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The effects of NaCl concentration on bleomycin-induced cleavages of single-strand and double-strand DNA fragments containing the phage G4 origin of complementary DNA strand synthesis were investigated. It was found that bleomycin could be used as a reagent to analyze secondary and tertiary structures and subtle changes of DNA structures. The effects of NaCl concentration on cleavages of single-stranded DNA were distinct at every target site, indicating that the diversity of topological properties of DNA might change the selectivity of the bleomycin-induced DNA cleavage. These results showed alternative secondary structures within and close to the G4 origin of complementary DNA strand synthesis.

In the primase-dependent oriC determinant sequences among the related phages of G4, not only the nucleotide sequences but also potential secondary structures are well conserved.13 Three adjoining stem-and-loop structures within the G4 oriC sequence prove to formed under low-salt conditions.23 In the direct interaction of primase-dependent oriC with primase, the tertiary structure including stem-loop-structures are believed to be involved in its function.34

Bleomycin is a glycopeptide antitumor antibiotic and its cleavage of DNA is thought to contribute to its antitumor activity. The bleomycin–Fe(II) complex selectively recognizes some dinucleotide sequences in double-stranded DNA,56 and abstracts hydrogen preciously from the C4′ position of the second nucleotide in the target dinucleotide sequence.78 We have investigated the DNA-breaking reaction of bleomycin with DNA fragments containing the oriC of phage G4, and have reported that double-stranded DNA is more susceptible to bleomycin than single-stranded DNA and secondary and tertiary structures of DNA affect the bleomycin–DNA interaction.2

In this paper, we describe the effects of NaCl concentration on the strand-breaking reaction of bleomycin with DNA fragments containing the G4 oriC, which contains secondary and tertiary structures. It is found that bleomycin can be used as a reagent for analyzing subtle changes of DNA structures. Alternative secondary structures within and closed to the minimal region of G4 oriC were also shown. Possible functional contributions of these secondary structures are suggested.

Materials and Methods

Chemicals and enzymes. Purified bleomycin A2 was a gift from Dr. Yukio Sugiyama. FeSO4 solution was prepared immediately before use, and then mixed with bleomycin A2 at concentrations of 1 mM to prepare bleomycin A2–Fe(II) complex. Enzymes were purchased from Takara Shuzo Co., Ltd. [γ-32P]ATP (specific activity >111 TBS/mmol) were obtained from Amersham International.

DNA substrates. The 278-bp EcoRI fragment, which contains the G4 oriC, and has an AATT sequence at both 5′-termini, was prepared from the R199/G4 RF DNA.23 The resulting fragment was labeled by extension of the 3′-termini with Klenow fragment in the presence of [γ-32P]ATP. The 178-bp ds DNA labeled singly at the 3′-end was prepared by digestion with HaeIII, and the 280-nt ss viral DNA labeled at the 3′-end was prepared by strand separation as shown in Fig. 1.

Reaction of end-labeled DNA with bleomycin. Reaction mixtures of 100 μl contained 25 mM Tris–HCl buffer (pH 8.1), 25 mM bleomycin A2–Fe(II) complex, 3′-end-labeled DNA fragments (50–70 ng), various concentrations of NaCl, and 10 μl of DTT, added last to start the reaction. The reaction mixture was incubated at 37°C for 5 min, and then the reaction was stopped by the addition of EDTA (the final concentration to 10 mM) and 2 μg of tRNA. DNA was precipitated by adding sodium acetate and ethanol.

Analysis of produced oligonucleotides. The precipitated DNA was dissolved in 3 μl of 80% (v/v) formamide–10 mM NaOH loading buffer. The DNA was denatured by heating at 90°C for 1 min and put on a 6% or 8% polyacrylamide slab gel for sequence analysis. The amount of the radioactivity in each product was measured by liquid scintillation counting of the gel slice. The relative amounts of products were also measured by scanning the autoradiograms with a microdensitometer as reported previously.31

Results

DNA cleavages of ss DNAs

The 280-nt 3′-end-labeled ss DNA shown in Figs. 1 and 2 corresponds to the G4 viral DNA strand, and contains G4 oriC which is directly recognized by primase in the

