Characterization of *Chlorella* virus PBCV-1 CviAII restriction and modification system

Yanping Zhang, Michael Nelson, Joseph W. Nietfeldt, Dwight E. Burbank and James L. Van Etten*

Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583-0722, USA

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ABSTRACT

A second DNA site-specific (restriction) endonuclease (R.CviAII) and its cognate adenine DNA methyltransferase (M.CviAII) were isolated from virus PBCV-1 infected *Chlorella* strain NC64A cells. R.CviAII, a heteroschizomer of the bacterial restriction endonuclease *NlaIII*, recognizes the sequence CATG, and does not cleave C^mATG sequences. However, unlike *NlaIII*, which cleaves after the G and does not cleave either C^mATG or ^mCATG sequences, CviAII cleaves between the C and A and is unaffected by ^mCATG methylation. The M.CviAII and R.CviAII genes were cloned and their DNA sequences were determined. These genes are tandemly arranged head-to-tail such that the TAA termination codon of the M.CviAII methyltransferase gene overlaps the ATG translational start site of R.CviAII endonuclease. R.CviAII is the first *Chlorella* virus site-specific endonuclease gene to be cloned and sequenced.

INTRODUCTION

PBCV-1 is a large (ca. 190 nm in diameter), polyhedral, plaque-forming, phycodnavirus (1), that replicates in a unicellular, eukaryotic green alga, *Chlorella* strain NC64A (2). The PBCV-1 genome is a linear, nonpermuted, 333 kbp dsDNA molecule with covalently closed hairpin ends (3). Phycodnavirus genomic DNAs contain 5-methylcytosine (5mC), which varies from 0.1% to 47.5% of the total cytosine, and many also contain N⁶-methyladenine (6mA), which varies from 1.45% to 37% of the total adenine (4). The finding that methylation is sequence-specific led to the discovery that these viruses encode DNA methyltransferases and DNA site-specific (restriction) endonucleases, some of which resemble type II restriction endonucleases (4).

We reported previously that PBCV-1 infected cells contain a restriction endonuclease, named R.CviAI, which cleaves /GATC sequences (5) and its cognate DNA methyltransferase, M.CviAI (G^mATC) (6). At that time we assumed that M.CviAI was the only adenine methyltransferase in PBCV-1 infected cells, and that M.CviAI activity accounted for the 1.5% 6mA present in PBCV-1 DNA (7). However, we have recently discovered

that many *Chlorella* virus DNA methyltransferases and restriction endonucleases are inhibited by chloride ions and require mild alkaline conditions for activity (Nelson and Van Etten, unpublished data). With this knowledge, PBCV-1 infected cells were re-examined for additional DNA methyltransferase and endonuclease activities. This report describes: (i) the discovery of a second restriction endonuclease, named R.CviAII, which cleaves C/ATG sequences, (ii) characterization of the M.CviAII sequence-specific modification, and (iii) the cloning and sequencing of the M.CviAII and R.CviAII genes from PBCV-1 DNA.

MATERIALS AND METHODS

Viruses, vectors, and host strains

The growth of the host alga, *Chlorella* strain NC64A, on MBBM medium, the production and purification of virus PBCV-1 (2), and the isolation of PBCV-1 DNA (8), have been described. *Chlorella* cells, infected with PBCV-1 at a multiplicity of infection of five, were collected by centrifugation at 4 hr post infection and frozen at -80°C.

E. coli strains DH5 α MCR (mcrA⁻, mcrB⁻, mrr⁻) and MY1193 served as hosts for plasmids pUC19 and pBluescript KS(+/-), respectively.

Enzyme isolation and assays

The frozen virus-infected cells were suspended in 20 mM Tris-acetate, pH 8.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, 500 mM potassium acetate, 20 μ g/ml phenylmethylsulfonylfluoride and disrupted with 0.1 mm glass beads in a Braun MSK mechanical homogenizer (5). Following centrifugation (30,000 rpm for 1 hr in a Beckman Ti 50.2 rotor), clarified extracts were applied in succession to the following columns and eluted as indicated: heparin sepharose, 0.1 to 0.8 M potassium acetate, pH 7.6 (R.CviAII elutes ca. 0.25 M); phosphocellulose, 0.1 to 1.0 M potassium acetate, pH 7.0 (R.CviAII elutes ca. 0.35 M); hydroxylapatite, 0 to 0.5 M KPO₄ (R.CviAII elutes ca. 0.3 M); dialyzed against 20 mM Tris-acetate, pH 8.0, 150 mM potassium acetate, 1 mM EDTA, 10 mM 2-mercaptoethanol and 5% glycerol and applied to blue sepharose (Cibacron blue), 0.15 M to 2 M potassium acetate, pH 8.0 (R.CviAII elutes ca. 1.3 M).

