A single amino acid change restores DNA cytosine methyltransferase activity in a cloned *Chlorella* virus pseudogene

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ABSTRACT

The *Chlorella* virus PBCV-1 contains an open reading frame, named P17-ORF4, which differs by eight amino acids from a DNA cytosine methyltransferase, M.CviJI, encoded by a different *Chlorella* virus IL-3A. Whereas IL-3A expresses M.CviJI, which methylates the central cytosine in (A/G)GC(T/C/G) sequences, P17-ORF4 is non-functional. Gene fusions between P17-ORF4 and M.CviJI and site-directed point mutations revealed that changing Gln¹⁸⁸ to Lys¹⁸⁸ abolishes M.CviJI methyltransferase activity. Conversely, changing Lys¹⁸⁸ in P17-ORF4 to Gln¹⁸⁸ results in M.CviJI activity. The other altered seven amino acids do not appear to affect M.CviJI activity.

INTRODUCTION

We have isolated and partially characterized 37 large (150 to 190 nm in diameter), polyhedral, dsDNA-containing (>300 kbp), plaque-forming viruses that replicate in a unicellular, eukaryotic exsymbiotic green alga, *Chlorella* strain NC64A (1,2,3). Although the viruses are morphologically similar and have a common host, they can be placed into 16 classes on the basis of plaque size, reaction with antibody, and the nature and abundance of methylated bases in their genomic DNAs (4). Each viral DNA contains 5-methylcytosine (5mC), which varies from 0.1% to 47.5% of the total cytosine. In addition, 25 of the 37 viral DNAs contain N⁶-methyladenine (6mA), which varies from 1.5% to 37% of the total adenine. This widespread DNA modification led to the discovery of virus encoded DNA methyltransferases and DNA site-specific (restriction) endonucleases (see review, 4). Four of the viral-encoded DNA methyltransferase genes have been isolated and sequenced, including the DNA cytosine methyltransferase M.CviJI from virus IL-3A (5). M.CviJI, which is one of at least two cytosine methyltransferases encoded by IL-3A (6), methylates the central cytosine in (G/A)GC(T/C/G) sequences; consequently IL-3A DNA contains a relatively high level of 5mC (9.7%) (7).

As expected, the cloned M.CviJI gene from IL-3A virus hybridized to DNA from 18 other *Chlorella* viruses whose DNAs contain high levels of 5mC and are resistant to the cognate

restriction endonuclease CviJI. Surprisingly, however, the cloned M.CviJI gene also hybridized at high stringency to DNA from the prototype *Chlorella* virus PBCV-1 [1.9% 5mC (7)], which lacks a functional M.CviJI gene (5) and whose DNA is sensitive to CviJI endonuclease. As described in this report, the M.CviJI-like region from PBCV-1 has a nonfunctional open reading frame (ORF), P17-ORF4, which differs by eight amino acids from functional M.CviJI. Gene fusions between P17-ORF4 and M.CviJI and reciprocal site-directed point mutations established that changing Gln¹⁸⁸ to Lys¹⁸⁸ abolishes M.CviJI activity. The other seven amino acid changes did not appear to affect M.CviJI methyltransferase activity.

MATERIALS AND METHODS

Cultural conditions and isolation of virus DNAs

Growth of the host alga, *Chlorella* strain NC64A, on MBBM medium, production and purification of viruses PBCV-1 and IL-3A (1,8), and isolation of virus DNAs (9) have been described. The terminal PBCV-1 restriction fragments were obtained by digesting viral DNA with restriction endonucleases and eluting appropriate fragments from agarose gels (10). Plasmid pIL-3A.22.8 containing the M.CviJI gene from virus IL-3A has been described (5).

Cloning and DNA sequencing

E. coli Sure strain (Stratagene) grown on 2×YT medium containing 100 µg/ml ampicillin (11) served as the host for pBluescript KS(+/-) (Stratagene) and recombinant plasmids containing virus DNA inserts. Nested deletions of the insert DNA were made with the 'Erase-a-Base' exonuclease III kit from Promega and the DNA was sequenced (12) using a Sequenase kit from United States Biochemicals Corp.

