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Viruses and Viruslike Particles of Eukaryotic Algae

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INTRODUCTION

Eukaryotic algae range in size and complexity from small unicellular organisms (microalgae) to large multicellular organisms (macroalgae), such as seaweeds. Most eukaryotic algae are free living, and members can grow and survive in many terrestrial and aquatic environments. A few, however, are found only in symbiotic association with other organisms such as molluscs, flatworms, coelenterates, protozoa, and fungi (lichens). Algae significantly influence aquatic environments, both as primary producers in the food chain and, conversely, as pollutants when growth becomes uncontrolled. Because most algae require only light, simple salts, and a nitrogen source for rapid growth, they are potential producers of proteins, carbohydrates, and fats for feeding humans and animals. In addition, algae are a source of useful compounds such as β-carotene, alginic acid, carrageenans, and agar (12, 137, 148). Algae such as *Chlamydomonas* and *Chlorella* spp. have also served as models for studying photosynthesis, cell cycle regulation, cell motility, and non-Mendelian (chloroplast) inheritance. Given the importance of eukaryotic algae, there is surprisingly little information about viruses or viruslike particles (VLP) in these organisms.

It is difficult to credit the first person who described a virus or VLP in a eukaryotic alga. The problem is complicated because early reports consisted solely of microscopic observations and, in some cases, cultures were not axenic. A few papers in the Russian literature as long as 30 years ago described a lytic activity in cultures of the green alga *Chlorella pyrenoidosa* (174, 224–226). This lytic activity was given the name "chlorellophage." However, these investigators acknowledged that their cultures were contaminated with bacteria. Furthermore, the chlorellophage had typical bacteriophage morphology (174, 224). Therefore, despite the name chlorellophage, the host for these particles is unclear.

Several investigators, almost simultaneously, reported the presence of VLPs in eukaryotic algae in the early 1970s. Lee (73) described 50- to 60-nm polyhedral particles in vegetative cells of the red alga *Sirodotia tenuissima*, Pickett-Heaps

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(123) described polyhedral particles (240 nm in diameter) in germlings of the green alga *Oedogonium* sp., and Toth and Wilce (176) described 170-nm polyhedral particles in spores of the marine brown alga *Chorda tomentosa*. Since these initial reports, VLPs or viruses have been reported in at least 44 taxa of eukaryotic algae (Table 1). These are distributed in 10 of the 14 recognized classes of eukaryotic algae (141). Particles have not been found in any member of the families Euglenophyceae, Bacillariophyceae, Raphidophyceae, and Tribophyceae. VLPs and viruses of eukaryotic algae have been reviewed previously by Lemke (74), Sherman and Brown (149), Dodds (27, 28), Martin and Benson (84), and Van Etten et al. (190, 192). The review by Sherman and Brown contains several electron micrographs of these particles, including some not published elsewhere. One other report is noteworthy. In 1970 Schnepf et al. (143) described large (200 nm in diameter) polyhedral particles in an *Aphelidium* sp. (which, although described as a fungus, may actually be a protozoan) that parasitized the green alga *Scenedesmus armatus*. These particles were much larger than most fungal viruses (68) but were similar in size and morphology to VLPs subsequently found in eukaryotic algae.

With few exceptions, the reports of VLPs in eukaryotic algae typically consist of single accounts of microscopic observations. Furthermore, many of them were with field-collected algae, which are not available for further study. Characterization of these particles was not pursued because of low concentrations. Several factors contributed to the low concentrations: (i) usually only a few algal cells contained particles, (ii) usually the cells contained particles only at one stage of the algal life cycle, (iii) usually cells containing particles did not lyse, and (iv) in most cases the particles were not infectious. In addition, some of the algae could not be cultured and others were inconvenient to work with (e.g., filamentous algae).

However, these difficulties are no longer a problem for a family of viruses which infect and replicate in certain strains of unicellular *Chlorella*-like green algae. The first of the *Chlorella* viruses was discovered about 10 years ago in *Chlorella*-like algae (zoochlorella) symbiotic with *Hydra viridis* (96, 186, 187) and subsequently in *Chlorella*-like algae in *Paramecium bursaria* (132, 181, 183, 187). The algae from *P. bursaria* can be grown free of the paramecium, and the cultured *Chlorella* strains NC64A and Pbi or their equivalents serve as hosts for many viruses. These viruses can be produced in large quantities, assayed by plaque formation, and analyzed by standard bacteriophage techniques.

This review will describe the biology of the *Chlorella* virus hosts, which normally exist as symbionts, and then focus on the discovery, biology, and molecular biology of the *Chlorella* viruses. Partially characterized viruses or VLPs of other eukaryotic algae are discussed at the end of the review.

CHARACTERISTICS OF *CHLORELLA* SPECIES

General Properties of *Chlorella* Species

The genus *Chlorella* contains small spherical or ellipsoidal, unicellular, nonmotile, asexually reproducing green algae. *Chlorella* species have a rigid cell wall and typically contain a single chloroplast, which may or may not contain a pyrenoid. They have a simple developmental cycle and reproduce by mitotic division. Vegetative cells increase in size and divide into two, four, eight, or more progeny (called autospores), which are released by rupture or enzymatic digestion of the parental walls. The autospores develop rapidly into vegetative cells. The number of progeny formed from a single vegetative cell varies with species and environmental conditions (200).

Chlorella species typically grow on a simple salts solution and utilize nitrate, ammonium, or amino nitrogen as a nitrogen source (116). However, they often grow best on casein hydrolysate (91, 151). Simple sugars such as sucrose or glucose increase the growth rate of some species but inhibit the growth of others. The doubling time varies with the strain or species, but many of them double in about 24 h.

Algae assigned to the genus *Chlorella* are more heterogeneous than their simple morphology suggests (25, 58, 65, 151). Huss et al. are examining the relationships among *Chlorella* species by using DNA base ratios, DNA hybridization, and DNA reassociation kinetics (see reference 58 and references cited therein). The guanosine-plus-cytosine content of nuclear DNA, which ranges from 43 to 79% (49), reflects the heterogeneity of algae assigned to the genus *Chlorella*. However, most isolates assigned to the same species have similar G+C contents.

The *Chlorella* base compositions were determined either by measuring DNA melting temperatures or by performing CsCl density equilibrium centrifugation. Recently, high levels of 5-methylcytosine (5mC) were found in the nuclear DNAs of *Chlorella* strain NC64A (21% of the cytosines were methylated [189]) and *Chlorella sorokiniana* (16% of the cytosines were methylated [22]). If high concentrations of 5mC are a common feature of *Chlorella* DNAs, many of the published *Chlorella* G+C contents may be low because 5mC can lower DNA melting temperatures and buoyant densities.

The DNA content of *Chlorella* cells was reported to range from about 15 fg per cell for cultured *Chlorella* strains 211/1e and NC64A to 47 fg per cell for symbiotic *Chlorella* strains isolated from hydras (88). We estimate that *Chlorella* strain NC64A contains about 40 fg (40 Mbp) of DNA per cell (180), which is almost three times higher than that found by McAuley and Muscatine (88). Assuming the higher value and assuming haploidy, the *Chlorella* strain NC64A genome is about twice the size of the *Saccharomyces cerevisiae* genome and one-half the size of the *Arabidopsis thaliana* genome. Repetitive sequences make up only a small fraction (about 5 to 8%) of the total *Chlorella* DNA (58).

The cell wall composition of *Chlorella* species also varies widely (see, e.g., references 3, 76, 114, 171, 172, and 219). Typically, *Chlorella* cell walls contain polysaccharides with a variety of component sugars as well as lesser amounts of protein and lipid. In addition, walls of some *Chlorella* species contain a layer of sporopollenin (3), a polymer of carotenoid derivatives resistant to most chemicals (13). The difficulty of classifying *Chlorella* species is illustrated by a report that the chemical compositions of the cell walls of some isolates assigned to the same species differ markedly, e.g., for *Chlorella vulgaris* and *Chlorella ellipsoidea* (219). Because of the heterogeneity in the genus *Chlorella*, properties discovered in one *Chlorella* species or strain may not always apply to another.

As noted below, viruses can distinguish at least two exsymbiotic *Chlorella* strains from each other and from other free-living *Chlorella* species. If viruses infecting additional *Chlorella* species are found, viruses could be useful in classifying *Chlorella* species in the same way that bacteriophages are useful in bacterial classification (see, e.g., reference 2).

TABLE 1. Viruses and VLPs of eukaryotic algae

Host species[a]	Location of virogenic stroma	Nucleic acid	Morphological characteristics	Infection and replication facts	Key references
Rhodophyceae					
Porphyridium purpureum[b]	Nucleus and cytoplasm	Unk[c]	Polyhedral, 40 nm in diameter	Unk	18
Sirodotia tenuissima[d]	Cytoplasm	Unk	Polyhedral, 50–60 nm in diameter	Unk	73
Cryptophyceae					
**Cryptomonas* sp.[e]	Nucleus and cytoplasm	Unk	Two types of particles were observed in the same cell: (i) polyhedral, 99 nm in diameter; (ii) stalked particle, head 120 nm diameter, length 240 nm; surrounded by a membrane	Unk	124
Dinophyceae					
Gymnodinium uberrimun[f]	Cytoplasm	Unk	Polyhedral, 385 nm in diameter	Unk	152
**Gyrodinium resplendens*[g]	Cytoplasm	Unk	Two types of particles: (i) tubular arrays, with particles 35 nm in diameter; (ii) particles 20 nm in diameter	Unk	34, 162
Chrysophyceae					
Chromophysomonas cornuta[h]	Cytoplasm	Unk	Two polyhedral particles, 50–60 nm and 150–180 nm, in diameter	Unk	127
Chrysophycean-like[i]	Cytoplasm	Unk	Polyhedral, 35 nm in diameter	Unk	175
Dinobryon sp.[j]	Cytoplasm	Unk	Polyhedral, 100 nm in diameter	Unk	Carson and Brown (unpublished) cited in 149
Hydrurus foetidus[k]	Cytoplasm	Unk	Polyhedral, 50–60 nm in diameter	Unk	55
Mallomonas sp.	Cytoplasm	Unk	Polyhedral, 175 nm in diameter	Unk	152
Paraphysomonas caelifrica[l]	Cytoplasm	Unk	Polyhedral, 150–180 nm in diameter	Unk	127
Paraphysomonas corynephora[m]	Cytoplasm	Unk	Three polyhedral particles, 50–60 nm, 150–180 nm, and 270–300 nm in diameter	Unk	127
Paraphysomonas bourrellyi	Cytoplasm	Unk	Two polyhedral particles, 50–60 nm and 150–180 nm in diameter	Unk	127
Prymnesiophyceae (Haptophyceae)					
**Chrysochromulina* sp.	Unk	Unk	Polyhedral, 22 nm in diameter	Unk	82
**Coccolithus huxleyi*	Unk	Unk	Polyhedral, 22 nm in diameter	Unk	82
**Hymenomonas carterae*					
Strain 1[n]	Nucleus?	Unk	Polyhedral, 65 nm in diameter	Unk	124
Strain 2	Cytoplasm	Unk	Polyhedral, 400 nm in diameter	Unk	Cooper and Brown (unpublished) cited in 149
Eustigmatophyceae					
Monodus sp.[o]	Nucleus?	Unk	Polyhedral, 400 nm in diameter	Unk	Huang and Hommersand (unpublished) cited in 149
Phaeophyceae					
**Chorda tomentosa*[p]	Cytoplasm?	Unk	Polyhedral, 170 nm in diameter	Unk	176
**Ectocarpus fasciculatis*[q]	Nucleus?	Unk	Polyhedral, 170 nm in diameter	Leads to cell lysis	4, 21
**Ectocarpus siliculosus*[r]	Cytoplasm	dsDNA	Polyhedral, 130 nm in diameter	Infects swimming zoospores	103
**Feldmannia* sp.[s]	Nucleus?	dsDNA	Polyhedral, 150 nm in diameter	Replicates only in meiotic sporangia	51
**Pylaiella littoralis*[t]	Nucleus then cytoplasm?	Unk	Polyhedral, 130–170 nm in diameter	Unk	83
**Sorocarpus uvaeformis*[u]	Cytoplasm	Unk	Polyhedral, 170 nm in diameter	Infectious	117
**Streblonema* sp.[v]	Cytoplasm	Unk	Polyhedral, 135–150 nm in diameter	Unk	71

Continued on following page

TABLE 1—*Continued*

Host species[a]	Location of virogenic stroma	Nucleic acid	Morphological characteristics	Infection and replication facts	Key references
Prasinophyceae					
**Heteromastix* sp.	Nucleus?	Unk	Polyhedral, 320 nm, with complex membranes	Unk	Ott and Hommersand (unpublished) cited in 149
Mesostigma viride[w]	Cytoplasm	Unk	Polyhedral, 130 nm in diameter	Unk	97
**Micromonas pusilla*[x]	Cytoplasm	dsDNA	Polyhedral, 130–135 nm in diameter	Virus adheres to the surface of host cells, not known if uncoating occurs at the surface or internally	87, 124, 168, 199
**Platymonas* sp.[y]	Nucleus	Unk	Polyhedral, 51–58 nm in diameter	Unk	122
**Pyramimonas orientalis*[z]	Nucleus	Unk	Two polyhedral particles, 60 and 200 nm in diameter	Unk	101
Chlorophyceae					
Aulacomonas sp.[aa]	Cytoplasm?	Unk	Polyhedral, 200–230 nm in diameter, with tail 150–200 nm long	Unk	170
**Brachiomonas* sp.	Cytoplasm?	Unk	Polyhedral, 380–400 nm in diameter; some had long tails (500 nm)	Unk	54
Chlorella spp.[bb]	Unk	Unk	Polyhedral head, 41 nm in diameter, with a 24-nm-long, 10-nm-wide tail	Unk	174
Chlorella spp.[cc]	Cytoplasm	dsDNA	Polyhedral, 170–180 nm	Unk	96, 186, 187
Chlorella strain NC64A[dd]	Cytoplasm	dsDNA	Polyhedral, 150–190 nm	Attaches specifically to host walls and uncoats at surface	63, 181, 184, 187
Chlorella strain Pbi[ee]	Cytoplasm	dsDNA	Polyhedral, 140–150 nm	Attaches specifically to host walls and uncoats at surface	132, 134
Chlorococcum minutum[ff]	Cytoplasm	dsDNA	Polyhedral, 180–220 nm in diameter, has a tail	Virus infects only zoospores, progeny viruses appear in ca. 8 h, viruses released by lysis of cell	46, 47
Cylindrocapsa geminella[gg]	Nucleus, then cytoplasm?	dsDNA	Polyhedral, 200–230 nm in diameter with a membranous coat	Particles may lead to cell lysis	56, 163, 164
Oedogonium sp.[hh]	Cytoplasm	Unk	Polyhedral, 240 nm in diameter	Unk	123
Radiofilum transversale[ii]	Nucleus	Unk	Polyhedral, 41 nm in diameter	Unk	86
Stigeoclonium farctum[jj]	Chloroplast?	Unk	Tubular? 16.3 nm in cross-section	Unk	86
Uronema gigas[kk]	Cytoplasm?	dsDNA	Polyhedral, 390 nm in diameter, some have long tails (1,000 nm)	Unk	23, 29, 86
Charophyceae					
Chara corallina[ll]	Cytoplasm?	ssRNA	Tubular rods, 532 nm long, 18 nm wide, with basic pitch of 2.75 nm	By artificial injection of virus into uninfected *Chara* cells; chlorosis and death in 10–12 days	38, 155
Coleochaete scutata	Nucleus	Unk	Polyhedral, 41 nm in diameter	Unk	86

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[a] Classification from reference 141. Asterisks denote marine algae.
[b] The intranuclear particles (termed concentrosomes) are probably not VLPs. The polyhedral particles are present only in the cytoplasm of a small number of cells.
[c] Unk, unknown.
[d] The virogenic stroma is prominent in the apical cell and appears to be transmitted to the daughter cells during division of the apical cell.
[e] The host cells with viral particles may be scenescent.
[f] Cells with particles lack a nucleus.
[g] Franca (34) reported two types of VLPs. Soyer (162) suggested that the V2 particles are tricocystoid filaments. The V1 particles do not have helical symmetry and are unlikely to be VLPs.
[h] Two classes of VLPs were sometimes present in the same cell.
[i] The unidentified chrysophycean alga was an endosymbiont in the dinophycean alga *Peridinium balticum*.
[j] This VLP may be responsible for rapid cell lysis when natural collections are brought to elevated temperatures of the laboratory, or in late spring when the water temperature increases. This may explain the mass-scale lysis of *Dinobryon* spp. in major water supplies, giving rise to subsequent blooms of other algae.
[k] VLPs were probably present in zoospores and were not seen in vegetative cells.
[l] The VLPs may lyse the alga. The VLPs were seen in 75% of the algal cells after enrichment for the alga, which was followed by the disappearance of the alga.
[m] Three size classes of VLPs were observed in the alga; the two smaller particles were occasionally present in the same cell.
[n] Cells containing VLPs were greatly enlarged and contained proliferating chloroplasts.
[o] The host could be xanthophycean or intermediate between the Xanthophyceae and Eustigmatophyceae.
[p] VLPs found in settled zoospores of *Chorda*; spores with VLPs do not synthesize a cell wall and cell organelles were disrupted.
[q] VLPs found in the plurilocular sporangia of native material. VLPs also found in developing zoospores; thus the VLPs may have been transmitted through the zoosporangium to the zoospore by subsequent cleavage. Particles are associated with tubular elements.
[r] The particles appear only in gametangia of certain isolates. They have a dsDNA of ca. 350 kbp.
[s] No nuclear membrane is present in sporangia with VLPs, but surrounding matrix appears to be nucleoplasm. They have a dsDNA of about 190 kbp.
[t] VLPs are present only in sporangia and are not seen in vegetative cells.
[u] VLPs are usually present in zoospores but are also seen in vegetative cells of the alga.
[v] VLPs are present in vegetative cells.
[w] VLPs are present in about 5% of the cells.
[x] The alga does not have a cell wall; the particles are infectious.
[y] Approximately 30% of the cells have particles.
[z] Both size classes of VLPs were found in the same cell.
[aa] *Aulacomonas* sp. is a colorless flagellate, lacking a chloroplast. The VLPs are surrounded by an envelope about 30 nm thick. Smaller polyhedral particles (50 nm in diameter) were present in the nucleus of one cell.
[bb] The presence of viruses in these *Chlorella* cells is questionable since the cultures were contaminated with bacteria and the particles may be bacteriophage.
[cc] The host *Chlorella* cell is normally a symbiont of *H. viridis*. The VLP dsDNA is at least 250 kbp.
[dd] The host *Chlorella* cell is normally a symbiont of a protozoan, *P. bursaria*, collected in the United States. The alga can be grown in culture, and a family of viruses which replicate in the alga can be plaque assayed.
[ee] The host *Chlorella* cell is normally a symbiont of a protozoan, *P. bursaria*, collected in Europe. The alga can be grown in culture, and a family of viruses which replicate in the alga can be plaque assayed.
[ff] Virus may have a retractable tail.
[gg] VLPs confined to single-cell germlings, and a heat shock of 40°C, 6–24 h, is necessary to induce particle formation. The heat shock, applied to the zoospores, produces germlings in which 3 to 10% of the cells are infected with VLPs. dsDNA is 275 to 300 kbp, particles contain at least 10 proteins.
[hh] Detected only in developing germlings.
[ii] No pictures of these particles have been published, but they are reported to resemble the VLPs of *Coleochaete* spp.
[jj] Probably not a VLP.
[kk] About 1% of the cells in the germling or young filament stage contain particles. A heat shock treatment leads to increased VLP production.
[ll] The virus was the first experimental infection of a eukaryotic algal cell. Physiochemical data: $s_{20,w}$ = 230S; molecular weight, 3.6×10^6; base ratio of G 24.5, A 28.0, C 20.0, U 27.5; 5% RNA; protein coat molecular weight, ca. 17.5×10^3.

Symbiotic *Chlorella* Species

Most algae assigned to the genus *Chlorella* are free living in nature. However, some forms called zoochlorellae or *Chlorella*-like algae, live as hereditary endosymbionts within freshwater and, to a lesser extent, marine animals (see, e.g., references 40, 105, 159, and 178). For convenience, we will use the term zoochlorellae in this review. Douglas and Huss (31) have shown that the DNA from several zoochlorellae hybridize with free-living *C. vulgaris* DNA. Because of the similarity of zoochlorella DNA and *C. vulgaris* DNA and because some zoochlorellae can be cultured, two groups of investigators (31, 62) have suggested that zoochlorella symbiotic associations may have arisen relatively recently.

The green coelenterate *H. viridis* (106) and the unicellular green protozoan *P. bursaria* (61) are among the most widely studied organisms containing zoochlorellae. Each gastrodermal cell of *H. viridis* contains 10 to 20 zoochlorellae and a single hydra contains 1.5×10^5 zoochlorellae under optimum laboratory conditions. *P. bursaria* typically contains several hundred zoochlorellae.

