Effect of netropsin on plasmid DNA cleavage by BAL 31 nuclease

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BAL 31 nuclease is known to possess two sorts of catalytic activities: one is a single-strand-specific endonuclease activity that converts negatively supercoiled DNA to the unit-length linear form, the other being a quasi-processive exonuclease activity that simultaneously degrades both 3'- and 5'-termini of linear duplex DNA. Netropsin, a bactericidal and antiviral compound, was found to enhance the former activity but inhibit the latter. Netropsin-bound supercoiled plasmid DNA had a tendency to be fragmented by BAL 31 into several species of linear DNAs smaller than full-length size. Size reduction of linear plasmid DNA by BAL 31 was significantly inhibited by netropsin binding.

Netropsin *Bactericidal antibiotic* *Antiviral antibiotic* *BAL 31 nuclease* *S1 nuclease* *Plasmid DNA structure distortion*

1. INTRODUCTION

The bactericidal and antiviral compound netropsin binds in a minor groove of the double helix of DNA interacting with A·T-rich sequences [1–3] by a non-intercalative mechanism [4,5]. It is a powerful inhibitor of RNA and DNA polymerases [6] as well as restriction endonucleases [7]. Netropsin exhibits the specificity for the right-handed B conformation of DNA, and can induce reversal from the Z-form and other non-B-forms to the B-form [8]. The data on the sedimentation coefficient [2] and NMR chemical shift [9] suggest that netropsin and intercalating drug have opposite effects on DNA. Snounou and Malcolm [5] reported that, in the presence of netropsin, positively superhelical molecules are produced by topoisomerase I action. To characterize the distortion of the DNA structure induced by netropsin binding, we have studied the change in single-strand-specific nuclease S1 susceptibility of negatively supercoiled DNA [10]. It is known that negative supercoiling introduces localized unwinding of helical base pairs into DNA molecules [11]. S1 can cleave both strands at one of the unbase-paired sites to yield full-length linear molecules [11]. It is interesting to note that netropsin-bound DNA has a tendency to be cleaved by S1 simultaneously at multiple sites, producing several species of linear DNAs smaller than full-length size [10]. This result, however, was obtained at acid pH at which S1 nuclease exhibits its activity. It is uncertain whether netropsin has a similar effect on the supercoiled DNA at neutral pH. To solve this problem, we attempted to use BAL 31 nuclease which works efficiently at pH 8.0.

2. MATERIALS AND METHODS

2.1. *DNAs*

Negatively supercoiled plasmid DNAs of pUB110 and pTP-4 were prepared from *Bacillus subtilis* as described in [12].

2.2. *Enzymes and reactions*

Substrate DNA (2.5 μg) was incubated without or with various concentrations of netropsin at 37°C for 15 min in 10 μl reaction buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl and 0.5 mM EDTA. Netropsin was a generous gift

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from Laboratory of Antibiotics, Riken Institute, Saitama, Japan. Incubation was carried out in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 1 mM EDTA in a total volume of 20 μl at 30°C for 15 min, unless indicated otherwise. BAL 31 was purchased from Takara Shuzo, Kyoto. One unit of BAL 31 activity is defined as the amount of enzyme that releases 1 μg acid-soluble nucleotide in 1 min at 30°C in the reaction mixture containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 12 mM $MgCl_2$, 12 mM $CaCl_2$, 1 mM EDTA, 0.65 mg/ml alkali-denatured DNA. The BAL 31 reaction was stopped by addition of 1/10 vol. of 0.5 M EDTA. The S1 reaction was done as in [10]. Linear DNA was prepared by digesting to completion 40 μg pTP-4 with *Hin*dIII (Takara Shuzo) restriction endonuclease. The DNA digest was extracted twice with an equal volume of phenol, treated several times with ether, precipitated with 2 vols ethanol and dissolved in 10 mM Tris-HCl (pH 7.5) and 0.5 mM EDTA.

