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Induction of double-strand breaks by S1 nuclease, mung bean nuclease and nuclease P1 in DNA containing abasic sites and nicks

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ABSTRACT

Defined DNA substrates containing discrete abasic sites or paired abasic sites set 1, 3, 5 and 7 bases apart on opposite strands were constructed to examine the reactivity of S1, mung bean and P1 nucleases towards abasic sites. None of the enzymes acted on the substrate containing discrete abasic sites. Under conditions where little or no non-specific DNA degradation was observed, all three nucleases were able to generate double-strand breaks when the bistranded abasic sites were 1 and 3 base pairs apart. However, when the abasic sites were further apart, the enzymes again failed to cleave the DNA. These results indicate that single abasic sites do not cause sufficient denaturation of the DNA to allow incision by these single-strand specific endonucleases. The reactivity of these enzymes was also investigated on DNA substrates that were nicked by DNaseI or more site-specifically by endonuclease III incision at the discrete abasic sites. The three nucleases readily induced a strand break opposite such nicks.

INTRODUCTION

S1 nuclease from *Aspergillus oryzae*, mung bean nuclease and nuclease P1 from *Penicillium citrinum* are endonucleases that exhibit high selectivity for single stranded nucleic acids, producing mono- and oligo-nucleotides terminating in 5′-phosphoryl and 3′-hydroxyl groups (reviewed in ref. 1). S1 nuclease can hydrolyse single-stranded regions in duplex DNA and also detect locally altered structures (e.g. minor distortions) introduced by physical and chemical procedures (1,2). In some instances, the enzyme can convert superhelical DNA to a nicked circular molecule and then to a unit-length linear molecule (3).

Mung bean nuclease similarly cleaves single-stranded regions of DNA such as single-stranded tails (4) and internal single-stranded gaps (5). The presence of regions of altered secondary structures, i.e., transiently localized unwound regions, is considered to be responsible for a limited number of incisions in superhelical DNA (6,7). Supercoiled DNA is converted to singly nicked circular DNA. The nicked-circular DNA is comparatively resistant to linearization (8). Nuclease P1 shows a substrate specificity similar to that of S1 and mung bean nucleases (1,9).

S1 nuclease, in particular, has been used to monitor chemical-induced disruption of DNA secondary structure. Enhanced susceptibility to digestion by S1 nuclease has been observed with DNA treated with UV light (10), alkylating agents (11,12), cisplatin (13) and *N*-acetoxy-*N*-2-acetylaminofluorene (14). DNA double-strand breaks (DSB) are observed following incubation of γ-irradiated DNA with S1 nuclease (15,16). These DSB are attributed to cleavage opposite single-strand breaks and to localized DNA denaturation caused by radiation-induced base damage or possibly base loss.

Introduction of an abasic site results in localised denaturation of duplex DNA (17). Shishido and Ando (18) investigated the action of S1 on T7 DNA that had been subjected to limited depurination at acidic pH. Nuclease-induced DNA degradation, which was measured by neutral sedimentation, was shown to be dependent on the degree of depurination, but it appeared that only a small fraction of the abasic sites gave rise to DSB. The action of nuclease P1 and mung bean nuclease on DNA containing abasic sites is unknown. BAL 31 nuclease, another well-characterized single-strand specific endonuclease, has been shown to react rapidly with nonsupercoiled, covalently closed cicular (ccc) phage PM2 DNA containing a single abasic site (19).

Abasic sites can arise spontaneously due to acid-catalyzed hydrolysis, by the action of DNA glycosylases, by exposure to chemicals (particularly alkylating agents) or by radiation (20). Because of the frequent occurence of abasic sites and the wide spread use of the three nucleases, we have examined the response of these enzymes towards DNA containing abasic sites using well-defined model substrates. One possible explanation for the low frequency of DSB induction observed by Shishido and Ando (18) is that a single abasic site may not cause sufficient denaturation of the DNA and DSBs were produced only at locations with two AP-sites in close proximity. We have, therefore, compared nuclease activity on substrates with single abasic sites or pairs of abasic sites on opposite strands in set loci. In addition, we have investigated the ability of these nucleases to produce a strand break opposite a nick in the DNA.

