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# Cleavage and sequence recognition of 2,6-diaminopurine-containing DNA by site-specific endonucleases

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The susceptibility of 2,6-diaminopurine (DAP)-containing bacteriophage DNA to several restriction and other endonucleases was examined. With the only exception of *Taq*I, these enzymes did not accept the modified base as a substitute for adenine. The phage DNA was extensively fragmented by the restriction endonucleases which recognize only G and C-containing sites. 5'-terminal analysis of the *Msp*I and *Sma*I fragments revealed that d(DAP-T) basepairs can be mistaken by some enzymes for d(G-C) pairs.

DNA; Modified nucleotide; Restriction endonuclease; Sequence recognition; (Bacteriophage)

## 1. INTRODUCTION

Bacteriophage DNAs often contain modified nucleotide bases which, in some cases, completely replace one of the four bases found commonly in DNA (review [1]). The effect of such modified bases on the sensitivity of both natural and synthetic substrates to restriction endonuclease cleavage has been studied in detail [2,3] and the data obtained contributed to the elucidation of how these enzymes recognize their specific target sequences.

The DNAs of bacteriophages with total base replacement generally contain modified pyrimidine nucleotides. The only known exception is phage S-2L of the cyanobacterium *Synechococcus elongatus* which contains DNA where 2,6-diaminopurine is substituted for adenine [4]. In DAP the 2-amino group forms an extra (third) hydrogen bond with the complementary thymine base and this interaction has been shown to alter duplex stability and conformation [4,5]. Here we report that DAP substantially influences the susceptibility of DNA to site-specific endonuclease cleavage and that different enzymes seem to follow common patterns in the recognition of DAP-containing sites.

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*Abbreviation:* DAP, 2,6-diaminopurine

## 2. MATERIALS AND METHODS

### 2.1. *Preparation of bacteriophage DNA*

Phage S-2L DNA was purified according to [4]. Phage λcI857 $S_7$ DNA was prepared as described in [6].

### 2.2. *Endonucleases and assay conditions*

Most of the restriction endonucleases used in this work were purchased from Reanal (Hungary). *Hae*II, *Hha*I, and *Hin*fI were obtained from New England Biolabs; *Taq*I was from Boehringer-Mannheim; *Acy*I, *Aha*I, *Aha*III, *Asu*I, *Ava*I, *Bsu*RI, *Fsp*I and *Fsp*II were prepared in our laboratory. Cleavage reactions were performed ac-

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cording to the conditions specified by the suppliers. Usually 1 $\mu$g DNA was treated for 1 h with 10 units of the enzymes. The activity and specificity of the nuclease preparations were tested on phage $\lambda$ DNA. Reaction products were analyzed on 1 to 2% agarose gels as in [6].

Micrococcal nuclease was obtained from P-L Biochemicals. Assay conditions were favorable for specific cleavage [7]. Reaction mixtures contained 100 $\mu$g DNA and one unit of the enzyme. Purification and reaction conditions of AS-1 endonuclease were as in [6]. The acid soluble material released during incubation was measured spectrophotometrically [8].

### 2.3. *Determination of the 5'-terminal nucleotides*

The procedure followed was as in [2]. DNA fragments labeled at the 5'-ends were subjected to enzymatic hydrolysis and the mononucleotides obtained were separated chromatographically on polyethylenimine cellulose thin layers. Radioactivity of the separated products was determined by scintillation counting. [$^{32}$P]ATP (spec. act. 150 TBq·mmol$^{-1}$) was purchased from Izinta (Hungary). Nucleotide standards were from Sigma, whereas 2'-deoxy-2,6-diaminopurine 5'-monophosphate was prepared in our laboratory by enzymatic hydrolysis of S-2L DNA.

## 3. RESULTS

### 3.1. *Sensitivity of S-2L DNA to restriction endonucleases*

We tested the ability of 38 restriction nucleases to cleave phage S-2L DNA (table 1).

