APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1991, p. 3433–3437
0099-2240/91/123433-05$02.00/0

Vol. 57, No. 12

# Screening of Natural Waters for Viruses Which Infect *Chlorella* Cells

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Received 28 June 1991/Accepted 18 September 1991

**By using a plaque assay with the unicellular green alga *Chlorella* sp. strain NC64A as a host, viruses were screened from natural pond waters collected in Kyoto and Higashi-Hiroshima, Japan. From some samples tested, two kinds of plaques, large ($\phi$ = 6 to 10 mm) and small ($\phi$ = 2 to 3 mm), were detected with various frequencies. The frequency of plaques in each of the water sources was seasonal; generally, it reached a peak value (8,000 PFU/ml) in May and gradually decreased to the limit of detection (<1) in November before increasing again in early spring. Electron microscopy revealed that the purified and negatively stained viruses were very large (125 to 200 nm) icosahedral particles. The genome isolated from these particles was always a linear double-stranded DNA of 340 to 370 kbp. Electrophoresis patterns of the DNA fragments produced by digestion with restriction enzymes differed considerably from plaque to plaque, even for plaques from the same water source. However, Southern hybridization showed strong homology among all of the virus DNAs tested, indicating relatedness of those viruses. A possible use of the *Chlorella* virus assay system to monitor the natural population of algal cells and water quality is discussed.**

In the natural aquatic environment, there are a huge number of viruses infecting a variety of organisms. Although viruses in natural waters have attracted wide attention, the size of their populations and their properties, natural hosts, infective processes, and ecological effects remain largely unknown. Human viruses in tap water, seawater, and sewage have been detected and quantitated by various methods to evaluate the safety of water supplies from viral diseases (19). In connection with the eutrophication of waters caused by algal blooms, cyanophages that infect blue-green algae (cyanobacteria) have been systematically surveyed in all parts of the world (12). A recent report (15) demonstrated that viruses in seawater ($10^6$ to $10^9$ virus particles per ml) could be a factor regulating phytoplankton community structure and primary productivity in the oceans. For eukaryotic algae, there have been several descriptions of viruslike particles (3, 5) but few examples of infectious viruses. A virus that infects the marine nanoflagellate *Micromonas pusilla* (11) has been isolated. Some freshwater *Chlorella*-like algae, which are endosymbionts of *Hydra* and *Paramecium* spp., also are virus hosts (17, 18). In the latter case, the viruses seem to play a key role in the symbiosis between the algae and their hosts.

The unicellular green alga chlorella is one of the most widely distributed and most frequently encountered eukaryotic algae on earth (6). In fact, *Chlorella* species are ubiquitous in all water habitats, in oligotrophic or eutrophic waters as well as dystrophic or saprobic water communities (2). Usually, coloration of water by growth of *Chlorella* organisms occurs in polluted waters. In order to better understand the role of *Chlorella* organisms in polluted waters, it is of interest to investigate their interaction in nature with *Chlorella* viruses. In this report, we have examined the numbers and characteristics of viruses infecting *Chlorella* sp. strain NC64A that were isolated in Japan from ponds with various degrees of eutrophication.

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## MATERIALS AND METHODS

***Chlorella* strains and culture conditions.** *Chlorella* sp. strain NC64A (16) was kindly given by J. Van Etten, Lincoln, Nebr. All other strains of *Chlorella ellipsoidea* (C-87), *Chlorella saccharophila* (C-211), and *Chlorella vulgaris* (C-135, C-150, and C-169) were obtained from the algal culture collection of the Institute of Applied Microbiology, University of Tokyo. Cells of *Chlorella* sp. strain NC64A were cultured in a modified Bold's basal medium (MBBM) as described elsewhere (16). Cells of other *Chlorella* strains were grown in a modified Bristol medium supplemented with 0.1% proteose peptone (20). The cultivation was carried out as described previously (22).

**Water samples.** Water samples for virus sources were collected from natural ponds, one in the Kyoto Prefectural Botanical Garden, Kyoto, Japan, and four in geographically separated areas (Budo, Gagara, Seisei, and Yamanaka Ponds) around the Saijo campus of Hiroshima University, Higashi-Hiroshima, Japan. Sampling was carried out almost every month to detect the seasonal variation of the virus populations over a 1-year period. After filtration through a nitrocellulose membrane filter (0.45-μm pore size; Sartorius GmbH), 100 μl of each water sample was subjected to a virus plaque assay.

