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# Unusual Life Style of Giant Chlorella Viruses

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# Unusual Life Style of Giant *Chlorella* Viruses

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## Abstract

*Paramecium bursaria* chlorella virus (PBCV-1) is the prototype of a family of large, icosahedral, plaque-forming, dsDNA viruses that replicate in certain unicellular, eukaryotic chlorella-like green algae. Its 330-kb genome contains ~373 protein-encoding genes and 11 tRNA genes. The predicted gene products of ~50% of these genes resemble proteins of known function, including many that are unexpected for a virus, e.g., ornithine decarboxylase, hyaluronan synthase, GDP-D-mannose 4,6 dehydratase, and a potassium ion channel protein. In addition to their large genome size, the chlorella viruses have other features that distinguish them from most viruses. These features include: (a) The viruses encode multiple DNA methyltransferases and DNA site-specific endonucleases. (b) The viruses encode at least some, if not all, of the enzymes required to glycosylate their proteins. (c) PBCV-1 has at least three types of introns, a self-splicing intron in a transcription factor-like gene, a spliceosomal processed intron in its DNA polymerase gene, and a small intron in one of its tRNA genes. (d) Many chlorella virus-encoded proteins are either the smallest or among the smallest proteins of their class. (e) Accumulating evidence indicates that the chlorella viruses have a very long evolutionary history.

**Keywords:** chlorella viruses, PBCV-1, algal viruses, protein glycosylation, ion channel proteins

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## Introduction

Typically, one thinks of viruses as containing small genomes that encode only a few genes. However, several families of viruses have large dsDNA genomes that encode many proteins (Table 1). Examples include bacteriophage G that infects *Bacillus megaterium* and has a genome of ~670 kb (77), phycodnaviruses and putative phycodnaviruses that infect eukaryotic algae and have genomes up to 560 kb

**Table 1.** Representative large dsDNA viruses

Virus <sup>a</sup>	Virus family	Host	Genome size (bp)	Minimum no. of codons <sup>b</sup>	No. of protein-encoding genes	Reference
Phage G	<i>Myoviridae</i>	<i>Bacillus megaterium</i>	~670,000	—	—	(77)
PBCV-1	<i>Phycodnaviridae</i>	<i>Chlorella NC64A</i>	330,744	65	373	(105)
WSV	Unclassified	Shrimp	292,967	51	184	(215)
MsEPV	<i>Poxviridae</i>	Grasshopper	236,120	60	267	(1)
MCV	<i>Poxviridae</i>	Human	190,289	60	182	(173)
ASFV	<i>Asfarviridae</i>	Swine	170,101	60	151	(238)
Phage T4	<i>Myoviridae</i>	<i>E. coli</i>	168,903	29	289	(124)
HSV-2	<i>Herpesviridae</i>	Human	154,746	—	74 <sup>c</sup>	(37)
AcNPV	<i>Baculoviridae</i>	Insects	133,894	50	154	(4)
LCDV	<i>Iridoviridae</i>	Flounder	102,653	40	110	(198)

a G, Giant; PBCV-1, *Paramecium bursaria chlorella virus 1*; WSV, white spots virus; MsEPV, *Melanoplus sanguinipes* entomopoxvirus; MCV, *Molluscum contagiosum virus*; ASFV, African swine fever virus; HSV-2, Herpes simplex virus type 2; AcNPV, *Autographa californica* multinucleocapsid nuclear polyhedrosis virus; LCDV, lymphocystis disease virus.

b Minimum number of codons used by the authors to calculate an open reading frame (ORF).

c HSV-2 has 473 met-initiated ORFs of 50 codons or longer of which 74 are known to be functional genes.

(203, 207), and some insect poxviruses with genomes as large as 365 kb (96). Other large, dsDNA-containing viruses, such as white spot virus (WSV) (215), herpesviruses (37), African swine fever virus (ASFV) (238), coliphage T4 (124), baculoviruses (4), and iridoviruses (198), have genomes ranging from 100 to 235 kb. With the exception of bacteriophage G, the genome of at least one representative from each of these dsDNA-containing viruses has been sequenced. The number of putative protein-encoding genes in these viruses range from 74 for the 154-kb herpes simplex virus-2 (HSV-2) to ~373 for the 330-kb phycodnavirus *Paramecium bursaria* chlorella virus (PBCV-1).

To put the size of these viral genomes into perspective, the smallest bacterium, *Mycoplasma genitalium*, encodes 470 proteins and estimates of the minimum genome size required to support life are ~250 protein-encoding genes (78, 133). A recent comparative analysis of protein sequences encoded by poxviruses, ASFV, iridoviruses, and phycodnaviruses identified 9 genes that are shared by all of these viruses and 19 more genes that are present in at least three of these viral families (79). Cladistic analysis, using the genes shared by at least two viral families as evolutionary characters, led Iyer et al. (79) to propose that viruses in all four families arose from a common ancestor. However, there are significant differences among these four groups of viruses (207), and the common ancestor would have been ancient.

In addition to their large genomes, chlorella viruses have other distinctive features: (a) they encode multiple type II DNA methyltransferases and DNA site-specific (restriction) endonucleases (142, 143). (b) Unlike other glycoprotein-containing viruses, chlorella viruses encode most, if not all, of the components required to glycosylate their proteins (58, 219). (c) Chlorella viruses are the first viruses discovered with more than one type of intron. Three types of introns have been described in PBCV-1: a self-splicing intron in a transcription factor TFIIS-like gene (104, 236), a spliceosomal-processed intron in the DNA polymerase gene (57, 239), and a small intron in one of the tRNA genes (145). (d) Many chlorella virus-encoded proteins are either the smallest or among the smallest proteins of their class and some may represent the minimal catalytic unit. In addition, genes shared by chlorella virus isolates can differ in nucleotide sequence by as much as 40%; proteins can differ in amino acid sequence by 35%. Therefore, comparative gene sequence analyses can identify conserved amino acids in proteins, as well as regions that tolerate amino acid changes. The small sizes and the finding that many virus-encoded recombinant proteins are "laboratory friendly" have resulted in the biochemical and structural characterization of several PBCV-1 enzymes. Examples include: (a) The smallest known eukaryotic ATP-dependent DNA ligase (75), which is the subject of intensive mechanistic and structural studies (183 and references cited therein). (b) The smallest known type II DNA topoisomerase (99). The PBCV-1 enzyme cleaves dsDNAs ~30 times faster than the human type II DNA topoisomerase (46, 47); consequently, the virus enzyme is a model to study topoisomerase II DNA cleavage. (c) An RNA capping guanylyltransferase (74) that was the first enzyme of its type to have its crystal structure resolved (62, 63). (d) The smallest known protein (94 amino acids) to form a func-

tional K<sup>+</sup> channel (153). (e) The smallest enzyme (120 amino acids) known to post-translationally modify histone proteins by methylating histone H3 at Lys<sup>27</sup> (115).

The history and ecological aspects of algal viruses can be found in other reviews (35, 101, 132, 178, 190, 207–209, 224). This review focuses mainly on the properties of the chlorella viruses, primarily PBCV-1. More information, including a complete list of chlorella virus publications and additional images of the viruses, is available on the “World of Chlorella Viruses” web page at <http://www.ianr.unl.edu/plantpath/facilities/Virology>.

## Classification of Chlorella Viruses and Their Hosts

It is difficult to credit the first person to mention chlorella viruses. A few papers in the Russian literature more than 40 years ago describe a lytic activity in cultures of *Chlorella pyrenoidosa* (see 208). However, the cultures were contaminated with bacteria and the viruses pictured have bacteriophage morphology. In 1978, Kawakami & Kawakami (86) described the appearance of large, lytic viruses in chlorella after the algae were released from the protozoan *Paramecium bursaria*, but no virus particles were detected in chlorella growing symbiotically inside the paramecium. Independently, lytic viruses were described in chlorella isolated from the green coelenterate *Hydra viridis* (122, 210) and also from *P. bursaria* (211). As in the 1978 report, viruses appeared only after the chlorella were separated from their hosts. Fortunately for the experimental biologist, the chlorella from *P. bursaria* can be grown free of the paramecium in culture, and ensuing experiments have revealed that these cultured algae are hosts for what have become known as the chlorella viruses. The one disadvantage to studying these viruses has been the inability to develop molecular procedures to manipulate the virus genome. To date all attempts to transform the host chlorella have been unsuccessful.

Chlorella viruses are large (1900 Å along the fivefold axes), icosahedral, plaque-forming, dsDNA viruses that infect certain unicellular, chlorella-like green algae. The chlorella viruses are included in the genus *Chlorovirus* that consists of three species (203). (a) Viruses that infect *Chlorella* isolate NC64A (NC64A viruses). (b) Viruses that infect *Chlorella Pbi* (Pbi viruses). (c) Viruses that infect symbiotic chlorella in the coelenterate *Hydra viridis*. This latter chlorella host has not been cultured free of the virus and consequently, the virus can be isolated only from chlorella cells freshly released from hydra.

Algae included in the genus *Chlorella* are small spherical or ellipsoidal, unicellular, nonmotile, asexually reproducing green algae and are among the most widely distributed and frequently encountered algae on earth (148, 177). Chlorella species have a rigid cell wall and typically have a single chloroplast, which sometimes contains a pyrenoid body. They have a simple developmental cycle and reproduce by mitotic division. Vegetative cells increase in size and, depending on the species and environmental conditions, divide into two, four, eight, or

more progeny, which are released by rupture or enzymatic digestion of the parental walls. Electrophoretic karyotyping of several chlorella isolates, including *Chlorella NC64A*, indicates that the algae have multiple chromosomes ranging in size from ~1 mb to ~6.5 mb, with a total genome size of ~40 mb (70, 116).

However, algae assigned to the genus *Chlorella* are more heterogeneous than their simple morphology suggests. Because of this diversity, properties discovered in one *Chlorella* species or isolate may not apply to another species. Two examples illustrate the degree of heterogeneity. (a) The G+C contents of the nuclear DNAs of *Chlorella* species range from 43% to 79% (67). However, most isolates assigned to the same species have similar G+C contents. The PBCV-1 host, *Chlorella NC64A*, has a G+C content of 67%. The genome of *Chlorella NC64A* also contains methylated nucleotides; 21% of the cytosines are 5-methylcytosine (m5C) and 0.6% of the adenines are N6-methyladenine (m6A) (212). (b) The cell wall polysaccharides of *Chlorella* species vary widely (108, 194, 235), even among isolates assigned to the same species (235). Unexpectedly, lipopolysaccharide (LPS)-like components were discovered recently in the cell walls of some chlorella isolates (167). This finding was a surprise because LPS is typically only found in Gram-negative bacteria.

Most chlorella species are naturally free-living. However, some live as hereditary endosymbionts (also referred to as zoochlorella) within freshwater and, to lesser extent, marine animals (160, 200). The only known hosts for the chlorella viruses are symbiotic chlorella, some of which can be cultured.