Each algal cell is enclosed within an individual, host-derived vacuole, known as the perialgal vacuole (193). The algae are always located in a well-defined position in the cells of the host hydra or paramecium (159). The zoochlorellae grow and divide in concert with the host so that the population of zoochlorellae is maintained at a constant level. When green hydras and green paramecia are placed under nongrowing conditions, algal growth also ceases. Thus the zoochlorellae and host grow coordinately (see, e.g., reference 32).

Symbiotic zoochlorellae fix CO_2 in light and can transfer 30 to 40% of their total photosynthate to the host hydra or paramecium (33, 98, 131). The symbiotic relationship can also be maintained for at least a short time in dark-grown hydras (120) or paramecia (89).

Symbiont-free *Hydra viridis* can be obtained in the laboratory by growing hydras in glycerol or in high-intensity light either alone or in the presence of a photosynthetic inhibitor such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (121). These treatments produce a white, aposymbiotic hydra. The aposymbiotic hydra can be grown in culture indefinitely if appropriately fed. In contrast, most investigators have had trouble growing hydra zoochlorellae in culture. Although there is one report of the successful culturing of a hydra zoochlorella (59), others believe that this alga may have been a surface contaminant (66).

Aposymbiotic *P. bursaria* organisms are produced by growth in the dark at high growth rates for several months

(130, 154). Paramecium zoochlorellae are apparently easier to culture than those of hydras, since they have been cultured on several occasions (see, e.g., references 75, 119, 154, 201).

Cultured zoochlorellae release as much as 80% of their photosynthate to the environment (17), primarily in the form of the disaccharide maltose, whereas free-living *Chlorella* species do not. Maltose release is pH dependent; it is maximal at about pH 4.0 and declines with increasing pH to nearly zero at pH 7.0. The excreted maltose apparently does not arise directly from starch degradation; instead, Cernichiari et al. (17) suggested that a specific glucosyltransferase condenses glucose at the algal cell surface to produce maltose.

The symbiotic relationships of hydras or paramecia and their zoochlorellae can be reestablished by coculture of the host and its symbiont. Symbionts are individually taken up into perialgal vacuoles, which avoid fusion with lysosomes. The fate of nonsymbiotic *Chlorella* cells taken in by hydras or paramecia varies with the *Chlorella* species and host. Typically, the nonsymbiotic *Chlorella* cells enter food vacuoles and are digested as the vacuoles fuse with lysosomes or else the *Chlorella* cells fail to grow and divide (see reference 90 and references cited therein).

Hereditary endosymbiotic associations are usually, but not always, host specific. For example, reassociation studies with different *Chlorella* species and aposymbiotic paramecia indicate that the colorless paramecium can take up not only its original symbiotic partners, but also a limited number of other *Chlorella* species, and can form green paramecia under special conditions. However, only the original paramecium zoochlorellae can reestablish a long-term, stable symbiosis with the protozoan (131). Paramecium zoochlorellae can also form a symbiotic association with hydras which is stable under laboratory conditions (91, 106).

Kessler et al. (66) reported that a variety of free-living *Chlorella* strains could form a symbiotic relationship with hydras. The single characteristic correlated with this property was the ability of the algae to grow under acidic (pH 4) conditions. This finding, if verified, suggests that specific surface recognition may not be as important in establishing symbiosis as was once thought (see, e.g., references 95 and 125). However, maintaining a stable symbiotic relationship is more complex. The ability of the alga to supply the host with carbon, in the form of maltose (159), and to utilize nitrogen and other host nutrients is probably important. Understanding symbiosis will require discovering the details of the physiological adaptations.

Chlorella Strains NC64A, N1a, Pbi, and SAG-241-80

The hosts for two groups of *Chlorella* viruses are the exsymbiotic *Chlorella* strains NC64A and N1a, isolated almost 30 years apart from *P. bursaria* collected in the United States, and *Chlorella* strains Pbi and SAG-241-80, isolated from paramecia collected in Europe. *Chlorella* strains NC64A and N1a have identical mitochondrial and chloroplast genomes (91), and thus the algae are probably identical. We have used NC64A and N1a interchangeably in our virus studies, and to simplify the discussion we will refer to both strains as NC64A in this review.

Chlorella strain NC64A can be grown easily in the laboratory on a simple salt solution (Bold's basal medium [111]) modified by adding 0.5% sucrose and 0.1% protease peptone at 25°C in continuous light (ca. 30 microeinsteins m^{-2} s^{-1}) with gentle shaking. Under these conditions the alga produces four autospores about every 48 h. The algae are oval and measure about 4 by 5 μm. They have a single cup-shaped chloroplast with a prominent pyrenoid.

Commercially available enzymes digest the cell walls of some free-living *Chlorella* species to give viable protoplasts, e.g., *C. vulgaris*, *C. saccharophila*, and *C. ellipsoidea* (1, 7, 220). *Chlorella* strain NC64A is resistant to commercial cell-wall-degrading enzymes (91). However, we have partially purified an enzyme(s) from virus lysates which degrades *Chlorella* strain NC64A cell walls. This enzyme(s) (called lysin) can produce protoplasts of *Chlorella* strain NC64A. As measured by vital stains, the protoplasts live for up to 4 weeks, but attempts to regenerate colonies from these protoplasts have so far been unsuccessful (91).

Isolated *Chlorella* strain NC64A cell walls were analyzed for component monosaccharides (92). Glucose and rhamnose made up 51 and 16% of the total sugars, respectively, with galactose, xylose, arabinose, mannose, and glucosamine each accounting for 5 to 10% of the sugars. These monosaccharides made up about 50% (by weight) of the solubilized wall material. The nature of the remaining wall material is unknown.

Chlorella strain NC64A nuclear DNA is 67% G+C (189), and mitochondrial DNA (195) and chloroplast DNA (147) are 32 and 38% G+C, respectively. The nuclear DNA contains methylated bases; 5mC makes up 21% of the cytosines, and N^6-methyladenine (6mA) makes up 0.6% of the adenines (189). No 4-methylcytosine (4mC) was detected in the nuclear DNA (179).

A lambda genomic library of *Chlorella* strain NC64A (N1a) nuclear DNA has recently been prepared in *Escherichia coli* K803 (91). The nuclear DNA was not clonable in other *E. coli* strains, presumably because of its high 5mC content (194). A single-copy β-tubulin gene, which hybridized to a *Chlamydomonas* tubulin gene, was identified in the *Chlorella* strain NC64A library and sequenced. The *Chlorella* tubulin gene has more introns (twelve) than any other known tubulin gene and shares sequence and gene structure with both fungi and higher plants (91). Vectors to transform *Chlorella* strain NC64A are being constructed with the promoter region of this tubulin gene.

Chlorella strain NC64A (N1a) contains a 120-kbp circular chloroplast DNA (147), which is smaller than the 175-kbp chloroplast DNA present in free-living *C. ellipsoidea* (217, 218). Typically, chloroplast DNAs, including those of *C. ellipsoidea*, contain an inverted repetitive sequence of about 20 kbp, containing a copy of the 23S, 16S, and 5S rRNA genes. However, *Chlorella* strain NC64A chloroplast DNA contains only a single rRNA gene.

The mitochondrial DNA from *Chlorella* strain NC64A (N1a) is a 76-kbp circle (195). The mitochondrial DNA was physically mapped, and some of the mitochondrial genes were placed on the map. Beyond these studies, nothing is known about the molecular properties of *Chlorella* strain NC64A. There is even less information about the molecular properties of *Chlorella* strains Pbi and SAG-241-80.

DISCOVERY OF *CHLORELLA* VIRUSES

Initial Observations

A fortuitous conversation between two of us (R.H.M. and J.V.E.) about 11 years ago led to a simple experiment, which produced an exciting result. We discovered that large (185 nm in diameter) polyhedral VLPs accumulated in zoochlorellae within a few hours after they were isolated from *H.*

viridis (Fig. 1). Replication of these particles lysed the entire population of zoochlorellae within 24 h following isolation from hydras (96). These VLPs, which were absent from intracellular zoochlorellae, were named HVCV-1 for *H. viridis Chlorella* virus.

HVCV-1 was isolated and partially characterized. The particles sedimented at 2,600S in sucrose density gradients and had a density of 1.295 g/ml in CsCl. HVCV-1 contained a double-stranded DNA (dsDNA) genome of at least 250 kbp and at least 19 structural proteins varying in apparent size from 10.3 to 82 kDa. The major viral protein of 46 kDa stained with Schiff's reagent, suggesting that it was a glycoprotein (186).

Our initial experiments were conducted with zoochlorellae isolated from a hydra strain (Florida strain) which had been maintained in culture for over 20 years. Subsequently we examined zoochlorellae isolated from six other hydra isolates for VLPs. In addition, zoochlorellae were isolated from *P. bursaria*. In each case, large dsDNA containing VLPs appeared within 24 h of zoochlorella isolation (187). Restriction endonuclease analysis of VLP DNAs revealed four distinct VLPs. The relationship of the VLPs to the source of the hydra was especially interesting. A green hydra obtained from a Massachusetts lake in 1981 yielded a VLP identical to HVCV-1. Green hydras collected in 1981 from lakes in North Carolina, Nebraska, and Massachusetts yielded a second VLP, designated HVCV-2. Two green hydras collected independently in England yielded a third VLP, designated HVCV-3. The presence of identical genomes in VLPs isolated from zoochlorellae from such diverse geographic locations suggested that the VLPs had a remarkably stable relationship with their zoochlorella host.

The source of the VLPs in the hydra-zoochlorellae systems is still unknown. Particles were never observed in intracellular zoochlorellae in the hydras. The particles could result from an external infection which is maintained by the natural death of hydras and release of associated zoochlorellae. Another possibility is that association with hydras suppressed viruses lysogenic with zoochlorellae. These lysogenic viruses might even code for a gene product(s) that mediates symbiosis between the zoochlorellae and the hydras. An additional possibility is that the particles replicate in hydra cells and infect the zoochlorellae during their isolation. Although we never observed VLPs in hydra cells, there is one report of VLPs in a hydra species (*Hydra vulgaris*) which lacks zoochlorellae (11). These polyhedral particles were smaller (70 to 75 nm) than the VLPs subsequently found in *H. viridis* zoochlorellae. The possibility of lysogeny in the zoochlorellae or viral replication in the hydras could be determined experimentally; however, these experiments have not been conducted.

The DNA restriction fragment pattern of the VLP from the *P. bursaria* zoochlorellae differed from those of HVCV VLPs, and this VLP was designated PBCV-1 for *P. bursaria Chlorella* virus. Kawakami and Kawakami (63) had previously observed VLPs infecting zoochlorella in a *P. bursaria* strain isolated in Japan. Zoochlorellae growing symbiotically inside the paramecia did not contain VLPs. However, the algae became infected immediately after release from the paramecia. The VLPs observed by Kawakami and Kawakami were neither isolated nor physically characterized; however, they were similar in size and morphology to PBCV-1.

In summary, VLP infections are common in zoochlorellae shortly after isolation from hydras and paramecia. This can explain the difficulty in culturing zoochlorellae from hydras. Although the VLP association may contribute to the symbiosis, the relationship in *P. bursaria* is not as strong, since zoochlorellae have been cultured free of paramecia on several occasions (75, 119, 130, 154, 201).

Development of a Culturable System for PBCV-1

We tested several cultured zoochlorellae, reputedly derived from invertebrate hosts, as well as two free-living *Chlorella* strains, to see whether they could serve as hosts for the three HVCV VLPs and for PBCV-1. Two of the zoochlorella isolates (NC64A and ATCC 30562) and, more recently, N1a, originally from United States isolates of *P. bursaria*, supported virus growth (181). The progeny particles were identical to PBCV-1, and they were infectious. This fulfilled Koch's postulates, and PBCV-1 is an authentic virus. More importantly, PBCV-1 formed plaques on lawns of *Chlorella* strain NC64A (Fig. 2), providing a sensitive bioassay for PBCV-1. None of the HVCV viruses replicated in the zoochlorellae from paramecia, and neither PBCV-1 nor HVCV replicated in any of ca. 50 additional free-living *Chlorella* or *Chlamydomonas* strains tested to date.

The plaque assay for PBCV-1, together with the ability to synchronously infect the alga, allows one to study PBCV-1 by using bacteriophage technology. For example, one-step growth studies, life cycle studies, and genetic studies are feasible. The lack of a sexual stage in the host *Chlorella* organism and the current absence of an effective transformation system for the alga are disadvantages of the system.

After developing the PBCV-1 plaque assay, we assayed freshwater samples collected throughout the United States and other parts of the world for plaque-forming viruses. As will be elaborated in the sections on properties of *Chlorella* strain NC64A viruses in addition to PBCV-1 and on the natural history of the *Chlorella* viruses, plaque-forming viruses on *Chlorella* strain NC64A are common in continental American, Chinese, and Japanese, but not European, fresh water. The viruses infecting *Chlorella* strain NC64A are referred to as NC64A viruses.

Once we realized that NC64A viruses were common, we examined our original culture of paramecia for virus by the plaque assay. This culture, which had been stored at room temperature for about 18 months, still had viable green paramecia. However, no plaque-forming viruses were detected in the culture, nor could they be detected in zoochlorellae following isolation of the zoochlorellae from these paramecia (179). Consequently, the appearance of VLPs in the original, isolated paramecium zoochlorellae probably resulted from an external infection.

Plaque-forming viruses which infect *Chlorella* strain SAG-241-80 (69, 70) and *Chlorella* strain Pbi (referred to as Pbi viruses) (132, 134) have also been discovered in Soviet and European fresh water. *Chlorella* strains SAG-241-80 and Pbi are zoochlorellae originally cultured from European isolates of *P. bursaria*.

PROPERTIES OF *CHLORELLA* VIRUSES

General Characteristics of PBCV-1

PBCV-1, the prototype NC64A virus, is easy to purify, and 80 to 105 A_{260} units (8 to 10 mg of PBCV-1) and 1.4×10^{12} to 2×10^{12} PFU of purified PBCV-1 are obtained per liter of lysate. Purified virus can be stored at 4°C for at least 1 year without detectable loss of infectivity. However, freezing inactivates PBCV-1.

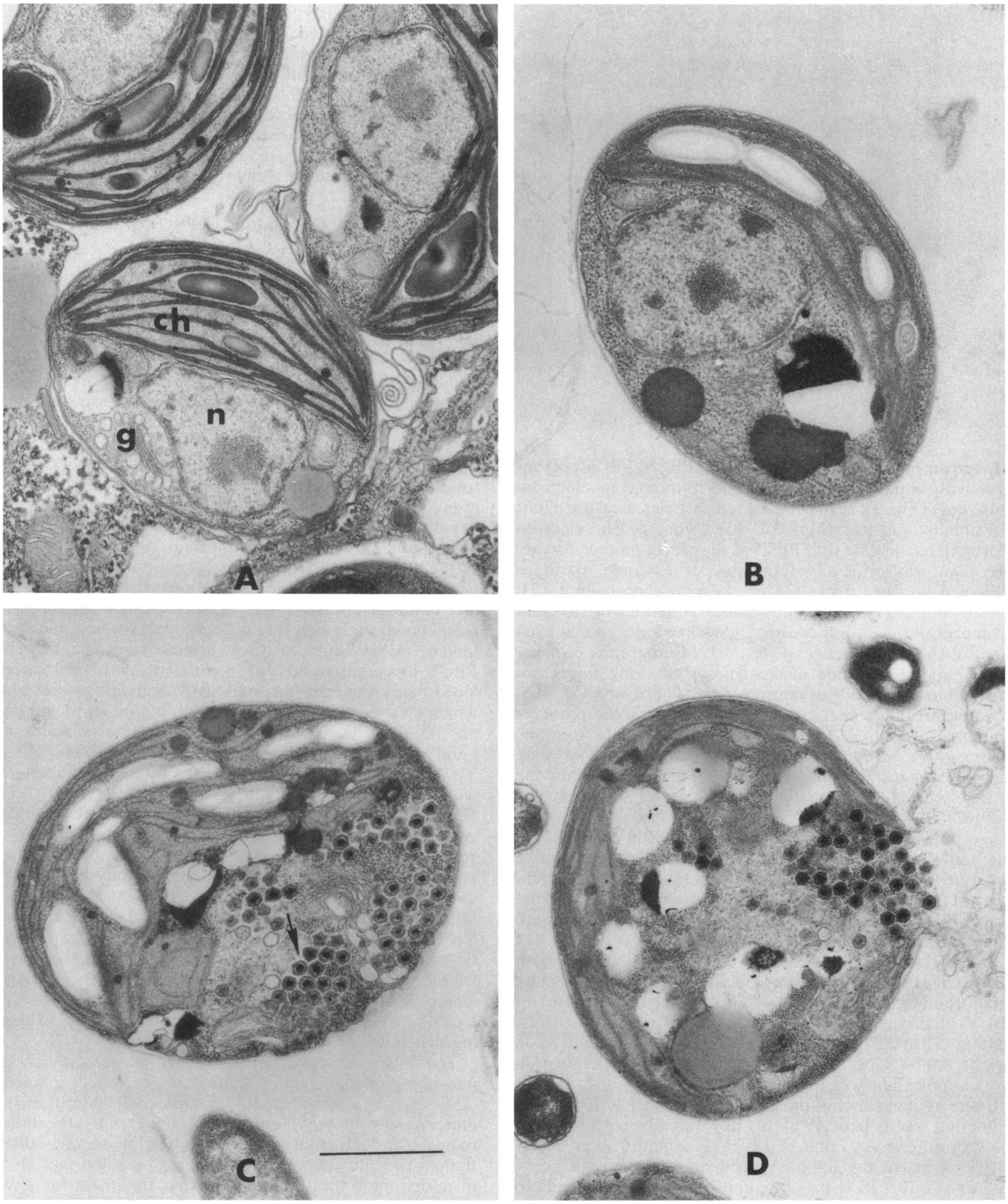


FIG. 1. Electron micrographs of the zoochlorellae present in *H. viridis* (A), immediately after isolation from the hydra (B), and after incubation of the isolated algae at 20°C for 6 h (C) and 20 h (D). Note the appearance of VLPs (arrow in panel C) in the algae at 6 and 20 h after isolation. Abbreviations: g, Golgi; n, nucleus; ch, chloroplast. The bar in C represents 1 μm. Reproduced from reference 96 with permission from Academic Press, Inc.

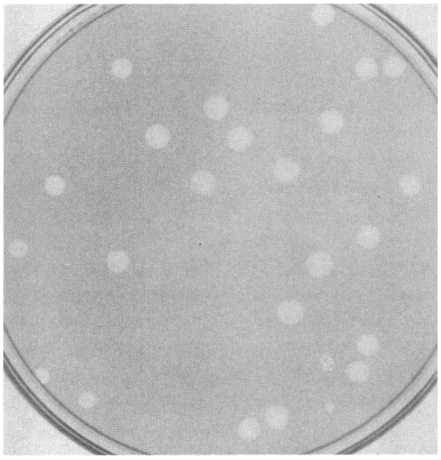


FIG. 2. Plaque assay of PBCV-1 on a lawn of *Chlorella* strain NC64A. The photograph was taken 4 days after the plate was prepared. However, plaques can be observed as early as 24 h after plating.

Electron micrographs indicate that PBCV-1 is a polyhedron with a multilaminate shell surrounding an electron-dense core (Fig. 3). Frozen hydrated particles measure about 175 nm in diameter (Fig. 3A) (5). More recent electron micrographs indicate that PBCV-1 may have more substructure than was originally thought. For example, particles subjected to the quick-freeze, deep-etch procedure (53) contain flexible hairlike appendages (fibers) with swollen structures at the end; these appendages extend from at least some of the virus vertices (Fig. 3B). Micrographs of negatively stained particles also suggest that long fibers are attached to the outside of the capsid (small arrow in Fig. 3C). In addition, these micrographs suggest that some particles contain a distinctive 20- to 25-nm spike extending from one vertex (large arrow in Fig. 3C). The proportion of such particles suggests a unique vertex (50). DNA within the particle seems to be retracted from this vertex. The diameter of PBCV-1 is about 160 nm by comparison with lambda phage on the same grid (50). Speculation on the possible function of both the hairlike appendages and the spike structure will be mentioned in the section on virus attachment. It is obvious that additional research on the ultrastructure of PBCV-1 is required and that the particle may be more complex than a simple polyhedron.

PBCV-1 sediments in sucrose density gradients at about 2,300S. The virus is disrupted on CsCl and Cs_2SO_4 density equilibrium gradients (187). The molecular weight of PBCV-1 has been estimated by two methods. From the DNA content (21 to 25%) and size (2.1×10^8 Da), the virion has a weight of 0.84×10^9 to 1×10^9 (184). Field flow fractionation gives a molecular weight of 1×10^9 (222).

If one assumes a viral molecular weight of 1×10^9 and an extinction coefficient $A^{260}_{0.1\%}$ of 10.7 (uncorrected for light scattering), one A_{260} unit of PBCV-1 contains about 5.6×10^{10} virus particles (184). Experimentally we obtain 1.5×10^{10} to 3×10^{10} PFU per A_{260} unit of PBCV-1. Thus, 25 to 50% of the viral particles form plaques.

