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Phycodnaviridae – large DNA algal viruses

Brief Review

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Summary. Members and prospective members of the family *Phycodnaviridae* are large icosahedral, dsDNA (180 to 560 kb) viruses that infect eukaryotic algae. The genomes of two phycodnaviruses have been sequenced: the 331 kb genome of *Paramecium bursaria* chlorella virus (PBCV-1) and more recently, the 336 kb genome of the *Ectocarpus siliculosus* virus (EsV-1). EsV-1 has ∼ 231 protein-encoding genes whereas, the slightly smaller PBCV-1 genome has 11 tRNA genes and ∼ 375 protein-encoding genes. Surprisingly, the two viruses only have 33 genes in common, of which 17 have no counterparts in the databases. The low number of homologous genes between the two viruses can probably be attributed to their different life styles. PBCV-1 is a lytic virus that infects a unicellular, endosymbiotic freshwater green alga whereas, EsV-1 is a lysogenic virus that infects a free-living filamentous marine brown alga. Furthermore, accumulating evidence indicates that the phycodnaviruses and their genes are ancient, thus allowing significant differences to have evolved. This review briefly describes some of the biological properties of the phycodnaviruses, focusing on PBCV-1 and EsV-1, and then compares their genomes.

Introduction

The largest virus genome sequenced to date was described recently, 335,593 bp from *Ectocarpus siliculosus* virus (EsV-1) that infects the marine filamentous brown alga, *E. siliculosus* [23]. The EsV-1 genome is ∼ 5 kb larger than the 330,744 bp genome of another algal virus, *Paramecium bursaria* chlorella virus

(PBCV-1). Both viruses are members of the family *Phycodnaviridae*. Members of this family share icosahedral morphology, an internal lipid membrane and large dsDNA genomes of 160 to 380 kb. However, larger dsDNA viruses are known. Some recently discovered marine algal viruses, tentatively assigned to the *Phycodnaviridae*, have genomes as large as $\sim$ 560 kb [66, 146]. Currently, four genera are included in the phycodnavirus family: *Chlorovirus*, *Prasinovirus*, *Prymnesiovirus*, and *Phaeovirus* [171]. These genera are distinguished by the taxonomic affiliation of the algal host infected by the viruses. PBCV-1 and related chlorella viruses comprise the genus *Chlorovirus*. EsV-1 and other brown algal viruses are assigned to the genus *Phaeovirus*.

The EsV-1 genome is predicted to contain 231 protein encoding genes whereas, the slightly smaller PBCV-1 genome contains 11 tRNA genes and $\sim$ 375 protein encoding genes. To put these numbers in prospective, 250–350 protein encoding genes is the estimated minimal number required to support life [62, 63, 117]. Surprisingly, the two phycodnaviruses only have 33 genes in common. Thus most viral genes are unique to one of the two viruses. Among these 33 common protein-coding open reading frames (ORFs), 17 have no counterparts in the databases. Despite these large genetic differences, phylogenetic analyses of their DNA polymerases [15, 87] indicate that all large algal viruses, including viruses that infect *Micromonas pusilla* (genus *Prasinovirus*) and *Chrysochromulina brevifilum* (genus *Prymnesiovirus*), are more closely related to each other than to other dsDNA viruses.

Several properties may contribute to the low number of homologous genes between PBCV-1 and EsV-1. First, three observations indicate that the phycodnaviruses have a long evolutionary history. i) Phylogenic analyses of DNA polymerases place the phycodnavirus enzymes near the root of all eukaryotic δ DNA polymerases [181, 182]. ii) Despite the fact that PBCV-1 and EsV-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) throughout their respective genomes are remarkably uniform. This pattern suggests that the genes have existed in the viruses for a long time. iii) 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 [e.g. 50, 147].

Second, even though the chlorella viruses and brown algal viruses have many common features, they have significant biological differences. i) The chlorella viruses infect their unicellular host by digesting the cell wall at the point of attachment, similar to bacteriophages (Fig. 1D, E). In contrast, EsV-1 and its relatives bind to the plasmalemma of the host's spores or gametes that lack a cell wall [98, 99]. ii) The 320- to 380-kb chlorella virus genomes are linear, nonpermuted molecules with cross-linked hairpin ends. The structure of the brown algal virus genomes, however, remains ambiguous. Electron microscopy and restriction analysis indicate that the genomes of EsV-1, the *Feldmannia* species

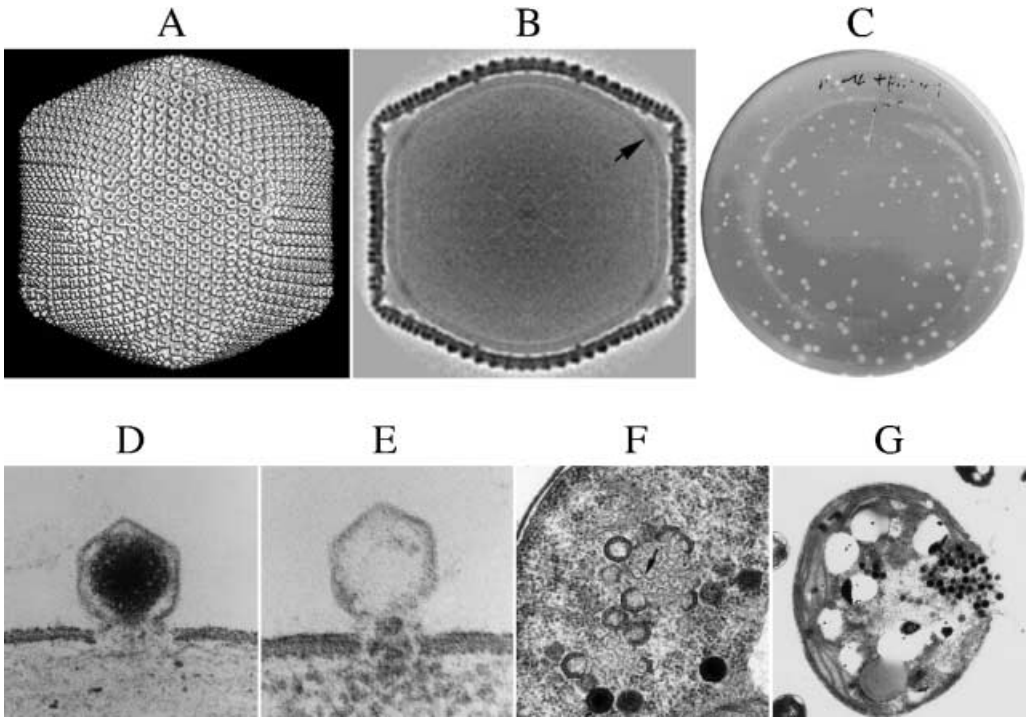


Fig. 1. A Three-dimensional reconstruction of the 190 nm PBCV-1 virion from electron micrographs of vitrified virus particles. **B** Cross-section of a three dimensional reconstruction in which the virus internal membrane is visible. **C** PBCV-1 plaques on a lawn of *Chlorella* NC64A. **D–G** PBCV-1 infection of *Chlorella* NC64A. **D** Digestion of the wall at the point of virus attachment. **E** The contents of the virus are released into the cell. **F** Cytoplasmic virus assembly centers at 3–4 hr p.i. **G** Release of progeny virus by lysis of host cell at 6–8 hr p.i. Photographs **D** and **E** were reproduced from Meints et al. [104], photograph **F** from Meints et al. [105], and photograph **G** from Meints et al. [106]; all reproduced with permission from Academic Press, Inc

virus (FsV-1) and the *Feldmannia simplex* virus (FlexV-1) are circular [37, 64, 84]. Sequencing of the EsV-1 genome, however, revealed a linear contig with nearly identical inverted terminal repeat regions [23]. Circularity could indicate non-covalent linkage of the inverted terminal repeats. iii) 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 of their hosts [115]. In contrast, the chlorella viruses have no obvious lysogenic phase, although a carrier- or pseudolysogeny-state has been observed in the laboratory on occasion [176].

Taken together, these properties indicate that the phycodnaviruses and their genes have a long evolutionary history. Consequently, studies on these viruses may reveal interesting aspects about the evolution of genes and genomes. This review will describe some of the biological properties of the chlorella viruses and brown algal viruses and then compare their genomes.

Chlorella viruses

The chlorella viruses are large (1900 Å in diameter, Fig. 1A) icosahedral, plaque-forming (Fig. 1C), dsDNA viruses that infect certain unicellular, chlorella-like green algae [176, 177]. Currently, the genus consists of three species. i) Viruses that infect *Chlorella* isolate NC64A (NC64A viruses) have been isolated from freshwater collected in the United States [174], China [201], Japan, Brazil [196], Argentina, Australia, Israel, and Italy (Van Etten, unpublished results). ii) Viruses that infect *Chlorella* Pbi initially were discovered in freshwater collected in Europe [141], and more recently in water collected in Australia, Canada, and the northern United States and at higher altitudes in the western United States (Nelson and Van Etten, unpublished results). Typically, the Pbi and NC64A virus titer in indigenous waters is 1 to 100 PFU per ml but titers as high as 100,000 PFU per ml have been obtained (Nelson and Van Etten, unpublished results). Virus concentrations are not static, but fluctuate with the seasons. Typically, the highest titers are found in the spring [180, 196]. Although the NC64A and Pbi viruses are morphologically, biologically and biochemically similar, two features distinguish them from one another. i) Viruses that infect *Chlorella* NC64A neither infect nor attach to *Chlorella* Pbi, and vice versa. ii) The G+C content of the NC64A and Pbi virus genomes are about 40% and 46%, respectively, indicating considerable evolutionary separation.

Chlorella NC64A and *Chlorella* Pbi are normally hereditary endosymbionts in green isolates of the protozoan *Paramecium bursaria*. In the symbiotic unit, individual algae are surrounded by a host-derived membrane [139]. The endosymbiotic chlorella, also called zoochlorella, are resistant to virus infection and are only infected when they are separated from the ciliate [140]. Fortunately, both *Chlorella* species can be grown in the laboratory free of the paramecium and virus.

Members of the third species of Chloroviruses infect symbiotic chlorella in the coelenterate *Hydra viridis* [178]. These lytic viruses also have icosahedral morphology and large dsDNA genomes. However, the chlorella host has not been cultured free of the virus and consequently, the virus only can be isolated from chlorella cells freshly released from hydra.