## Structure of Chlorella Viruses

Chlorella virus particles are complex (molecular weight  $\sim 1 \times 10^9$  Da) and the PBCV-1 virion contains ~50 different proteins (180), including several that bind DNA (231). The PBCV-1 54-kDa major capsid protein Vp54 is a glycoprotein and comprises ~40% of the virus protein. Vp54 consists of two eight-stranded, anti-parallel  $\beta$ -barrel, jelly-roll domains related by a pseudo-sixfold rotation (140). The major capsid proteins from many viruses have a similar jelly-roll structure including those from dsDNA bacterial (e.g., PRD1) and animal viruses (e.g., adenoviruses) (140, 166). This structural similarity belies a lack of amino acid identity in many of these capsid proteins.

Cryo-electron microscopy (cryoEM) and three-dimensional (3D) image reconstruction of PBCV-1 (26 Å resolution) indicate that the outer glycoprotein capsid is icosahedral and surrounds a lipid bilayer membrane (Figure 1A,B [page 197]) (237). The membrane, which contains phosphatidyl choline and phosphatidyl ethanolamine, is required for infectivity because the virus loses infectivity rapidly in chloroform and more slowly in ethyl ether or toluene (180). The membrane is connected to the outer shell by regularly spaced proteins (Figure 1C). The outer diameter of the viral capsid varies from a minimum of 1650 Å along the two- and threefold axes to a maximum of 1900 Å along the fivefold axes. The capsid shell consists of 1680 donut-shaped trimeric capsomers plus 12 pentameric capsomers

at each icosahedral vertex. The trimeric capsomers are arranged into 20 triangular facets (trisymmetrons, each containing 66 trimers) and 12 pentagonal facets (pentasymmetrons, each containing 30 trimers and one pentamer at the icosahedral vertices) (Figure 1A). Assuming all the trimeric capsomers are identical (but see below), the outer capsid of the virus contains 5040 copies of the major capsid protein Vp54. The triangulation number (T) for the virus is 169 and the virus has a right-handed, skew class of T lattice (16).

Most of the trimeric capsomers have a central, concave depression surrounded by three protruding towers. The trimeric capsomers are 72 Å in diameter and ~75 Å high. The prominent, cylinder portion of each trimer extends ~50 Å above the surface of the capsid shell. The capsomers interconnect at their bases in a contiguous shell that is 20–25 Å thick. Within each trimer, the three monomers connect in the middle; thus each trimer has the appearance of the letter “H” when viewed in cross section (Figure 1D). This structure agrees with the Vp54 structural studies (140). Cross-sectional views of 3D reconstructions at 20 Å resolution reveal a “cement” protein(s) that connects the bases of the trimeric capsomers (arrow in Figure 1D) (X. Yan, V. Bowman, N. H. Olson, M. V. Graves, L. C. Lane, M. G. Rossmann, J. L. Van Etten & T. S. Baker, manuscript in preparation).

Twelve pentamer capsomers, each ~70 Å in diameter, exist at the fivefold vertices of the virus and probably consist of a different protein. Each pentamer is surrounded by radially distributed trimers, and the axis of each of these trimers tilts away from the pentamer. Each pentamer has a cone-shaped, axial channel at its base (Figure 1E). One or more proteins appear below the axial channel and outside the inner membrane (Figure 1E). This protein(s) may digest the host cell wall during infection. Presumably, contact between the virus and its host receptor alters the channel sufficiently to release the cell wall-degrading enzyme(s).

Stereo views indicate that PBCV-1 attaches to the host cell wall by hair-like fibers that originate from the virus particle (208). Similar views of isolated virus particles suggest that the fibers are flexible with terminal swollen structures. Although 3D reconstructions of PBCV-1 at 26 Å resolution suggested that all the capsomers in the virion trisymmetrons were identical (237), more refined PBCV-1 3D reconstructions reveal additional surface features (Figure 1F). Three regularly spaced trimeric capsomers in each PBCV-1 trisymmetron have an oval-shaped structure that resembles the top of a mushroom (Figure 1D,F). We suspect that these structures and the hair-like fibers mentioned above are related. Our interpretation of these results is that the hair-like fibers exist as coiled “springs” in the virion and that the fibers extend when they contact the host.

## PBCV-1 Genome

The PBCV-1 genome consists of a linear 330,744-bp, nonpermuted dsDNA molecule with covalently closed hairpin termini (154, 165). The termini consist of 35-nucleotide-long, incompletely base-paired, covalently closed hairpin loops that exist in one of two forms; the two forms are complementary when the 35-nucleo-

tide sequences are inverted (flip-flop) (243). Identical 2221-bp inverted repeats are adjacent to each hairpin end (184). The remainder of the PBCV-1 genome contains primarily single-copy DNA (54). PBCV-1 and all other NC64A virus genomes are ~40% G + C. Yamada & Higashiyama (232) suggested that the inverted repeat region of another chlorella virus, CVK1, contains a site-specific nick. If so, this nick could serve as an initiation point for DNA replication.

The PBCV-1 genome contains methylated nucleotides. For example, 1.9% of the cytosines are m5C and 1.5% of the adenines are m6A (212). In fact, the genomes of all the chlorella viruses have methylated bases and the viruses can be distinguished from one another by the site-specificity and amount of DNA methylation (142, 143, 208).

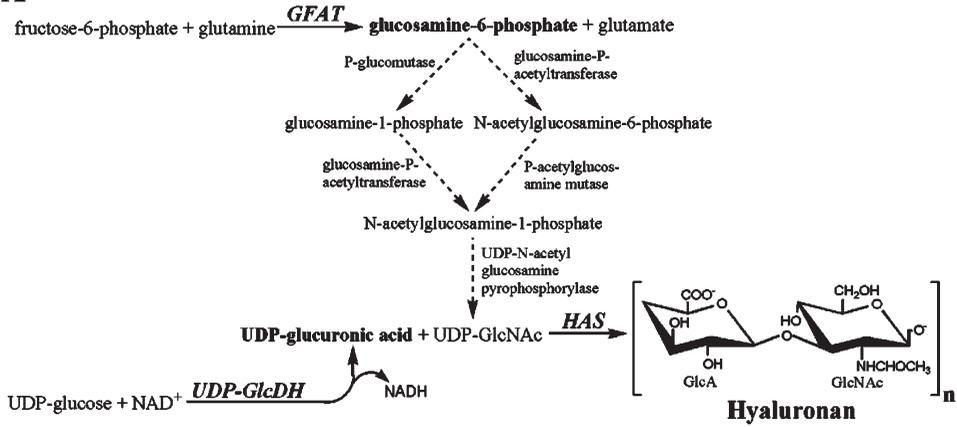
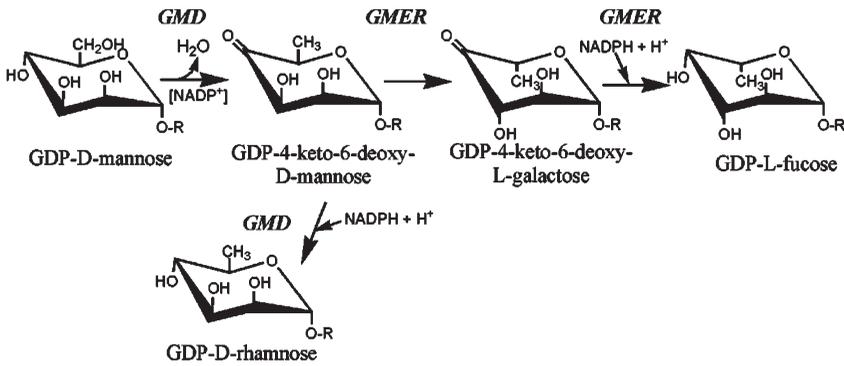
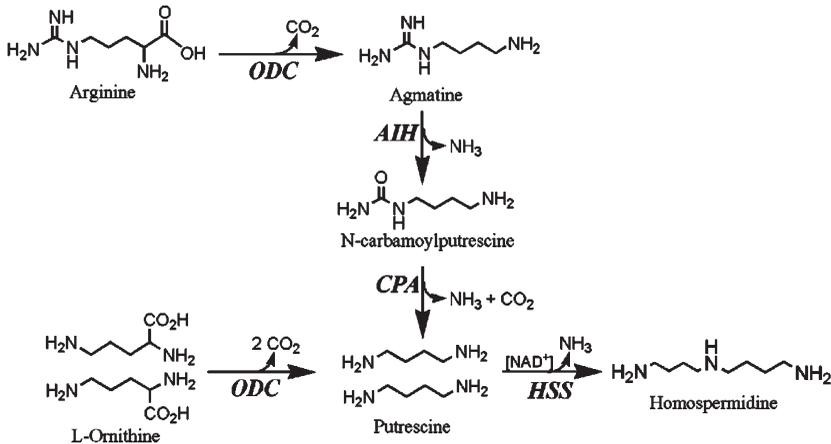
The sequence of the PBCV-1 genome revealed 697 ORFs of 65 codons or larger. About 373 of these ORFs probably encode proteins (92, 104, 105, 110, 111). Initially, PBCV-1 protein-encoding genes were identified by the following criteria: (a) A minimal size of 65 codons initiated by an ATG codon. (b) The largest ORF was chosen when competing ORFs overlapped. (c) ORFs with AT-rich (>70%) sequences in the 50 nucleotides upstream of the putative initiation codons. To date, all protein-encoding genes have met these criteria.

Unlike the poxviruses, in which genes near the terminal regions are transcribed toward the termini (130), the 373 putative protein-encoding genes are evenly distributed on both strands and, with one exception, intergenic space is minimal. In fact, 275 ORFs are separated by less than 100 nucleotides. The exception is a 1788-nucleotide sequence near the middle of the genome. This DNA region, which contains many stop codons in all reading frames, encodes 11 tRNA genes. In addition, 900 nucleotides in this region have two properties characteristic of CpG islands (2). (a) The region has greater than 60% G + C, whereas the flanking regions contain less than 40% G + C. (b) The observed-to-expected ratio of CpG dinucleotides is 1.45 (50, 98). The 2.2-kb inverted terminal repeat region of the PBCV-1 genome contains 4 ORFs; these 4 ORFs are duplicated (111, 184).

Approximately 50% of the 373 PBCV-1 gene products have been tentatively identified, including some that seem irrelevant to virus replication. Some PBCV-1 genes are closely related to genes of bacteria and their viruses, whereas other PBCV-1 genes appear eukaryotic in origin. Some PBCV-1-encoded enzymes can be assembled into metabolic pathways, e.g., hyaluronan synthesis (Figure 2A), fucose synthesis (Figure 2B), and polyamine synthesis (Figure 2C). Surprisingly, given the large number of PBCV-1 genes, the virus lacks a recognizable RNA polymerase gene. Therefore, PBCV-1 must depend on host enzymes for transcription.

## PBCV-1 Attachment and Infection

PBCV-1 infects its host *Chlorella NC64A* by attaching rapidly, possibly by the hairlike fibers mentioned above, to the algal cell wall (120). The virus then contacts the cell wall via one of its vertices, presumably producing a conformational change in the virus, which leads to the release of a precise amount of cell wall-

**A****B****C**

digesting enzyme(s). Following digestion of the wall, the internal membrane of PBCV-1 presumably fuses with the host membrane to translocate virus DNA and probably associated proteins (e.g., transcription factors?) to the inside of the host, leaving an empty capsid on the surface.