With the only exception of *Taq*I the substrate DNA was refractory to cleavage by those enzymes which are specific for A-containing recognition se-

Table 1

Susceptibility of phage S-2L DNA to restriction endonuclease cleavage

| | Enzymes unable to hydrolyse S-2L DNA[a] | | | | Enzymes hydrolysing S-2L DNA[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Enzyme | Sequence | Enzyme | Sequence | Enzyme | Sequence | Enzyme | Sequence |
| Recognition sites containing A | *Aha*III | TTTAAA | *Pvu*I | CGATCG | *Taq*I | TCGA | | |
| | *Avr*II | CCTAGG | *Sac*I | GAGCTC | | | | |
| | *Bam*HI | GAATTC | *Sal*I | GTCGAC | | | | |
| | *Bgl*II | AGATCT | *Xba*I | TCTAGA | | | | |
| | *Eco*RI | GAATTC | *Xho*I | CTCGAG | | | | |
| | *Eco*RV | GATATC | *Atu*BI | CC$^{A}_{T}$GG | | | | |
| | *Fsp*I | TGCGCA | *Ava*II | GG$^{A}_{T}$CC | | | | |
| | *Fsp*II | TTCGAA | *Hin*fI | GANTC | | | | |
| | *Hin*dIII | AAGCTT | *Mbo*II | GAAGA | | | | |
| | *Hpa*I | GTTAAC | *Alu*I | AGCT | | | | |
| | *Kpn*I | GGTACC | *Mbo*I | GATC | | | | |
| | *Pst*I | CTGCAG | *Sau*3AI | GATC | | | | |
| Degenerate recognition sites | | | | | *Acy*I | GPuCGPyC | | |
| | | | | | *Ava*I | CPyCGPuG | | |
| | | | | | *Hae*II | PuGCGCPy | | |
| Recognition sites containing only G and C | | | | | *Bgl*I | GCCN$_5$GGC | *Asu*I | GGNCC |
| | | | | | *Sac*II | CCGCGG | *Bsp*RI | GGCC |
| | | | | | *Sma*I | CCCGGG | *Bsu*RI | GGCC |
| | | | | | *Xma*III | CGGCCG | *Hha*I | GCGC |
| | | | | | *Aha*I | CC$^{G}_{C}$GG | *Msp*I | CCGG |

[a] No detectable change of phage DNA (1 $\mu$g) is observed after incubation with 10 units of the enzymes for one hour

[b] No detectable amount of intact phage DNA remains after incubation of 1 $\mu$g DNA with 10 units of the enzymes for one hour

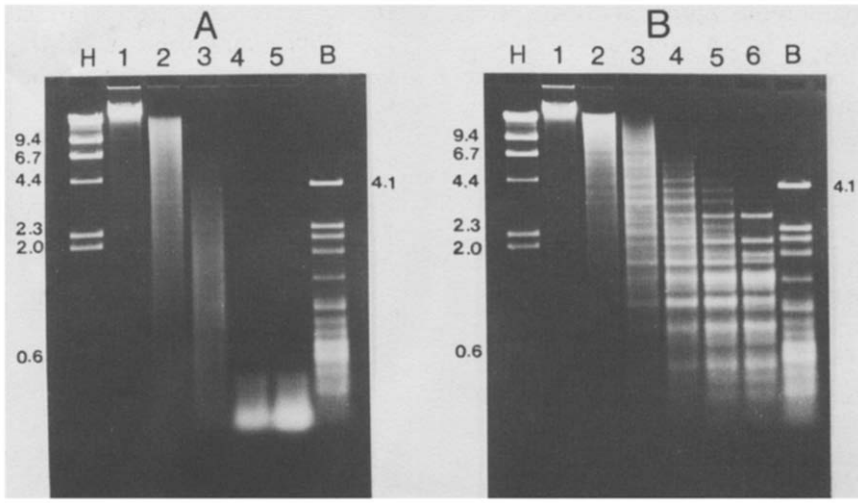


Fig.1. Agarose gel electrophoretogram of *Bsp*RI (A) and *Sma*I (B) endonuclease-treated S-2L DNA (1 $\mu$g). Lanes 1–6 show patterns obtained with no enzyme added, 1, 2, 5, 10 and 20 units of the enzymes, respectively. Lane H contains *Hin*dIII, while lane B contains *Bsp*RI-digested λ DNA markers. Fragment sizes are expressed in kilobase pairs.

quences. Since restriction sites of 4 to 5 bp in length can normally be expected to occur in the 42 kbp long phage genome these data suggest that DAP is usually not tolerated as a substitute for A by this group of enzymes.

As anticipated, the rest of the restriction enzymes with only G and C-containing or degenerate recognition sequences accepted and cleaved S-2L DNA. Gel electrophoretic analysis revealed that the DNA fragments generated were of lower molecular masses than expected indicating that sensitive sites in the substrate occur with higher frequency than predicted on a statistical basis. Fig.1 shows typical cleavage patterns of a 6 bp specific (*Sma*I) and a 4 bp specific (*Bsp*RI) enzyme. This figure also shows that limited digestion of 1 $\mu$g DNA could only be reached by using large amounts of the enzymes. In our experiments this varied from 10 (*Bsp*RI, *Bsu*RI and *Msp*I) to 50 (*Xma*III) activity units.