Structure of chlorella viruses

Chlorella virus particles are complex and the PBCV-1 virion contains at least 50 different proteins [154]. The most abundant protein is the major capsid glycoprotein Vp54. A three-dimensional image reconstruction of PBCV-1 indicates that Vp54 is arranged in homotrimeric capsomers that cover the surface of the virus. The capsomers are doughnut-shaped ($\sim$ 70 Å in diameter and $\sim$ 75 Å high) and most contain axial channels of $\sim$ 17 Å in diameter (Fig. 1A). Assuming all hexavalent capsomers are identical chemically, the outer capsid of the virus contains 5,040 copies of Vp54, associated as 1,680 trimers [199]. The structure of the 12 capsomers at the virus vertices differ slightly from the structure of the hexavalent capsomers and probably are composed of a different protein.

A lipid bilayer membrane located inside the outer capsid shell (Fig. 1B) [199] is required for infectivity [154]. The source of this membrane is unknown. Chlorella virus genomes are linear molecules exceeding 330 kb with terminal inverted repeat regions and incompletely base-paired, covalently closed hairpin ends [144, 203]. An unusual feature of chlorella virus genomes is the presence of both 5-methylcytosine (5mC) and N^6-methyladenine (6mA) [179]. In fact, viruses can be distinguished from one another by the site-specificity and amount of DNA methylation [176, 177].

PBCV-1 life cycle

PBCV-1 infects its host by attaching rapidly and irreversibly to the external surface of the algal cell wall (Fig. 1D) [104]. Attachment always occurs at a virus vertex and is followed by degradation of the host wall at the attachment point and entry of the viral DNA and probably associated proteins (e.g. transcription factors) into the cell (Fig. 1E). Circumstantial evidence indicates the viral DNA and putative DNA-associated proteins quickly move to the nucleus where early transcription can be detected within 5–10 min p.i. [148]. Virus DNA replication begins 60–90 min after infection and is followed by transcription of late virus genes [148, 172]. Host nuclear and chloroplast DNAs are degraded beginning $\sim$ 60 min p.i.; however, because of extensive viral DNA synthesis, total DNA in the cell increases 4- to 10-fold by 4 hr p.i. [172]. Ultrastructural studies of PBCV-1 infected chlorella suggest that the nuclear membrane remains intact, at least during early stages of replication [105]. However, virus replication does not require a functional host nucleus because PBCV-1 replicates, albeit poorly and with a small burst size, in UV-irradiated cells [173]. Approximately 2 hr p.i., capsid assembly begins in localized regions in the cytoplasm, called virus assembly centers, which become prominent at 3–4 hr p.i. (Fig. 1F) [105]. By five hr p.i., the cytoplasm is filled with infectious progeny virus particles ($\sim$ 700 particles/cell) [175] and by 6–8 hr p.i. lysis of the host cell releases progeny (Fig. 1G). About 50% of the progeny particles are infectious, i.e. each infected cell yields $\sim$ 350 plaque-forming units (PFU) [175].

PBCV-1 DNA sequence analysis

The sequence of the 330,744-bp PBCV-1 genome revealed 700 ORFs of 65 codons or larger. About 375 of these ORFs probably encode proteins. PBCV-1 also encodes 11 tRNA genes [79, 91, 92, 96, 97]. Four PBCV-1 ORFs reside in the 2.2 kb inverted terminal repeat region of the PBCV-1 genome; consequently these 4 ORFs are duplicated in the PBCV-1 genome [96, 162]. The 375 putative protein-encoding genes are evenly distributed on both strands and, with one exception, intergenic space is minimal. The exception is a 1788-bp sequence near the middle of the genome. This DNA region, which contains many stop codons in all reading frames, contains the 11 tRNA genes.

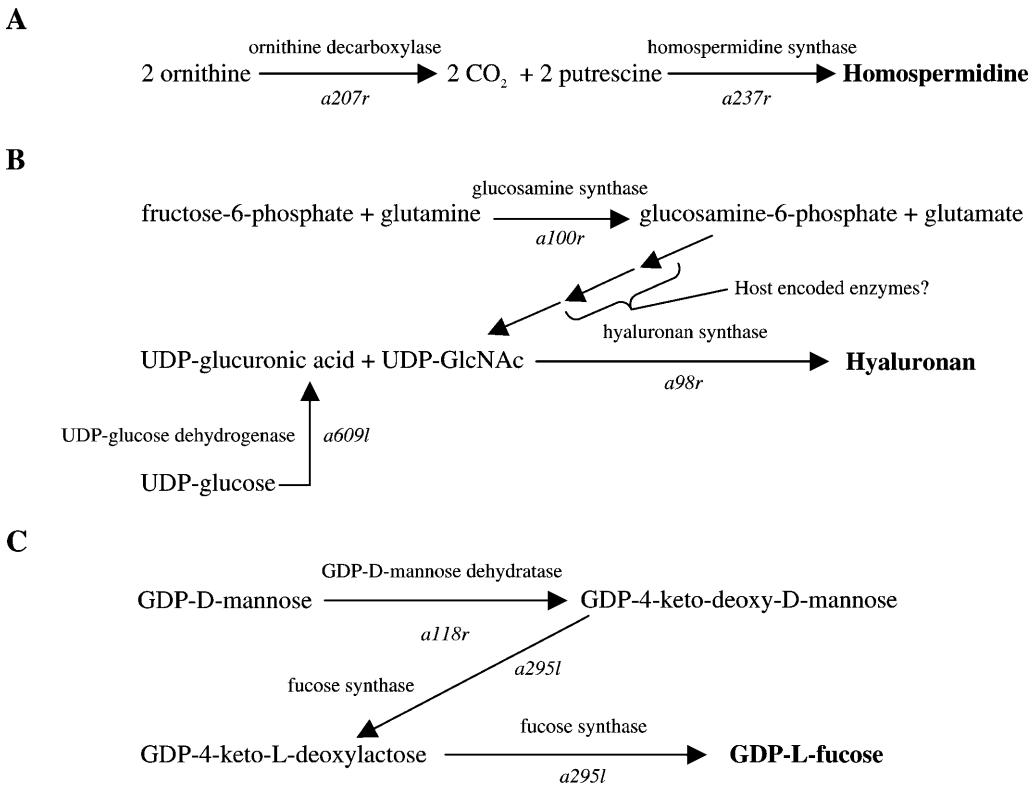


Fig. 2. Biosynthetic pathways encoded by chlorella virus PBCV-1. **A** Homospermidine, **B** hyaluronan (also called hyaluronic acid) and **C** GDP-L-fucose. The PBCV-1 encoded enzymes are indicated. Recombinant proteins have been produced and shown to be biochemically active for the three pathways

Approximately 50% of the 375 PBCV-1 gene products have been tentatively identified; they consist of both prokaryotic- and eukaryotic-like proteins, many of which have not previously been seen in viruses. Some PBCV-1 encoded enzymes can be assembled into metabolic pathways, e.g. polyamine biosynthesis (Fig. 2A), hyaluronan biosynthesis (Fig. 2B) and fucose synthesis (Fig. 2C). Surprisingly, given the large number of PBCV-1 genes, the virus lacks a recognizable RNA polymerase gene. Therefore, PBCV-1 must depend on at least some host enzymes for transcription.

Chlorella virus encoded proteins

Many PBCV-1 encoded enzymes are either the smallest or among the smallest proteins of their class. In addition, genes shared by chlorovirus isolates may differ in nucleotide sequence by as much as 40%, which translates into amino acid sequence differences of 25 to 30%. 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 “user friendly” in the laboratory have resulted in the biochemical and structural characterization of several PBCV-1 enzymes.

Examples include: i) The smallest known eukaryotic ATP-dependent DNA ligase [58], which is the subject of intensive mechanistic and structural studies [129, 130, 157–159]. ii) The smallest known type II DNA topoisomerase [85]. The PBCV-1 enzyme cleaves dsDNAs about 30 times faster than the human type II DNA topoisomerase [35]; consequently, the virus enzyme is being used as a model enzyme to study the topoisomerase II DNA cleavage process. iii) An RNA capping guanylyltransferase [57] that was the first enzyme of its type to have its crystal structure resolved [48, 49]. iv) The smallest known protein (94 amino acids) to form a functional voltage-gated K^+ channel [135].

Genetic variability

Several years ago 37 NC64A viruses were grouped into 16 classes on the basis of plaque size, virion antiserum sensitivity, DNA restriction patterns, sensitivity of the DNAs to restriction endonucleases and the nature and abundance of methylated bases in their DNAs [176]. For example, the levels of methylated bases in the NC64A virus genomes range from 0.1% 5 mC and undetectable levels of 6 mA to 45% 5 mC and 37% 6 mA. There is no obvious correlation between the classification of viruses and their geographic distributions. For example, four of six plaques originally picked from a water sample collected in New York fell into different classes. In other collection sites, most of the viruses belong to a single class.

We routinely hybridize individual PBCV-1 genes with DNAs from these 37 NC64A viruses. Interestingly, not every PBCV-1 gene is present in all NC64A viruses. However, in general, DNA hybridization patterns support the above classification scheme. That is, if one member in a class lacks a particular gene, other members of the class also lack the gene; e.g., see the hyaluronan synthase gene [46]. Therefore, not all chlorella viruses encode the same genes; consequently the total virus gene pool exceeds that of a single virus.

Other unique features of the chlorella viruses

In addition to their large genomes, chlorella viruses have other distinctive features: i) they encode multiple type II DNA methyltransferases and DNA site-specific (restriction) endonucleases (see below). ii) PBCV-1 has three types of introns: a self-splicing intron in a transcription factor TFIIS-like gene [91, 198], a spliceosomal-processed intron in the DNA polymerase gene [44, 200], and a small intron in one of the tRNA genes [125]. iii) Unlike other glycoprotein-containing viruses, PBCV-1 encodes most, if not all, of the components required to glycosylate its proteins (see below).

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/index.htm

Brown algal viruses

E. siliculosus is just one of many marine filamentous brown algal species and each of eight species that have been examined contains its own phaeovirus [115].

