset of those observed for the corresponding DNA strand of a DNA duplex of identical sequence. The sites of cleavage of the RNA strand of the heteroduplex involved both purines and pyrimidines and were found to be different than the sites of cleavage of the 5S rRNA alone, demonstrating that cleavage of the former must actually have involved heteroduplex recognition by Fe^{II}·BLM A_2. Both the DNA and RNA strands of the heteroduplex were cleaved by Fe^{II}·BLM A_2 in the presence of physiological concentrations of Mg^{2+}, consistent with the possibility that DNA–RNA heteroduplexes may be therapeutically relevant targets for bleomycin.

Bleomycin A_2 is a member of a family of structurally related glycopeptide antitumor antibiotics (the bleomycins, BLMs[1]) that were first isolated by Umezawa and his co-workers (Umezawa et al., 1966a,b). The assigned structure (Figure 1) was verified by total synthesis (Takita et al., 1982; Aoyagi et al., 1982). The bleomycins are used clinically as antineoplastic agents, e.g. for the treatment of testicular carcinomas (Sikic, 1985), germ cell ovarian cancer (Einhorn & Donohue, 1977), non-Hodgkin's lymphomas (Carlson et al., 1983), and squamous cell carcinomas of the head, neck, skin, and cervix (Bennett & Reich, 1979).

While bleomycin can effect the degradation of both ribonucleic (RNA) and deoxyribonucleic (DNA) acids, the majority of the investigations of the mechanism of BLM action have centered on DNA degradation (Burger et al., 1981, 1986; Hecht, 1986, 1994; Stubbe & Kozarich, 1987; Natrajan et al., 1990, 1994). Bleomycin-mediated nucleic acid degradation has been found to be metal ion dependent, oxidative in nature and sequence-selective (Hecht, 1986, 1994; Stubbe & Kozarich, 1987; Natrajan et al., 1994). The lesions typically involve oxidative destruction of the pyrimidine nucleotides in 5′-GC-3′ and 5′-GT(U)-3′ sequences (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Mirabelli et al., 1982; Carter et al., 1990; Holmes et al., 1993; Hecht, 1994). It is thought that bleomycin-mediated DNA and RNA damage is initiated by H abstraction from the sugar moieties in the minor groove of these substrates (Hecht, 1986, 1994; Stubbe & Kozarich, 1987).

The effects of bleomycin on a variety of nucleic acid structures have been reported. The types of structures investigated include chromosomal DNA (Twentyman, 1984; Berry et al., 1985), supercoiled plasmid DNA and linearized plasmid DNA (Mirabelli et al., 1983), methylated DNA

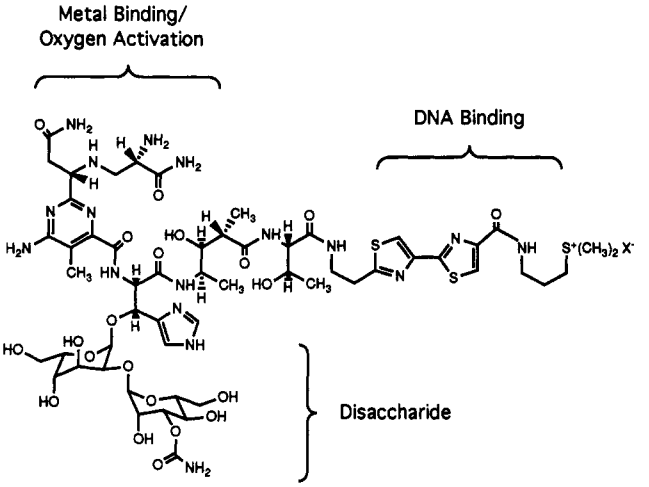

FIGURE 1: Structure of bleomycin A_2.

(Hertzberg et al., 1985, 1988; Long et al., 1990), platinated DNA (Mascharak et al., 1983; Gold et al., 1988), duplex DNA containing bulged regions (Williams & Goldberg, 1988), triplex DNA (Kane, 1993), transfer RNA and transfer RNA precursors (Magliozzo et al., 1989; Carter et al., 1990, 1991a,b; Hüttenhofer et al., 1992; Holmes et al., 1993), messenger RNA (Carter et al., 1990; Dix et al., 1993), ribosomal RNA (Holmes et al., 1993), and DNA–RNA heteroduplexes (Haidle & Bearden, 1975; Krishnamoorthy et al., 1988; Absalon et al., 1992). Of all these types of structures, only the RNA strands of DNA–RNA heteroduplexes were reported not to act as substrates for degradation by Fe·BLM (Haidle & Bearden, 1975; Krishnamoorthy et al., 1988; Absalon et al., 1992). Overall, bleomycin-mediated degradation of DNA is more efficient but less selective than that of RNA (Holmes et al., 1993; Hecht, 1994).

There are several features which make the analysis of the structure and biological functions of heteroduplexes quite interesting. These chimeric duplexes are formed during transcription of the genetic information stored in chromosomal and mitochondrial DNAs, as a consequence of the synthesis of DNA from RNA during retroviral replication, and during the formation of Okazaki fragments, which serve as intermediates for lagging strand synthesis as one step in DNA replication. The structural characterization of heteroduplexes

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[1] Abbreviations: BLM, bleomycin; nt, nucleotide; bp, base pair; PAGE, polyacrylamide gel electrophoresis; mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; cDNA, complementary DNA; pur, purine; pyr, pyrimidine; Tris, tris(hydroxymethyl)aminomethane; DMT, dimethoxytrityl; EDTA, ethylenediaminetetraacetic acid.

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is also of considerable interest in the context of the ongoing development of antisense technologies (Uhlmann & Peyman, 1990).