The virus also attaches to and digests cell-free wall fragments that have been boiled or extracted by harsh procedures, supporting the conclusion that the wall-degrading enzymes(s) are packaged in the virus particles (120, 163). Release of virus DNA requires a host function because virus attachment and digestion of isolated walls does not release DNA. Experiments to identify the virus receptor in *Chlorella NC64A* indicate that it is probably a carbohydrate (119).

Two observations suggest that the infecting PBCV-1 DNA and probably DNA-associated proteins quickly move to the nucleus and commandeer at least some of the host transcription machinery to initiate early viral RNA synthesis; early virus transcripts can be detected within 5–10 min post infection (p.i.). (a) PBCV-1 does not encode a recognizable RNA polymerase gene(s) nor were we able to detect RNA polymerase activity in isolated virions (J. Rohozinski & J. L. Van Etten, unpublished results). (b) A small intron with splice-site sequences characteristic of nuclear-spliced mRNAs exists in the PBCV-1 DNA polymerase gene (57). Presumably, this intron would need to be excised in the infected cell nucleus.

Ultrastructural studies indicate that an apparently intact nuclear membrane often can be seen in infected cells, at least during early stages of virus replication (121). However, virus replication does not require a functional host nucleus because PBCV-1 replicates, albeit slowly and with a small burst size, in UV-irradiated cells; such cells are unable to form colonies and endogenous RNA and DNA

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**Figure 2.** (page 160) Unexpected biosynthetic pathways encoded by chlorella virus PBCV-1. **A. Hyaluronan:** PBCV-1 contains genes that encode three enzymes involved in hyaluronan biosynthesis, glucosamine synthase (GFAT), UDP-glucose dehydrogenase (UDP-GlcDH), and hyaluronan synthase (HAS). **B. GDP-L-fucose and GDP-D-rhamnose:** The virus encodes GDP-D-mannose 4,6 dehydratase (GMD), which catalyzes both the dehydration of GDP-D-mannose to the intermediate GDP-4-keto-6-deoxy-D-mannose and the NADPH-dependent reduction of this latter compound to GDP-D-rhamnose. GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (GMER) catalyzes the final two steps to GDP-L-fucose. **C. Polyamines:** PBCV-1 encodes the enzymes for two pathways to make putrescine. Ornithine decarboxylase (ODC) converts ornithine directly to putrescine. However, the same ODC also decarboxylates arginine to agmatine (A. E. Pegg, personal communication). PBCV-1 also encodes the two enzymes [agmatine iminohydrolase (AIH) and N-carbamoylputrescine amidohydrolase (CPA)], that convert agmatine to putrescine (M. Piotrowski, personal communication). Finally, the virus encodes homospermidine synthase that converts two putrescines to homospermidine. The PBCV-1-encoded enzymes are in bold. Recombinant proteins have been produced for each of these enzymes and shown to be biochemically active.

syntheses are reduced to background levels (205). PBCV-1 replication also does not require labile host factors since the virus replicates in UV-inactivated cells incubated in the dark for up to 8 h before infection.

PBCV-1 DNA replication begins 60–90 min p.i., presumably in the nucleus, and is followed by transcription of late virus genes (170, 204). Progeny viruses are first released 4–5 h p.i. and the majority of infectious virus particles are released by 8 h p.i. via localized lysis of the cell wall. The virus has a burst size of 200 to 350 plaque-forming units (PFU); 25% to 50% of the released virions form plaques (206).

Mechanical disruption of the cells releases infectious virus 30 to 50 min prior to spontaneous lysis. Thus, infectious PBCV-1 is assembled inside the host and does not acquire its membrane and glycoprotein capsid by budding through an exterior host membrane. PBCV-1 also replicates in dark-grown chlorella cells or in light-grown cells treated with the photosynthetic inhibitor DCMU prior to virus infection (206). In both situations the time course of virus release is similar to that of untreated light-grown cells, which implies that PBCV-1 replication does not require host photosynthesis. However, the burst size is reduced ~50% in dark-grown cells. PBCV-1 infection rapidly inhibits host CO<sub>2</sub> fixation (172, 206). Like most bacterial viruses (64), PBCV-1 replicates most efficiently in actively growing host cells and poorly in stationary-phase cells.

The infection process of the chlorella viruses differs from that of all DNA viruses known to infect eukaryotes, but resembles that of bacterial viruses in that uncoating occurs at the cell surface. In terms of structure (e.g., an internal membrane and the major capsid protein), attachment, and penetration, the chlorella viruses resemble bacteriophages of the *Tectiviridae* family (5, 125). However, tectiviruses are much smaller (~650 Å in diameter) with a 40-kd genome.

### *Cell Wall-Degrading Enzymes*

PBCV-1 and another NC64A virus (CVK2) encode several enzymes that could aid in host cell wall degradation either during virus infection or virus release. These enzymes include two chitinases, a chitosanase, a  $\beta$ -1,3-glucanase, and another enzyme that partially degrades NC64A cell walls. Recombinant proteins produced from these five genes have the expected enzyme activities (71, 72, 185–188, 234). Enzymes packaged in the virion and involved in virus infection are expected to be expressed late in the infection cycle. One of the chitinases and the chitosanase meet this requirement and are packaged in the PBCV-1 virion. However, the  $\beta$ -1,3-glucanase is expressed early in infected cells and the protein disappears by 90 min p.i., i.e., before virus assembly and host cell lysis (188). Incubation of host cells with all five recombinant enzymes does not produce algal protoplasts, indicating that cell wall degradation requires at least one additional enzyme activity. In contrast, a crude enzyme extract made from virus lysates, called lysin, completely digests *Chlorella NC64A* cell walls (119; R. H. Meints, unpublished results).

The chitosanase gene, which is expressed late in infection, has been characterized from virus CVK2 (234). The CVK2 gene encodes two proteins with chi-

tosanase activity, a 37-kDa protein of the expected size from the DNA gene sequence and a 65-kDa protein. The larger protein is packaged in nascent virions, whereas the smaller protein occurs only in infected cells. Interestingly, PBCV-1 contains the same chitosanase gene flanked by two ORFs common to virus CVK2. However, CVK2 has an extra ORF inserted immediately downstream of its chitosanase gene, which contributes to the bigger 65-kDa protein, presumably as a read-through product.

The discovery of chitinase and chitosanase genes in the chlorella viruses was initially unexpected because chitin is rare in algae (69). Chitin is a normal component of fungal cell walls and the exoskeleton of insects and crustaceans (56). However, chitin or chitin-like material has been reported in cell walls of some chlorella species (195), including *Chlorella Pbi* (84, 85) and possibly *Chlorella NC64A*. Support for this possibility is that ~10% of the component monosaccharides in *Chlorella NC64A* cell walls is glucosamine (119). Glucose and rhamnose make up 51% and 16% of the total sugars, respectively, in *Chlorella NC64A* walls, with galactose, xylose, arabinose, and mannose each accounting for 5% to 10% of the sugars. Trace amounts of fucose have also been detected in *Chlorella NC64A* walls.

### **PBCV-1 DNA Replication, Recombination, Repair, and Methylation**

PBCV-1 encodes at least 10 putative proteins involved in either DNA replication or recombination. Presumably, these genes are transcribed in the nucleus, translated in the cytoplasm and then returned to the nucleus to initiate virus DNA synthesis, which begins at 60–90 min p.i. The PBCV-1 DNA polymerase enzyme (57) belongs to the DNA polymerase- $\delta$  family (217) and contains a proof-reading 3'-5' exonuclease domain (76). PBCV-1 also encodes two sliding clamp processivity factor proteins (PCNA). The two viral PCNA proteins resemble PCNAs from other organisms more than they resemble each other (26% amino acid identity), suggesting independent acquisition of the two genes rather than gene duplication. PCNA not only interacts with proteins involved in DNA replication, but proteins involved in DNA repair and post-replicative processing such as DNA methyltransferases and DNA transposases (220). Because PBCV-1 encodes proteins involved in DNA repair, DNA methylation, and a possible transposase, the two PCNAs may serve different functions in PBCV-1 replication.

A protein complex, called replication factor C (RFC) in Archae and eukaryotes, is responsible for the ATP-dependent loading of PCNA onto DNA (41, 131). RFCs from eukaryotes are heteropentamers. The clamp loading equivalent, called  $\gamma$ -complex, from bacteria also is a heteropentamer, whereas RFC complexes from Archae are heterodimers (88, 152). PBCV-1 encodes a single protein that resembles one of the two proteins that comprise Archae RFC (L. Fitzgerald, M. Griep, & J. L. Van Etten, unpublished results). It is unknown if the PBCV-1 protein can function by itself as an RFC protein or requires other host proteins for activity.

PBCV-1 also encodes other proteins involved in DNA replication including an ATP-dependent DNA ligase (75), an RNase H (J. L. Van Etten, unpublished results), and a type II DNA topoisomerase (99). The PBCV-1 topoisomerase II is smaller than other topoisomerase II enzymes, partially because it lacks a nuclear localization signal. The computer program PSORT, a cellular protein localization prediction program (139), predicts that the protein resides in the cytoplasm. If the topoisomerase II is in the cytoplasm and the host nucleus remains intact during virus replication, the enzyme may process viral DNA during packaging into nascent virions.

Thus PBCV-1 encodes genes for many of the essential elements of eukaryotic DNA replication, but does not have a full complement of DNA replication proteins and must partially rely on host enzymes. For example, PBCV-1 lacks a DNA primase encoding gene. Primase initiates synthesis of new DNA strands by synthesizing short RNA oligomers on ssDNA (3). However, PBCV-1 encodes a protein (ORF A456L) that displays amino acid sequence similarity to a special class of trifunctional primase-helicase-origin recognition proteins (M. Griep, personal communication). The helicase portion of the viral protein resembles the superfamily III helicases. Therefore, we predict that this viral protein participates in viral DNA replication.

PBCV-1 also encodes a 5'-3' exonuclease homolog (ORF A166R) that is probably involved in DNA recombination (18). Using PBCV-1 temperature-sensitive mutants, Tessman (197) established that genetic recombination occurs at a frequency of ~2%. Finally, PBCV-1 encodes a homolog of the bacteriophage T4 pyrimidine dimer-specific glycosylase (PDG), a well-characterized DNA repair enzyme involved in pyrimidine photodimer excision (49). Comparative studies with the T4 enzyme revealed that PBCV-1 PDG cleaves both *cis-syn* and *trans-syn-II* cyclobutane pyrimidine dimers, whereas the T4 enzyme only cleaves the *cis-syn* form (117). The PBCV-1 PDG also excises two other monomeric products induced by UV radiation or hydroxylradicals, 4,6-diamino-5-formamidopyrimidine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (81). The PBCV-1 PDG is more processive than the T4 enzyme and is the first *trans-syn-II*-specific glycosylase identified to date.

### *DNA Methyltransferases and Site-Specific Endonucleases*

DNA from each of 37 chlorella viruses contains m5C in amounts varying from 0.12% to 47.5% of the total cytosine. In addition, 24 of the 37 viral DNAs contain m6A in amounts varying from 1.5% to 37% of the total adenine (142, 212). At one end of the spectrum is a virus with 0.12% m5C and no m6A and at the other end, a virus with 45% m5C and 37% m6A. Because these viruses differ greatly in DNA methylation, they offer a unique opportunity to study the effect of methylated bases on common enzymes and proteins, such as transcription factors, that interact with DNA.