PBCV-1 was treated with several reagents under a variety of conditions in an attempt to produce empty capsids and/or stable subviral core particles (156, 188). PBCV-1 is stable at pH 4 to 11. Virus infectivity is rapidly lost if PBCV-1 is stored above 45°C. PBCV-1 infectivity was stable for at least 1 week at 22°C in 5 mM EDTA or ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA); however, infectivity decreased slowly in 50 mM EDTA or EGTA. Virus treated with either 5 mM dithiothreitol or 5 mM dithioerythritol, but not 5 mM mercaptoethanol, rapidly lost infectivity. The dithiothreitol- or dithioerythritol-treated virus sedimented at the normal rate in sucrose gradients; however, electron micrographs of uranyl acetate-stained preparations revealed disrupted virus. To date, none of the treatments which decreased PBCV-1 infectivity have yielded either empty capsids or stable subviral core particles.

Composition of PBCV-1

PBCV-1 contains about 64% protein, 21 to 25% dsDNA, and 5 to 10% lipid (156, 184). Analysis of PBCV-1 proteins by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4A) indicates more than 50 structural proteins ranging in apparent size from 10 to more than 200 kDa. Two-dimensional PAGE revealed additional proteins. However, some of the spots (especially the major capsid protein) smear on isofocusing gels, and repeated attempts to obtain "clean" two-dimensional patterns have been unsuccessful (157).

One 54 kDa protein (Vp54) makes up about 40% of the total viral protein. If PBCV-1 is heated in denaturing buffer at 60°C, instead of 100°C, Vp54 migrates as a dimer with an apparent molecular weight of 104,000 (Fig. 4A, compare lanes 1 and 2). Vp54 is a glycoprotein. After deglycosylation of the protein with trifluoromethanesulfonic acid or hydrogen fluoride-pyridine, the protein migrates with an apparent size of about 49 kDa (196). The nature of the glycan linkage is unknown, although two observations suggest that it is O linked. Monensin, but not tunicamycin, inhibits both PBCV-1 synthesis and Vp54 synthesis (198). Incubation of Vp54 with the enzyme endo-β-*N*-acetylglucosaminidase, which cleaves N-linked sugars, does not affect its migration on SDS-gels. A second protein, Vp135, also stains with Schiff's reagent and is probably a glycoprotein. Vp135, Vp71, Vp54, and Vp14.5 are labeled by treating intact virus with ^{125}I and are believed to be surface proteins (156). Serological experiments, described below, indicate that the glycan is on the virus surface. The resistance of PBCV-1 to several proteases is consistent with this interpretation.

PBCV-1 proteins were also analyzed for myristylation and phosphorylation by infecting cells in the presence of [^{3}H]myristic acid or $^{32}PO_4^{3-}$ (128). Four proteins (Vp135, Vp55, Vp54, and Vp27.5) were specifically labeled by myristic acid. The myristic acid label was resistant to hydroxylamine (pH 8.5) and alkaline methanolysis, suggesting an amide linkage. Six PBCV-1 proteins (Vp60, Vp45, Vp36, Vp29, Vp20, and Vp14) were labeled with $^{32}PO_4^{3-}$ suggesting that they are phosphoproteins.

The major lipids in PBCV-1 are phosphatidylcholine, phosphatidylethanolamine, and an unidentified component (156). PBCV-1 loses infectivity rapidly in chloroform and more slowly in ethyl ether or toluene; thus the lipid is required for infectivity. Sedimentation of organic solvent-treated particles on sucrose gradients revealed that chloroform disrupted the virus. Ethyl ether treatment for 1 week did not alter the sedimentation properties of the virus. However, the ether-treated virus was swollen and partially disrupted after staining with uranyl acetate.

PBCV-1 infectivity is unaffected by incubation with several detergents. For example, 2% Triton X-100, 2% Nonidet P-40, and 2% sodium deoxycholate have no effect on viral

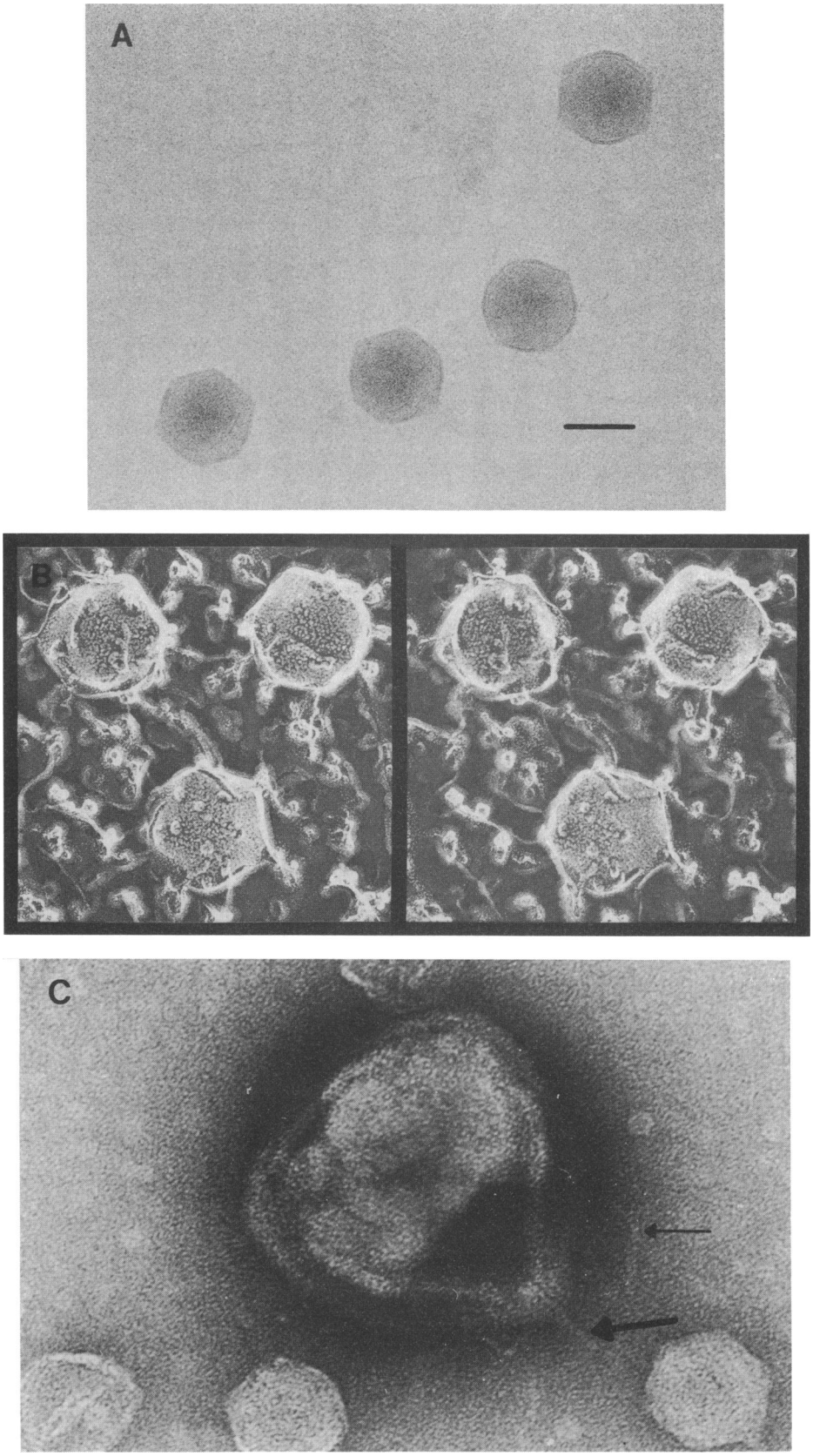


FIG. 3. Morphology of PBCV-1. (A) Frozen-hydrated particles (photograph kindly provided by Tim Baker). Note that the capsid appears as a double-layered structure in some of the particles. Bar, 200 nm. (B) PBCV-1 subjected to the quick-freeze, deep-etch procedure (53) (photograph kindly provided by John Heuser). Note the presence of flexible hairlike fibers, which appear swollen at their ends, on the surface of the particles. (C) PBCV-1 negatively stained with uranyl acetate (photograph kindly provided by Roger Hendrix). The smaller particles are lambda phage. Note that (i) long fibers are associated with the particles (small arrow), (ii) a distinctive 20- to 25-nm spike structure (large arrow) extends from one vertex of the particle, (iii) the particle appears to be packed asymmetrically, and (iv) an interior membrane appears to be located inside the capsid which extends to the unique vertex.

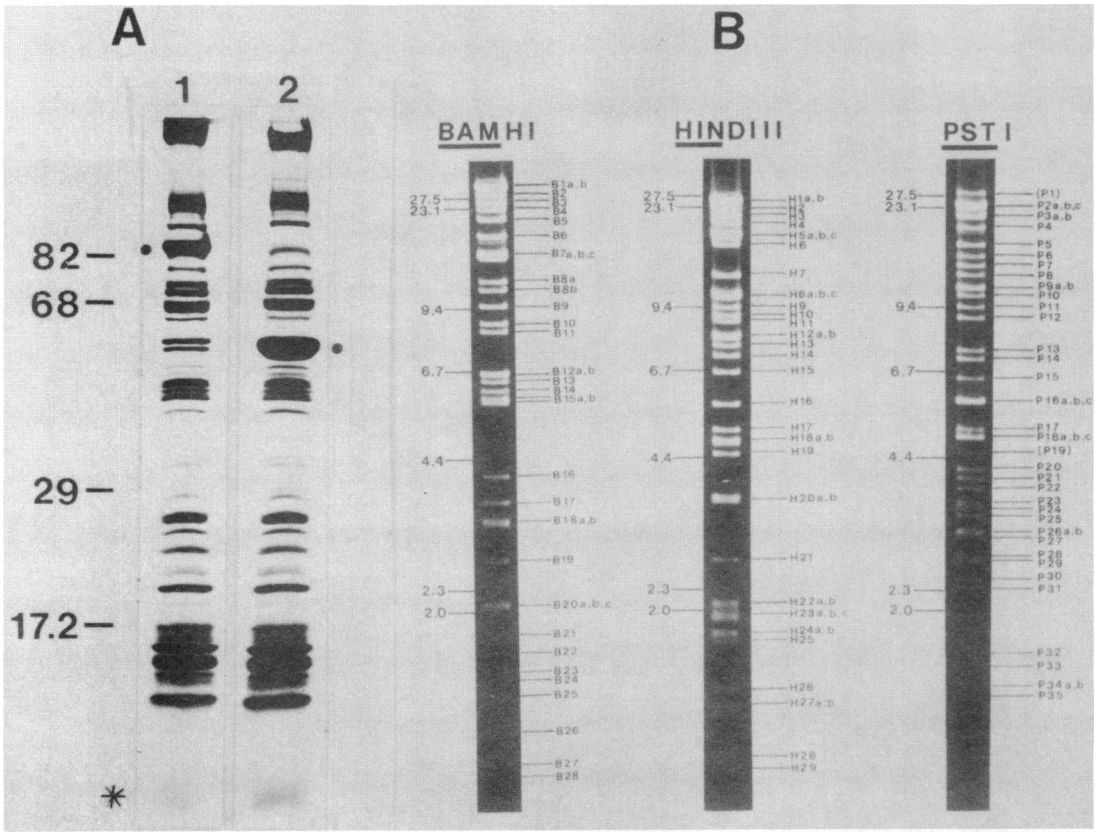


FIG. 4. SDS-polyacrylamide gel of PBCV-1 proteins and PBCV-1 DNA restriction fragments. (A) Fluorogram of ^{14}C-labeled PBCV-1 structural proteins following incubation at 60°C for 1 h (lane 1) or at 60°C for 55 min followed by 100°C for 5 min (lane 2). The material marked with an asterisk is presumed to be lipid. Note that the major viral protein, Vp54 (●), exists as a dimer in lane 1 and as a monomer in lane 2. Reproduced from reference 156 with permission from Academic Press, Inc. (B) PBCV-1 DNA restriction fragments: *Bam*HIII (lane 1), *Hin*dIII (lane 2), and *Pst*I (lane 3). Reproduced from reference 39 with permission from Martinus Nijhoff Publishers.

infectivity. However, PBCV-1 loses infectivity slowly in 2% sodium *N*-lauroyl sarcosine and rapidly in 2% SDS. These data, together with the ultrastructural data (i.e., an internal lipid layer is apparent in Fig. 3C), suggest that the lipid component is located inside the outer glycoprotein capsid.

PBCV-1 Genome

PBCV-1 contains a large dsDNA genome with a 40% G+C content (189). PBCV-1 DNA contains methylated bases. 5mC makes up 1.9% of the total cytosines, and 6mA makes up 1.5% of the total adenines.

The size of the PBCV-1 genome, 333 kbp, was derived initially from summing restriction fragments. PBCV-1 DNA contains at least 41 *Bam*HI fragments, 44 *Hin*dIII fragments, and 50 *Pst*I fragments (Fig. 4B) (39). Analysis of intact DNA by pulsed-field electrophoresis verified the size of PBCV-1 DNA at about 333 kbp (138).

We initially reported that the PBCV-1 DNA restriction map was circular (39). This map was based on analyses of both cloned DNA and Southern transfers probed in some cases with gel-purified fragments that could not be cloned. From these experiments we concluded that PBCV-1 DNA was either a covalently closed circle, circularly permuted, or linear with terminal repetition. Subsequent experiments have established that the genome is a linear nonpermuted molecule and that the termini contain identical inverted repeats. Furthermore, the termini have covalently closed hairpin ends (138, 167).

PBCV-1 DNA terminal fragments were identified by incubating intact DNA with *Bal*31 exonuclease, which can initiate digestion in the single-stranded region of hairpin ends (see, e.g., references 6, 9, and 221), for various times before digestion with either *Hin*dIII, *Bam*HI, or *Pst*I restriction endonucleases (138). Two *Hin*dIII fragments (H4 and H17), two *Bam*HI fragments (B10 and B15a), and two *Pst*I fragments (P17 and P31) disappeared with increased *Bal* 31 exonuclease treatment, suggesting that these fragments were termini. Hybridization studies established that restriction fragments B15a, H4, and P31 (arbitrarily called the left end) were at one terminus and that B10, H17, and P17 were at the other terminus. A revised restriction map beginning with B15a at zero and ending with B10 at 333 kbp is shown in Fig. 5.

The terminal fragments, e.g., P31 and P17, hybridize with one another, indicating terminal repetition. The termini (minus the hairpin) have recently been cloned and sequenced to within about 40 to 60 bases of the end (167). The termini contain identical inverted repeats of at least 2,185 bases, after which the sequence diverges. The inverted repeats contain two small open reading frames (ORFs) and several direct repeats. However, neither of the ORFs nor the remainder of the inverted repeats appear to be transcribed

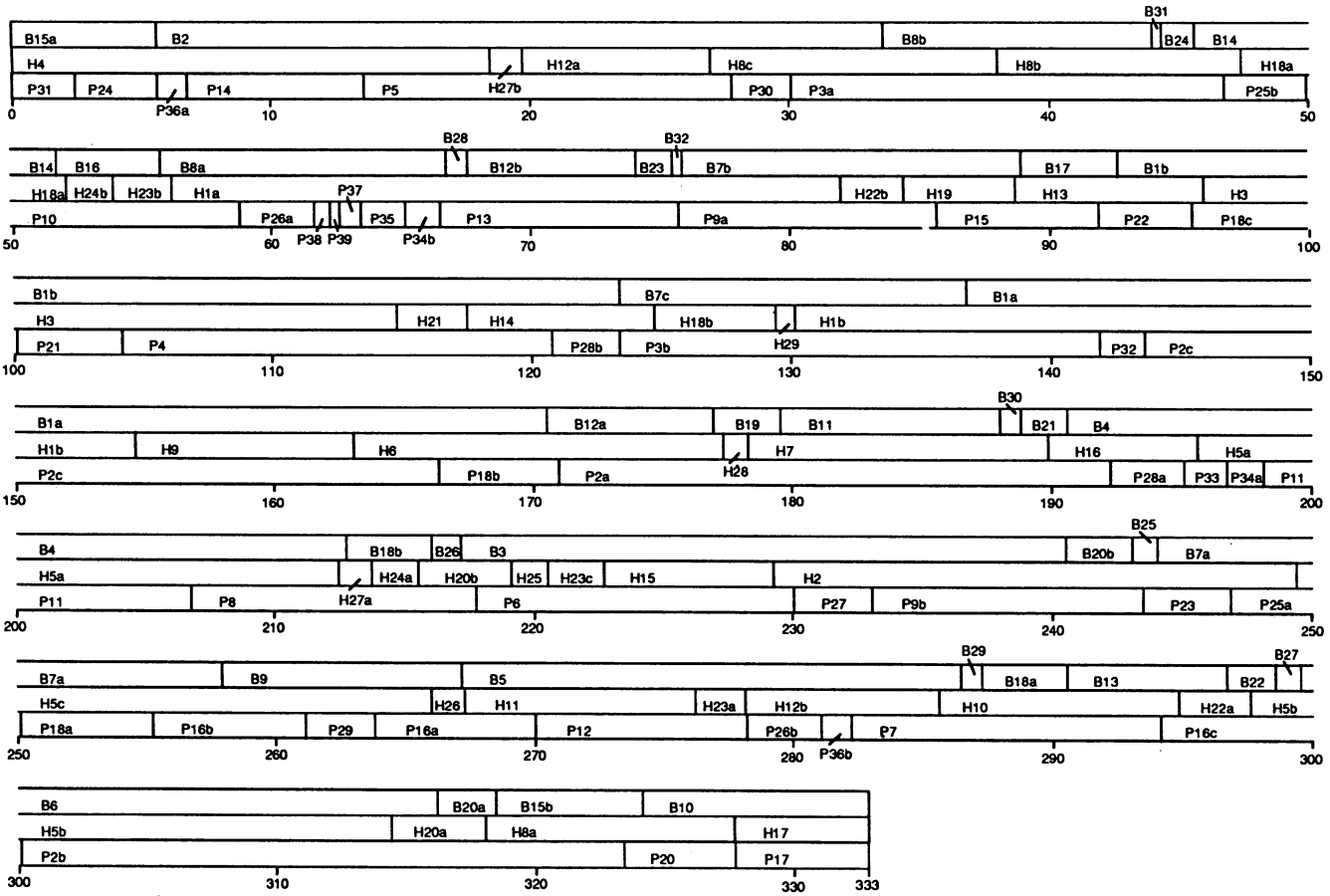


FIG. 5. Revised PBCV-1 restriction map. The restriction sites for *Bam*HI, *Hin*dIII, and *Pst*I are shown. Fragments B15a, H4, and P31 were placed at the left terminus, coordinate zero, and fragments B10, H17, and P17 were placed at the right terminus.

during PBCV-1 replication. The remainder of the PBCV-1 genome contains primarily unique DNA and has the potential to code for several hundred proteins (39).

Serology of PBCV-1

Polyclonal antiserum prepared against intact PBCV-1 completely inhibits PBCV-1 infection by agglutinating the particles even at a dilution of 1:500. Western immunoblot analysis indicates that although the antiserum reacts with many of the virus proteins, it reacts most strongly with the major capsid protein Vp54 (197). If Vp54 is deglycosylated with trifluoromethanesulfonic acid or treated with periodic acid after transfer to nitrocellulose, the protein no longer reacts with PBCV-1 antiserum.

Spontaneously derived antiserum-resistant mutants of PBCV-1 can be isolated at a frequency of about 10^{-6}. Polyclonal antiserum was initially prepared against one of these mutants, named EPA-1. EPA-1 antiserum completely inhibits EPA-1 infection (dilution of 1:500), but has no effect (dilution of 1:10) on PBCV-1 infection (19). EPA-1 major capsid protein migrates slightly faster, with an apparent molecular mass of 53 kDa, than PBCV-1 Vp54. Like antiserum to PBCV-1, antiserum to EPA-1 reacts with the glycosylated but not the deglycosylated form of the major capsid protein on Western blot analysis.

Subsequently, we isolated mutants resistant to both PBCV-1 and EPA-1 antiserum and prepared polyclonal antiserum against some of these mutants (197). The antiserum-resistant mutants have several interesting properties. (i) Four PBCV-1 serotypes have been identified to date. All antisera reacted only with the virus which induced the antiserum. (ii) The major capsid proteins from all the mutants migrated slightly faster on acrylamide gels than did those of the strains from which they were derived. All major capsid proteins comigrated on SDS-gels after deglycosylation. (iii) Western blot analysis of the major capsid protein from the mutants, before and after deglycosylation, indicates that the serological differences among the viruses is due to a change in the carbohydrate moiety(ies) of the major capsid protein. (iv) The ratio of seven sugars (fucose, galactose, glucose, xylose, mannose, arabinose, and rhamnose) associated with the major capsid protein of PBCV-1 and the mutants varied in a predictable manner which was related to their serotype and the migration of the major capsid protein. (v) Each of the four serotypes arises from apparent single mutations in PBCV-1. This suggests that at least four virus gene products influence the PBCV-1 serotypes and that these genes are in a common pathway involved in glycosylation.