### 3.2. *Sensitivity to micrococcal nuclease and AS-1 endonuclease*

As the majority of restriction enzymes behaved fairly predictably in the presence of DAP-containing substrate we were interested in testing the ability of two endonucleases, with very limited sequence specificity, to degrade phage S-2L DNA. Micrococcal nuclease, under appropriate conditions, recognizes and cleaves dsDNA at various sites containing d(A-T) [7]. The micrococcal nuclease degradation profiles of S-2L DNA compared to that of phage λ DNA (45 kbp) is given in fig.2A. As can be seen, the fragmentation susceptibility of phage λ DNA was 30-times higher than that of S-2L DNA. AS-1 endonuclease is specific

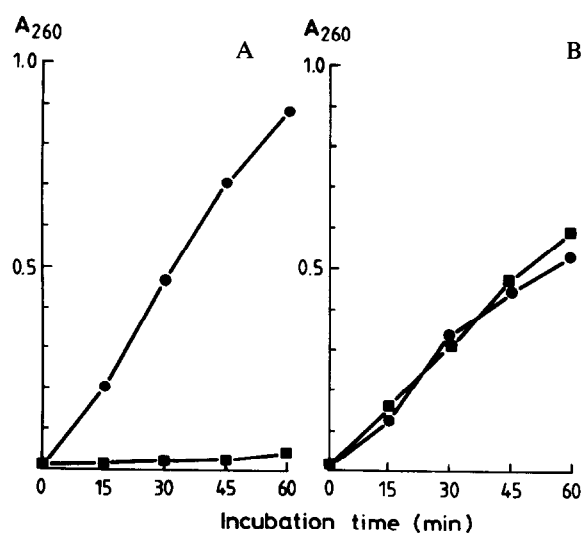


Fig.2. Release of trichloroacetic acid-soluble material during degradation of S-2L (■) and λ (●) DNA, 100 $\mu$g each, with 1 unit micrococcal nuclease (A) and $10^3$ units of AS-1 endonuclease (B).

Table 2

Distribution of radioactivity among the 5′-terminal nucleotides of the S-2L DNA fragments generated by restriction endonucleases

| Enzyme | Nucleotide | Radioactivity | |
|---|---|---|---|
| | | Counts/min | % |
| *Asu*I | dDAPMP | 8 237 | 3 |
| | dGMP | 240 567 | 90 |
| | dCMP | 10 296 | 4 |
| | dTMP | 7 116 | 3 |
| *Bsp*RI | dDAPMP | 2 433 | 1 |
| | dGMP | 3 294 | 2 |
| | dCMP | 172 235 | 96 |
| | dTMP | 2 078 | 1 |
| *Msp*I | dDAPMP | 10 671 | 3 |
| | dGMP | 12 981 | 3 |
| | dCMP | 244 311 | 61 |
| | dTMP | 133 856 | 33 |
| *Sma*I | dDAPMP | 43 744 | 21 |
| | dGMP | 153 514 | 76 |
| | dCMP | 3 491 | 2 |
| | dTMP | 1 749 | 1 |

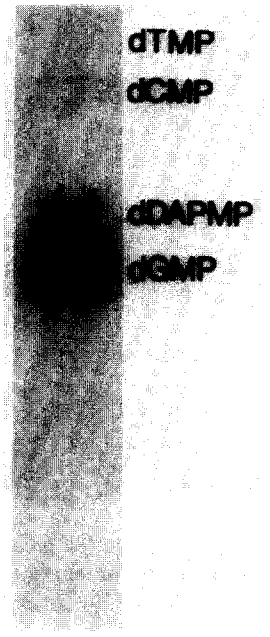


Fig.3. Radioautogram of thin layer chromatographically separated 5′-terminal nucleotides of S-2L DNA fragments cleaved by *Sma*I endonuclease.

for the trinucleotide sequence PuGC [9]. Comparison of the reaction rates measured with S-2L and λ DNA indicated an equal vulnerability of these substrates to AS-1 endonuclease cleavage (fig.2B).

These results confirm that the 2-amino group of DAP may have a general effect on the sequence recognition of site-specific endonucleases.

