Restriction endonuclease activity induced by NC-1A virus infection of a *Chlorella*-like green alga

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ABSTRACT

A type II restriction endonuclease, CviBI, was isolated from a eukaryotic, *Chlorella*-like green alga infected with the dsDNA containing virus NC-1A. The enzyme recognizes the sequence GATC and cleaves DNA between the G and A. Methylation of deoxyadenosine in the GATC sequence probably inhibits enzyme activity. *In vitro* CviBI cleaves host nuclear DNA but not viral DNA. A survey of 18 other viruses which infect the same *Chlorella* sp. revealed that infection with 5 of these viruses also induced a restriction endonuclease which cleaves DNA into the same size fragments as CviBI.

INTRODUCTION

We have isolated and partially characterized 30 large, polyhedral, dsDNA containing (ca. 300 kbp in size), plaque forming viruses which infect the unicellular, eukaryotic *Chlorella*-like green alga, strain NC64A (1,2). These viruses can be placed into 11 classes on the basis of plaque size, reaction with antibody, and the nature and abundance of methylated bases in their genomic DNAs (2). All of the viral DNAs contain 5-methyldeoxycytosine (m^5dC); the concentration of m^5dC as a percentage of deoxycytosine, varies from 0.12% to 47.5% (2,3). In addition, 18 of the 30 viral DNAs also contain N⁶-methyldeoxyadenosine (m^6dA); the concentration of m^6dA , as a percentage of deoxyadenosine, varies from 1.45% to 37%.

Infection of the alga with one of these viruses, PBCV-1, induces the synthesis of a methyltransferase which specifically methylates adenosines in the sequence GATC (4). PBCV-1 infection also induces a restriction endonuclease which recognizes the same GATC sequence and cleaves DNA 5' to the G; the enzyme does not cleave DNAs containing G^mATC sequences (5). Since host nuclear DNA contains GATC sequences and PBCV-1 DNA contains G^mATC (3), the PBCV-1 induced restriction and

modification system can explain how host DNA is degraded during viral infection while, at the same time, PBCV-1 DNA is synthesized.

This report describes a different restriction endonuclease which appears after infection of the alga with another of these viruses, NC-1A. NC-1A DNA contains substantially more m^5dC (7.1%) and m^6dA (7.3%) than does PBCV-1 DNA (1.9% m^5dC ; 1.5% m^6dA) (3). The NC-1A induced restriction endonuclease recognizes the sequence GATC and cleaves DNA between G and A.

MATERIALS AND METHODS

Growth and infection of the alga. The production and isolation of NC-1A and the other viruses, and the growth of *Chlorella* NC64A on MBBM medium, have been described (1,2,6). *Chlorella* cells (1 to 2×10^7 cells/ml) were infected with NC-1A at a multiplicity of infection of 5 and cells were collected at 3 hr postinfection by centrifugation and either used immediately or frozen at $-80^\circ C$.

Enzyme extracts. Enzyme extracts were prepared by the same procedure used to extract the PBCV-1 induced restriction endonuclease (5). Cells were disrupted in 0.01 M Tris-HCl (pH 7.9), 0.01 M 2-mercaptoethanol and 50 $\mu g/ml$ phenylmethylsulfonyl fluoride and centrifuged at $10,000 \times g$ for 20 min. The supernatant was frozen at $-20^\circ C$, thawed at $4^\circ C$ and centrifuged at $16,000 \times g$ for 30 min (Fraction I). The following components were added per ml of supernatant: 0.5 ml of denatured salmon sperm DNA at 5 mg/ml in 0.01 M Tris-HCl, pH 7.9, 0.001 M EDTA, 0.6 gm of polymer concentrate [7% (wt/wt) dextran T500, 28% (wt/wt) polyethylene glycol 6000 (7)] and 0.64 ml of 4 M NaCl. The samples were mixed, centrifuged, and the upper layer was dialyzed overnight against 0.01 M KPO₄ (pH 7.4), 0.5 mM sodium EDTA, 7 mM 2-mercaptoethanol and 5% glycerol (Fraction II). The samples were either assayed for enzyme activity at this stage or chromatographed on a phosphocellulose column equilibrated with 0.01 M KPO₄ (pH 7.4). Protein was eluted stepwise with 0.01 M KPO₄ (pH 7.4) containing KCl from 0 to 0.8 M in 0.1 M increments. Fractions containing enzyme activity (0.3 to 0.6 M KCl) were pooled (Fraction III) and the enzyme was precipitated with 70% (NH₄)₂SO₄. The precipitate was taken up in 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.1 mM sodium EDTA, 1 mM dithiothreitol, 500 $\mu g/ml$ bovine serum albumin and 50% (v/v)

glycerol (Fraction IV) and stored at -20°C .