Typically, carbohydrate moieties of virus glycoproteins are added in the rough endoplasmic reticulum and Golgi apparatus by host glycosyltransferases (142). Growing a virus in a different host (e.g., herpesvirus [77, 78], and influenza virus [26, 107]) can change the nature of the glycan.

Mutations that alter the polypeptide directly, e.g., changing a serine or asparagine to another amino acid (e.g., vesicular stomatitis virus [79]), can eliminate the glycan. We are presently testing the mutation possibility by cloning and sequencing the major capsid protein from PBCV-1 and some of the serological mutants. If the major capsid protein in PBCV-1 and the serotypes have identical amino acid sequences, this will confirm direct participation of viral enzymes in glycosylation.

Additional *Chlorella* Strain NC64A Viruses

Hundreds of plaque-forming viruses, all of which infect *Chlorella* strain NC64A, have been isolated from fresh water collected throughout the continental United States and from China. Thirty-six of these viruses have been partially characterized and can be grouped into 16 classes (Table 2). Like PBCV-1, each of these viruses contains many structural proteins and a large dsDNA genome (at least 300 kbp) with 40% G+C, and all are chloroform sensitive. The viruses are polyhedra, with diameters of 150 to 190 nm. The DNAs of some hybridize extensively with PBCV-1 DNA, whereas others hybridize poorly (144, 227).

The viruses can be distinguished from PBCV-1 and from each other by plaque size, antiserum sensitivity, DNA restriction patterns, sensitivity of the DNAs to restriction endonucleases, and the nature and abundance of methylated bases. DNA from each of the viruses contains 5mC in amounts varying from 0.12 to 47.5% of the total cytosine (Table 2). In addition, 24 of the 36 viral DNAs contain 6mA in amounts varying from 1.45 to 37% of the total adenine. The virus DNAs do not contain 4mC. Thus these viruses are a rich source of methylated DNAs. Such DNAs may be useful for studying the biological and physical properties of methylated DNAs.

Growth curves revealed that viruses that form plaques similar in size to PBCV-1 (e.g., NC-1A and IL-3A) replicate at about the same rate as PBCV-1, whereas viruses that form small plaques (e.g., SC-1A, CA-4A, and NY-2A) take about two to three times longer to replicate (190). The burst size for each of the viruses is typically 200 to 350 PFU.

Viruses Infecting *Chlorella* Strain Pbi

Plaque-forming viruses that infect an exsymbiotic *Chlorella* strain (*Chlorella* strain Pbi) of a European isolate of *P. bursaria* have also been isolated from European fresh water (132, 134). The Pbi viruses, like the NC64A viruses, are large polyhedra with diameters of 140 to 150 nm, are chloroform sensitive, have many structural proteins, and have large dsDNA genomes of at least 300 kbp that contain methylated bases (Table 3). However, the Pbi viruses are serologically distinct from the NC64A viruses, and their DNAs hybridize poorly with the NC64A virus DNAs. The Pbi virus DNAs have a higher G+C content (46%) than do the NC64A virus DNAs (40% G+C). Pbi viruses neither infect nor attach to *Chlorella* strain NC64A and the NC64A viruses neither infect nor attach to *Chlorella* strain Pbi (133).

Additional viruses infecting *Chlorella* strain SAG-241-80 have been isolated and characterized from Soviet fresh water (69, 70). These viruses also infect *Chlorella* strain Pbi. Thus *Chlorella* strains Pbi and SAG-241-80 may be closely related or identical.

REPLICATION OF PBCV-1

One-step growth experiments revealed that progeny PBCV-1 is first released about 3 to 4 h after infection and that virus release is complete by 8 to 10 h (Fig. 6A) (184). PBCV-1 release involves localized lysis of the cell wall. Thus one or more late virus gene products presumably degrade cell walls. Mechanical disruption of the cells releases infectious virus 30 to 50 min prior to spontaneous lysis. Consequently, infectious PBCV-1 is completely assembled inside the host and does not acquire lipids by budding through the host membrane. PBCV-1 also replicates in dark-grown *Chlorella* cells (Fig. 6B) or in light-grown cells treated with the photosynthetic inhibitor DCMU prior to virus infection. In both cases the time course of virus release is similar to that of untreated light-grown cells, and so PBCV-1 replication does not require host photosynthesis. However, the burst size, which is 200 to 350 PFU per cell in light-grown cells, is reduced about 50% in dark-grown cells. PBCV-1 infection rapidly inhibits host CO_2 fixation. PBCV-1 replicates most efficiently in actively growing host cells and poorly in stationary-phase cells.

Attachment of PBCV-1 to *Chlorella* Strain NC64A

PBCV-1 attaches rapidly and irreversibly to the external surface of cell walls, but not to protoplasts, of *Chlorella* strain NC64A with an adsorption rate of 5×10^{-9}/ml/min. PBCV-1 attachment is specific since the virus attaches only to its host (93). Attachment occurs between pH 3.5 and 9 and between 5 and 25 mM $MgCl_2$ or 5 and 100 mM NaCl (92). Each cell contains at least 5×10^4 binding sites, and Scatchard analysis indicates that 5,000 PBCV-1 particles can adsorb to each cell. Assuming that the alga is a sphere with a diameter of 5 μm (94), approximately 2,500 virus particles of 175 nm would cover the surface of a single cell. Other estimates of the size of PBCV-1 have been smaller, 160 nm (50) or 139 nm (222), which means that more virus particles could fit on the surface of a single cell. From these data we concluded that (i) the entire surface of the *Chlorella* cell wall contains PBCV-1 receptors, (ii) individual receptor sites have similar affinities, and (iii) at most only a small percentage of the binding sites are used at any one time.

The virus receptor is stable to heat and organic solvents since PBCV-1 attaches equally well to isolated walls of *Chlorella* strain NC64A even if the walls are first heated to 100°C or extracted with organic solvents, detergents, or high-salt solutions. The receptor is also resistant to many proteases, cellulase, and pectinase. However, exposure of the walls to HCl, pH 1, 0.5 M H_2SO_4, or 0.2 M NaOH for 1 h at 100°C inactivates the receptor (92). Treating walls with the lectins wheat germ agglutinin, ricin, *Lens culinaris* lectin, or concanavalin A in the presence or absence of their specific hapten inhibitors also prevents virus attachment (91). This suggests that the lectins bind nonspecifically to the algal walls and that the lectin binding merely occludes viral attachment. With the exception of high concentrations (400 mM) of glucosamine and mannosamine, simple sugars such as glucose, *N*-acetylglucosamine, galactose, maltose, sucrose, fucose, rhamnose, arabinose, xylose, and *N*-acetylgalactosamine had no effect on PBCV-1 attachment (92).

Attachment and infection of *Chlorella* strain NC64A by PBCV-1 is shown in Fig. 7A to E and in stereo views in Fig. 8. Attachment always occurs at a virus vertex. It is not known whether all virus vertices are identical and can serve as points of attachment. As mentioned above in the section

TABLE 2. Separation of *Chlorella* strain NC64A viruses into 16 classes[a]

Class	Virus	Plaque size (mm)	Reaction with antibody to[b]:				Restriction group[c]	Level of methylation	
			PBCV-1	NY-2C	NYs-1	NY-2A		5mC[d]	6mA[e]
1	NE-8D	3	Yes	No	No	No	A	0.44	ND[f]
	NYb-1	3	Yes	No	No	No	A	1.60	ND
	CA-4B	3	Yes	No	No	No	A	0.12	ND
2	AL-1A	3	No	Yes	Partial	Partial	A	0.45	ND
	NY-2C	3	No	Yes	Partial	Partial	A	0.39	ND
	NC-1D	3	No	Yes	Partial	Partial	A	0.33	ND
3	PBCV-1	3	Yes	No	No	No	C	1.86	1.5
	NC-1C	3	Yes	No	No	No	C	1.72	1.6
4	CA-1A	3	Yes	No	No	No	B	10.0	ND
	CA-2A	3	Yes	No	No	No	B		
	IL-2A	3	Yes	No	No	No	B	9.4	ND
	IL-2B	3	Yes	No	No	No	B	10.9	ND
	IL-3A	3	Yes	No	No	No	B	9.7	ND
	IL-3D	3	Yes	No	No	No	B	12.6	ND
5	SC-1A	1	Yes	No	No	No	D	1.94	7.3
	SC-1B	1	Yes	No	No	No	D	2.04	7.5
6	NC-1A	3	Yes	No	No	No	D	7.1	7.3
7	NE-8A	3	Yes	No	No	No	E	14.3	8.1
	AL-2C	3	Yes	No	No	No	E		
	MA-1E	3	Yes	No	No	No	E	14.9	8.1
	NY-2F	3	Yes	No	No	No	E	14.6	8.1
	CA-1D	3	Yes	No	No	No	E		
	NC-1B	3	Yes	No	No	No	E	13.4	8.2
8	NYs-1	1	No	Partial	Yes	Yes	F	47.5	11.3
9	IL-5-2s1	1	No	Partial	Yes	Yes	G	45.0	16.1
	AL-2A	1	No	Partial	Yes	Yes	G	35.8	14.6
	MA-1D	1	No	Partial	Yes	Yes	G	47.0	16.7
	NY-2B	1	No	Partial	Yes	Yes	G	36.5	16.2
10	CA-4A	1	No	Partial	Yes	Yes	H	39.8	19.6
11	NY-2A	0.5	No	Partial	Yes	Yes	I	44.9	37.0
12	XZ-3A	1	Yes	No	No	No	J	12.8	2.2
13	SH-6A	1	Yes	No	No	No	K	12.6	10.3
	BJ-2C	1	Yes	No	No	No	K	12.8	11.5
14	XZ-6E	1	Yes	No	No	No	E	21.2	15.2
15	XZ-4C	1	No	No	Yes	Yes	G	46.7	20.8
16	XZ-5C	1	No	No	Yes	Yes	H	42.7	27.9
	XZ-4A	1	No	No	Yes	Yes	H	44.1	28.3

[a] The separation is based on at least one of the following criteria: plaque size, reaction with four viral antisera, sensitivity of DNA to 13 restriction endonucleases, on percentage of 5mC and 6mA in genomic DNA.

[b] A 5-μg sample of purified virus was mixed with twofold serial dilutions of antiserum, and the precipitate was monitored 2 h later. Yes means that a reaction was obtained at an antibody dilution of 1:64 or greater; Partial means a dilution of 1:8 to 1:32; and No means a dilution of 0 to 1:4.

[c] Viral DNAs were tested for susceptibility to the 13 restriction endonucleases *Bgl*II, *Eco*RI, *Bam*HI, *Sal*I, *Xho*I, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, *Mbo*I, *Dpn*I, *Msp*I, and *Hap*II. The DNAs were grouped according to the resistance of the DNAs to these enzymes: group A, *Dpn*I; group B, *Hin*dIII, *Sst*I, and *Dpn*I; group C, *Bcl*I and *Mbo*I; group D, *Sal*I, *Xho*I, *Bcl*I, *Pst*I, and *Mbo*I; group E, *Sal*I, *Xho*I, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, and *Mbo*I; group F, *Bam*HI, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, *Mbo*I, *Msp*I and *Hpa*II; group G, *Bam*HI, *Sal*I, *Xho*I, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, *Mbo*I, *Msp*I, and *Hpa*II; group H, *Eco*RI, *Bam*HI, *Sal*I, *Xho*I, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, *Mbo*I, *Msp*I, and *Hpa*II; group I, *Bgl*II, *Eco*RI, *Bam*HI, *Sal*I, *Xho*I, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, *Mbo*I, *Mps*I, and *Hpa*II; group J, *Bcl*I, *Hin*dIII, *Sst*I, and *Mbo*I; group K, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, and *Mbo*I.

[d] Percentage of 5mC per C plus 5mC plus deoxyuridine.

[e] Percentage of 6mA per A plus 6mA plus deoxyinosine.

[f] ND, not detected.

TABLE 3. Properties of five *Chlorella* strain Pbi viruses[a]

Virus	Plaque size (mm)	Restriction group[b]	Level of methylation	
			5mC[c]	6mA[d]
CVA-1	1	M	43.1	ND[e]
CVB-1	1	N	42.7	17.7
CVG-1	1	L	19.2	ND
CVM-1	1	F	41.9	10.1
CVR-1	1	L	14.2	ND

[a] None of these viruses reacted with polyclonal antisera to the *Chlorella* strain NC64A viruses.

[b] Viral DNAs were tested for susceptibility to the 13 restriction endonucleases *Bgl*II, *Eco*RI, *Bam*HI, *Sal*I, *Xho*I, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, *Mbo*I, *Dpn*I, *Msp*I, and *Hpa*II. The DNAs were grouped according to the resistance of the DNAs to these enzymes: group F, *Bam*HI, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, *Mbo*I, *Msp*I, and *Hpa*II; group L, *Sal*I and *Dpn*I; group M, *Bam*HI, *Sal*I, *Hin*dIII, *Sst*I, *Dpn*I, *Msp*I, and *Hpa*II; group N, *Eco*RI, *Bam*HI, *Sal*I, *Bcl*I, *Pst*I, *Hin*dIII, *Sst*I, *Mbo*I, *Msp*I, and *Hpa*II.

[c] Percentage of 5mC per C plus 5mC plus deoxyuridine.

[d] Percentage of 6mA per A plus 6mA plus deoxyinosine.

[e] ND, none detected.

on general characteristics of PBCV-1, certain negatively stained preparations of PBCV-1 suggest that one vertex contains a spike structure which is consistent with a unique vertex. The stereo views (Fig. 8) indicate that the virus is attached to the wall by hairlike fibers which originate from at least some virus vertices. These interesting photographs suggest that the tips of the hairlike fibers are responsible for the initial recognition and attachment of the virus to the host. They could also be involved in orienting a unique virus vertex to the host surface. A scanning electron micrograph of numerous PBCV-1 particles attached to the host is shown in Fig. 9.

PBCV-1 attachment is followed by dissolution of the host wall at the point of attachment (Fig. 7B to D) and entry of the viral DNA and associated proteins into the cell, leaving an empty capsid on the host surface (Fig. 7E) (93). Since the virus also attaches to and digests *Chlorella* strain NC64A wall fragments which have previously been heated to 100°C, the virus carries the hydrolytic enzyme(s) (Fig. 7F). The cell-wall-degrading activity is located inside the virus particle (possibly in the spike structure), because (i) intact, protease-treated PBCV-1 particles still attach and digest a hole in the host wall and (ii) cell-wall-digesting activity can be isolated from purified, osmotically disrupted PBCV-1 particles (20). This suggests that attachment might trigger a change in the virus structure. However, attachment to and digestion of wall fragments does not lead to empty PBCV-1 capsids; thus DNA release appears to require a host function(s) (e.g., perhaps the virus internal membrane fuses with the host membrane). Attempts to release DNA from PBCV-1 attached to isolated walls by adding various agents and cell fractions have so far been unsuccessful (179).

Intracellular Site of PBCV-1 Replication

The destination of the infecting DNA and the intracellular site of PBCV-1 transcription and DNA replication are unknown. PBCV-1 proteins are synthesized on cytoplasmic ribosomes and not organelle ribosomes, since cycloheximide, but not chloramphenicol, inhibits viral replication (156). PBCV-1 replication does not require host transcription, since PBCV-1 replicates in UV-irradiated cells (182). Such cells are unable to form colonies, and endogenous host RNA and DNA syntheses are reduced to background levels. However, PBCV-1 replication is slow (latent period of 16 to 20 h) and the burst size is small (2 to 10 PFU per cell) in

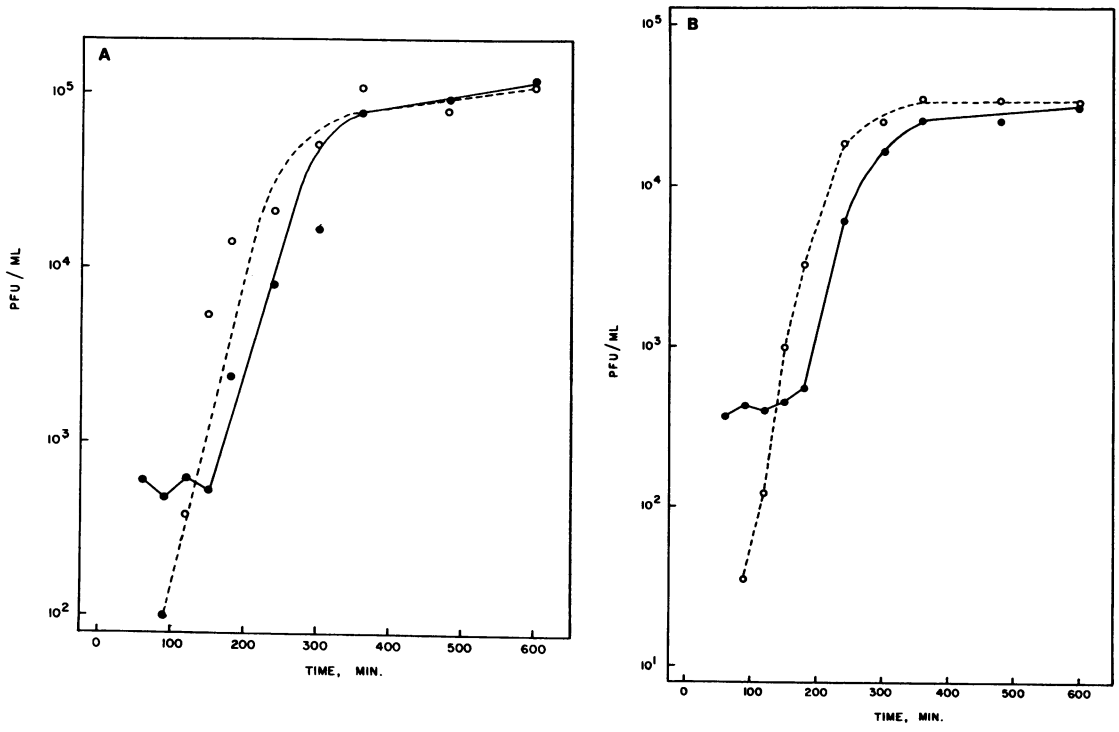


FIG. 6. One-step growth curves of PBCV-1 on *Chlorella* strain NC64A grown in the light (A) or in the dark (B). Symbols: ●, spontaneous lysis; ○, mechanically disrupted algae. Reproduced from reference 184 with permission from Academic Press, Inc.

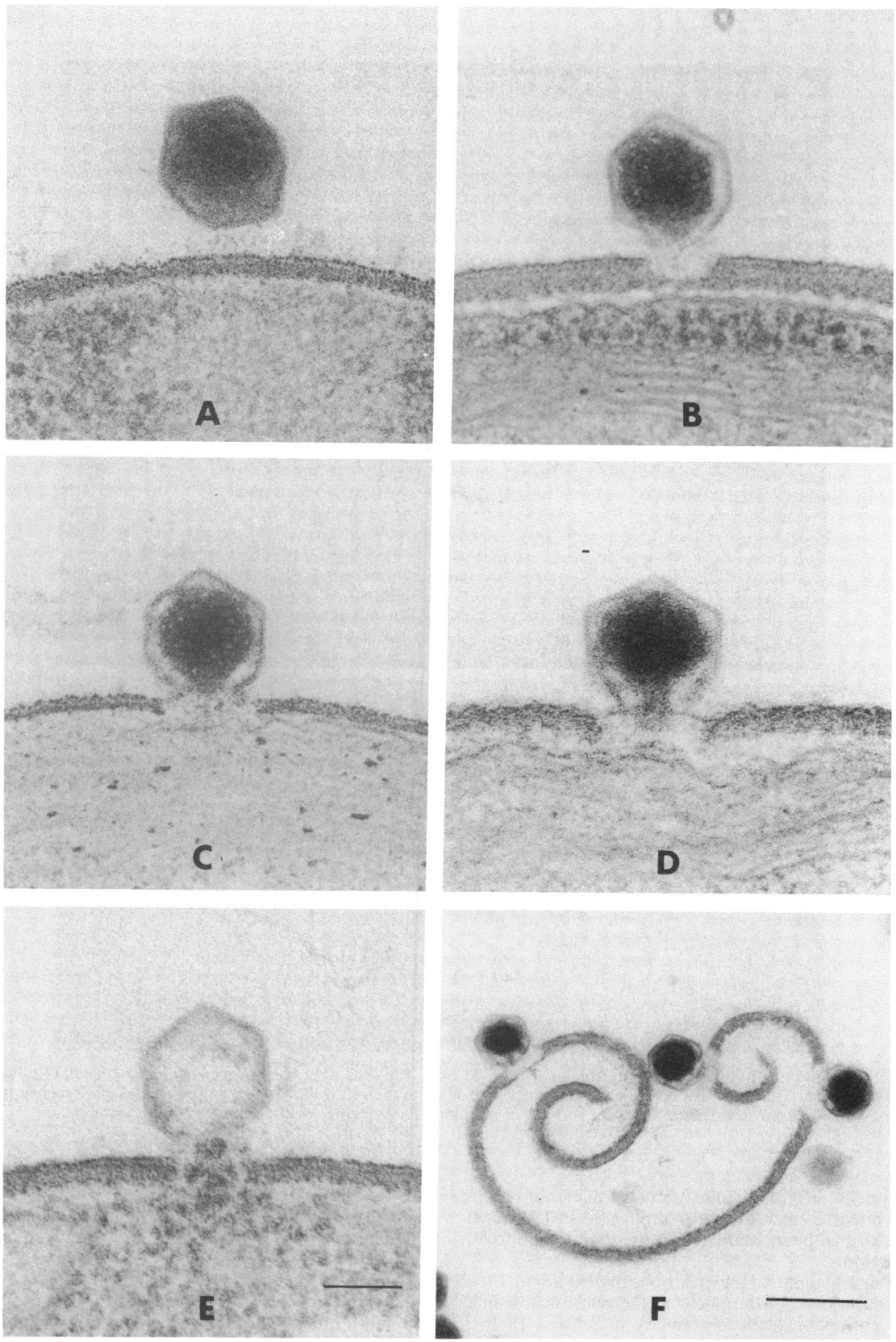


FIG. 7. Infection of *Chlorella* strain NC64A by PBCV-1. (A) Viral particle in close proximity to the alga. (B and C) Attachment of PBCV-1 to the algal wall and digestion of the wall at the point of attachment. (D) Viral DNA beginning to enter the host. (E) An empty viral capsid remaining on the surface of the host. (F) PBCV-1 attachment and dissolution of a *Chlorella* cell wall fragment. Note that (i) viral attachment always occurs on the external side of the wall (i.e., the internal side of the wall curls inward) and (ii) DNA is not released from viral particles attached to wall fragments. The size markers in panels E and F represent 100 nm and 200 nm, respectively. Reproduced from reference 93 with permission from Academic Press, Inc.

