Interaction of the Periplasmic dG-Selective *Streptomyces antibioticus* Nuclease with Oligodeoxynucleotide Substrates[†]

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ABSTRACT: The interaction of a periplasmic nuclease, isolated from *Streptomyces antibioticus*, with several oligodeoxynucleotide substrates has been studied. Double-stranded oligonucleotides that contain sequences of four or more consecutive deoxyguanosine residues are preferentially hydrolyzed, with the strongest cutting site occurring at GGG↓G. The enzyme does not hydrolyze these sequences in single-stranded DNA. However the sequence selectivity of the nuclease is far from absolute. Other sequences can also be cut, albeit more poorly, and differences in cutting rates are observed for runs of dG bases that differ in their flanking sequences. An oligonucleotide, thirty-six bases in length, that contains a central run of five dG bases has been used to evaluate the importance of the individual deoxyguanosines in recognition and cleavage. With this oligonucleotide cutting takes place at GG▼G↓G▼G (↓, most prominent cut; ▼, less prominent cuts). The use of dG base analogues revealed that two bases, one and two steps removed from the cleavage site in the 5′ direction (*G*GG↓), were of most importance in the determination of the nuclease DNA cleavage selectivity. Of these the inner starred dG was the most critical. The use of 5-methyldeoxycytidine also showed that the dC, base paired to this critical dG, influenced cleavage specificity. The overall pattern of results seen with the base analogues suggested that the nuclease interacted with both strands of the DNA and also contacted the nucleic acid in both the major and minor grooves. Gel retardation analysis together with footprinting experiments using hydroxyl radicals, dimethyl sulfate, and ethylnitrosourea indicated that the nuclease does not form a tight and specific complex with sequences containing dG runs, at least in the absence of the essential co-factor, Mg^{2+}.

Nucleases are attractive models for the study of protein−DNA interactions due to their uncomplicated reaction requirements (usually only Mg^{2+}) and the very broad range of DNA hydrolysis selectivities that they show. The most intensively studied nucleases are the restriction endonucleases, which have an exceedingly high specificity for a particular DNA sequence usually four, six, or eight bases in length (Roberts & Halford, 1993). Other nucleases recognize both sequence and structural elements in DNA and are involved in genetic recombination (West, 1993) and in altering the topological state of DNA (Hsieh, 1993). A most important class are nucleases that are capable of recognizing damaged DNA and initiating DNA repair (Lloyd & Linn, 1993; Sancar, 1994). In addition several nucleases are known that have a very low selectivity for DNA and can hydrolyze most of the phosphodiester bonds in the duplex. One of the best characterized is bovine pancreatic DNase I (Moore, 1981; Drew & Travers, 1984, 1985). Others include the *Serratia marcescens* endonuclease (Meiss *et al.,* 1995) and several secreted fungal endonucleases, e.g., from *Aspergillus nidulans* (Campbell & Winder, 1983) and *Neurospora crassa* (Fraser, 1979). However, no known endonuclease is completely nonspecific and even low-selectivity nucleases show some degree of sequence and/or structure preference.

Our group has previously demonstrated the existence, in several species of *Streptomyces*, of nutritionally-controlled endonucleases. The synthesis of these DNases is repressed by rich nitrogen sources, which promote high growth rates (Aparicio *et al.,* 1988, 1991; De los Reyes-Gavilán *et al.,* 1988a,b, 1991). In surface cultures, the appearance of these nucleases always precedes aerial mycelium formation, and the nutritional conditions which impair the production of these reproductive hyphae also suppress the synthesis of the nucleases (Aparicio *et al.,* 1991; De los Reyes-Gavilán *et al.,* 1991). These enzymes are located in the cell wall cytoplasmic membrane space, from which they are released after protoplast production. All the DNases tested require Mg^{2+} and (with the exception of the *S. antibioticus* nuclease) a low ionic strength for optimal activity. Although they can circumstantially restrict the growth of actinophages (Aparicio *et al.,* 1988; De los Reyes-Gavilán *et al.,* 1988a,b), they are not restriction endonucleases, and a possible role in differentiation has been suggested (De los Reyes-Gavilán *et al.,* 1991; Aparicio *et al.,* 1991). Two of these nucleases (those from *S. antibioticus* and *S. glaucescens*) have been

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purified to homogeneity. Both are active as monomers and nick one of the DNA strands to give either 5′ or 3′ short single-stranded overhangs with 3′-hydroxyl and 5′-phosphate termini (Aparicio *et al.*, 1992a,b; Cal *et al.*, 1995). The *S. antibioticus* nuclease shows a preference for cutting after the third dG in sequences of three or more dG/dC tracts (Cal *et al.*, 1995). This specificity is not absolute, and other sequences are also hydrolyzed in the absence of the preferred ones, or following the initial hydrolysis of well-cut sites. Therefore a sequential pattern of definite nucleic acid fragments is observed during the course of DNA degradation and small oligonucleotides are the ultimate products. Due to this striking behavior, the *S. antibioticus* nuclease can be considered to be intermediate between the highly sequence-specific restriction endonucleases and low-selectivity nucleases like DNase I. Thus the *S. antibioticus* enzyme could share mechanisms of binding and/or catalysis with both groups of enzymes. However, some of its properties, such as the preference for dG runs and the production of a specific band pattern followed by the progressive degradation of the intermediate forms, are reminiscent of eukaryotic nucleases, especially endonuclease G (Côté *et al.*, 1989, 1993) and DNases located in mammalian heart mitochondria (Cummings *et al.*, 1987; Low *et al.*, 1987, 1988).

In order to better define the properties of the *S. antibioticus* nuclease and to probe its recognition of DNA, we have started an analysis of the interaction of the enzyme with several oligonucleotide substrates. This investigation follows on from an earlier one, in which long-stranded plasmid DNA was used as a substrate and in which the sequence preferences of the nuclease were defined (Cal *et al.*, 1995). We have analyzed, in detail, the capability of the nuclease to digest single-stranded DNA, which was previously only investigated with denatured DNA. Furthermore, oligonucleotides containing dG[1] and dC base analogues have been used to define both the actual bases important for nuclease DNA recognition and to try to elucidate some of the protein−DNA contacts relevant for the hydrolysis reaction. Attempts have also been made to analyze the specificity of binding of the nuclease in the absence of Mg^{2+}, by carrying out gel retardation analysis and several footprinting experiments. These approaches have enabled us to define some of the structural and sequence features of the DNA−nuclease interaction and shed light on the properties of this unusual enzyme.

