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Evidence for a Restriction/Modification-Like System in *Anacystis nidulans* Infected by Cyanophage AS-1

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Anacystis nidulans infected by AS-1 cyanophage contains an endonuclease (AS-1 endonuclease) which splits host DNA but not AS-1 phage DNA [Szekeres, M. (1981) *Virology*, *111*, 1–10]. AS-1 phage DNA proved to be resistant not only to AS-1 endonuclease but also to a number of restriction endonucleases the recognition sites of which contain a central dG-dC dinucleotide. Since an unmodified 5′dG-dC dinucleotide was shown to be present at the sites at which DNA is cleaved by AS-1 endonuclease, the results suggest that the sites attacked preferentially by the AS-1 endonuclease are specifically protected on the AS-1 DNA molecule. The modification of AS-1 DNA was shown to occur specifically in infected *Anacystis* because AS-1 DNA fragments which are normally resistant to AS-1 endonuclease became susceptible to this enzyme if inserted into pBR322 plasmid and cloned in *Escherichia coli*. AS-1 DNA was shown to contain about 5% of a modified nucleotide which was not 5-methyldeoxycytidylic acid. Results presented and our earlier data suggest that in *Anacystis* infected by AS-1 phage, a restriction/modification-like system operates which is able to eliminate 'unwanted' (host) DNA selectively.

Specific endodeoxyribonucleases and the modification enzymes conveying protection against them provide a possibility for the selective degradation of 'foreign' DNA molecules in the cell. The function of these enzymes *in vivo* is still highly debated, since in those cases in which the widely studied bacterial restriction and modification methylases are involved, the occurrence of foreign DNA is fortuitous [1, 2].

We have recently been studying a restriction/modification-like system which appears to be involved in the selective breakdown of 'unwanted' DNA. We have shown *in vitro* that the DNA of *Anacystis nidulans*, a unicellular cyanobacterium, is readily degraded by an endonuclease induced in *Anacystis* by cyanophage AS-1, while the DNA of the infecting phage is completely protected against the enzyme [3]. These and further data suggested the involvement of AS-1 endonuclease in the selective breakdown of host DNA [3].

The virus-induced processes taking place in *Escherichia coli* infected with T even phages are different. In the infected *E. coli* cell cytosine-specific endonucleases appear which are responsible for the breakdown of host DNA. This breakdown is also selective because the phage DNA, in which cytosine is completely replaced by 5-hydroxymethylcytosine, is protected against these endonucleases [4, 5]. This system, as far as its function is concerned, is similar to that found in phage-infected *Anacystis*. There are, however, significant differences. In *E. coli* the protection of phage DNA is based on the complete substitution of one of the four DNA bases and the modification is acquired at the mononucleotide level. The present paper will show that in *Anacystis* both DNA cleavage and protection are sequence-specific, suggesting post-replicational modification of the phage DNA. In the infected cell this restriction/modification-like system may play a regulatory role at the DNA level by eliminating host genes.

Abbreviations. Nucleotides are abbreviated according to IUPAC-IUB Recommendations, see *Eur. J. Biochem. 15*, 203–208 (1970).

Enzymes. Phage T4-induced DNA ligase (EC 6.5.1.1); DNase from bovine pancreas (EC 3.1.21.1); snake venom phosphodiesterase (EC 3.1.4.1); restriction endonucleases (EC 3.1.23); AS-1 endonuclease (EC 3.1.23.–).

MATERIALS AND METHODS

Cyanobacterium, Cyanophage and Growth Conditions

These were the same as previously described [3].

DNA Samples

AS-1 DNA was obtained from phage preparations, purified on a linear CsCl gradient, by treatment with proteinase K (Merck), followed by phenol extraction. pBR322 plasmid DNA obtained by CsCl equilibrium ultracentrifugation was kindly supplied by Dr P. Horvath (Institute of Biochemistry, Biological Research Center, Szeged). A hybrid plasmid carrying the 56H8 DNA fragment of *Drosophila* [6], and a recombinant plasmid prepared by inserting the 1200-residue *Sal* I fragment of plasmid 122 [7] into the *Sal* I site of pBR322 were prepared as described by Török and Karch [6]. DNA concentrations were calculated from the A_{260} values.