The methylated bases occur in specific DNA sequences; thus it is not surprising that the chlorella viruses encode multiple m5C and m6A DNA methyltransferases (MTases). However, we were surprised to discover that ~25% of the

virus-encoded DNA MTases have a companion DNA site-specific (restriction) endonuclease. Thus, virus-infected chlorella are the first nonprokaryotic source of type II DNA restriction endonucleases (for reviews see 142, 143).

Some virus-encoded endonucleases have recognition and cleavage site specificities identical to bacterial type II restriction endonucleases [e.g., R.CviAI (/GATC) (225)], others are heteroschizomers of bacterial endonucleases [e.g., R.CviAII (C/ATG) (240)], and still others have novel recognition sites [e.g., R.CviJI (RG/CY) (226)]. Statistically, R.CviJI recognizes a 3-bp sequence, and under certain reaction conditions, called R.CviJI\* ("star" conditions), the enzyme also cleaves RGCR and YGCY sequences (44, 193, 226). Two of the chlorella virus site-specific endonucleases cleave only one strand of dsDNA [NYs1-nickase cleaves /CC sites (227) and NY2A-nickase cleaves R/AG sites (241)]. Like bacterial restriction endonucleases, viral endonucleases are inhibited by m5C or m6A in their recognition sites.

A summary of other characteristics of the chlorella virus restriction-modification (R-M) systems follows: (a) Some viruses, e.g., NY-2A, encode as many as ten distinct DNA MTases (seven m6A MTases and three m5C MTases) and at least two site-specific endonucleases (241). (b) Viruses with high levels of methylated bases encode some MTases that recognize short (2- to 3-bp) target sites, e.g., M.CviPI from virus NYs-1 methylates GC sequences (229). (c) Like bacterial type II R-M genes (222), the chlorella virus R-M genes are located near one another, although the spacing and relative orientation of the two genes can vary. (d) The virus m6A- and m5C-MTases contain the same conserved amino acid motifs found in bacterial m6A MTases (114) and m5C MTases (91, 154). (e) In some instances the amino acid sequence of virus MTases with the same site-specificities differ by as much as 60%, suggesting that the gene has either evolved extensively or been acquired by the viruses from more than one source (157).

The biological function(s) of the chlorella virus-encoded DNA site-specific endonucleases and MTases is unknown. Bacterial R-M systems confer resistance to foreign DNAs and DNA viruses. Two functions have been considered for the chlorella virus R-M enzymes. (a) The endonucleases help degrade host DNA, thus providing deoxynucleotides for incorporation into virus DNA. Methylation of nascent virus DNAs by the cognate MTases protects the DNA from self-digestion. (b) The endonucleases prevent infection of a cell by a second virus.

Several observations are consistent with the first hypothesis. (a) Host nuclear and chloroplast DNAs, but not virus DNA, are digested by virus-encoded site-specific endonuclease(s) *in vitro*. (b) Host DNA degradation does not occur if cycloheximide is added at the time of virus infection (204), indicating that DNA degradation requires protein synthesis. (c) *In vivo* degradation of host nuclear and chloroplast DNAs coincides with the appearance of DNA site-specific endonuclease activity. (d) Initiation of virus DNA synthesis *in vivo* coincides with the appearance of DNA MTase activity (225, 228).

The isolation of three independently derived deletion mutants of virus IL-3A, which had lost their MTase (M.CviJI) and site-specific endonuclease (R.CviJI) ac-

tivities, allowed us to test the host DNA degradation hypothesis (14). If 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 DNA levels decreased at similar rates following infection with each of the four viruses (14). Therefore, CviJI activity was not essential for host DNA degradation. However, these results do not exclude participation of the enzyme in the degradation process.

To determine whether the endonuclease(s) excludes 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 (20). These experiments revealed that the chlorella viruses, like certain bacteriophages (see 36) exclude one another. However, this exclusion was independent of the site-specific endonuclease activities.

Therefore, the biological function of the DNA MTases and site-specific endonucleases remains unknown. However, if a virus encodes a functional cytosine MTase gene, then its expression is necessary for virus growth. There is a direct correlation between increasing m5C concentrations in the virus genome and sensitivity of virus replication to the cytidine methylation inhibitor, 5-azacytidine (14).

PBCV-1, containing only 1.9% m5C and 1.5% m6A, encodes 7 R-M enzymes, including two R-M systems that involve m6A and three m5C MTases. Interestingly, one of these latter genes encodes an inactive enzyme that differs by 8 amino acids from M.CviJI from another virus. Domain swapping and site-directed mutations established that a single base change that creates a single amino acid substitution made the inactive enzyme active (242).

As discussed below, the natural history of the chlorella viruses is poorly understood. Presumably, the DNA MTases and endonucleases confer an evolutionary advantage to the viruses in their native environment.

### *Nucleotide Metabolism Enzymes*

PBCV-1 DNA replication begins 60–90 min p.i. at the same time that host nuclear and chloroplast DNAs begin degrading (204). Total DNA in the cell increases four- to tenfold by 4 h p.i. due to viral DNA synthesis. Viral DNA synthesis requires large quantities of dNTPs that cannot be accounted for simply by recycling deoxynucleotides from host DNA. To guarantee a supply of deoxynucleotides in non-proliferating host cells, large DNA viruses frequently encode deoxynucleotide synthesis enzymes, including both subunits of ribonucleotide reductase. PBCV-1 encodes at least 13 nucleotide metabolic enzymes, aspartate transcarbamylase, both subunits of ribonucleotide reductase, dUTP pyrophosphatase, dCMP deaminase, thymidylate kinase, nucleotide triphosphatase, cytidine deaminase, thymidylate synthase, glutaredoxin, thioredoxin, and two ATPases.

Two of the PBCV-1-encoded enzymes, dUTP pyrophosphatase and dCMP deaminase, synthesize dUMP, the substrate for thymidylate synthetase. PBCV-1 lacks

a traditional thymidylate synthetase A. Instead, PBCV-1 encodes a protein that is a member of a newly recognized family of flavin-dependent thymidylate synthetases called X (134). A thymidylate synthetase X encoding gene is also found in *Dictyostelium* (40) and in ~25% of sequenced bacteria and Archae genomes (134).

## PBCV-1 Transcription

PBCV-1 gene expression is temporally regulated and can be divided into two distinct stages, early and late. However, a few genes are transcribed both early and late (e.g., elongation factor 3 and ornithine decarboxylase). The junction between these stages is 60 to 90 min p.i., which coincides with the start of virus DNA synthesis. The incorporation of [<sup>3</sup>H]-adenine into polyadenylated RNA also decreases abruptly at about 60 min p.i. Assuming this decrease is not due to dilution from a large increase in an ATP pool, we predict that polyadenylate segments are rare in late virus transcripts, but likely common in early virus transcripts (213).

Two studies (170, 171) have examined PBCV-1 RNA synthesis and the following general statements can be made about virus transcription. (a) Viral infection rapidly inhibits host RNA synthesis. Chloroplast, but not cytoplasmic, rRNAs are degraded beginning at about 30 min p.i. (b) PBCV-1 transcription is programmed, and the first transcripts appear within 5–10 min p.i. A few, but not all, early virus transcripts are synthesized in the absence of de novo protein synthesis. The synthesis of late transcripts requires translation of an early virus gene(s). (c) Early and late virus genes are interspersed throughout the PBCV-1 genome. (d) Gene full-length viral DNA probes often hybridize to mRNA transcripts that are 40–60% larger than the gene itself, suggesting that PBCV-1 contains overlapping genes, transcribes both strands of DNA, or posttranscriptionally modifies RNA, or that some transcripts are polycistronic. Transcription studies with individual PBCV-1 genes indicate that some genes produce the expected size mRNA as either an early or late gene, whereas other genes produce complex RNA patterns; these complex patterns occur even using ssDNA probes (e.g., the K<sup>+</sup> channel gene). (e) Transcription mapping of 7 PBCV-1 genes revealed that the virus has mRNAs with 5' untranslated regions as small as 14 nucleotides and as large as 149 nucleotides. Transcripts also often extend beyond the translational stop codon (60, 61, 171). To date, no early or late PBCV-1 promoters have been identified, although the 50 bases preceding most of the major ORFs are at least 70% AT. In addition, several genes including UDP-glucose dehydrogenase (95) and hyaluronan synthase (59) genes have -35 and -10 sites with spacing characteristic of *Escherichia coli* promoters (103).

PBCV-1 does not encode a recognizable RNA polymerase or RNA polymerase subunit. The lack of a virus-encoded RNA polymerase suggests that infecting viral DNAs are targeted to the cell nucleus and that a host RNA polymerase initiates viral transcription, possibly in conjunction with virus-packaged transcription factors. Consistent with this possibility, PBCV-1 encodes at least four transcrip-

tion factor-like elements, TFIIB, TFIID, TFIIS, and VLTf-2. However, it is unknown if these proteins are active and if they are packaged in the virion.

PBCV-1 also encodes two enzymes involved in forming mRNA cap structure, an RNA triphosphatase (73) and an RNA guanylyltransferase (74). The size, amino acid sequence, and biochemical properties of the PBCV-1 capping enzymes resemble yeast-capping enzymes more than poxvirus and ASFV multifunctional RNA capping enzymes (55, 178). PBCV-1 also encodes an active RNase III that presumably processes either virus mRNAs or tRNAs (Y. Zhang, I. Calin-Jageman, J. R. Gurnon, B. Adams, A. W. Nicholson, & J. L. Van Etten, manuscript in preparation). Finally, PBCV-1 encodes two proteins that contain sequence elements of superfamily II helicases. Superfamily II helicases are involved in transcription (196).

### *Set Domain-Containing Protein*

PBCV-1 infection rapidly inhibits most, if not all, host transcription. Furthermore, at least some host transcription machinery is subverted to initiate virus transcription. The recent discovery that PBCV-1 encodes a small 120-amino acid protein (referred to as vSET) that methylates Lys<sup>27</sup> in histone H3 (115) provides a possible mechanism for virus inhibition of host transcription. Proteins with ~120 amino acid SET domains often methylate histones [this process, including histone acetylation and phosphorylation, is often referred to as chromatin remodeling (e.g., 6)]. These methylating enzymes typically contain one or two SET domain(s) near their C terminus preceded by several hundred amino acids. Presumably, the N-terminal region confers histone specificity for SET-containing protein activity. Since the PBCV-1 enzyme consists solely of a SET domain, we predict that vSET methylates Lys<sup>27</sup> residues in specific histone H3s and that such methylation results in the inhibition of most, if not all, host transcription. A recent report that the polycomb group of proteins inhibit expression of HOX genes in *Drosophila* by methylating Lys<sup>27</sup> in histone H3 proteins (15) is consistent with histone H3 Lys<sup>27</sup> methylation inhibiting transcription.

### **PBCV-1 Protein Synthesis, Modification, And Degradation**

PBCV-1 infection rapidly inhibits host protein synthesis and some early virus proteins appear within 15 min p.i. PBCV-1 proteins are synthesized on cytoplasmic ribosomes and not organellar ribosomes since cycloheximide, but not chloramphenicol, inhibits viral replication (180). How the virus takes over the host translational machinery forcing it to translate virus mRNAs is not known.