* To whom correspondence should be addressed

Active column fractions were pooled and diluted to 0.1 M potassium acetate using 20 mM Tris-acetate, 1 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.6 between chromatographic steps.

R. CviAII was assayed at 23°C by adding 3 µl of enzyme extract to 40 µl reactions containing 20 mM glycyl-glycine pH 8.5, 10 mM magnesium acetate, 1 mM 2-mercaptoethanol, 50 µg/ml acetylated bovine serum albumin, and 1 µg of phage T7 or pUC19 dam⁺ DNA.

Co-purifying M. CviAI (6) and M. CviAII methyltransferase activities were distinguished from each other using defined polynucleotide substrates; 5'-phosphorylated, polymerized oligonucleotides (pCGGATCCG)_n and (pGGCATGCC)_n prepared by self-ligation using T4 ligase, served as substrates for M. CviAI and M. CviAII, respectively (9). M. CviAII was assayed at 23°C in 30 µl reactions containing 50 mM glycyl-glycine pH 8.2, 10 mM EDTA, 200 µg/ml acetylated bovine serum albumin, 2 µmol ³H-S-adenosylmethionine (SAM) (80 Ci/mmol), and 0.3 µg of polymerized oligonucleotide substrate. In some cases, 1.5 µg of phage λ, Xp12, or T4 DNA served as the methyltransferase substrate.

Figure 1. (A) Heparin sepharose column chromatography of an enzyme extract prepared from PBCV-1 infected cells at 4 hr p.i. The column was eluted with a 0.1 to 0.8 M Tris acetate/potassium acetate gradient. Five ml fractions were collected and 3 µl of each fraction were incubated with 1 µg phage T7 DNA at 23°C for 2 hr. The peak which elutes at about 0.25 M potassium acetate (fractions C–G) is R. CviAII endonuclease and the peak eluting at about 0.4 M potassium acetate (K–M) is the previously described R. CviAI endonuclease (5). (B) R. CviAII (lanes 1, 3, and 5) and *Nla*III (lanes 2, 4, and 6) digestion of pUC19 (lanes 1 and 2), phage φX174 (lanes 3 and 4) and virus SV40 (lanes 5 and 6) DNAs. (C) R. CviAII (lane x) cleaves between the C and A in CATG sequences.

Cloning and sequencing the M. CviAII and R. CviAII genes

A PBCV-1 DNA library was created by ligating *Sau*3A partially digested PBCV-1 DNA into the *Bam*HI site of pUC19 DNA and then transforming *E. coli* DH5αMCR (10). Clones expressing the M. CviAII gene were selected from the PBCV-1 DNA library by digesting the pooled ampicillin resistant plasmid DNAs with *Sph*I (GCATGC) prior to DH5αMCR transformation. *Sph*I is inhibited by adenine methylation of its recognition sequence but is unaffected by cytosine methylation (11); plasmid DNAs resistant to *Sph*I cleavage are therefore C^mATG methylation proficient.

For DNA sequencing, pUC19 inserts were cloned into pBluescript KS(+/-) vectors grown in *E. coli* strain MY1193. Viral DNA subclones and nested deletions of cloned viral DNA, made with an 'Erase-a-Base' exonuclease III kit (Promega), were sequenced (12) in both strands using a Sequenase kit (version 2.0, United States Biochemical Corp.). The putative R. CviAII endonuclease gene was truncated by unidirectional exonuclease III digestion from the *Eco*RV site of a *Eco*RV/*Kpn*I DNA fragment from pJN4A (Fig. 2B).