Enzyme assays. The enzyme was assayed at 37°C for 2 hr in 20 μl reaction mixtures containing: 10 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 50 mM KCl, 1 mM dithiothreitol, 1 μg of substrate DNA and 2.5 to 5 μl of enzyme extract. In some cases core buffer [50 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 50 mM NaCl] was used for the DNA digestion. The reaction products were electrophoresed either on 1.2% agarose gels or on 7.5 to 15% linear gradient polyacrylamide gels as described (5). One unit of enzyme is the amount to completely digest 1 μg of unmethylated lambda DNA in 1 hr at 37°C in 20 μl of assay buffer.

Determination of cleavage site by DNA sequencing. Dideoxy chain termination sequencing reactions were conducted on plasmid pBR322 DNA linearized with EcoRI. The sequencing primer T2.7, which contains a sequence 5'-CTGGGCTGTGTGCACG-3' complementary to the 2779-2794 pBR322 region, and primer T2.8, which contains a sequence 5'-GCCTACATACCTCGCT-3' complementary to the 2925-2910 pBR322 region, were used for sequencing the DNA in clockwise and counterclockwise directions, respectively. Linearized pBR322 DNA (1.7 μg) was mixed with 0.17 μg of oligonucleotide primer in 10 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 30 mM NaCl in a total volume of 27 μl . The mixtures were sealed in glass capillaries, heated for 5 min at 100°C , cooled in ice/ethanol, and transferred to Eppendorf vials. Using these template-primers, dideoxynucleotide sequencing reactions were carried out as described in the Bethesda Research Lab M13 Cloning and Sequencing Manual. DNA primed synthesis products used for restriction endonuclease digestions were prepared in the same way as the "A reaction" except that dideoxyATP was omitted. After the chase reaction with unlabeled dATP, the mixture (mixture AA) comprising 43 μl was heated for 15 min at 65°C . Ten μl of this mixture AA was added to 1.5 μl of 10 x core buffer and 3.5 μl of water; 5 μl aliquots were removed and incubated at 37°C for 60 min with either HinfI (10 units) or the NC-1A induced restriction endonuclease (0.6 units). An aliquot without enzyme served as a control. The reactions were terminated with 10 μl of formamide-dye mix (0.1% xylene cyanol FF, 0.1% bromophenol blue, 10 mM EDTA, 95% deionized formamide) and 2 to 3 μl samples were applied to the sequencing gel.

The remainder of mixture AA was extracted with phenol after adding 1 μg of linearized pBR322 carrier DNA; the DNA was

precipitated with ethanol, washed, dried and resuspended in water. Samples were digested with the two restriction endonucleases as described above. All samples were electrophoresed on 8% polyacrylamide/7 M urea sequencing gels adjacent to the products of a set of the dideoxynucleotide DNA sequencing reactions. After electrophoresis the gels were soaked in 5% ethanol/5% acetic acid for 10 min to remove urea and then dried on Whatman 3 MM paper and autoradiographed using Kodak XAR-5 film.

Sources of DNAs. Host nuclear DNA and viral DNAs were isolated as described previously (3). Unmethylated pBR322 plasmid DNA was prepared by growing the plasmid in the dam, dcm, E. coli strain GM2163 (8) as described (3). Unmethylated lambda DNA was purchased from New England Nuclear Corp. SV40 DNA (Form I) isolated from BSC-1 monkey cells, adenovirus-2 DNA isolated from HeLa cells, ϕ X174 DNA (Form I), and sequencing primers T2-7 and T2-8 were from Bethesda Research Labs.

Other procedures. Ligation and recleaving of SV40 DNA restriction fragments produced by either HinfI or the NC-1A induced restriction endonuclease were determined as previously described (5). The DNAs were analyzed by equilibrium sedimentation on CsCl gradients as described (9). The DNAs were treated with restriction endonucleases according to the protocols provided by the suppliers and electrophoresed on either agarose or polyacrylamide gels.