The chlorella viruses are the first viruses known to encode a translation elongation factor (EF) enzyme (230). The putative protein from PBCV-1 has ~45% amino acid identity to an EF-3 protein from fungi (7, 19). The fungal protein stimulates EF-1 $\alpha$ -dependent binding of aminoacyl-tRNA to the A site of the ribosome.

Like fungal EF-3 proteins, the PBCV-1 protein has an ABC transporter family signature and two ATP/GTP-binding site motifs.

PBCV-1 codon usage is biased to codons ending in A/U (63%) over those ending in C/G (37%) (171). This bias is expected because PBCV-1 DNA is 40% G + C, whereas host nuclear DNA is 67% G + C (212). Therefore, finding that PBCV-1 encodes 11 tRNA genes may not be surprising: 3 for Lys, 2 each for Asn and Leu, and 1 each for Ile, Tyr, Arg, and Val. None of the tRNAs has a CCA sequence encoded at the 3' end of the acceptor stem. Typically these 3 nucleotides are added separately to tRNAs. Some chlorella viruses encode as many as 16 tRNAs (22, 145). There is a strong correlation between the abundance of virus-encoded tRNAs and the virus gene codon use (145).

The virus-encoded tRNAs contain internal A and B boxes characteristic of RNA polymerase III promoter elements, suggesting the tRNAs might be transcribed individually by RNA polymerase III (145). However, the tRNA genes are transcribed as a large precursor RNA and processed via intermediates to mature tRNAs at both early and late stages of virus replication. Some, if not all, of the tRNAs are aminoacylated *in vivo*, suggesting they probably function in viral protein synthesis (145). Possibly, the virus-encoded EF-3 in combination with the virus-encoded tRNAs alter the host protein synthetic machinery to preferentially translate viral mRNAs.

Including glycosylation (see below), PBCV-1 encodes several enzymes involved in posttranslational modification. Like several large dsDNA viruses, PBCV-1 encodes an ERV/ALR protein that functions as a protein thiol oxidoreductase (174). PBCV-1 also encodes a putative protein disulfide isomerase and a prolyl 4-hydroxylase that converts Pro-containing peptides into hydroxyl-Pro-containing peptides (42). Finally, PBCV-1 encodes 7 putative Ser/Thr-protein kinases, 1 Tyr-protein kinase, and a Tyr phosphatase. With the exception of one of the Ser/Thr protein kinases (156), these protein kinase/phosphatase enzymes have not been studied. Three protein kinases are packaged in the related chlorella virus (CVK2) virion (231).

PBCV-1 encodes two putative proteins that interact with ubiquitin, a ubiquitin C-terminal hydrolase and a Skp1 protein. Skp1 proteins belong to the SCF-E3 ubiquitin ligase family that targets cell cycle and other regulatory factors for degradation (33). Finally, PBCV-1 encodes at least one putative serine proteinase.

### *Protein Glycosylation*

Structural proteins of many viruses, such as herpesviruses, poxviruses, and paramyxoviruses, as well as the chlorella viruses, are glycosylated. Typically, viral proteins are glycosylated by host-encoded glycosyltransferases located in the endoplasmic reticulum (ER) and Golgi and then transported to a host membrane (38, 89, 149). Nascent viruses acquire the glycoprotein(s) and only become infectious by budding through the membrane, usually as they are released from the cell. Consequently, the glycan portion of virus glycoproteins is host specific.

However, glycosylation of PBCV-1 major capsid protein Vp54 differs from this paradigm. This conclusion originally arose from antibody studies. Polyclonal antiserum prepared against intact PBCV-1 virions inhibits virus plaque formation by agglutinating particles. Spontaneously derived, antiserum-resistant variants of PBCV-1 arise at a frequency of  $\sim 10^{-6}$ . These antiserum-resistant variants fall into five serologically distinct classes (219; M. V. Graves & J. L. Van Etten, unpublished results). Polyclonal antisera prepared against members of each of these antigenic classes react exclusively with the Vp54 equivalent from the viruses in the class used in the immunization. The Vp54 equivalents from the variants migrate in a distinctive fashion on SDS-PAGE; the size of Vp54 in each variant is smaller than wild-type Vp54. Western blot analyses of Vp54 proteins, before and after removing the glycans, established that the antigenic variants reflect differences in the Vp54 glycans. In addition, the ratio of the 7 neutral sugars [glucose, fucose, galactose, mannose, xylose, rhamnose and arabinose (219)] associated with Vp54 from PBCV-1 and the variants change in a manner that correlates with antigenicity and Vp54 migration on SDS-PAGE.

Additional observations indicate that Vp54 glycosylation is unusual. (a) Unlike viruses that acquire their glycoprotein(s) by budding through a membrane, intact infectious PBCV-1 particles accumulate inside the host 30–40 min before virus release (206). (b) The nucleotide sequence of the *vp54* gene in each of the variants is identical to PBCV-1 (219). (c) All of the variants are grown in the same host. (d) Antibodies to the virus do not react with host oligosaccharides. (e) Compounds that inhibit ER-Golgi localized N-linked glycosylation of proteins (tunicamycin) and ER to Golgi protein transport Brefeldin A (39, 107) neither affect PBCV-1 replication nor the  $M_r$  of Vp54 at concentrations lethal to the host (155). (f) Variants from different classes can complement and recombine in dual infection experiments to produce wild-type progeny, indicating that the enzymes involved in glycosylation reside in different virus-encoded complementation groups (58). (g) The Vp54 glycans lack N-acetylglucosamine (GlcNAc), a sugar commonly found in Asn-linked (N-linked) and many O-linked glycoproteins produced via the cellular ER-Golgi pathway (164). (h) Unlike most glycoproteins that exhibit size microheterogeneity, Vp54 appears homogeneous on SDS-PAGES; also mass spectrometry analysis only reveals one satellite peak. (i) The ability to easily crystallize Vp54 as a homotrimer provides additional evidence that the protein is homogeneous (140).

The Vp54 crystal structure also revealed the location of the glycans (140). Comparing the molecular mass of Vp54 with its predicted molecular weight from the amino acid sequence indicates that the protein contains  $\sim 30$  sugar moieties, of which 20 could be ordered in the density map. Single sugars are attached to Ser<sup>57</sup> and Ser<sup>387</sup>; six- and seven-branched chain sugar moieties are attached to Asn<sup>280</sup> and Asn<sup>302</sup>, respectively, as well as three and two sugar residues at Asn<sup>399</sup> and Asn<sup>406</sup>, respectively. As predicted from the antibody results, the four N-linked glycans face the external surface of the virus. GlcNAc fits the carbohydrate electron density at residues Asn<sup>280</sup>, Asn<sup>302</sup>, and Asn<sup>399</sup>. However, the inability to detect GlcNAc as one of the sugars associated with Vp54 suggests that another modified sugar might be in this position, possibly glucose with an O-linked acetate.

The identification of the glycan-linked Asn residues in Vp54 provided additional evidence that glycosylation of Vp54 does not involve host glycosyltransferases. Asn<sup>302</sup>, Asn<sup>399</sup>, and Asn<sup>406</sup> occur in the amino acid sequence motif (A/G)NTXT, and Asn<sup>280</sup> occurs in an ANIPG sequence. None of these Asn residues resides in a NX(T/S) sequence commonly recognized by endoplasmic reticulum- and Golgi-located glycosyltransferases (164). This also explains why previous tests for N-glycosylation were negative (155). Taken together, the results suggest that PBCV-1 encodes the enzymes involved in constructing the glycans attached to Vp54.

Comparison of PBCV-1 ORFs to proteins in the databases identified seven possible glycosyltransferase-encoding genes, *a64r*, *a111r*, *a114r*, *a222-226r*, *a328l*, *a473l*, and *a546l*. None of these putative PBCV-1-encoded glycosyltransferases has an identifiable signal peptide that would target them to the ER. Furthermore, the cellular protein localization program PSORT predicts that all of these proteins, with the exception of A473L, are cytoplasmic. A473L is predicted to be in a membrane.

The *a64r* gene encodes a 638 amino acid protein that has 4 motifs conserved in fringe-type glycosyltransferases. Analysis of 13 PBCV-1 antigenic variants revealed mutations in *a64r* that correlated with a specific antigenic variation. Dual infection experiments with different antigenic variants indicated viruses that contained wild-type *a64r* could complement and recombine with viruses that contained variant *a64r* to form wild-type virus. Therefore, we concluded that *a64r* encodes a glycosyltransferase involved in synthesizing the Vp54 glycan (58).

Collectively, the results indicate that glycosylation of the PBCV-1 major capsid protein differs from that of other viruses and that glycosylation is independent of the ER and Golgi apparatus. Could Vp54 glycosylation reflect an ancestral pathway that existed prior to ER and Golgi formation? Among many questions to be addressed, it will be important to determine if the Vp54 glycan precursor is attached to a lipid carrier such as undecaprenol-phosphate, which is the carrier for bacterial peptidoglycans and cell surface polysaccharides (158), or dolichol diphosphate, which is the carrier for eukaryotes (164).

## PBCV-1 Assembly and Release

Like poxviruses, iridoviruses, and ASFV, PBCV-1 assembly occurs in localized regions in the cytoplasm, referred to as virus assembly centers. Therefore, one might expect the cytoskeleton to participate in either targeting the virus proteins to the virus assembly centers and/or assembling virus proteins into capsids. However, 13 cytoskeleton-disrupting agents, which inhibit either tubulin or actin functions, had little effect on formation of PBCV-1 virus assembly centers or PBCV-1 replication, even at concentrations higher than required to inhibit host chlorella growth (144). PBCV-1 empty capsids appear prior to filling with DNA (121).

The origin of the PBCV-1 internal membrane is unknown. Other large ds-DNA viruses with internal membranes, including a brown algal virus that infects *Hinckia hinckia* (223) or ASFV (24), acquire their membrane by a pinching off of

the ER, and PBCV-1 may use a similar mechanism. In support of this hypothesis, Songsri et al. (182) identified two urea-soluble proteins from virus CVK2 that contain an N-terminal ~20 amino acid hydrophobic sequence followed by a lysine. These sequences, which are absent in the native virion protein, resemble signal peptides that are cleaved in the lumen of the ER.

PBCV-1 also contains genes that encode putative lipid-metabolizing enzymes, glycerophosphoryl diesterase, 2-hydroxyacid dehydrogenase, and lysophospholipase. These enzymes could be involved in either synthesis or modification of the internal virus membrane. However, PBCV-1 deletion mutants, which lack the neighboring glycerophosphoryl diesterase and the 2-hydroxyacid dehydrogenase genes, still replicate in chlorella grown in the laboratory (94). The product of another PBCV-1 gene has a GNAT-N-acetyltransferase domain. This may be significant because three proteins associated with the PBCV-1 virion, including the major capsid protein, contain an amide-linked myristic acid (155). Typically, N-linked myristic acids are attached to the N-terminal amino acid, usually a glycine, after removal of the terminal methionine (10). However, the myristic acid(s) attached to the PBCV-1 major capsid protein is linked to an internal amino acid, probably a lysine (155). The putative PBCV-1-encoded N-acetyltransferase could be responsible for this rare modification.