MATERIALS AND METHODS

Dimethyl sulfate, piperidine, *N*-ethyl-*N*-nitrosourea, cetylpyridinium bromide, and tRNA were purchased from Sigma (Madrid, Spain). Chemicals used for the generation of hydroxyl radicals (ferrous ammonium sulfate, EDTA, and hydrogen peroxide) were obtained from Aldrich (Madrid, Spain). T4 polynucleotide kinase and snake venom phosphodiesterase were the products of Boehringer Mannheim. [γ-^{32}P]ATP was bought from Amersham International. The preparation of the *S. antibioticus* nuclease has been described (Cal *et al.*, 1995).

Oligonucleotide Substrates and Modified Bases. All oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer using standard phosphoramidite chemistry. Reagents for DNA synthesis were obtained from Cruachem Ltd. (Glasgow, U.K.). Appropriately protected phosphoramidite derivatives of 2-aminopurine-(1-β-D)-2′-deoxyriboside ($d^{2am}P$), 6-thiodeoxyguanosine ($d^{6S}G$), 7-deazadeoxyguanosine ($d^{7C}G$), deoxyinosine (dI), 3-deazadeoxyguanosine ($d^{3C}G$), and 5-methyldeoxycytidine ($d^{5Me}C$) were purchased from either Cruachem Inc. or Glen Research (Sterling, VA) or else prepared as described (Connolly, 1991, 1992; Waters & Connolly, 1994). Oligonucleotides were purified twice by reverse phase high-pressure liquid chromatography (HPLC), first trityl-on and second, following detritylation, trityl-off (Connolly, 1991, 1992). Alternatively, purification was carried out using denaturing 15% polyacrylamide gel electrophoresis and UV shadowing. The desired bands were eluted from the gel slices using 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and the detergent was removed by extraction with *n*-butanol (Sambrook *et al.*, 1989). Following purification, all oligodeoxynucleotides were desalted using NAP-25 columns (Pharmacia), and their concentrations were determined by absorbance at 260 nm (Newman *et al.*, 1990a; Connolly, 1991, 1992). Labeling at the 5′ terminus was carried out using T4 polynucleotide kinase and [γ-^{32}P]ATP on about 10 pmol of the oligonucleotide (Sambrook *et al.*, 1989). Following phosphorylation the excess of nonincorporated radioactivity was removed by two rounds of precipitation with ethanol. When required oligonucleotides were hybridized by mixing *n* pmol of the radioactively labeled strand with 2.5*n* pmol of the complementary unlabeled strand in 20 mM Tris pH 7, containing 50 mM NaCl. The solution was heated to 85 °C and allowed to cool slowly to 30 °C. Double-stranded radioactively labeled oligonucleotides were purified by non-denaturing electrophoresis in a 15% polyacrylamide gel. Radiolabeled single-stranded oligonucleotides were purified by denaturing gel electrophoresis using 15% polyacrylamide. Following the extraction of the oligonucleotides from gel slices by the crush and soak method an ethanol precipitation was carried out to give nucleic acids free from gel materials (Sambrook *et al.*, 1989).

Cleavage of Oligonucleotides by the S. antibioticus Nuclease. The hydrolysis of both the single-stranded and double-stranded oligonucleotides was carried out in 10 μL of 10 mM Tris-HCl, pH 7, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol at a temperature of 37 °C. Oligonucleotide amounts were typically 0.4−2.5 pmol. Two different concentrations of the nuclease were used, 0.26 and 2.6 units [units as defined by Cal *et al.* (1995)]. The DNA hydrolysis was carried out for 30 or 60 min, and the reaction was terminated by heating at 80−90 °C. The hydrolytic products were analyzed by denaturing gel electrophoresis using 20% polyacrylamide gels containing 7 M urea. Product bands were detected by autoradiography. The positions at which nuclease catalysed hydrolysis occurred were determined by co-electrophoresis of either a snake venom phosphodiesterase digest of the oligonucleotide or a dG specific chemical cleavage (Maxam & Gilbert, 1980).

Gel Retardation Experiments. The double-stranded 36′-mer, formed from the hybridization of the oligonucleotides

[1] Abbreviations: dG, deoxyguanosine; dC deoxycytidine; tRNA, transfer RNA, $d^{2am}P$, 2-aminopurine-(1-β-D)-2′-deoxyriboside; $d^{6S}G$, 6-thiodeoxyguanosine; $d^{7C}G$, 7-deazadeoxyguanosine; dI, deoxyinosine; $d^{3C}G$, 3-deazadeoxyguanosine; $d^{5Me}C$, 5-methyldeoxycytidine; HPLC, high-pressure liquid chromatography; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DNase, deoxyribonuclease.