It has been reported previously that only the DNA strands of poly(rA):poly(dT), poly(dA):poly(rU), poly(rI):poly(dC), and poly(dI):poly(rC) hybrid duplexes were damaged after incubation with Fe^{II}·BLM in the presence of oxygen (Haidle & Bearden, 1975; Krishnamoorthy et al., 1988; Absalon et al., 1992). However, because these homopolymeric species do not constitute an adequate representation of the sequence diversity found in the nucleic acids present in living systems, we have investigated a more representative example. Presently, we demonstrate that a 120-bp heteroduplex of mixed sequence formed by reverse transcription of *E. coli* 5S ribosomal RNA undergoes Fe^{II}·BLM A_2-mediated degradation of both strands. The strands of the heteroduplex were both cleaved with an efficiency comparable to that of B-DNA; the DNA strand was cleaved with the same 5′-G-pyr-3′ sequence selectivity noted previously for other substrates. Sites of RNA cleavage, in comparison, involved both purines and pyrimidines with no obvious common two-base recognition motif. Cleavage of the DNA and RNA strands persisted in the presence of physiological concentrations of Mg^{2+}, suggesting that heteroduplex cleavage could occur upon administration of bleomycin in a therapeutic situation.

EXPERIMENTAL PROCEDURES

Materials. Blenoxane was obtained from Bristol Laboratories and fractionated as described (Chien et al., 1977; Oppenheimer et al., 1979) to afford bleomycin A_2. Synthetic DNA oligonucleotides were purchased from Midland Certified Reagent Co. *E. coli* 5S rRNA, calf intestinal phosphatase, dNTP's, and Quick-Spin columns were purchased from Boehringer-Mannheim. Moloney-murine leukemia virus reverse transcriptase, RNase H minus, was from Promega. T4 polynucleotide kinase and the base-specific ribonucleases used for RNA sequencing were purchased from U.S. Biochemicals. [γ-^{32}P]ATP (7000 Ci/mmol) was from ICN Radiochemicals. Sep-Pak C18 cartridges were purchased from Waters Chromatography.

Methods. Purification of Synthetic Oligonucleotides. Synthetic oligonucleotides were purified by 20% denaturing (8 M urea) PAGE and the bands were visualized by UV shadowing. The bands of interest were excised from the gel, recovered from the gel slice by electroelution, and then desalted on Sep-Pak C18 cartridges.

5′-^{32}P End Labeling of E. coli 5S rRNA. *E. coli* 5S rRNA (4 μg) was dephosphorylated using calf intestinal alkaline phosphatase; the incubation mixture included 0.4 unit of enzyme in 20 μL of 50 mM Tris-HCl, pH 9.0, containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 6% polyethylene glycol, and 10 mM spermidine. Following incubation at 37 °C for 45 min, the dephosphorylated 5′-ends of the *E. coli* 5S rRNA were radiolabeled using 12 units of T4 polynucleotide kinase (50 μL solution of 50 mM Tris-HCl, pH 7.5, containing 5 mM dithiothreitol, 10 mM $MgCl_2$, 0.01 mM spermidine, 0.01 mM EDTA, and 400 μCi of [γ-^{32}P]ATP (7000 Ci/mmol) at 37 °C for 1 h. The RNA was then purified by 20% denaturing PAGE and recovered by the "crush and soak" method (Sambrook et al., 1989).

Reverse Transcription Reaction. An amount of 5 μg of *E. coli* 5S rRNA and 5 μg of synthetic primer were incubated for 60 min at 37 °C in a solution containing 50 μL of 50 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 20 mM dithiothreitol, 75 mM KCl, 1.2 mM each dNTP's, and 1200 units of Moloney-murine leukemia virus reverse transcriptase, RNase H$^-$. Small aliquots of the reactions were analyzed either by 10% nondenaturing PAGE or on 5% nondenaturing agarose gels. The oligonucleotides were visualized by staining with ethidium bromide and viewing under a UV lamp. The reaction mixtures were purified on Sephadex G-50 Quick-Spin columns, and the products were then radiolabeled.

5′-^{32}P End Labeling of the DNA Strand of the Heteroduplex. The DNA strand (2 μg) was specifically radiolabeled using 12 units of T4 polynucleotide kinase in 50 μL of 50 mM Tris-HCl, pH 7.5, containing 5 mM dithiothreitol, 10 mM $MgCl_2$, 0.01 mM spermidine, 6% polyethylene glycol, 0.01 mM EDTA, and 400 μCi of [γ-^{32}P]ATP (7000 Ci/mmol). The incubation mixture was maintained at 37 °C for 1 h, and the end labeled heteroduplex was then purified by 10% native PAGE and recovered by the "crush and soak" method (Sambrook et al., 1989).

5′-^{32}P End Labeling of the RNA Strand of the Heteroduplex. The reverse transcription product, prepared using a DNA primer blocked at the 5′-end with a dimethoxytrityl group, was purified on a Sephadex G-50 Quick-Spin column. The RNA strand of the heteroduplex was dephosphorylated using 0.4 unit of calf intestinal phosphatase in 20 μL of 50 mM Tris-HCl, pH 9.0, containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 6% polyethylene glycol, and 10 mM spermidine. The incubation mixture was maintained at 37 °C for 45 min and the enzyme was then denatured by heating the reaction solution at 75 °C for 10 min. The RNA strand of the heteroduplex ($\sim$5 μg) was then 5′-^{32}P end labeled under the same conditions as described above for the DNA strand of the heteroduplex.

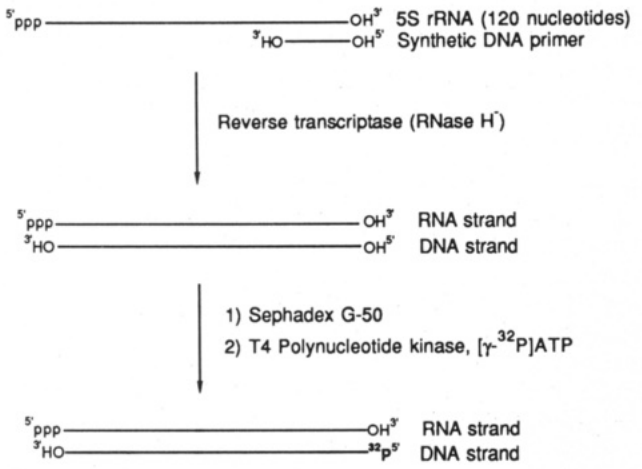
DNA and RNA Sequencing. Maxam–Gilbert DNA sequencing was carried out essentially as described (Sambrook et al., 1989). RNA sequencing was carried out using base specific ribonucleases with a kit purchased from U.S. Biochemicals.

