NUCLEOTIDE SEQUENCE OF SN-07 CHROMOPHORE BINDING SITE

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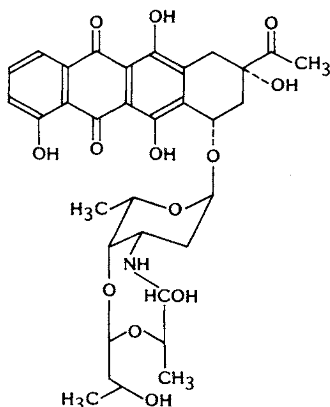
(Received for publication May 31, 1989)

DNase I footprinting was used to investigate binding sites for SN-07 chromophore on DNA fragments prepared from pBR322. Six sites were protected on about 150 base pair DNA fragments by SN-07 chromophore, but not by related anthracycline antibiotics from DNase I digestion. All the protected sites contained the dinucleotide sequence 5′-GC-3′, but no other regularities could be discerned. A drug-induced conformational change of DNA was suggested by enhancement of DNase I sensitivity between the protected sites. These results support covalent interaction of the carbinolamine function of SN-07 chromophore to 2-amino group of guanine residues.

SN-07 chromophore (barminomycin I) is an anthracycline antibiotic. It is atypical of this group in that it contains an eight-membered ring with the carbinolamine structure[1~4] (Fig. 1). The carbinolamine of SN-07 chromophore is similar to those found in the covalent binding antibiotics such as pyrrolo(1,4)benzodiazepine antibiotics (*e.g.*, anthramycin, tomaymycin, sibiromycin and neothramycin)[5~9], saframycins[10] and naphthyridinomycin[11]. SN-07 chromophore binds covalently to the DNA containing guanine and is intercalated into the DNA not containing guanine[12,13]. Based on our results of restriction enzyme digestion of SN-07 chromophore-λDNA complex, we predicted that SN-07 chromophore preferentially binds to 5′-GC-3′ sequence[13].

DNase I footprinting analysis has been employed to investigate DNA binding characteristics of intercalative anthracycline antibiotics such as daunomycin and nogalamycin. Daunomycin did not show a clear pattern of protection because it easily dissociated from DNA[14]. Recently, it was shown that the binding site for daunomycin contained adjacent GC base pairs on binding at 4°C[15]. Nogalamycin interacted with DNA with a low sequence specificity[14,16]. On the other hand, pyrrolo(1,4)benzodiazepine antitumor antibiotics that bound covalently to the 2-amino group of guanine showed a sequence-selective binding ability. The most preferred binding sequence for these drugs was 5′-PuGPu-3′ and the least was 5′-PyGPy-3′[17].

Fig. 1. Structure of SN-07 chromophore (carbinolamine type).



The experiments described in this paper confirmed that an anthracycline antibiotic SN-07 chromophore bound to DNA in a sequence-selective fashion and the preferred sequence was 5′-GC-3′. In our knowledge, SN-07 chromophore was the first anthracycline antibiotic which bound to DNA covalently in a sequence-selective manner.

Materials and Methods

Antibiotics, Enzymes and Chemicals

SN-07 chromophore was purified as described previously[1]. Carminomycin III and the reduction product were obtained by reacting SN-07 chromophore with sodium cyanoborohydride[2]. Solution of each antibiotic was prepared by diluting the stock solution stored in methanol (1 mg/ml) at −20°C. T4 polynucleotide kinase was purchased from Boehringer Mannheim (Germany) and deoxyribonuclease I (DNase I) was purchased from Sigma Chemical Company (St. Louis, U.S.A.). Bacterial alkaline phosphatase, restriction enzymes and pBR322 were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). All chemicals used were reagent grade. Chemicals for polyacrylamide gel were electrophoretic grade. [γ-^{32}P]ATP (>5,000 Ci/mmol) was purchased from Amersham.

