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Specificity of Deoxyribonucleic Acid Cleavage by Bleomycin, Phleomycin, and Tallysomycin†

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ABSTRACT: The sites of cleavage of DNA by bleomycin A_2, bleomycin B_2, phleomycin, tallysomycin A, and Blenoxane (Bristol-Meyers) in reactions containing equimolar Fe^{2+} and atmospheric oxygen were analyzed by gel electrophoresis of ^{32}P end labeled DNA fragments. Bleomycin A_2 and bleomycin B_2 reactions cleaved DNA at all sites with a frequency equal to that of Blenoxane. At high concentrations of bleomycin the site specificity of cleavage was unchanged. Bleomycin cleavage sites and phleomycin cleavage sites are a subset of sites cleaved in reactions containing tallysomycin A. The nature of 5′ and 3′ termini induced by bleomycin cleavage was investigated. Electrophoresis of bleomycin-induced fragments after alkaline phosphatase or polynucleotide kinase treatment indicated that 5′ termini are phosphoryl groups but 3′ termini are not simple phosphoryl groups. Analysis of bleomycin cleavage of single-stranded DNA substrate showed that cleavage occurs only in regions of potentially double-stranded looped-back sequences. Possible mechanisms for determination of bleomycin cleavage sequence specificity are discussed.

Bleomycin is a glycoprotein antibiotic that is used clinically in the treatment of certain tumors (Blum et al., 1973; Hecht, 1979; Crooke & Bradner, 1976; Suzuki et al., 1969; Umezawa, 1978). The antibiotic is produced by *Streptomyces verticillus* (Umezawa et al., 1966). The clinical preparation in common use, Blenoxane (Bristol-Meyers), is a complex mixture of several different bleomycin species that differ from one another in the structure of the terminal amine group. Blenoxane is comprised predominantly of bleomycin A_2 (approximately 70%) and bleomycin B_2 (approximately 30%). The structure of these compounds is indicated in Figure 1.

Several other antibiotics structurally related to bleomycin have also been tested for antitumor effectiveness. These include the phleomycins. The phleomycin antibiotics differ from bleomycin in the bithiazole ring structure. One of the bithiazole rings is reduced in phleomycin. Recently, a new bleomycin-like compound, tallysomycin, has been introduced into clinical trials (Bradner, 1978; Kawaguchi et al., 1977). Tallysomycins differ from the bleomycins structurally in a few ways but principally by an additional sugar attached to the (aminoethyl)bithiazole moiety (Figure 1).

The bleomycin-like antibiotics bind to and break DNA (Suzuki et al., 1969; Haidle, 1971; Muller et al., 1972; Povirk et al., 1977; Strong & Crooke, 1978; Mirabelli et al., 1979, 1980). Strand scission by these compounds requires both ferrous ion and molecular oxygen (Sausville et al., 1976, 1978a; Lown & Sim, 1977; Gupta et al., 1979; Dabrowiak et al., 1979; Burger et al., 1979).

The available evidence supports a mechanism whereby a complex of DNA–bleomycin–chelated ferrous ion and molecular oxygen is formed. Oxidation of the bound ferrous ion occurs in this complex with concomitant reduction of oxygen to produce a reactive species (Burger et al., 1979; Povirk et al., 1979; Huang et al., 1980; Suguira, 1980). However, the precise details of the strand scission reaction have yet to be elucidated.

Strand scission by bleomycin is known to occur preferentially at specific sequences. D'Andrea & Haseltine (1978) and Takeshita et al. (1978) have demonstrated that under conditions of limited cleavage, DNA breaks occur preferentially at guanosine–cytidine (GC) and guanosine–thymidine (GT)

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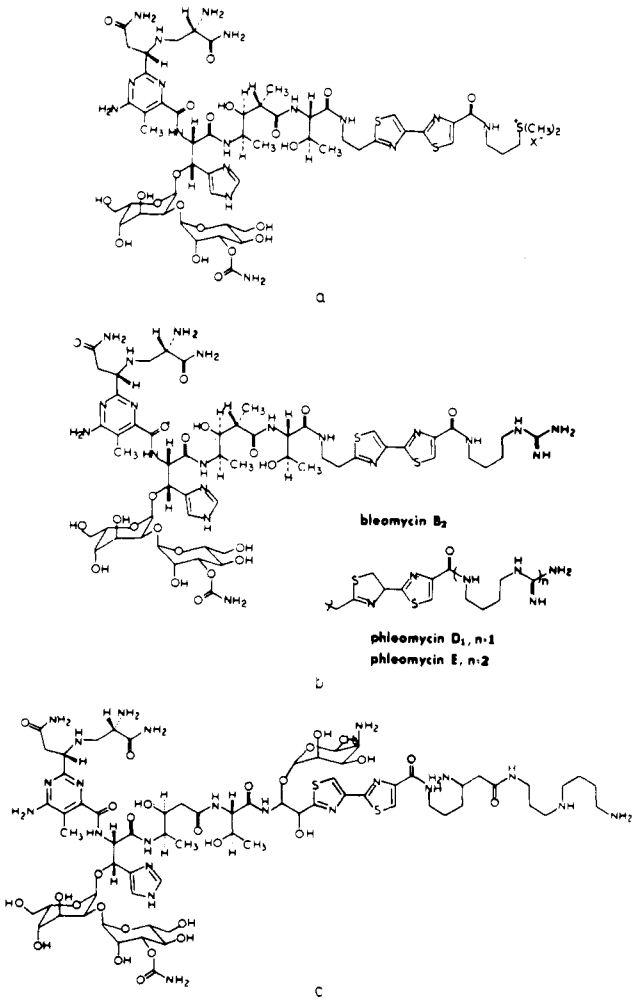

FIGURE 1: Structural diagrams of (a) bleomycin A_2, (b) bleomycin B_2 and phleomycin, and (c) tallysomycin A (Takita et al., 1978; Kawaguchi et al., 1977). The assignment of stereochemistry to tallysomycin A is made without proof, on the assumption that it is the same as that found for bleomycin and phleomycin.

sequences. The specificity for guanosine–cytidine is of particular interest as cleavage at this sequence leads to a higher fraction of double-stranded breaks than would be anticipated upon random nicking of DNA, as the complementary sequence of GC is also GC.

As it is not unlikely that the sequence specificity of the DNA damage is important for the cytotoxic activity of these antibiotics, we have examined the cleavage specificity of several of the bleomycin-like antibiotics. The cleavage specificity of these antibiotics was addressed by examining the site of cleavage of DNA fragments of defined sequence. We have also investigated the structure of the DNA at the termini of the breaks created by bleomycin.

