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Viruses of Eukaryotic Green Algae
Final Technical Report
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James L. Van Etten
Department of Plant Pathology
University of Nebraska
Lincoln, NE 68583-0722

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Summary Statement

We have isolated and partially characterized many large, polyhedral, dsDNA (>330 kb) containing, plaque forming viruses which infect certain unicellular, eukaryotic, chlorella-like green algae. These viruses have several unique features, including the fact that they code for DNA site-specific (restriction) endonucleases and DNA methyltransferases (R/M systems). The primary objectives of this contract were to identify, clone, and characterize some of the virus-encoded DNA methyltransferases and DNA restriction endonucleases in order to understand their biological function. Considerable progress was made during the tenure of this contract: three DNA restriction endonucleases, which recognize or cleave at unique sites, were discovered and characterized, five new DNA methyltransferases were discovered and characterized, and three DNA methyltransferases were cloned and sequenced. We also made considerable effort at trying to understand the biological function of these methylating and cleavage enzymes. However, their biological function is still unknown.

We also made several unanticipated discoveries about these viruses:

- (i) The virus genome is a linear, nonpermuted molecule with covalently linked hairpin termini. The termini contain identical inverted repeats of at least 2,185 bases.
- (ii) The viruses typically encode three to eight different R/M systems and are among the most complex and densely packed source of R/M systems known.
- (iii) More restriction endonucleases can be detected in virus infected cells if the isolation and assay conditions are changed.
- (iv) Two viral-encoded endonucleases cleave only one strand of duplex DNA, i.e. they are base-specific nicking enzymes.
- (v) The DNA polymerase gene for two of the viruses contains a small intron that resembles eukaryotic nuclear-spliced mRNA introns.

Progress Report

Our understanding of the chlorella viruses improved greatly during the tenure of this contract. Specific findings that occurred as a result of this contract are described along with the publication(s) that describe the findings in more detail. Reprints of manuscripts not sent previously are enclosed.

1. New Chlorella viruses.

A group of viruses (called Pbi viruses) were found in European fresh water that specifically infect another exsymbiotic, eukaryotic chlorella-like green algae Chlorella strain Pbi. Preliminary characterization of these viruses indicate that they are related to the NC64A viruses (the ones originally discovered by us). Some of the characteristics of the Pbi viruses are described in the Reisser et al., 1991 (publication number 8). This finding, plus discoveries from both our laboratory and other laboratories, of NC64A viruses in fresh water from China, Japan, South America, and Australia indicate that chlorella viruses are present in fresh water throughout the world.

2. Genome of the chlorella viruses.

The 330 kb dsDNA genome of the prototype chlorella virus PBCV-1 is a linear, nonpermuted molecule and the termini contain identical inverted repeats of at least 2,185 bases, after which the sequence diverges (Strasser et al., 1991; number 7). The remainder of the genome, ca. 325 kb, consists of single copy DNA and so the virus has the potential to encode several hundred proteins. The ends of the PBCV-1 DNA are covalently closed hairpin (Rohozinski et al., 1989; numbers 1).

3. DNA polymerase gene.

A DNA polymerase gene was identified in the prototype chlorella virus PBCV-1 [contains 1.9% 5-methylcytosine (5mC) and 1.5% N⁶-methyladenine (6mA)] and the most heavily methylated virus NY-2A (contains 45% 5mC and 37% 6mA) using a degenerate oligonucleotide probe to a region conserved in all DNA polymerases. Sequencing the gene from

the two viruses revealed that both virus genes contained the expected conserved six motifs present in other DNA polymerases (Grabherr *et al.*, 1992; number 13). However, motifs IV, II, VI, III, and I were in one reading frame and motif V was in another reading frame for both viruses. This led to the discovery of a small intron (101 bases for PBCV-1 and 86 bases for NY-2A) at the same location in the gene in both viruses. Interestingly, both introns have a 5' splice site (AG/GUGAGY) characteristic of nuclear mRNA splicing. Nuclear precursor mRNAs are processed by a ribonucleoprotein particle called a spliceosome. This discovery suggests that either the chlorella viruses encode their own splicing machinery, or more likely, virus replication has a nuclear phase.