Bleomycin Reactions. Radiolabeled oligonucleotides were incubated with Fe^{II}·BLM A_2 in 5-μL reaction mixtures that contained 10 mM sodium cacodylate buffer, pH 7.6, calf thymus DNA (5 μM final nucleotide concentration) and varying concentrations of Fe^{II}·BLM A_2. The reactions were initiated by the simultaneous addition of equal amounts of Fe^{2+} and BLM A_2 from freshly prepared solutions, to the final concentrations indicated in the figure legends. The reaction mixtures were incubated at 0 °C for 30 min, and then quenched by addition of 3 μL of a solution of 50 mM Tris-borate, pH 8.3, containing 1 mM EDTA, 80% (w/v) deionized formamide, 0.1% (w/v) xylene cyanol, and 0.1% (w/v) bromophenol blue prior to being analyzed by 20% denaturing PAGE (containing 8M urea) and visualized by autoradiography.

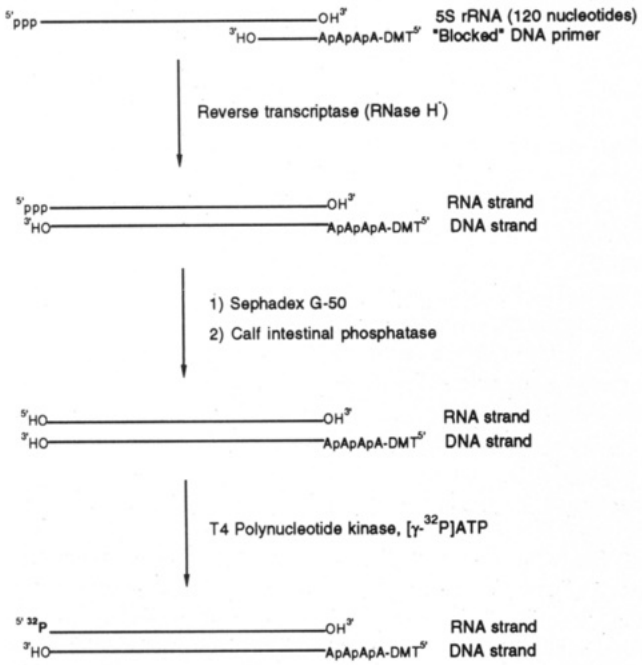
RESULTS

The formation of the DNA–RNA heteroduplex was accomplished by reverse transcription of *E. coli* 5S rRNA. In order to investigate the Fe(II)·BLM A_2-mediated degradation of each strand of the heteroduplex, it was essential to be able to radiolabel each strand uniquely in separate experiments. The strategy for accomplishing this selective labeling is outlined in Schemes 1 and 2. In both cases, the heteroduplex was formed via reverse transcription of *E. coli* 5S rRNA. As shown in Scheme 1, the DNA strand of the formed heteroduplex was preferentially 5′-^{32}P end labeled by using T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Since the original RNA strand had been obtained by transcription, it possessed a 5′-terminal triphosphate and thus was not

Scheme 1: Strategy for the Preparation of a DNA–RNA Heteroduplex Having a 5′-^{32}P End Labeled DNA Strand



Scheme 2: Strategy for the Preparation of a DNA–RNA Heteroduplex Having a 5′-^{32}P End Labeled RNA Strand



phosphorylated. To permit the selective radiolabeling of the 5′-end of the RNA strand of the heteroduplex, a synthetic DNA primer that was blocked at the 5′-end with a dimethoxytrityl group was used in the reverse transcription reaction (Scheme 2). The phosphate groups on the 5′-end of the RNA strand were then removed with calf intestinal phosphatase, and the RNA was selectively radiolabeled at the 5′-end via the agency of T4 polynucleotide kinase. As can be seen in Figure 2, these radiolabeling procedures were successful, producing species that comigrated as single bands when analyzed by 10% nondenaturing PAGE.

As one might have anticipated based on the wealth of information dealing with BLM-induced degradation of duplex DNA (Hecht, 1986; Stubbe & Kozarich, 1987), Fe^{II}·BLM A_2-mediated oxidative destruction of the DNA strand of the heteroduplex was sequence-specific. Every lane of the 20% denaturing (8 M urea) polyacrylamide gel depicted in Figure 3 contained the DNA–RNA heteroduplex having the ^{32}P label at the 5′-end of the DNA strand. As the figure illustrates, three major cleavage sites were found; sequence analysis indicated that these occurred at the pyrimidine bases of 5′-G_3C_4-3′, 5′-G_8C_9-3′, and 5′-$G_{11}T_{12}$-3′. The last of these bands

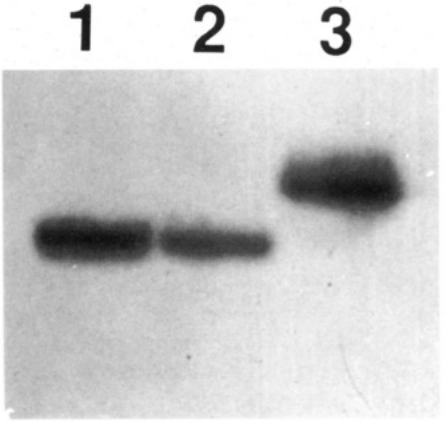


FIGURE 2: Nondenaturing 10% PAGE analysis of 5′-^{32}P end labeled 120-bp heteroduplexes in which either the RNA strand (lane 1) or DNA strand (lane 2) was radiolabeled selectively. Lane 3 contained a 5′-^{32}P end labeled 159-bp DNA duplex.