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MATERIALS AND METHODS

Enzymes

S1 nuclease, mung bean nuclease, *Hin*dIII and uracil–DNA glycosylase were purchased from Life Technologies Inc and DNaseI from Sigma. Nuclease P1, *Bam*HI and *Sal*I were obtained from Pharmacia, and *Acc*65 I, *Hin*cII, *Nhe*I and phage T4 DNA ligase from New England Biolabs. Unit (U) definitions of the three enzymes under investigation are as follows: S1 and mung bean nucleases, 1 U hydrolyzes 1 μg of denatured DNA to acid-soluble material in 1 min at 37°C; nuclease P1, 1 U catalyzes the hydrolysis of 1 μmol of 3′-AMP per min at 37°C (at pH 7.2). *E.coli* endonuclease III (fraction IV) was purified from the strain $\lambda N99_{C1857}$ carrying the pHIT1 plasmid (kindly provided by Dr R.P. Cunningham of SUNY, Albany, NY) according to the procedure of Asahara *et al.* (21).

Preparation of substrates

Plasmid oligomers with single isolated AP-sites. Plasmid DNA was prepared as described before (22). Plasmid pUC18 DNA was digested with *Hin*cII followed by *Hin*dIII. The restriction enzymes were then removed by phenol/chloroform extraction. The overhanging DNA termini produced by *Hin*dIII were enzymatically filled in by 15 min incubation at room temperature with 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 μg/ml BSA, 75 μM each of dGTP, dCTP, dATP and dUTP and 1 U of Klenow fragment of *E.coli* DNA polymerase I per μg of DNA. The reaction was terminated by the addition of 0.5 M EDTA to a final concentration of 25 mM and the DNA was extracted once with phenol/chloroform and precipitated. Blunt-end ligations were carried out in a total volume of 200 μl containing 20 μg of DNA, 10% polyethylene glycol, the reaction buffer provided by the supplier of the ligase (New England Biolabs) and 400 U T4 DNA ligase. Ligation of these blunt-ended molecules can occur in three ways depending on the termini that are joined, that is *Hin*cII/*Hin*cII, *Hin*dIII/*Hin*dIII and *Hin*cII/*Hin*dIII. The symmetrical junctions were selectively cleaved by incubation with *Hin*cII and *Nhe*I, respectively. The uracil bases were removed by incubation with uracil–DNA glycosylase in 30 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$ at 37°C for 1 h to produce abasic sites.

Plasmid oligomers with paired AP-sites. These substrates were prepared as previously described (23). Briefly, pUC18 plasmid DNA was singly digested with *Hin*dIII, *Bam*HI, *Acc*65 I or *Sal*I. The termini were enzymatically filled in with dATP, dGTP, dCTP and dUTP, using the Klenow fragment of DNA polymerase I, followed by blunt end ligation with T4 DNA ligase. This procedure places dUTP on opposite strands of the DNA set at 1, 3, 5 and 7 bases apart, respectively. After ligation, the DNA was incubated with uracil–DNA glycosylase to produce abasic sites. The removal of uracil from the DNA was monitored in parallel experiments by the incorporation of [^{3}H]dUTP (Amersham International) and the subsequent release of uracil in the supernatant by scintillation counting after completion of the reaction and precipitation of the DNA.

Nicked DNA substrates. One substrate was prepared by nicking form I plasmid DNA with DNaseI (24). The DNA was incubated with DNaseI (1 U/μg of DNA) at 30°C for 90 min in the presence of 330 μg/ml of ethidium bromide. The reaction was terminated by the addition of EDTA to a final concentration of 20 mM. The DNA was then extracted with phenol, phenol/chloroform and chloroform before being precipitated.