Timing the release of nascent virions from the host is critical and presumably involves at least some of the virus-encoded cell wall-digesting enzymes. Perhaps the chlorella viruses have something similar to holins, which time the release of dsDNA bacteriophages (218).

### *Host Cell Surface Changes*

The discovery that PBCV-1 infection leads to host cells becoming covered with the linear polysaccharide hyaluronan, also called hyaluronic acid, was completely unexpected (59). Furthermore, PBCV-1 encodes the enzyme, hyaluronan synthase (HAS), responsible for its synthesis (30). Hyaluronan, a ubiquitous constituent of the extracellular matrix in vertebrates, is composed of ~20,000 alternating  $\beta$ -1,4-glucuronic acid and  $\beta$ -1,3-N-acetylglucosamine residues (28). Until the *has* gene was discovered in PBCV-1, hyaluronan was thought to occur only in vertebrates and a few pathogenic bacteria, where it forms an extracellular capsule, presumably to avoid the immune system (28, 29).

PBCV-1 also encodes two enzymes involved in the biosynthesis of hyaluronan precursors, glutamine:fructose-6-phosphate amidotransferase (GFAT) and UDP-glucose dehydrogenase (UDP-GlcDH) (Figure 2A) (95). All three proteins are produced early in PBCV-1 infection and hyaluronan begins to accumulate as hyaluronan lyase-sensitive, hair-like fibers on the outside of the chlorella cell wall by 15-30 min p.i.; by 240 min p.i. the infected cells are covered with a dense fibrous hyaluronan network (59). The biosynthesis of a single hyaluronan chain requires a lot of energy; five ATP equivalents, two NAD cofactors, one acetyl CoA group and the two monosaccharide components are required to form each individual

disaccharide unit (28). Therefore, hyaluronan presumably serves some essential purpose or provides a selective advantage to the virus.

The PBCV-1-encoded GFAT and UDP-GlcDH enzymes most closely resemble bacterial enzymes, whereas PBCV-1-encoded HAS resembles vertebrate enzymes. These observations suggest that the viruses may have acquired the *gfat* and *udp-glcdh* genes separately from the *has* gene. One might expect these three genes to be clustered on the viral genome. However, this is not the case, whereas *has* and *gfat* genes are colinear and separated by only 99 nucleotides, they are located 240 kb from the *udp-glcdh* gene.

We have considered three biological functions for the extracellular hyaluronan: (a) The polysaccharide prevents uptake of virus-infected chlorella by the paramecium. Presumably, such infected algae would lyse inside the paramecium and the released virions would be digested by the protozoan. This scenario would be detrimental to virus survival. (b) The viruses have another host that acquires virus by taking up the hyaluronan-covered algae. (c) Virus-infected cells aggregate, presumably due to the extracellular polysaccharide. This aggregation, which can trap uninfected cells, may aid the virus in finding its next host.

A complicating factor in understanding the biological importance of hyaluronan, however, is the discovery that not all chlorella viruses encode *has* and at least some virus-infected cells lack a surface polysaccharide (59). The story became even more bizarre with the recent discovery by Kawasaki et al. (87) that some chlorella viruses have a gene that encodes chitin synthase (*chs*) instead of a *has* gene. Chitin is a linear polysaccharide composed of  $\beta$ -1,4-linked N-acetylglucosamine residues. Cells infected with viruses that have a *chs* gene accumulate chitin on their surface. A few viruses contain both a *has* gene and a *chs* gene and both polysaccharides appear on the surface of cells infected with these viruses (87).

The discovery of viral polysaccharide synthesis leads to many questions such as: (a) What is the function of these extracellular polysaccharides? (b) Why do infected cells expend huge amounts of energy for the processes when they are going to lyse in a few hours? (c) How were these genes acquired by the viruses?

## Additional PBCV-1 Genes

### *Fucose Synthesis*

As mentioned above, PBCV-1 encodes at least 7 putative glycosyltransferases and 3 enzymes involved in hyaluronan biosynthesis. In addition, PBCV-1 is the first virus known to encode enzymes involved in nucleotide sugar metabolism. The virus encodes two enzymes, GDP-D-mannose 4,6 dehydratase (GMD) and the bifunctional GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (GMER), that comprise the highly conserved pathway that converts GDP-D-mannose to GDP-L-fucose (Figure 2B) (199). Experiments with recombinant GMD and GMER established that both virus proteins have properties comparable to human and bacterial enzymes. However, the PBCV-1 GMD is more stable than recombinant

GMDs from other organisms. In addition to the dehydratase activity, the PBCV-1 GMD also catalyzes NADPH-dependent reduction of the intermediate GDP-4-keto-6-deoxy-D-mannose, forming GDP-D-rhamnose. As a consequence, in vitro reconstruction of the biosynthetic pathway using PBCV-1 GMD and GMER produces both GDP-L-fucose and GDP-D-rhamnose in the presence of NADPH (Figure 2B). Both fucose and rhamnose are present in the glycans attached to the virus major capsid protein Vp54. However, uninfected *Chlorella NC64A* cell walls contain rhamnose and trace amounts of fucose (119). Thus the uninfected *Chlorella* synthesizes these two sugars. Presumably, the virus-encoded pathway could circumvent a limited supply of GDP-sugars by the host.

### *Polyamine Biosynthetic Enzymes*

PBCV-1 is the first virus known to code for polyamine biosynthetic enzymes, including two separate pathways to synthesize putrescine (Figure 2C). Ornithine decarboxylase (ODC) catalyzes decarboxylation of ornithine to putrescine, which is the first and rate-limiting enzymatic step in the classical polyamine biosynthetic pathway (25, 27). Typically, ODC is subject to a variety of control mechanisms and the protein turns over rapidly in vivo in other organisms (25). Turnover is mediated by an antizyme protein that, at least in mammals, binds to a PEST sequence in ODC, and targets the enzyme for proteolysis by the 26S proteasome (25, 45, 66). The PBCV-1-encoded ODC is smaller (372 amino acids) than other ODCs. Despite its small size, the recombinant protein has good ODC activity (128). The virus enzyme lacks the two PEST sequences present in mammalian enzymes and thus may lack the regulation of more advanced ODCs. This suggestion is supported by phylogenetic analyses; the PBCV-1 ODC resides near the origin of the clade containing eukaryotic ODCs.

PBCV-1 ODC is more sensitive to the irreversible inhibitor difluoromethylarginine (DFMA) than to the specific ODC irreversible inhibitor difluoromethylornithine (DFMO) (128). This observation led to the discovery that the virus enzyme also has arginine decarboxylase activity (A. E. Pegg, unpublished results). The product of arginine decarboxylase is agmatine. Interestingly, PBCV-1 also encodes two enzymes, agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase, that convert agmatine to putrescine (Figure 2C) (138; M. Piotrowski, personal communication). PBCV-1 also encodes a functional NAD<sup>+</sup>-dependent enzyme (homospermidine synthase) that synthesizes the rare polyamine homospermidine from two molecules of putrescine (82).

The polyamines putrescine, spermidine, and spermine are common in cells and also are structural components of many viruses, where they help neutralize viral nucleic acids (25, 202). PBCV-1 virions, as well as uninfected and virus-infected *Chlorella NC64A* cells, contain putrescine, cadaverine, spermidine, and homospermidine. However, it is unlikely that these polyamines are important in neutralizing DNA because the number of polyamine molecules per virion could neutralize only ~0.2% of DNA phosphate residues (82). Furthermore, the functional significance of polyamines in the PBCV-1 particle must be limited since

they can be displaced by washing the particles in a polyamine-free buffer without affecting virus infectivity.

Thus it is unknown why PBCV-1 encodes so many polyamine biosynthetic enzymes. The PBCV-1 *odc* gene is expressed at both early and late times during virus infection (128), whereas the homospermidine synthase gene is only expressed late (82). Expression patterns of the other polyamine biosynthetic enzymes remain to be determined.

Finally, PBCV-1 encodes two other putative enzymes involved in amine metabolism, monoamine oxidase and histidine decarboxylase. Functions of these enzymes remain unclear.

### *Ion Channel Proteins*

PBCV-1 was the first virus discovered to encode a functional K<sup>+</sup> channel protein. The 94-amino acid protein (called Kcv) produces a K<sup>+</sup>-selective and slightly voltage-sensitive conductance in *Xenopus* oocytes (153) and mammalian HEK293 (129) and CHO cells (52). Kcv is the smallest protein known to form a functional K<sup>+</sup> selective channel and its transmembrane (TM1)-pore-TM2 structure corresponds to the "pore module" of K<sup>+</sup> channels. Because of its small size, Kcv is a model protein to study channel assembly and function (51). The K<sup>+</sup> channel inhibitors amantadine and Ba<sup>2+</sup>, but not Cs<sup>+</sup>, inhibit K<sup>+</sup> conductance in oocytes, as well as PBCV-1 plaque formation. Thus PBCV-1 Kcv activity is probably important in some aspect of virus replication.

One possible scenario is that the internal membrane of PBCV-1 contains Kcv and that after digestion of the cell wall during infection, the virus membrane fuses with the host plasma membrane, triggering the release of virus DNA and associated proteins into the host. The fusion activates the Kcv channel and rapidly changes the host internal K<sup>+</sup> concentration, creating an altered membrane potential. This signal spreads rapidly to alter the entire host plasma membrane potential and prevents membrane fusion by a second virus, thus restricting infection by a second virus. This hypothesis is consistent with several observations: (a) Virus infection rapidly depolarizes the host plasma membrane (118). This depolarization displays the same sensitivity to inhibitors as Kcv conductance in oocytes. (b) Multiple virus particles can attach to and degrade the host cell wall without releasing their DNA (J. L. Van Etten, unpublished results). (c) The host exhibits an exclusion phenomenon (see section on DNA MTases and site-specific endonucleases) (20).

We have isolated genes encoding Kcv proteins from 40 additional chlorella viruses. In total, differences in 16 of the 94 amino acids have been observed, resulting in 6 new Kcv-like proteins. Amino acid differences occur in all the Kcv functional domains. The 6 new Kcv-like proteins produced K<sup>+</sup> selective currents in *Xenopus* oocytes with new properties, e.g., current kinetics and inhibition by Cs<sup>+</sup> (M. Kang, S. Gazzarrini, M. Severino, D. DiFrancesco, G. Thiel, J. L. Van Etten, & A. Moroni, manuscript in preparation). Single and multiple amino acid substitutions are being produced in Kcv to identify key amino acids that influence the K<sup>+</sup> currents.