Gene fusions and site-directed mutagenesis

Gene fusions were constructed between functional M.CviJI and P17-ORF4 by ligating agarose separated DNA restriction fragments. Site-directed point mutations were constructed using mutant synthetic oligonucleotides and an *in vitro* mutagenesis kit from United States Biochemicals, following manufacturer's

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instructions, except that the bacterial host was *E. coli* Sure strain. Plasmids containing site-directed point mutations were verified by sequencing appropriate regions of plasmid insert DNA. Plasmid DNAs resulting from gene fusions and site-directed point mutations were treated with 5mC methylation-sensitive restriction endonucleases, *Hae*III, *Alu*I, and *Sst*I, to detect expression of M.CviJI methyltransferase activity.

Purification of PBCV-1 cytosine methyltransferase

Enzyme extracts were prepared and assayed from PBCV-1 infected cells by procedures similar to those used for isolating the two PBCV-1 encoded adenine methyltransferases M.CviAI [methylates G^mATC; (13)] and M.CviAII [methylates C^mATG; (14)]. Cells were disrupted in [20 mM Tris acetate, 1 mM EDTA, 0.5 M potassium acetate, 10 mM 2-mercaptoethanol, 20 µg/ml phenylmethylsulfonyl fluoride (pH 8.0)] with glass beads in a Bronwill MSK homogenizer with CO₂ cooling, and the homogenate was centrifuged at 10,000×g for 20 min. The supernatant was treated with 7% (w/v) polyethylene glycol (PEG) 8000 (Sigma) for 4 hr and centrifuged at 10,000×g for 2 hr at 4°C. PEG clarified supernatant was diluted to 0.17 M potassium acetate with 2 volumes of [20 mM Tris, 1 mM EDTA (pH 8.0)] buffer, applied in succession to the following columns, and eluted as indicated: phosphocellulose, 0.1 to 1.0 M potassium acetate (pH 7.0); heparin Sepharose, 0.1 to 0.8 M potassium acetate (pH 7.5); hydroxylapatite, 0.1 to 0.5 M KPO₄ (pH 7.0); and Q Sepharose, 0.1 to 0.5 M potassium acetate (pH 8.2). Chloride-free buffers were employed throughout extraction and purification steps because many chloroella virus restriction/modification enzymes are inhibited by chloride ion.

The two adenine methyltransferases, M.CviAI (G^mATC) and M.CviAII (C^mATG), co-purified with the cytosine methyltransferase, named M.CviAIII, through phosphocellulose and heparin sepharose columns. However, M.CviAIII separated from the two adenine methyltransferases on hydroxylapatite and Q sepharose columns. M.CviAIII was judged to be free of M.CviAI and M.CviAII activities because: (i) M.CviAIII did not incorporate S-adenosyl[methyl-³H] methionine (SAM) into polymerized oligonucleotides (pCGGATCCG)_n and (pGGCATGCC)_n which are substrates for M.CviAI and M.CviAII, respectively, (ii) DNA methylated *in vitro* by M.CviAIII was digested by *Mbo*I (does not cleave G^mATC) and *Sph*I (does not cleave G^mATGC), and (iii) M.CviAIII did not incorporate ³H-SAM into 5mC containing phage XP12 DNA (15) or 5-hydroxymethylcytosine containing T4 DNA (16), whereas M.CviAI and M.CviAII incorporated ³H-SAM into both of these modified DNA substrates.

Other procedures

DNA was electrophoresed on agarose gels in TPE buffer [80 mM Tris-phosphate, 8 mM EDTA, (pH 8.0)] (17), stained with 0.5 µg/ml ethidium bromide, and visualized by mid-range ultraviolet illumination. DNA was transferred to nitrocellulose filters by standard protocols (17). Total RNA was isolated from PBCV-1 infected cells and analyzed as described previously (18). Radioactive DNA probes were prepared by nick translation with a Bethesda Research Labs kit. Restriction endonucleases were obtained from New England Biolabs, Boehringer Mannheim, or Bethesda Research Labs and were used according to manufacturer's recommendations, except for CviJI endonuclease which was isolated and assayed as described previously (19).