EsV-1, the type phaeovirus, is pandemic in *E. siliculosus* populations on the coasts of all oceans in the temperate climate zones of both hemispheres [116, 149]. Laboratory studies indicate that some phaeoviruses can infect hosts on interspecific, intergeneric and even interordinal levels, opening the possibility of gene transfer between eukaryotic hosts [115]. The phaeovirus particles, 1300–1800 Å in diameter, consist of an electron-dense core surrounded by a mono- or bilayered shell. Like chlorella viruses, they also have an internal lipid-containing bilayered membrane. Although the origin of chlorovirus membranes is unknown, phaeovirus membranes are derived from the endoplasmic reticulum (Fig. 3E, 3G) [188–190]. The phaeoviruses possess large dsDNA genomes, ranging in size from

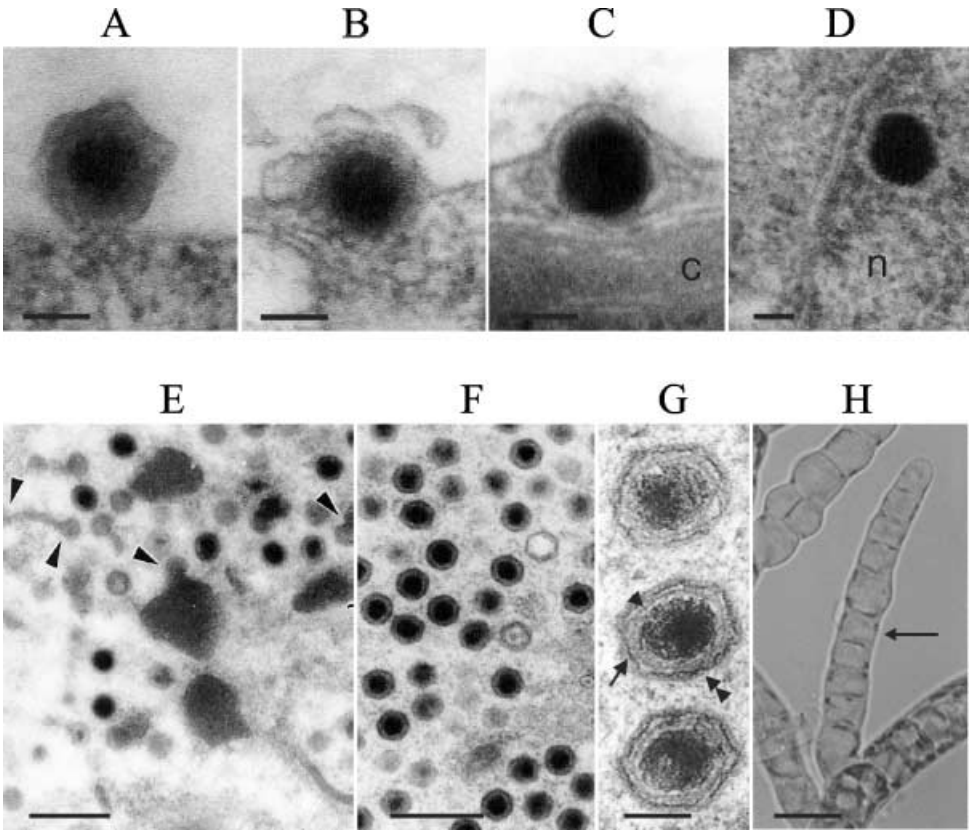


Fig. 3. A–D. Infection of *Ectocarpus fasciculatus* spores by EfasV-1. **A** Virus fusion with the host cell membrane. **B**, **C** Capsid release and internalization of the viral nucleoprotein core. **D** Viral nucleoprotein core in the nucleus. Scale bars **A–D**: 0.1 μm. **E–H**. EsV-1 assembly in *Ectocarpus siliculosus*. **E** Virus capsids are detached from storage bodies and modified endoplasmic reticulum (arrowheads). **F** Late stage of assembly with densely packed mature virions. Scale bar **E–F**: 0.5 μm. **G** Virus particles showing a nucleoprotein core surrounded by a core shell containing a lamina (arrowhead), a membrane layer inherited from the endoplasmic reticulum (arrow) and a capsid layer (double arrowhead). Scale bar: 0.1 μm. **H** Infected gametophyte with abnormal plurilocular structures (arrow). Scale bar: 25 μm. From Mueller [115], Wolf [188] and Maier et al. [99]

160 kb for FsV-1 to 340 kb for MclaV-1 [115]. In contrast to the lytic chlorella viruses, phaeoviruses are lysogenic.

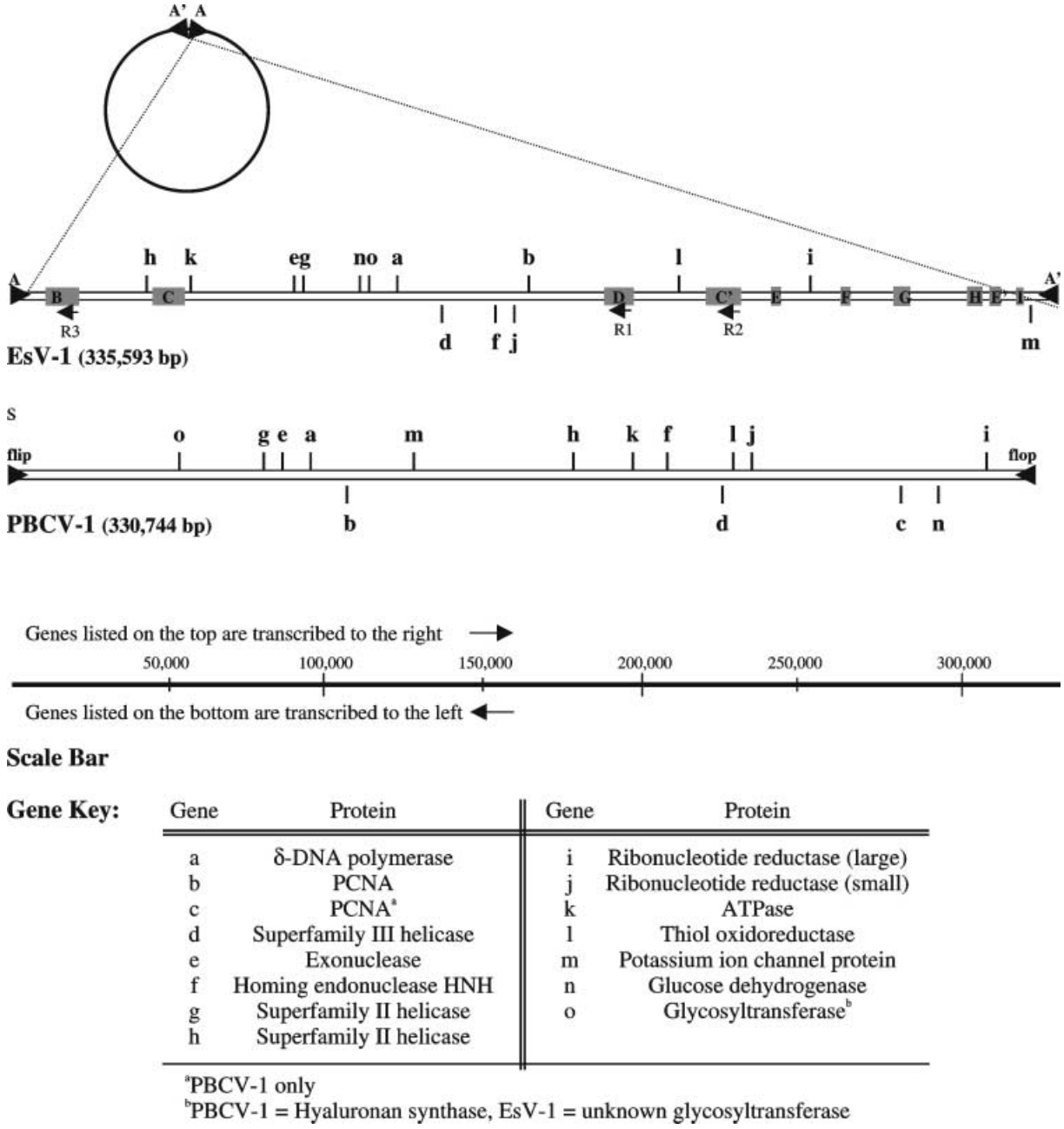
The viruses initiate their life cycle by infecting free-swimming, wall-less gametes or spores of their host. Phaeovirus particles enter the cells by fusion with the host cell plasma membrane (Fig. 3A–C) and release a nucleoprotein core particle into the cytoplasm, leaving remnants of the outer capsid at the surface. Within five min p.i., virus core particles are seen in the nucleus (Fig. 3D) [99]. The viral DNA becomes integrated into the host genome and is transmitted to all cells of the developing filamentous alga [10, 22, 113]. The viral genome remains latent in vegetative cells, but is expressed in the reproductive algal cells, the sporangia and gametangia. Massive replication of viral DNA occurs in hypertrophic nuclei and is followed by nuclear breakdown and viral assembly which continues until the cell becomes densely packed with virus particles (Fig. 3E–H) [115, 189, 190]. The same factors that induce the discharge of spores or gametes from the host, e.g. changes in temperature, light and water composition [114], also stimulate release of the phaeoviruses. This synchronization facilitates interaction of viruses with susceptible host cells.

EsV-1 virion structure

EsV-1 virions contain more than 13 proteins ranging in molecular mass from $\sim$ 20 to $>$ 150 kDa. The major capsid protein has a predicted molecular weight of 53.2 kDa. At least 3 of the virion proteins are glycosylated including glycoprotein-1 (gp1) which has been characterized [72]. Recent homology searches indicate that gp1 resembles bacterial alginate mannuronan C-5-epimerases (see below). Two other viral proteins, vp55 and vp74, are components of the EsV-1 nucleoprotein core [24]. Surprisingly, another virion constituent resembles plant and bacterial histidine protein kinases, elements of two-component signal transduction pathways. This is the first report of a His-protein kinase in a viral system [25].