A second substrate was generated by incubating the isolated abasic site-containing species described above with *E.coli* endonuclease III. Approximately 1 μg of DNA was incubated with 6 ng of the enzyme in 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM EDTA. 0.1 mM DTT at 37°C for 1 h.

Reaction with S1 nuclease, mung bean nuclease and nuclease P1

The S1 nuclease reaction was carried out in 50 mM sodium acetate (pH 4.5), 200 mM NaCl, 1 mM $ZnSO_4$, 0.5% glycerol, 300–400 ng of DNA and 100 U of S1 nuclease in a total volume of 10 μl, at 37°C for 15 min The mung bean reaction was performed in the same buffer as for S1 with 147 U of mung bean nuclease and 37°C incubation for 30 min. The nuclease P1 reaction was also carried out in the S1 reaction buffer at 37°C for 15 min with 0.2 U of nuclease P1. To induce cleavage opposite a nick, 0.06 U of the nuclease P1 were used.

Analysis of DNA products

Production of double-strand breaks and ligation reactions were monitored by neutral agarose gel electrophoresis followed by ethidium bromide staining and visualization of gels under UV light. The relative quantity of DNA in each gel band was determined by densitometric scanning of the negatives of the Polaroid type 665 film on a PDI DNA 35 scanner (PDI, Huntington Station, NY) using the PDI Quantity One software.

RESULTS

Reactivity of S1 nuclease, mung bean nuclease and nuclease P1 on the plasmid DNA containing AP-sites

Action at single isolated abasic sites. A substrate containing isolated AP-sites (Table 1, substrate A) was prepared by a five-step procedure: (i) digestion of ccc pUC18 DNA with *Hin*cII and *Hin*dIII; (ii) DNA polymerase-catalysed conversion of the *Hin*dIII terminus to a blunt end and incorporation of a deoxyuridine; (iii) ligation of the blunt ended molecule; (iv) cleavage of *Hin*cII/*Hin*cII junctions by incubation with *Hin*cII and cleavage of blunt-ended *Hin*dIII/*Hin*dIII junctions by incubation with *Nhe*I; and (v) creation of AP-sites by removal of uracil with uracil–DNA glycosylase. Ligation of the plasmid DNA generated linear oligomers of pUC18 DNA up to at least four monomers in length (Fig. 1, lane 1). Cleavage by the nucleases at or near the abasic site, followed by incision opposite the newly introduced nick, would be anticipated to reduce the pUC18 oligomers to unit-length plasmid. The reaction products were analysed by agarose gel electrophoresis at neutral pH. Figure 1 (lanes 3, 5 and 7) demonstrates that none of the enzymes was able to incise the oligomers. Reducing the high salt concentration of the digestion buffer (200 mM NaCl) to 130 mM and 60 mM NaCl led to an increase in non-specific degradation, but did not enhance cleavage at the abasic sites (data not shown). As a positive control for enzyme activity, a substrate was prepared by introducing single-strand breaks at the abasic sites by incubation with *E.coli* endonuclease III. Subsequent incubation with S1 nuclease, mung

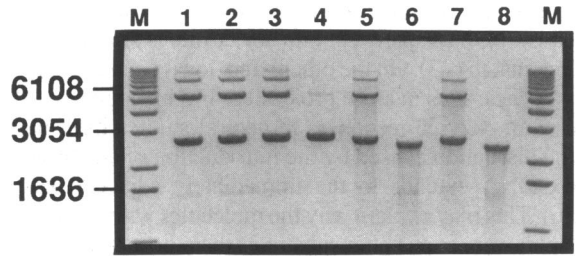

Figure 1. Action of nucleases on DNA substrates containing discrete abasic sites or single-strand breaks. Lane 1 shows the oligomeric pUC18 substrate containing abasic sites. Lanes 3, 5 and 7 show the result of incubation of this substrate with S1 nuclease, mung bean nuclease and nuclease P1, respectively. Lane 2 shows that treatment with endonuclease III to nick the DNA at the abasic sites does not alter the mobility of the plasmid molecules. Lanes 4, 6 and 8 show the result of incubation of the nicked substrate with S1 nuclease, mung bean nuclease and nuclease P1, respectively. Lanes marked M show a 1 kb linear double-stranded DNA ladder (Life Technologies Inc.) with some of the sizes indicated.

bean nuclease or nuclease P1 (Fig. 1, lanes 4, 6 and 8) produced double-strand breaks (see below).