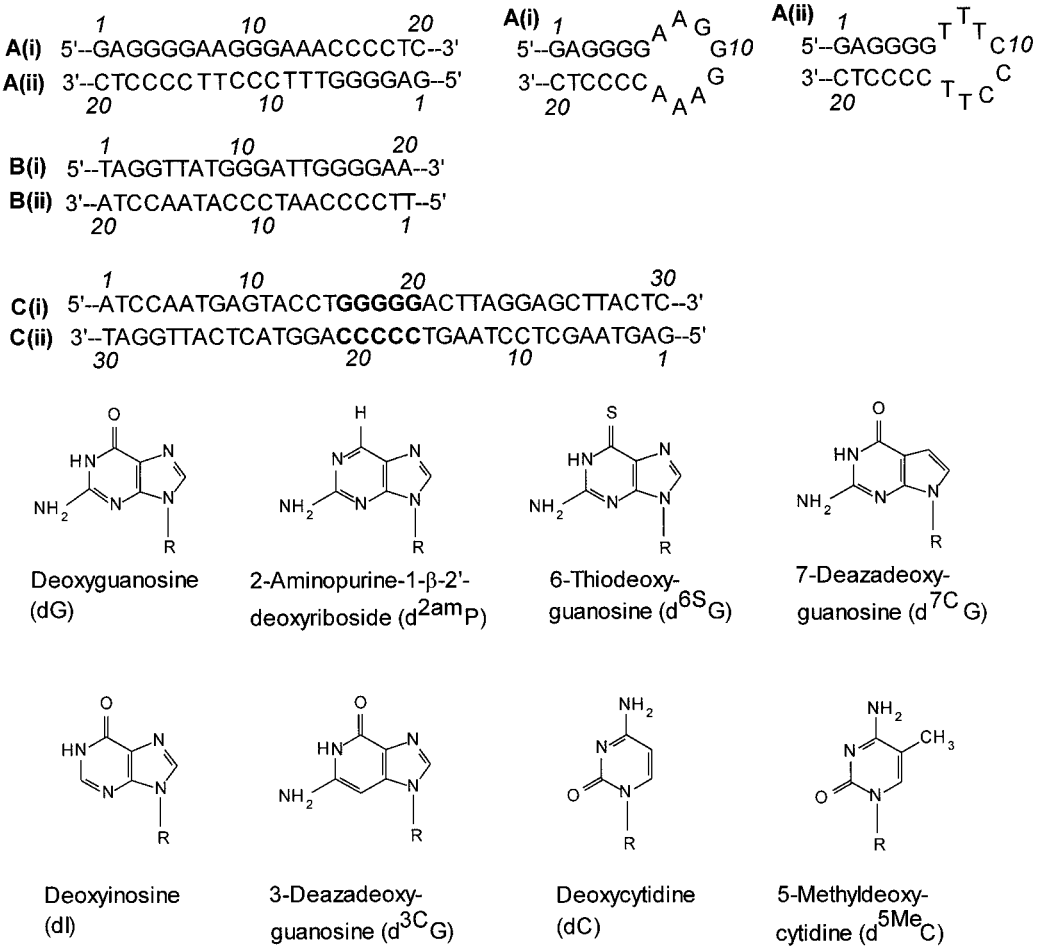


FIGURE 1: Oligonucleotide substrates and dG and dC analogues used in the work. A(i) and A(ii) form a 20-base pair duplex when mixed but can also form the stem−loop structures in the absence of their complementary strand. B(i) and B(ii) produce a duplex structure but cannot give rise to stem−loops. C(i) and C(ii) form a double-stranded 36-mer that contains a sequence of five dG bases at a central position. The dG analogues illustrated in this Figure were incorporated at this run of five bases ($d^{5Me}C$ was incorporated at the complementary dC bases on the opposite strand). R = 1-β-D-(2′-deoxyribosyl).

C(i) and C(ii) (Figure 1) was used for these experiments. The 5′-end-labeled duplex (prepared as described above) was mixed with different amounts of the endonuclease (0.3−1.5 μg of protein, corresponding to between 10 and 50 pmol for a 29 000 dalton protein) in 10 μL of 50 mM Tris-HCl, pH 7.5, 100 mM $NaCl_2$, 7 mM 2-mercaptoethanol, 1 mM EDTA. Typically 0.2 pmol of the double-stranded oligonucleotide was present. After 10 min at room temperature 2.5% sucrose was added and the samples were electrophoresed in a 10.8% polyacrylamide gel in a 90 mM Tris-boric acid buffer at 4 °C. The bands were visualized by autoradiography.

Footprinting Experiments. Protection footprinting was carried out using two approaches: dimethyl sulfate followed by piperidine cleavage (Maxam & Gilbert, 1980) and hydroxyl radical cleavage (Dixon *et al.*, 1991; Price & Tullius, 1992). Complexes between the 36′-mer duplex oligonucleotide and the nuclease were prepared as for the gel retardation experiments (see above), and any protection was evaluated by the methods outlined in the references. Interference footprinting was carried out using the 36-mer and ethylnitrosourea (phosphate ethylation) or dimethyl sulfate (purine methylation) as described (Siebenlist & Gilbert, 1980; Szczelkun *et al.*, 1995). The protein and oligonucleotide levels and the buffer used were as for the gel retardation experiments.

RESULTS

Oligonucleotides Used. The oligonucleotides that have been used in this study are given in Figure 1. The first double-stranded 20′-mer, formed by annealing the two complementary 20-mers A(i) and A(ii), has two sequences of four consecutive dG residues and one sequence of three. However, both A(i) and A(ii), in the absence of their complementary partners, can produce hairpin structures, with six base pairs in the double-stranded stem (five of which are dG/dC) and eight bases in the single-stranded loop. As shown in Figure 1, A(i) gives a run of four dG bases in the stem and three in the loop, whereas A(ii) produces only four dG residues in the stem. An oligonucleotide with similar properties, albeit of different sequence, has previously been used in the study of the structural and sequence preferences of several nucleases (Drew, 1984). Non-denaturing gel electrophoresis showed that equimolar mixtures of A(i) and A(ii) only gave rise to duplex structures, and no, or very few, hairpins were produced under these conditions. A second duplex 20-mer that contains a run of both three and four dG bases was produced from the sequences B(i) and B(ii). These single-stranded oligonucleotides were designed to contain either no dC bases, B(i), or no dG bases, B(ii), and therefore to have no possibility of producing hairpins. The effects that modified bases have on the nuclease-

catalyzed hydrolysis of DNA was studied using a double-stranded oligonucleotide 36 bases in length, produced by the hybridization of C(i) and C(ii) (Figure 1). This duplex has a run of five consecutive dG bases, in a central location, and so is expected to be a very good substrate for the *S. antibioticus* nuclease. The dG base analogues, illustrated in Figure 1, were introduced, at defined locations, within this run of dG bases. In addition 5-methyldeoxycytidine was also substituted into the complementary sequence of five dC bases. The gel retardation and footprinting experiments were also carried out with this 36-base pair duplex.