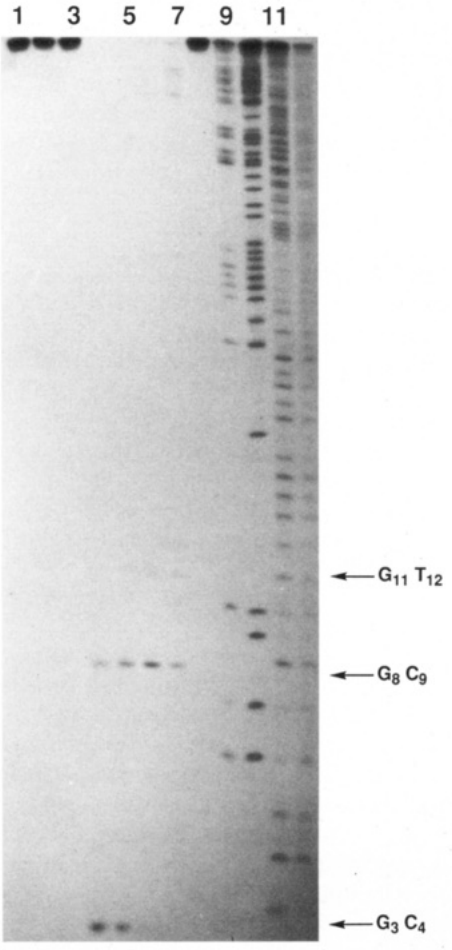


FIGURE 3: Fe^{II}·BLM A_2-mediated cleavage of a 120-bp DNA–RNA heteroduplex 5′-^{32}P end labeled on the DNA strand. The heteroduplex was treated with Fe^{II}·BLM A_2 as described in the Experimental Procedures: lane 1, heteroduplex alone; lane 2, 125 μM BLM A_2; lane 3, 125 μM Fe^{2+}; lanes 4–8, 125, 50, 10, 5, and 1 μM Fe^{II}·BLM A_2; lanes 9–12, G, G+A, T+C, and C-lanes, respectively.

was not prominent in this specific experiment, but was much more readily apparent in other experiments (*vide infra*). Also apparent in Figure 3, lanes 6 and 7, and in other experiments where the DNA substrate was only partially degraded, were the appearance of additional DNA cleavage bands of lesser intensity. These included the second bases of the sequences 5′-$A_{26}T_{27}$-3′, 5′-$G_{31}A_{32}$-3′, 5′-$G_{51}C_{52}$-3′, 5′-$G_{53}C_{54}$-3′, 5′-$G_{59}C_{60}$-3′, 5′-$G_{74}T_{75}$-3′, and 5′-$G_{91}T_{92}$-3′. The sequence specificity of DNA cleavage was entirely consistent

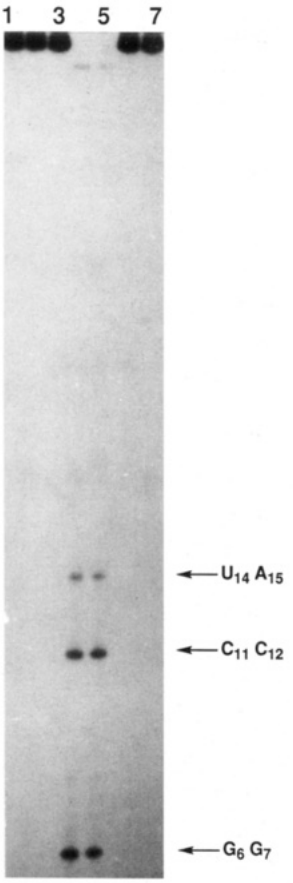


FIGURE 4: Fe^{II}·BLM A_2-mediated cleavage of a 120-bp DNA–RNA heteroduplex 5′-^{32}P end labeled on the RNA strand. The heteroduplex was treated with Fe^{II}·BLM A_2 as described in the Experimental Procedures: lane 1, heteroduplex alone; lane 2, 5 μM BLM A_2; lane 3, 5 μM Fe^{2+}; lanes 4–7, 10, 5, 2.5, and 1 μM Fe^{II}·BLM A_2. Three of the four cleavage sites are indicated by arrows; a cleavage band near the 5′-end of the RNA migrated off the bottom of this gel during electrophoresis.

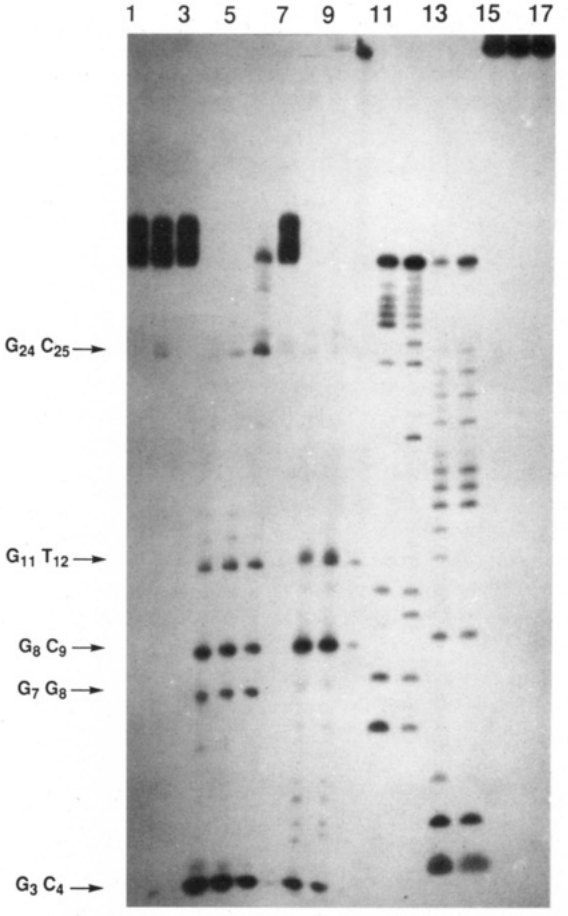


FIGURE 5: Comparison of Fe^{II}·BLM A_2-mediated cleavage of a 120-bp DNA–RNA heteroduplex 5′-^{32}P end labeled on the DNA strand and a 36-bp DNA duplex 5′-^{32}P end labeled on the DNA strand identical in sequence with the first 36 nt of the DNA strand of the heteroduplex. The substrates were treated with Fe^{II}·BLM A_2 as described in the Experimental Procedures: lanes 1–7 and 11–14 contained the 36-bp DNA duplex; lanes 8–10 and 15–17 contained the 120-bp heteroduplex. Lane 1, DNA duplex alone; lane 2, 10 μM BLM A_2; lane 3, 10 μM Fe^{2+}; lanes 4–7, 10, 5, 2.5, and 1 μM Fe^{II}·BLM A_2, respectively; lanes 8–10, 5, 2.5, and 1 μM Fe^{II}·BLM A_2, respectively; lanes 11–14, G, G+A, T+C and C-lanes, respectively; lane 15, DNA–RNA heteroduplex alone; lane 16, 5 μM BLM A_2; lane 17, 5 μM Fe^{2+}. The multiple bands at the top of lanes 1, 2, 3, and 7 reflect incomplete denaturation of the DNA substrate.

with the sequence selectivity determined previously for cleavage of duplex DNA by activated bleomycin. This is, however, the first demonstration of the sequence selective cleavage of a heteroduplex by Fe·BLM.