RESULTS

Hybridization of the M.CviJI gene to PBCV-1 DNA

A 770 base pair *Eco*RI/*Sst*I DNA fragment from plasmid pIL-3A.22.8 (Fig. 1B), containing part of the cytosine methyltransferase gene M.CviJI from virus IL-3A, hybridized strongly to single *Bam*HI (B10), *Pst*I (P17), and *Hind*III (H17) fragments from virus PBCV-1 (data not shown). The M.CviJI-like region is at the right terminus of the PBCV-1 physical map immediately upstream of the previously sequenced 2.2 kb inverted terminal repeat (Fig. 1A) (10). The P17 fragment (4.8 kb) was isolated from an agarose gel, digested with *Apa*I to create a cloning site, and cloned into pBluescript KS(−) using the *Pst*I site and the *Apa*I site in the inverted terminal repeat region of P17 (called pYZ-411). The remaining 2.6 kb of the P17 fragment was sequenced (Fig. 2). The region contains two ORFs: (i) a 1,101 base ORF (P17-ORF4) begins at base 1,400 and ends at base 2,500, (ii) a 486 base ORF (P17-ORF3) begins at base 780 and ends at base 1,265. Interestingly, the DNA sequence from bases 388 to 2,629, which includes P17-ORF3 and P17-ORF4, is nearly identical to the M.CviJI region of virus IL-3A (5) (differences are indicated in Fig. 2). The predicted amino acid sequence of P17-ORF4 differs from authentic M.CviJI by only eight amino acids (boxed in Fig. 2). The predicted amino acid sequence of P17-ORF3 differs from a small ORF 134 bases upstream of the M.CviJI gene in IL-3A by two amino acids (boxed in Fig. 2). Outside the two homologous ORFs, DNA sequences of PBCV-1/P17 and pIL-3A.22.8 diverge completely (Fig. 2).

P17-ORF4 does not encode a PBCV-1 DNA cytosine methyltransferase

The similarity between P17-ORF4 and M.CviJI suggested that P17-ORF4 might encode a cytosine DNA methyltransferase. Several possibilities existed: (i) P17-ORF4 might encode the M.CviJI DNA modification, (A/G)G^mC(T/C/G), (ii) P17-ORF4 might code for a cytosine methyltransferase with an altered sequence specificity, or (iii) P17-ORF4 might be a non-functional gene. We investigated these three alternatives.

The first alternative was eliminated because both plasmid pYZ-411 (contains P17-ORF4) and virus PBCV-1 DNAs were digested by CviJI, *Sst*I, *Hae*III, *Alu*I, and *Hind*III restriction endonucleases, all of which are inhibited by M.CviJI (A/G)G^mC(T/C/G) cytosine methylation (20). The inability of plasmid pYZ-411 to express a gene with M.CviJI modification activity in *E. coli* does not result from a defective promoter because the active M.CviJI gene is expressed with the upstream region of P17-ORF4 (see section on gene fusions).

Three sets of experiments were conducted to identify the sequence(s) containing 5mC in virus PBCV-1 DNA (1.9% 5mC) and to determine if a P17-ORF4 gene product was responsible for this methylation. First, PBCV-1 and plasmid pYZ-411 DNAs were treated with more than 60 restriction endonucleases, known to be inhibited by cytosine methylation (20); however, both DNAs were cleaved by all enzymes tested. Second, a DNA cytosine methyltransferase activity, named M.CviAIII, was isolated from PBCV-1 infected cells. M.CviAIII incorporated ³H-SAM into G+C rich DNAs including Adenovirus-2, phage N3, and the polylinker (pGCGGCCGC)_n, but poorly into phage T7 and lambda DNAs. M.CviAIII also methylated virus IL-3A DNA and plasmid DNAs containing P17-ORF4 and M.CviJI but not virus PBCV-1 DNA. M.CviAIII activity was unstable when

stored at -20°C in Tris-EDTA buffered 50% glycerol; over 75% of its activity was lost in 2–3 weeks. Although we have not determined the sequence specificity of M.CviAIII, it is a short G+C rich sequence that differs from the M.CviJI specificity.

Third, to determine if P17-ORF4 and P17-ORF3 were transcribed, total RNA was isolated from *Chlorella* NC64A cells at 0, 15, 30, 60, 90, 120, 240, and 360 min after PBCV-1 infection and hybridized with plasmid clones containing P17-ORF4 or P17-ORF3. As a control, viral RNAs were also hybridized to a plasmid containing the PBCV-1 B7b DNA fragment (plasmid pLG125) which contains both early and late PBCV-1 transcripts (18). No P17-ORF4 or P17-ORF3 transcripts were detected even after extended (10 days) film exposure. In contrast, early and late transcripts hybridizing to pLG125 were clearly visible after a one day film exposure. Thus neither P17-ORF4 nor P17-ORF3 appear to be transcribed.