EsV-1 genome structure

Pulsed field electrophoresis, DNA restriction mapping and electron microscopy experiments initially suggested that the EsV-1 genome was a 330-kb circular molecule with multiple single-stranded regions, some of which were site-specific [73, 84]. However, the DNA sequencing results produced an enigma. Alignments of overlapping DNA fragments produced one 335-kb linear contig with termini containing almost perfect inverted repeats of 1.8 and 1.6 kb [23]. Attempts to link the termini (i.e. to complete a circle) by PCR using primers corresponding to sequences within the inverted repeat regions were unsuccessful. Thus, the inverted repeats apparently mark the ends of a linear molecule. These apparent conflicting results on the genome structure can be resolved if the complementary sequences in the right inverted terminal repeat (ITR-A) anneal with the left ITR-A$'$ to form a cruciform structure which effectively closes the DNA circle (Fig. 4) [23].



Gene Key:

Gene	Protein	Gene	Protein
a	δ-DNA polymerase	i	Ribonucleotide reductase (large)
b	PCNA	j	Ribonucleotide reductase (small)
c	PCNA[a]	k	ATPase
d	Superfamily III helicase	l	Thiol oxidoreductase
e	Exonuclease	m	Potassium ion channel protein
f	Homing endonuclease HNH	n	Glucose dehydrogenase
g	Superfamily II helicase	o	Glycosyltransferase[b]
h	Superfamily II helicase		

[a]PBCV-1 only
[b]PBCV-1 = Hyaluronan synthase, EsV-1 = unknown glycosyltransferase

Fig. 4. Map of EsV-1 and PBCV-1 genomes indicating the location of 14 common genes (shown as letters a–n above and below the map). Genes listed above the map are transcribed right to left and genes listed below the map are transcribed from left to right. Note: the genes are not co-linear. Repetive elements in the EsV-1 genome are indicated as letters in shaded regions of the map

Comparison of PBCV-1 and EsV-1 genomes

Several properties distinguish the PBCV-1 and EsV-1 genomes. i) Although the EsV-1 genome resembles the PBCV-1 genome in having inverted terminal repeats, the sequence of the PBCV-1 inverted repeat regions (2.2 kb) are 100% identical [162] whereas, the EsV-1 terminal repeats contain short non-identical regions [23]. ii) The PBCV-1 genome has cross-linked hairpin ends consisting of 35 incompletely paired nucleotides [144, 203]. The terminal structure of the EsV-1 genome is unknown; however, the ends are probably not cross-linked. iii) In addition to the terminal regions, the EsV-1 genome contains large regions of repeated sequences (Fig. 4). Collectively these repeats occupy $\sim$ 12% of the EsV-1 genome. An additional 22% of the EsV-1 genome has no apparent coding potential, such as the $\sim$ 11 kb region H (Fig. 4) [23]. Thus, only $\sim$ 67% of the EsV-1 genome contains protein encoding genes. The presence of these numerous repeats could account for size variation among phaeovirus genomes. The brown algal virus FsV-1, infecting a *Feldmannia* sp., also contains 173-bp direct repeats which comprises 12% and 6% of the large and small viral genomes, respectively [86]. Another latent form of the FsV genome contains a $>$ 50 kb region of small repeated sequences that interrupts an ORF encoding a Ser/Thr protein kinase [88]. In contrast, except for the terminal inverted repeat regions, the PBCV-1 genome consists primarily of single copy DNA. iv) EsV-1 does not encode tRNAs whereas, PBCV-1 encodes 11 tRNAs. v) The EsV-1 genome appears to lack introns. In contrast, the PBCV-1 genome contains three types of introns. vi) The PBCV-1 genome has a G+C content of 40% whereas, the EsV-1 genome is 52% G+C. vii) Methylated bases are common in chlorella virus genomes whereas, EsV-1 DNA contains only a low level of methylated bases [84].

Comparison of EsV-1 and PBCV-1 genes

Putative protein-encoding genes for PBCV-1 and EsV-1 were identified by similar criteria, namely a minimal size of 65 codons initiated by an ATG codon and AT-rich ($>$ 70%) sequences in the 50 bp upstream of the putative initiation codons. As indicated above, one surprising finding was that the two viruses only have 33 common genes (Table 1). Of these 33 gene products, 17 have no counterparts in databases. Both viruses contain common genes that encode proteins that have domains found in proteins with variable functions such as transcription factors, structural proteins, and chaperones. The amino acid identity between these 33 common proteins ranges from 22 to 47%. For example, the major capsid proteins from the two viruses have 28% amino acid identity. There is no evidence of colinearity of the common genes between the two viruses (Fig. 4).

DNA replication, recombination, and repair proteins

Five of the common proteins in PBCV-1 and EsV-1 are involved in DNA replication or recombination. In a pairwise comparison, the PBCV-1-encoded DNA polymerase has 32% amino acid identity to EsV-1 DNA polymerase. The enzymes

Table 1. Selected putative ORFs encoded by chlorella virus PBCV-1[a] and brown algal virus EsV-1. ORFs in common are shaded

	PBCV-1	EsV-1
DNA Replication, Recombination and Repair		
δ–DNA polymerase	(E) A185R (913)	93 (1046)
ATP-dependent DNA ligase	(E) A544R (298)*	—
DNA topoisomerase type II	(E) A583L (1061)*	—
PCNA	A193L (262)	132 (301)
" "	A574L (264)	—
RNase H	A399R (194)	—
Replication factor C-Archae large subunit	—	138 (402)
Replication factor C-small subunit 1	—	87 (324)
Replication factor C-small subunit 2	—	182 (326)
Replication factor C-small subunit 3	—	187 (324)
Replication factor C-small subunit 4	—	224 (324)
Replication factor C-Archae large subunit	A417L (429)	—
Superfamily III helicase	A456L (654)	109 (606)
Rec BCD-like helicase	—	29 (675)
Exonuclease	—	126 (206)
Exonuclease	(E) A166R (268)*	64 (208)
Pyrimidine dimer-specific glycosylase	(E,L) A50L (141)*	—
Integration and Transposition		
Integrase	—	213 (786)
Protelomerase	—	175 (350)
Anti-repressor of lysogeny	—	117 (524)
Repressor of lysogeny	—	197 (458)
Transposase	—	155 (383)
" "	—	170 (383)
" "	A625R (433)	—
Homing endonuclease GIY-YIG	A163L (165)	—
" "	A287R (251)	—
" "	A315L (246)	—
" "	A351L (358)	—
" "	A495R (221)	—
" "	A539R (173)	—
" "	A651L (230)	—
Homing endonuclease HNH	A422R (342)	119 (427)
" "	A87R (456)	
" "	A267L (314)	—
" "	A478L (310)	——
" "	A490L (310)	—
" "	—	16 (188)
" "		
DNA Methyltransferases and Site-specific Endonucleases		
Cytosine DNA methylase	(E) A517L (344)*	—
Cytosine DNA methylase	(L) A530R (335)*	—
Cytosine DNA methylase	A683L (367)	—
Adenine DNA methylase	A251R (326)*	—
DNA site-specific endonuclease	A252R (342)*	—
Adenine DNA methylase	(E) A581R (265)*	—
DNA site-specific endonuclease	(E) A579L (183)*	—
Transcription		
Transcription factor IIB	(E) A107L (290)	—
Transcription factor IID	(E) A552R (270)	—
Transcription factor IIS	A125L (180)	—
VLTF2-Type transcription factor	A482R (215)	96 (294)
RNA triphosphatase	A449R (193)*	—
RNA guanylyltransferase	(E) A103R (330)*	—
Superfamily II helicase	A153R (459)	66 (477)
" "	A363R (811)	23 (612)
SWI/SNF helicase	A548L (458)	—
Ski1 helicase	A241R (725)	—
RNase III	A464R (245)	—
Oligoribonuclease	—	139 (274)

	PBCV-1	EsV-1
Nucleotide Metabolism		
Aspartate transcarbamylase	(E) A169R (323)*	—
Ribonucleotide reductase large subunit	A629R (771)	180 (765)
Ribonucleotide reductase small subunit	A476R (324)	128 (419)
Thioredoxin	A427L (119)	—
Glutaredoxin	A438L (78)	—
dUTP pyrophosphatase	A551L (141)	—
dCMP deaminase	A596R (142)	—
Thymidylate kinase	A416R (188)	—
Nucleotide triphosphatase	A326L (209)	—
Cytidine deaminase	A200R (118)	—
Thymidylate synthase complement	A674R (216)	—
ATPase	A392R (258)	26 (290)
" "	A554L (271)	—
Protein Synthesis, Modification, and Degradation		
Translation elongation factor-3	A666L (918)	—
Prolyl-4-hydroxylase	(L) A85R (242)*	
Thiol oxidoreductase	A465R (118)	161 (180)
Protein disulfide isomerase	A448L (106)	—
Ubiquitin C-terminal hydrolyase	A105L (284)	—
SKP-1 protein	A39L (151)	—
Zn metallopeptidase	A604L (134)	—
Cysteine protease	—	75 (393)
Signaling		
Potassium ion channel protein	(E,L) A250R (94)*	223 (124)
Ligand-gated channel protein	A162L (411)	—
" "	A163R (433)	—
Ser/Thr protein kinase	A34R (308)	—
" "	(L) A248R (308)*	—
" "	A277L (303)	—
" "	A278L (610)	—
" "	A282L (569)	—
" "	A289L (283)	—
" "	A614L (577)	—
" "	—	82 (304)
" "	—	104 (284)
" "	—	111 (447)
" "	—	156 (317)
Tyr-protein kinase	A617R (321)	—
Tyr phosphatase	A305L (204)	—
Hybrid His-protein kinase	—	14 (573)
" "	—	65 (767)
" "	—	88 (461)
" "	—	112 (583)
" "	—	181 (933)
" "	—	186 (680)
Phosphoshuttle	—	113 (108)
Sugar Manipulating Enzymes		
Glucosamine synthase	(E) A100R (595)*	—
Glucose dehydrogenase	(E) A609L (389)*	83 (261)
Hyaluronan synthase	(E) A98R (568)*	84 (493)
GDP-D-mannose dehydratase	(E) A118R (345)*	—
Fucose synthase	(E) A295L (317)*	—
Alginate mannuronan epimerase	—	226 (661)
Mannosyltransferase	(E,L) A64R (638)	—
Glycosyltransferase	A111R (389)	—
Fucosyltransferase	A114R (485)	—
Glycosyltransferase	A222/226R (432)	—
Glycosyltransferase	A328L (355)	—
Glycosyltransferase	(E) A473L (517)	—
Glycosyltransferase	A546L (321)	—

(*continued*)