Table 1. DNA sequence and position of abasic sites at ligated pUC18 termini

Substrate	Distribution of AP-site(s)	DNA construct[a]	Sequence[b]
A	Single	*Hinc*II/*Hin*dIII	AAGC-GACCT
			TTCGACTGGA
B	Bi-stranded, 1 bp apart	*Hin*dIII/*Hin*dIII	AAGC-AGCTT
			TTCGA-CGAA
C	Bi-stranded, 3 bp apart	*Bam*HI/*Bam*HI	GGA-CGATCC
			CCTAGC-AGG
D	Bi-stranded, 5 bp apart	*Acc*65I/*Acc*65I	GG-ACGTACC
			CCATGCA-GG
E	Bi-stranded, 7 bp apart	*Sal*I/*Sal*I	G-CGATCGAC
			CAGCTAGC-G

[a]Denotes junction of blunt-ended termini. dUTP was incorporated opposite protruding termini and after ligation, uracil was removed by uracil–DNA glycosylase.
[b]Abasic sites are indicated by a dash.

Action at paired abasic sites. The preparation of substrates B–E (Table 1) containing AP-sites set 1, 3, 5 and 7 bp apart, respectively, on opposite strands has been more fully described elsewhere (23). Ligation of the blunt-ended linear monomers of pUC18 generated linear oligomers of pUC18 ranging from two to at least six in length, with relatively few circular monomers evident (Fig. 2a and b, lanes 1, 2, 6 and 7). These substrates were incubated with S1 nuclease, mung bean nuclease and nuclease P1. Figure 2 shows the results obtained with S1 nuclease. Double-strand breaks were readily induced in the plasmid DNA containing AP-sites at *Hin*dIII and *Bam*HI filled in sites, i.e., set 1 and 3 bp apart, respectively, (Fig. 2a, lanes 4 and 9), while no cleavage was observed in the controls in which either the enzyme was incubated with a substrate containing deoxyuridine in place of the AP-site (Fig. 2a, lanes 3 and 8) or the AP-site containing molecules were incubated in buffer alone (Fig. 2a, lanes 5 and