Materials and Methods

Blenoxane, a clinical bleomycin sulfate (70% bleomycin A_2 and 30% bleomycin B_2), tallysomycin A, and phleomycin were provided by Bristol Laboratories through the courtesy of Drs. William Bradner and Stanley Crooke. Bleomycin A_2 and bleomycin B_2 were obtained by fractionation of Blenoxane as previously described (Chien et al., 1977).

Enzymes. Bacterial alkaline phosphatase and T4 polynucleotide kinase were obtained from P-L Biochemicals, Milwaukee, WI. The Klenow fragment of DNA polymerase I of *Escherichia coli* was obtained from New England Biolabs, Beverly, MA.

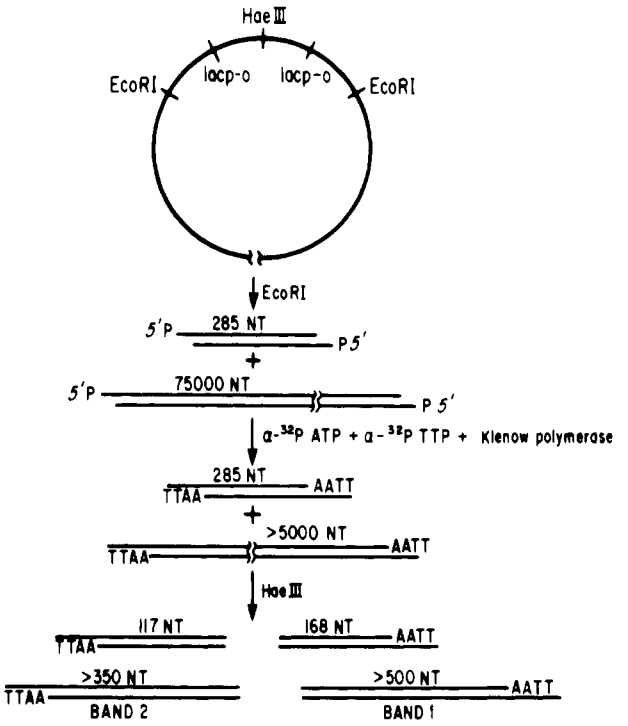

FIGURE 2: Preparation of defined-sequence DNA fragments from pLJ3 plasmid and 3′-end ^{32}P labeling with Klenow polymerase.

Double-Stranded DNA Substrates. Three DNA fragments of defined sequence were obtained from the pLJ3 plasmid (Haseltine et al., 1980). The plasmid was grown in *E. coli* strain NM294 and isolated by the methods of Tanaka & Weisblum (1974), and a 285 base pair insert of a lactose operon promoter–operator sequence was excised by a restriction endonuclease *Eco*RI cleavage of the purified plasmid DNA. The DNA was labeled by an enzymatic extension of the 3′ termini with Klenow polymerase (Klenow & Henningsen, 1970) in the presence of [α-^{32}P]dATP[1] and [α-^{32}P]TTP (S.A. 2800 Ci/mmol) (Sanger & Coulson, 1975). [5′-^{32}P]DNA fragments were obtained by incubation of the DNA with [γ-^{32}P]ATP (S.A. 3200 Ci/mmol) in a reaction that contained T4 polynucleotide kinase and ADP (Chaconas et al., 1975). A treatment with *Hae*III restriction enzyme, followed by DNA isolation on a nondenaturing 10% polyacrylamide gel, yielded four DNA fragments labeled at a single terminus of which three have been sequenced (Figure 2).

A 30 base pair fragment was isolated from pMC1 plasmid grown in *E. coli* strain S90C (Calos et al., 1978). After digestion of pMC1 plasmid DNA with restriction endonuclease *Bst*III, the 3′ terminus was labeled by utilizing the Klenow polymerase and all four [α-^{32}P]dNTPs (S.A. 3000 Ci/mmol). Singly 3′-end labeled fragments were then obtained by digestion with restriction endonuclease *Sau*96I, followed by DNA isolation on a nondenaturing 10% polyacrylamide gel to yield a 30 base pair DNA fragment.

A Single-Stranded DNA Substrate. The 5′-end labeled 168 base pair DNA fragment prepared as described above was resuspended in 0.05 N NaOH–8 M urea loading buffer, heat denatured at 90 °C, and loaded onto a thin 20% polyacrylamide–5 M urea denaturing gel. Electrophoresis was per-

[1] Abbreviations: dATP, 2′-deoxyadenosine 5′-triphosphate; TTP, thymidine 5′-triphosphate; dNTP, 2′-deoxynucleoside 5′-triphosphates; Py, pyrimidine; S.A., specific activity; DMS, dimethyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

formed at 1000 V for 12 h. Such a procedure separates the two strands, as the labeled 168 DNA strand is four nucleotides longer than the unlabeled complementary strand. The labeled strand was located by autoradiography and eluted from the gel.

Reaction Conditions. All bleomycin, tallysomycin A, and phleomycin reactions were done under the following conditions. ^{32}P-Labeled DNA [approximately 1 ng/reaction (S.A. 10^4 cpm/ng)] was suspended in 100 μL of a 25 mM Tris-HCl, pH 7.4, solution. A concentration of a freshly prepared solution of ferrous sulfate equal to the concentration of the antibiotic was included in each reaction. After a 10-min incubation at 37 °C, the reactions were terminated by the addition of 10 μL of a mixture that contained 100 mM EDTA. Reactions that included subsequent treatment with T4 polynucleotide kinase were stopped with a buffer that contained 2.5×10^{-6} M $CoCl_2$, 1 mM magnesium acetate, and 4 M sodium acetate.

For analysis on high-resolution polyacrylamide gels, the DNA products were first precipitated with ethanol. The pellets were washed with cold ethanol, lyophilized, dissolved in a 0.05 N NaOH–7 M urea loading buffer, and heat denatured at 90 °C prior to loading onto thin 8% or 20% polyacrylamide–5 M urea denaturing gels. Electrophoresis was at 1000 V. Autoradiography was done on Kodak XR-1 Exomat film with an intensifying screen at −70 °C. The amount of radioactivity in each scission product was quantitated by measurement of Cerenkov radiation in gel slices with a Beckman LS 7000 scintillation counter. The extent of strand scission is expressed as the fraction of the radioactivity in a lane that migrates faster than intact DNA: (cpm faster than unbroken DNA/total cpm loaded) × 100.

Analysis of 5′ Termini by Electrophoretic Mobility. The 5′ termini of the DNA fragments resulting from bleomycin and dimethyl sulfate reactions were analyzed for the presence of phosphoryl groups by using bacterial alkaline phosphatase (Gordon & Haseltine, 1981). DNA from the reactions was ethanol precipitated and resuspended in 10 μL of 10 mM Tris-HCl–2 mM EDTA, pH 8.0, buffer. The DNA was heat denatured at 90 °C for 5 min. After the samples were chilled on ice, 14 units of bacterial alkaline phosphatase was added to each sample, and the samples were incubated at 37 °C for 12 h. The reactions were terminated by ethanol precipitation, washed in cold ethanol, and lyophilized. Pellets were resuspended in 9 μL of 0.05 N NaOH–7 M urea loading buffer for gel electrophoresis.

