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A site-specific single strand endonuclease activity induced by NYs-1 virus infection of a *Chlorella*-like green alga

Yuannan Xia+, Richard Morgan[1], Ira Schildkraut[1] and James L.Van Etten

Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583-0722 and [1]New England Biolabs, 32 Tozer Road, Beverly, MA 01915-9990, USA

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ABSTRACT

A site-specific endonuclease was isolated from a eukaryotic Chlorella-like green alga infected with the dsDNA-containing virus NYs-1. The enzyme recognizes the sequence 5'-CC-3'and cleaves 5' to the first C. It cleaves 5'-C^{m}C-3' sequences but not 5'-^{m}CC-3' sequences. The enzyme creates breaks in dsDNA whenever two 5'-CC-3' sequences on opposite strands are close enough for the two strands to separate; when the 5'-CC-3' sequences on opposite strands are further apart only a portion of the strands separate. Consequently, NYs-1 endonuclease does not produce a completely stable DNA digestion pattern. The enzyme probably does not cleave ssDNA and definitely does not cleave ssRNA or dsRNA.

INTRODUCTION

We have isolated and partially characterized 30 large, polyhedral, dsDNA-containing, plaque forming viruses which infect a unicellular, eukaryotic, Chlorella-like green alga, strain NC64A (1,2). Although the viruses are morphologically similar and have a common host, they can be grouped into 11 classes on the basis of plaque morphology, reaction with antibody, resistance of the virus DNAs to restriction endonucleases, and the nature and abundance of methylated bases in their genomic DNAs (2). Each of the viral DNAs contain 5-methylcytosine (m^5C); the concentration of m^5C, as a percentage of cytosine, varies from 0.1% to 47.5%. In addition, 18 of the 30 virus DNAs also contain N^6-methyladenine (m^6A); the concentration of m^6A, as a percentage of adenine, varies from 1.5% to 37% (2,3).

The finding that methylation was sequence specific led to the discovery of virus specified DNA methyltransferases and DNA site-specific (restriction) endonucleases (see review 4). In fact, the virus-infected alga is the first eukaryotic source

of type II DNA restriction endonucleases. The restriction endonucleases CviAI, CviBI, CviQI (formly named CviII) and CviJI appear after infection with the viruses PBCV-1, NC-1A, NY-2A, and IL-3A, respectively. CviAI, like the bacterial restriction endonuclease MboI, recognizes the sequence GATC, cleaves DNA 5' to the G, and does not cleave $G^{m}ATC$ sequences (5). CviBI is similar to the bacterial restriction endonuclease HinfI because it recognizes the sequence GANTC, cleaves DNA between the G and A, and does not cleave $G^{m}ANTC$ sequences (6). CviQI is a Rsa1 isoschizomer which recognizes the sequence GTAC (7). Unlike Rsa1, however, CviQI cleaves DNA between the G and T. The restriction endonuclease CviJI appears after infection with the virus IL-3A (8). CviJI is the first restriction endonuclease to recognize the sequence PuGCPy, cleaving between the G and C.

This report describes the characterization of a different type of DNA site-specific endonuclease which appears after infection with another of these viruses, NYs-1. The NYs-1 endonuclease recognizes the two base sequence 5'-CC-3' and cleaves DNA 5' to the first cytosine.

MATERIALS AND METHODS

Growth and infection of the alga.

The production and purification of NYs-1 and the other viruses, and the growth of Chlorella NC64A on MBBM medium, have been described (1,2,9). Chlorella cells (1 to 2 x 10^{7} cells/ml) were infected with NYs-1 at a multiplicity of infection of 10 and cells were collected by centrifugation at 12 hr post infection and used immediately or frozen at -80°C.

DNAs.

Chlorella NC64A nuclear DNA and virus DNAs were isolated as described previously (3). Unmethylated pBR322 plasmid DNA was propagated in dam^{-}, dcm^{-} E. coli strain GM2163 (10) as described (3). Bacteriophage DNAs were from New England Biolabs.

Enzyme extracts.