RESULTS

Properties of NC-1A. A one step growth curve of NC-1A revealed that the virus was first released about 5 hr after infection and virus release was complete at about 10 hr after infection (unpublished results). Like PBCV-1 (9), NC-1A infection resulted in the degradation of the host nuclear DNA beginning between 1 and 2 hr after infection (unpublished results).

Restriction endonuclease activity. To determine if NC-1A infected cells contained restriction endonuclease activity, extracts were prepared from both uninfected and 3 hr infected cells and chromatographed on a phosphocellulose column. The extract from viral infected cells (Fig. 1), but not from uninfected cells (data not shown), contained activity eluting at 0.3 to 0.6 M KCl which cleaved unmethylated lambda DNA into unique fragments. Fractions containing activity were pooled and

Figure 1. Phosphocellulose column chromatography of an enzyme extract prepared from NC-1A infected *Chlorella* cells at 3 hr post infection. Thirty mg of protein were applied to the column and eluted stepwise with 0 to 0.8 M KCl. Fractions (2.5 ml) were collected and 5 μ l of each fraction were incubated with 1 μ g of unmethylated lambda DNA for 2 hr. Samples were electrophoresed on 1.2% agarose gels.

concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation.

As shown in Fig. 2⁴ (lane 3) unmethylated lambda DNA was cleaved into many discrete fragments by the enzyme after a 2 hr incubation. No additional DNA fragments appeared if the DNA was incubated with enzyme for 16 hr (lane 4) or if the DNA was treated with a second aliquot of enzyme for 2 more hr after the 16 hr incubation (lane 5). Thus the enzyme cleaved lambda DNA at a limited number of sites. Heating the enzyme to 65°C for 10

Figure 2. Ability of an enzyme extract (Fraction IV) from NC-1A infected *Chlorella* cells to degrade unmethylated lambda DNA. Lane 1 is untreated lambda DNA, lane 2 is lambda DNA after a 2 hr incubation with an extract from uninfected cells, and lane 3 is lambda DNA after a 2 hr incubation with an extract from 3 hr infected cells. Lane 4 is the same as lane 3 only the enzyme reaction was incubated for 16 hr; lane 5, same as lane 4 but incubated an additional 2 hr with a second aliquot of enzyme; lane 6 is the same as lane 3 but $MgCl_2$ was omitted from the assay; lane 7 is the same as lane 3 except that the enzyme extract was heated to $65^\circ C$ for 10 min prior to adding it to the DNA. The DNAs were electrophoresed on a 1.2% agarose gel.

min partially inactivated the enzyme (Fig. 2, lane 7).

Enzyme activity was not stimulated by ATP and/or S-adenosylmethionine (data not shown) but required Mg^{++} (Fig. 2, lane 6); thus the enzyme is a type II restriction endonuclease

Figure 3. Ability of the NC-1A induced restriction endonuclease to degrade unmethylated plasmid pBR322 DNA. Untreated DNA (lane 1), treated with *Taq*I (lane 2), *Mbo*I (lane 3), *Hinf*I (lane 4), and the NC-1A induced restriction endonuclease (lane 5). Band 7 in lanes 4 and 5 consists of two fragments. **Note:** the size of the DNA fragments in lanes 4 and 5 are identical.

(10, 11, 12, 13). The enzyme was most active in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 50 mM KCl. The temperature optimum was 37 °C. The enzyme can be stored at -20 °C in buffer containing 50% glycerol and 500 µg/ml bovine serum albumin for at least 1 year without loss of activity. The specific activity of the purified enzyme was about 10^5 units/mg protein. About 2000 units of activity were obtained per liter (2×10^{10} cells) of infected cells.

Specificity of DNA recognition and cleavage. The base sequence recognized by the NC-1A induced restriction endonuclease was examined with unmethylated pBR322 DNA as a substrate. Plasmid pBR322 DNA was cleaved into 9 fragments larger than 140 bp by the enzyme (Fig. 3, lane 5). These fragments were identical in size to those produced by the bacterial restriction endonuclease *Hinf*I (Fig. 3, lane 4).

The cleavage patterns produced by *Hinf*I and the NC-1A induced restriction endonuclease with four other DNAs, lambda DNA, adenovirus-2 DNA, SV40 DNA and ϕ X174 DNA (Form I) also were identical (results not shown). Since *Hinf*I recognizes the sequence GATC, the NC-1A restriction endonuclease most likely recognizes the same sequence.