Preparation of ^{32}P-Labelled AS-1 DNA

Anacystis nidulans cells were grown as described in [3]. Phosphorus was withdrawn from the cultures in the logarithmic phase of growth (5×10^7 cells · ml^{-1}) for two generation times (8 h). Then, [^{32}P]K_3PO_4 was added to the cultures (80 kBq · ml^{-1}), the cells were infected with purified AS-1 phage at a multiplicity of infection of 5, and the cultures grown until lysis occurred. From the lysate, phage DNA was obtained as described above (spec. act. $1-2.5 \times 10^4$ dis. · min^{-1} · μg DNA^{-1}).

Endonucleases

Purification of AS-1 endonuclease, assay conditions and definition of the unit of enzyme activity have been described in [3]. Restriction endonucleases were prepared in the Institute of Biochemistry, Hungarian Academy of Sciences, Szeged.

Determination of the Cleavage Sites of AS-1 Endonuclease

DNA fragments of known sequences, ^{32}P-labelled at one of their 5′ ends [6], were digested with AS-1 endonuclease for various periods of time. The fragments were precipitated and washed several times with ethanol. Then, the preparations were run on 8 % polyacrylamide sequencing gel containing 7 M urea [8]. Products of the reactions for adenine > cytosine and guanine, obtained with the original DNA fragments, were run as standards [9].

Cloning of AS-1 DNA Fragments

Purified AS-1 DNA and pBR322 plasmid DNA were digested with *Eco*RV endonuclease. The digests were pooled, the endonuclease was inactivated by heat treatment and the sample was treated with T4 DNA ligase prepared in our laboratory. The ligation products were then used for the transformation of *Escherichia coli* HB 101 cultures. The transformants were selected for ampicillin resistance. Plasmid DNA was prepared from the tetracycline-sensitive colonies according to Klein et al. [10]. The plasmid preparations were treated with *Eco*RV endonuclease. The phage DNA fragments incorporated into the plasmid were thus released and could be identified by screening the samples on agarose gels, on the basis of their electrophoretic mobilities as compared to fragments of native AS-1 DNA digested with *Eco*RV endonuclease. The AS-1 DNA origin of the DNA fragments derived from the plasmid was established by nitrocellulose filter hybridization with ^{32}P-labelled authentic AS-1 DNA, as described by Denhardt [11].

Thin-Layer Chromatography of 5′-Nucleotides

Preparation, thin-layer chromatography and quantitative assay of the 5′-nucleotides obtained from ^{32}P-labelled AS-1 DNA were carried out as described earlier [3].

RESULTS

Sensitivity of AS-1 DNA to Restriction Endonucleases

Since the DNA of AS-1 phage has been found to be resistant to the site-specific endonuclease formed in AS-1-infected *Anacystis* cells [3], we have also tested the sensitivity of AS-1 DNA to other site-specific endonucleases. Several endonucleases did attack the AS-1 DNA (e. g. *Eco*RV, *Eco*RI, *Bgl*II, *Xba*I, *Ava*I, *Bam*HI). However, the AS-1 phage DNA proved to be resistant to all restriction endonucleases the recognition sites of which contain a central dG-dC dinucleotide (Table 1). This suggests that the dG-dC sequence is involved in the protection of AS-1 DNA against the endonucleases tested. This protection appears to be specific because (a) it is unlikely that the recognition sequences of the endonucleases endowed with a comparatively low specificity (*Alu*I, *Bsp*I, *Bsu*I) would not occur on a DNA chain with an M_r of 80×10^6 (the M_r of the AS-1 DNA; unpublished data) and (b) *Bsu*I*, the low specificity form of *Bsu*I [12], did not split AS-1 DNA either.