Analysis of 3′ Termini by Electrophoretic Mobility. The 3′ termini of the DNA fragments resulting from bleomycin and dimethyl sulfate reactions were analyzed for the presence of a phosphoryl group. [5′-^{32}P]DNA treated with these agents was precipitated with ethanol and resuspended in 50 μL of H_2O. The DNA was heat denatured at 90 °C for 5 min and chilled on ice, and 50 μL of a buffer containing 10 mM β-mercaptoethanol, 20 mM Tris-HCl (pH 6.5), and 20 mM magnesium acetate and 2 μL of T4 polynucleotide kinase (10 units/μL) were added to each sample. To ensure that no further DNA degradation occurred after this point, we also added 5 μL of 5×10^{-5} M $CoCl_2$ (Sausville et al., 1978b) to the reaction to inhibit the activity of the bleomycin. A 10-μL aliquot of 5×10^{-5} M $CoCl_2$ was added to the dimethyl sulfate treated DNA sample to verify that $CoCl_2$ did not inhibit T4 polynucleotide kinase 3′-phosphatase activity. The reactions were incubated at 37 °C for 12 h and terminated by ethanol precipitation, washed in ice-cold ethanol, and lyophilized. Each pellet was resuspended in 9 μL of 0.05 N NaOH–7 M urea loading buffer and analyzed by gel electrophoresis.

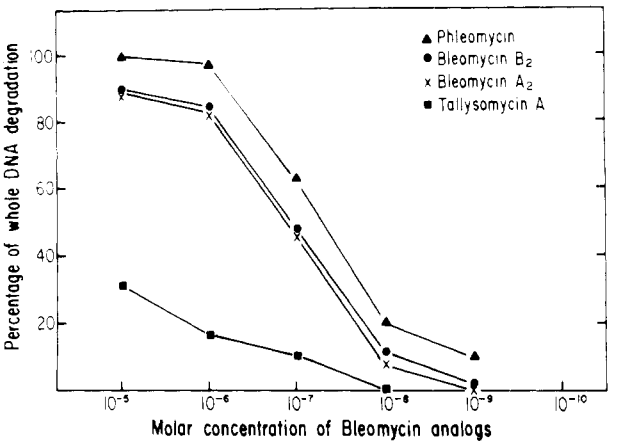

FIGURE 3: Concentration dependence of DNA degradation by equimolar bleomycin analogue–Fe^{2+}. The 168 base pair 3′-end labeled [^{32}P]DNA (10 000 cpm, 1 ng of DNA/reaction) was incubated in the indicated equimolar concentrations of bleomycin A_2–Fe^{2+} (×), bleomycin B_2–Fe^{2+} (●), phleomycin–Fe^{2+} (▲), or tallysomycin A–Fe^{2+} (■) for 10 min at 37 °C. Reactions were stopped with 10 mM EDTA and, after ethanol precipitation, the [^{32}P]DNA was loaded and electrophoresed on a denaturing 8% polyacrylamide gel. Quantitation is described under Materials and Methods.

Results

Extent of Strand Cleavage as a Function of Antibiotic Concentration. The extent of strand scission as a function of the concentration of the bleomycin-related antibiotics was measured by using end-labeled DNA molecules of defined sequence as substrates. For these experiments, an equimolar mixture of the compounds with ferrous ion was prepared immediately prior to the reactions. The reactions were incubated at 37 °C for 10 min at ambient atmosphere. The reactions were complete after 10 min, and further incubation did not result in an increased extent of strand scission (not shown). The reactions were terminated by addition of EDTA to chelate the ferrous ion. DNA was denatured and analyzed on high-resolution polyacrylamide gels of the type used for DNA sequencing. Such gels separate DNA molecules on the basis of the charge and mass of the molecule. DNA molecules that differ in length by a single nucleotide are resolved on such gels. The location of the DNA molecules on the gel was determined by autoradiography. The extent of strand scission in each reaction was determined by measurement of the amount of radioactivity in intact, full-length molecules, as well as the amount of radioactivity in the scission products that migrated more rapidly than the unbroken substrates. The percent breakage was calculated as the fraction of total ^{32}P-labeled molecules that migrated more rapidly than the unbroken molecules. This assay reflects the actual number of strand scission events only when the total number of breaks is less than one break per 168 nucleotides, the length of the end-labeled DNA substrate used in the experiment.

Figure 3 shows that the activities of purified bleomycin A_2, bleomycin B_2, and phleomycin are roughly equivalent. A concentration of 1×10^{-7} M of each compound is required to introduce strand breaks into about half of the labeled DNA molecules. In contrast, tallysomycin A was much less active in the strand scission reaction than was either bleomycin or phleomycin. A concentration of tallysomycin of 1×10^{-5} M resulted in breakage of less than half of the substrate molecules. The lesser efficiency of the tallysomycin A reaction has been reported previously (Strong & Crooke, 1978).

The addition of ferrous ion has led to higher levels of DNA breakage by bleomycin observed in the present experiments