Table 1 (*continued*)

Cell Wall Degrading Enzymes	PBCV-1	EsV-1
Chitinase	(E) A181/182R (830)*	—
	(L) A260R (484)*	—
Chitosanase	(L) A292L (328)*	—
α-1,3-glucanase	(E) A94L (364)*	—
Alginate lyase	(E,L) A215L (321)*	—
Lipid Manipulating Enzymes		
Glycerophosphoryl diesterase	A49L (219)	—
2-Hydroxyacid dehydrogenase	A53R (363)	—
Lysophospholipase	A271L (159)	—
N-acetyltransferase	A654L (197)	—
Lipase	—	185 (257)
Miscellaneous		
	PBCV-1	**EsV-1**
Ornithine decarboxylase	(E,L) A207R (372)*	—
Homospermidine synthase	(L) A237R (518)*	—
Monomine oxidase	A217L (394)	—
O-methyltransferase	A61L (209)	—
α-alanine synthase	A78R (298)	—
Cu/Zn-superoxide dismutase	A245R (187)	—
Amidase	A284L (279)	—
Histidine decarboxylase	A598L (363)	—
BCS1 protein	A44L (599)	—
Thaumatin	—	169 (393)

Other Common Proteins		
PBCV-1	EsV-1	Feature
A564L (351)	7 (378)	—
A154L (347)	" "	—
A129R (358)	" "	—
A481L (224)	20 (333)	Ring finger
A408L (177)	42 (174)	—
A410L (110)	" "	—
A468R (443)	45 (566)	—
A14R (1369)	50 (422)	—
A400R (118)	59 (141)	—
A306L (86)	61 (89)	—
A623L (67)	73 (81)	AN1-like zinc finger
A312L (238)	76 (289)	—
A533R (374)	91 (551)	
A494R (360)	101 (320)	—
A324L (453)	103 (465)	—
A154L (347)	115 (528)	
(L) A430L (437)	116 (476)	Major capsid protein
(L) A622L (437)	" "	
(L) A11L (403)	" "	
(L) A558L (400)	" "	
(L) A10R (401)	" "	
A383R (301)	" "	
A470R (203)	137 (143)	—
A225L (90)	140 (275)	—
A471R (173)	141 (208)	—
A540L (1176)	160 (1207)	—

[a]The number in brackets refers to the number of codons in the ORF. *E* and *L* refer to early and late genes, respectively. An asterisk means that the gene encodes a functional enzyme as determined by either complementation or enzyme activity of a recombinant protein

belong to the DNA polymerase-δ family [182]. Each DNA polymerase contains a proof-reading 3′–5′ exonuclease domain [60]. EsV-1 also possesses an additional gene that could encode a proof-reading exonuclease (ORF 126). This putative exonuclease displays 30% amino acid identity with the ε-subunit of bacterial DNA polymerase III [23].

Both PBCV-1 and EsV-1 encode a sliding clamp processivity factor protein (PCNA). Surprisingly, PBCV-1 encodes two PCNA proteins that resemble PCNAs from other organisms more than each other (26% amino acid identity), suggesting independent acquisition of the two PCNA genes. PCNA not only interacts with proteins involved in DNA replication, but proteins involved in DNA repair and post-replicative processing such as DNA methylases and DNA transposases [184]. Because PBCV-1 encodes proteins involved in both DNA repair and DNA methylation (see below), the two PCNAs may serve different functions in the PBCV-1 life cycle.

EsV-1 encodes five proteins that collectively could form a heteropentameric replication factor C (RFC) complex; RFC is responsible for the ATP-dependent loading of PCNA onto DNA [32, 112]. RFCs from eukaryotes have a heteropentameric structure. The clamp loading RFC complexes from bacteria and Archae contain five different peptides and two different peptides, respectively [71, 134]. Surprisingly, the four EsV-1 small subunit proteins resemble those of eukaryotes whereas the large subunit resembles an Archae subunit. In contrast to EsV-1,

PBCV-1 encodes a single protein that resembles one of the two proteins that comprise the Archae RFC complex (Fitzgerald, Griep, and Van Etten, unpublished results). PBCV-1, but not EsV-1, encodes other proteins involved in DNA replication including an ATP-dependent DNA ligase [58], a type II DNA topoisomerase [35, 85], and RNase H (Van Etten, unpublished results).

Thus each virus encodes genes for essential elements of eukaryotic DNA replication, but neither virus encodes a full complement of DNA replication proteins. In particular, both viruses lack a DNA primase gene. Primase initiates synthesis of new DNA strands by synthesizing short RNA oligomers on ssDNA [1]. However, both viruses encode a protein (ORF 109 in EsV-1 and A456L in PBCV-1) which displays amino acid sequence similarity to a special class of trifunctional primase–helicase-origin recognition proteins (Griep, personal communication). The helicase portion of the two viral proteins resembles the superfamily III helicases. Therefore, one predicts that these two viral proteins also participate in viral DNA replication.

Both viruses encode a 5′–3′ exonuclease homolog (ORF 64 in EsV-1 and A166R in PBCV-1) that is probably involved in DNA recombination [12]. EsV-1 also encodes a putative helicase (ORF 29) that is related to the RecD subunit of the bacterial RecBCD enzyme involved in recombination [118]. 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 [38]. 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 [102]. The PBCV-1 PDG also is more processive than the T4 enzyme and is the first *trans-syn-II* glycosylase identified to date.

DNA methyltransferases and site-specific endonucleases

Chlorella virus genomes contain different levels of 5 mC and 6 mA [179]. Therefore, it is not surprising that these viruses encode multiple 5 mC and 6 mA DNA methyltransferases (Mtases). However, we were surprised to discover that $\sim$ 25% of the virus-encoded DNA Mtases have a companion DNA site-specific (restriction) endonuclease. Thus, virus-infected chlorella are a source of type II DNA restriction endonucleases and the first nonprokaryotic source [for reviews see 123, 124]. Some of the virus-encoded endonucleases have novel recognition sites, e.g. R.CviJI cleaves RG/CY sites [192]; other endonucleases cleave only one strand of dsDNA, e.g. NYs1-nickase cleaves/CC sites [193] and NY2A-nickase cleaves R/AG sites [202]. Like bacterial restriction endonucleases, the virus endonucleases do not cleave DNA with methylated nucleotides in their recognition sites.

Other characteristics of the chlorella virus restriction-modification (R-M) systems can be summarized as follows: i) Some viruses, e.g. NY-2A, encode as many as 10 distinct DNA Mtases (seven 6 mA Mtases and three 5 mC Mtases) and at least two site-specific endonucleases [202]. ii) Viruses with high levels of methylated

bases encode some Mtases that recognize short (2- to 3-bp) target sites, including M.*Cvi*PI from virus NYs-1, which methylates GC sequences [194]. iii) Like bacterial type II R-M genes [187], the chlorella virus R-M genes are located near one another, although the spacing and relative orientation of the two genes can vary. iv) The virus 6 mA- and 5 mC-Mtases contain the same conserved amino acid motifs found in bacterial 6 mA Mtases [100] and 5 mC Mtases [78, 136].

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. i) Chlorella virus endonucleases help degrade host DNA, thus providing deoxynucleotides for recycling into virus DNA. Methylation of nascent virus DNAs by the cognate Mtases protects the DNA from self-digestion. ii) Chlorella virus endonucleases prevent infection of a cell by a second virus. However, experimental tests of these two hypotheses have yielded ambiquous results [11, 14]. In addition, the fact that, under laboratory conditions, at least some of the R-M genes can be deleted without affecting viral replication in the chlorella host [11] makes it difficult to ascertain the role(s) of these enzymes in the viral life cycle.

In contrast to the chlorella viruses, EsV-1 lacks a DNA Mtase gene even though EsV-1 DNA contains a low level of methylated nucleotides [84]. However, the EsV-1 genome does encode 4 other putative viral nucleases (ORFs 16, 119, 139, and 168) including a thermostable nuclease (ORF 168) and an oligoribonuclease (ORF 139, see below). The EsV-1 nucleases may help degrade host cell nucleic acid upon induction of EsV-1 replication in the reproductive cells of the infected host.

Integration

One feature of the EsV-1 life cycle is a long latency period after infection when the viral genome is mitotically transmitted from cell to cell during host development. Studies with EsV-1 [22] and FsV-1 [177] indicate that the viral DNAs are integrated into their host genomes. EsV-1 encodes a large protein (ORF 213) with a C-terminal 200-amino acid region that resembles the catalytic domain of the integrase family of site-specific recombinases. Integrases cooperate with other proteins to integrate and excise large DNAs from host genomes [83]. The EsV-1 integrase has the two domains characteristic of bacteriophage-type integrases including 4 conserved amino acid residues Arg, His, Arg and Tyr [128]. Another EsV-1 protein (ORF 175) contains a putative Int-catalytic site with the same 4 conserved amino acids residues and shares similarity with the *Borrelia burgdorferi* cp26 protein and the bacteriophage N15 protelomerase. This latter enzyme is responsible for linearizing bacteriophage N15 plasmid DNA [26]. The EsV-1 protein might be part of a pathway that converts circular EsV-1 DNA into linear DNA, and visa versa. Two additional EsV-1 proteins (ORFs 117 and 197) which resemble bacteriophage regulators of lysogeny may aid in maintaining the EsV-1 latent state.

Transposons

The EsV-1 genome has three large dispersed repeat regions, termed R1, R2, and R3 (Fig. 4), that contain ORFs which presently are not counted as genes because they lack upstream AT-rich sequence elements; also these ORFs do not match any genes in the databases. Interestingly, repeats R1 and R2 are located downstream of genes encoding putative bacterial IS4 family transposases (IS, insertion sequence) [143]. The R1- and R2-transposase units are bracketed by imperfect inverted repeats of 20 bp which resemble the ends of bacterial IS4 elements. Furthermore, R1 and R2 frame a genomic 29 kb segment forming a transposon-like structure. Like bacterial transposons, the EsV-1 transposon could confer advantages to the host. For example, the putative EsV-1 transposon contains a gene (*orf169*) encoding a putative pathogenesis-related 5 protein (PR5), also called thaumatin-like protein, one of the factors known to be involved in plant defense against fungi [59].