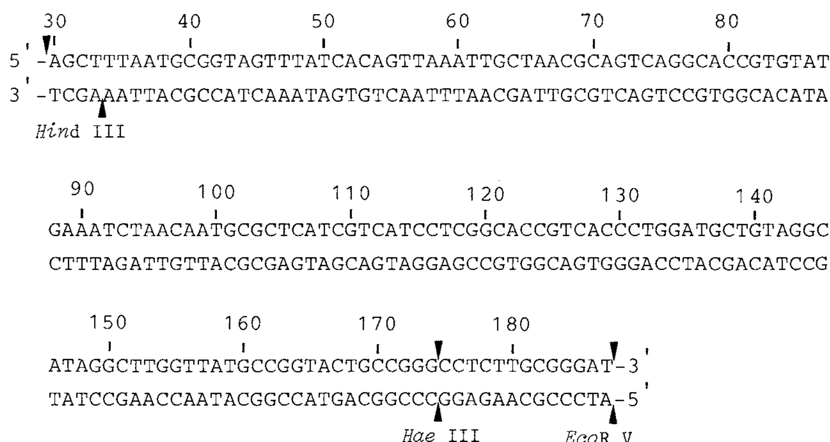
DNA Substrates

The *Hin*d III - *Hae* III (position 30 ~ 174†, 145 base-pairs (bp)) and *Eco*R V-*Hin*d III (position 187 ~ 34†, 154 bp) fragments from plasmid pBR322 were used (Fig. 2). The minus (−) strand probe was prepared by cutting pBR322 DNA first with *Hin*d III and the terminal 5′ phosphates were removed with bacterial alkaline phosphatase. The DNA was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified by Sephadex G-50 column chromatography. The ^{32}P-labeled DNA fragment was cut by *Hae* III and the *Hin*d III-*Hae* III fragment of 145 bp long (Fig. 2) was separated by 8% polyacrylamide gel electrophoresis. The fragment was extracted from the gel with elution buffer (4×10^7 cpm/μg). The same procedure was employed for labeling plus (+) strand of pBR322 DNA except that pBR322 DNA was cut first by *Eco*R V instead of *Hin*d III. Specific activity of *Eco*R V-*Hin*d III DNA fragment of 154 bp (Fig. 2) was 3×10^7 cpm/μg.

DNase I Footprinting

SN-07 chromophore was mixed with one of the ^{32}P-labeled DNA fragment in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) according to the method described previously[12]. The mixture was incubated for 2 days at room temperature and 1 μg of poly(dI)-poly(dC) was added to the mixture. SN-07 chromophore-DNA complex thus made was then digested with DNase I in 25 μl of DNase I buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 5% glycerol). The final DNase I concentration was 0.4 μg/ml and the solution was incubated for 1 minute at room temperature. The reaction mixture was dried under vacuum after adding 1 μl of 0.5 M EDTA. The DNA was resuspended

Fig. 2. Sequence and numbering scheme for the pBR322 DNA fragment.



† The nucleotides are numbered in a clockwise direction from the single *Eco*R I site. Nucleotide 1 is the first tymidine residue in the *Eco*R I site.

in 80% formamide - 10 mM NaOH - 1 mM EDTA - 0.1% xylene cyanol - 0.1% bromophenol blue and incubated at 90°C for 5 minutes prior to gel electrophoresis.