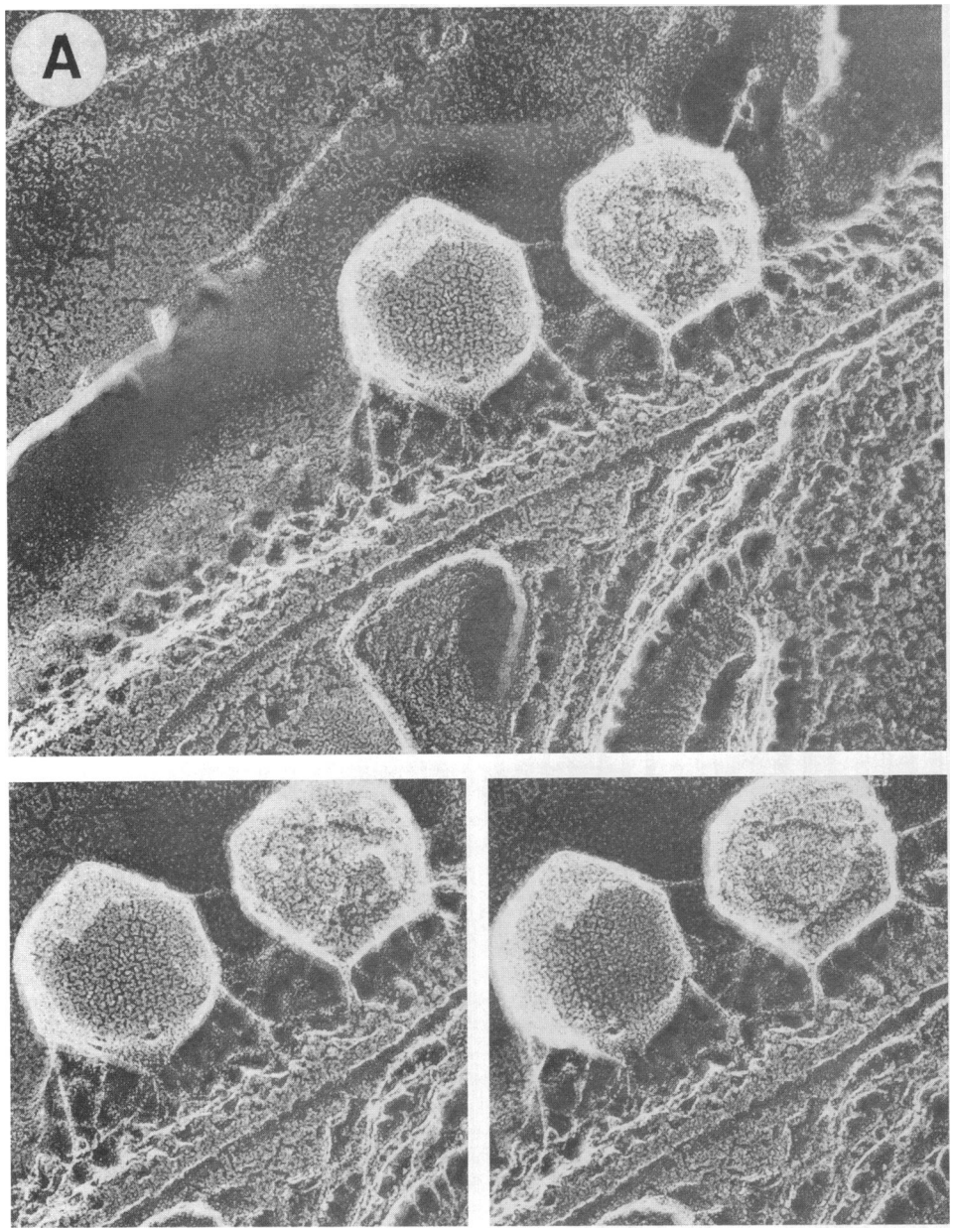


FIG. 8. Stereo views of PBCV-1 attached to *Chlorella* strain NC64A. The cells were quick-frozen and deep-etched. (A) Side view of the particles attached to the cell wall; (B) top view of the particles attached to the cell wall. Note that the particles are attached to the wall via hairlike fibers, which appear to originate from the vertices of the virus. Photographs kindly provided by John Heuser.

UV-treated cells. PBCV-1 replication also does not require labile host factors, since the virus replicated in UV-irradiated cells that had been incubated in the dark for up to 8 h before infection.

The host cells change cytologically beginning about 1 to 2 h after infection (94). In the nucleus, the nucleolus disintegrates and chromatin heterochromicity increases. The nucleus, mitochondria, and Golgi apparatus become appressed to the chloroplast, leaving one or more finely granulated electron-translucent areas in the cytoplasm (labeled V in Fig. 10A to D). Since PBCV-1 ultimately forms in these areas, we have designated these areas virus assembly centers.

Early stages of PBCV-1 morphogenesis in the virus assembly centers are seen in a few cells by 1 h postinfection (p.i.) and are common by 2 h p.i. (Fig. 10 A and B). Short membranous structures, which are vesicular or angular in shape (arrow in Fig. 10 B and C), first appear in the virus assembly centers. These membranes appear to function as templates for capsid assembly. More dense material (presumably protein) appears on the exterior surface of these membranes, giving rise to angular configurations (Fig. 10A and B). These developing capsids are bounded by a distinct electron-dense layer; a thicker translucent region is visible directly underneath this dense outer layer (Fig. 10C and D). Capsids are usually formed at the periphery of the virus assembly centers, producing the rosette pattern seen in Fig.

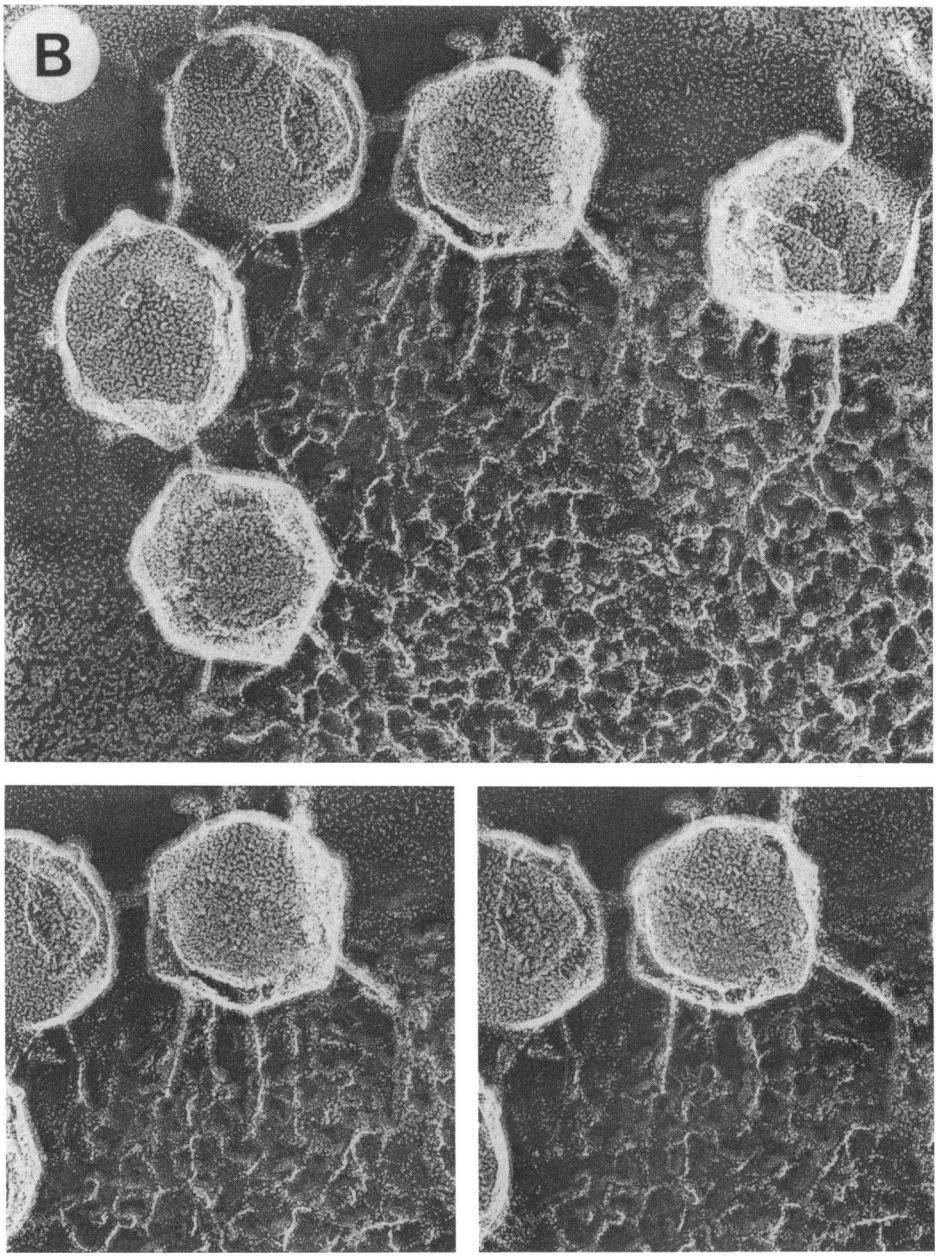


FIG. 8—*Continued.*

10B. Apparent complete capsids are assembled prior to DNA accumulation. A ring of virus particles surrounding a virus assembly center, which are in various stages of packaging DNA, are seen in Fig. 10D. These observations suggest that PBCV-1 probably packages DNA through an opening in the capsid. By 4 to 5 h p.i. the cells contain numerous filled virus particles, which are distributed throughout the cytoplasm.

Sometimes a nuclear membrane is still visible at late stages of infection (Fig. 10E). Mitochondria and the chloroplast disintegrate in late stages of PBCV-1 replication. So far there is no evidence for nuclear or organellar roles in viral replication. If the nucleus is not involved, transcription-associated enzymes, such as RNA polymerase(s) and processing enzymes, must be imported into the cell with the infecting DNA.

PBCV-1 has not been effectively assayed for these enzymes. We have been unsuccessful in brief attempts to detect RNA polymerase activity either in intact PBCV-1 or in PBCV-1 that had been disrupted osmotically or by freeze-thawing (15). Although PBCV-1 may not contain a functional RNA polymerase, we suspect that our failure may reflect unsatisfactory disruption methods. We predict that PBCV-1 imports at least some of the enzymes involved in its replication.

Since PBCV-1 is assembled in specific regions in the cytoplasm, the cytoskeleton might be expected to actively target virus proteins, including the glycoproteins, to the

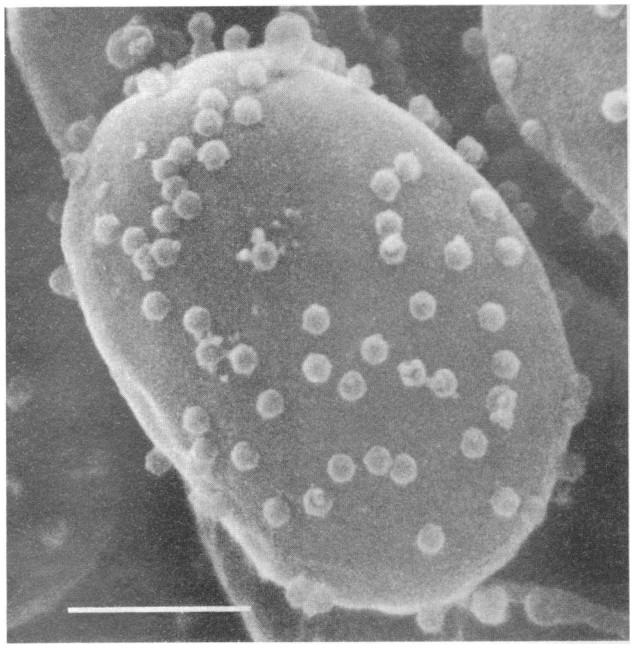


FIG. 9. Scanning electron micrograph of PBCV-1 particles attached to *Chlorella* strain NC64A (photograph kindly provided by Kit Lee). Bar, 1 μm.

assembly center. However, several cytoskeleton-disrupting agents, which inhibit either tubulin or actin functions, had no effect on PBCV-1 replication even at concentrations much higher than those required to inhibit growth of the host *Chlorella* cell (112).

Effect of PBCV-1 Infection on Host DNA, RNA, and Protein Syntheses

PBCV-1 infection dramatically changes host macromolecular syntheses. The following changes occur in DNA metabolism (180). (i) Virus infection almost immediately inhibits host DNA synthesis. (ii) PBCV-1 DNA synthesis begins 50 to 60 min after infection and requires de novo synthesis of a protein(s). This implies that PBCV-1 directs the synthesis of at least some of its own DNA synthetic machinery. New DNA polymerase activities appear in infected cells (139, 140). (iii) PBCV-1 infection leads to the degradation of host chloroplast and nuclear DNAs within 1 to 2 h p.i. (Fig. 11). Presumably a virus-encoded protein(s) mediates this response since cycloheximide added at the time of infection prevents host DNA degradation. Host nuclear and chloroplast DNA levels also decrease, albeit more slowly, if UV-inactivated algae are inoculated with PBCV-1 (209). (iv) The total DNA level in the host cell increases 3- to 10-fold by 5 h after PBCV-1 infection. Thus PBCV-1 replication requires a large increase in deoxynucleotide intermediates. Although recycling from host DNA may supply some of these intermediates, it can supply only 10 to 25% of the requirement. Consequently, virus-infected cells may be an excellent model system for studying nucleotide metabolism.

One obvious test for recycling is to label host DNA prior to infection and determine whether the label accumulates in virus DNA. Unfortunately, *Chlorella* strain NC64A lacks thymidine kinase (180) and so host DNA cannot be labeled specifically.

PBCV-1 infection also alters RNA metabolism (145). (i) Viral infection rapidly inhibits host RNA synthesis. (ii) Chloroplast rRNAs, but not cytoplasmic rRNAs, are degraded beginning about 30 min p.i. Presumably a virus-encoded protein(s) contributes to this process, since cycloheximide added at the time of PBCV-1 infection prevents chloroplast rRNA degradation. (iii) PBCV-1 transcription is programmed, and early transcripts appear within 5 to 10 min after infection. (iv) Some, but not all, early viral transcripts are synthesized in the absence of de novo protein synthesis. The synthesis of later transcripts requires translation of an early gene product(s). (v) Late viral transcription begins about 1 h p.i., at the same time viral DNA synthesis begins (Fig. 12). (vi) A few early transcripts continue to be synthesized after virus DNA synthesis begins (45).

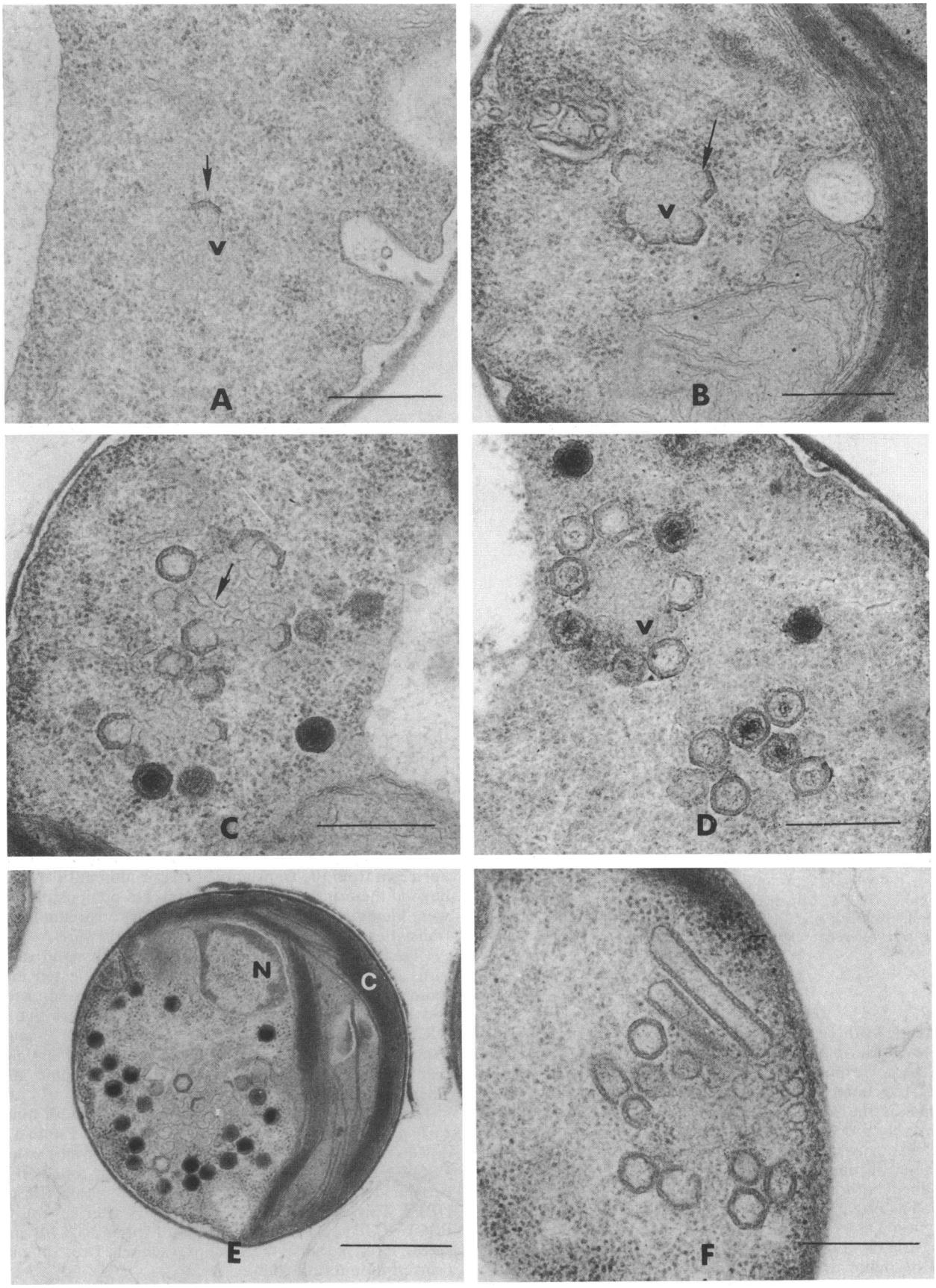
Little is known about protein synthesis following virus infection. PBCV-1 infection rapidly inhibits host protein synthesis by at least 75%. We have briefly investigated virus protein synthesis in PBCV-1-infected cells by examining proteins on polyacrylamide gels (215). These experiments were less than satisfactory because (i) the synthesis of some host proteins continued (at least briefly) after infection and (ii) the specific labeling of viral proteins was low, even after long labeling periods (more than 1 h) with high-specific-activity amino acids. This latter finding suggests that PBCV-1 infection may expand amino acid pools. The increase could reflect recycling from degraded host protein or from increased amino acid synthesis. A complicating factor is that PBCV-1 has the potential to code for several hundred proteins, which would require two-dimensional gels for adequate resolution.