Figure 4 illustrates the products formed by the selective scission of the RNA strand of the heteroduplex incubated in the presence of Fe^{II}·BLM A_2. All of the lanes in this gel contained the heteroduplex that had been ^{32}P-labeled at the 5′-end of the RNA strand. As the figure shows, complete destruction of the RNA strand of the heteroduplex was effected in the presence of 5 μM Fe^{II}·BLM A_2, which indicated that the RNA strand of the heteroduplex was as susceptible to cleavage by Fe·BLM as B-form DNA. This represents the first time that the RNA strand of a DNA–RNA heteroduplex has been shown to be cleaved by Fe·BLM. Further, as is apparent from lanes 4 and 5 in Figure 4, it is the first RNA substrate found to be converted essentially completely to cleavage products by activated Fe·BLM. The sites of bleomycin-mediated cleavage of the RNA strand were determined by RNA sequence analysis using base-specific ribonucleases; these involved the second nucleotides of the sequences 5′-C_4U_5-3′, 5′-G_6G_7-3′, 5′-$C_{11}C_{12}$-3′, and 5′-$U_{14}A_{15}$-3′ (Donis-Keller et al., 1977) (supplementary material, Figure 1). The first of these bands was not apparent in Figure 4, but was established in analogous experiments (supplementary material, Figure 1). As in the case of cleavage of the DNA strand of the heteroduplex, additional minor RNA cleavage bands were also noted, especially in those cases in which the heteroduplex was not completely degraded.

Having determined the sequences at which Fe^{II}·BLM A_2 cleaved the DNA strand of the heteroduplex, we were interested in comparing these with the products resulting from cleavage of a DNA duplex having the same sequence. Since all of the major sites of cleavage of the DNA strand of the DNA–RNA heteroduplex were localized at the 5′-end of the DNA strand, a DNA duplex 36-bp in length corresponding to this region was prepared. This DNA duplex was 5′-^{32}P end labeled on the strand having the same sequence as the 5′-end of the DNA strand of the heteroduplex. As shown in Figure 5, the DNA duplex was cleaved efficiently at five sites, three of which were identical with the sites of cleavage of the DNA strand of the heteroduplex. The two additional sites of cleavage of the DNA duplex were at 5′-G_7G_8-3′ and 5′-$G_{24}C_{25}$-3′. The DNA duplex also contained additional minor cleavage sites not shared in common with the DNA strand of the heteroduplex (data not shown). Comparison of the polyacrylamide gels in Figures 3 and 5 reveals that the cleavage of the

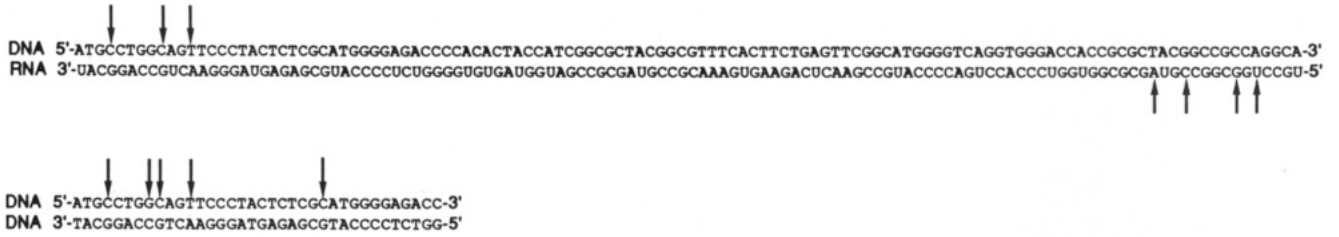


FIGURE 6: Sites of Fe^{II}·BLM A_2-mediated cleavage of the DNA–RNA heteroduplex and the 36-bp DNA duplex.

heteroduplex at 5′-$G_{11}T_{12}$-3′ was more prominent in Figure 5, although it was still the weakest of the three bands.

The cleavage products resulting from cleavage of the DNA heteroduplex in this experiment were analyzed using a Molecular Dynamics PhosphorImager. As anticipated, the products derived from the DNA strand of the heteroduplex included two bands at each cleavage site, which had the gel mobilities associated with oligonucleotide 3′-phosphates and 3′-phosphoroglycolates (Henner et al., 1983; Hertzberg & Dervan, 1984). Thus the chemical products resulting from cleavage of the DNA strand of the heteroduplex are the same as those known to be produced from duplex DNA upon treatment with Fe·BLM (Takeshita et al., 1978; Kross et al., 1982a,b). This finding was entirely consistent with the results reported for DNA–RNA heteroduplexes prepared using homopolymeric substrates (Krishnamoorthy et al., 1988; Absalon et al., 1992). Figure 6 gives a summary of the major sites of cleavage of the DNA and RNA strands of the heteroduplex and of the labeled strand of the DNA duplex.