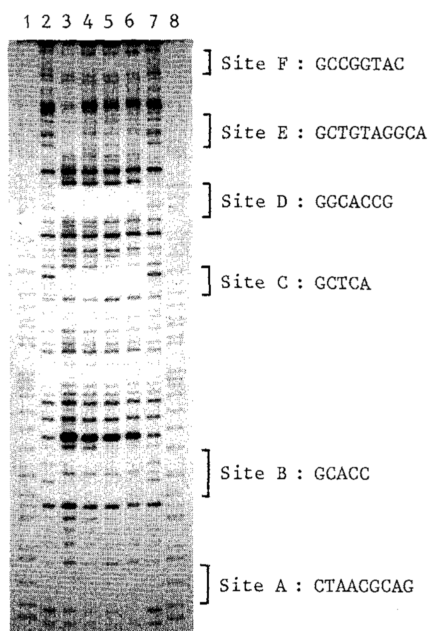
Gel Electrophoresis

The digestion products of *Hin*d III-*Hae* III and *Eco*R V-*Hin*d III fragments were analyzed on a 0.4-mm thick 8% (w/w) polyacrylamide gel containing 7 M urea in Tris-borate-EDTA buffer (pH 8.3). After electrophoresis at 1,700 V for 3 hours, the gel was dried and subjected to autoradiography at −80°C with an intensifying screen. Bands in the digestion pattern were assigned by using formic acid-piperidine markers specific for guanine and adenine[18].

Results

Footprinting patterns for the 145 bp *Hin*d III-*Hae* III DNA fragment in the presence and absence of SN-07 chromophore are displayed in Fig. 3. It was immediately apparent that the cleavage pattern in the presence of SN-07 chromophore was substantially different from that of the DNA alone. At molar ratios of SN-07 chromophore to DNA fragment of 0.5 : 1 (lanes 3 and 4 in Fig. 3) and 50 : 1 (lanes 5 and 6), six sites near positions 68 (site A), 79 (site B), 105 (site C), 122 (site D), 141 (site E) and 163 (site F) from *Eco*R I site were protected with SN-07

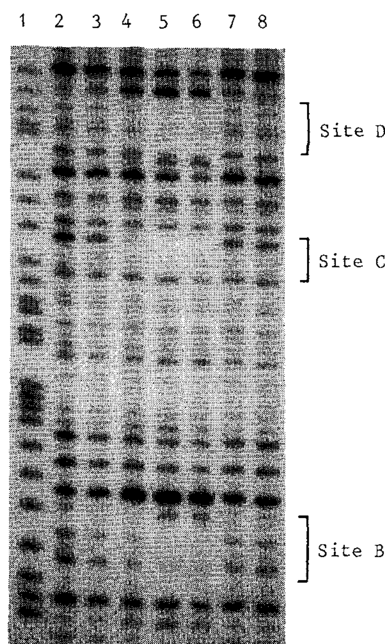
Fig. 3. DNase I digestion patterns for the 145 bp pBR322 DNA (labeled at the 5′ end of the upper strand) in the presence of SN-07 chromophore.



The digestion was performed as described under the Materials and Methods.

Lanes 1 and 8: Maxam-Gilbert A+G reactions. Lanes 2 and 7: Control digestions without SN-07 chromophore. Lanes 3 and 4: SN-07 chromophore-DNA (0.5 : 1). Lanes 5 and 6: SN-07 chromophore-DNA (50 : 1).

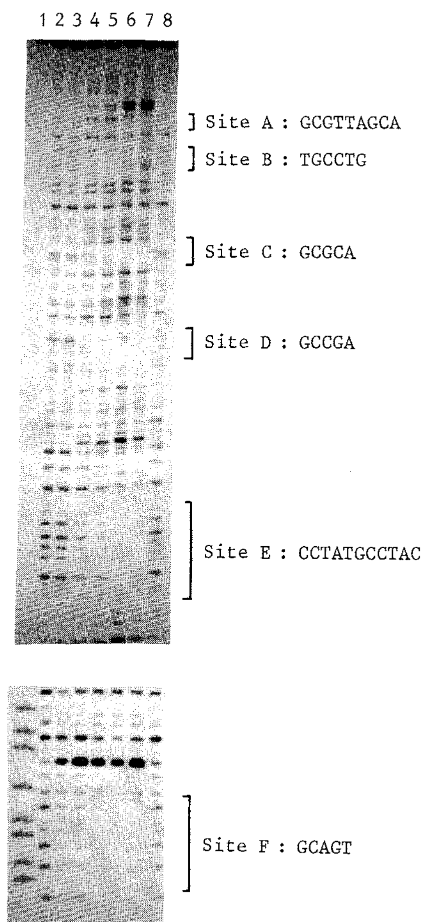
Fig. 4. DNase I digestion patterns for the 145 bp pBR322 DNA (labeled at the 5′ end of the upper strand) in the presence of SN-07 chromophore and related compounds.