TRANSCRIPTION OF PBCV-1

As mentioned in the preceding section, PBCV-1 transcription can be conveniently divided into early and late stages. The junction between these stages is about 1 h p.i., which coincides with the start of virus DNA synthesis. Interestingly, the incorporation of [^{3}H]adenine into polyadenylate-containing RNA also changes abruptly at about 1 h after PBCV-1 infection. Polyadenylate segments are virtually absent from late transcripts, whereas at least some early virus transcripts are probably polyadenylated (190).

RNA isolated at various times after PBCV-1 infection was hybridized with each of four cloned fragments of PBCV-1 DNA (Fig. 12) (145). Both early and late genes are dispersed within the PBCV-1 genome, since each of the four cloned fragments hybridized to both early and late transcripts.

FIG. 10. *Chlorella* cells at 2 h (A to C), 5 h (D), and 6 h (E) after PBCV-1 infection. Parts of newly forming capsids (arrow in panel A) were located in defined areas in the cytoplasm (labeled v for virus assembly center in panels A to D) by 2 h p.i. Membranous materials which assume vesicular or angular shapes were usually seen in the virus assembly centers (arrow in panels B and C). The newly forming capsids can have a rosette appearance, which surrounds the virus assembly center (panel B). By 5 h p.i. (panel D) the cells contained a mixture of filled and partially filled capsids, empty capsids, and incomplete capsids. By 6 h p.i. (panel E), the cells contained many filled capsids which were distributed throughout the cytoplasm. Occasionally, cells contained aberrant virus capsids (F). Bars: 500 nm (panels A to D and F) and 1,000 nm (panel E). Reproduced from reference 94 with permission from Academic Press, Inc.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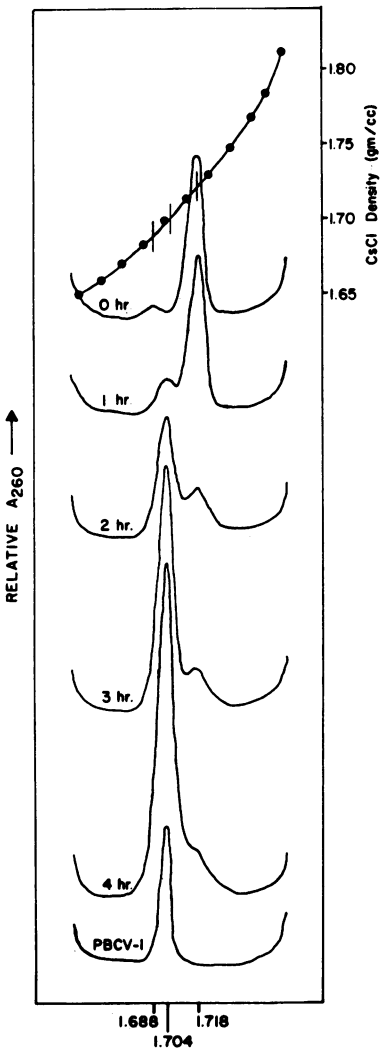

FIG. 11. CsCl equilibrium centrifugation of DNA isolated from an equal number of cells at various times after infection with PBCV-1. The top drawing is from uninfected cells, and the ones below, in descending order, are from cells at 1, 2, 3, and 4 h after infection. The bottom line is DNA isolated from purified virus. Nuclear DNA bands at 1.718 g/ml, chloroplast DNA at 1.688 g/ml, and viral DNA at 1.704 g/ml. Note that viral DNA clearly begins to accumulate by 2 h p.i. and host nuclear DNA decreases. Reproduced from reference 180 with permission from Academic Press, Inc.

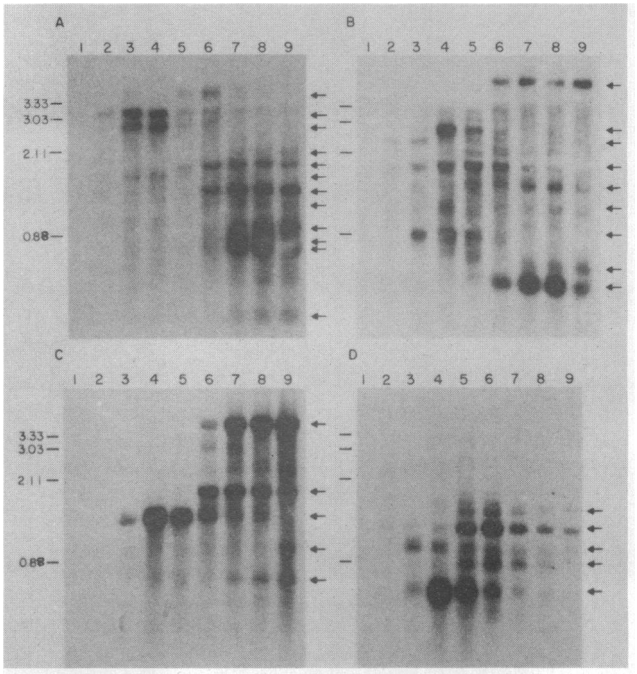

FIG. 12. Hybridization of four cloned fragments of PBCV-1 DNA to Northern blots of total RNA isolated at various times after PBCV-1 infection. RNA was isolated at 0, 5, 15, 30, 45, 60, 90, 120, and 240 min after infection (lanes 1 to 9, respectively). The PBCV-1 DNA probes were isolated from clones containing (A) fragment B7a, (B) fragment B7b, (C) fragment B13, and (D) fragment B18b. Sizes of brome mosaic virus RNAs are indicated on the left. Note the abrupt change in the transcripts at about 1 h p.i. Reproduced from reference 145 with permission from Academic Press, Inc.

Surprisingly, three of the four PBCV-1 DNA clones hybridized to transcripts which additively were 40 to 60% larger than the corresponding DNA probe. This suggests that the PBCV-1 genome contains overlapping genes, that both strands of the DNA are transcribed, or that RNAs are extensively processed after transcription.

To distinguish among these possibilities, a 4.3-kbp subfragment of one of the cloned fragments (B7a), which contained both early and late transcripts, was sequenced (146). The fragment contained five major ORFs (Fig. 13) and six minor ORFs; only the major ORFs were transcribed. Both strands contained ORFs, in both orientations, separated by minimal sequence. Some of the ORFs were transcribed into a complex pattern of RNAs. The total length of transcribed RNA exceeded that of the ORFs, indicating either posttranscriptional modification or multiple transcription start-stop sites. The transcripts for major ORFs 3 and 4 overlap. Two of the minor ORFs also overlap with major ORFs.

Transcription studies with a series of M13 clones corresponding to the ORFs suggest that the mRNAs start just upstream from the translational start codon but extend well beyond the translational stop codon. No obvious promoters were identified, although the regions just upstream from the translation start codons for the major ORFs were A+T rich. A+T-rich regions also precede the start codons in vaccinia virus and fowlpox virus genes and are implicated in promoter function (102). The A+T-rich ORF 3 promoter region was situated closer to the start codon than were the other ORFs. The lack of a good promoter for ORF 3 may be explained by the Northern (RNA) analyses, which suggest that the ORF 3 transcript is part of a dicistronic mRNA which includes the ORF 4 transcript.

The TTTTTNT transcriptional termination motif found in early genes of vaccinia virus (223) was present 230 to 479 bp downstream of the translation stop codon in all five major PBCV-1 ORFs. The TTTTTNT motif was found in both early and late PBCV-1 genes but never within the major ORFs. This same sequence also occurs after several other PBCV-1 ORFs, as well as genes of other NC64A viruses (228). It is possible that, as in vaccinia virus, it signals transcription termination.

Codon usage by PBCV-1 was strongly biased to codons

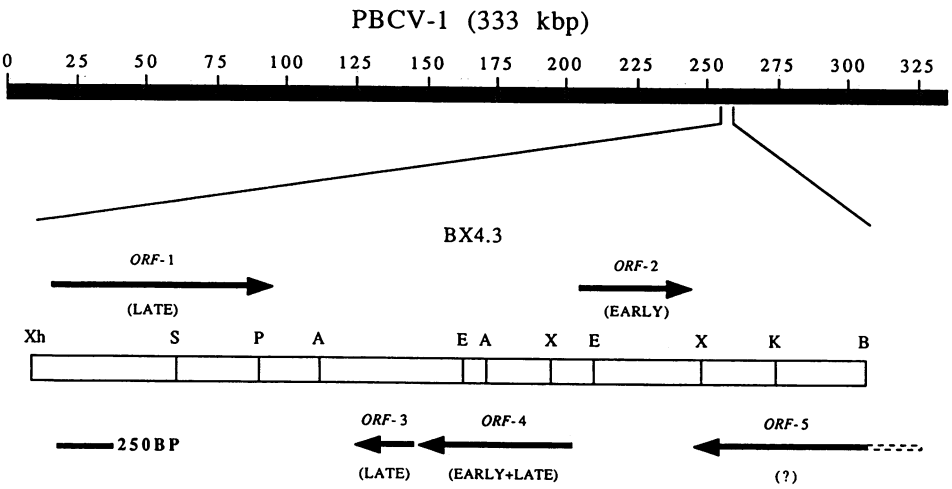

FIG. 13. Transcription map of a 4.3-kbp fragment of PBCV-1 DNA. Five ORFs and their time and direction of transcription are indicated.

ending in XXA/U (63%) over those ending in XXC/G (37%) (146). This is probably not unexpected, since the DNA of PBCV-1 is 40% G+C. In contrast, the codon usage of the host tubulin gene has a strong bias to codons ending in XXC/G (67%) (91). These results suggest that PBCV-1 might encode many of its tRNAs.

There were no obvious sequence differences in the upstream and downstream regions between early and late PBCV-1 genes. Also, early and late genes contained no obvious polyadenylation sequence signals.

VIRUSES ENCODE DNA METHYLTRANSFERASES AND SITE-SPECIFIC (RESTRICTION) ENDONUCLEASES

PBCV-1 DNA Methyltransferases and DNA Site-Specific Endonucleases

Given that the *Chlorella* virus DNAs contain 5mC and 6mA, it is not surprising that the viruses encode DNA methyltransferases. However, the discovery of virus-encoded DNA site-specific (restriction) endonucleases was unexpected. The first virus-encoded DNA methyltransferase was found in PBCV-1-infected cells and resulted from the discovery that PBCV-1 DNA methylation differs from host DNA methylation (189). For example, PBCV-1 DNA contains 6mA in the sequence GATC, whereas the host DNA does not contain 6mA in this sequence.

Consequently, PBCV-1-infected cells were examined for an adenine DNA methyltransferase that methylated GATC sequences (216). The enzyme, named M.*Cv*iAI, appeared about 30 min p.i., and its activity increased until 3 to 4 h p.i. Thus M.*Cv*iAI is an early-gene product. Since PBCV-1 DNA also contains 1.9% 5mC, PBCV-1 probably also codes for a cytosine methyltransferase.

PBCV-1 infection also induces a DNA site-specific endonuclease, *Cv*iAI, which recognizes the same GATC sequence as M.*Cv*iAI (209). *Cv*iAI cleaves DNA 5′ to G and does not cleave DNAs containing G^{m}ATC sequences. Thus *Cv*iAI is an isoschizomer of the bacterial restriction endonucleases *Mbo*I and *Sau*3AI. Like bacterial type II restriction endonucleases, *Cv*iAI activity absolutely requires Mg^{2+} but is unaffected by ATP or *S*-adenosylmethionine. In vitro, *Cv*iAI cleaves *Chlorella* strain NC64A DNA, but not PBCV-1 DNA, because host DNA contains GATC sequences whereas PBCV-1 DNA contains G^{m}ATC sequences. *Cv*iAI activity is first detected between 30 and 60 min p.i., coinciding with the onset of host DNA degradation.

Several observations indicated that PBCV-1 encodes *Cv*iAI and M.*Cv*iAI. (i) Both activities first appeared 30 to 60 min after PBCV-1 infection. (ii) The appearance of both enzyme activities required de novo protein synthesis. (iii) Both enzyme activities appeared in UV-irradiated *Chlorella* cells inoculated with PBCV-1, in which presumably only virus-encoded genes were expressed (209, 216). (iv) Infection of *Chlorella* strain NC64A with viruses other than PBCV-1 induced the synthesis of other DNA methyltransferases and site-specific endonucleases with different sequence specificities (see below). (v) As reported below, three DNA methyltransferase genes from other NC64A viruses have been cloned from viral DNAs.

Additional Viral DNA Methyltransferases and DNA Site-Specific Endonucleases

Each of the NC64A virus genomes contains 5mC, and 25 virus DNAs also contain 6mA (Table 2). Methylation specificities are unknown for most of these viruses. However, the resistance of viral DNA to methylation-sensitive restriction endonucleases is evidence for the site of methylation. Some of the viruses must encode several DNA methyltransferases. For example, virus NC-1A DNA contains 7.1% 5mC and 7.3% 6mA. If each DNA sequence containing 5mC and/or 6mA is methylated by a distinct methyltransferase that recognizes a 4-base sequence, one predicts that 8 to 10 methyltransferases are needed in NC-1A-infected cells (4 to 5 enzymes for methylating cytosines and 4 to 5 enzymes for methylating adenines). Preliminary experiments indicate that NC-1A induces at least three distinct adenine methyltransferases; i.e., separate enzymes methylate adenine in TCGA, GATC, and GANTC sequences (214).

For cells infected with viruses such as NY-2A, which has 45% 5mC and 37% 6mA in its genomic DNA, we predict that at least some of the virus-induced DNA methyltransferases recognize either 3- or 2-base sequences. Enzymes recognizing 3 bases or 2 bases could account, respectively, for 6.3 or 25% of the 5mC and 6mA. DNA methyltransferases encoded

TABLE 4. DNA methyltransferases and DNA site-specific endonucleases from virus-infected *Chlorella* strain NC64A cells

Virus	DNA methyl-transferase	Sequence[a]	DNA site-specific endo-nuclease	Sequence[b]
PBCV-1	M.*Cvi*AI	G*ATC	*Cvi*AI	/GATC
NC-1A	M.*Cvi*BI	G*ANTC	*Cvi*BI	G/ANTC
	M.*Cvi*BII	G*ATC		
	M.*Cvi*BIII[c]	TCG*A		
NY-2A	M.*Cvi*QI	GT*AC?	*Cvi*QI	G/TAC
				Pu/AG[d]
IL-3A	M.*Cvi*JI[c]	(G/A)G*C(T/C/G)	*Cvi*JI	PuG/CPy
XZ-6E	M.*Cvi*RI[c]	TGC*A	*Cvi*RI	TG/CA
			*Cvi*RII	G/TAC
NYs-1		*CC?		/CC[d]

[a] The asterisk indicates the base methylated.
[b] The slash indicates the cleavage site.
[c] Methyltransferases that have been cloned and sequenced.
[d] Cleaves only one strand of dsDNA.

by viruses with highly methylated DNAs are just beginning to be characterized.

Since the NC64A viruses vary greatly in DNA methylation, they provide a unique system with which to study the effect of methylation on DNA replication and transcription. Questions that can be asked include the following. (i) Is the transcription machinery adapted to the level of methylation? (ii) Is it possible to isolate 5-azacytidine-resistant mutants (5-azacytidine is an inhibitor of cytosine methylation [60]) of viruses containing high levels of 5mC (e.g., NYs-1, which has 47.5% 5mC)? (iii) If such mutants can be obtained, do they exhibit altered DNA replication and/or gene expression?

In this regard, spontaneously derived 5-azacytidine-resistant mutants of virus IL-3A were obtained at a frequency of about 10^{-6} to 10^{-7}. Wild-type IL-3A DNA contains about 10% 5mC, whereas each of the mutants had 1.6% 5mC (16). The growth and burst sizes of the mutants and the parent virus were identical. Thus, in at least this case, the factors involved in DNA replication and gene expression were probably not affected by the 5mC in the DNA. Otherwise, the virus would have to contain or encode a second set of factors which functioned on DNA with low levels of methylated bases.

Some of the virus-infected cells have also been assayed for DNA site-specific endonucleases. Many, if not all, of these viruses code for one or more DNA site-specific endonucleases (Table 4). For example, cells infected with virus NC-1A contain a GANTC-specific endonuclease named *Cvi*BI. *Cvi*BI, like the bacterial restriction endonucleases *Hin*fI and *Hha*II, cleaves DNA between G and A, and methylation of the A prevents cleavage (211). *Cvi*BI requires only Mg^{2+} for activity.

Virus NY-2A induces at least two site-specific endonucleases. One of these enzymes, named *Cvi*QI (previously named *Cvi*II), is an isoschizomer of the bacterial enzyme *Rsa*I (213). However, unlike *Rsa*I, which cleaves between T and A in the sequence GTAC, producing a blunt end, *Cvi*QI is the first enzyme recognizing this sequence to cleave between G and T. *Cvi*QI does not cleave GT^{m}AC sequences. The other site-specific endonuclease present in NY-2A-infected cells cleaves DNA into small fragments. This enzyme is a base-specific nicking enzyme which cleaves between Pu and A in PuAG sequences but not Pu^{m}AG sequences (166).

The site-specific endonuclease, named *Cvi*JI, induced by virus IL-3A is the first enzyme to recognize the sequence PuGCPy; it cleaves between the G and C (210). *Cvi*JI does not cleave PuG^{m}CPy sequences. On a statistical basis, *Cvi*JI is the first site-specific endonuclease to recognize a 3-base sequence, and it should produce fragments with an average size of 64 bases. Thus *Cvi*JI cleaves DNA frequently, making it ideal for characterizing short DNA fragments such as polymerase chain reaction products.

*Cvi*JI behaves as a type II restriction endonuclease in the presence of Mg^{2+} and specifically recognizes the sequence PuGCPy. When ATP is included in the reaction mixture, the cleavage products are smaller than in its absence. Apparently, ATP relaxes the sequence specificity to (close to) 2 bp, and we term this activity *Cvi*JI* (star activity). Conditions which induce *Cvi*JI* activity differ from those which favor star activity of bacterial type II restriction endonucleases. *Cvi*JI* activity absolutely requires ATP; related compounds such as ADP, dATP, ATP-α-S, and ATP-γ-S induce some star activity; whereas GTP, CTP, UTP, AMP-PNP, and AMP-PCP do not. Reducing agents and *S*-adenosylmethionine stimulate *Cvi*JI*, but these compounds do not, by themselves, induce star activity. Therefore, *Cvi*JI* resembles a type III restriction enzyme in its dependence on Mg^{2+} and ATP and its stimulation by *S*-adenosylmethionine and reducing agents (210). Although circumstantial evidence suggests that *Cvi*JI and *Cvi*JI* activities result from the same protein, purification of *Cvi*JI to homogeneity is required to eliminate the unlikely possibility that *Cvi*JI and *Cvi*JI* activities are separate proteins.

Virus XZ-6E induces two site-specific endonucleases (177). One of these enzymes, named *Cvi*RI, is the first restriction endonuclease to recognize the sequence TGCA. *Cvi*RI cleaves between the G and C and is inhibited by adenine methylation. The second enzyme, *Cvi*RII, has the same specificity as *Cvi*QI; i.e., it cleaves at G/TAC sequences. *Cvi*RII does not cleave GT^{m}AC sequences.

The site-specific endonuclease induced by virus NYs-1 is a base-specific nicking enzyme that recognizes the sequence 5′-CC-3′ and cleaves 5′ to the first C (212). It cleaves 5′-C^{m}C-3′ sequences but not 5′-^{m}CC-3′ sequences.

The remainder of the viruses listed in Table 2 have not been examined for site-specific endonucleases. However, some of these viruses probably induce interesting and potentially useful enzymes. For example, virus CA-4B might induce a site-specific endonuclease(s) which requires methylation for activity. CA-4B DNA contains no 6mA and only 0.12% 5mC (Table 2), whereas host *Chlorella* strain NC64A DNA has 20% 5mC and traces of 6mA. If CA-4B infected *Chlorella* cells contain a site-specific endonuclease that digests host DNA, but not CA-4B DNA, the enzyme would have to require 5mC and/or 6mA in its recognition site for activity. Otherwise the enzyme would not be specific for the host DNA. Note that the level of 5mC in CA-4B DNA is such that the virus could encode a 6-base-recognizing site-specific endonuclease, which does not require 5mC. At present there are no known bacterial restriction endonucleases which require 5mC in specific sequences.

The Pbi viruses, like the NC64A viruses, also contain various levels of 5mC and 6mA in their genomes (Table 3), indicating that they also code for DNA methyltransferases. Preliminary experiments indicate that infected cells of *Chlorella* strain Pbi, like *Chlorella* strain NC64A, also contain DNA site-specific endonucleases (24).

Thus, the virus-infected *Chlorella* cells are a rich source of DNA methyltransferases and DNA site-specific endonu-

cleases, and some of the enzymes differ in specificity from known bacterial enzymes. The two DNA site specific nicking enzymes are new types of endonucleases and await purification and characterization.

The presence of DNA-methylating and site-specific endonuclease enzymes in *Chlorella* virus-infected cells leads to two obvious questions. (i) What is the evolutionary origin of these enzymes? (ii) What is the function of these enzymes? Experiments designed to address these questions are discussed in the next two sections.