2.3. *Agarose gel electrophoresis*

After addition of 1/10 vol. of 0.05% bromphenol blue-80% glycerol, DNA samples were analyzed electrophoretically on agarose horizontal slab gels as in [13] under the conditions described in the figure legends. DNA staining and photography were done as in [13].

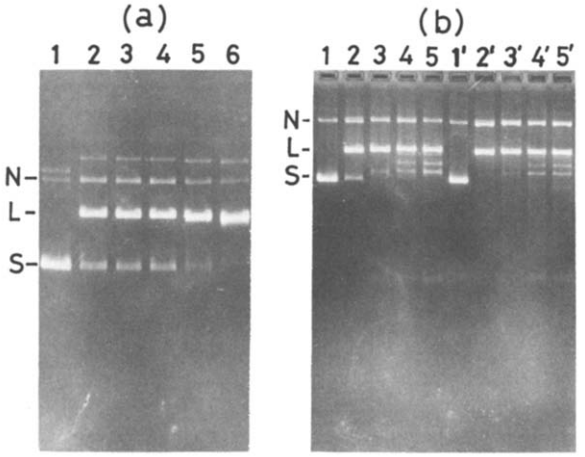

Fig.1. Nuclease digestion patterns of untreated (a) and netropsin-treated (b) supercoiled pUB110 DNA. (a) BAL 31 digests (2.5 μg each). Lanes: 1, DNA alone; 2, 0.1; 3, 0.12; 4, 0.15; 5, 0.20; 6, 0.25 unit. (b) DNA (2.5 μg) treated with the following amounts of netropsin was digested with 0.15 unit BAL 31 (lanes 1–5) and 5 units S1 (lanes 1′–5′). Lanes: 1, 1′, DNA alone; 2, 2′, 0; 3, 3′, 0.04; 4, 4′, 0.10; 5, 5′, 0.20 μg. Two sets each of the same reactions in (b) were done in parallel, combined, extracted twice with phenol and treated with ether. Electrophoreses were in 1% (a) and 1.5% (b) agarose at 3–4 V/cm for ~3 h. N, L, S, nicked, full-length linear and supercoiled forms, respectively. DNA bands seen above the nicked form are the nuclease digests of the dimer DNA species copurified in the DNA preparation.

3. RESULTS AND DISCUSSION

Fig.1a shows the cleavage patterns of natural plasmid pUB110 DNA by BAL 31 as a function of enzyme concentration. BAL 31 nuclease is known to possess, in addition to a single-strand-specific endonuclease activity that converts supercoiled DNA to the full-length linear form, quasi-processive exonuclease activity that simultaneously degrades both 3′- and 5′-termini of linear duplex DNA [14]. The efficiency of cleavage of the DNA was proportional to the concentration of the enzyme used. With 0.1–0.15 unit (lanes 2–4), approx. 70% of the DNA was converted to the full-length linear form, the rest being non-digested, supercoiled and nicked, circular forms. With 0.2 unit (lane 5) and 0.25 unit (lane 6), the extent of linearization of the DNA increased. However, size reduction of the linearized DNA was observed owing to the above-mentioned exonuclease activity of BAL 31. To characterize the binding of the drug, netropsin-bound pUB110 was digested with 0.15 unit BAL 31 (lanes 3–5 in fig.1b). It was evident that several species of the fragmented linear DNAs were produced in addition to the full-length linear form. Production of DNA fragments was dependent upon the amount of netropsin. Without netropsin (lane 2), fragmented linear DNA was not generated. These results indicate that BAL 31 introduces multiple double-strand cuts into the netropsin-bound DNA. Compared to BAL 31, netropsin-bound pUB110 was also digested with S1 nuclease (lanes 3′–5′ in fig.1b). Almost the same cleavage patterns were obtained by both enzymes, although, in the case of S1, a few minor DNA fragments of intermediate molecular size were additionally detectable on the gels.

The effect of netropsin on DNA cleavage by

BAL 31 was tested with other plasmid DNA, i.e. pTP-4 (fig.2). Two size-defined DNA fragments were produced, indicating a unique cleavage depending on the DNA source.