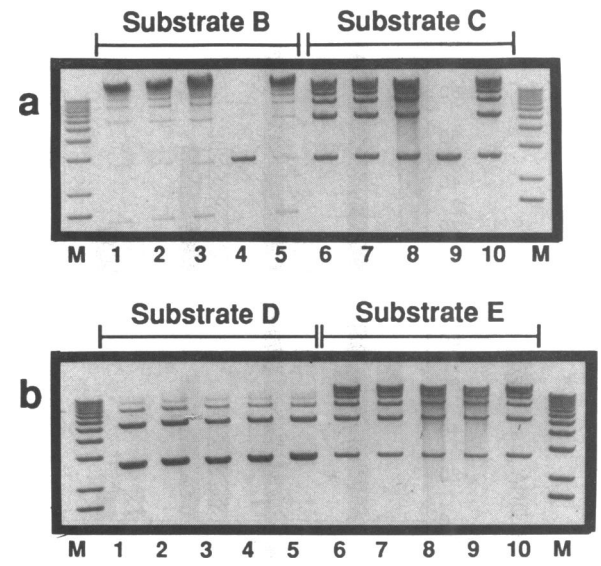

Figure 2. S1 nuclease reaction with plasmid DNA containing paired AP-sites. (**a**) Lane 1 shows ligated plasmid DNA filled in with dUTP at the *Hin*dIII cleavage site, lane 2 is this DNA incubated with uracil–DNA glycosylase to produce the AP-site containing substrate (Substrate B), lane 3 and 4 are the S1 treated dU and AP-site containing plasmid DNA, respectively and lane 5 is the AP-site containing DNA incubated with S1 nuclease reaction buffer alone. The same sequence of DNA samples are shown in lanes 6–10 except that this shows the products resulting from *Bam*HI cleavage of pUC18 (Substrate C). Lanes marked M show the 1 kb linear double-stranded DNA ladder. (**b**) The same sequence of products for the substrates prepared from *Acc*65 I (Substrate D, lanes 1–5) and *Sal*I (Substrate E, lanes 6–10) cleaved pUC18 DNA.

10). When the DNA substrates with AP-sites positioned at 5 and 7 bp apart were reacted with S1 nuclease no DNA cleavage at these sites was observed (Fig. 2b). Mung bean nuclease and nuclease P1 gave nearly identical results to S1 nuclease as indicated in Figure 3, which shows the quantitative data obtained by densitometry. Decreasing the salt concentration to 130 and 60 mM NaCl did not increase the level of DSB formation after incubation of substrate E with S1 nuclease (data not shown).

The action of S1 nuclease, mung bean nuclease and nuclease P1 opposite a nick in the DNA

The ability of S1 nuclease to cleave opposite a nick is well known, however, the action of mung bean nuclease and nuclease P1 at such sites has not been clearly established. A nicked substrate (form II DNA) was prepared by incubation of ccc pUC18 with DNaseI in the presence of ethidium bromide (24). The three nucleases readily converted this substrate to the linear form of the plasmid (Fig. 4, lanes 4–6). However, this observation does not prove conclusively that the nucleases cleaved the DNA opposite the nick generated by DNaseI, since cleavage could be occuring elsewhere in the plasmid DNA. That the nucleases cleave opposite a nick is evident from Figure 1, where it is shown that the nucleases incise the oligomer plasmid molecules containing nicks induced by endonuclease III to uniformly generate unit-length plasmid products.

DISCUSSION

This study was designed to characterise the interaction of S1 nuclease, mung bean nuclease and nuclease P1 with DNA

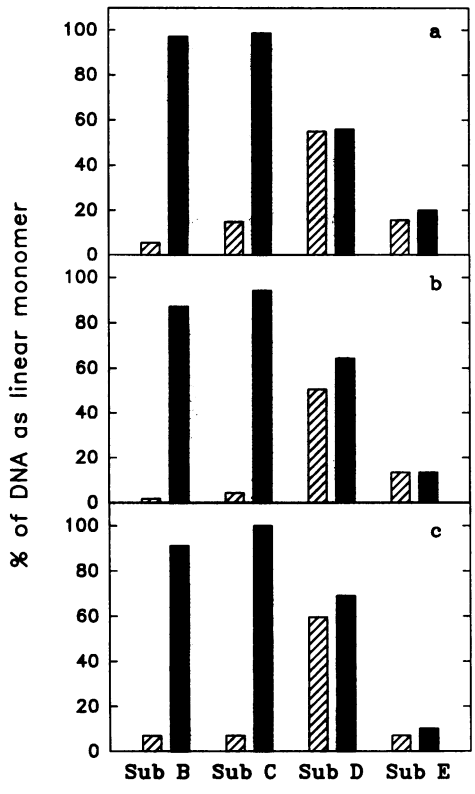

Figure 3. Monomerization of pUC18 oligomers containing paired abasic sites by S1 nuclease (**a**), mung bean nuclease (**b**) and nuclease P1 (**c**). The hatched bars represent DNA incubated with digestion buffer alone, while the solid bars represent DNA incubated with the enzyme.