Cleavage of Double- and Single-Stranded Oligonucleotides by the S. antibioticus Nuclease. Previous data (Cal *et al.*, 1995) have shown that the enzyme degrades double-stranded DNA more readily than denatured nucleic acids. Thus denatured DNA had a K_m value about three times higher than that of native DNA. This suggests that double-stranded DNA is the preferred substrate, but the undefined character of denatured DNA (i.e., is it completely single-stranded or are double-stranded regions still present) prevents one from drawing firm conclusions.

In order to analyze this feature in more detail the 20-mers illustrated in Figure 1 were used. The results observed when the oligonucleotides A(i) and A(ii), hybridized both singly and together, were treated with the nuclease are shown in Figure 2. With the duplex the preferred cutting sites occur after bases 5, 10, and 11 for the A(i) strand, which are at the sequences GGG↓G and GG↓G↓A, respectively (Figure 2). The A(ii) strand in the duplex is cut after base 5, i.e., at the sequence GGG↓G. This confirms that the nuclease prefers to cut double-stranded DNA at dG rich sequences. When A(i), on its own, was treated with the nuclease, the strong cutting site after base 5 was retained and, in addition, weaker hydrolysis took place immediately following bases 2, 3, and 4. Inspection of Figure 2 shows that these sites, at the sequence GA↓G↓G↓G↓G, occur at a dG rich area in the double-stranded stem region. Most importantly the previously observed hydrolysis sites after dG 10 and dG 11 are abolished. As shown in Figures 1 and 2 these two dG bases occur in the single-stranded loop, and so these results suggest that the nuclease has a very strong preference for double-stranded structures. As expected, single-stranded A(ii) retained its strong cutting site after dG 5 but also showed weaker hydrolysis at positions 2−4. As with A(i) these are located in a double-stranded dG region.

The preference of the nuclease for double-stranded DNA was confirmed using the oligonucleotides B(i) and B(ii). With the duplex formed from these oligonucleotides the strongest cutting takes place on strand B(i) after the two dG residues at positions 10 and 11 (Figure 2), with the site at 11 being preferred. As above cleavage occurs at a dG rich double-stranded region. The four dG residues toward the 3′ end of the B(i) strand are in a poorly resolved area of the gel, but a good hydrolysis site at dG 17 can just be made out. The B(ii) strand lacks dG residues and is not hydrolyzed (not shown). Single-stranded B(i) was not cut by the endonuclease under these conditions, and this establishes that the nuclease shows very poor activity toward dG runs in single-stranded nucleic acids.

Oligonucleotides Containing Modified Bases As Substrates for the S. antibioticus Nuclease. In order to gain further insights into the structural aspects of the interaction of the nuclease with its DNA substrates, base analogues were used.

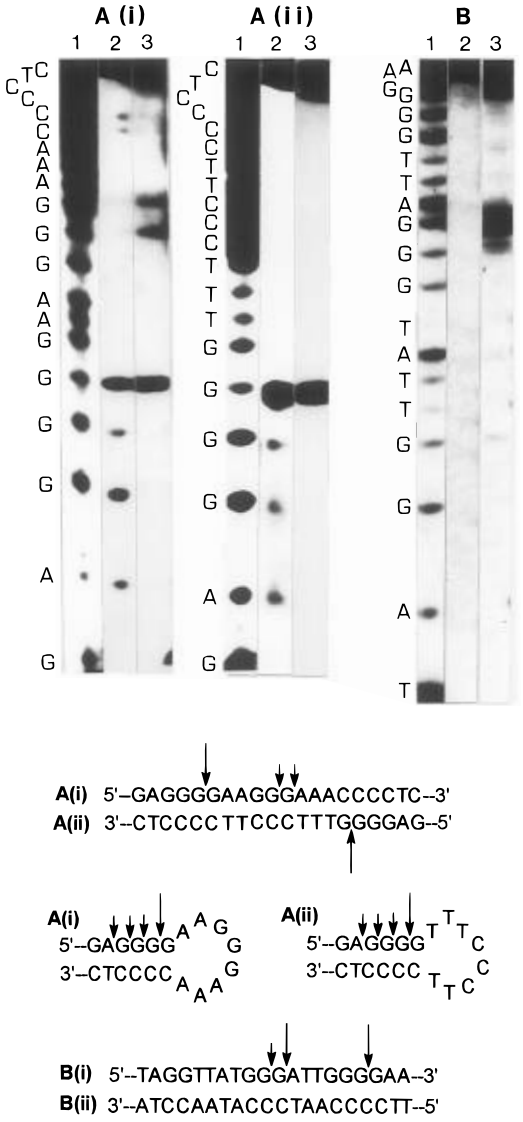
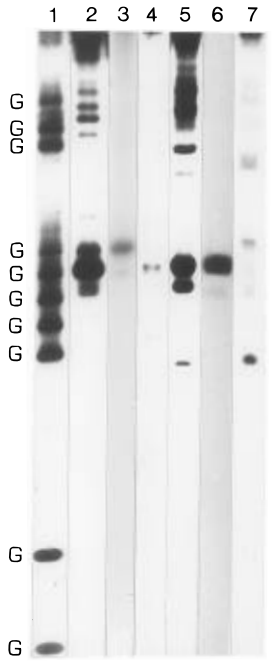


FIGURE 2: Cutting of dG rich regions in single- and double-stranded DNA by *S. antibioticus* nuclease. A(i). Hydrolysis of single-stranded oligonucleotide A(i) with snake venom phosphodiesterase (lane 1) and *S. antibioticus* nuclease (lane 2); hydrolysis of oligonucleotide A(i) in a duplex with A(ii) (lane 3). A(ii). Hydrolysis of single-stranded oligonucleotide A(ii) with snake venom phosphodiesterase (lane 1) and *S. antibioticus* nuclease (lane 2); hydrolysis of oligonucleotide A(ii) in a duplex with A(i) (lane 3). B. Hydrolysis of single-stranded oligonucleotide B(i) with snake venom phosphodiesterase (lane 1) and *S. antibioticus* nuclease (lane 2); hydrolysis of oligonucleotide B(i) in a duplex with B(ii) (lane 3). Oligonucleotide B(ii), which lacks dG, was not cut by the *S. antibioticus* nuclease (not shown). In all cases the strand, having its hydrolysis measured, was labeled at the 5′ end with ^{32}P. Digestion was carried out with 2.6 units of the nuclease (Cal *et al.*, 1995) for 30 min. Similar results were seen when 0.26 units of the nuclease was used (although the bands were much weaker) and when the digestion was carried out for 1 h. The positions of the bases are indicated. The intensity of the cuts is indicated by arrows of different size in the sequences.