Table 1. *List of the restriction endonucleases which do not split the DNA of AS-1 phage*

Endonuclease	Recognition sequence
*Hin*dIII	d(A-A-G-C-T-T)
*Pst*I	d(C-T-G-C-A-G)
*Sac*I	d(G-A-G-C-T-C)
*Sac*II	d(C-C-G-C-G-G)
*Alu*I	d(A-G-C-T)
*Bsp*I	d(G-G-C-C)
*Bsu*I	d(G-G-C-C)
*Bsu*I*	d(G-C)

It may be assumed that the specific regions of AS-1 DNA, which protect it against the AS-1 endonuclease [3], are the same regions that protect the AS-1 DNA against the restriction endonucleases listed in Table 1. If this is the case, the cleavage of substrate DNA by AS-1 endonuclease should also exhibit a low level of site-specificity. In line with this idea, prolonged digestion of various DNA samples (e.g. λ DNA, pBR322 DNA) with the AS-1 endonuclease resulted in the formation of DNA fragments with an average size shorter than 100 base pairs [3]. This observation and the results presented in Table 1, when taken together, strongly suggest that the minimum specificity of the AS-1-phage-induced endonuclease can not include more than three residues including a dG-dC dinucleotide. Therefore, efforts were made to determine the cleavage sites of AS-1 endonuclease.

Determination of the Cleavage Sites of AS-1 Endonuclease

To determine the cleavage sites of AS-1 endonuclease, DNA fragments of known nucleotide sequence, carrying a ^{32}P label at one of their 5′ ends, were partially digested with AS-1 endonuclease and electrophoresed on sequencing gels. After autoradiography, the size of the digestion products were determined from the sequence ladder obtained by the adenine > cytosine and guanine reactions of the initial (substrate) fragments (Fig. 1), considering that chemical cleavage of the DNA results in a one-nucleotide mobility increase of the reference ladder compared to the mobility of enzymatically generated fragments. By this method, 5′d(R-G$^{\downarrow}$C) and 5′d (G$^{\downarrow}$C-Y) were obtained, almost invariably, as cleavage sites (Fig. 1, Fig. 2, Table 2). Since these sites represent trinucleotides of complementary sequences, one may suppose that the two strands of the DNA molecule are attacked by the enzyme in the following way:

$$\begin{array}{l} 5'\text{d(R-G}^{\downarrow}\text{C)} \\ \quad\quad\cdot\quad\cdot\quad\cdot \\ 3'\text{d(Y-C}_{\uparrow}\text{G)}. \end{array}$$

On the one hand, these data confirm the presence of a 5′dG-dC dinucleotide sequence in the recognition site of the enzyme. On the other hand, since the data were obtained by partial digestion, they do not show whether or not the endonuclease is able to split other sequences, albeit with a lower frequency and a lower specificity.

Studies on Cloned AS-1 DNA

In order to test whether or not the AS-1 DNA retains its resistance to AS-1 endonuclease after cloning, both the original AS-1 fragments and the same fragments cloned in *Escherichia*