To rule out the possibility that netropsin binds to the nuclease protein and changes its cleavage specificity, both BAL 31 and S1 were incubated with a sufficient amount of netropsin, fully dialyzed and then the enzyme activities were determined. The results showed that BAL 31 and S1 retained their original cleavage specificity to generate only unit-length linear DNA. Thus, the data above confirm that netropsin alters the overall conformation of supercoiled plasmid DNA at both pH 5 (S1) and 8 (BAL 31) so that simultaneous cleavage occurs at specific, multiple sites on each DNA molecule. However, it was noticeable that simultaneous cleavage is not so efficient. Since introduction of a single cut instantly converts supercoiled DNA to the nicked, circular form, the chance that all the DNA molecules are cleaved at multiple sites is limited. Recently, Kopka et al. [15] have reported that netropsin alters the backbone of DNA in such a way as to give a small additional helical twist. This constraint of the DNA molecule is favourable for stabilizing the localized unwinding of the double helix in closed, circular DNA whose polynucleotide strands cannot rotate freely. Thus, the susceptibility of supercoiled DNA to a single-strand-specific nuclease is enhanced by netropsin.

The effect of netropsin on the action of BAL 31 on linear duplex pTP-4 DNA was investigated (fig.3). The results show that netropsin inhibits significantly BAL 31 exonuclease-catalyzed shortening of the linear DNA. Shishido has observed that spermine enhances BAL 31 exonuclease activity dramatically (unpublished). Spermine is known to bind in the minor groove of the double helix interacting with phosphate groups of the DNA backbone, stabilizing the double helix [16,17], showing an analogous characteristic to netropsin. The contrasting effect on BAL 31 exonuclease activity between both bioactive substances is interesting. Moreover, spermine-bound supercoiled DNA is not cleaved by both BAL 31 and S1 simultaneously at multiple sites on each DNA molecule (unpublished).

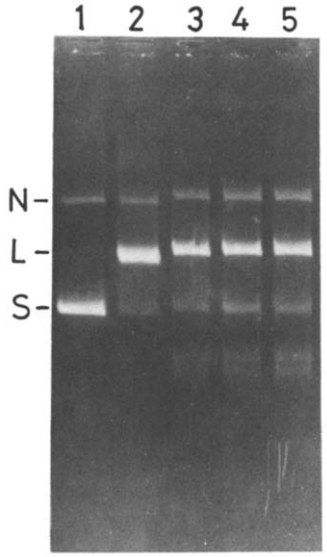

Fig.2. BAL 31 digestion patterns of supercoiled pTP-4 DNA treated with netropsin. DNA (2.5 μg) was digested with 0.15 unit. Lanes: 1, DNA alone; 2, 0; 3, 0.02; 4, 0.05; 5, 0.15 μg drug. DNA samples were extracted with phenol and electrophoresed in 1% agarose as in fig.1. N, L, S as in fig.1.

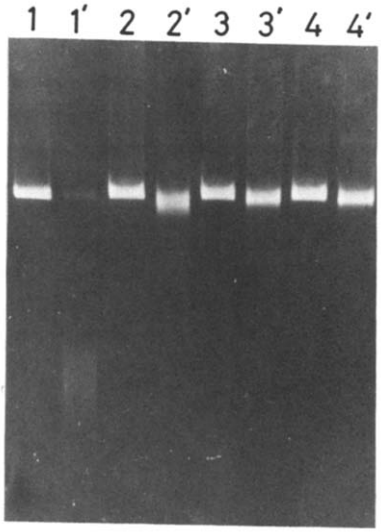

Fig.3. BAL 31 digestion patterns of linear pTP-4 DNA treated with netropsin. *Hin*dIII-generated linear DNA (2.5 μg) was incubated without (lanes 1–4) and with 0.7 unit (lanes 1'–4') for 30 min. Lanes: 1, 1', 0; 2, 2', 0.02; 3, 3', 0.05; 4, 4', 0.10 μg drug. DNA samples were extracted with phenol and electrophoresed in 1% agarose as in fig.1.

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