**Plaque assay.** *Chlorella* viruses were detected by the plaque assay method described by Van Etten et al. (16) with *Chlorella* sp. strain NC64A as a host strain. A 100-μl sample of water was mixed with 200 μl of the host cells at a concentration of $1 \times 10^8$ to $2 \times 10^8$ cells per ml. The mixture was poured with 2.5 ml of 0.75% soft-agar MBBM onto a 1.5% MBBM agar plate and incubated in the light at 25°C.

**Purification and characterization of the viruses.** Each virus isolated from a single plaque on the algal lawn was propagated in liquid cultures of the same host for 48 h at 25°C in continuous light. After the cell debris was spun down at 7,000 × *g* for 10 min, virus particles were precipitated by centrifugation at 27,000 × *g* for 30 min. Viruses were further purified by linear 10 to 40% sucrose gradient centrifugation (18). The purified virus was stained with 1.5% uranyl acetate

TABLE 1. Concentration of *Chlorella* viruses in pond water[a]

| Sampling date | Virus titer (PFU/ml) for sample from the following pond[b]: | | | | |
|---|---|---|---|---|---|
| | Budo | Seisei | Yamanaka | Gagara | Kyoto |
| 1990 | | | | | |
| June | 50 | 4,000 | 1 | ND[c] | 20 |
| July | 15 | 300 | <1 | 45 | ND |
| August | 10 | 140 | <1 | 3 | ND |
| September | 2 | 90 | <1 | <1 | ND |
| October | <1 | 15 | <1 | <1 | ND |
| November | <1 | <1 | <1 | <1 | ND |
| December | <1 | <1 | <1 | <1 | ND |
| 1991 | | | | | |
| March | 20 | 1,300 | <1 | 10 | 90 |
| April | 30 | 7,700 | 1 | 100 | 90 |
| May | 20 | 8,000 | <1 | 130 | ND |

[a] *Chlorella* sp. strain NC64A was used as a host for the plaque assay.
[b] Means of duplicated experiments. The location of each water source is described in the text.
[c] ND, not determined.

before observation with a Hitachi H600A electron microscope.

**Isolation and characterization of nucleic acids from the virus particles.** Nucleic acids were isolated from the virus preparations by phenol extraction after treatment with proteinase K (1 mg/ml; Nippon Gene Co., Ltd.) in 0.1 M NaCl–0.01 M Tris-HCl (pH 7.4)–1 mM EDTA–0.1% Sarkosyl (Fluka AG) at 37°C for 1 h. Digestion of DNAs with restriction enzymes, agarose gel electrophoresis, and Southern hybridization were carried out by the method of Maniatis et al. (9). *Eco*RI digests of CVK1 virus DNA were labeled with nonradioactive digoxygenin-dUTP for a hybridization probe. Labeling by a random priming method and immunodetection were carried out with the Boehringer kit (Boehringer Mannheim) according to the manufacturer's manual. Hybridization was performed in a mixture containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5% blocking reagents, 0.1% sodium lauroyl sarcosinate, and 0.02% sodium dodecyl sulfate for 20 h at 42°C. All restriction enzymes used were purchased from Nippon Gene Co., Ltd.

**Pulsed-field gel electrophoresis.** The purified virus particles were embedded in 0.7% low-melting-point agarose (InCert agarose; FMC Corp.) prepared in 0.125 M EDTA (pH 7.5) and cooled to 42°C. After solidification, the gel was treated overnight at 50°C with a lysis solution containing proteinase K (1 mg/ml), 10 mM Tris-HCl (pH 8.0), and 1% Sarkosyl. DNA samples were cut from the agarose to fit into the gel wells of an electrophoresis agarose plate (1% agarose in 0.5× TBE [45 mM Tris-borate (pH 8.0) and 1 mM EDTA]). Contour-clamped homogeneous electric field (CHEF) gel electrophoresis (4) was carried out at 13°C with a switching interval of 30 s at 6.6 V/cm for 24 h in 0.5× TBE.