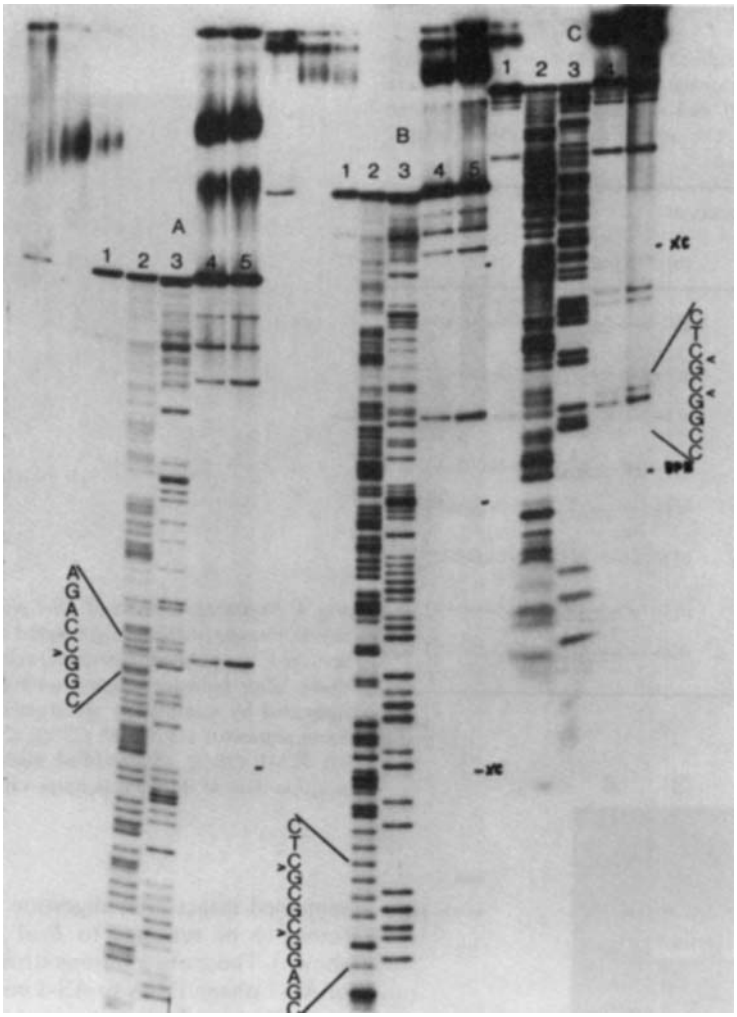

Fig. 1. *Determination of cleavage sites of AS-1 endonuclease using* Drosophila *56H8 DNA as substrate.* 2 µg of the cloned 345-base-pair *SalI/BglI* fragment of 56H8 DNA labelled at the *SalI* 5′ terminus [6] was digested with 5 units of AS-1 endonuclease. Aliquots were withdrawn at different times during digestion and analyzed on 8 % sequencing gel [8]. Channels 1, 4 and 5 show samples taken at 1, 2 and 4 h, respectively. Channels 2 and 3 show the reaction products of the adenine > cytosine and guanine reactions [9], respectively, obtained with another aliquot of the same DNA fragment. A, B and C gels were run for 6, 4 and 2 h, respectively. The cleavage sites of AS-1 endonuclease are indicated by arrows. The sequence of the same DNA region is shown in Fig. 2

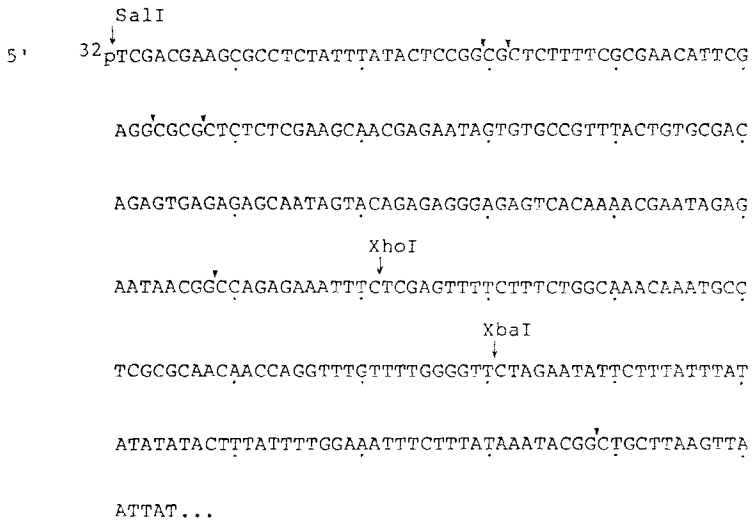

Fig. 2. *Cleavage sites of AS-1 endonuclease on* Drosophila *56H8 DNA*. Part of the sequence of the 345-base-pair *SalI/BglI* fragment [16]. The cleavage sites are shown by arrows. The hyphens representing phosphodiester bonds and the d representing deoxy have been omitted to save space

Table 2. *The cleavage sites of AS-1 endonuclease on cloned* Drosophila *DNA fragments*
The sequences shown in (a) were obtained on the 345-base-pair *SalI/BglI* fragment of the 56H8 region of *Drosophila* DNA; those shown in (b) were obtained on the 453-base-pair *SalI/AluI* subfragment of the 1300-base *SalI* fragment of 122 plasmid [7]. The arrows show the cleavage sites, recognition sequences are underlined