It is also interesting that the intron nucleotide sequences between the two DNA polymerase genes were more similar (83% nucleotide identity) than were the coding regions (72% nucleotide identity). Usually intron sequences diverge more rapidly than exon sequences. The conservation of the viral intron might imply that insertion of the intron into the DNA polymerase gene was a recent event or that the intron serves an important function in the virus life cycle.

The nuclear-type intron, which is the intron found in a chlorella virus gene, was unexpected because PBCV-1 does not require a functional nucleus for replication. Furthermore, neither poxviruses nor iridoviruses, which have some similarities to the chlorella viruses, have introns.

4. New DNA site-specific endonucleases.

When the proposal was written we had just discovered that chlorella virus PBCV-1 encoded a DNA methyltransferase (M.CviAI) and a type II restriction endonuclease (R.CviAI). During the tenure of the contract we have characterized two virus encoded restriction endonucleases which either recognize new sequences or which are isoschizomers of known bacterial enzymes, but cleave at different positions in the recognition sequence. XZ-6E infected cells contain both of these restriction endonucleases (manuscript in preparation): (i) R.CviRI is the first enzyme to recognize the sequence TGCA; the enzyme cleaves between the G and C, and is inhibited by 6mA. (ii) R.CviRII, like the bacterial isoschizomer RsaI, recognizes the sequence GTAC and is inhibited by 6mA. However,

unlike RsaI which cleaves between the T and A, R.CviRII cleaves between the G and T.

In addition to type II restriction endonucleases, some of the viruses code for base-specific endonucleases which cleave one strand of duplex DNA. Virus NYs-1 infected cells produce an endonuclease, NYs-1 endo, which recognizes the sequence 5'-CC-3' and cleaves 5' to the first C. NYs-1 endo cleaves 5'-C^mC-3' sequences but not 5'-mCC-3' sequences. Virus NY-2A also induces a base specific nicking enzyme, NY-2A endo, which recognizes the sequence RAG and cleaves between the R and A (manuscript in preparation).

5. Cloning and sequencing DNA methyltransferases and DNA restriction endonucleases.

One adenine [M.CviRI methylates TGC^mA sequences (Stefan *et al.*, 1991); number 9] and one cytosine [M.CviJI methylates RG^mCY sequences (Shields *et al.*, 1990; number 5)] chlorella virus DNA methyltransferases were cloned by expression in *E. coli*. The predicted amino acid sequences of these DNA methyltransferases were compared to each other and to bacterial DNA methyltransferase sequences using the NBRF computer program ALIGN. Incidentally, the adenine methyltransferase is the first adenine methyltransferases to be cloned from a eukaryotic organism.

Like bacterial DNA methyltransferases, the chlorella virus adenine DNA methyltransferase appears to be unrelated to the cytosine family. However, the chlorella virus and bacterial 5mC methyltransferases have similarities. Bacterial cytosine methyltransferases contain ten conserved amino acid motifs. The chlorella virus virus enzyme M.CviJI contains all ten motifs which are in the same order as the bacterial motifs (Shields *et al.*, 1990; number 5). However, M.CviJI and the bacterial enzymes differ in the spacing between motif VIII and motif IX. The bacterial enzymes contain a long variable region of 80 to 250 amino acids, the proposed target recognition domain, between these two motifs. In contrast, these two motifs are immediately adjacent to one another in M.CviJI. This arrangement implies that the M.CviJI target recognition domain must be located elsewhere in the protein, possibly in the 68 amino acid stretch between motifs IX and X or in the 102 amino acids following

motif X.

Chlorella virus and bacterial adenine DNA methyltransferases contain fewer conserved motifs. However, bacterial adenine methyltransferases and the chlorella virus adenine methyltransferase contains the motif (Asp/Asn)-Pro-Pro-(Tyr/Phe).