These experiments establish that: (i) M.CviAIII is responsible for at least part of the PBCV-1 DNA cytosine methylation, although its exact sequence specificity is unknown, (ii) P17-ORF4

does not code for M.CviAIII, and (iii) P17-ORF4 is not transcribed in PBCV-1 infected cells. Therefore, P17-ORF4 appears to be a cytosine DNA methyltransferase pseudogene.

Gene fusions between a functional and non-functional methyltransferase

Since the predicted gene products of M.CviJI and P17-ORF4 differ by only eight amino acids, we determined which of these alterations affected M.CviJI methyltransferase activity. Smaller recombinant plasmids containing M.CviJI and P17-ORF4 were constructed for these experiments. A 1,669 base *Dra*I fragment from pIL-3A.22.8 (Fig. 1B) was subcloned into pBluescript KS(–) digested with *Eco*RV. This plasmid, named pYZ-710, contained an insert beginning 115 bases upstream from the M.CviJI start codon and ending 453 bases downstream from the M.CviJI stop codon. pYZ-710 expressed sufficient M.CviJI methyltransferase activity in *E. coli* to protect pYZ-710 DNA from digestion with *Sst*I, *Alu*I, and *Hae*III (Fig. 4A, lanes 1–3). A 1,366 base *Dra*I/*Pst*I fragment from pYZ-411 (Fig. 1A) was

Figure 1. (A) Restriction map of PBCV-1 fragment P17 and placement on the PBCV-1 physical map. The 2.2 kp inverted terminal repeat (ITR) region (shaded area) of P17 was sequenced previously (10). The region sequenced in this manuscript is indicated by the open area. ORFs, with the predicted number of amino acids, are indicated. (B) Map of pIL-3A.22.8 containing a functional M.CviJI gene (5).

Figure 3. Gene fusions between the functional M.CviJI methyltransferase (shaded areas) and non-functional P17-ORF4 (open areas) methyltransferase. The eight amino acid differences between M.CviJI and P17-ORF4 are indicated as well as the ten conserved amino acid motifs in cytosine methyltransferases (hatched areas). M.CviJI methyltransferase expression of the hybrid genes are indicated on the right side of the figure.

Figure 4. (A) Restriction endonuclease sensitivity of pYZ-710 DNA (contains the active M.CviJI gene) (lanes 1 to 3) and pYZ-710K DNA in which Gln¹⁸⁸ was changed to Lys¹⁸⁸ (lanes 4 to 6). (B) Restriction endonuclease sensitivity of pYZ-701 (contains P17-ORF4) DNA (lanes 1 to 3) and pYZ-701Q DNA in which Lys¹⁸⁸ was changed to Gln¹⁸⁸ (lanes 4–6). Lanes 1 and 4, 2 and 5, and 3 and 6 were treated with *XhoI/SstI*, *XhoI/AluI* and *XhoI/HaeIII*, respectively.

subcloned into pBluescript KS(–) digested with *EcoRV/PstI*. This plasmid, named pYZ-701, contained an insert beginning 115 bases upstream from the potential translational start codon of P17-ORF4 and ending 150 bases downstream from the potential stop codon. pYZ-701 did not express M.CviJI methyltransferase activity because plasmid DNA was sensitive to *SstI*, *AluI*, and *HaeIII* (Fig. 4B, lanes 1–3).

Gene fusions were constructed between pYZ-710 and pYZ-701, and hybrid plasmid DNAs were treated with *SstI*, *AluI*, or *HaeIII*. The results, summarized in Fig. 3, indicate that changing Lys¹⁸⁸ to Gln¹⁸⁸ abolishes M.CviJI methyltransferase activity. Substitution of the other seven amino acids, alone or in combinations, did not affect M.CviJI activity.

Reciprocal site-directed point mutations made at position 188 in pYZ-710 and pYZ-701 verified that a single amino acid substitution at position 188 could alter M.CviJI methyltransferase activity. pYZ-710 DNA, containing functional M.CviJI, was resistant to restriction endonucleases *SstI*, *AluI*, or *HaeIII* (Fig. 4A, lanes 1–3), whereas pYZ-710 DNA was sensitive to these same restriction endonucleases when the Gln¹⁸⁸ was changed to a Lys (named pYZ-710K) (Fig. 4A, lanes 4–6). Figure 4B shows the results of the reciprocal mutagenesis experiment. Plasmid pYZ-701 DNA containing P17-ORF4 was sensitive to *SstI*, *AluI*, and *HaeIII* (Fig. 4B, lanes 1–3), whereas the plasmid DNA was resistant to these three restriction endonucleases after changing the Lys¹⁸⁸ to Gln¹⁸⁸ (named pYZ-701Q) (Fig. 4B, lanes 4–6). Thus, a single amino acid substitution at position 188 completely alters M.CviJI methyltransferase activity.