The dG analogues utilized, most of which have a potential hydrogen bond donor or acceptor removed, are illustrated in Figure 1. These analogues have been embedded into the double-stranded 36-mer formed by mixing the single strands C(i) and C(ii) (Figure 1). This duplex contains a central run of five dG bases, supplied from C(i), and it is here that the modified dG bases have been placed. In order to study any influence of the complementary dC bases, we have also made use of 5-methyldeoxycytidine.



C(i) 5'-ATCCAATGAGTACCT**GGGGG**ACTTAGGAGCTTACTC-3'
C(ii) 3'- TAGGTTACTCATGGA**CCCCC**TGAATCCTCGAATGAG-5'

FIGURE 3: Oligonucleotides containing deoxyinosine as substrates for the *S. antibioticus* endonuclease. A 36-mer double-stranded oligonucleotide formed by the hybridization of C(i) and C(ii) that contains a central $d(G)_5 \cdot d(C)_5$ sequence preferentially cut by the nuclease was used. Deoxyinosine (dI) was introduced within this central sequence (underlined in the lower part of the figure). The effect of dI substitution was determined by hydrolysis of the nucleic acid with the nuclease (2.6 units, 30 min) with reference to the unsubstituted control. Identical experiments were carried out with all the other base analogues (not shown), and these results are summarized in Table 1. Lane 1, dG positions determined by Maxam and Gilbert chemical sequencing with dimethyl sulfate and piperidine; lane 2, digestion of the unmodified oligonucleotide by the endonuclease; lanes 3−7, digestion of the oligonucleotide with deoxyinosine (dI) in the first, second, third, fourth, and second-plus-fourth positions, respectively, of the same sequence. The bands produced as a result of the hydrolysis by the nuclease appear displaced one position upward (in the 3′ direction) with respect to the position of the dG as revealed by the chemical sequencing reaction, due to the different nature of the 3′ termini generated (Sollner-Webb & Reeder, 1979). The main cuts produced in the unmodified oligonucleotide substrate (control) and their intensity are indicated by arrows of different size in the sequence of the lower part. The labeled strand is indicated by an asterisk. Only a part of the sequence (that visualized in the gel) is represented.

As expected, nuclease-catalyzed hydrolysis of the control (no modified bases) duplex, 36 bases in length, resulted in cutting in the dG rich region. At the central GGGGG sequence the most strongly cleaved site was GGG↓GG. However, hydrolysis, albeit at a reduced rate, was also observed at the immediate flanking sites, i.e., GG↓GG↓G. These results, obtained by analyzing the products of hydrolysis by denaturing gel electrophoresis followed by autoradiography, are shown in Figure 3. The 36-mer also contains a dG rich sequence, GGAG, at its 3′-end, and some cleavage was also seen here. However, this is not relevant to the base analogue results and so is not discussed.

The electrophoretograms obtained when dI substitutions were introduced at the central run of the five dG bases are also shown in Figure 3. In order to obtain a semiquantitative measure of how this analogue affects the activity of the enzyme, the bands corresponding to the major cut (GGG↓GG) have been scanned and their values are given in Table 1. This table also gives the values, for this major cutting site, found with the other base analogues. The data with these modified bases were obtained from electrophoretograms similar to those found with dI (not shown). The percentages in Table 1 represent the amount of oligonucleotide (all at the same concentrations and specific activities) cleaved in 30 min by identical concentrations (2.6 units) of the enzyme. Similar results were seen when the hydrolysis reaction was extended to 1 h. When 0.26 units of the enzyme was used, the results were again similar, although only the most strongly cut bonds gave easily visible bands. As shown in Figure 3 and Table 1, some modifications to the first dG (*GGG↓GG) of the pentameric sequence strongly reduce cutting after the third base. In particular the base analogues $d^{2am}P$ and dI practically abolish catalysis. A large reduction

Table 1: Effects of Base Analogue Substitutions on the *S. antibioticus* Nuclease[a]

base changed	base analogue	DNA groove affected	activity (%)
5′-GGGGG-3′	none	none	100
5′-*GGGGG-3′	dI	minor	1
5′-*GGGGG-3′	$d^{7C}G$	major	73
5′-*GGGGG-3′	$d^{6S}G$	major	34
5′-*GGGGG-3′	$d^{2am}P$	major	1
5′-G*GGGG-3′	dI	minor	5
5′-G*GGGG-3′	$d^{7C}G$	major	9
5′-G*GGGG-3′	$d^{6S}G$	major	41
5′-G*GGGG-3′	$d^{2am}P$	major	3
5′-GG*GGG-3′	dI	minor	85
5′-GG*GGG-3′	$d^{7C}G$	major	87
5′-GG*GGG-3′	$d^{6S}G$	major	103
5′-GG*GGG-3′	$d^{2am}P$	major	100
5′-GGG*GG-3′	dI	minor	182
5′-GGG*GG-3′	$d^{7C}G$	major	25
5′-GGG*GG-3′	$d^{6S}G$	major	89
5′-GGG*GG-3′	$d^{2am}P$	major	13
5′-G*GG*GG-3′	dI	minor	0
5′-G*GG*GG-3′	$d^{7C}G$	major	7
5′-G*GG*GG-3′	$d^{6S}G$	major	6
5′-G*GG*GG-3′	$d^{2am}P$	major	1
3′-*CCCCC-5′	$d^{5Me}C$	major	48
3′-C*CCCC-5′	$d^{5Me}C$	major	12
3′-CC*CCC-5′	$d^{5Me}C$	major	55
3′-CCC*CC-5′	$d^{5Me}C$	major	29
3′-*C*C*C*C*C-5′	$d^{5Me}C$	major	6