Other procedures

DNA was electrophoresed on 1% or 1.6% agarose gels in TPE buffer (80 mM Tris-phosphate, 8 mM EDTA) or TBE buffer (80 mM Tris-borate, 2 mM EDTA) (10), stained with 0.5 µg/ml ethidium bromide, and visualized with mid-range ultraviolet illumination. Southern transfer of DNA to nylon membranes employed standard protocols (10). *Chlorella* virus DNAs used for dot blots were denatured and applied to nitrocellulose membranes as described (13). Radioactive DNA probes were prepared by nick translation using a Bethesda Research Labs kit. The R. CviAII endonuclease cleavage site was determined by digesting a radiolabelled DNA-primed synthesis product with R. CviAII and comparing the cleavage products to G, A, T, and C dideoxy chain termination products.

RESULTS AND DISCUSSION

Isolation of R. CviAII

Chloride-free enzyme extracts were prepared from *Chlorella* cells 4 hr after PBCV-1 infection, chromatographed on a heparin sepharose column, and enzyme activity was eluted with a 0.1 to 0.8 M Tris-acetate/potassium acetate gradient. The previously described restriction endonuclease R. CviAI (/GATC) eluted at about 0.4 M potassium acetate. Unexpectedly, a second prominent peak of endonuclease activity, named R. CviAII, eluted at about 0.25 M potassium acetate (Fig. 1A). R. CviAII cleaved bacteriophage T7 DNA into more than 40 fragments, whereas R. CviAI produced 7 well-defined large fragments. R. CviAII was further purified over phosphocellulose, hydroxylapatite, and blue sepharose columns; enzyme activity was eluted with chloride-free gradients.

Recognition sequence, cleavage site, and assay conditions of R. CviAII endonuclease

R. CviAII and the bacterial restriction endonuclease *Nla*III (CATG) cleaved pUC19, phage φX174, and SV40 DNAs (Fig. 1B) into identical size fragments after prolonged (18 hr) digestion. R. CviAII and *Nla*III did not cleave PBCV-1 DNA or bacteriophage T4 DNA, which contains glycosylated hydroxymethylcytosine in place of cytosine (14). However, R. CviAII, but not *Nla*III, cleaved phage Xp12 DNA, in which

all DNA cytosines are replaced by 5mC (15). The two endonucleases also differed in their cleavage site; R.CviAII cleaved between the C and A in CATG sequences (Fig. 1C), whereas *Nla*III cleaves after the G (16).

R.CviAII was most active in low ionic strength buffers and was inhibited by potassium or sodium ion concentrations above 50 mM. The temperature optimum was 23°C and very little R.CviAII activity was observed above 30°C. The pH optimum was 8.5 to 9.0 using glycylglycine, Tris, or N,N-Bis-(2-hydroxyethyl)2-hydroxypropane sulfonic acid buffers. R.CviAII activity was unstable; active enzyme could not be stored longer than two weeks on ice or 4–6 weeks in Tris-EDTA buffered 50% glycerol at –20°C.

Isolation and assay of M.CviAII methyltransferase

Virus PBCV-1 DNA was resistant to the 6mA sensitive restriction endonucleases, *Sph*I (GC^mATGC), *Bbu*I (GC^mATGC), *Bsp*HI (TC^mATGA) and *Nsp*I (PuC^mATGPy), but was cleaved by 6mA insensitive *Nco*I (CCATGG) (11). Therefore, PBCV-1 DNA has 6mA in CATG sequences, presumably reflecting adenine methylation by M.CviAII.

M.CviAII activity was detected in crude or partially purified enzyme preparations. M.CviAII co-eluted with M.CviAI activity on heparin sepharose and phosphocellulose columns. However, the two enzyme activities could be distinguished by using selective DNA substrates (see Materials and Methods). M.CviAII also incorporated ³H-SAM into *Mbo*I-digested phage Xp12 DNA indicating that M.CviAII adenine methylation is not affected by 5mC methylation. M.CviAII activity was unstable, with activity diminishing rapidly two to three days after phosphocellulose column chromatography. Like R.CviAII endonuclease, M.CviAII activity was sensitive to moderate salt concentrations (> 50 mM KCl or NaCl) and had a pH optimum of 8.5 to 9.0.

The extreme sensitivity of R.CviAII and M.CviAII to salt—especially chloride ions, their high pH optima, and their inherent

instability explain why R.CviAII and M.CviAII activities were not detected in earlier studies (5,6). However, M.CviAII obviously functions *in vivo* in PBCV-1 infected chlorella because viral DNA is resistant to cleavage by R.CviAII and related endonucleases (*Sph*I, *Bbu*I, *Bsp*HI, *Nsp*I and *Nla*III), all of which are sensitive to C^mATG adenine modification.