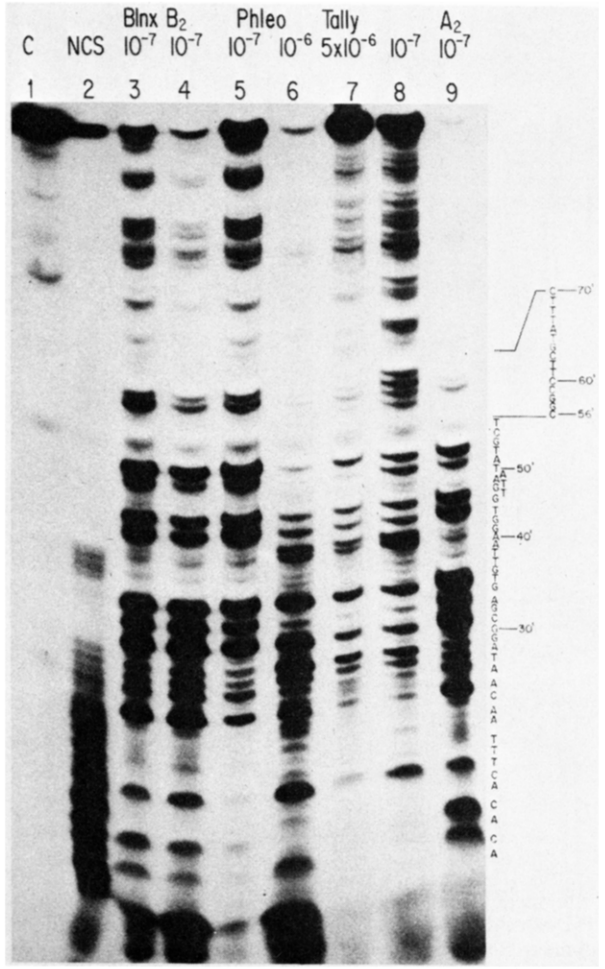

FIGURE 4: Specificity of DNA degradation by bleomycin analogues. The 168 base pair 3′-end labeled [^{32}P]DNA (10000 cpm, 1 ng of DNA/reaction) was incubated with 10^{-7} M equimolar bleomycin analogue–Fe^{2+} for 10 min at 37 °C. After stopping the reactions with 10 mM EDTA and ethanol precipitating the [^{32}P]DNA, we loaded and ran each on a denaturing 8% polyacrylamide gel. Products of reactions with 10^{-7} M Blenoxane–Fe^{2+}, bleomycin B_2–Fe^{2+}, phleomycin–Fe^{2+}, tallysomycin–Fe^{2+}, and bleomycin A_2–Fe^{2+} were in lanes 3, 4, 5, 8, and 9, respectively. Lanes 2, 6, and 7 contained products of reactions with neocarzinostatin (D'Andrea & Haseltine, 1978), 10^{-6} M phleomycin–Fe^{2+}, and 5×10^{-7} M tallysomycin A–Fe^{2+}. Lane 1 contained untreated 168 base pair 3′-end labeled [^{32}P]DNA.

relative to our earlier experiments (D'Andrea & Haseltine, 1978), which were done under conditions of limiting ferrous ion (Burger et al., 1979).

Sites of Strand Scission. To determine the cleavage specificity of the antibiotics, we used end-labeled DNA fragments of defined sequence as substrates. The site of strand scission was determined by direct comparison of the antibiotic scission products with products produced by treatment of the same DNA with reagents that produced breaks at known sequences. In all cases, the DNA was treated with equimolar mixtures of ferrous ion and antibiotics. A concentration of 1×10^{-7} M of the antibiotic was used for each compound. In the case of phleomycin and tallysomycin A, additional reactions with 1×10^{-6} and 5×10^{-6} M were also done. Inspection of Figure 4 reveals that the sites of strand scission are the same for the reactions that contained 1×10^{-7} M Blenoxane, purified bleomycin A_2, and purified bleomycin B_2. Moreover, the same pattern of strand scission products was observed in a reaction that contained 1×10^{-7} M phleomycin as was observed in the reactions that contained the bleomycin compounds. The relative frequency of strand scission at individual scission sites,

Table I: Comparison of Site Specificity of DNA Cleavage by Bleomycin and Tallysomycin A on 168 and 117 Base Pair 3′-End Labeled [^{32}P]DNA

	cleaved		uncleaved	
site[a]	bleomycin	tallysomycin A	bleomycin	tallysomycin A
G(T)	6	6	0	0
G(C)	6	7	1	0
G(G)A	3	4	1	0
G(A)	2	2	0	0
PyPy(T)	2	4	2	0
TA(T)	3	3	0	0
CA(T)	1	1	0	0
GA(T)	1	1	0	0
AA(T)	0	0	4	4

[a] Dinucleotide or trinucleotide sequences are presented in 5′ to 3′ orientation. Site refers to cleavage occurring 5′ to the deoxynucleoside listed in parentheses.

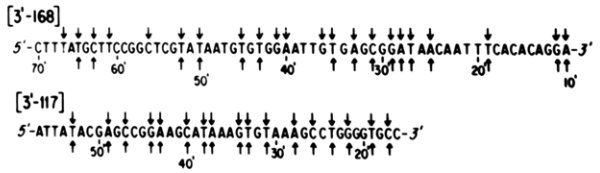

FIGURE 5: Sites of specific DNA cleavage by Blenoxane–Fe^{2+}, bleomycin A_2–Fe^{2+}, and bleomycin B_2–Fe^{2+} (↑) and tallysomycin A (↓) on 168 and 117 base pair 3′-end labeled [^{32}P]DNA.

as judged by the relative intensity of the bands in the autoradiography, was indistinguishable in the phleomycin and bleomycin reactions.

Figure 4 demonstrates that in the tallysomycin A reactions, the DNA was cleaved at sites that were not apparent in other reactions. Moreover, the relative frequency of strand scission at individual sites was somewhat different in reactions that contained tallysomycin A from those that contained the other antibiotics. A similar result was observed for another 3′-end labeled DNA fragment (not shown).

The sites at which strand scission occurred for the antibiotics are summarized in Figure 5 and in Table I. The majority of the scission sites were found to be common to all the antibiotics. However, the sites of strand scission of bleomycin and phleomycin are a subset of the sites of cleavage by tallysomycin A.

Previous studies of bleomycin demonstrate that strand scission occurred preferentially at sites of GC or GT (D'Andrea & Haseltine, 1978; Takeshita et al., 1978). The results of this study are in agreement with earlier findings. In the bleomycin and phleomycin reactions strand scission events were observed at all GT sequences and all but one GC sequence. The GC sequence that was not cleaved by the bleomycin reaction was a target for tallysomycin A (Table I). The cleavage specificity noted here is somewhat broader than that observed in the earlier experiments (D'Andrea & Haseltine, 1978; Takeshita et al., 1978). Under the conditions used here strand scission by bleomycin is not restricted to GC and GT sequences. Breaks occur at some GA and AT sequences as well.

Some of the loss in specificity is only apparent as autoradiography was more prolonged in order to demonstrate minor cleavage sites. The inclusion of ferrous ion in the reactions might also have led to broadened specificity. Some of the additional cleavage sites observed in the tallysomycin A reaction occurred at GC and GA sequences, sequences that are sometimes cleaved by the bleomycin compounds. However, cleavage was also observed in the tallysomycin A reaction at