## RESULTS

**Detection of *Chlorella* viruses.** All the samples collected from the initial June 1990 sampling yielded virus plaques on the algal lawn which varied from source to source and ranged from 1 to 4,000 plaques per ml of water (Table 1). Viruses isolated from some sources produced two plaque sizes: the larger ones were 6 to 10 mm in diameter, and the smaller ones were 2 to 3 mm in diameter. The Kyoto sample averaged 4 small plaques and 16 large ones per ml of water. The values in Table 1 represent the sums of the numbers of small and large plaques. The highest value was obtained for the water sample from Seisei Pond (4,000 $m^2$ in area), which is located beside the dairy farm of the Faculty of Applied Biological Science, Hiroshima University. The dairy sewage was flowing into this pond. Duckweeds covered most of the water surface, and photomicroscopical observations revealed various kinds of phytoplanktons as well as zooplanktons, some of which are typical indicators of eutrophication, such as *Euglena*, *Nitzschia*, *Oscillatoria*, *Chlamydomonas*, and *Chlorella* spp. (13). In contrast, Yamanaka Pond (13,000 $m^2$ in area), about 800 m northwest of Seisei Pond, gave only one plaque per ml of water (Table 1). Yamanaka Pond water was transparent and contained very few planktons. Budo Pond (12,500 $m^2$ in area), located 800 m downstream from and connected to Yamanaka Pond, gave a moderate number of plaques (50 PFU/ml). The water of Budo Pond was turbid (muddy and sometimes greenish), probably because of the construction of buildings beside this pond. Phytoplanktons such as green algae (including chlorellas), diatoms, and flagellates dominated the population observed by photomicroscopy. Gagara Pond (200 $m^2$ in area), which is at the foot of Gagara Hill and geographically isolated from any of the other ponds studied, also gave approximately 45 plaques per ml of water in July. Gagara Pond water was also turbid (white) and contained precipitated refuse. In addition to phytoplanktons, protozoa such as paramecia, euglena, and amoebae were observed in the water samples. These data from June 1990 indicate that viruses infecting *Chlorella* cells are ubiquitously distributed in Japan and flourish in eutrophic waters. In order to understand the ecological role of these viruses, the seasonal changes in the number of plaques were surveyed for a period of 1 year. As shown in Table 1, the *Chlorella* viruses generally showed a characteristic cycle of population fluctuation in which the viral counts gradually decreased from June through October. In the November samples, no viruses were detected in any water source. Changes in the ponds during this period were as follows. In Seisei Pond, water hyacinths started to grow in July and flourished and covered the surface in August and September. After flowering at the end of September, the water hyacinths were collected from the pond in October. In Budo Pond, the greenish color of the water which occurred in July and August disappeared in September. For Gagara and Yamanaka ponds, eminent changes in water status were not observed during this period.

By March of the following year, considerable numbers of plaques were again detected, and the viral counts increased to the highest levels in April and May. At this stage, duckweeds grew over the surface of Seisei Pond again, although there was nothing on the water surface in the winter season. This general pattern of virus fluctuation fits all of the water sources used in this study with the exception of Yamanaka Pond, from which water samples consistently bore very few virus plaques throughout the year. Yamanaka Pond water was consistently clear and contained very few planktons.

**Isolation and comparison of *Chlorella* viruses.** Single virus plaques were picked from the *Chlorella* sp. strain NC64A lawn, propagated in the same host liquid cultures, and purified by sucrose gradient centrifugation (18). Electron microscopy revealed large icosahedral particles from all purified viral preparations (Fig. 1). The particle sizes of viruses from each purified plaque were homogeneous. However, the virus sizes varied from 125 to 200 nm, depending on