The digestion was performed as described under the Materials and Methods.

Lane 1: Maxam-Gilbert A+G reaction. Lane 2: control digestion without SN-07 chromophore. Lane 3: SN-07 chromophore-DNA (0.004 : 1). Lane 4: SN-07 chromophore-DNA (0.02 : 1). Lane 5: SN-07 chromophore-DNA (0.1 : 1). Lane 6: SN-07 chromophore-DNA (0.5 : 1). Lane 7: carminomycin III-DNA (0.5 : 1). Lane 8: reduction product-DNA (0.5 : 1).

Fig. 5. DNase I digestion patterns for the 154 bp pBR322 DNA (labeled at the 5′ end of the lower strand) in the presence of SN-07 chromophore and carminomycin III.



The digestion was performed as described under the Materials and Methods.

Lane 1: Maxam-Gilbert A+G reaction. Lane 2: control digestion without SN-07 chromophore. Lane 3: SN-07 chromophore-DNA (0.004:1). Lane 4: SN-07 chromophore-DNA (0.02:1). Lane 5: SN-07 chromophore-DNA (0.1:1). Lane 6: SN-07 chromophore-DNA (0.5:1). Lane 7: SN-07 chromophore-DNA (50:1). Lane 8: carminomycin III-DNA (0.5:1).

chromophore. The protected regions were five to ten base pairs long. An increased DNase I sensitivity was observed at the regions adjacent to the protected sites (Fig. 3).

The pattern of cleavage varies with the antibiotic concentration (Fig. 4). The protection was observed at the molar ratio of SN-07 chromophore to DNA of 0.02:1 (lane 4 in Fig. 4). The inhibition of restriction enzyme reaction was also observed at the same SN-07 chromophore to DNA ratio[13].

On the other hand, related anthracycline antibiotic, carminomycin III and the reduction product of SN-07 chromophore[2] that did not contain a carbinolamine structure, had almost no effect on the cleavage pattern in the above conditions (lanes 7 and 8 in Fig. 4).

A typical DNase I footprinting pattern for the 154 bp *Eco*R V-*Hin*d III DNA fragment is shown in Fig. 5. At molar ratio of SN-07 chromophore to DNA fragment of 0.5:1 (lane 6 in Fig. 5), six sites near positions 66 (site A), 76 (site B), 102 (site C), 119 (site D), 145 (site E) and 168 (site F) from *Eco*R I site were protected with SN-07 chromophore. The protected region at each site was five to eleven base pairs long. Enhanced sensitivity to DNase I was observed at the regions adjacent to the binding sites (Fig. 5).

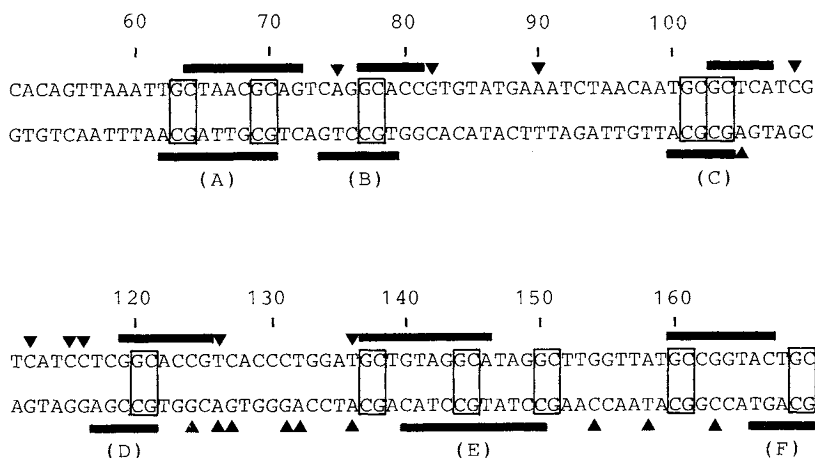
The results described above are summarized in Fig. 6. Almost all the 5′-GC-3′ sequences in the fragment were located in the DNase I resistant sites. In some sites (sites A, B, C and D), the block staggered across the two strands by about two to three bases toward the 3′ end, as previously observed for DNase I footprinting with other antibiotics[19].