### 3.3. *Analysis of the 5′-terminal nucleotides of the restriction fragments*

The cleavage patterns presented in fig.1 and others generated by restriction nucleases with G and C-containing sites imply that fragmentation by these enzymes may occur in a manner less specific than usual, that is sites other than the canonical ones may also be cleaved. In order to test this possibility we investigated the fragment termini obtained after *Asu*I, *Bsp*RI, *Msp*I and *Sma*I cleavage. Ligation of the fragments of these enzymes with $T_4$ DNA ligase was normal and repeated restriction restored the original banding patterns (not shown). This demonstrates that the enzymes introduced double-strand cleavages into phage S-2L DNA in the usual fashion. Results of the 5′-terminal nucleotide analysis are presented in table 2. The distribution of the 5′-label among the four nucleotides separated by thin layer chromatography (see fig.3) shows that *Msp*I and *Sma*I cleavage generates heterogeneous termini where both purine and both pyrimidine nucleotides occur, respectively. These data indicating recognition degeneracies are in line with the observed anomalous cleavage patterns. Though the gel patterns of *Asu*I and *Bsp*RI cleaved S-2L DNA were similar in appearance no inaccurate recognition was evidenced at the nucleotides forming the 5′-termini with these nucleases.

## 4. DISCUSSION

Cleavage sensitivity of restriction sites containing modified nucleotides has been reported to vary greatly depending on the restriction nucleases used [2]. Our results reveal that the susceptibility of S-2L DNA to most of the enzymes investigated depends on the A requirement of the recognition event.

The restriction endonucleases seem to belong to two major categories in this respect suggesting that several of these enzymes may have common recognition mechanisms for the purine nucleotides.

The restriction endonucleases with a definite A requirement within the recognition sequence failed to fragment S-2L DNA. Though some 6 bp sites may not be represented in the phage genome, we identified *Bam*HI, *Pst*I and *Xho*I sites in cloned S-2L DNA replicated in *Escherichia coli* (not shown). *Eco*RI has been reported to cleave its site, though with decreased efficiency, when only the central adenines were replaced by DAP [3]. In our experiments *Eco*RI was totally inactive with S-2L DNA even under relaxed specificity (*Eco*RI*) conditions. *Taq*I, the only enzyme of this specificity group which fragments S-2L DNA, is noted for its extreme tolerance for modified and mismatch-containing recognition sites [2,10]. Some other A-requiring enzymes may also react with DAP-substituted DNA but we wish to point out the remarkably similar behavior of the restriction nucleases tested. We are of the opinion that the 2-amino group of DAP, which differentiates it from A and makes it resemble G, may mean a strong general signal influencing sequence recognition.

The apparent specificity decrease during fragmentation of S-2L DNA with restriction endonucleases of the G and C specificity group was shown to be associated with the relaxation of sequence recognition in the case of *Msp*I and *Sma*I. The nucleotide heterogeneity at the 5′-ends indicates that the presence of the 2-amino group, also present in G, may delude these enzymes and make them recognize d(DAP-T) base pairs as d(G-C) pairs. Thymines appear to be well tolerated at these positions. This implies that pyrimidine nucleotides could play a subordinate role in the recognition process as has been demonstrated in the case of *Eco*RI nuclease [11]. We propose that most enzymes of the G and C recognition group may cleave DAP-containing sites.

The degradation of S-2L DNA to very small fragments and the large amounts of enzyme activity required to achieve complete digestion support this hypothesis. Our failure to detect nucleotide heterogeneity at the 5′-termini of *Asu*I and *Bsp*RI products could be due to the sensitivity of these restrictases to methylation at these base pairs. Therefore the 5-methyl group of T may present a protection signal at these positions. We note here that the terminal cytosines of the *Sma*I fragments are also the sites of methylation in the *Sma*I restriction/modification system but their methylated state does not necessarily prevent restriction [12].

In DAP-containing dsDNA the 2-amino group lies in the minor groove of the duplex structure. This region of the DNA is believed to have no direct contact with the *Eco*RI endonuclease during the recognition process [3]. Nevertheless, the potential of the DNA substrate to adopt the active conformation necessary for productive interaction with the enzyme is greatly influenced by the substituents of the minor groove [3,5]. We presume that the relatively uniform and predictable reactions of various restriction and other endonucleases to DAP-containing sequences may be explained by the general distortion by this base of the active DNA structure.

The physiological significance of the S-2L DNA modification is not clearly understood. Complete substitution of 5-hydroxymethylcytosine for cytosine in bacteriophage $T_4$ DNA provides the molecular basis of the preferential breakdown of cytosine-containing host DNA by the phage-encoded endonucleases II and IV [13]. On the basis of this analogy we assume that the presence of DAP may protect S-2L DNA against the phage-induced endonuclease(s) which initiate host DNA degradation. In addition, DAP-containing DNA can be advantageous for the phage when infecting certain restrictive cyanobacterial hosts.

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