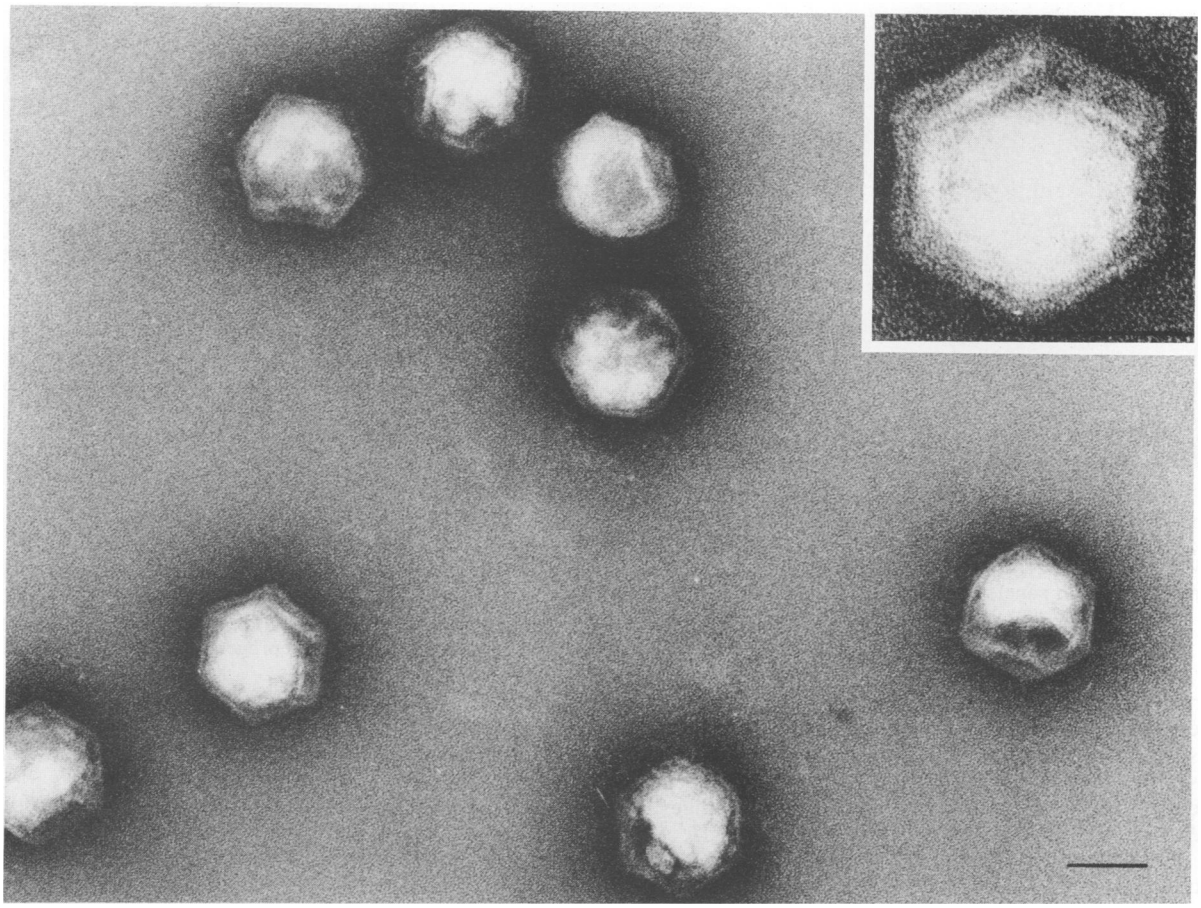

FIG. 1. Electron micrograph of *Chlorella* viruses. As a representative, the CVK1 virus particles negatively stained with uranyl acetate are shown. Bar, 100 nm.

the water source from which they were isolated. The smallest particles ($\phi$ = 125 nm) were obtained from plaques on the plates with Budo Pond water. Particles with similar sizes were also obtained from the Kyoto pond (from large plaques). Gagara, Kyoto (small plaques), and Yamanaka samples gave moderate-size particles ($\phi$ = 180 nm). The largest particles ($\phi$ = 200 nm) were obtained from Seisei Pond. In spite of the size variation, the morphologies and dimensions of these viruses are somewhat comparable to those of algae from *Hydra* and *Paramecium* spp. (18).

In order to characterize the *Chlorella* viruses, genomic material was isolated. Pulsed-field gel (CHEF) electrophoresis of the material revealed that the virus genome was a linear double-stranded DNA (dsDNA) in all cases (Fig. 2). Usually, a single band from each preparation appeared, ranging from 340 to 370 kbp. The size on CHEF gel electrophoresis is in good agreement with the sum of the fragments produced by digestion with several restriction enzymes (data not shown). As seen here, the viruses isolated in this study possess a common feature with minor differences, suggesting that in spite of their distant sources (Kyoto is 350 km from Higashi-Hiroshima), the viruses are closely related to one another. To demonstrate the degree of relatedness, the viral DNAs were digested with several restriction enzymes, including *Bam*HI, *Bcl*I, *Eco*RI, *Hin*dIII, *Nar*I, *Not*I, and *Sfi*I, and their digestion patterns were compared. Interestingly, the restriction enzyme fragmentation patterns varied significantly among the viruses, including those from the same sources. For example, the *Bam*HI patterns are shown in Fig. 3A. Among eight Budo Pond virus DNAs (lanes 2 through 7, 12, and 13), only CVB2, CVB3, and CVB5 gave similar patterns. Four Kyoto virus DNAs (lanes 8 to 11) were very different from each other. DNAs giving similar

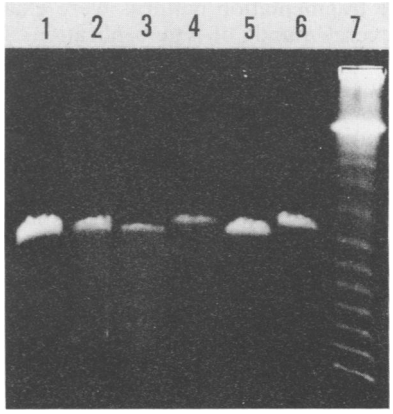


FIG. 2. CHEF gel electrophoresis of virus DNAs. Lanes: 1, CVB1; 2, CVK1; 3, CVK2; 4, CVS1; 5, CVY1; 6, CVG1; 7, ladders of $\lambda$ DNA.