Cloning of the M.CviAII gene

A PBCV-1 DNA library, containing *Sau*3A partially digested DNA fragments, was incubated with *Sph*I prior to transforming *E. coli* DH5 α MCR. Forty-eight ampicillin resistant clones were obtained after transformation and these clones were tested individually for sensitivity to *Sph*I (GCATGC), *Nla*III (CATG), *Nsp*I (PuCATGPy) and R.CviAII (CATG) endonucleases. Plasmid DNA from one clone, containing a 2.1 kb DNA insert (named pJN1A), was resistant to these four restriction endonucleases, but was sensitive to several other restriction endonucleases, including *Hinf*I, *Sau*3A and *Taq*I (Fig. 2A). Furthermore, pJN1A DNA was resistant to *Sph*I, *Nla*III, and R.CviAII endonucleases when the inserted 2.1 kb fragment was cloned in the opposite orientation with respect to the cloning vector's lac promoter. From this sequence-specific methylation phenotype and the DNA sequence data, we conclude that (i) plasmid pJN1A contains the M.CviAII methyltransferase gene and (ii) M.CviAII transcription in *E. coli* uses a promoter within the inserted viral DNA.

DNA sequence of the M.CviAII methyltransferase gene

Sequencing the 2.1 kb insert DNA from pJN1A revealed an open reading frame (ORF) of 978 bases, which could code for a protein with 326 amino acids and a predicted molecular weight of 37,499 kDa. This ORF has two amino acid motifs which are characteristic of adenine methyltransferases (Fig. 4): (i) an N-terminal DLFaGsC sequence beginning at amino acid position 34 which is probably the SAM binding site (17) and (ii) a DPPY

Figure 2. (A) Sensitivity of *Sst*I linearized plasmid pJN1A DNA to several restriction endonucleases. pJN1A DNA was incubated with *Sst*I, *Sst*I/*Sph*I, *Sst*I/*Nla*III, *Sst*I/*Nsp*I, *Sst*I/R.CviAII, *Sst*I/*Hinf*I, *Sst*I/*Sau*3A, and *Sst*I/*Taq*I (lanes 1 to 8, respectively). (B) Restriction map of pJN1A containing the M.CviAII gene, pJN4A containing the M.CviAII and R.CviAII endonuclease genes, and placement of the genes on the PBCV-1 genome map. ITR refers to the inverted terminal repeat region. The *Clal*/*Acc*I fragment in the M.CviAII gene and the combined *Ssp*I fragments in the R.CviAII gene of pJN4A were used as probes in Fig. 3.

sequence beginning at amino acid position 208 that is probably the methyltransferase site (17).

The 50 bases preceding the ATG translation start site contain 82% adenines and thymidines. Previous studies have established that A+T rich regions precede functional chloroella virus genes (18). Two possible promoter sequences (TTCTAA₂₀TATAAT) and (TTTACA₁₆TTTATT) beginning 107 and 93 bp upstream, respectively, from the ATG start codon 1, resemble the -35 and -10 regions of the *E. coli* promoter consensus sequence (19).

The terminal adenine in the M.CviAII TAA translation stop codon coincides with the first base of a potential ATG start codon of an ORF that extends to the 3' end of the pJN1A insert DNA (Fig. 2B). In bacteria, DNA restriction endonuclease genes always accompany cognate DNA methyltransferase genes, although spacing and relative orientation of the two genes vary (20). Therefore, we suspected that this truncated ORF might encode the R.CviAII endonuclease gene.

Cloning and DNA sequence of the R.CviAII gene

The pJN1A *Clal/AccI* fragment (Fig. 2B) was hybridized to PBCV-1 DNA cleaved with *Bam*HI, *Hind*III, or *Pst*I to locate the M.CviAII gene on the PBCV-1 physical map (4) and to identify a larger DNA fragment containing the suspected R.CviAII endonuclease gene. The pJN1A *Clal/AccI* fragment hybridized to single *Bam*HI (B7c), *Hind*III (H18b), and *Pst*I (P3b) fragments. This places the single-copy M.CviAII gene between map positions 125 and 130 on the PBCV-1 physical map (Fig. 2B).