Often other proteins or compounds influence the assembly and/or function of  $K^+$  channels, and PBCV-1 encodes several proteins that could mediate these processes: (a) Potassium ion channel activity is often modulated by phosphorylation and dephosphorylation (102) and PBCV-1 encodes eight protein kinases. In fact, a Ser/Thr protein kinase gene is adjacent to the *kcv* gene in PBCV-1 as well as in many other chlorella viruses (M. Kang, M. V. Graves, M. Mehmel, A. Moroni, S. Gazzarrini, D. DiFrancesco, G. Thiel, J. R. Gurnon, & J. L. Van Etten manuscript in preparation). (b) Polyamines inhibit biological activity of Kir type  $K^+$  channels (43, 106, 109). PBCV-1 encodes at least four enzymes associated with polyamine biosynthesis (Figure 2C). (c) PBCV-1 encodes a putative histidine decarboxylase. Histamine, the product of histidine decarboxylase, is an important neurotransmitter of photoreceptors in insects and other arthropods (141). As a photoreceptor transmitter in insects, histamine acts on ligand-gated chloride ion channels. (d) PBCV-1 encodes two adjacent but divergent ORFs (A162L and A163R) that resemble ligand-gated ion channel proteins (83). The A162L and A163R proteins have three properly spaced transmembrane domains that are typical of glutamate receptor ion channel proteins (34). Glutamate receptor channels mediate influx of cations ( $K^+$ ,  $Na^+$ , and  $Ca^{2+}$ ) across membranes (34, 123). However, the two PBCV-1 putative proteins lack recognizable glutamate binding sites. Expression of cRNAs from these two genes in oocytes failed to produce currents (83).

The connection, if any, between the putative PBCV-1-encoded protein kinases, polyamine biosynthetic enzymes, putative histidine decarboxylase, putative ligand-gated ion channel proteins, and Kcv is unknown. However, all of these components could be part of a regulated system for balancing ions and/or membrane potential within the host during virus replication.

### **PBCV-1 Gene Families And Gene Duplications**

Eighty-four of the PBCV-1 ORFs resemble one or more other PBCV-1 ORFs forming 26 families. Thirteen families have two members, eight families have three members, three families have six members, and two families have eight members. One six-member family contains multiple ankyrin-like repeats (150). Five members in another family encode proteins that resemble the PBCV-1 major capsid protein Vp54. Like the Vp54 gene, the genes for four of these proteins are transcribed late in the infection cycle; expression of the fifth gene was not detected (M. V. Graves & J. L. Van Etten, unpublished results). Although hybridization analysis indicates that these five additional capsid protein-like genes are not highly conserved among the chlorella viruses, the major capsid protein of a brown algal virus EsV-1 most closely resembles one of these other PBCV-1 major capsid-like proteins.

### **Diversity of Chlorella Virus Genomes**

Not all PBCV-1 genes are required for virus replication in the laboratory. For example, four spontaneously derived, PBCV-1 mutants were isolated that contain 27- to 37-kb deletions at the left end of the 330-kb genome (94). Two of these mu-

tants, which probably resulted from recombination, have deletions beginning at nucleotide coordinates 4.9 or 16 kb and ending at 42 kb. The other two deletion mutants, which probably arose from nonhomologous recombination, lack the entire left terminal 37-kb of the PBCV-1 genome, including the 2.2-kb terminal inverted repeat region. The deleted left terminus is replaced by the transposition of an inverted 7.7- or 18.5-kb copy from the right end of the PBCV-1 genome. Taken together, ~40 kb of single-copy DNA encoding 31 ORFs at the left end of the genome, or 12% of the PBCV-1 genome, is unnecessary for PBCV-1 replication in the laboratory. However, replication of the PBCV-1 deletion mutants is attenuated, i.e., their burst sizes are about half those of wild-type virus.

The deletion mutants also indicate that the size and sequence of the inverted terminal repeat regions are not conserved among chlorella viruses (181, 184, 232). This nonconservation is somewhat surprising because one predicts that DNA termini are essential for either virus DNA replication and/or DNA packaging. These results also indicate that the virus DNA packaging machinery tolerates significant differences in genome size, e.g., the largest deletion in PBCV-1 creates a genome of ~302 kb, whereas another chlorella virus NY-2A has a genome of ~380 kb.

Large insertions also occur in the chlorella viruses. Comparison of PBCV-1 and CVK2 genomes revealed that an ~15-kb region in the PBCV-1 left terminal region is absent in the CVK2 genome. However, CVK2 contains a 22.2-kb insert in this region that contains five gene copies of a homolog of PBCV-1 glycoprotein Vp260; this 22.2-kb sequence is absent in the PBCV-1 genome (23, 146).

The sizes and locations of the deletions and transpositions found in the chlorella viruses resemble poxvirus (201) and ASFV (11) deletion mutants. Like PBCV-1, poxviruses and ASFV have inverted terminal repeats and covalently closed hairpin ends. Models proposed to explain the generation of deletions and deletion/transpositions in the poxvirus genomes (175, 201) may be relevant to the chlorella viruses.

Several years ago we grouped 37 viruses that infect *Chlorella NC64A* into 16 classes on the basis of plaque size, virion antiserum sensitivity, DNA restriction patterns, sensitivity of the DNAs to restriction endonucleases, and the amount and location of methylated bases in their DNAs (208). Hybridization of individual PBCV-1 genes with DNAs from these 37 chlorella viruses indicate that not every PBCV-1 gene is present in all NC64A viruses.

Several other observations reflect the diversity of chlorella virus genomes. (a) The virus NY-2A genome is about 50 kb larger than the PBCV-1 330-kb genome (165). (b) An extra ORF is often inserted between colinear genes (157). The insertion of extra genetic elements, termed "morons," between adjacent genes also occurs in related lambda phages (68). (c) PBCV-1 encodes 11 tRNA genes that are clustered with less than 33 intergenic nucleotides; other NC64A viruses encode clusters of up to 16 tRNA genes (22, 145). Clustering of tRNAs is common in bacteria and rare in eukaryotes. Some of the tRNA genes contain introns as large as 1 kb (145). (d) Sequence analyses of a gene (*pdg*) encoding a UV-specific DNA repair enzyme from 42 NC64A viruses revealed that 15 contain a 98-nucleotide, spliceosomal-processed intron that is 100% conserved; four other viruses contain

an identically positioned 81-nucleotide intron that is nearly 100% identical (189). In contrast, the nucleotides in the *pdg* coding regions (exons) from the intron-containing viruses are 84% to 100% identical.

The 100% identity of the 98-nucleotide intron sequence in 15 viruses and the near 100% identity of an 81-nucleotide intron sequence in another 4 viruses implies that either the intron was acquired recently or there is strong selective pressure to maintain the DNA sequence of the intron once it is in the *pdg* gene. However, the abilities of intron-containing and intron-lacking viruses to repair UV-damaged DNA in the dark were indistinguishable (189). These findings contradict the dogma that intron sequences are more variable than exon sequences (103).

(e) Yamada and his colleagues (147, 236) reported that 8% of the NC64A viruses isolated in Japan contain a self-splicing group I intron. The intron is inserted in the gene encoding either transcriptional elongation factor TFIIS (~60% of the viruses) or an unidentified ORF encoding a 14.2-kDa polypeptide (~40%). Divergence of the self-splicing intron sequences is constrained by the exon regions, i.e., introns in the same gene have >99% sequence identity, whereas introns in different genes have only 72–78% identity. Phylogenetic analyses indicate these introns are distantly related to those found in rRNA genes in various microorganisms, including green algae, red algae, yeast, fungi, and protozoa, suggesting horizontal gene transfer between these distantly related organisms (93). Yamada et al. (236) suggested that the self-splicing intron might function as a mobile genetic element, e.g., transferring genes between algae and protists. In this context, it is noteworthy that 1 putative transposase and 12 putative group I intron homing endonuclease encoding genes are in PBCV-1.

To summarize, considerable variation occurs in the chlorella virus genomes, and the total number of genes in the chlorella virus gene pool exceeds that of a single isolate. The different sizes of the chlorella virus genomes as well as the large deletions and insertions suggest that dynamic and frequent rearrangements of virus genomes occur in natural environments. The fact that the left end of the chlorella virus genome is tolerant to deletions/insertions/rearrangements suggests that a recombinational “hotspot” in this region allows viruses to exchange genes among themselves and possibly with host(s). However, despite these differences, the location of 22 PBCV-1 genes, many of which are housekeeping genes, are nearly colinear in the 350-kb NC64A virus CVK2 (146), suggesting similar overall genome organization between the two chlorella virus isolates.

## Natural History of the Chlorella Viruses

The natural history of the chlorella viruses is poorly understood, and major advances are needed in our understanding of their biology before we can fully appreciate their ecology and explain their evolutionary origin. The hosts for the chlorella NC64A and Pbi viruses, *Chlorella NC64A* and *Chlorella Pbi*, respectively, normally exist as hereditary endosymbionts in green isolates of the protozoan *P.*

*bursaria*. However, the algae are resistant to virus infection when they are in the symbiotic relationship and are infected only when they are separated from the ciliate (86, 161). In the symbiotic unit, algae are enclosed individually in perialgal vacuoles surrounded by a host-derived membrane (160). The initial establishment of a successful symbiotic relationship and the long-term maintenance of symbiosis require that the algae resist digestion by the paramecium. Reassociation studies with different chlorella species and algae-free *P. bursaria* indicate that only symbiotic algae readily re-establish symbiosis with the paramecium (160). Other chlorella are digested. The chlorella provide an advantage to the paramecium in the symbiotic relationship because they fix CO<sub>2</sub> and transfer sugars to the paramecium (159). The finding that paramecia protect chlorella from virus infection in the symbiotic relationship provides an advantage to the algae (161).

*Chlorella NC64A* and *Chlorella Pbi* were originally isolated from American and European strains of *P. bursaria*, respectively. Although the NC64A and Pbi viruses are morphologically, biologically, and biochemically similar, viruses that infect *Chlorella NC64A* neither infect nor attach to *Chlorella Pbi*, and Pbi viruses neither infect nor attach to *Chlorella NC64A* (161). The component sugars in the cell walls of *Chlorella NC64A* and *Chlorella Pbi* differ considerably (84). Because the viruses can distinguish the two chlorella isolates, we predicted that the host receptor for the viruses might also be the recognition factor for the paramecia. This hypothesis was incorrect, however, because both *Chlorella NC64A* and *Chlorella Pbi* established stable symbiotic relationships with both American and European isolates of *P. bursaria* (161).

NC64A viruses have been isolated from freshwater collected in the United States, South America, Australia, China, Japan, South Korea, Israel, and Italy. Pbi viruses initially were discovered in freshwater collected in Europe (162), and more recently in water collected in Australia, Canada, and the northern United States or at higher elevations in the western United States (M. Nelson, unpublished results). Therefore, the initial assumption that Pbi viruses were limited to Europe and the NC64A viruses were present only in the Americas and eastern Asia is incorrect. In fact, two water samples collected in Australia contained both NC64A and Pbi viruses (J. L. Van Etten & J. Rohozinski, unpublished results). The most important factors influencing the distribution of NC64A and Pbi viruses are probably latitude and altitude.

Typically, the chlorella virus titer in indigenous water is 1–100 plaque-forming units (PFU) per ml, but titers as high as  $1 \times 10^5$  PFU per ml have been obtained. The concentration of chlorella viruses fluctuates with the seasons; the highest titers are typically found in late spring (214, 233). However, a water sample was recently collected through the ice, with a titer of >1000 PFU per ml (M. Nelson, unpublished results).