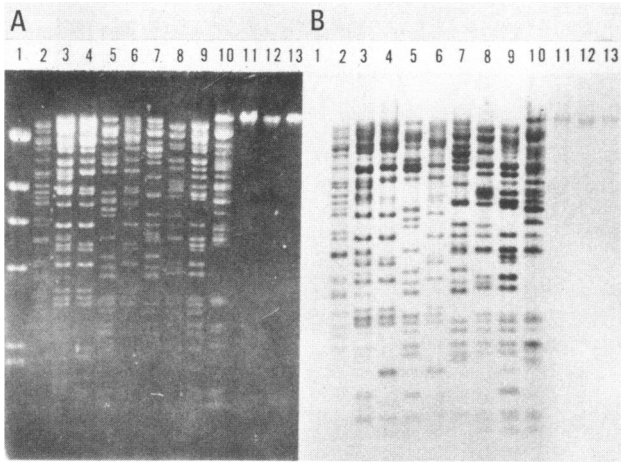


FIG. 3. (A) Electrophoretic separation patterns of *Bam*HI digests of virus DNAs. Lanes: 1, λ-*Hin*dIII size marker; 2, CVB1; 3, CVB2; 4, CVB3; 5, CVB4; 6, CVB5; 7, CVB6; 8, CVK1; 9, CVK2; 10, CVK3; 11, CVK4; 12, CVB21; 13, CVB22. (B) Hybridization patterns of the gel in panel A with labeled CVK1 DNA.

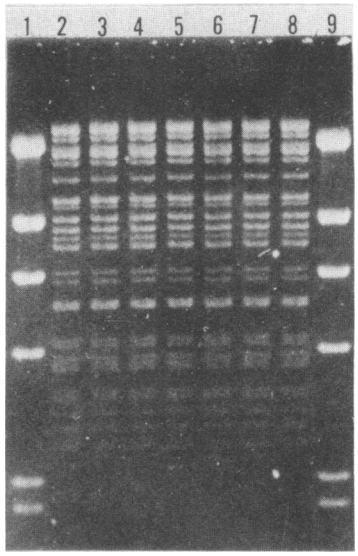


FIG. 4. Electrophoretic separation patterns of *Bam*HI digests of CVB1 DNA before and after six rounds of the lytic cycle. Lanes 1 and 9 contain λ-*Hin*dIII size markers. The original CVB1 DNA (lane 2) and DNAs of six individual progenies after lytic cycles (lanes 3 to 8) are shown.

patterns with one restriction enzyme often yielded very different fragments with other enzymes. In some cases, viral DNAs could not be digested with specific restriction enzymes (Fig. 3A, lanes 11 to 13). DNAs of two viruses isolated from Seisei Pond (CVS1 and CVS2) were digestible with *Eco*RI and *Bam*HI but not with *Bcl*I, *Hin*dIII, *Nar*I, *Not*I, or *Sfi*I (data not shown). For further comparison, the restriction fragments of viral DNAs were blotted onto a nylon filter and hybridized with labeled CVK1 virus (a small plaque-type virus from Kyoto) DNA as a probe. Figure 3B shows the result of hybridization of the *Bam*HI fragments. All the viral DNAs showed strong hybridization with CVK1 DNA, notwithstanding their different fragmentation patterns, indicating relatedness not only to CVK1 DNA but also to each other. However, it should be noted that there was variation in the signal strength for each virus species; for example, most of the bands from CVB5 DNA (Fig. 3B, lane 6) showed strong hybridization, but the 7th, 10th, 11th, and 14th bands from the top of the gel were very faint.