[a] The 36-mer double-stranded oligonucleotides formed from C(i) and C(ii) (or these sequences containing a base analogue, Figure 1) were used as substrates. The effects of modified dG or dC bases within the central $d(G)_5 \cdot d(C)_5$ sequence were investigated by hydrolysis of the appropriate oligonucleotide with 2.6 units of the nuclease for 30 min. The hydrolysis products were separated by gel electrophoresis (as shown in Figure 3 for the control and dI-containing oligonucleotides), and quantitative values were obtained by densitometric analysis of the autoradiographic films produced. For the above table only the major band produced by cutting after the third dG (GGG↓GG) has been analyzed, and this is the cutting site that the percentages refer to. Percentages were calculated with reference to the value obtained with the unsubstituted oligonucleotide (100%). Values are the mean of at least two separate experiments. The modified base is preceded by an asterisk. The base analogue 3-deazadeoxyguanosine, $d^{3C}G$, was also introduced in the third position (5′-GG*GGG-3′), and the resulting oligonucleotide was hydrolyzed at a similar rate to that seen with the control. Introduction of $d^{3C}G$ at both the second and forth positions completely inhibited cleavage. The modifications to the dC positions are in the complementary strand.

in rate is also seen with $d^{6S}G$. It should be noted that $d^{2am}P$ and $d^{6S}G$ both probe the same locus of the dG base, the 6-keto oxygen atom. However, $d^{2am}P$ completely removes the possibility of a protein−DNA interaction by replacing the oxygen with hydrogen, whereas $d^{6S}G$, in which the oxygen is replaced by sulfur, would be expected to weaken, rather than completely abolish, such a contact. Thus in the case where a protein makes a hydrogen bond with the 6-oxygen of a dG, one might expect more severe effects with $d^{2am}P$ than $d^{6S}G$, as is observed here. Only with $d^{7C}G$ at the first dG position is a reasonable rate seen. The second dG (G*GG↓GG) is the most critical for DNA recognition and hydrolysis by the nuclease. As shown in Figure 3 and Table 1, $d^{2am}P$, dI, and $d^{7C}G$ all severely reduce hydrolysis. The base $d^{6S}G$ is not quite as inhibitory, but this is probably due to reducing rather than eliminating protein−DNA interactions, as discussed above. Remarkably, the third dG (GG*G↓GG), which actually flanks the cutting site, does not seem to be important for enzyme activity. Oligonucleotides containing all four base analogues, at this location, are cleaved with an efficiency approaching that of the control nucleic acid. Finally the dG immediately following the major site of cutting (GGG↓*GG), showed some slight effects. The presence of the bases dI and $d^{6S}G$ did not impede cleavage, whereas $d^{7C}G$ and $d^{2am}P$ reduced cutting to 25% and 13%, respectively. Very strong inhibition was seen when two modified bases were introduced at positions 2 and 4 (G*GG↓*GG). It is likely that this arises mainly from alteration to the critical second base. A few experiments (not shown) were carried out with $d^{3C}G$, although difficulties in obtaining the protected phosphoramidite of this base prevented a full study. In agreement with the above results, the presence of $d^{3C}G$ at position 3 had little effect, whereas to $d^{3C}G$ bases at sites 2 and 4 completely inhibited hydrolysis.

The above experiments suggest that the presence of two consecutive dG residues, one and two bases removed from the cutting site in the 5′-direction (*G*GN↓), are of most importance in *S. antibioticus* nuclease-catalyzed hydrolysis of DNA. This is confirmed, to a certain extent, by a consideration of the consequences that the base analogues have on the minor cleavage sites (GG↓GG↓G). Thus with dI in the first position the weak cut after the fourth dG is retained. Introducing dI into the second site abolishes this minor hydrolysis site. The presence of dI in either position also eliminates cleavage after the second dG. Also, analogues in the third site have no influence on the preferred cutting that takes place after this base. However, with these oligonucleotides the cut after the fourth dG is impeded (Figure 3). These results, once again, support the idea that dG residues preceding the site of hydrolysis are important for the nuclease−DNA interaction.

In light of the fact that the nuclease interacts with double-stranded rather than single-stranded DNA, we have also examined the influence of the dC residues base paired to the run of the five dG bases. This has been carried out using 5-methyldeoxycytidine ($d^{5Me}C$). The results found are summarized in Table 1. As can be seen, in general, the presence of a single dC only reduces the hydrolysis by about 50%. The only exception is for the fourth dC (CCC*CC), where cutting is reduced by about an order of magnitude. This dC will be base paired to second dG (G*GGGG) in the complementary strand, and this was the dG identified as most important in the above experiments. These results corroborate the importance of this second deoxyguanosine for the contacts with the nuclease and suggest that the interaction of the enzyme takes place with both strands of DNA. The presence of five dC residues was very inhibitory, in agreement with earlier studies that hemimethylated M13 DNA, in which one strand contained all 5-methyldeoxycytidine residues, was not digested (Cal *et al.*, 1995).

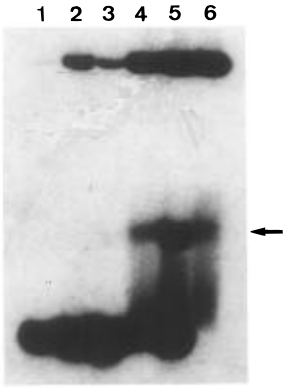


FIGURE 4: Gel retardation of *S. antibioticus* nuclease−DNA complexes. The double-stranded 36-mer, composed of a mixture of oligonucleotides C(i) and C(ii), was used at a concentration of 20 nM and incubated with increasing amounts of the nuclease prior to running on a non-denaturing gel. Lane 1, oligonucleotide alone; lane 2, 0.3 μg (1 μM) of nuclease; lane 3, 0.39 μg (1.3 μM) of nuclease; lane 4, 0.57 μg (1.9 μM) of nuclease; lane 5, 0.66 μg (2.2 μM) of nuclease; lane 6, 1.5 μg (5 μM) of nuclease. The arrow points to the expected position of a band corresponding to a nuclease−oligonucleotide complex with a 1:1 stoichiometry. Bands seen at the top of the gel are at the positions of the wells and so have not entered the gel. These are probably due to insoluble protein−DNA aggregates.