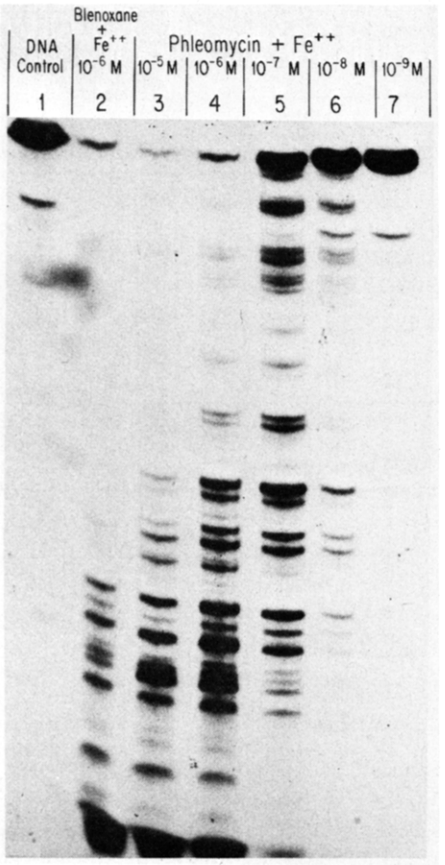

FIGURE 6: DNA degradation over a concentration range of 10^{-9}–10^{-5} M equimolar phleomycin–Fe^{2+}. The 168 base pair 3′-end labeled [^{32}P]DNA (10000 cpm, 1 ng of DNA/reaction) was incubated with equimolar phleomycin–Fe^{2+} and electrophoresed on a denaturing 8% polyacrylamide gel as described under Materials and Methods. Products of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M equimolar phleomycin–Fe^{2+} were contained in lanes 3, 4, 5, 6, and 7, respectively. Lane 2 contained products of 10^{-6} M equimolar Blenoxane–Fe^{2+}. Lane 1 contained untreated 168 base pair 3′-end labeled [^{32}P]DNA.

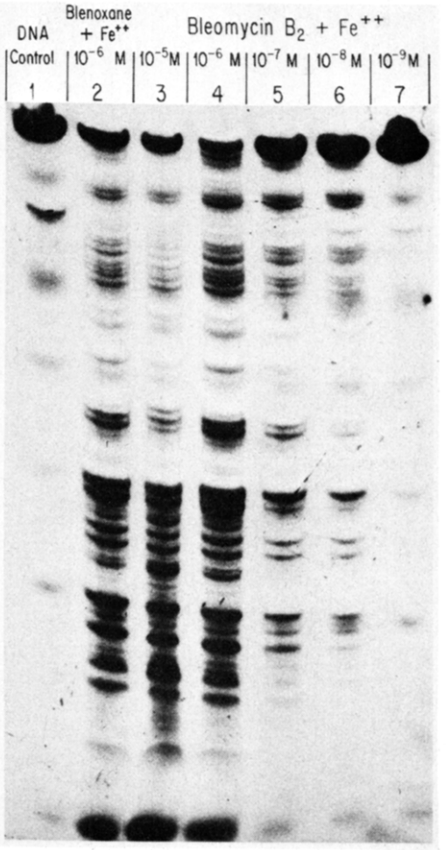

FIGURE 7: DNA degradation over a concentration range of 10^{-9}–10^{-5} M equimolar bleomycin B_2–Fe^{2+}. The 168 base pair 3′-end labeled [^{32}P]DNA (10000 cpm, 1 ng of DNA/reaction) was incubated with equimolar bleomycin B_2–Fe^{2+} and electrophoresed on a denaturing 8% polyacrylamide gel as described under Materials and Methods. Products of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M equimolar bleomycin B_2–Fe^{2+} were contained in lanes 3, 4, 5, 6, and 7, respectively. Lane 2 contained products of 10^{-6} M equimolar Blenoxane–Fe^{2+}. Lane 1 contained untreated 168 base pair 3′-end labeled [^{32}P]DNA.

pyrimidine–thymidine sequences, sequences that are not sites of cleavage for bleomycin.

The altered specificity of tallysomycin compared to bleomycin and phleomycin is not simply a result of the higher concentrations of tallysomycin required to achieve equal amounts of cleavage. While increasing concentrations of phleomycin (Figure 6) or bleomycin B_2 (Figure 7) lead to changes in the pattern of fragments, these changes are due to intensification of minor bands and at very high concentrations to secondary breakage of longer fragments. For example, cleavage by tallysomycin occurs at sites 67, 61, and 36, but DNA cleavage is not seen at these sites by bleomycin or phleomycin.

Structure of Termini of Bleomycin-Induced Breaks. The enzymatic repair of bleomycin-induced strand scission events will depend, at least in part, upon the structure of the termini of the breaks. We noticed that the electrophoretic mobilities of DNA fragments produced by treatment of 3′-end labeled DNA with bleomycin were the same as some of those produced by treatment of the same DNA fragment with hydrazine or dimethyl sulfate, the reagents used in the Maxam–Gilbert sequencing protocols (Maxam & Gilbert, 1977). These sequencing reagents produce fragments that are phosphorylated at the 5′ terminus (Maxam & Gilbert, 1980). Since the electrophoretic mobility of a DNA fragment depends upon both the charge and the mass of the molecule, comigration of the products suggested that the 5′ terminus of the bleomycin-induced nick might be phosphorylated.

To test this possibility, we denatured DNA that had been treated with bleomycin and incubated it with bacterial alkaline phosphatase. This enzyme removes both 3′- and 5′-phosphoryl groups (Cameron & Uhlenbeck, 1977). Removal of a 5′-phosphoryl group from the scission products would be expected to alter the charge/mass ratio of the molecule and thus retard the electrophoretic mobility of the fragment relative to that of the untreated control. Such retardation cannot be the result of removal of the 3′-phosphoryl from such molecules, as the 3′-end labeled substrate terminates in a hydroxyl group, not a phosphoryl group.

Figure 8 demonstrates that treatment of the products of a dimethyl sulfate reaction with bacteria alkaline phosphatase does result in the expected shift in the electrophoretic mobility of the scission products. Treatment of the products of the bleomycin reaction results in a similar shift. Moreover, in both cases, the reactions are quantitative. These results demonstrate that the 5′ termini of the bleomycin-induced nicks contain phosphoryl groups.

Similar methods were used to probe the structure of the 3′ termini of the nicks. For these experiments, 5′-end labeled substrates were used. Initial experiments suggested that the predominant product of bleomycin cleavage did not terminate in a 3′-phosphoryl group as the migration of bleomycin fragments during electrophoresis is slightly more rapid than migration of fragments of equal base length created by Maxam and Gilbert DNA sequencing protocols (D'Andrea & Ha-