**Stability of the virus genome.** To estimate the rearrangement frequency of the *Chlorella* virus genomes, the restriction enzyme fragmentation patterns were examined before and after several rounds of lytic replication. The host alga (*Chlorella* sp. strain NC64A) in the liquid culture (10 ml) was infected with CVB1 virus (Budo Pond origin), and after the complete lysis of the host cells, a fresh culture of host cell was inoculated with 0.1 ml of the lysate. The same lytic cycle was repeated six times, and after the final round, the cell lysate was subjected to the plaque assay. Six well-separated plaques were selected, and virus particles and DNAs were isolated. The virus DNAs were digested with *Bam*HI and compared with the original CVB1 *Bam*HI-digested DNA. The result is shown in Fig. 4. There is no discernible change in any progeny DNAs. This was true with other restriction enzymes, including *Eco*RI, *Hin*dIII, and *Pst*I (data not shown). Thus, the virus genomes seem to be stable during the usual lytic cycles. Recently, we found that one of the viruses (CVK1) had a possible lysogenization property and after a provirus state at least one part of its genome changed (21). However, this mechanism could not solely explain the extended varieties observed in the *Chlorella* virus DNA structures.

**Host specificity of the *Chlorella* viruses.** None of the *Chlorella* viruses isolated in this study could infect standard laboratory strains of *Chlorella* in liquid or plate cultures, including *C. ellipsoidea* C-87; *C. vulgaris* C-135, C-150, and C-169; and *C. saccharophila* C-211. In the natural environment, the viruses must infect *Chlorella* cells living in their habitats. So far, four kinds of *Chlorella*-like algae that are possible viral host candidates have been isolated from the ponds where the viruses were found. The properties and taxonomy of these algae are now under investigation.

## DISCUSSION

**Characteristics of the *Chlorella* viruses.** It is now evident that *Chlorella* viruses are ubiquitously distributed in freshwater bodies in Japan. The virus particles isolated from several different water sources share common molecular characteristics, such as a large size ($\phi$ = 125 to 200 nm), an icosahedral shape, and a dsDNA genome. These features are also similar to those reported for the viruses of algae from *Hydra* and *Paramecium* spp. isolated in the United States (18). In spite of the general homology of the viral genomic DNAs revealed by Southern hybridization, restriction fragmentation patterns were considerably different from each other, indicating some dynamics of DNA rearrangements. Since the DNA structure hardly changes during the normal lytic cycle (Fig. 4), some specific mechanisms could be involved in the DNA rearrangements, such as lysogenization-excision, DNA methylation and demethylation, recombination between chromosomes and virus DNAs, and intra- or intermolecular recombinations. Comparing the detailed physical and genetic maps of the viral DNAs will reveal conserved and divergent regions of the genomes.

Among the unique characteristics of the *Chlorella* viruses, the following two are worthy of special mention: the linear structure of the dsDNA genome and the large size of the

genome. The replication of linear dsDNA molecules requires various specific terminal structures, such as cohesive ends, direct or inverted repeats, hairpins, and terminal proteins (8). The *Chlorella* virus assay system should serve as a good model to study the replication of linear DNA molecules and the roles of the DNA terminal structures. The large size of the dsDNA genome is unique among plant viruses. There are many types of plant viruses, but most of those studied have a genome made of RNA (10). Cauliflower mosaic virus represents an exceptional type of virus that contains a circular dsDNA. However, it has a small genome (8 kbp) (7) that does not integrate into the host genome, giving it limited usability as a molecular biological tool. Since the *Chlorella* viruses contain very large DNA genomes (340 to 370 kbp), the *Chlorella* virus assay system may provide a novel and useful host-vector system for cloning very large DNA molecules.

**Ecological significance of the *Chlorella* viruses.** *Chlorella* cells can grow mixotrophically and heterotrophically as well as autotrophically, so they propagate well in eutrophicated waters (13). Coloration of water by the growth of *Chlorella* cells occurs usually in polluted waters (6). The seasonal and geographical variations of the virus concentrations may reflect the fluctuations of the host, *Chlorella* cells. In general, it is labor-intensive and time-consuming to quantitate the populations of specific organisms in the natural environment. The plaque assay used in this work is very easy, requiring only 2 days, so the *Chlorella* virus assay system should provide a convenient way to monitor natural water quality. In this connection, it may be of interest that the *Chlorella* virus frequencies observed are comparable to bacteriophage titers in sewage (1) and to frequencies of cyanophage populations in natural waters (14).

## ACKNOWLEDGMENTS

We thank J. L. Van Etten for providing a culture of *Chlorella* sp. strain NC64A. We also thank Tokichi Miyakawa and Eiko Tsuchiya for helpful discussions and Atsuko Shimomae and Seiji Furukawa for water samplings and plaque assays.

This work was supported in part by grants from the Nissan Science Foundation.

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