6. Function of the chlorella virus R/M systems.

The biological function of the chlorella virus encoded DNA site-specific endonucleases and DNA methyltransferases is unknown. Bacterial restriction endonucleases are believed to confer resistance to virus infection. In fact the name "restriction" refers to their role in excluding foreign DNA. Bacterial DNA methyltransferases serve to prevent self digestion of their DNA. We have considered two possible functions for the chlorella virus enzymes. (i) The site-specific endonucleases help degrade host DNA so that the resultant deoxynucleotides can be recycled into virus DNA. Methylation of nascent virus DNA by the cognate DNA methyltransferase protects it from "self-digestion". (ii) The site-specific endonucleases are involved in preventing infection of a cell by a second virus.

Several observations are consistent with the first hypothesis. (i) Host nuclear and chloroplast DNAs, but not virus DNA, are digested by the virus encoded site-specific endonuclease(s) in vitro. (ii) In vivo degradation of host nuclear and chloroplast DNA coincides with the appearance of site-specific endonuclease activity, and (iii) initiation of virus DNA synthesis in vivo coincides with the appearance of DNA methyltransferase activity.

This hypothesis was tested with IL-3A virus. Three independently derived deletion mutants of virus IL-3A were isolated. Each mutant lacked DNA methyltransferase (M.CviJI) and DNA site-specific endonuclease (R.CviJI) activity. If R.CviJI activity was essential for host DNA degradation, nuclear and/or chloroplast DNA should be preserved or at least degraded more slowly in cells infected with the mutants than in cells infected with wild type IL-3A. However, both nuclear and chloroplast DNAs decreased at nearly identical rates following infection

with each of the four viruses (Burbank *et al.*, 1990; number 6). Thus we conclude that R.CviJI activity is not essential for host DNA degradation. However, this does not exclude participation of the enzyme in the degradation process. It is possible that IL-3A infected cells contain a second DNA site-specific endonuclease which is responsible for degrading the host DNA. However, a search for such activity was unsuccessful.

To determine if the site-specific endonuclease(s) are involved in preventing infection of a cell by a second virus, chlorella cells were dually inoculated with different viruses and plaques arising from infective centers were distinguished by immunoblotting. At least 90% of the plaques resulting from single cells inoculated with two viruses contained only one of the viruses (Chase *et al.*, 1989; number 3). Thus the chlorella viruses, like bacteriophage, mutually exclude one another. If the virus-encoded endonucleases mediate exclusion one virus should dominate in certain combinations. For example, viruses PBCV-1 and NC-1A produce the site-specific endonucleases R.CviAI and R.CviBI, respectively. *In vitro*, R.CviBI digests PBCV-1 DNA but R.CviAI does not digest NC-1A DNA. Consequently, if the site-specific endonucleases mediate exclusion, NC-1A should dominate in a dual infection. That did not occur - about 40% of the infective centers were NC-1A and 60% were PBCV-1 (Chase *et al.*, 1989; number 3). It is always possible that PBCV-1 and NC-1A code for additional site-specific endonucleases which influence the results. However, we have been unable to detect additional site-specific endonucleases in these virus infected cells.

Therefore, the biological function of the DNA methyltransferases and DNA site-specific endonucleases remains unknown. It should be noted, however, that nothing is known about the natural history of these viruses and it is very likely that the DNA methyltransferases and DNA site-specific endonucleases serve an important role for the viruses in their native environment.

Publications resulting from the contract

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- *11. **Van Etten, J. L., L. C. Lane, and R. H. Meints.** 1991. Viruses and virus-like particles of eukaryotic algae. *Microbiological Reviews* **55**:586-620
- *12. **Nietfeldt, J. W., K. Lee, and J. L. Van Etten.** 1992. Chlorella virus PBCV-1 replication is not affected by cytoskeletal disruptors. *Intervirology* **33**:116-120..
- *13. **Grabherr, R., P. Strasser, and J. L. Van Etten.** 1992. The DNA polymerase gene from chlorella viruses PBCV-1 and NY-2A contains an intron with nuclear splicing sequence. *Virology* **188**:721-731.
- *14. **Nelson, M., Y. Zhang, and J. L. Van Etten.** 1993. DNA methyltransferases and DNA site-specific endonucleases encoded by chlorella viruses. In: DNA Methylation: Molecular Biology and Biological Significance. J. P. Jost and H. P. Saluz (eds). Birkhauser Verlag Publ., Basel. pp. 186-211.

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