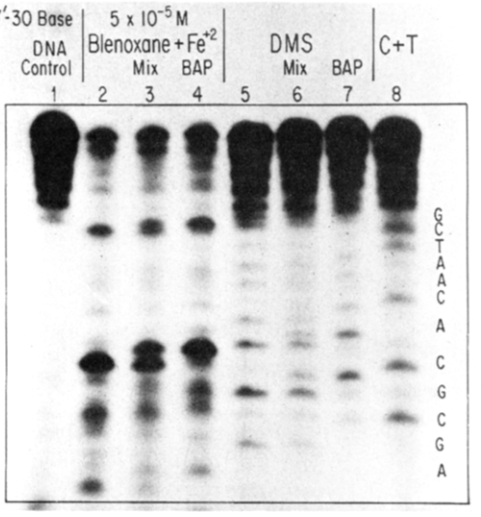

FIGURE 8: Presence of 5′-phosphate at site of bleomycin strand scission as determined by bacterial alkaline phosphatase. The 30 base pair 3′-end labeled [^{32}P]DNA (20000 cpm, 2 ng of DNA/reaction) was incubated with 5×10^{-8} M equimolar Blenoxane–Fe^{2+}, ethanol precipitated, and resuspended as described under Materials and Methods. An identical DNA sample was also cleaved in a Maxam & Gilbert (1980) DMS sequencing reaction in which 0.1 N NaOH hydrolysis, followed by pH neutralization, replaced piperidine treatment. The DMS products were ethanol precipitated and resuspended. Half of both of the Blenoxane–Fe^{2+} and DMS reactions were then reincubated in the presence of bacterial alkaline phosphatase. These reactions were again ethanol precipitated, and one-third of the [^{32}P]DNA products were mixed with one-third of their original Blenoxane–Fe^{2+} or DMS [^{32}P]DNA strand scission products. Each sample was then loaded and run on a denaturing 20% polyacrylamide gel. Lanes 2 and 5 contain the original Blenoxane–Fe^{2+} and DMS reactions, respectively. Lanes 4 and 7 contain the bacterial alkaline phosphatase treated blenoxane–Fe^{2+} and DMS reactions, respectively. Lanes 3 and 6 contain mixtures of the original and bacterial alkaline phosphatase treated blenoxane–Fe^{2+} and DMS reactions, respectively. Lane 8 contains a Maxam & Gilbert (1980) hydrazine reaction, which cleaves at pyrimidines. Lane 1 contains untreated 30 base pair 3′-end labeled [^{32}P]DNA.

seltine, 1978; Hecht, 1979). Electrophoresis of a mixture of bleomycin and sequencing protocol induced fragments confirmed the altered mobility of the bleomycin-induced fragments (not shown). In most experiments a minor product of bleomycin cleavage with a mobility indistinguishable from that of DNA sequencing fragments was also detected (D'Andrea & Haseltine, 1978; Figure 9).

As a further test of this hypothesis, 5′-end labeled DNA that was treated with bleomycin was denatured and subsequently treated with T4 polynucleotide kinase in the absence of ATP or ADP, under conditions where T4 polynucleotide kinase acts as a 3′-phosphatase (Cameron & Uhlenbeck, 1977). The 3′-phosphatase activity of the enzyme was confirmed by treatment of the scission products of a dimethyl sulfate reaction. As expected (Figure 9) the electrophoretic mobility of the 5′-end labeled fragments was retarded by such treatment. In contrast, the electrophoretic mobilities of the major bleomycin-induced scission product were unaffected by incubation with T4 polynucleotide kinase. We conclude that the 3′ terminus of the bleomycin-induced nick is *not* a simple phosphoryl group.

Activity on Single-Stranded DNA. During DNA replication and especially at replication forks a substantial fraction of DNA may be present in a single-strand form. Therefore, it is of interest to determine whether or not bleomycin can cleave single-stranded molecules. To investigate this question, we prepared a single-stranded substrate. The 5′-end labeled 168 nucleotide long fragment of the *lac* p-o region was denatured

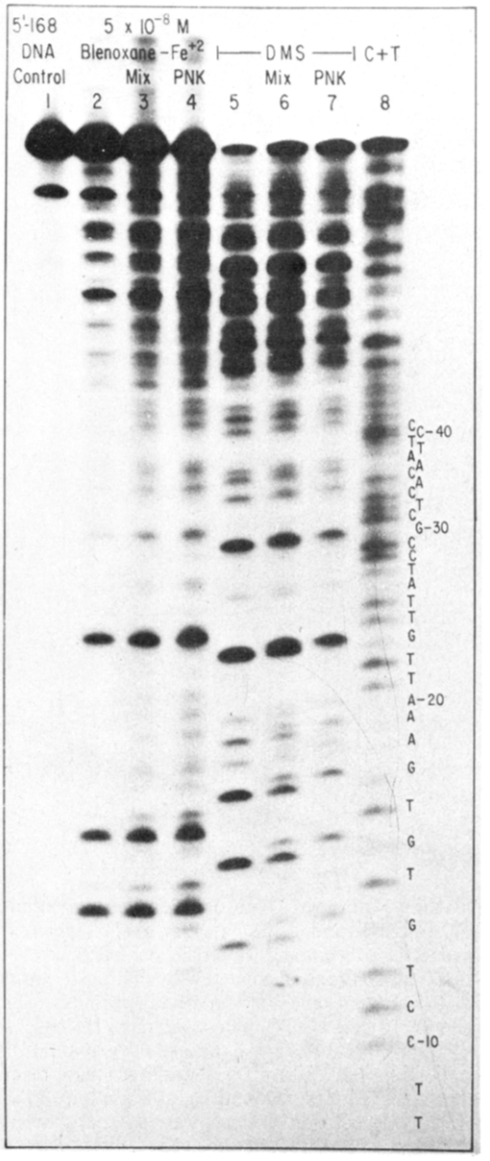

FIGURE 9: Absence of 3′-phosphate at site of bleomycin strand scission as determined by 3′-phosphatase activity of T4 polynucleotide kinase. Two solutions of 168 base pair 5′-end labeled [^{32}P]DNA (10000 cpm, 1 ng of DNA/reaction) were incubated in 5×10^{-8} M equimolar Blenoxane–Fe^{2+} for 10 min at 37 °C. After each was stopped with 1.6×10^{-6} M $CoCl_2$, its [^{32}P]DNA scission products were ethanol precipitated. Two solutions of 168 base pair 5′-end labeled [^{32}P]DNA (10000 cpm, 1 ng of DNA/reaction) were also cleaved in a Maxam and Gilbert DMS reaction (see legend to Figure 8). One-half of both the Blenoxane–Fe^{2+} and DMS reactions was then reincubated in the presence of T4 polynucleotide kinase under conditions for 3′-phosphatase activity. These reactions were again ethanol precipitated and one-third of the [^{32}P]DNA products were mixed with one-third of their original Blenoxane–Fe^{2+} or DMS [^{32}P]DNA strand scission products. Each sample was then loaded and run on a denaturing 20% polyacrylamide gel. Lanes 2 and 5 contain the original Blenoxane–Fe^{2+} and DMS reactions, respectively. Lanes 3 and 6 contain mixtures of the original and T4 polynucleotide kinase treated Blenoxane–Fe^{2+} and DMS reactions, respectively. Lanes 4 and 7 contain the T4 polynucleotide kinase treated Blenoxane–Fe^{2+} and DMS reactions, respectively. Lane 8 contains a Maxam and Gilbert hydrazine reaction. Lane 1 contains untreated 168 base pair 5′-end labeled [^{32}P]DNA.