SEQUENCE OF VIRUS-ENCODED DNA METHYLTRANSFERASES

The fact that the virus-induced DNA methyltransferases and DNA site-specific endonucleases are similar to bacterial enzymes suggests that they might share a common ancestry. One way to analyze the viral and bacterial enzymes is to compare their predicted amino acid sequences. Many bacterial cytosine and adenine DNA methyltransferases have been cloned in *E. coli* by using a strategy first proposed by Mann et al. (81). A gene library is screened for expression of a particular methyltransferase by digesting the recombinant plasmid DNAs with the cognate restriction endonuclease prior to transforming *E. coli*. Plasmids expressing the cognate methyltransferase are resistant to the restriction endonuclease and are isolated as transformants.

We have used this strategy to clone three *Chlorella* strain NC64A virus DNA methyltransferases in *E. coli* (Table 4). The two adenine methyltransferases, M.*Cv*iBIII (methylates TCGA sequences [110]) from virus NC-1A and M.*Cv*iRI (methylates TGCA sequences [165]) from virus XZ-6E, contain 377 and 379 amino acids, respectively. Both genes are expressed in both orientations in pUC vectors, suggesting transcription from a virus promoter. Comparison of M.*Cv*iBIII and M.*Cv*iRI with each other and with bacterial adenine methyltransferases revealed that the *Chlorella* virus enzymes were most similar to each other: 40% of the amino acids were identical. The two *Chlorella* virus enzymes, which are the first adenine methyltransferases to be cloned from a eukaryotic system, also contain the bacterial adenine methyltransferase motif (Asp/Asn)-Pro-Pro-(Tyr/Phe) about 100 to 120 amino acids from the amino-terminal end of the protein. M.*Cv*iBIII was also similar to the bacterial isoschizomer M.*Taq*I (39% of the amino acids were identical) and M.*Pae*R7, whose recognition sequence (CTCGAG) contains the subset TCGA (109).

The M.*Cv*iBIII transcript was detected within 40 min p.i., indicating that it is an early gene (108). The M.*Cv*iBIII gene is not essential for virus NC-1A replication, since a spontaneously derived mutant of the virus, with the M.*Cv*iBIII gene deleted, also replicated in *Chlorella* strain NC64A.

The *Chlorella* virus IL-3A gene encoding the cytosine methyltransferase M.*Cv*iJI [methylates (G/A)GC(T/C/G) sequences] has also been cloned and sequenced (150). The M.*Cv*iJI protein contains 367 amino acids. The M.*Cv*iJI amino acid sequence had no obvious similarity to the viral adenine methyltransferases M.*Cv*iBIII and M.*Cv*iRI. This is not surprising since bacterial adenine and cytosine methyltransferases differ substantially (see, e.g., references 126 and 158, but see references 67 and 71a for some evidence of similarity).

However, M.*Cv*iJI shares motifs with bacterial cytosine methyltransferases. Lauster et al. (72) and Posfai et al. (126) reported that several bacterial cytosine methyltransferases and a cytosine methyltransferase from mouse cells (8) contain conserved amino acid sequences. Each of the bacterial enzymes contains 10 motifs which are in the same order in each enzyme (126). The M.*Cv*iJI gene contains amino acid sequences which resemble each of the bacterial motifs. Motif IV (11 of 12 amino acids) was the most highly conserved motif in M.*Cv*iJI, and the least conserved sequences were the three motifs (motif VIII [5 of 13 amino acids], motif IX [3 of 9 amino acids], and motif X [4 of 11 amino acids]) toward the C terminus of the protein. Of the 21 invariant amino acids found in the 13 bacterial enzymes (126), M.*Cv*iJI contained 17.

One apparent difference between M.*Cv*iJI and bacterial cytosine methyltransferases was the spacing between motif VIII and motif IX. The bacterial enzymes contain a long variable region of 80 to more than 250 amino acids between these two motifs (126). This region has been proposed to contain the sequence specificity domain (72, 126), i.e., the target recognition domain. However, these two motifs are immediately adjacent to one another in M.*Cv*iJI. This means that the M.*Cv*iJI target recognition domain is located elsewhere in the protein. Two possible candidates for the M.*Cv*iJI target recognition domain are (i) the 68-amino-acid stretch between motifs IX and X and (ii) the 102 amino acids following motif X.

To date none of the *Chlorella* virus-encoded DNA site-specific endonucleases have been cloned. We have isolated spontaneously derived deletion mutants of virus IL-3A which have simultaneously lost both M.*Cv*iJI and *Cv*iJI activities (16). Assuming that the mutants arose from a single deletion, this suggests that the two virus genes are located close together. If so, the arrangement of these virus genes would be similar to that of bacteria. In bacteria the DNA methyltransferase gene is always located near its cognate DNA restriction endonuclease gene (160).

In conclusion, the similarity between the *Chlorella* virus and bacterial DNA methyltransferases suggests that they belong to the same protein family.

FUNCTION OF VIRUS-ENCODED DNA SITE-SPECIFIC ENDONUCLEASES AND DNA METHYLTRANSFERASES

The biological functions of the virus-encoded DNA site-specific endonucleases and DNA methyltransferases are unknown. Bacterial restriction-methylation systems confer resistance to foreign DNAs and DNA viruses (see, e.g., reference 206). In fact, the name "restriction" refers to their role in excluding foreign DNA. Bacterial DNA methyltransferases serve to prevent self-digestion of bacterial DNA. We have considered two possible functions for the *Chlorella* virus enzymes. (i) The site-specific endonucleases might help degrade host DNA so that the resultant deoxynucleotides are recycled into viral DNA. Methylation of nascent virus DNA by the cognate methyltransferase would protect it from self-digestion. (ii) The endonucleases might prevent infection of a cell by a second virus.

Several observations are consistent with the first hypothesis. (i) Host nuclear and chloroplast DNAs, but not virus DNA, are digested by the virus-encoded site-specific endonuclease(s) in vitro. (ii) In vivo degradation of host nuclear and chloroplast DNAs coincides with the appearance of DNA site-specific endonuclease activity. (iii) Initiation of virus DNA synthesis in vivo coincides with the appearance of DNA methyltransferase activity (209, 216).

The isolation of three independently derived deletion mutants of virus IL-3A, which had lost their methyltransferase (M.*Cv*iJI) and site-specific endonuclease (*Cv*iJI) ac-

tivities, allowed us to test the host DNA degradation hypothesis directly (16). If *C*viJI 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 nearly identical rates following infection with each of the four viruses (16). Therefore we concluded that *C*viJI activity was not essential for host DNA degradation. However, this does not exclude participation of the enzyme in the degradation process. It is possible that IL-3A-infected cells contain additional DNA site-specific endonucleases which also degrade host DNA. However, a search for such activities has been unsuccessful.

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 (19). These experiments led to several conclusions. (i) At least 90% of the plaques resulting from single cells inoculated with two viruses contained only one of the viruses. Thus the *Chlorella* viruses, like bacteriophages (see, e.g., reference 30 for a review), exclude one another. (ii) Infection of the alga by one virus did not prevent attachment of a second virus to the host. (iii) Cells inoculated with one virus 30 min before inoculation with a second virus preferentially replicated the first virus. Thus the exclusion mechanism is probably triggered within 30 to 45 min p.i. (iv) A faster-growing virus does not necessarily dominate in a dual inoculation since virus SC-1B (replicates in 8 to 15 h) competed very well with NY-2C (replicates in 4 to 9 h) even though it replicates much more slowly. (v) Some viruses dominated in certain combinations. For example, IL-3A dominated NY-2C and the PBCV-1 serotype EPA-1. However, the dominance could not be predicted from the site-specific endonucleases. For example, viruses PBCV-1 and NC-1A produce the site-specific endonucleases *C*viAI and *C*viBI, respectively. In vitro, *C*viBI digests PBCV-1 DNA but *C*viAI does not digest NC-1A DNA. Consequently, if the site-specific endonucleases mediate exclusion, NC-1A should dominate in a mixed infection. This did not occur; about 40% of the infective centers contained only NC-1A and 60% contained only PBCV-1. We have also found exclusion in cells inoculated with the isogenic viruses PBCV-1 and EPA-1, which have the same site-specific endonuclease. Exclusion among isogenic viruses probably explains the low level of recombination (1 to 2%) between temperature-sensitive mutants of PBCV-1 (173). In summary, exclusion appears to be independent of known site-specific endonucleases.

Therefore, the biological function of the DNA methyltransferases and DNA site-specific endonucleases remains unknown. However, if a virus contains a functional cytosine methyltransferase gene, its expression is apparently necessary for virus growth since 5-azacytidine inhibits virus replication. There was a direct correlation between increasing 5mC concentrations in the virus genome and sensitivity of virus replication to 5-azacytidine (16). It should also be noted that some of the virus DNAs are more heavily methylated than is necessary for protection from their site-specific endonucleases, e.g., virus NY-2A. The reason for this apparent excess methylation is unknown.

As discussed in the next section, the natural history of the *Chlorella* viruses is poorly understood. Presumably the DNA-methylating and endonuclease enzymes must confer an evolutionary advantage to the viruses in their native environment. We have conducted one simple experiment to determine whether the enzymes confer an advantage to the virus in a laboratory setting. A culture of *Chlorella* strain NC64A was inoculated with a 1:1 mixture of virus IL-3A and an IL-3A mutant deficient in *C*viJI and M.*C*viJI activities (one-step growth curves for the two viruses are identical). A fresh culture of *Chlorella* strain NC64A was inoculated with a portion of the lysate. After five serial transfers, the titer of the lysate was determined. The mutant made up the majority of the plaques (113). Thus *C*viJI and M.*C*viJI conferred no selective advantage to the virus under these conditions.

NATURAL HISTORY OF THE *CHLORELLA* VIRUSES

As noted in the introduction, eukaryotic algae are important components of both freshwater and marine environments; however, the significance of viruses or VLPs in these systems is just beginning to be explored. Two recent reports (153, 169) suggest that viruses may strongly influence the marine phytoplankton community.

Algal viruses in native waters have traditionally been ignored because of the lack of a sensitive biological assay. However, plaque assays for at least NC64A viruses and Pbi viruses now make such experiments feasible. As mentioned in the section on properties of *Chlorella* strain NC64A viruses other than PBCV-1, *Chlorella* strain NC64A-infecting viruses are ubiquitous in United States and Chinese fresh water (144, 183, 227). Typically, the virus titer is 1 to 100 PFU/ml but occasionally the titer is much higher. For example, a water sample collected from the Waccamaw River, N.C., in 1983 contained 4×10^4 PFU/ml (183). Although less is known about Pbi viruses, their titer in European fresh water appears to be similar to the titer of NC64A viruses (132). Incidentally, the type of filter used to remove bacteria from the freshwater samples prior to titer determination is important. We have found that Millipore filters but not Nuclepore filters disrupt the NC64A viruses and result in a reduced titer (179).

In 1984 we collected water samples monthly from seven locations in central Illinois over an 8-month period to determine whether the titer of the NC64A viruses fluctuated (191). Both qualitative and quantitative changes were found. For example, the titer in one location decreased from 3.2×10^3 to 100 PFU/ml in 1 month. The virus titer also fluctuated, but less dramatically, in other locations. In addition, some water samples contained viruses of two plaque types in one sampling period and only one type in another. Nothing is known about the stability or replication of these viruses in nature.

Not only do natural sources contain NC64A viruses that form different plaque types, but also the virus genomes are highly diverse as judged by restriction patterns. In some instances this diversity may reflect genetic recombination; however, as noted above, mutual exclusion apparently inhibits recombination. The variability of the NC64A virus genomes contrasts dramatically with the uniformity of the genomes of VLPs isolated from *H. viridis* zoochlorellae (see the section above on initial observations of *Chlorella* viruses).

There is no obvious correlation between the level of methylation in NC64A virus DNAs and their geographic origin. For example, four of six plaques originally picked from a water sample collected in New York fell into different classes (Table 2). The level of methylation ranged from NY-2C, which contained 0.4% 5mC and no detectable 6mA, to NY-2A, which contained 45% 5mC and 37% 6mA.

It is not known whether NC64A viruses replicate exclu-

sively in *Paramecium* zoochlorellae or whether they have other host(s). It is also not known whether zoochlorellae can exist free of their hosts in natural environments. It is known, however, that symbiosis protects *P. bursaria* zoochlorellae from infection and that zoochlorellae become infected only when they are released from their host (63). Reisser et al. (136) placed *P. bursaria* containing zoochlorellae in a dense solution of virus (5×10^6 viruses per ml) and found that symbiotic zoochlorellae were maintained in the paramecia. However, the zoochlorellae were infected by virus as soon as they were isolated from the paramecia. A single protozoan contains as many as 1000 zoochlorellae, and if each zoochlorella produces 300 virus particles, zoochlorellae released from a single paramecium could produce 300,000 virus particles. This could account for the occasional high virus titer observed in nature. However, this number is artificially high since not all zoochlorellae would be infected in dilute solutions of virus and algae. For example, Wiggins and Alexander (202) have demonstrated that bacterial concentrations must exceed a threshold value of about 10^4 cells per ml to consistently support virus replication.

The finding that paramecia protect zoochlorellae from virus infection led Reisser et al. (133) to propose that the evolutionary forces which typically favor symbiosis may not function solely at the physiological level. Establishing a protective habitat for one partner, e.g., protecting the zoochlorellae from virus infection, could also favor symbiosis.

As far as we are aware, zoochlorellae or their equivalents isolated from other freshwater organisms, have not been examined for viruses or VLPs. However, zoochlorellae from *Stentor polymorphus* (129) and from *Climacostomum virens* (135) have been cultured. An attempt to detect VLPs in isolated dinoflagellate algae symbiotic with the sea anemone *Anthopleura xanthogrammica* was unsuccessful (115).

Once it was established that NC64A viruses were widespread in nature, we assumed that plaque-forming viruses of other eukaryotic algae might also be common. However, freshwater samples did not contain viruses lytic for other *Chlorella* or *Chlamydomonas* species (179, 183, 191). Besides testing filter-sterilized water directly for viruses, we also enriched for viruses of other algae by adding high concentrations of one or more algae to several liters of nonsterile fresh water and incubating for several days. Aliquots were then filter sterilized, and titers were determined on the same algae used to seed the culture. This simple procedure increased the titer of NC64A viruses from 1–100 to 10^8–10^9 PFU/ml. However, viruses on other hosts were not detected. Thus, lytic viruses may be limited to a few algae, they may exist as lysogens in nature and only infrequently become lytic, or we did not use the appropriate hosts.

Lysogeny is consistent with the observation by many investigators that VLPs appear infrequently in eukaryotic algae and that they are limited to certain stages of algal development. The apparent lack of infectivity of the previously observed VLPs is also consistent with lysogeny. The VLPs might either infect the host and resume a lysogenic relationship or be excluded by preexisting lysogenic viruses, as are bacteriophages (see, e.g., reference 80). However, at present there is no direct evidence that the *Chlorella* viruses have a lysogenic phase. Additional comments about lysogeny of VLPs of other eukaryotic algae are mentioned below in the section on properties of viruses or VLPs of other algae.

Algae might also harbor viruses in a carrier state relationship (also called pseudolysogeny), in which at any one time a small population of algae are continually infected by virus. This type of relationship occurs with bacteriophages (see, e.g., reference 48). On two occasions we have observed a carrier state relationship between PBCV-1 and *Chlorella* strain NC64A in the laboratory. That is, colonies of *Chlorella* strain NC64A occasionally grow in PBCV-1 plaques. Colonies picked from these plaques continually produced low concentrations of PBCV-1 (ca. 10^5 PFU/ml), even after repeated subculturing over several months. PBCV-1 DNA did not hybridize to DNA isolated from single-colony isolates of these *Chlorella* cells, which rules out lysogeny. Furthermore, single-colony isolates from these cultures did not produce PBCV-1; however, about 25% of these isolates could reestablish the carrier state relationship when reinoculated with PBCV-1. The remaining colonies were either sensitive or resistant to PBCV-1 (214).

In conclusion, very little is known about the natural history of the *Chlorella* viruses or VLPs of other eukaryotic algae, and this would appear to be a fruitful research area.

COMPARISON OF *CHLORELLA* VIRUSES WITH OTHER VIRUSES

Chlorella viruses share some features with certain other viruses that infect eukaryotic organisms. Table 5 compares PBCV-1 with African swine fever virus (ASFV) (recently removed from the iridoviruses), vaccinia virus (a poxvirus), frog virus 3 (FV3) (an iridovirus), and Chilo iridescent virus (an iridovirus). PBCV-1 has the largest genome among these viruses. Incidentally, PBCV-1 DNA does not hybridize with FV3 or vaccinia virus DNA (185).

The *Chlorella* viruses have several properties in common with the iridoviruses, also called icosahedral cytoplasmic deoxyriboviruses (42, 85, 203). Iridoviruses are widely distributed in nature and commonly infect insects and some animals. Shared properties include polyhedral morphology, a large dsDNA genome, and an internal lipid component which makes up 5 to 10% of the viral weight. FV3 is the most widely studied iridovirus. FV3, like PBCV-1, is one of the few viruses that infects a eukaryotic organism and contains methylated bases (205); FV3 also encodes a cytosine methyltransferase enzyme (204). However, FV3 and PBCV-1 differ in several ways. (i) The recent finding that PBCV-1 probably has specialized attachment structures (see the section on general characteristics of PBCV-1) suggests that the morphologies of PBCV-1 and FV3 are different. (ii) FV3 is typically uncoated inside host cells (57, 64), whereas PBCV-1 is uncoated at the cell surface (93). (iii) FV3 cannot initiate replication in UV-inactivated host cells (44), whereas PBCV-1 initiates and completes viral replication (albeit slowly and with a small burst size) in UV-inactivated *Chlorella* cells (182). (iv) FV3 does not contain glycoproteins, whereas at least two PBCV-1 structural proteins are glycoproteins. (v) FV3 mRNAs are not polyadenylated, whereas PBCV-1 early, but probably not all late, mRNAs are polyadenylated. (vi) Most importantly, the structures of the DNAs are completely different. FV3 DNA is linear and circularly permuted (43), whereas PBCV-1 DNA is linear and nonpermuted with covalently closed hairpin ends.

Thus, even though vaccinia virus (a poxvirus) and the *Chlorella* viruses are morphologically distinct, PBCV-1 DNA has two characteristics in common with vaccinia virus DNA. Like PBCV-1, vaccinia virus DNA contains both covalently closed hairpin termini and inverted terminal repetition (35, 36, 207). However, the terminal repetitive region

TABLE 5. Comparative properties of PBCV-1, ASFV, vaccinia virus, FV3 and Chilo irridescent virus

Property	Property in:				
	PBCV-1	ASFV	Vaccinia virus	FV3	Chilo irridescent virus
Structure	Icosahedral	Icosahedral	Ovoid	Icosahedral	Icosahedral
Diameter	190 nm	200 nm	365 × 260 nm	130 nm	130 nm
Mol mass	1 GDa		3.3 GDa	0.5 GDa	1 GDa
Sedimentation coefficient	2,300S	3,500S		1,300S	2,200S
Nonionic detergent	Resistant	Sensitive	Sensitive	Sensitive	Sensitive
Infection	Attachment-injection	Endocytosis	Endocytosis	Endocytosis	Endocytosis
Virus assembly area	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
Membrane	Internal	External + Internal	External + Internal	Internal	Internal
Lipid content	8%		5%	9%	9%
DNA content	23%	20%	5%	20%	12%
Genome size	333 kbp	170 kbp	190 kbp	166 kbp	240 kbp
G+C content	40%	41%	36–37%	53%	29%
DNA methylation	1.9% 5mC, 1.5% 6mA	None	None	20% 5mC	None
DNA termini	Hairpin	Hairpin	Hairpin	Terminally redundant	Terminally redundant
Glycoproteins	Major capsid protein	None	Several in membrane	None	None

of vaccinia virus DNA is larger (ca. 10 kbp) than that of PBCV-1 DNA (2.2 kbp), even though the PBCV-1 genome is about twice the size of the vaccinia virus genome. A second difference is that many identical direct tandem repeats of 50 to 125 bases occur in vaccinia virus termini (208). Although PBCV-1 termini contain several direct repeats, with the exception of 118- and 39-base repeats they are much smaller and the same repeat occurs only a few times.

Preliminary analysis indicates some similarities between PBCV-1 and vaccinia virus transcription: (i) the region just upstream from the translation start codons of both viruses is A+T rich and (ii) the TTTTTNT transcription termination motif present in vaccinia virus early genes (223) occurs 230 to 480 bp downstream of the translation stop codon in both PBCV-1 early and late genes. However, it is not known whether this sequence is involved in PBCV-1 transcription termination. Additional similarities and differences between vaccinia virus and PBCV-1 are listed in Table 5.

ASFV is the only other large polyhedral virus containing dsDNA with hairpin termini and inverted terminal repetition (10, 41, 118, 161). There are several differences between PBCV-1 and ASFV, however (Table 5). Most significantly, (i) they infect different hosts; (ii) PBCV-1 DNA is roughly twice the size of ASFV DNA; (iii) PBCV-1 DNA contains methylated bases, whereas ASFV does not; and (iv) ASFV lacks glycoproteins, whereas PBCV-1 has at least two glycoproteins.