Gel Retardation Analysis. In an attempt to detect specific complexes of the *S. antibioticus* endonuclease with a preferred site (i.e., a dG rich double-stranded oligonucleotide) gel retardation assays were carried out with the 36-mer duplex formed from C(i) and C(ii). In order to prevent hydrolysis these experiments were carried out in the absence of the essential co-factor Mg^{2+}. A retarded band could be distinguished in the middle of the gel, at about the position expected for a complex between a 29 kDa protein and a 36-mer (Figure 4). This was produced at protein concentrations of about 1 μM. At about 2.5 μM levels of enzyme very little free DNA remained. However a continuous smear of radioactivity always appeared below the band. This smearing is characteristic of weak complexes that dissociate during electrophoresis. Increasing the protein concentration produced little difference in the appearance of these gels, and a discrete, non-smeared, retarded band was never observed. Furthermore, much of the DNA did not enter the gel and appears as a band at the top of the gel in the positions of the wells. This may be due to high concentrations of the nuclease being insoluble and forming aggregates with the nucleic acid.

Footprinting Experiments. Protection experiments were carried with two reagents, dimethyl sulfate and hydroxyl radicals. The conditions used were identical to those shown for the gel retardation experiments in Figure 4, i.e., protein levels were about 1−2.5 μM and most or all of the DNA was complexed. These experiments were carried out with the 36-mer duplex in the absence of Mg^{2+}. No protein dependent protection of the oligonucleotide, from the two reagents, was observed, and the electrophoresis ladders

produced from the free nucleic acid and the oligonucleotide−nuclease mixture were identical (not shown). In order to avoid the possibility of dissociation of any DNA−nuclease complexes due to the reagents used for the protection experiments, interference assays were also carried out. These used the 36-mer and dimethyl sulfate or ethylnitrosourea. It was possible to gel shift these modified oligonucleotides although 2.5 times higher concentrations of nuclease were required, as compared to the unmodified duplex. However, an analysis of the unbound and protein bound oligonucleotides, following electrophoretic separation, showed absolutely no evidence for any interference (not shown). In particular the results with dimethyl sulfate, which is specific for dG bases, failed to give any indication that the modification of this base within the run of five dG residues prevented nuclease binding. Similarly no specific phosphate interference footprint, localized, for example, to the dG run, was seen with ethylnitrosourea. These results suggest that the nuclease does not bind specifically and tightly to regions of the DNA that are its preferred hydrolysis sites, at least in the absence of Mg^{2+}.

DISCUSSION

In this publication we have commenced the enzymological characterization of a periplasmic nuclease isolated from *S. antibioticus.* Previous experiments suggested that the *S. antibioticus* nuclease has a preference for sequences of three or more contiguous dG residues in double-stranded DNA (Cal *et al.,* 1995). The use of the oligonucleotides A(i), A(ii), B(i), and B(ii) has confirmed that runs of dG bases in duplex DNA are the preferred substrates. Continuous sequences of dG residues in single-stranded DNA are not, or at best very poorly, cut. The employment of defined oligonucleotides allows this conclusion to be drawn with far greater certainty than from earlier experiments with native and denatured DNA. Here the precise nature of the DNA vis-à-vis single and double strands is unclear. Drew (1984) has used oligonucleotides similar to A(i) and A(ii) to study the sequence and structural preferences of DNase I, DNase II, nuclease S1, and micrococcal nuclease. It was concluded that DNase I interacted with the minor groove of duplex DNA, whereas nuclease S1 preferred single-stranded regions. Micrococcal nuclease required exposed single strands with an unpaired dA or T base, and DNase II attacked partially exposed single strands. These features have been confirmed by crystallography for DNase I (Suck *et al.,* 1988; Lahm & Suck, 1991; Weston *et al.,* 1992) and micrococcal nuclease (Cotton *et al.,* 1979; Loll & Lattman, 1989). However, the cutting patterns observed with these four nucleases are rather complicated. This indicates that the four enzymes interact with nucleic acids in an intricate fashion that involves both DNA structure and deformability and also shows some dependence on base sequence. The different, and much simpler, hydrolysis patterns seen with the *S. antibioticus* nuclease are much more reminiscent of those seen with restriction endonucleases. Here interaction can be understood in terms of recognizing a short sequence of bases (Roberts & Halford, 1993). However, it is clear that the selectivity of the *S. antibioticus* nuclease is much lower than those of restriction enzymes.

The interaction of the *S. antibioticus* nuclease with runs of dG bases has been further probed using base analogues. The most powerful use of this technique is to study the energetics of a protein−DNA interaction, when a crystal structure is available (Jen-Jacobson, 1995). The approach can also be used to get an idea of which bases are involved in interacting with a protein and also to help elucidate which particular groups on the base participate in these contacts (Aiken & Gumport, 1991). However, the use of base analogues, in the absence of structural information, has some well-recognized limitations. Thus the loss of a base functional group that is not directly contacted by the protein can also inhibit binding or catalysis by having an indirect effect on the overall DNA structure. In a study with the *Eco*RV endonuclease the use of modified bases led to the proposition of several protein−DNA contacts (Newman *et al.,* 1990a,b; Waters & Connolly, 1994). With the benefit of crystal structures (Winkler *et al.,* 1993; Kostrewa & Winkler, 1995) it was observed that many, but not all, of the proposed contacts were correct. With this proviso in mind the dG analogues show that, for the best cut site GGG↓GG, the order of significance for efficient catalysis is dG2 > dG1 > dG4. Remarkably, the dG at position 3, which is at the cutting site, appears to be unimportant. This gives a consensus sequence of GGN↓G, which is in reasonable agreement with the results obtained from an analysis of many cutting sites on long-strand DNA (Cal *et al.,* 1995). All of the dG analogues used, with the exception of $d^{6S}G$, delete a group on the base that has the potential to make a hydrogen bond to the protein. Thus the simplest explanation of the results is that when poor hydrolysis is seen with the modified bases (Table 1), it is as a result of the disruption of a critical protein−DNA interaction. However the alternative, that the presence of the analogue changes the structure of the oligonucleotide and this results in poor cutting, cannot be excluded (this is discussed further below). We note that for both dG1 and dG2, preceding the cleavage site, base analogues that change functions in both the major ($d^{2am}P$, $d^{7C}G$) and the minor (dI, $d^{3C}G$) grooves inhibit hydrolysis. This suggests that the *S. antibioticus* nuclease might make contacts in both DNA grooves. Highly specific restriction endonucleases such as *Eco*RV (Winkler *et al.,* 1993; Kostrewa & Winkler, 1995), *Eco*RI (Kim *et al.,* 1990), *Bam*HI (Newman *et al.,* 1994), and *Pvu*II (Cheng *et al.,* 1994) make most of their contacts to the bases via the major groove. The relatively nonspecific DNase I interacts with DNA through the minor groove (Suck *et al.,* 1988; Lahm & Suck, 1991; Weston *et al.,* 1992). This is because the hydrogen bonding functions of individual base pairs are rather different when viewed from the major groove but similar when present in the minor groove (Seeman *et al.,* 1976). Specific nucleases bind in the major groove to make use of the great differences in base pair determinants and so achieve selectivity. DNase I, a digestive enzyme which is required to cut as many phosphodiester bonds as possible, uses the minor groove to allow it to interact well with most base sequences. The *S. antibioticus* enzyme has a selectivity between these two extremes. It might use a judicious mixture of high-specificity interactions (major groove) and lower-specificity contacts (minor groove) over a short sequence to generate its observed selectivity.