Five to 10 x 10^{10} infected cells were suspended in 15 ml of buffer A [0.01 M Tris-HCl (pH 7.9), 0.01 M 2-mercaptoethanol and 50 μg/ml phenylmethylsulfonyl fluoride] plus 10 gm of 0.3 mm glass beads and homogenized for 80 sec at 4000 rpm in a Bronwill MSK mechanical homogenizer with cooling from a CO_2

tank. The homogenate was centrifuged at 10,000 x g for 20 min and protein was precipitated from the supernatant at 70% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in buffer A and treated with dextran T500 and polyethylene glycol 6000 as described (5,11). The resulting upper phase was removed and adjusted to 20% (v/v) glycerol and was dialyzed overnight at 4°C against three changes of buffer B [20 mM Tris-HCl (pH 7.5), 0.5 mM sodium EDTA, 7 mM 2-mercaptoethanol and 10% (v/v) glycerol]. The dialysate was diluted 5-fold with buffer B and applied to a heparin sepharose (Pharmacia) column; the column was eluted with a 0.2 to 1.0 M gradient of NaCl in buffer B. Enzyme activity was pooled and adjusted to 0.1 M NaCl with buffer C [10 mM KPO_4 (pH 7.4), 0.5 mM EDTA, 7 mM 2-mercaptoethanol, 10% (v/v) glycerol]. The sample was applied to a phosphocellulose (Whatman P11) column and eluted with a 0.2 to 1.0 M gradient of KCl in buffer C. The pooled active fractions were dialyzed against buffer B containing 50 mM KCl and 50% (v/v) glycerol and then stored at -20°C.

Enzyme assays.

Unless noted otherwise, NYs-1 endonuclease was assayed at 37°C for 2 hr in 20µl reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 2 mM ATP, 1 µg of substrate DNA and 1 to 3 µl of enzyme extract. The reaction products were electrophoresed either on 2% agarose gels in 90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA (pH 8.0) (12) or on 7.5 to 15% linear gradient polyacrylamide gels as described (5,13). DNA was stained with ethidium bromide and viewed under UV light.

Ligation of linker oligonucleotides.

Linker oligonucleotides (New England Biolabs.) were phosphorylated by T4 polynucleotide kinase and ligated with T4 DNA ligase at 12°C for 18 hrs. Twenty five µl of the ligase reaction was heated at 90°C for 10 min, reannealed at room temperature for 5 min, and adjusted to 50 mM NaCl. The ligated linker oligonucleotides (0.05 A_{260} units) were digested with 3 µl of enzyme extract at 37°C for 4 hrs and then electrophoresed on 20% polyacrylamide/7 M urea gels in 40 mM Tris-acetate (pH 8.3), 20 mM sodium acetate and 2 mM EDTA.

Determination of recognition sequence and cleavage site by DNA sequencing.

Dideoxy chain termination sequencing reactions were carried out with single-stranded M13mp8 DNA and a 5' end

labeled 19-base primer by a modification of the method of Sanger, et al (14). The 19-base oligonucleotide 5'-CATTGACAGGAGGTTGAGG-3', complementary to the M13mp8 region at position 2281 to 2263, served as a primer for sequencing the DNA from position 2263 in a counterclockwise direction. The primer was labeled at the 5' end by the method of McGraw (15) in 10 μl reaction mixtures containing: 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 12.5 μCi γ-^{32}P ATP, 25 ng of primer DNA and 10 units of T4 polynucleotide kinase. The mixture was incubated at 37°C for 35 min and the reaction was stopped by heating in a boiling water bath for 3 min.

DNA primed synthesis products for NYs-1 endonuclease digestions were prepared in a 30μl reaction mixture as follows: 2.5 μg of M13mp8 plus strand DNA pre-annealed with 2.5 ng of 5' end labeled primer was extended in the same way as a standard dideoxy sequencing "A" reaction except that dideoxy ATP was omitted. After the chase reaction with 6 μl of deoxynucleotide triphosphates at 250 μM, the mixture was drawn into a 100 μl glass capillary pipet and the ends were sealed. The capillary was immersed in a 70°C water bath for 25 min and then cooled at room temperature for 15 min. Seven μl of the mixture were incubated with either 0.5 or 1.5 μl of enzyme extract at 37°C for 20 min. Three μl of the digestion reaction were then added to 4.5 μl of stop solution (formamide-dye mixture). Three μl of this reaction mixture were placed on an 8% polyacrylamide sequencing gel beside the four sequencing reactions and electrophoresed.