The 13 kb PBCV-1 *Bam*HI B7c fragment was cloned into pBluescript KS(-) (named pJN2A). Hybridization of radiolabelled pJN1A DNA to pJN2A DNA digested with several restriction enzymes, localized the M.CviAII gene, and the neighboring suspected R.CviAII gene, to a 2.6 kb *Hind*III/*Kpn*I fragment (Fig. 2B). This DNA fragment, which expressed M.CviAII activity, was cloned into pBluescript KS(-) (named pJN4A).

To determine if pJN4A contained and expressed the R.CviAII endonuclease gene, chloride-free enzyme extracts were prepared from *E. coli* DH5 α MCR cells containing pJN4A, and chromatographed on a heparin sepharose column using a 0.1 to 0.8 M potassium acetate gradient. Column fractions were assayed immediately for R.CviAII activity using either of two substrates: pUC19-dam⁺ DNA or a ³⁵S-radiolabelled DNA-primed synthesis product. Fractions eluting at about 0.25 M potassium acetate partially cleaved pUC19 DNA, but not C^mATG modified DNA from PBCV-1 or plasmid pJN1A, into fragments which were identical to those produced by low enzyme concentrations (1 to 2 units for 2 hr) of *Nla*III endonuclease. These same enzyme fractions also specifically cleaved a DNA-primed synthesis product between the C and A at CATG sequences, as detected on a denaturing DNA sequencing gel. Like the R.CviAII enzyme from PBCV-1 infected chloroella, the cloned R.CviAII enzyme was unstable and activity was detected only if enzyme extracts were assayed shortly after cell disruption. We conclude that plasmid pJN4A contains the R.CviAII endonuclease and M.CviAII methyltransferase genes, and that both genes are expressed in *E. coli*.

Sequencing the remainder of the pJN4A insert DNA identified an ORF that could code for a protein of 342 amino acids (predicted molecular weight 39,923 kDa) if the ATG codon that overlaps the M.CviAII TAA stop codon is the authentic

Figure 3. Hybridization of the *Clal/AccI* fragment of the M.CviAII gene and a ligated 0.73 kb *SspI* fragment of the R.CviAII gene to DNA isolated from 37 chloroella viruses. The percentage of 5mC (as total cytosine) and 6mA (as total adenine) in the DNA of each virus (4) and sensitivity of the DNAs to R.CviAII are also indicated. The blots contain 1, 0.5, 0.25, and 0.12 μ g of DNA, left to right, respectively.

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M.Chaill MNRIGYIGSKLKLKDWIFEEISKRTDDTYTK*FADLFAGSCIMTHEALE 48
      :.|||||||:|:|. :|||. :..... |.|||||..|:.. .
M.Nail ***MNYIGSKLKLNSWLETEISNVAGHSLSDKVFCDLFAGTGIVGRKFKT 47

KKYECISNDLETYPYIMVNLG*KCPFSDKLQNIETLDDDLDTKDMVIPG 96
      :.:||:|:|. |||:|. . . . . :|:|:|:|:|:|:|:|:|:|
NVKQVIANDMEYYSVYLNRYIGNCQSIKAGELLQRLQLPPREGLI** 95

FVTLTYSRPNRMFYFTEDIAMRIDIIRENIERMKER*VSTDEYNFLLAS 144
      :. .... :|| ||:|:|. :|:| :| :|| :| :| :|||
YQHYCLGSGSERQYFSDENGKKIDAVRIQIEEWKNTRYIEDEDTYFLLAT 145

LLTSADSVKNTSVVYGYAYLKKFKKTALKRMVFAPLHTRSTTVTLETFNED 194
      ||:|:|. ||. |||:|:|:|:|:|:|:|. :| . . . :|:|.
LLEGADKVANTASVYGAFLKNLKKSAKLPLSLEPALFEIGSDGHQVYQAD 195

ATEL*EIKTDIAYDPFPNYSRQYGANYFVLNLI*LTPKEIGNVT 237
      |:| :|:|. |:|||:|:|:|:|:|:|:|:|:|:|:|:| :|| :|
ANQLIKNISGDILYDPFPNARQYGANYHLLNSIALYDDFTPK***GKT 241

GLPEYKXSSFCRKQEVAMSFHKMLKNVSAARLFVISYSSSELKSGDMVAL 287
      ||:|:|:|:|:|. | :|. :|:|:|:|:|:|:|:|:|:|:|:| :|:|.
GLREYSRSKYCSKSDVVPVFEALIRDADFYIFLSYNNEGLMSVGVQVREI 291