Yeast 5S ribosomal RNA has been shown to be a substrate for cleavage by Fe^{II}·BLM A_2 (Holmes et al., 1993); since the sequence and secondary structure of yeast and *E. coli* 5S rRNAs are known to be quite similar (Garret et al., 1981; Pieler & Erdman, 1982; Nazar, 1991), it seemed reasonable to believe that the latter would also act as a substrate for cleavage by Fe^{II}·BLM. Therefore, it was necessary to demonstrate that cleavage of the RNA strand of the heteroduplex actually occurred while this strand was bound to its DNA complement. Accordingly, a 5′-^{32}P end labeled *E. coli* 5S rRNA was studied in comparison with a sample of the DNA–RNA heteroduplex labeled at the 5′-end of the RNA strand; both radiolabeled RNA strands had the same specific activity. As shown in Figure 7, *E. coli* 5S rRNA was only a weak substrate for cleavage by Fe^{II}·BLM A_2 in comparison with the RNA strand of the heteroduplex. The same results were obtained both at 0 °C and at 25 °C. Although the exact sites of cleavage of the *E. coli* 5S rRNA have not been established as yet, it is clear from a darker exposure of Figure 7 and from other experiments (not shown) that they are quite different than the sites of cleavage of the RNA strand of the heteroduplex, primarily involving several sites in the region encompassing nucleotides 80–100 of the ribosomal RNA. Further, admixture of the labeled samples of *E. coli* 5S rRNA and DNA–RNA heteroduplex in the same reaction mixture prior to addition of Fe^{II}·BLM A_2 afforded products no different than those observed when the two species were treated separately with Fe·BLM A_2. Thus, the sites of cleavage summarized for the RNA strand in Figure 6 must represent cleavage of the RNA bound to its complementary DNA strand.

Since it had been shown that Mg^{2+} can affect the facility with which bleomycin induces strand scission of polynucleotides (Hüttenhoffer et al., 1992; Holmes et al., 1993), it seemed important to investigate the effect that Mg^{2+} had on Fe^{II}·BLM A_2-mediated scission of the DNA and RNA strands of the heteroduplex. Cleavage of the DNA strand of the

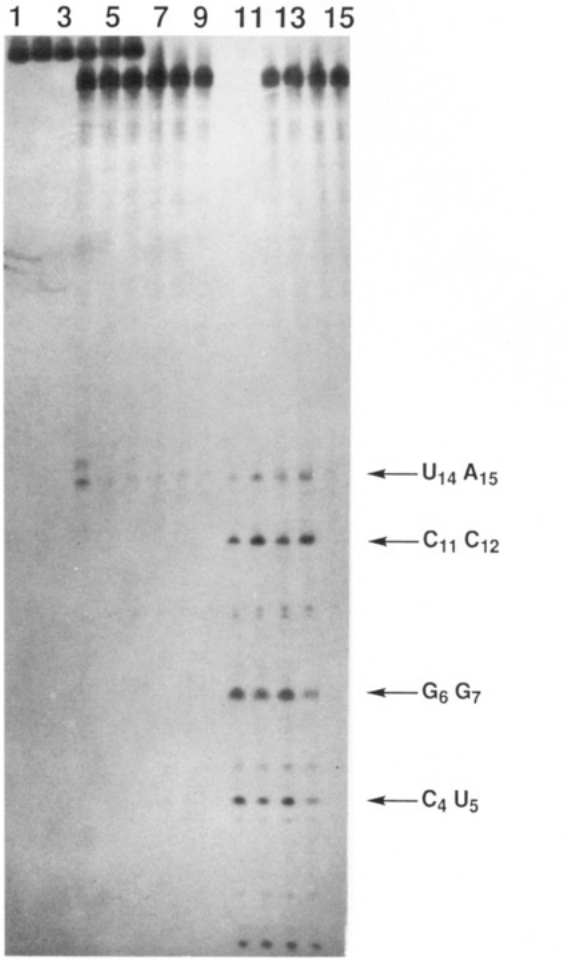


FIGURE 7: Comparison of Fe^{II}·BLM A_2-mediated cleavage of the RNA strand of the 120-bp DNA–RNA heteroduplex and 5′-^{32}P end labeled *E. coli* 5S rRNA. The substrates were treated with Fe^{II}·BLM A_2 as described in the Experimental Procedures. Lanes 1–3, 10, and 11 contained the DNA–RNA heteroduplex 5′-^{32}P end labeled on the RNA strand; lanes 7–9, 14, and 15 contained 5′-^{32}P end labeled 5S rRNA; lanes 4–6, 12, and 13 contained both substrates. Lane 1, heteroduplex alone; lane 2, heteroduplex + 5 μM BLM A_2; lane 3, heteroduplex + 5 μM Fe^{2+}; lane 4, mixture of both substrates; lane 5, both substrates + 5 μM BLM A_2; lane 6, both substrates + 5 μM Fe^{2+}; lane 7, 5S rRNA alone; lane 8, 5S rRNA + 5 μM BLM A_2; lane 9, 5S rRNA + 5 μM Fe^{2+}; lanes 10 and 11, heteroduplex + 5 μM or 2.5 μM Fe^{II}·BLM A_2, respectively; lanes 12 and 13, both substrates + 5 μM or 2.5 μM Fe^{II}·BLM A_2, respectively; lanes 14 and 15; 5S rRNA + 5 μM or 2.5 μM Fe^{II}·BLM A_2, respectively.

heteroduplex was inhibited when 0.5–1 mM Mg^{2+} was present (supplementary material, Figure 2, lanes 10 and 11), while cleavage of the RNA strand was more refractory to added Mg^{2+}, exhibiting complete inhibition only at concentrations >1 mM Mg^{2+} (Figure 8, lanes 11 and 12). Thus both strands of the heteroduplex were cleaved by Fe·BLM at physiological concentrations of Mg^{2+}, consistent with the possibility that heteroduplexes may serve as therapeutically relevant targets for bleomycin.