RESULTS AND DISCUSSION

Site specific endonuclease activity.

Enzyme extracts were prepared from uninfected *Chlorella* cells or cells at 12 hr after NYs-1 infection and assayed for DNA site specific endonuclease activity. Enzyme activity was detected in extracts from the virus infected cells but not from uninfected cells. A single peak of endonuclease activity appeared after chromatography on heparin sepharose (at 0.4 to 0.6 M NaCl) and phosphocellulose (at 0.5 to 0.65 M KCl) columns. The purification steps increased the endonuclease specific activity at least 100 fold.

As shown in Fig. 1 (lane 6) the enzyme cleaved unmethylated plasmid pBR322 DNA into many small fragments. The

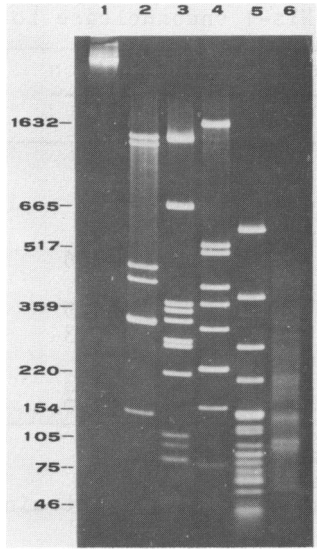

Figure 1. Ability of NYs-1 endonuclease to digest unmethylated pBR322 plasmid DNA. Untreated DNA (lane 1) and DNA treated with TaqI, CviAI, CviBI, CviJI, and NYs-1 endonuclease (lanes 2 to 6, respectively).

DNA digestion pattern was never constant - even after 16 hr incubations or adding fresh enzyme after 16 hr - and minor bands continually appeared between a few stable characteristic major bands. Because the size of the major bands did not change after prolonged incubation we conclude that the endonuclease was free of non-specific nucleases and that the enzyme cleaved pBR322 DNA at many specific sites.

The NYs-1 endonuclease required Mg^{++} for activity. The enzyme was most active at 37°C in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 50 mM NaCl and was stimulated several fold by 2 mM ATP. Neither S-adenosylmethionine nor reducing agents affected the reaction. In contrast to the other Chlorella virus induced site-specific endonucleases, heating NYs-1 endonuclease to 65°C for 10 min or storing it at room temperature for several days reduced activity only slightly. The enzyme can be stored at -20°C in buffer B containing 50 mM KCl and 50% glycerol for at least 1 year without loss of activity.

Sensitivity of DNAs to NYs-1 endonuclease.

The sensitivity of various DNAs to NYs-1 endonuclease are reported in Table 1. All susceptible DNAs were cleaved into

Table 1. Ability of NYs-1 endonuclease to digest various DNAs.

DNA	Level of methylation		Susceptibility of DNA to NYs-1 endonuclease
	m^5C[a]	m^6A[b]	
pBR322	0	0	yes
Bacteriophage			
Lambda[c]	?	0	yes
Xp12	100	0	no
Chlorella NC64A	21	0.6	yes
Chlorella viruses			
PBCV-1	1.9	1.5	yes
NC-1A	7.1	7.3	yes
IL-3A	9.7	0	yes
NYs-1	47.5	11.3	no
NY-2A	44.9	37.0	no

[a]Percent m^5C of total C plus m^5C.
[b]Percent m^6A of total A plus m^6A.
[c]The phage was grown in an *E. coli* strain containing *dcm* activity.

smaller fragments by NYs-1 endonuclease than with the other *Chlorella* virus induced site-specific endonucleases including *Cv*iJI (e.g. compare the size of the fragments in lane 5 and lane 6 in Fig. 1). *Cv*iJI recognizes PuGCPy sequences, an equivalent of a three base sequence on a probability basis (8). Since the NYs-1 endonuclease cleaves DNAs so frequently we assumed that it probably recognized a two base sequence.