LSQYGKCEVVVRNHKRFKAQISAVGNDVEE*YLFFVYIEQ* 326
      :.:|:|:|:|:|. :|:|:|:|:|. . . . :|:|. :|:|.
FERFGKYDVLQTEYRRFKADKTENRNHNKANSTEYHLHILEKTF* 334

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Figure 4. Maximum alignment between the amino acid sequences for M.CviAII and M.NlaIII using the NBRF computer program ALIGN (27).

translational start site. Alternatively, if the next downstream ATG codon is the translational start codon, then a protein of 250 amino acids would result. Either of these potential ATG start codons could encode a protein in the size range of most type II bacterial restriction endonucleases which range in size from 157 to 576 amino acids (20).

Identification of the R.CviAII translational start site

Two observations indicate that the ATG codon which overlaps the M.CviAII stop codon is the translational start site for R.CviAII. (i) Repeated attempts to clone the *EcoRV/KpnI* fragment from pJN4A (Fig. 2B) were unsuccessful; presumably this fragment expresses the lethal R.CviAII gene in the absence of its cognate DNA methyltransferase. In contrast, exonuclease III produced truncated DNA fragments with 5' termini between the two potential ATG start codons, were easily cloned in *E. coli* DH5 α MCR. One truncated fragment beginning at base 1143 was sequenced, ruling out any other base alterations in the ORF. (ii) The larger construction contains a sequence (TTGAA-Gx₁₇TATATT) that begins 37 bases from the ATG start codon and resembles the consensus -35 and -10 regions of *E. coli* promoter sequences (19).

The chlorella virus restriction/modification system resembles bacterial restriction/modification systems in two ways: (i) the M.CviAII methyltransferase and R.CviAII endonuclease genes are tightly linked and, in fact, overlap by one base. (ii) There is no obvious similarity between the restriction endonuclease gene and the methyltransferase gene.

Occurrence of the M.CviAII and R.CviAII genes in other *Chlorella* viruses

We have partially characterized 36 other viruses which infect the same host, *Chlorella* NC64A, and placed these viruses into 16 classes (4). PBCV-1 is one of two members, placed in class 3, whose DNA contains 1.7 to 1.9% 5mC and 1.5 to 1.6% 6mA. To determine whether other *chlorella* virus genomes contain

In summary, the previously undetected M.CviAII 6mA modification is widespread among the chlorella viruses. Many, but not all of these viruses, also contain the R.CviAII endonuclease gene.

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Comparison of M.CviAII/R.CviAII to bacterial restriction/modification amino acid sequences

The predicted amino acid sequence of M.CviAII was compared to 19 adenine methyltransferases available in GenBank using the GAP program (21). M.CviAII has the greatest homology to its bacterial isomethylomer M.NlaIII from *Neisseria lactamica* (22), sharing 58% amino acid similarity and 36% amino acid identity (Fig. 4). M.CviAII also has 55% amino acid similarity and 36% amino acid identity to M.FokI which has two asymmetric adenine methylation sites, GG^mATG and C^mATCC (23,24,25) that resemble the M.CviAII (C^mATG) modification site.

The predicted amino acid sequence of R.CviAII endonuclease did not match any amino acid sequences in GenBank, including those of over 20 bacterial restriction endonucleases. However, as will be reported in a separate paper, R.CviAII has some sequence similarity to its heteroschizomer R.NlaIII (R. Morgan, S. Xu, R. Camp, and G. Wilson, manuscript in preparation). R.CviAII is unusual in that it contains 55 lysines (16% of the total amino acids) and has a predicted isoelectric point of 9.65.

CONCLUSIONS

We have previously reported that the prototype chloroella virus PBCV-1 codes for the restriction endonuclease R.CviAI (/GATC) (5), its cognate adenine DNA methyltransferase M.CviAI (G^mATC) (6), a cytosine DNA methyltransferase M.CviAIII of unknown specificity (26), and a cytosine DNA methyltransferase pseudogene (26). We have shown here that PBCV-1 also encodes another restriction/modification system, named CviAII. R.CviAII endonuclease (C/ATG) is a heteroschizomer of *Nla*III (16). Altogether, including the pseudogene, virus PBCV-1 encodes at least six genes (2 to 3% of the 333 kbp genome) involved in DNA restriction and modification.

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