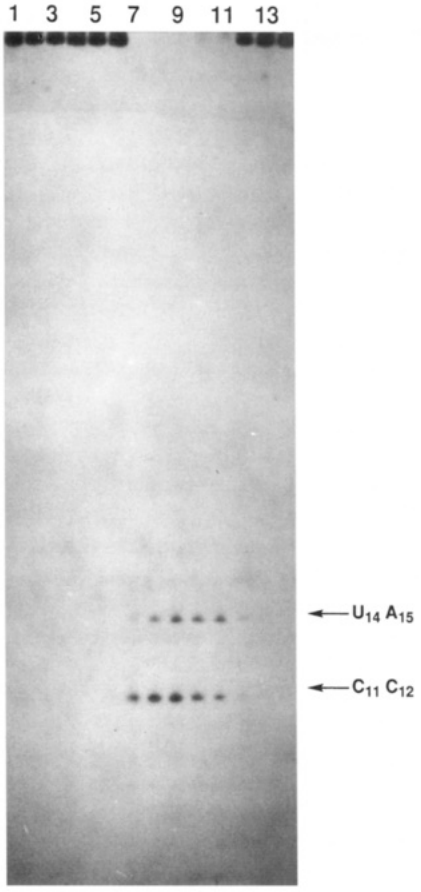


FIGURE 8: Effect of Mg^{2+} on Fe·BLM-mediated cleavage of the RNA strand of the DNA–RNA heteroduplex. Samples of the heteroduplex 5′-^{32}P end labeled on the RNA strand were treated with Fe^{II}·BLM A_2 as described in the Experimental Procedures. Lane 1, heteroduplex alone; lane 2, 5 μM BLM A_2; lane 3, 5 μM Fe^{2+}; lane 4, 25 mM Mg^{2+}; lane 5, 5 μM BLM A_2 + 25 mM Mg^{2+}; lane 6, 5 μM Fe^{2+} + 25 mM Mg^{2+}; lane 7, 5 μM Fe^{II}·BLM A_2; lanes 8–14, 5 μM Fe^{II}·BLM A_2 + 0.1, 0.25, 0.5, 1, 5, 10, and 25 mM Mg^{2+}, respectively.

DISCUSSION

The ability of Fe^{II}·BLM to mediate the destruction of nucleic acid oligo- and polynucleotides has been studied using several different types of substrates (*vide supra*). The cleavage of these species have a number of features in common, including the oxidative nature of the transformation and the sequence selectivity of cleavage, which involves 5′-GC-3′ and 5′-GT-(U)-3′ sites more frequently than others (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Mirabelli et al., 1982; Carter et al., 1990; Holmes et al., 1993; Hecht, 1994). Strand scission can occur with enhanced efficiency at the junction between single- and double-stranded regions (Carter et al., 1990; Holmes et al., 1993; Holmes & Hecht, 1993, Hecht, 1994), including both DNA and RNA bulges (Williams & Goldberg, 1988; Holmes et al., 1993).

While bleomycin-mediated RNA strand scission has many features in common with DNA cleavage, previous studies of RNA cleavage have suggested at least a few characteristics not shared for the analogous cleavage of DNA. These include a much greater selectivity of cleavage of RNA (Carter et al., 1990; Holmes et al., 1993), and the absence of any example of double-strand cleavage of RNA or cleavage of RNA near the end of a strand (Holmes et al., 1993; Hecht, 1994). By the use of a tRNA precursor and its corresponding tDNA as substrates for Fe^{II}·BLM, Holmes and Hecht (1993) have shown that at least some of the unusual cleavage sites observed for tRNA's and tRNA precursors derive from the unique conformations assumed by the RNA's.

The actual chemistry of RNA cleavage is less well defined than that of DNA. Oxidative destruction of *B. subtilis* $tRNA^{His}$ precursor by Fe^{II}·BLM A_2 afforded strand scission primarily at U_{35} (Carter et al., 1990); strand breaks were shown to be formed to essentially the same extent as free uracil (Holmes, 1993). That the strand scission products were analogous to those resulting from cleavage of DNA was suggested both by the migration of Fe^{II}·BLM-induced cleavage bands of 5′- and 3′-^{32}P end labeled yeast 5S ribosomal RNA (Holmes et al., 1993) and from the chemical products observed following treatment of C_3-*ribo* CGCTAGCG with Fe^{II}·BLM (Duff et al., 1993). The latter included $CpGp_{CH_2COOH}$, indicative of oxidative destruction of $cytidine_3$ via initial abstraction of C-4′H. In the present case, the cleavage bands also migrated ahead of the bands produced by base-specific nucleases, consistent with the same chemical products observed previously for RNA and DNA (supplementary materials, Figure 1). At present it is not known whether abstraction of C-4′H in RNA leads to the formation of an alkali-labile lesion comparable to that formed from DNA. It is conceivable that if the extent of alkali lesion formation were disproportionately greater for RNA than for DNA, it could contribute to the greater observed selectivity of cleavage of RNA.

RNA and DNA are obviously different structurally. At the level of constituent nucleosides, the differences involve the absence of a 2′-OH group on the sugars of DNA nucleotides and the presence of uridine in RNA versus a methylated uridine (thymidine) in DNA. These differences in constituent nucleotides result in different sugar puckers within the derived polynucleotides and unique structures for DNA and RNA. DNA tends to adopt a B-form helix whereas RNA generally adopts an A-form helix in solution. These two forms of helixes differ in several ways (Saenger, 1984). Overall, the A-form helix, having the sugars in a C-3′-endo conformation, is a tighter helix with a wider, shallower minor groove as compared to the B-form helix, in which the sugars are in a C-2′-endo conformation. Crystallographic studies of A-DNA and B-DNA helixes have demonstrated that C-1′H is located prominently in the minor grooves of both types of helixes (Drew et al., 1982; McCall et al., 1985). In contrast, C-4′H is located prominently within the minor groove only for B-DNA. As noted previously (Hecht, 1994), the lesser accessibility of C-4′H within the minor groove of an A-form helix could constitute the molecular basis for the observed abstraction of C-1′H from two chimeric oligonucleotides by Fe·BLM (Duff et al., 1993) and for the greater selectivity of RNA strand scission by Fe·BLM.

The structure of the DNA–RNA heteroduplex has not been defined as rigorously as that of B-DNA and A-DNA. Rich (1960) reported optical density measurements of poly(rA)–poly(dT). In the three decades since this publication appeared, the reported data concerning the structure of hybrid duplexes has been conflicting. It has been reported that heteroduplexes more closely resemble double-stranded RNA (i.e., A-type helixes) (Tunis & Hearst, 1968; O'Brien & MacEwan, 1970; Pardi et al., 1981), that both strands of hybrid duplexes form B-type helixes (Reid et al., 1983; Gupta et al., 1985), that the RNA strand adopts an A-type helix, while the DNA strand adopts a B-type helix (Zimmerman & Pheiffer, 1981; Shindo & Matsumoto, 1984; Arnott et al., 1986) and, finally, that the RNA strand adopts an A-type helix and the DNA strand adopts neither an A- nor B-type helix, but forms an intermediate, O4′-endo conformation (Salazar et al., 1993).