Discussion

Footprinting is a technique that has been developed to identify the precise binding sites for ligands on DNA fragments of defined sequence. Sites to which the antibiotic is bound are protected from enzymatic (or chemical) cleavage and are visualized at a single band resolution as gaps in the autoradiogram of a denaturing polyacrylamide gel[20].

Biochemical target for many antitumor antibiotics are DNA. They recognize DNA sequence with varying degrees of specificity. Among them are several antibiotics such as actinomycin which binds to

Fig. 6. Summary of footprinting results for SN-07 chromophore.



Bars indicate protected regions while triangles show positions of enhanced DNase I sensitivity. The upper sequence represents the 'Watson' (−) strand and the lower sequence represents the 'Crick' (+) strand. These maps were complied from visual inspection of numerous gels and may be considered as a set of averaged values.

sequence 5'-GC-3'[21,22], echinomycin to 5'-CG-3'[19,23], mithramycin to 5'-GG-3'[24,25], pyrrolo(1,4)benzodiazepine antibiotics to 5'-PuGPu-3'[17], and distamycin to AT rich regions[21]. On the other hand, sequence-selectivity of the intercalative anthracycline antibiotic daunomycin was shown only under the restricted conditions[15]. Nogalamycin binds to preferential regions of mixed sequence containing purines and pyrimidines[16].

SN-07 chromophore is an anthracycline antibiotic. But it is unique because it has two binding sites for DNA, namely, carminomycinone(intercalation) and carbinolamine(covalent binding) structures (Fig. 1). We have shown by restriction enzyme digestion studies that SN-07 chromophore has a preferred sequence selectivity for 5'-GC-3'. The sequence selectivity was presumably due to the carbinolamine function of SN-07 chromophore which differed from the related anthracycline antibiotics[12,13].

The results presented in this paper show that SN-07 chromophore did induce considerable changes in the susceptibility of particular nucleotide sequences to the attack by DNase I. Observed DNase I sensitive sites (Fig. 6) presumably resulted from conformational changes in the DNA induced by the binding of the ligand[16,19,21]. Similar enhancement of DNase I cleavage has previously been observed with other sequence selective ligands[24,26]. In the E and F regions, the block staggered across the two strands toward the 5' end differing from A, B, C and D regions. This might depend on the conformational changes of E and F resistance regions.

DNase I footprinting patterns produced by SN-07 chromophore bound on pBR322 DNA fragment revealed that all six DNase I resistance resions contained 5'-GC-3' sequence. Some site protected with SN-07 chromophore was longer than those protected by other antibiotics. More than 2 molecules of SN-07 chromophore might bind to the A, E and F regions.

These results were entirely consistent with our previous results and the hypothetical binding model of SN-07 chromophore to DNA, that is, SN-07 chromophore covalently bind to 2-amino group of guanine[12]. SN-07 chromophore was the first anthracycline antibiotic that bind to DNA covalently in a sequence-selective manner by carbinolamine function.

Acknowledgments

We are grateful to Prof. S. MIZUNO, Tohoku University, Sendai, and Dr. A. YUKI, Research Institute of Life Science, Snow Brand Milk Products Co., Ltd., for their helpful advice on this study.

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