Site	Sequences obtained on fragments of	
	(a) *Drosophila* 56H8 region	(b) 122 plasmid
1.	d(T-C-C-G-G↓C-G-C-T-C-T-T)	d(G-G-C-G-G↓C-C-A-A-T-C-C)
2.	d(C-G-G-C-G↓C-T-C-T-T-T-T)	d(A-A-C-C-G↓C-C-C-A-G-T-G)
3.	d(C-G-A-G-G↓C-G-C-G-C-T-C)	d(G-T-C-G-G↓C-T-A-G-T-C-A)
4.	d(G-C-G-C-G↓C-T-C-T-C-T-C)	d(A-T-C-A-G↓C-A-G-T-C-A-A)
5.	d(A-A-C-G-G↓C-C-A-G-A-G-A)	d(C-T-A-A-G↓C-A-T-A-C-A-T)
6.	d(T-A-C-G-G↓C-T-G-C-T-T-A)	d(A-C-C-C-G↓C-T-T-C-A-G-C)
7.		d(T-T-C-A-G↓C-G-A-C-A-A-C)
8.		d(C-A-G-A-G↓C-T-T-C-G-T-G)

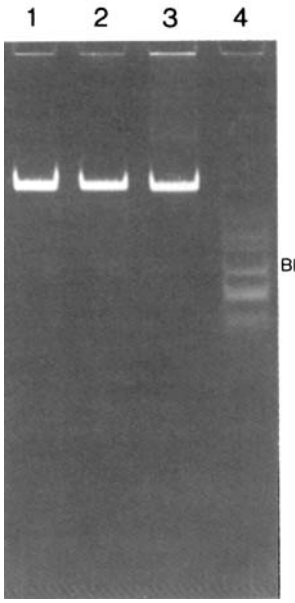

Fig. 3. *Digestibility of native and cloned AS-1 DNA with AS-1 endonuclease.* The 900-base-pair *Eco*RV fragment was isolated from native AS-1 DNA and AS-1 DNA cloned in the plasmid vector pBR322, respectively. 1 μg of each DNA sample was digested with AS-1 endonuclease and was run on 2% agarose slab gel. 1st channel: untreated phage DNA; 2nd channel: native phage DNA treated with 3 units of AS-1 endonuclease; 3rd channel: cloned untreated phage DNA; 4th channel: cloned AS-1 DNA treated with 3 units of AS-1 endonuclease. The position of the bromophenolblue marker dye is indicated on the right side of the gel (BPB)

coli were digested with AS-1 endonuclease. Fig. 3 shows the electrophoretic patterns obtained by the digestion of the 900-base-pair *Eco*RV fragment. It may be seen that the cloned fragment was split by the enzyme into characteristic products of well defined sizes, whereas the 'original' AS-1 DNA fragment

Fig. 4. *Nucleotide analysis of AS-1 phage DNA.* AS-1 DNA, ^{32}P-labelled *in vivo*, was enzymatically hydrolyzed and the resulting 5′-nucleotides were analyzed by poly(ethyleneimine)-cellulose thin-layer chromatography. Spots, after radioautography, were cut out and their ^{32}P content was measured by scintillation spectrometry. On the autoradiogram shown spots represent: (1) dGMP (22%), (2) dAMP (27%), (3) dCMP (18%), (4) dTMP (28%), (5) modified nucleotide (5%). The nucleotide composition data of different samples varied within an error of 1%

remained intact after digestion. Cloned fragments were also found to be sensitive to *Bsu*I and *Alu*I cleavage (data not shown). These observations strongly suggest that the resistance of AS-1 phage DNA to AS-1 endonuclease is acquired during viral DNA multiplication in the host cell.

Nucleotide Analysis of AS-1 DNA

Conventional thin-layer chromatographic analysis of the AS-1 DNA hydrolysate has suggested that the AS-1 DNA does not contain large amounts of an unusual nucleotide [3]; i.e. the protection of AS-1 DNA against AS-1 endonuclease must be based on specific modification of the phage DNA, rather than on its general structural features.

In order to detect small amounts of modified nucleotides in the AS-1 DNA, isotope methods were used. AS-1 DNA labelled by ^{32}P *in vivo* was hydrolyzed to 5′-nucleotides and the products obtained were separated by poly(ethyleneimine)-cellulose thin-layer chromatography (Fig. 4). The spots were identified by using authentic standards (Sigma), eluted and quantified by scintillation spectrometry. The results summarized in Fig. 4 show that the ratio dG:dC in the hydrolysate was higher than one. A fifth spot, presumably a modified dCMP derivative, was also present in small amounts on the chromatograms (Fig. 4). The ^{32}P recovered from this fifth spot, if added to the amount of ^{32}P found in dCMP, was enough to obtain the complementary dG:dC = 1:1 ratio.