PBCV-1 also encodes a protein (ORF A625R) that resembles bacterial transposases; however, the PBCV-1 ORF differs from the two putative EsV-1 encoded transposases. In addition, PBCV-1 encodes 12 ORFs that contain motifs common to homing endonucleases [76]. Homing endonucleases, which are rare DNA-cleaving enzymes typically encoded by introns and inteins, are classified into four families [4]. Seven of the PBCV-1 ORFs are members of the GIY-YIG family and five are members of the HNH family. It is unknown if the putative PBCV-1 encoded transposase and homing endonucleases are functional or if they play an essential role in the virus life cycle. EsV-1 also encodes two ORFs with an endonuclease HNH motif (ORF 16 and ORF 119). In total, including the enzymes involved in DNA recombination mentioned above, both viruses encode several proteins that could fascilitate DNA rearrangements either within or between virus, and possibly host, genomes.

Transcription-associated proteins

Neither virus encodes a recognizable RNA polymerase nor RNA polymerase component. The lack of a virus-encoded RNA polymerase supports the notion that the 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. For example, PBCV-1 encodes at least four transcription factor-like elements, TFIIB (A107L), TFIID (A552R), TFIIS (A125L), and a VLTF-2 type transcription factor (A482R). EsV-1 also encodes an A482R homolog, two subunits (ORFs 193 and 196) of the eukaryote transcription complex TFIID, and several proteins with domains found in transcription factors (see below). In addition, PBCV-1, but not EsV-1, encodes at least two enzymes involved in forming a mRNA cap structure, an RNA triphosphatase [56] and an RNA guanylyltransferase [57]. The size, amino acid sequence, and biochemical properties of the PBCV-1 enzymes are more closely related to yeast enzymes than to poxvirus multifunctional RNA capping enzymes [152]. PBCV-1 also encodes

an RNase III that is presumably involved in processing of virus mRNAs. The protein encoded by EsV-1 ORF 139 may be involved in breakdown of host mRNA during viral replication as reported in *E. coli* [41]. Finally, two EsV-1 proteins (ORFs 23 and 66) and their PBCV-1 homologs (A363R and A153R, respectively) contain sequence elements of superfamily II helicases. Superfamily II helicases are required for transcription [169].

Protein synthesis, modification and degradation

The chlorella viruses are the first known viruses to encode a translation elongation factor (EF) [195]. ORF A666L from PBCV-1 has $\sim$ 45% amino acid identity to an EF-3-like protein from fungi [3, 13]. Fungi require EF-3 for growth, and the protein stimulates EF-1α-dependent binding of aminoacyl-tRNA to 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 also codes for 11 tRNAs: 3 for Lys, 2 for Asn and Leu, and 1 each for Ile, Tyr, Arg, and Val. None of these tRNAs have a CCA sequence encoded at the 3′ end of the acceptor stem. Typically these 3 nucleotides are added separately to tRNAs. Some NC64A viruses encode up to 15 tRNAs [125]. The tRNA genes are co-transcribed at both early and late stages of virus replication as a large precursor RNA and processed via intermediates. Codon usage analysis of virus-encoded proteins reveals a strong correlation between the abundance of virus-encoded tRNAs and the virus gene codons [125]. Possibly, the virus-encoded EF-3 in combination with the viruses encoded tRNAs alters the host protein synthetic machinery so that viral mRNAs are preferentially translated.

Both PBCV-1 and EsV-1 encode an ERV/ALR protein homolog. The ERV/ALR protein, which is encoded by several large dsDNA viruses, recently has been shown to be a protein thiol oxidoreductase [150]. Excluding phosphorylation and glycosylation (see below), PBCV-1 encodes two other proteins involved in post-translational modification, a prolyl 4-hydroxylase that converts Pro-containing peptides into hydroxyl-Pro-containing peptides [33] and a protein disulfide isomerase (A448L). Finally, PBCV-1 encodes two putative proteins that interact with ubiquitin, a ubiquitin C-terminal hydrolase (A105L) and a Skp1 protein (A39L). Skp1 proteins belong to the SCF-E3 ubiquitin ligase family that targets cell cycle and other regulatory factors for degradation [27].

Nucleotide metabolism-associated proteins

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 follows this pattern and encodes at least 13 nucleotide metabolic enzymes. The PBCV-1 encoded enzymes are important because the DNA concentration in a virus-infected cell increases 4- to 10-fold after infection [172]. Consequently, virus DNA synthesis requires large quantities of dNTPs that cannot be accounted for simply by recycling dNTPs from host DNA.

Two of the PBCV-1 encoded enzymes, dUTP pyrophosphatase and dCMP deaminase, produce dUMP, the substrate for thymidylate synthetase. Interestingly, PBCV-1 does not encode a recognizable thymidylate synthetase. However, PBCV-1 ORF A674R is about the same size and has 29% amino acid identity to the Thy1 protein from *Dictyostelium discoideum*. Thy1 complements the thymidine growth requirement of a *Dictyostelium* mutant [31]. A674R also has 51% amino acid identity to an ORF from a *Synechocystis* sp. [68] and about 25% amino acid identity to ORFs from *Brevibacterium lactofermentum* and *Corynebacterium glutamicum* [133]. It seems likely that these putative proteins participate in a thymidine synthesis pathway that lacks a traditional thymidylate synthetase.

In contrast, EsV-1 only encodes an ATPase and both subunits of ribonucleotide reductase. Because EsV-1 replicates in cells that normally undergo extensive DNA replication (to form gametes), presumably the host supplies most of the enzymes for deoxynucleotide synthesis.

Signal transduction

PBCV-1 and EsV-1 each encode many protein kinases that may be involved in signal transduction. EsV-1 encodes six hybrid His-protein kinases including one, vhk-1, that is a component of the virion internal membrane [25]. Hybrid His-protein kinases are members of a large family of two-component signalling systems that serve as stimulus-response coupling mechanisms in many organisms [161]. Their primary function is to sense changes in the environment and to induce appropriate physiological responses [161]. To this end, five of the six EsV-1 His-kinase proteins contain long N-terminal domains that could detect external stimuli such as temperature, light, and medium changes, stimuli known to trigger virus replication and virus release [23, 115]. For example, the hybrid kinase encoded by ORF 181 has a N-terminal extension that resembles the phytochrome chromophore-binding domain found in plant enzymes and some bacterial proteins [19, 75]. EsV-1 also encodes a small protein (ORF 113) related to His-containing phosphotransfer (HPt) domains. In bacteria, HPt domains are usually modules of hybrid kinases, but in eukaryotes HPts are separate proteins serving as intermediates in phosphoryl-transfer relay systems [161].

In contrast to EsV-1, PBCV-1 has no His kinase-like protein encoding genes. However, PBCV-1 encodes 7 putative Ser/Thr-protein kinases, one Tyr-protein kinase and a Tyr phosphatase. EsV-1 also contains four genes that could encode Ser/Thr-protein kinases of which one has been previously described for another brown algal virus, FsV-1 [88]. Thus the chloroviruses and the phaeoviruses encode complex, but distinct phosphate transfer systems. These systems may not only mediate viral and host functions, but also may transmit external and internal stimuli.

Ion channel proteins

The algal viruses are the first viruses known to encode K^+ channel proteins. PBCV-1 encodes a 94 amino acid protein (called Kcv) that produces a K^+-selective

and voltage-sensitive conductance in *Xenopus oocytes* [135] as well as in human HEK293 and CHO cells (Moroni, Van Etten and Thiel, manuscript in preparation). The K^+ channel inhibitors amantadine and Ba^{2+}, but not Cs^+, inhibit this K^+ conductance, as well as PBCV-1 plaque formation. Thus PBCV-1 Kcv channel activity is important for virus replication.

Often other proteins or compounds influence the assembly and/or function of K^+ channels and PBCV-1 encodes several proteins that could be involved in these processes. For examples: i) Potassium channel activity is often modulated by phosphorylation and dephosphorylation [89] and PBCV-1 encodes eight protein kinases. ii) PBCV-1 encodes two enzymes involved in polyamine biosynthesis, ornithine decarboxylase (ODC) and homospermidine synthase [67]. Polyamines inhibit biological activity of Kir type K^+ channels [e.g. 34, 93, 95]. iii) PBCV-1 encodes a putative histidine decarboxylase. The product of histidine decarboxylase, histamine, is an important neurotransmitter of photoreceptors in insects and other arthopods [122]. As a photoreceptor transmitter in insects, histamine acts on ligand-gated chloride channels. iv) PBCV-1 encodes two adjacent but divergent ORFs (A162L and A163R) that resemble ligand-gated ion channel proteins. The A162L and A163R proteins are predicted to have the three properly spaced transmembrane domains that are typical of glutamate receptor ion channel proteins [28]. Glutamate receptor channels mediate influx of cations (K^+, Na^+, and Ca^{2+}) across membranes [28, 107] whereas, inward rectifying K^+ channels mediate K^+ eflux. The connection, if any, between the putative PBCV-1 encoded protein kinases, polyamine biosynthetic enzymes, histidine decarboxylase, and ligand-gated ion channel proteins and Kcv is unknown. However, all of these components could be part of a regulated system for maintaining ionic balance and/or electrical signalling within the chlorella during virus replication.

EsV-1 encodes a 124 codon ORF that has significant amino acid identity to PBCV-1 Kcv (41% amino acid identity over 77 residues). However, the putative EsV-1 protein has a longer N-terminus (35 amino acids) containing 2 consensus protein kinase C sites and it has three transmembrane domains. It will be interesting to determine if the EsV-1 protein forms a functional channel in heterologous cells. The EsV-1 genome also encodes several proteins with hydrophobic amino acid rich regions that resemble helical transmembrane domains. Among these proteins, the input domain of the putative hybrid His-kinase 186 and the ORF 188 resemble ion channel proteins.