The NYs-1 endonuclease digested the host *Chlorella* DNA but not virus NYs-1 DNA. DNAs isolated from the other *Chlorella* viruses differed in their sensitivity to the enzyme. Viral DNAs, which contained low to moderate concentrations of methylated bases such as PBCV-1, NC-1A, and IL-3A, were

Table 2. Cleavage of ligated and unligated oligonucleotide linkers by NYs-1 endonuclease.

Oligonucleotide linker	Sequence	Susceptibility to NYs-1 endonuclease	
		before ligation	after ligation
*Pst*I	GCTGCAGC	no	no
*Nhe*I	CTAGCTAGCTAG	no	no
*Pst*I + *Nhe*I		no	yes
*Pvu*I	CCGATCGG	no	yes
*Aat*II	GGACGTCC	yes	yes
*Eco*RI	CCGGAATTCCGG	yes	yes

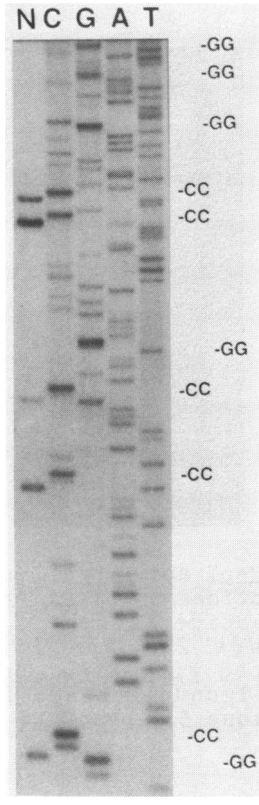

Figure 2. NYs-1 endonuclease recognition and cleavage sites in M13mp8 DNA. A stretch of M13mp8 DNA was sequenced in a counterclockwise direction by dideoxy sequencing using a 5' end labeled 19-base primer annealed to M13mp8 template at position 2281 to 2263 (lanes C,G,A,T). In lane (N) the reaction contained no dideoxynucleotides and the DNA was partially digested with NYs-1 endonuclease.

digested by the endonuclease. Viral DNAs, which contained high concentrations of methylated bases, e.g. NY-2A, were resistant to it. NYs-1 endonuclease also did not cleave DNA from the Xanthomonas oryzae bacteriophage Xp12; all cytosines in Xp12 DNA are methylated (16). This finding suggested that cytosine methylation inhibits enzyme activity and that cytosine was in the recognition sequence.

The NYs-1 endonuclease digested bacteriophage M13 ssDNA into a few fragments. However, because of the large size of the fragments, these cleavage sites probably occurred where the

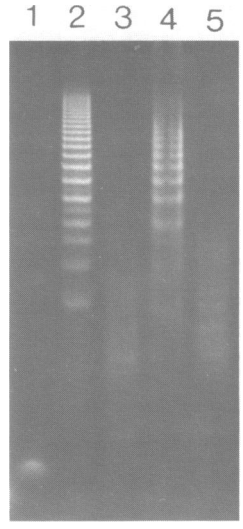

Figure 3. Effect of M.MspI and M.HpaII methylation of ligated EcoR1 linker oligonucleotides on NYs-1 endonuclease activity. EcoRI linker oligonucleotide (lane 1), ligated EcoRI linker oligonucleotides (lane 2), and ligated EcoRI linker oligonucleotides incubated with NYs-1 endonuclease (lane 3). Ligated EcoRI linker oligonucleotides incubated with M.MspI (lane 4), and M.HpaII (lane 5), prior to incubation with NYs-1 endonuclease.

ssDNA formed duplexs (17). Thus, we do not believe that NYs-1 endonuclease cleaves ssDNA per se. The enzyme did not cleave brome mosaic virus ssRNA or bacteriophage phi 6 dsRNA.

NYs-1 endonuclease recognition and cleavage site.