The modified nucleotide of the AS-1 DNA does not appear to be 5-methyldeoxycytidylic acid since its mobility differed from that of the authentic standard in both the system used for running the sample shown in Fig. 4 and in two-dimensional cellulose thin-layer chromatography (not shown), as described by Cedar et al. [13].

DISCUSSION

The purpose of the present work was to investigate the mechanism by which the DNA of cyanophage AS-1 inducing the AS-1 endonuclease, supposed to be involved in the breakdown of host DNA [3], escapes nucleolytic breakdown by the same enzyme. AS-1 endonuclease appears to be an enzyme which splits DNA at specific sites. However, the average size of fragments gradually decreases with the progress of digestion; i.e. limit digestion is difficult to reach with this enzyme [3]. No similar virus-induced nuclease has so far been described. Recently, however, a mammalian endonuclease has been reported which behaves similarly [14].

As to the mode of action of both enzymes, the properties of *Eco*RI*, a reduced-specificity form of the *Eco*RI enzyme, is relevant. The sequence of DNA regions flanking the minimal recognition sequence of *Eco*RI* greatly affects the frequency of splitting at the recognition site. The affinity of the enzyme with *Eco*RI* activity to its recognition sites located in different 'environments' has been studied in detail [15]. On the basis of preliminary data, we have suggested that the cleavage of AS-1 sites is similarly influenced by neighbouring nucleotides [3].

Current data show that the AS-1 enzyme splits the substrate DNA at different sequences [d(A-G-C) and d(G-G-C)] but do not indicate the frequency of splitting at these two sequences. The occurrence of d(R-G-C-Y) sequences in the substrate DNA may provide an additional variability in the sense that two recognition sites [d(R-G↓C), d(G↓C-Y)] occur twice within a tetranucleotide. Since in this case the two recognition sites belong to the same cleavage site [d(R-G↓C-Y)], splitting of the chain might be more frequent at such regions. (This shows most clearly that the AS-1 endonuclease recognizes palindrome-like sequences.) Although the presence of the d(R-G-C), d(G-C-Y) sequences seems to be required for cleavage by the AS-1 endonuclease, some of these sites in the DNA region analysed appear to be cleaved less readily or not at all (Fig. 1, Fig. 2). Since this enzyme, like *Eco*RI*, shows different affinities for its potential substrate sites, one may expect that several possible partial fragments will not accumulate in detectable amounts [15].

Since the specificity of AS-1 endonuclease is low, a comparatively high number of modified sites have to be present on the AS-1 DNA. The resistance of AS-1 DNA to a number of restriction endonucleases can most probably be explained by supposing that the modified sequences are parts of the recognition sites of these enzymes.

*Bsu*I*, the low-specificity form of *Bsu*I, which recognizes the 5′dG-dC sequence is, under ideal conditions, able to split a DNA chain at all 5′dG-dC sequences [12]. *Bsu*I* could not attack AS-1 DNA even under conditions supposed to approach limit digestion. This observation suggests that perhaps all dG-dC dinucleotides of AS-1 DNA are protected. Since we have found about 5% unusual nucleotide in the AS-1 DNA, there is a statistical possibility for such a complete protection. If this were true, the AS-1 endonuclease might be able to cleave all unprotected 5′dG-dC sites. It cannot be ruled out, however, that the specificity of AS-1 DNA modification is less than that of the AS-1 endonuclease.

The fact that the protection of AS-1 DNA against AS-1 endonuclease and restriction endonucleases from other sources is site-specific suggests that it might well be due to postreplicational modification. The usual way to achieve such a protection is methylation performed by methyltransferases. The nucleotide analysis of AS-1 DNA, however, appears to exclude the presence of 5-methyldeoxycytidylic acid. In addition to 5-methylcytosine, the only known natural cytosine component in DNA is 5-hydroxymethylcytosine. Hydroxymethylation is, however, unlikely to occur in a site-specific manner since in T even phage it occurs at the nucleotide level before DNA synthesis.

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