The cleavage site for the NC-1A induced restriction endonuclease was established by two methods. First, two sets of SV40 DNA restriction fragments, generated by digestion with either *Hinf*I or the NC-1A restriction endonuclease, were allowed to ligate. Both sets of DNA fragments hybridized and ligated equally well (>90%) with the other. Furthermore, the ligated products were recleaved equally well (>90%) by both restriction endonucleases. This suggests that the NC-1A induced restriction endonuclease cleaves DNA at the same position as *Hinf*I, i.e., between G and A in the GATC sequence. In addition, the high ligation efficiency indicates that the NC-1A induced restriction endonuclease generates a free phosphate at the 5' termini like most other restriction endonucleases.

Figure 4. Cleavage site in pBR322 DNA by the NC-1A induced restriction endonuclease. A stretch of pBR322 DNA was sequenced by the dideoxy sequencing method using 16-base primers annealed to linearized heat denatured pBR322 DNA (lanes C, A, T, G). ³⁵S-labelled pBR322 DNA prepared analogous to the sample in the "A" lane (except that dideoxyATP was omitted) was treated with either the NC-1A induced restriction endonuclease (lanes 3 and 4), *Hinf*I (lanes 2 and 5), or no restriction endonuclease (lanes 1 and 6). The pBR322 DNA used in lanes 4 to 6 was phenol extracted prior to restriction enzyme digestion, whereas the pBR322 DNA used in lanes 1 to 3 was not phenol extracted.

A. Sequencing in clockwise direction using the primer T2.7 which anneals at position 2779-2794 of pBR322 DNA.

B. Sequencing in counterclockwise direction using the primer T2.8 which anneals at position 2925-2910 of pBR322 DNA.

The bands in lanes 2, 3, 4 and 5 which coincided with the G are indicated by an arrow head (>).

Figure 5. Sensitivity of *Chlorella* nuclear DNA (lanes 1, 2, and 3) and NC-1A DNA (lanes 4, 5, and 6) to *Hinf*I (lanes 2 and 5) and the NC-1A induced restriction endonuclease (lanes 3 and 6). DNAs in lanes 1 and 4 were untreated. The DNAs were electrophoresed on a 1.2% agarose gel.

Second, the site of cleavage was confirmed by sequencing stretches of pBR322 DNA and determining the position of the fragment generated by the NC-1A induced restriction endonuclease on sequencing gels. Plasmid pBR322 DNA contains a sequence GAGTC at position 2845-2849, which is recognized by *Hinf*I, and the NC-1A induced restriction endonuclease (Fig. 4). A major band in lanes 4 and 5 coincides with the G just below the sequence AGTC (Fig. 4A) or ACTC (Fig. 4B), respectively. Thus, the NC-1A induced restriction endonuclease cleaves DNA between

the G and the A in the GAGTC and GACTC sequences, exactly as does *Hinf*I. The 65°C treatment, which does not completely inactivate *E. coli* DNA polymerase I (Klenow fragment), resulted in an end-filling reaction during the restriction endonuclease digestion. The appearance of three additional, slower migrating, bands on top of the G band (lanes 2 and 3 in Fig. 4A and 4B) provides additional support for the generation of a 3 nucleotide long staggered end with the sequence 5'-AGT-3' or 5'-ACT-3'. Thus, the recognition and cleavage site for the NC-1A induced restriction endonuclease is

Sensitivity of DNAs to the NC-1A induced enzyme. As shown in Fig. 5 both the NC-1A induced restriction endonuclease and *Hinf*I cleaved host nuclear DNA into numerous fragments. In contrast NC-1A DNA was resistant to both enzymes. Since *Hinf*I cleaves GANTC and GANT^mC sequences but not G^mANTC sequences (14), NC-1A DNA contains either G^mANTC or G^mANT^mC sequences. Like *Hinf*I, the NC-1A enzyme is apparently inhibited by m⁶dA in the recognition sequence.

As mentioned previously we have partially characterized 30 viruses (19 of these viruses are listed in Table 1) which infect this same *Chlorella* sp.; these viruses were grouped into 11 classes (2). One criterion used to classify these viruses was the amount of m⁵dC and m⁶dA in their genomic DNAs. DNAs isolated from representative viruses in each of these classes were treated with *Hinf*I and the NC-1A induced enzyme to determine their susceptibility to the two enzymes. All 8 viral DNAs cleaved by *Hinf*I were also cleaved by the NC-1A induced enzyme (Table 1); the DNA fragment patterns were always identical for the 2 enzymes (data not shown).