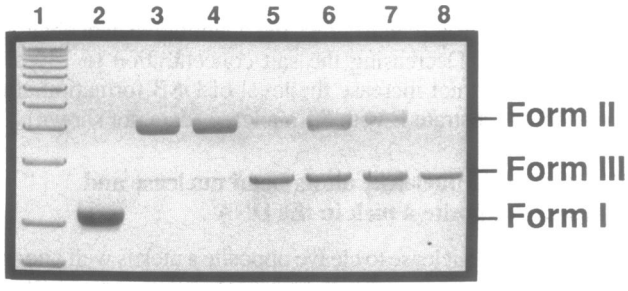

Figure 4. Action of S1, mung bean and P1 nucleases towards DNaseI nicked pUC18 DNA. Lane 1 is the 1 kb molecular weight ladder; lane 2 is the untreated plasmid pUC18 DNA; lane 3 is the DNaseI nicked substrate; lanes 4–7 show the DNaseI nicked plasmid incubated with reaction buffer alone, with S1, with mung bean nuclease and with P1 nuclease, respectively; lane 8 is restriction enzyme linearized pUC18 DNA.

substrates containing abasic sites or nicks. Previous studies have shown that although these nucleases are specific for single-stranded DNA, DNA with locally altered structures (minor distortions, transient unwinding, etc.) is recognised by these nucleases leading to DNA strand break formation (1). Hypochromicity (17), optical density (25) and NMR (25,26) analyses have demonstrated that abasic sites destabilize duplex DNA molecules. Our data indicates, however, that a single abasic site, within the sequence context we have examined, does not destabilize the DNA sufficiently to allow digestion by the nucleases. In this regard a single abasic site is similar to a single base mismatch (27). On the other hand, local denaturation caused by two abasic sites in close proximity (1–3 bp apart) is sufficient to elicit an enzyme response. Structural studies have indicated that the disruption caused by the introduction of an abasic site in duplex DNA extends to the immediately adjacent base pairs (25,26). This may explain why the molecules with abasic sites set 3 bp apart were even better substrates than those with abasic sites 1 bp apart and why little DSB formation was seen when the abasic sites were 5 or 7 bp apart. With the abasic sites set 1 and 3 bp apart, destabilization of the duplex would effectively extend over 4 and 6 base pairs, respectively. When the abasic sites are further apart, they have to be considered as individual abasic sites.

Our results with single and double AP-sites may also explain the observation by Shishido and Ando (18). Employing phage T7 DNA (38 000 bp) that had been treated at low pH to release ~1.65% of the bases (3.3% of the purines), they determined, by neutral sucrose sedimentation, that the average moleular weight of the molecule following S1 nuclease digestion was ~1/6 of the intact phage DNA. This is considerably larger than the anticipated molecular weight (~1/630 intact size) if it is assumed that the enzyme reaction went to completion and that double-strand incision occured at every abasic site. If the S1 nuclease requires two opposing abasic sites for cleavage, such sites can be calculated to occur with a frequency of the square of 1.65% or 0.027%, of the bases. Of course this is an oversimplification. A more complete model would take into account such features as the distance between abasic sites required for nucleolytic cleavage, which would result in smaller fragments. Nonetheless, in the simplest case, digestion with S1 nuclease would then be expected to generate products ~1/10 the size of the intact phage DNA, which falls much closer to the observed outcome.

We have presented direct evidence that all three nucleases preferentially cleave DNA opposite a nick. For S1 nuclease this is in accord with published reports (28,29). In the case of nuclease P1 this is, to our knowledge, the first time this question has been addressed. Our observations with mung bean nuclease, on the other hand, appear to contradict those of Kroeker and Kowalski (5) who found that the enzyme failed to preferentially incise phage T5 DNA opposite naturally occuring nicks. However, it should be noted that the concentration of enzyme in our experiments was ~30-fold greater than that used in the earlier investigation. In addition, Kroeker and Kowalski noted that if the nicks in the T5 DNA were expanded into gaps, by removal of a few nucleotides with exonuclease III, the opposite strand became highly susceptible to cleavage. We cannot rule out the possibility that the nucleases themselves slightly enlarge the nick before incising the opposite strand.

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