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Abbreviations: ds, double-strand(ed); oriC, origin of complementary DNA strand synthesis; RF, replicative form (double-stranded closed circular); ss, single-strand(ed); SSB, E. coli single-strand DNA binding protein(s); ssi, single-strand initiation.
presence of SSB. We have reported\(^2\) that three stem-and-loop structures, I, II, and III, are formed within the G4 \(ori_c\) minimal region in the 25 mm Tris–HCl buffer (pH 8.1) (Fig. 2). These stem-and-loop structures have been shown to be essential for the function of G4 \(ori_c\) \textit{in vivo}.\(^{10-12}\) To analyze the fine structures of DNA, the bleomycin-induced DNA cleavages of the 3'-end-labeled ss DNA were investigated with various NaCl concentrations (Fig. 3). The amounts of oligonucleotides were drastically changed, depending on the NaCl concentration, when they were measured by liquid scintillation counting of gel slices (Fig. 4). A distinct effect of the NaCl concentration on the produced amount of each oligonucleotide was observed. The maximal amounts of two oligonucleotides, \(b\) and \(c\), which were produced by cleavages at stem-and-loop III (see Fig. 2), were obtained at the NaCl concentration of 10 mm (Fig. 4(A)), while those of the three oligonucleotides \(f, g,\) and \(h\) produced by cleavages at stem-and-loop I were gotten in the absence of NaCl (Fig. 4(B)). The amounts of the oligonucleotide \(e\) obtained by the cleavage at stem-and-loop II and of the oligonucleotide \(a\) by the cleavage outside of the three stem-and-loops, close to the 5'-end of the ss DNA, became maximum when the NaCl concentration was 50 mm (Fig. 4(A)). The cleavage in the spacer region between stem-and-loops II and III, which would produce the oligonucleotide \(d\), was only slightly affected by the NaCl concentration (Fig. 4(A)). These results showed that each target site of the bleomycin–induced DNA cleavage had its own optimum NaCl concentration, indicating that topological structures at the bleomycin–DNA interaction sites were distinct from each other. Moreover, different topological structures of the stems of stem-and-loops I and III were suggested.

Fig. 2. Potential Secondary Structures within the G4 \(ori_c\) in ss DNA, and Sites of Selective DNA Cleavages by Bleomycin.

Arrows represent the sites of DNA cleavages and their sizes represent the relative amounts of oligonucleotides produced from the 3'-end-labeled DNA. The arrows in parentheses indicate the amounts that were variable. \(a-k\) correspond to cleavage sites which produce oligonucleotides \(a-k\) shown in Figs. 3 and 4.

DNA cleavages of ds DNA fragment

The sequence preference of cleavages by bleomycin–Fe(II) complex was investigated with the 178-bp 3'-end-labeled ds DNA fragment. The dinucleotide sequences G-T, G-C, A-T, and A-C were preferentially cleaved in the presence of DTT (Fig. 5) as reported previously.\(^{2,5,60}\) We examined the effects of the NaCl concentration on the bleomycin-induced DNA-breaking reaction. The sequence preference of DNA cleavages was not affected by the NaCl concentration between 0—500 mm. The amounts of the produced oligonucleotides, \(G, H,\) and \(M,\) measured by the liquid scintillation counting of gel slices, passed through a sharp maximum when the NaCl concentration was varied (Fig. 6), the
optimum being 10 mM for all of them.

**Discussion**

We have studied the effects of the salt concentration on the interaction between bleomycin and a DNA fragment which contains both the primase-dependent oriC and the ribosome-binding site of gene G, and is composed of secondary and tertiary structures (Fig. 2). The DNA-reading reaction of bleomycin-Fe(II) complex in the presence of DTT depended on the NaCl concentration, and the degree of cleavages was drastically changed in the range of 0—50 mM NaCl. DNA cleavages of the ds DNA fragment were greater in the presence of 10 mM NaCl than in the absence of NaCl at all target sites, and were inhibited by 50 mM or higher concentrations of NaCl (Figs. 5 and 6). It has been reported that increasing the ionic strength significantly decreases the activity of bleomycin-induced DNA cleavage, and the strongest change is observed between 50—300 mM buffer components. The increased ionic strength may hinder the ionic interaction between the sulfonium group of bleomycin and a phosphate residue in DNA, and decrease the binding of bleomycin to DNA.

However, the enhancement of the bleomycin-induced DNA cleavage at 10 mM NaCl could not be simply explained by the change of these ionic interactions. It is not likely that the salt at 0—50 mM influences the conformation and/or the
affinity of the antibiotic for iron. The absorption spectra of the bleomycin-iron complex did not change drastically in the range of the NaCl concentration between 0 and 50 mM (data not shown). Because double-stranded DNA is more susceptible to bleomycin than single-stranded DNA, these results suggest that double-stranded DNA was stabilized in the presence of 10 mM NaCl to be more susceptible to bleomycin than in the absence of salt.