Additional viruses induce a restriction endonuclease which cleaves GANTC sequences. To determine if other viruses which infect *Chlorella* NC64A also induce a NC-1A type restriction endonuclease, *Chlorella* cells were infected with each of the viruses listed in Table 1. Fraction II extracts were prepared from cells at either 3 hr after infection (for viruses which replicate at the same rate as NC-1A) or at 8 hr after infection (for viruses that replicate slower than NC-1A) and assayed for

Table 1. Ability of *Hin*FI and the NC-1A induced restriction endonuclease to cleave *Chlorella* virus DNAs. The site recognized by the various viral induced restriction endonucleases, if known, is also indicated.

Virus	Class ^a	Level of methylation ^b		Sensitivity of DNA to		Site recognized by the viral induced restriction endonuclease
		m5dC	m6dC	<i>Hin</i> FI	NC-1A	
NE-8D	1	0.44	nd ^c	yes	yes	-
NY-2C	2	0.39	nd	yes	yes	-
NC-1D		0.33	nd	yes	yes	-
PBCV-1	3	1.86	1.45	yes	yes	GATC ^d
CA-1A	4	10	nd	yes	yes	? ^e
IL-3A		9.67	nd	yes	yes	?
IL-3D		12.62	nd	yes	yes	?
SC-1A	5	1.94	7.34	yes	yes	-
SC-1B		2.04	7.45	yes	yes	-
NC-1A	6	7.1	7.3	no	no	GANTC
NE-8A	7	14.3	8.1	no	no	GANTC
AL-2C		-	-	no	no	GANTC
MA-1E		14.9	8.1	no	no	GANTC
NY-2F		14.6	8.1	no	no	GANTC
CA-1D		-	-	no	no	GANTC
NYs-1		47.5	11.3	no	no	?
AL-2A	9	35.8	14.6	no	no	-
CA-4A	10	39.8	19.6	no	no	-
NY-2A	11	44.9	37.0	no	no	?

^a See reference 2 for the criteria used to group the viruses into different classes.

^b Data from references 2,3.

^c nd is not detected

^d Data from reference 5.

^e A question mark means that restriction endonuclease-like activity was detected but the cleavage site has not been determined.

restriction endonuclease activity. Although some extracts were slightly contaminated with DNase activity, each of the 5 viruses, NE-8A, AL-2C, MA-1E, NY-2F, and CA-1D, grouped in class 7 (NC-1A is the only member in class 6) induced an enzyme which cleaved pBR322 DNA into fragments identical in size to those produced by the NC-1A enzyme. Extracts from cells

infected with other viruses either produced DNA fragments of different sizes, e.g. IL-3A and NY-2A or they failed to produce distinct fragments.

DISCUSSION

This report establishes that infection of Chlorella strain NC64A with the virus, NC-1A, results in the appearance of a restriction endonuclease which recognizes the sequence GANTC. We have named the enzyme CviBI. Cvi refers to Chlorella strain NC64A virus. The B signifies that this is the second virus which produces a restriction endonuclease upon infection of the NC64A strain of Chlorella. Accordingly, the restriction endonuclease induced by PBCV-1 infection of the same Chlorella sp. (5) will be named CviAI.

CviBI has several properties in common with the bacterial restriction endonuclease HinfI: (i) both enzymes recognize the same GANTC sequence, (ii) both enzymes cleave DNA between G and A, and (iii) both enzymes are apparently inactive on G^mANTC sequences. However, the origins of the enzymes are entirely different; CviBI is probably viral encoded and it is synthesized in a eukaryotic organism. From an evolutionary standpoint it will be interesting to compare the gene encoding HinfI with the gene encoding CviBI.

A preliminary survey of Chlorella NC64A infected with 18 additional viruses revealed that 5 of them also produced a restriction endonuclease which cleaved DNA into the same sized fragments as CviBI. Extracts from cells infected with the other viruses either produced different DNA fragments than the PBCV-1 (CviAI) and NC-1A (CviBI) induced enzymes, e.g. IL-3A and NY-2A, or no specific fragments were obtained.⁶ Since all of these viral genomes contain distinct levels of m⁶dA and/or m⁵dC (2,3) and host nuclear DNA is degraded following infection with each of the viruses (unpublished results), we believe that Chlorella sp. infected with these viruses may serve as an important new source of both DNA restriction endonucleases and DNA methyltransferases.

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