Table 1: Analysis of Cleavage of the DNA Strand of the Heteroduplex at 5′-G-pyr-pur-pur-3′ and 5′-G-pyr-pyr-pyr-3′ Sequences

cleaved strongly by Fe^{II}·BLM A_2	cleaved weakly by Fe^{II}·BLM A_2
ATGCCT	GAGTTC
TGGCAG	AGGTGG
CAGTTC	

These apparent inconsistencies may well be a function of the specific sequences of the oligonucleotides that were studied as well as the methods by which the structural determinations were conducted. It has been reported, for example, that altering the relative humidity of the nucleic acid environment can alter the helix type that an oligonucleotide adopts. At relative humidities below 87%, chimeric duplexes have been reported to form A-type helixes, but two distinct backbone conformations were observed at relative humidities above 92% (Zimmerman & Pheiffer, 1981; Shindo & Matsumoto, 1984).

The present study is the first to document the sequence selectivity of Fe^{II}·BLM-mediated oxidative destruction of the DNA strand of a heteroduplex. In agreement with the preferred cleavage sites determined previously for duplex DNA, the DNA strand of the heteroduplex was degraded selectively primarily at the pyrimidine residues of 5′-GC-3′ and 5′-GT-3′ sequences. It is interesting that the Fe^{II}·BLM A_2 cleavage sites produced from the DNA strand of the heteroduplex were a subset of those derived from the DNA strand of a DNA duplex of identical sequence (Figures 5 and 6). At one level, this suggests that bleomycin recognizes the structure of the DNA strand of a heteroduplex as being similar to that of a DNA strand of a DNA duplex. However, the complex of the DNA strand with an RNA strand imparts a greater selectivity of cleavage in the case of the heteroduplex, which parallels the greater selectivity of cleavage of RNA substrates by Fe·BLM. Also of interest was the production of different minor cleavage products from these two substrates. This provides a further indication of the ability of bleomycin to discriminate between nucleic acid structures and also provides an additional type of data that characterizes the nature of DNA–RNA heteroduplex structure (*vide supra*).

The DNA strand of the heteroduplex contains five sites having the sequences 5′-G-pyr-pur-pur-3′ or 5′-G-pyr-pyr-pyr-3′. As shown in Table 1, the only three of these cleaved strongly by Fe^{II}·BLM A_2 were those preceded by one purine and one pyrimidine.

The present study also provides the first report of the degradation of a DNA–RNA heteroduplex of mixed sequence by Fe·BLM and the first evidence of degradation of the RNA strand of a heteroduplex (Figure 4, lanes 4–7). Although it has been reported previously that only the DNA strands of heteroduplexes were degraded by bleomycin (Haidle & Bearden, 1975; Krishnamoorthy et al., 1988; Absalon et al., 1992), the substrates used involved only heteroduplexes whose individual strands were homopolymers. While these earlier findings of DNA strand-specific cleavage are interesting at a mechanistic level, the substrates employed do not adequately reflect the diversity of sequences, and presumably of structural types, present within heteroduplexes in living systems.

The RNA strand of the heteroduplex is the best RNA substrate for bleomycin reported to date; complete degradation was effected in the presence of 5 μM Fe^{II}·BLM A_2. To date, this RNA substrate is the only one found to be converted to strand scission products completely by bleomycin, even though other efficient substrates have been treated with much higher concentrations of Fe^{II}·BLM (Holmes et al., 1993; Holmes & Hecht, 1993; Hecht, 1994). The efficiency of cleavage of both strands of this DNA–RNA heteroduplex is so pronounced that to date it has been difficult to repeatedly effect only partial destruction of either strand under a predetermined set of experimental conditions. The fact that most of the sites of cleavage on both the DNA and RNA strands were closest to the sites of attachment of the radiolabel might be thought to argue that multiple cleavage events have occurred on each strand. However, a number of additional experiments in which only partial cleavage of the DNA or RNA strands of the heteroduplex was obtained (see, e.g., supplementary materials, Figures 1 and 2) revealed only weak cleavage bands at sites other than those indicated in Figure 6. This was true both in the presence and absence of Mg^{2+}, reinforcing the generality of the observation.

It may also be noted that the cleavage of the RNA strand of the heteroduplex at uridine$_5$ constitutes the first example of the cleavage of an RNA near the end of a strand. This parallels the cleavage of several short DNA oligonucleotides by Fe^{II}·BLM (Sugiyama et al., 1985, 1986) and potentially provides a method to characterize the actual chemistry of RNA degradation by Fe·BLM with greater facility and precision.

The finding that the RNA strand of the DNA–RNA heteroduplex was cleaved with much greater efficiency, and at different sites, than the 5S rRNA from which the heteroduplex was derived lends support to the thesis that bleomycin recognizes and distinguishes between higher order nucleic acid structures (Holmes et al., 1993; Hecht, 1994). By the use of *E. coli* 5S rRNA and its derived DNA–RNA heteroduplex, we have demonstrated that changing the secondary and tertiary structure of an RNA molecule can result in an alteration in the sites as well as the efficiency of BLM-mediated degradation.

The finding that RNA's can act as efficient substrates for cleavage by Fe·BLM, under conditions that could occur in a therapeutic situation, has underscored the need to characterize all of those RNA species that constitute potential therapeutic targets for the antitumor agent (Hecht, 1994). The present study documents another viable locus for the antitumor activity exhibited by bleomycin. Both the DNA and RNA strands of the heteroduplex were degraded as efficiently as the DNA strands of double-stranded DNA. Heteroduplexes are formed in the nuclei of all eukaryotic cells as they undergo DNA transcription and replication; oxidative destruction of the resulting mRNA strands prior to translation, or of Okazaki fragments during replication, could easily be envisioned to lead to cell death. The highly efficient cleavage of the DNA–RNA heteroduplex studied here also suggests that the analogous structures formed as a consequence of reverse transcription of retroviral RNA's could be particularly susceptible to cleavage by bleomycin.

SUPPLEMENTARY MATERIAL AVAILABLE

Autoradiograms of Fe^{II}·BLM-mediated cleavage of RNA and DNA (3 pages). Ordering information is given on any current masthead page.

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