It is unknown whether NC64A and Pbi viruses replicate exclusively in paramecium chlorella or whether they have another host(s). In fact, it is not even known if the symbiotic chlorella exist free of paramecia in natural environments. A single paramecium contains as many as 1000 algae, and if each chlorella produces 300 vi-

rus particles, chlorella released from a single paramecium could produce 300,000 virus particles. This could account for the high virus titers occasionally observed. However, this number is artificially high, since not all chlorella would be infected in dilute solutions of virus and algae. For example, Wiggins & Alexander (221) demonstrated that bacterial concentrations must exceed a threshold of about  $10^4$  cells per ml to consistently support virus replication. It is also pertinent that anecdotal reports indicate green (symbiotic) paramecia are rare in nature.

The occasional high chlorella virus titers in indigenous waters are also surprising because the viruses are constantly exposed to solar radiation. Such radiation damages viral DNAs and inactivates viruses. For example, inactivation of bacteriophages and cyanophages occurs at rates of 0.4 to 0.8 per h in full sunlight (191, 192). The chlorella viruses have adapted to solar radiation by having access to two independent DNA repair systems (49). (a) PBCV-1 encodes a pyrimidine dimer-specific glycosylase (PDG) that initiates UV-induced thymidine dimer repair. This DNA repair system functions in both the light and the dark. (b) PBCV-1 also uses the host photolyase to repair UV-induced thymidine dimers (also called host cell reactivation) (49). The combined activities of these repair systems should enable PBCV-1 to effectively exploit their hosts under a range of environmental conditions and to withstand substantial solar radiation. We expected the PDG enzyme to be packaged in the virion and accompany the virus DNA into the host, where it could initiate DNA repair. However, attempts to detect the PDG protein in PBCV-1 virions were unsuccessful (49). The *pdg* gene is expressed early after virus infection, however. PBCV-1 also encodes a putative Cu/Zn superoxide dismutase that could help protect DNA from reactive oxygen species.

Once it was established that chlorella viruses were common in nature, we assumed that plaque-forming viruses of other eukaryotic algae might also be common. However, we were unable to detect plaque-forming viruses in freshwater on other *Chlorella* or *Chlamydomonas* species. Thus, lytic viruses may be limited to a few algae or they may exist as lysogens in nature and only infrequently become lytic.

Lysogeny is consistent with the observation by several investigators that virus-like particles (VLP) are observed infrequently in eukaryotic algae and that the particles are limited to certain stages of algal development (208). The apparent lack of infectivity of these VLPs is also consistent with lysogeny. The VLPs might either infect the host and resume a lysogenic relationship or be excluded by pre-existing proviruses, as are bacteriophages. However, there is no evidence that chlorella viruses have a lysogenic phase.

Algae might also harbor viruses in a carrier-state relationship (also called pseudolysogeny), in which at any one time a small population of algae is infected by virus. This type of relationship occurs with some bacteriophages. We have observed a carrier-state relationship between PBCV-1 and *Chlorella* NC64A in the laboratory. That is, colonies of *Chlorella* NC64A occasionally grow in PBCV-1 plaques. Colonies picked from these plaques produce low concentrations of virus in liquid culture, even after repeated subculturing over several months, without

lysing the culture. PBCV-1 DNA did not hybridize to DNA isolated from single-colony isolates of these chlorella cells, which eliminates lysogeny. Furthermore, single-colony isolates from these cultures did not produce PBCV-1; however, about 25% of these isolates re-established the carrier-state relationship when re-inoculated with PBCV-1. The remaining colonies were either sensitive or resistant to PBCV-1 (Y. Xia, M. P. Skrdla, & J. L. Van Etten, unpublished results).

## Other Phycodnaviruses and Putative Phycodnaviruses

Members and prospective members of the Phycodnavirus family are continually being discovered. They all have icosahedral morphology, an internal lipid membrane, and large dsDNA genomes of 160 to 560 kb (203, 207). The viruses infect both freshwater and marine eukaryotic algae.

The largest virus genome sequenced to date, the 336-kb genome from virus EsV-1 that infects the marine filamentous brown alga *Ectocarpus siliculosus*, is a phycodnavirus (32). EsV-1 contains ~231 putative protein-encoding genes, whereas the slightly smaller PBCV-1 genome contains 11 tRNA genes and ~373 protein-encoding genes. Surprisingly, these two phycodnaviruses only have 33 common genes. Among the 33 common protein-encoding genes, 17 have no counterparts in the databases. Despite these genetic differences, phylogenetic analysis of their DNA polymerases indicate that all large algal viruses, including viruses that infect *Micromonas pusilla*, *Emiliana huxleyi*, and *Chrysochromulina brevifilum*, are more closely related to each other than to other dsDNA viruses (17, 21, 100).

The low number of EsV-1 and PBCV-1 homologous genes can be attributed to several factors. First, four observations indicate that the phycodnaviruses have a long evolutionary history. (a) Phylogenetic analyses of DNA polymerases place the phycodnavirus enzymes near the root of all eukaryotic  $\delta$  DNA polymerases (216, 217). (b) Phylogenetic analyses of other PBCV-1-encoded proteins place the proteins near the root of their eukaryotic counterparts, e.g., the K<sup>+</sup> channel protein Kcv (153), ornithine decarboxylase (128), and GDP-D-mannose 4,6 dehydratase (G. Duncan & J. L. Van Etten, unpublished results). (c) Despite the fact that EsV-1 and PBCV-1 encode a mixture of prokaryotic- and eukaryotic-like proteins, except for one repetitive region in the EsV-1 genome, the G+C contents (40% for PBCV-1 and 52% for EsV-1) are quite uniform throughout their respective genomes. This pattern suggests that most of the genes have existed together in the viruses for a long time. (d) Some evolutionary biologists believe that the first eukaryotic cell resembled a unicellular green alga; consequently, if the algal viruses appeared and evolved with their hosts, their evolutionary history could date back more than 1.2 billion years (65, 169).

Second, even though the chlorella viruses and brown algal viruses have many common features, significant differences exist. (a) The chlorella viruses infect their unicellular host by digesting the cell wall at the point of attachment, similar to bac-

terioophages. In contrast, EsV-1 and its relatives bind to the plasmalemma of the host's spores or gametes that lack a cell wall (112, 113). (b) The 320- to 380-kb chlorella virus genomes are linear, nonpermuted molecules with cross-linked hairpin ends. The 335-kb genome of EsV-1 has complementary ends that anneal to form a circular molecule (32, 97). (c) Most importantly, the brown algal viruses have a lysogenic phase in their life cycle and produce virus particles only in modified gametangium or sporangium initials (the reproductive cells) of their hosts (31, 132). In contrast, the chlorella viruses have no obvious lysogenic phase. A recent review compared the biological properties and the genes of PBCV-1 and EsV-1 (207).

Eight genetically distinct lytic viruses that infect the nanoflagellate *Micromonas pusilla* (MpV) were isolated from five locations in the Pacific and Atlantic Oceans and the Gulf of Mexico (26). The proportion of identical nucleotides for all pairwise combinations of the *dnapol* gene among the eight MpV viruses ranged from 78% to 99%, indicating substantial differences in a portion of the gene that is typically highly conserved (21).

Other algal viruses have been studied primarily because of their importance in regulating phytoplankton communities in marine environments (9, 12, 190, 224). Viruses are reportedly associated with disappearance of "brown tides" caused by *Aureococcus anophagefferens* (179) and "red tides" caused by *Heterosigma akashiwo* (135-137). Likewise, Bratbak et al. (13) reported that virus lysis accounted for >25% of the mortality in *Emiliana huxleyi* blooms.

Bratbak and colleagues have isolated lytic viruses specific for *Emiliana huxleyi* (EhV) (17), *Phaeocystis pouchetii* (PpV) (80), *Chrysochromulina ericina* (CeV-01B) (168), and *Pyramimonas orientalis* (PoV-01B) (168) that have genomes of 412, 485, 510, and 560 kb, respectively. If the gene densities of these viruses are similar to PBCV-1, they have about 466, 549, 578, and 634 protein-encoding genes, respectively. These values are as high or higher than the 470 genes encoded by the smallest mycoplasma (48). Except for EhV, these viruses, which can be difficult to grow in culture, are largely uncharacterized. However, the sequence of the 412-kb EhV genome is nearly complete (W. Wilson, personal communication), and it will be fascinating to compare the genes encoded by this virus with those encoded by PBCV-1 and EsV.

## Final Comments

Research on the chlorella viruses is in its infancy and without doubt many more surprises await discovery. However, even with our limited knowledge many excellent scientific and economic reasons justify increased research on the chlorella viruses as well as other algal viruses. (a) The chlorella viruses encode 373 or more genes. Some of the other algal viruses probably have even more genes and the genomic complexity of these viruses approaches that of mycoplasma. (b) Algal viruses play a dynamic, albeit largely unknown, role in regulating phytoplankton communities in aqueous environments, such as the termination of algal blooms.

(c) The chlorella viruses, as well as other algal viruses, are sources of new and unexpected genes. The genes not only encode commercially important enzymes such as DNA restriction endonucleases, but many viral enzymes are the smallest in their class. Consequently, these proteins serve as models for mechanistic and structural studies. (d) The viruses are a novel source of genes and genetic elements that can be used to engineer other organisms. Examples include: 1. PBCV-1 promoter elements function well in higher plants, both in monocots and dicots (126). The promoter elements also work well in bacteria (127; Y. Xia & J. L. Van Etten, unpublished results) and mammalian cells (M. V. Graves, unpublished results). 2. Expression of the PBCV-1 gene encoding the pyrimidine dimer-specific glycosylase enzyme in baculovirus increased its resistance to UV inactivation (151). One disadvantage to using baculoviruses as biological control agents is their susceptibility to inactivation by UV-light. (e) The chlorella viruses contain a mosaic of prokaryotic and eukaryotic genes and may be related to the last common ancestor of these two kingdoms. In fact, accumulating evidence indicates the algal viruses and their genes have a long evolutionary history. Consequently, studies on these viruses could reveal interesting aspects about the evolution of genes and genomes. We encourage more investigators to study these viruses.

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**Figure 1.** (*page 197*) Three-dimensional image reconstruction of chlorella virus PBCV-1 from cryoelectron micrographs. **A.** The virion capsid consists of 12 pentasymmetrons and 20 trisymmetrons. Five trisymmetrons are highlighted in the reconstruction (*blue*) and a single pentasymmetron is colored yellow. A pentavalent capsomer (*white*) lies at the center of each pentasymmetron. Each pentasymmetron consists of one pentamer plus 30 trimers. Eleven capsomers form the edge of each trisymmetron (*black dots*) and therefore each trisymmetron has 66 trimers. **B.** Cross-sectional view of PBCV-1 along the twofold axis. A lipid bilayer, like a railway track, exists beneath the capsid shell (magenta arrows). Magnified views at two- and fivefold axes (outlined in *B* are shown in *C* and *E*, respectively.) **C.** Connections occur between the capsid shell and the lipid bilayer (*green arrows*). **D.** The capsomers are interconnected by "cement" proteins (*yellow arrows*). The red arrow indicates the fiber-like structure in one trimeric capsomer. **E.** Dense material (*blue arrow*) (cell wall-digesting enzyme(s)?) is present at each vertex (*red arrow*) between the vertex and the membrane. **F.** Close-up view of a portion of a trisymmetron. Three of the 66 capsomers that comprise a trisymmetron have a kidney-shaped structure (*green arrows*). A cross section of one of these structures is seen in *D* (*red arrow*).