and run on a preparative high-resolution urea-containing polyacrylamide gel. The 5′-end labeled strand migrated more slowly than the unlabeled complementary strand in this experiment, since the unlabeled strand was four bases shorter than the labeled strand. No double-stranded material was detected in this preparation after incubation under hybridizing conditions and analysis of the material on a nondenaturing gel

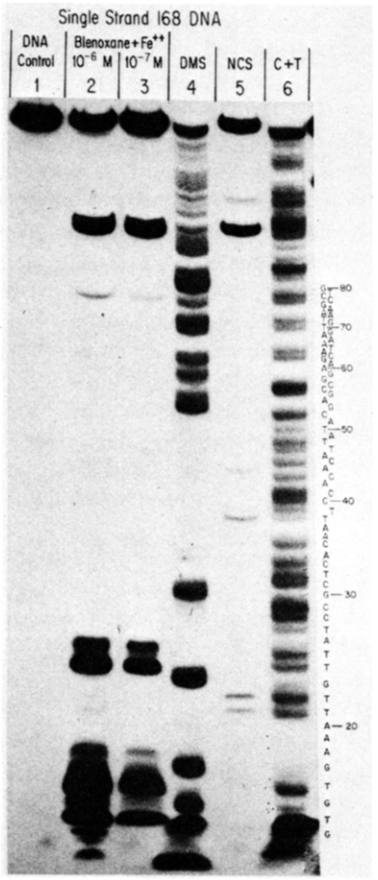

FIGURE 10: Single-stranded DNA degradation by equimolar bleomycin–Fe^{2+}. Single-stranded 168 base pair 5′-end labeled [^{32}P]DNA was prepared as described under Materials and Methods (10000 cpm, 1 ng of DNA/reaction) and incubated with equimolar concentrations of 10^{-6} and 10^{-7} M Blenoxane–Fe^{2+} for 10 min at 37 °C. The reaction was stopped with 10 mM EDTA. Single-stranded 168 base pair 5′-end labeled [^{32}P]DNA (10000 cpm, 1 ng of DNA/reaction) was also cleaved by Maxam and Gilbert DMS and hydrazine reactions and a neocarzinostatin reaction (D'Andrea & Haseltine, 1978). Each reaction's [^{32}P]DNA scission products were ethanol precipitated and run on a denaturing 8% polyacrylamide gel. Lanes 2 and 3 contained 10^{-7} and 10^{-6} M Blenoxane–Fe^{2+} reactions, respectively. Untreated single-stranded 168 base pair 5′-end labeled [^{32}P]DNA (lane 1) and products of reactions with DMS (lane 4), neocarzinostatin (lane 5), and hydrazine (lane 6) were electrophoresed in parallel.

that resolves single- from double-stranded DNA molecules (not shown).

For this experiment, both the single- and double-stranded 5′-end labeled 168 nucleotide long fragments were incubated with bleomycin, denatured, and layered on high-resolution polyacrylamide gels. Figure 10 shows that the cleavage sites observed in the single-stranded substrate were a subset of those observed upon treatment of the double-stranded molecule.

We thought it curious that only some of the sites on the single-stranded substrate were sensitive to bleomycin cleavage. Therefore, we examined the DNA sequence of the 168 nucleotide long fragment for possible secondary structures. Figure 11 demonstrates that secondary structure loops can be formed in this substrate. These secondary structure loops of double strandedness occur from nucleotide 13 to nucleotide 47 and from nucleotide 113 to nucleotide 144. The secondary structure loops pictured here should be stable under the conditions of incubation and are characteristic of the *lac* p-o region (Dickson et al., 1975). We note that the cleavage sites observed upon treatment of the single-stranded substrate with bleomycin are confined to regions that can assume the double-stranded configuration. Potentially looped-back regions

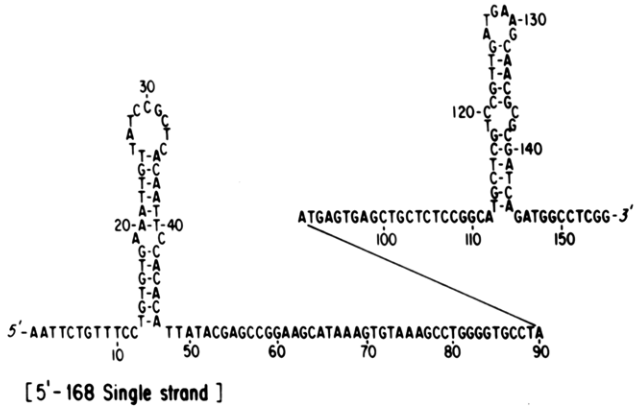

FIGURE 11: Structural diagram of potential double-stranded loop regions in single-stranded 168 base pair fragment.

of single-strand DNA are also sites for cleavage by *Micrococcus luteus* UV-specific endonuclease, which strongly prefers double-stranded DNA as substrate (Gordon & Haseltine, 1980). Therefore, we conclude that double-stranded DNA is a much better substrate for bleomycin cleavage than is single-stranded DNA.

Discussion

We have investigated the DNA cleavage specificity of several glycopeptide antibiotics that are structurally related to bleomycin. The results demonstrate that all the antibiotics cleave DNA at specific sites. The specificity of the scission of DNA by these compounds stands in marked contrast to the nonspecific DNA degradation observed upon treatment of the same substrates with 1 mM ferrous sulfate. Such specificity of the DNA degradation observed here implies that these compounds bind to DNA via specific site interactions. What is the nature of these interactions?

The cleavage specificity of purified bleomycin A_2 and bleomycin B2 is identical. These two compounds differ markedly in the structure of the C-terminal substituent attached to the bithiazole ring. Therefore, it seems unlikely that this region of the molecule plays an important role in the determination of the specific nature of the drug–DNA interaction. A similar conclusion was reached by Takeshita et al. (1978) for reactions carried out under conditions of limited breakage. However, the importance of this region of the molecule in overall DNA–drug interaction was suggested by inhibition studies with synthetic bithiazole analogues (Kross et al., 1982). These studies demonstrated that only those carboxyl-terminal substituents that are positively charged are effective inhibitors of bleomycin–DNA interactions. We conclude that although the nature of the substituents is probably not a key determinant of sequence specificity, it is likely that a positively charged substituent is required for efficient DNA–drug interaction.