The infection process of the *Chlorella* viruses differs from that of all DNA viruses that infect eukaryotes, but resembles that of bacterial viruses, in having to pass through a cell wall. In terms of structure, attachment, and penetration the *Chlorella* viruses resemble members of the bacteriophage family Tectiviridae (99). However, tectiviruses are much smaller (about 65 nm in diameter) and contain only about 40 kbp of DNA. The *Chlorella* viruses have recently been given family status with the name Phycodnaviridae.

PROPERTIES OF VIRUSES OR VLPs OF OTHER ALGAE

As mentioned in the introduction, at least 44 taxa of freshwater and marine eukaryotic algae contain viruses or VLPs (Table 1). Although it is difficult to generalize from these observations, which are primarily ultrastructural, several common themes emerge. (i) With a few exceptions, all the particles are polyhedra and the diameters vary from 20 to about 400 nm. Typically the particles have an external, multilaminate shell enclosing core material. Several investigators have mentioned that this shell resembles a membrane, although there is no direct evidence that the particles contain an external membrane. In fact, many of the particles look like the *Chlorella* viruses, which do not have an external membrane, but do have an interior lipid component. (ii) Although only one type of particle is usually present in a single alga, five species of algae (*Crytomonas* species [124], *Gyrodinium resplendens* [34], *Chromophysomonas cornuta* [127], *Paraphysomonas bourrellyi* [127], and *Pyramimonas orientalis* [101]) contain two sizes of particles, and, in a couple of cases, both particles have been seen in the same cell. Finally, three sizes of particles were present in *Paraphysomonas corynephora* (127). (iii) The intracellular location of the particles varies with the VLP and the alga. Particles have been found in the nucleus, in the cytoplasm, and in both locations. Several investigators mentioned that infected cells lacked a nucleus and that the particles appeared to form at the expense of the nucleus. In some cases, the other organelles, e.g., chloroplasts, mitochondria, and the Golgi apparatus, appeared normal in cells containing VLPs. (iv) Typically, vegetative cells of multicellular algae lacked VLPs, and the particles were seen only in the reproductive cells or the germlings produced from them. Because many of these algae have a wall-less free-swimming zoospore stage, several investigators have proposed that virus or VLP infection occurred in the zoospore stage. (v) With a couple of exceptions (*Platymonas* species [122] and *Paraphysomonas caelifrica* [127]), only a small percentage (1 to 5%) of the algal cells contained particles. (vi) Although it is generally assumed that cells that contained particles eventually lysed or died, lysis has been observed only in a few algae. (vii) The infrequent appearance of the VLPs, the presence of the VLPs in only certain differentiated cells, and their apparent lack of infectivity have led many investigators to propose that the VLPs represent a latent infection. However, to date, no direct evidence for lysogenic VLPs has been reported. This lack of evidence for lysogeny may change soon, since two groups of investigators (51, 104) have

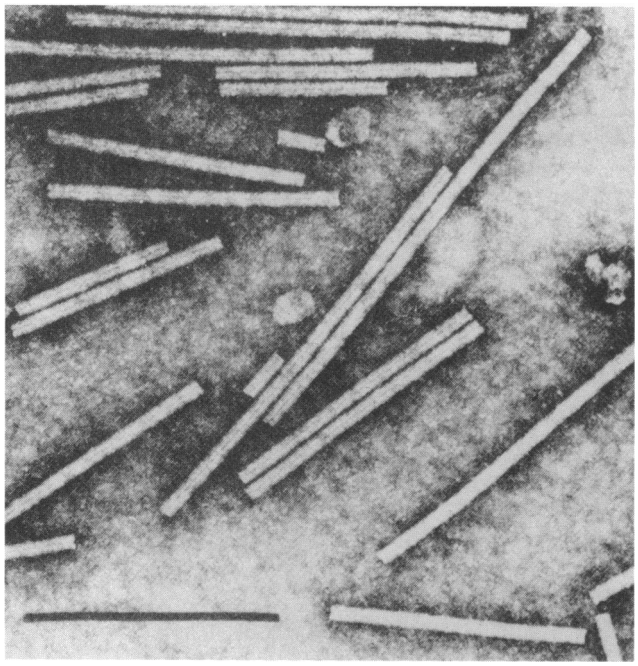

FIG. 14. Micrograph of an equal mixture of pure preparations of CCV (long rods) and tobacco mosaic virus (short rods). Bar, 300 nm. Reproduced from reference 38 with permission from Academic Press, Inc.

preliminary data suggesting that the DNAs from VLPs of two marine brown algae are integrated into the host DNA.

Viruses or VLPs from eukaryotic algae for which at least a small amount of information is available are described below.

Gibbs and colleagues (38, 155) isolated and characterized the first infectious virus in a eukaryotic alga. This single-stranded RNA (ssRNA)-containing virus, *Chara corallina* virus (CCV), which resembles tobacco mosaic virus, replicates in the large multicellular green alga *Chara corallina*. The particles are rigid helical rods, 532 nm long and 18 nm wide, with a basic pitch of 2.75 nm (Fig. 14). CCV is about twice the length of tobacco mosaic virus (300 nm) and contains a correspondingly larger ssRNA genome (3.6×10^6 Da versus 2.1×10^6 Da for tobacco mosaic virus RNA). Recently obtained nucleotide sequences (37) suggest that CCV is more closely related to beet necrotic yellow vein virus (a furovirus) than to tobamoviruses. Furoviruses (14) are rod-shaped, divided-genome viruses which are transmitted in a persistent manner by soilborne, zoosporic fungi. CCV is the only known algal virus which resembles vascular plant viruses and is therefore a potential key to the evolutionary origin of plant viruses. CCV can be injected into virus-free *C. corallina*, and the inoculated algae become chlorotic 7 to 8 days after infection. Progeny viruses have been isolated from the inoculated plants.

Dodds and colleagues (23, 29) characterized a large (6,300S), noninfectious VLP from the filamentous green alga *Uronema gigas* (Fig. 15). Low concentrations of VLPs accumulated in liquid growth medium at a rate which paralleled the rate of algal growth. About 1% of the cells in the germling or in the young filament stage contained particles; VLPs were not detected in cells of mature filaments. Purified VLPs were polyhedra, 390 nm in diameter; they have a 15-nm-thick multilaminate shell and a core of two distinct materials, which appears to be packed asymmetrically. About 10% of the VLPs contain a 1-μm-long tail with a central swelling. The particles contain dsDNA, reported to range in size from 13 to 120 kbp. However, these values probably reflect fragmented DNA, and presumably the DNA is larger than the reported values. The VLPs contain at least 10 structural proteins; the major protein weighs about 45 kDa.

Stanker and colleagues (56, 163, 164) partially characterized a noninfectious VLP from the multicellular filamentous green alga *Cylindrocapsa geminella*. VLPs were present in 5% of the cells at the single-celled germling stage. The appearance of the VLPs depended on heating the zoospores to 40°C for 6 h (heat shock) immediately after the zoospores had attached to a support. VLPs appeared about 18 to 24 h after the heat shock. Infected cells degenerated and presumably lysed. The particles are polyhedra, 200 to 239 nm in diameter, and have a 14- to 16-nm-thick multilaminate shell and a dense fibrillar core. Purified particles contain dsDNA (275 to 300 kbp) and at least 10 structural proteins.

Considerable effort was made to increase the titer of the VLPs in both *U. gigas* and *C. geminella* by varying factors such as light and temperature. Although a short heat treatment increased the titer severalfold in both algae, yields remained extremely low.

Gromov and Mamkaeva (46, 47) described a dsDNA-containing virus which infects zoospores, but not vegetative cells, of the green alga *Chlorococcum minutum*. This virus has a hexagonal head measuring 220 by 180 nm and, most interestingly, a tail which is reported to invaginate into the head of the virus particle. The tail is believed to extend from the virus head during infection of zoospores. Infection occurs through the flagellar canals and leads to loss of zoospore motility and eventually loss of the flagella. Progeny viruses appear in about 8 h and are released by cell lysis.

A virus which infects four isolates of the marine unicellular nanoflagellate *Micromonas pusilla* was described by Mayer and Taylor (87). Unlike most eukaryotic algae, *M. pusilla* lacks a discernible cell wall. The virus attaches to the surface of the host cells (Fig. 16), but details of the infection process are unknown. This virus, which contains a dsDNA genome (168), is a polyhedron, 130 to 135 nm in diameter. Waters and Chan (199) developed an end point dilution assay for the virus and demonstrated a latent period of 7 h and a burst size of about 70 infective particles. Virus infection reduces CO_2 fixation by the host, and progeny are released by localized rupture of the host plasma membrane. Host range mutants have been isolated, presumably reflecting changes in the host receptor specificity. Unfortunately, the host cannot be grown axenically; however, *M. pusilla* can be grown monaxenically in the presence of certain bacterial species.

Finally, an interesting story is emerging with certain filamentous marine brown algae. Polyhedral VLPs (150 nm in diameter) developed exclusively in the sporangia of a *Feldmannia* sp. isolated from the coast of New Zealand (Fig. 17) (52). The particles were purified from cultures of the alga and shown to contain a dsDNA genome of about 190 kbp. Pulsed-field electrophoresis of the DNA revealed two DNA molecules that differed by about 10 kbp. Newly prepared cultures arising from single vegetative cells continue to produce two viral genomes (51). This suggests either that the cells contain two VLPs or that two DNAs are packaged in the same virus particle. Of particular interest, the particles are found only in the meiotic sporangia and not in the mitotic

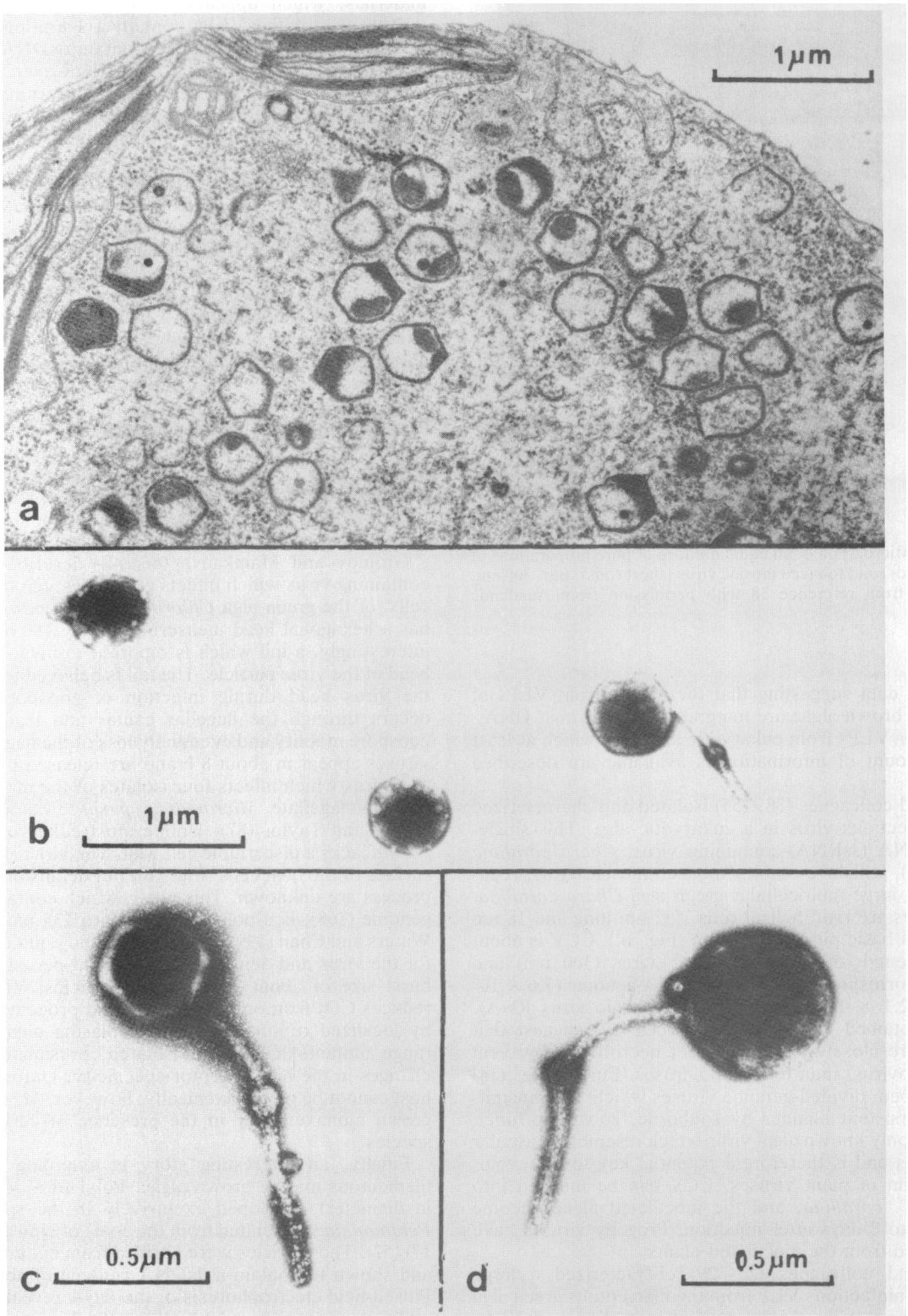

FIG. 15. Micrographs of the VLP from *U. gigas*. (a) Thin section of an infected cell containing hexagonal and pentagonal particles with two internal components in some particles. (b) Tailed and tailless VLPs negatively stained in 2% phosphotungstic acid. (c) Tailed particle with a hexagonal capsid. Note the characteristic swelling in the middle of the tail. (d) Tailed particle with a swollen spherical capsid. Reproduced from reference 29 with permission from Academic Press, Inc.

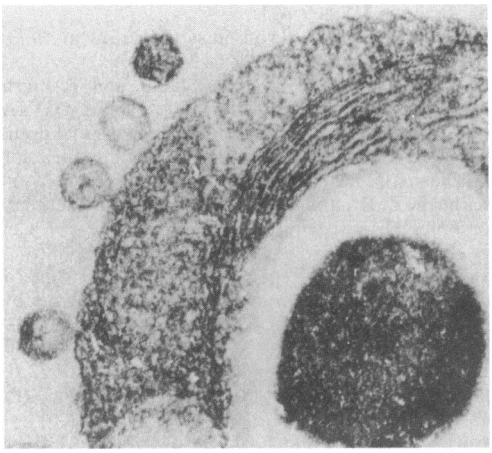

FIG. 16. VLPs near and attached to the surface of the marine nanoflagellate *M. pusilla* 15 min after inoculation. Unlike most eukaryotic algae, *M. pusilla* lacks a cell wall. Reproduced from reference 87 with permission from Macmillan Magazines Ltd.

sporangia (52), suggesting that VLP replication might be triggered by meiosis.

Muller et al. (103) found viruses in *Ectocarpus siliculosus*, a related ectocarpoid species also isolated near the coast of New Zealand, which contained particles about 130 nm in diameter. These particles were located exclusively in the gametangia. The particles contain a 350-kbp linear dsDNA, which is about twice the size of the DNA from the *Feldmannia* VLPs. The *Ectocarpus* particles are released into the medium when host cells lyse, and they can infect healthy zoospores. The infected zoospores become paralyzed and settle to the bottom of the container, where they develop into plants that produce viruses in their sporangia. Expression of the *Ectocarpus* virus is temperature sensitive and is linked to differentiation of reproductive cells of the host. Notably, both research groups have preliminary evidence suggesting that the *Feldmannia* and *Ectocarpus* virus DNAs are integrated into their host DNAs (51, 104).

CONCLUDING COMMENTS

Algal viruses, especially the *Chlorella* viruses, are interesting for several reasons and deserve investigation. The *Chlorella* viruses are examples of a new virus-host relationship, and their bacteriophagelike biology makes them excellent models for studying gene regulation and expression in photosynthetic eukaryotes. The inability to perform host genetics and to introduce foreign genes into the viruses remains a disadvantage. The genomes of these viruses are potential sources of control elements such as origin(s) of replication or promoters for plant genetic engineering. In fact, the upstream region of one of the *Chlorella* virus DNA methyltransferase genes (M.*C*viBIII) functions well as a promoter in both *Agrobacterium tumefaciens* and higher plants (100). Methylation levels of the *Chlorella* viral DNAs vary considerably; thus, some of these DNAs may be useful for molecular and biological studies on the effect of methylation on DNA structure and function. For example, it would be interesting to determine whether DNA-binding proteins

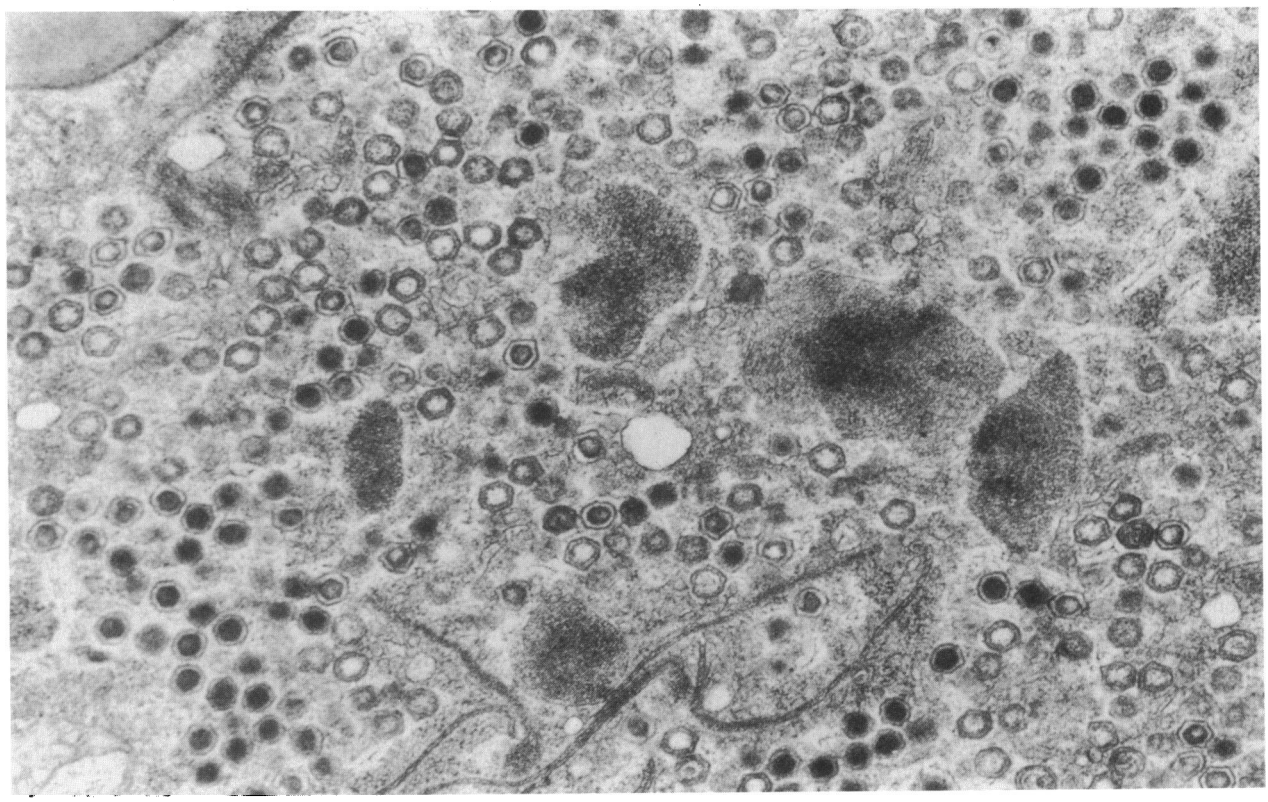

FIG. 17. Virus particles in *Ectocarpus siliculosus* (photograph kindly provided by Eric Henry). The particles are present only in the sporangia.

of the viruses are adapted to levels of methylation. The *Chlorella* viruses contain several genes of commercial interest such as DNA methyltransferases and DNA site-specific endonucleases, some of which have unique specificities. In addition, the viruses contain host-wall-degrading enzymes which could potentially digest plant cell walls. Finally, preliminary results suggest that the viruses may contain glycosyltransferase genes. The common association of VLPs with zoochlorellae isolated from hydras and paramecia suggests that these particles may influence symbiosis. Viruses that infect *Chlorella* strain NC64A are common in fresh water; however, nothing is known about their potential ecological impact on the algal community.

Although investigators are just beginning to look for viruses of other eukaryotic algae, it is already obvious that viruses are a major factor in marine algal ecology (153, 169). Besides limiting algal growth, the viruses could potentially mediate genetic exchange among native algae. Pearson and Norris (122) and Hoffman and Stanker (56) have also suggested that algae could serve as reservoirs or vectors for transmitting viruses to other forms of aquatic life, including animals. In this regard the *Chlorella* viruses have some similarities to important disease viruses such as the poxviruses and ASFV. Finally, at least some of these viruses, such as those infecting brown algae, appear to be expressed only in meiotic cells, suggesting highly specific coordination between the virus and the host life cycle. We encourage more investigators to work on these virus systems.

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