Oligonucleotides f, g, and h were produced by cleavages at the stem-and-loop I (Fig. 2). Those from the 3'-end-labeled ss DNA fragments were maximum in the absence of NaCl (Figs. 3 and 4(B)). In the DNA-breaking reaction of bleomycin with ds DNA, the sequence cGCa, at which the oligonucleotide G, equivalent to g in the case of ss DNAs, would be produced by the cleavage, was more susceptible than the sequence cGTCa, at which the oligonucleotide H, equivalent to h, would be produced by the cleavage, especially in the presence of 10 mM NaCl (Figs. 5 and 6). With the 3'-end-labeled ss DNA, on the contrary, the sequence cGTCa was a much better target site for bleomycin than the sequence cGCCa and other sequences in stem-and-loop I (Figs. 3 and 4(B)). These results indicate that the conformation of the stem of stem-and-loop I is not the normal B-form ds DNA, which affects the selectivity of the bleomycin-induced DNA cleavages in the ds DNA. It is also suggested that the effects of the salt concentration on the bleomycin-DNA interaction may be influenced by these changes of DNA conformation. This specific conformation of stem-and-loop I could be essential for the interaction between G4 *ori*c and primase, and this may explain the observation that changes of stem-and-loop I depress the function of G4 *ori*c.10,15

The production of the oligonucleotide e, which would be produced by the cleavage at stem-and-loop II, was enhanced at 50 mM NaCl (Fig. 4(A)), suggesting that stem-and-loop II is stable in the presence of 50 mM NaCl. The cleavage between stems of stem-and-loops II and III, which would produce the oligonucleotide d, was only slightly affected by the NaCl concentration, with the weak optimum being at 10—50 mM NaCl. The production of oligonucleotide d also suggested that the ss region flanked by two stems of stem-and-loop II and III may be recognized by bleomycin, or there exists another double helix. It is of interest to note that this is the equivalent region in the *ori*c sequence to that of φK, which is directly protected by primase.31 And, changes in this region as well as the bottom of stem-and-loop III in the G4 *ori*c sequence affect the function of G4 *ori*c.4,11 Both the nucleotide sequence and higher structures in this region may be critical for the interaction between *ori*c and primase.49

The oligonucleotide a was one of the major products. Oligonucleotides i, j, and k were also produced by cleavages at sites outside the predicted three stem-and-loops (Fig. 2) although their amounts were variable.2 These results suggest that the regions outside of the G4 *ori*c sequence also contain secondary and tertiary structures. The alternative secondary structure (stem-and-loop III') within G4 *ori*c and secondary structures (stem-and-loops I, R1, R2, and R3) outside the *ori*c sequence are shown in Fig. 7. All the target sites of bleomycin are involved in double helices in

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Fig. 7. Other Possible Secondary Structures.

••• the stop codon of gene F; ---- the start codon of gene G; ———, the initiation site of the primer RNA synthesis. The ribosome-binding site of gene G is boxed.

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these structures. From the location, it is speculated that stem-and-loops L and RI contribute to the biological functions. Stem-and-loop L seems to be stable and is located within, near the 3'-end of, the coding region of gene F; just outside of the minimal region of G4 oriC. 169 Stem-and-loop RI is 4 nt upstream from the initiation site of the primer RNA synthesis, and contains the ribosome-binding site of gene G. The nucleotide sequences in the region containing the A-bulged stem of stem-and-loop RI, which is on the boundary of the G4 oriC minimal region, are well conserved in the related phages φK, St-1, and α3. 170 All the secondary structures as well as the nucleotide sequences outside of oriC, both upstream and downstream, may affect the formation of the oriC-primase complex in the presence of SSB. If these are the cases, these can explain the observations that deletion or changes in the regions outside of G4 oriC 6,15 and of the ‘G4-type ssi signals’ 18 affect the efficiency of initiation, although they are not essential. It is indicated that various profiles of the effects of salt concentration may reflect diversities of topological properties and fine structures of DNA within and close to G4 oriC. Furthermore, it is suggested that not only secondary and tertiary structures but also conformational and topological properties within the oriC of G4 may play an important role in the function. It has also been demonstrated that alteration of DNA conformation can significantly change the pattern and extent of cleavage normally obtained with B-form DNA and bleomycin using DNA oligonucleotides as substrates. 19,20 Studies of the DNA-bleomycin interaction will provide not only the mode of antitumor action of bleomycin but also important insights into DNA-protein interactions.

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