Phleomycin differs generically from bleomycin in that the bithiazole moiety is replaced by a thiazolinylthiazole heterocycle. Povirk et al. (1981) have suggested that such a structural change either eliminates or substantially reduces the ability of phleomycin to intercalate into DNA, on the basis of the observation that the binding of bleomycin leads to DNA elongation while that of phleomycin did not. If this suggestion were correct, it would follow that intercalation of the bithiazole moiety does not play a major role in directing the specificity of strand scission since, under these conditions, bleomycin and phleomycin produce similar products.

The cleavage specificity of the purified preparations of bleomycin A_2 and bleomycin B_2 was found to be identical with

that of a mixture of bleomycin (Blenoxane) that is in common use. This result implies that mechanistic conclusions reached for the purified preparation are likely to be applicable to the clinical preparation as well. The results also demonstrate that the cleavage specificity of phleomycin is remarkably similar to that of the bleomycin. Both the extent and location of strand scission events by both antibiotics are similar over a wide concentration range of 5×10^{-6}–1×10^{-9} M.

Tallysomycin A differs from bleomycin in two important respects. The concentration of the tallysomycin needed to achieve a similar extent of degradation was much higher in accord with earlier observations (Strong & Crooke, 1978). Moreover, DNA products not observed in the bleomycin reaction were observed in reactions that contained tallysomycin A. A major difference in the structure of bleomycin and tallysomycin is the presence of an amino sugar attached to the (aminoethyl)bithiazole moiety of bleomycin. It seems likely that this sugar plays some role in determination of both the binding affinity and the specificity of the drug scission. Synthesis of other analogues modified in this region of the molecule should be of considerable interest. Mirabelli et al. (1979, 1980, 1981) have also concluded that the cleavage specificity of tallysomycin is somewhat different from that of bleomycin.

Our results are in agreement with the recent report of Takeshita et al. (1981) in which they measured DNA cleavage patterns by various bleomycin analogues. They also found that GT, GC, and AT sequences are preferred sites of attack for bleomycin and phleomycin and that tallysomycin shows significant differences in cleavage preference from the other bleomycin analogues tested. We cannot, however, confirm their observation that phleomycin showed less cleavage at purines than bleomycin. From both our results and those of Takeshita, it is evident that specificity of bleomycin cleavage is determined in part by DNA sequences longer than dinucleotides.

The results also demonstrate that single-stranded DNA is not a good substrate for bleomycin. Only the regions of a single-stranded substrate that can form double-stranded structures are effectively attacked by bleomycin. This result is in agreement with studies that demonstrate that bleomycin does not bind efficiently to single-stranded DNA (Umezawa, 1978). The lack of the requirement for intercalation (Povirk et al., 1979, 1981) and the requirement for double-stranded configuration suggest that site-specific binding to the major or minor grooves of DNA might occur. Such binding could also account for the quenching of the fluorescence of the bithiazole rings that is observed upon drug binding to DNA.

The structure of the termini of the bleomycin-induced single-stranded nicks has been the subject of intensive investigation. Our results demonstrate that, under the conditions used, greater than 90% of the 5′ termini of the nicks contain simple phosphoryl groups. This result is in agreement with most of the previous literature. The structure of the 3′ terminus is more complex. For each cleavage site, we observed cleavage products that differ from one another in electrophoretic mobility. The predominant product does not terminate in a simple 3′-phosphoryl group by criteria of altered mobility and resistance to 3′-phosphatase activity. These results are consistent with a 3′ terminus containing a fragment of deoxyribose esterified to phosphate. Work by Giloni et al. (1981) and Burger et al. (1980) suggests that the fragment is likely to contain C-4 and C-5 of the deoxyribose. We note that the presence of such 3′ termini may require specialized DNA repair activities.

The experiments reported here illustrate the ability of DNA fragments of defined sequences to serve as tools for the analysis of drug analogues. Comparative studies can be done rapidly and with great precision with such substrates. Moreover, details of drug action can be deduced by straightforward analysis of the reaction products.

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Nitrocellulose Filter Binding Studies of the Interactions of *Escherichia coli* RNA Polymerase Holoenzyme with Deoxyribonucleic Acid Restriction Fragments: Evidence for Multiple Classes of Nonpromoter Interactions, Some of Which Display Promoter-like Properties†

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ABSTRACT: The variety of detectable interactions between *Escherichia coli* RNA polymerase holoenzyme and an unfractionated *Hae*III digest of T7D111 DNA has been examined by using a filter binding gel electrophoresis assay, appropriately modified to study nonpromoter interactions. Filter-retainable complexes form rapidly on all fragments in the digest, either at 0 °C or at 37 °C. These complexes differ from the fast-forming specific complexes observed on promoter-containing fragments in that they are sensitive to competition by the polyanion heparin. From binding studies on two isolated sets of fragments of average size 800 and 2000 base pairs, we infer that the fast-forming, heparin-sensitive complexes occur at the ends of fragments. Binding constants for this class of interactions increase modestly with increasing temperature (yielding a van't Hoff $\Delta H°$ of 4 ± 1 kcal) and decrease strongly with increasing salt concentration; the large salt dependence is qualitatively similar to those observed previously for promoter and nonpromoter complexes. In addition, complexes which exhibit a slow, strongly temperature-dependent rate of formation are found on a subset of T7D111 fragments, as well as on some *Hae*III fragments of λCI47, P22, and SV40 DNA. These complexes are stable to a competition with heparin and are similar to the tight-binding (TB) complexes recently described by Kadesch et al. [Kadesch, T. R., Williams, R. C., & Chamberlin, M. J. (1980) *J. Mol. Biol. 136*, 79-93]. In addition to their stability, these complexes share with promoter complexes the ability to initiate transcription. In both cases, initiation is sensitive to rifampicin and displays specific nucleoside triphosphate requirements. We find that these TB complexes are formed in general at interior sites rather than at the ends of fragments. The extent of formation of TB complexes is very sensitive to salt concentration. Neither the kinetic nor thermodynamic aspects of this reaction have yielded to simple interpretation. Because (i) weak complexes can be eliminated by a short challenge with a competitor and (ii) RNAP forms tight complexes at TB sites much more slowly than at strong promoters, we conclude that filter binding can be used to quantify the interactions of polymerase with strong promoters carried on restriction fragments.

Escherichia coli RNA polymerase holoenzyme (RNAP),[1] like other genome regulatory proteins that act at specific target sites on DNA, exhibits a significant general affinity for other regions of DNA. These nonpromoter[2] interactions are im-

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[1] Abbreviations: RNAP, *E. coli* RNA polymerase holoenzyme; NTP, ribonucleoside 5'-triphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Bis, *N*,*N*'-methylenebis(acrylamide); BAC, diacrylylcystamine; Me_2SO, dimethyl sulfoxide; bp, base pair.

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