DISCUSSION

We have previously shown that the prototype chlorella virus PBCV-1 codes for two adenine DNA methyltransferases, M.CviAI (G^mATC) (13) and M.CviAII (C^mATG) (14), and a cytosine methyltransferase M.CviAIII of unknown specificity (this report). Both of the adenine DNA methyltransferases have a cognate restriction endonuclease partner: CviAI cleaves /GAT-C sequences (21) and CviAII cleaves C/ATG sequences (14).

The present results indicate that virus PBCV-1 not only encodes functional DNA methyltransferase and DNA site-specific endonuclease genes, but it also contains a nonfunctional, non-transcribed cytosine DNA methyltransferase gene (P17-ORF4). P17-ORF4 differs by eight amino acids from a functional cytosine methyltransferase gene M.CviJI from another chlorella virus, IL-3A. Thus P17-ORF4 is a pseudogene.

Gene fusions between the functional and nonfunctional M.CviJI methyltransferase genes and site-directed point mutations established that a single amino acid change, Gln¹⁸⁸ to Lys¹⁸⁸, abolished M.CviJI methyltransferase activity. The reciprocal experiment, changing Lys¹⁸⁸ in P17-ORF4 to Gln¹⁸⁸, restored M.CviJI methyltransferase activity. As noted previously (5), the M.CviJI amino acid sequence shares ten blocks of highly conserved amino acids (22) with several bacterial cytosine methyltransferases (indicated in Fig. 3). Posfai *et al.* (22) identified twenty-one amino acids that were invariant in thirteen bacterial enzymes, of which seventeen residues are conserved in M.CviJI. Of the eight amino acids which differed between M.CviJI and the P17-ORF4 pseudogene, one occurs in an 'invariant' position: Glu¹⁶⁷ is changed to a Lys¹⁶⁷. However, this change had no obvious effect on M.CviJI methyltransferase activity. The amino acid change that abolishes M.CviJI activity occurs outside the ten conserved motifs.

Using the Robson-Garnier algorithm (23), the predicted secondary structure for M.CviJI suggests that Gln¹⁸⁸ is located in a proline-rich beta turn. We speculate that this beta turn is important in allowing correct protein folding such that the N-terminal SAM-binding domain [E⁷LFxGxAG¹⁴] and the catalytic site [P⁶¹EDLx₇PC⁷³] are aligned with the putative C-terminal target recognition domain [G²²²x₇TTLTxGx₁₁G²⁴⁷]. If so, changing Gln¹⁸⁸ to Lys¹⁸⁸ could hinder the flexibility of this turn, sterically hindering essential interactions between the interdomain catalytic site, the N-terminal SAM binding site, and the C-terminal target recognition domain.

A non-trivial possibility is that the Q¹⁸⁸K substitution results in an unstable protein that is preferentially degraded in *E. coli*. However, SDS-PAGE protein profiles from *E. coli* cells, containing either the vector alone or active or inactive M.CviJI genes, were identical (data not shown). Presumably, functional M.CviJI protein is present at concentrations in *E. coli* too low to be easily detected by SDS-PAGE.

The presence of a M.CviJI DNA methyltransferase pseudogene in virus PBCV-1 is not an isolated occurrence; the M.CviJI gene also hybridizes strongly to DNA from two other chlorella viruses, SC-1A and SC-1B, which do not express the M.CviJI modification (5). We suspect that the chlorella viruses also contain DNA site-specific endonuclease pseudogenes. Thus the chlorella virus systems are a unique and versatile source of DNA methyltransferase pseudogenes and possibly site-specific endonuclease pseudogenes. Comparing nonfunctional and functional gene sequences from different viruses provides a powerful tool for determining functionally important regions of these enzymes.

Pseudo domains have recently been reported in the multispecific cytosine DNA methyltransferases from certain *Bacillus* phages (24). DNA methyltransferase pseudogenes are apparently widespread in nature and may represent intermediates in the evolution of new restriction/modification systems.

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