The use of base analogues has identified potential nuclease−DNA contacts in the dG rich sequences preferred by the protein. However, it is possible that this protein recognizes a peculiar DNA structure rather than making direct interactions with the dG bases. For this to take place

one would expect areas of DNA that have runs of dG bases to have an overall conformation different to that of "ideal" B-DNA. There is evidence that this does occur. A crystal structure of the sequence GGGGCCCC (McCall *et al.*, 1985) showed a conformation that was similar to A-form DNA. It was further suggested that DNA containing long runs of dG bases might generally exist in an A conformation. Thus it is possible that the *S. antibioticus* nuclease is recognizing an A structure rather than directly interacting with the dG bases. In this scenario the base analogues reduce hydrolysis by compromising the A structure of the DNA substrate. At present we cannot distinguish, unambiguously, between these two modes of interaction, i.e., direct readout of the dG bases or indirect readout of an A-like conformation consequent on a run of dG's. We suspect that this nuclease, like most DNA binding proteins, will use all the features of the target DNA sequence to achieve its selectivity. Thus it is most likely that the two recognition mechanisms will be occurring simultaneously and side by side.

We were unable to obtain any evidence for the specific binding of the *S. antibioticus* nuclease to runs of five dG bases. Although band shift experiments showed the formation of protein−DNA complexes, a variety of footprinting methods failed to localize the enzyme to the dG rich, or indeed any other, DNA sequence. To prevent turnover these experiments were conducted in the absence of Mg^{2+}. Two conclusions are possible. First, this cation might be essential for high-affinity, specific, DNA binding. This has been observed for several restriction endonucleases such as *Eco*RV (Taylor *et al.*, 1991; Thielking *et al.*, 1992; Vipond & Halford, 1995) and *Taq*I (Zebala *et al.*, 1992). Both of these nuclease show no binding selectivity for their cognate DNA sequences, unless Mg^{2+} is present. These proteins generate most of their specificity at a late stage of their catalytic cycle (Zebala *et al.*, 1992). However, other restriction endonucleases such as *Eco*RI (Lesser *et al.*, 1990) and *Rsr*I (Aiken *et al.*, 1991) are able to bind, with high selectivity, to their recognition sequences in the absence of the metal co-factor. Here much of the discrimination originates at the DNA binding step, early in the catalytic cycle. Alternatively, the *S. antibioticus* enzyme, which is not as specific as the restriction enzymes, may not form tight complexes with DNA under any conditions (i.e., $\pm Mg^{2+}$). Here the protein would associate weakly with all sequences (either by continuously binding and desorbing from the DNA or else by tracking alone the nucleic acid) but only cause hydrolysis when it encounters preferred sites. Such a mechanism may be most appropriate for nucleases of low to intermediate specificity. Unfortunately, the failure to produce specific complexes meant that footprinting techniques could not be used to map the protein−DNA interface.

Several nucleases are known which have a preference for cutting at runs of dG bases and so, at least in this respect, show a similarity to the *S. antibioticus* nuclease. These include the extracellular nuclease from *Serratia marcescens* (Meiss *et al.*, 1995) and endonuclease G isolated from mammalian nuclei and mitochondria (Ruiz-Carrillo & Renaud, 1987; Low *et al.*, 1988; Côté *et al.*, 1989; Côté & Ruiz-Carrillo, 1993). However other properties of these nucleases do not match those of the *S. antibioticus* enzyme. Thus the *S. marcescens* nuclease cuts both single- and double-stranded DNA, and when double-stranded nucleic acid is a substrate both strands are cut. Similarly, although endonuclease G recognizes dG/dC tracts, both the purine and the pyrimidine strands are equally well cut.

Finally it is pertinent to discuss the physiological role of the nuclease and how this relates to its DNA-cutting properties. The most likely role for the *S. antibioticus* enzyme is the recycling of DNA deoxynucleotides from the substrate mycelium to the aerial mycelium following the lysis of the vegetative hyphae during development (De los Reyes-Gavilán *et al.*, 1991; Méndez *et al.*, 1985). The periplasmic location of the nuclease would permit it to gain access to the DNA after the lysis of the mycelium. The preference for dG rich regions would make the *Streptomyces* DNA, which has a high dG + dC content, an excellent substrate for the nicking activity. According to this hypothesis, a second nuclease would be required in order to accomplish the degradation, to the deoxynucleotide level, of the nicked and fragmented DNA produced by the dG specific nuclease. Such an enzyme has been recently detected in *S. antibioticus* (R. Gonzalez and J. Sánchez, unpublished observations); it is located at the cell surface, and its synthesis responds to analogous nutritional signals to those governing the appearance of the periplasmic enzyme.

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