Sugar- and lipid-manipulating enzymes

Phycodnaviruses are also unusual because they encode enzymes involved in sugar metabolism. Two PBCV-1-encoded enzymes, GDP-D-mannose dehydratase and fucose synthase, comprise a three step pathway that converts GDP-D-mannose to GDP-L-fucose (Tonetti, Gurnon, and Van Etten, unpublished results) (Fig. 2C). Fucose, a rare sugar, is present in the glycan attached to the major capsid protein (see below). Three additional PBCV-1 encoded enzymes, glutamine:fructose-6-phosphate amindotransferase (GFAT), UDP-glucose dehydrogenase (UDP-GlcDH) and hyaluronan synthase, are involved in the synthesis of hyaluronan, a

linear polysaccharide composed of alternating β1,4-glucuronic acid and β1,3-N-acetylglucosamine residues (Fig. 2B) [21, 82]. All three genes are transcribed early in PBCV-1 infection, and hyaluronan accumulates on the external surface of the infected chlorella cells [46]. Hyaluronan was unexpected because, heretofore, it had been found only in the extracellular matrix of vertebrates and the extracellular capsules of a few pathogenic bacteria [20]. The PBCV-1-encoded GFAT and UDP-GlcDH enzymes most closely resemble bacterial enzymes whereas, the PBCV-1-encoded hyaluronan synthase resembles vertebrate enzymes. These observations suggest that the viruses may have acquired the GFAT and UDP-GlcDH genes separately from the hyaluronan synthase gene.

At least three EsV-1 genes encode proteins involved in polysaccharide metabolism. One protein (ORF 226), that is a component of the virus particle (gp1, see above), resembles bacterial alginate mannuronan C-5-epimerases. Another gene (*orf83*) is predicted to encode a GDP-mannose/UDP-glucose dehydrogenase. Members of this dehydrogenase family provide precursors for many glycosyltransferases like hyaluronan synthase, alginate synthase, and chitin synthase. In fact, an EsV-1 gene (*orf84*) encodes a glycosyltransferase-like protein that resembles these three polysaccharide synthases. Although the ORF 84 protein was initially considered to be a chitin synthase [115], it is more likely that it, together with ORF 83 and ORF 226 proteins, mediate alginate synthesis. A fourth EsV-1 ORF, number 166, encodes a protein with a cellulose-binding motif and it may belong to this group of possibly functionally related genes.

Three PBCV-1 genes encode proteins that resemble lipid-metabolizing enzymes, glycerophosphoryl diesterase, 2-hydroxyacid dehydrogenase, and lysophospholipase. PBCV-1 deletion mutants, which lack the neighboring glycerophosphoryl diesterase and the 2-hydroxyacid dehydrogenase genes, still replicate in chlorella grown in the laboratory [81]. The product of another PBCV-1 gene (*a654l*) has a GNAT-N-acetyltransferase domain. This may be significant because 3 proteins associated with the PBCV-1 virion, including the major capsid protein, contain an amide-linked myristic acid [137]. Typically, N-linked myristic acid is attached to the amino-terminal amino acid, usually a glycine after removal of terminal methionine [6]. However, the myristic acid attached to the PBCV-1 major capsid protein is attached to an internal amino acid, probably a lysine [137]. The putative PBCV-1 encoded N-acetyltransferase could be responsible for this rare modification.

Protein glycosylation

Structural proteins of many viruses, such as herpesviruses, poxviruses, and paramyxoviruses, as well as PBCV-1 and EsV-1, 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 [29, 74, 131, 160]. Nascent viruses acquire the glycoprotein(s), and only become infectious, by budding through the membrane, often 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 arose from antibody studies. Polyclonal antiserum prepared against intact PBCV-1 virions inhibits virus plaque formation by agglutinating particles. Spontaneously derived, antiserum-resistant mutants of PBCV-1 arise at a frequency of $\sim 10^{-6}$. These antiserum-resistant mutants fall into five serologically distinct classes [183], (Graves and 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 mutants migrate in a distinctive fashion on SDS-PAGE; the size of Vp54 in each mutant is smaller than wild-type Vp54. Western blot analyses of the Vp54 proteins, before and after removing the glycan, established that the antigenic classes reflect differences in the Vp54 glycan. In addition, the ratio of the 7 neutral sugars [glucose, fucose, galactose, mannose, xylose, rhamnose and arabinose [183]] associated with Vp54 from PBCV-1 and the mutants varies in a sequential manner that correlates with antigenicity and Vp54 migration on SDS-PAGE. Finally, the oligosaccharide lacks N-acetylglucosamine, a sugar commonly found in Asn-linked (N-linked) and many O-linked glycoproteins produced via the cellular ER-Golgi pathway [142].

Additional observations indicate that Vp54 glycosylation is atypical. i) Unlike viruses that acquire their glycoprotein(s) by budding through a membrane, intact infectious PBCV-1 particles accumulate inside the cell 30–40 min before virus release [175]. ii) The nucleotide sequences of the gene encoding Vp54 in each of the mutants are identical to PBCV-1 [183]. iii) All of the mutants are grown in the same host. iv) Antibodies to the virus do not react with host oligosaccharides. v) Compounds that inhibit ER-Golgi localized N-linked glycosylation of proteins (tunicamycin) and ER to Golgi protein transport (Brefeldin A) [30, 94] neither affect PBCV-1 replication nor the M_r of Vp54 at concentrations lethal to the host [137]. vi) Mutants 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 [45]. Taken together, these results imply that PBCV-1 encodes most, if not all, of the enzymes involved in constructing the complex oligosaccharide attached to Vp54.

Comparison of PBCV-1 ORFs to genes in the databases identified seven possible glycosyltransferase encoding genes, *a64r*, *a111r*, *a114r*, *a222-226r*, *a328l*, *a473l*, and *a546l*. Interestingly, none of these putative PBCV-1 encoded glycosyltransferases have an identifiable signal peptide that would target them to the ER. Furthermore, PSORT, a cellular protein localization prediction program, indicates that all of these proteins, with the exception of A473L, are cytoplasmic. A473L is predicted to be located in the plasma membrane. More specifically, the *a64r* gene encodes a 638 amino acid protein that has 4 motifs conserved in "Fringe type" glycosyltransferases. Analysis of 13 PBCV-1 antigenic mutants revealed mutations in *a64r* that correlated with a specific antigenic variation. Dual infection experiments with different antigenic mutants indicated viruses

that contained wild-type *a64r* could complement and recombine with viruses that contained mutant *a64r* to form wild-type virus. Therefore, we conclude that *a64r* encodes a glycosyltransferase involved in synthesizing the Vp54 glycan [45].

Virions of EsV-1 also contain glycoproteins. However, there is no evidence to indicate that glycosylation of these proteins occurs via virus encoded glycosyltransferases. Electron microscopic studies indicate that capsids of phaeoviruses are probably assembled in the ER [188–190], in the same manner as are African swine fever virus (ASFV) and poxviruses. Consequently, EsV-1 proteins most likely are glycosylated by host-encoded glycosyltransferases.

Cell wall-degrading enzymes

Digestion of the host cell wall is critical at two stages in the life cycle of the chlorella viruses, infection and virus release. Thus it is not surprising that PBCV-1 and another NC64A virus (CVK2) encode several enzymes that could degrade the host *Chlorella* NC64A cell wall, including two chitinases, a chitosanase, a β-1,3-glucanase, and one enzyme initially described as an alginase [163]. With the exception of the putative alginase, recombinant proteins produced from these five genes have the expected enzyme activities [54, 55, 163–166, 197]. Enzymes involved in either virus infection or virus release should be expressed late in the infection cycle; furthermore, enzymes involved in virus infection should be packaged in the virion. One of the chitinases and the chitosanase are packaged in the PBCV-1 virion and may facilitate virus entry into the host. However, β-1,3-glucanase cannot be involved in either process since it is expressed early in infected cells and disappears by 90 min p.i. Incubation of host cells with all five recombinant enzymes does not lead to algal protoplasts, indicating that cell wall degradation requires at least one additional enzyme activity.

The chitosanase gene, which is expressed late in infection, has been characterized from virus CVK2 [197]. The CVK2 gene encodes two proteins, 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 is found only in infected cells. Interestingly, PBCV-1 contains the same chitosanase gene flanked by two ORFs also 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 a read-through product. The presence of chitinase and chitosanase genes in the chlorella viruses was unexpected, because chitin is rare in algae [53]. Chitin is a normal component of fungal cell walls and the exoskeleton of insects and crustaceans [42]. However, Kapaun and Reisser [69] reported a chitin-like glycan in the cell walls of *Chlorella* Pbi, and *Chlorella* NC64A walls may also contain chitin; $\sim$ 10% of the sugars in NC64A cell walls are glucosamine [103].

EsV-1 encodes no cell wall degrading enzymes because they are not required for virus infection. Presumably, nascent EsV-1 virions are released from the host reproductive cells by the same host cell enzymes that release the walless spores.

Polyamine biosynthetic enzymes

PBCV-1 encodes two enzymes involved in polyamine biosynthesis, ODC and homospermidine synthase (HSS) (Fig. 2A). ODC catalyzes the decarboxylation of ornithine to putrescine, which is the first and also the rate limiting enzymatic step in the polyamine biosynthetic pathway [16, 18]. The predicted size of the PBCV-1-encoded ODC is smaller (372 amino acids) than all other known ODCs. Despite its small size, the recombinant protein has excellent enzyme activity (Morehead, Gurnon, Adams, Nickerson, Fitzgerald and Van Etten, manuscript submitted for publication).

The PBCV-1 encoded *hss* gene also encodes a functional enzyme that synthesizes the rare polyamine homospermidine from two molecules of putrescine [67]. The *hss* mRNA is expressed late during PBCV-1 infection and PBCV-1 virions contain homospermidine. However, homospermidine is not the primary virion polyamine. PBCV-1 also encodes a putative monomine oxidase that may be related to the polyamine biosynthetic enzymes. The biological significance of these PBCV-1-encoded polyamine metabolic enzymes is unknown.

Gene families and gene duplications

Eighty-eight of the PBCV-1 ORFs resemble 1 or more other PBCV-1 ORFs forming 28 families. Fifteen families have two members, 8 families have three members, 3 families have six members and 2 families have eight members. One six-member family contains multiple ankyrin-like repeats [132]. Five members in another PBCV-1 family resemble the PBCV-1 major capsid protein Vp54. The genes for four of these proteins are actually transcribed late in the infection cycle similar to the Vp54 gene; the fifth appears to be a pseudogene (Graves and Van Etten unpublished). 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 EsV-1 most closely resembles one (A622R) of these other PBCV-1 major capsid-like proteins.

Forty-two EsV-1 ORFs resemble 1 or more other EsV-1 ORFs forming 14 families. The largest family has 9 members with ankyrin-repeat motifs. Another family contains 4 members, each possessing a RING finger domain; some of these proteins have been described previously for EsV-1 [24] and for FsV-1 [77].