The specific base sequence recognized by NYs-1 endonuclease was examined with ligated linker oligonucleotide substrates (Table 2). The enzyme did not cleave ligated Pst1 linker oligonucleotides, which contains the sequence GCTGCAGC, or ligated NheI linker oligonucleotides, which contains the sequence CTAGCTAGCTAG. However, heterologous oligonucleotides formed by ligation of Pst1 linkers to Nhe1 linkers were cleaved by the enzyme. These heterologous oligonucleotides contain CC and GG sequences at the ligation junctions. Since we suspected that NYs-1 endonuclease recognized two bases and that C was part of its recognition sequence we hypothesized that the CC sequence was cleaved by the enzyme. Other ligated linker oligonucleotides such as PvuI, AatII and EcoRI, which contained CC sequences, were cleaved by NYs-1 endonuclease.

The cleavage site for NYs-1 endonuclease was confirmed by sequencing stretches of M13mp8 DNA and determining the positions of the fragments generated by partial digestion with the enzyme. These fragments were compared to standard dideoxy sequencing of M13mp8 using the same primer. To visualize the fragments on the sequencing gel, which included the primer and extended to the first site cleaved by the enzyme, the 5' end of the primer was labeled prior to polymerization. As seen in Fig. 2 (lane N) the NYs-1 endonuclease partial digestion products are one base smaller than the first C in each of the five 5'-CC-3' sequences. No products were present in the 5'-GG-3' sequences which is the complement of the 5'-CC-3' sequence. We interpret this to mean that the enzyme recognizes the two base sequence 5'-CC-3'and cleaves 5' to the first C.

As a consequence NYs-1 endonuclease would create breaks in dsDNA whenever two 5'-CC-3' sequences on opposite strands are close enough that the two strands separate. As the distance between CC dinucleotides on opposite strands increases, the melting temperature of the overlap between the strands increases. Thus a completely stable digestion pattern, where the sizes of all the fragments add up to the size of the uncleaved DNA, is not achieved.

NYs-1 endonuclease activity is inhibited by DNA methylation.

As mentioned above, NYs-1 endonuclease does not cleave bacteriophage Xp12 DNA; thus cytosine methylation inhibits its activity. To determine if the enzyme cleaved 5'-^{m}CC-3' or 5'-C^{m}C-3' sequences, ligated EcoR1 linker oligonucleotides were methylated with either M.MspI, which methylates the external C of CCGG sequences, or M.HpaII, which methylates the internal C of CCGG sequences. The oligonucleotides were then incubated with NYs-1 endonuclease. DNA methylated with M.MspI, but not with M.HpaII, was resistant to the enzyme (Fig. 3). Thus, methylation of the first C, but not the second C, in 5'-CC-3' sequences inhibits NYs-1 endonuclease activity.

NYs-1 endonuclease digested the Chlorella host nuclear DNA but not NYs-1 viral DNA. The sensitivities of the host and virus DNAs to restriction endonucleases support the conclusion that the 5' C in at least some 5'-CC-3' sequences in NYs-1 DNA, but not host DNA, is methylated. NYs-1 DNA was resistant to MspI and HaeIII restriction endonucleases indicating that NYs-1 DNA contains ^{m}CCGG and GG^{m}CC sequences. In contrast, host DNA was digested by both enzymes (data not shown).

Comparison to other endonucleases

DNA site-specific endonucleases, which produce single-stranded breaks in dsDNA, have been reported in bacteriophage T5 infected bacteria (18) and the green alga Chlamydomonas reinhardtii (19). However, the specific recognition and cleavage sites are not known for either enzyme. Furthermore, the two enzymes can be distinguished from the NYs-1 endonuclease. The bacteriophage enzyme is not stimuated by ATP and the Chlamydomonas enzyme produces regions of single stranded gaps in the dsDNA. Thus NYs-1 endonuclease is a new type of site-specific endonuclease.

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+Present Address: Service d'Infectiologie, Centre Hospitalier de Université Laval, Ste-Foy, Quebec G1V 4G2, Canada

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