PBCV-1 deletion mutants

Not all PBCV-1 genes are required for virus replication in the laboratory. For example, four spontaneously derived, antigenic variants of PBCV-1 have been isolated that contain 27- to 37-kb deletions at the left end of the 330-kb genome [81]. Two of these mutants have deletions beginning at position 4.9 or 16 kb and ending at 42 kb. In total, these two deleted regions, which probably resulted from recombination, encode 28 putative proteins. 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. These regions encode 26 single-copy ORFs, of which 23 are common to those deleted in the first two mutant viruses. 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 wildtype virus.

The deletion mutants also indicate that the size of the inverted terminal repeats in PBCV-1 can vary. Furthermore, the virus DNA packaging mechanism tolerates significant changes 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. Similar large deletion mutants were reported for NC64A virus CVK1 [156]. CVK1-infected cells exposed to UV radiation produced 30- to 45-kb deletions. Like PBCV-1, these deletions occurred in the left terminal portion of the virus genome. Finally, the sizes and locations of the deletions and deletion/transpositions found in the chlorella viruses resemble poxvirus [170] and ASFV [7] 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 [151, 170] may be relevant to the chlorella viruses.

Diversity of chlorella virus genomes

Several observations indicate that the chlorella virus genomes are more diverse than might be expected. For example, the NY-2A genome is about 50 kb larger than the PBCV-1 330-kb genome [144]. Assuming the density of PBCV-1 and NY-2A genes are similar, NY-2A should contain ∼ 55 more genes than PBCV-1 for a total of ∼ 430 genes. Southern hybridization and DNA sequence analyses indicate that not all PBCV-1 genes exist in all *Chlorella* NC64A virus isolates or that an extra ORF often is inserted between two otherwise co-linear genes [e.g., 138]. The insertion of extra genetic elements, termed “morons”, between adjacent genes also occurs in related lambda phages [51]. PBCV-1 encodes 11 tRNA genes that are clustered; other NC64A viruses encode clusters of 10–15 tRNA genes [125]. Some of these tRNA genes probably reflect gene duplications.

Sequence analyses of a gene (*pdg*) encoding a UV-specific DNA repair enzyme from 42 NC64A viruses revealed that 15 of them 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 [167]. In contrast, the nucleotides in the *pdg* coding regions (exons) from the intron-containing viruses range from 84 to 100% identical. In many instances intron-containing and intron-lacking viruses came from the same water sample. [Note: The conserved intron results contradict the widely accepted dogma that intron sequences are more variable than exon sequences [e.g. 90].]

Yamada and his colleagues [127, 198] have demonstrated 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 ($\sim$ 60% of the viruses) or an unidentified ORF encoding a 14.2 kDa polypeptide ($\sim$ 40%); however, in a few viruses the intron is in the major capsid protein gene. Sequence analyses indicate that 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 organisms including green algae, red algae, yeasts, fungi and protozoa, suggesting horizontal gene transfer between these distantly related organisms [80].

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 NC64A 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 prone to deletions/rearrangements and genes in this region tend to be less conserved suggests a recombinational "hotspot" exists in this region that allows viruses to exchange genes among themselves and possibly with hosts. However, despite these differences, the location of 22 PBCV-1 genes, many of which are housekeeping genes, are nearly co-linear in the 350 kb NC64A virus CVK2 [126], suggesting similar overall genome organization between the two chlorella virus isolates.

Diversity of other algal virus genomes

FsV-1 is another well-studied phaeovirus that infects an unclassified *Feldmannia* species. Although the entire FsV-1 180-kb genome sequence is unknown, some genomic fragments have been sequenced. These sequences allow some comparisons with EsV-1. i) Like EsV-1, FsV-1 contains numerous repeats; however, the DNA sequences of these repeats differ from those of EsV-1. ii) The similarity between FsV-1 and the EsV-1 genes is low. For example, EsV-1 DNA polymerase only has 45% amino acid identity to the FsV-1 enzyme [23]. iii) FsV-1 ORF 1 is absent in EsV-1. iv) The EsV-1 and FsV-1 genes are arranged differently, e.g. ORF 2 and ORF 3 of FsV-1 are separated by 78 bases and oriented in the same direction whereas, the corresponding EsV-1 genes are separated by ca. 15.5 kb and oriented in opposite directions [77]. Consequently, the overall structure of the two phaeovirus genomes differ although their life-cycles are similar and their hosts are closely related. The gene similarity/diversity among other brown algal viruses is unknown.

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 [17]. The proportion of identical nucleotides for all pair-wise combinations of the *dnapol* gene among the eight MpV viruses

ranged from 78 to 99%, indicating substantial differences in a portion of a gene that is typically highly conserved [15]. Thus, all available evidence indicates that algal virus genomes are quite diverse, probably reflecting their long evolutionary history.

Additional putative phycodnaviruses

Other algal viruses have been studied primarily because of their importance in regulating phytoplankton communities in marine environments [e.g., 5, 8, 168, 191]. Viruses are reportedly associated with the disappearance of "brown tides" caused by *Aureococcus anophagefferens* [108, 153] and "red tides" caused by *Heterosigma akashiwo* [119–121]. Likewise, Bratbak et al. [9] reported that virus lysis accounted for $>$ 25% of the mortality in blooms of *Emiliania huxleyi*.

Bratbak and colleagues have discovered four lytic viruses specific for *Emiliania huxleyi* (EhV) [11a], *Phaeocystis pochetii* (PpV) [66], *Chrysochromulina ericina* (CeV-01B) [146], and *Pyramimonas orientalis* (PoV-01B) [146] that have genomes estimated to be 412, 485, 510 and 560 kb, respectively. If the gene densities of these viruses are similar to PBCV-1, they have roughly 466, 549, 578, and 634 protein-encoding genes. These values are as high or higher than the 470 genes encoded by the smallest mycoplasma [36]. These four viruses, which are difficult to grow in culture, are largely uncharacterized. It will be fascinating to determine the genes encoded by these viruses.

Comparison of phycodnaviruses to other viruses

The chlorella viruses have some common properties with other large dsDNA-containing viruses that are assembled in the cytoplasm, including iridoviruses, poxviruses, and asfarviruses. Iridoviruses, which infect insects and aquatic animals, have an icosahedral morphology, an internal lipid membrane, and some have genomes with methylated bases, e.g. 20% of the cytosines in the frog virus 3 (FV3) genome are methylated [186]. FV3 also encodes a 5 mC DNA Mtase [185]. Finally, portions of the PBCV-1 and EsV-1 major capsid protein are similar to the FV3 major capsid protein [101]. However, there are numerous differences between FV3 and PBCV-1 [see 176]. Most importantly, their DNA structures differ. FV3 DNA is linear and circularly permuted [43] whereas, PBCV-1 DNA is linear and nonpermuted with covalently closed hairpin ends [144].

The PBCV-1 genome shares two characteristics with vaccinia virus (a poxvirus), even though the virions are morphologically distinct. Like PBCV-1, the vaccinia virus genome contains covalently closed hairpin termini and inverted terminal repeats [39, 40]. However, one difference between the viruses is that virtually all poxvirus genes located in the terminal 20 to 25 kb regions are transcribed toward the end of the genome [111] whereas, PBCV-1 genes located in these regions are transcribed in both directions.

ASFV, the single member of the family *Asfarviridae*, is the only other large icosahedral virus containing a dsDNA genome with hairpin termini and inverted

terminal repetition [7, 155]. However, PBCV-1 and also EsV-1 differ from ASFV in several aspects: i) the PBCV-1 and EsV-1 genomes are about twice the size of the ASFV genome. ii) The PBCV-1 and EsV-1 genomes contain methylated bases, while AFSV does not. iii) ASFV lacks glycoproteins. iv) ASFV infection occurs via endocytosis whereas, PBCV-completely uncoats and EsV-1 partially uncoats at the cell surface.

The infection process of the chlorella viruses differs from that of all DNA viruses known to infect eukaryotes, but resembles that of many bacterial viruses, in having to pass through a cell wall. In terms of structure, attachment, and penetration the chlorella viruses resemble the tectiviruses [2]. However, tectiviruses are much smaller ($\sim$ 650 Å in diameter) and contain only about 40 kb of DNA.

Although the exact molecular structure of the EsV-1 genome is unknown, EsV-1 particles contain linear DNA whose ends are probably not covalently linked. The phaeovirus genomes possess numerous repeat sequences like those in baculoviruses and herpesviruses. The phaeoviruses share two other properties with the herpesviruses. i) Herpesviruses penetrate the cells by fusing with the cell membrane, releasing the tegument-covered nucleocapsid in the cytoplasm [70]. ii) Herpesviruses also establish a lysogenic state in their hosts by integrating into the host genome [61, 145].

Finally a recent comparative analysis of the protein sequences encoded by poxviruses, asfarviruses, 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 [65]. Cladistic analysis using the genes shared by at least two viral families as evolutionary characters, led Iyer et al. [65], to conclude that viruses in all four families arose from a common ancestor.

Final comments

Research on the *Phycodnaviridae* is in its infancy and without doubt many more surprises await discovery. However, even with our limited knowledge there are excellent scientific and economic reasons that justify increased research on algal viruses including: i) The viruses play a dynamic, albeit largely unknown, role in regulating phytoplankton communities in aqueous environments, e.g. in the termination of algal blooms. ii) The viruses are an important source of enzymes, not only commercial enzymes such as DNA restriction endonucleases, but many viral enzymes are the smallest in their class. Consequently, these proteins can serve as models for mechanistic and structural studies. iii) The viruses are a novel source of genes and genetic elements that can be used to engineer other organisms. For example, PBCV-1 promoter elements function extremely well in both higher plants [109] and bacteria [110, Xia and Van Etten, unpublished results]. iv) EsV-1, or any of the other latent algal viruses, could be engineered to carry a foreign gene and used to produce large amounts of a gene product in their host. This could prove economically important since latently infected algae are somatically unaffected and can be mass-cultured at low cost [52, 115]. v) Accumulating evidence suggests that the phycodnaviruses are extremely old, which suggests

that their genes have a long evolutionary history. Therefore, studies on these viruses may reveal interesting aspects about the evolution of genes and genomes. We encourage more investigators to work on these exciting virus systems.

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