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A Survey for Viruses from Fresh Water That Infect a Eucaryotic *Chlorella*-Like Green Alga†

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Viruses which formed plaques on lawns of a eucaryotic, chlorella-like green alga were detected in 37% of the 35 freshwater samples surveyed. Virus populations, monitored in seven locations, fluctuated both qualitatively and quantitatively over an 8-month period.

Viruses which infect cyanobacteria (blue-green algae) are common in nature (e.g., see references 4 and 5), and some of these viruses have been characterized in detail (6). In contrast, little information is available on viruses or virus-like particles which infect eucaryotic algae (for reviews, see references 1-3, 6). Most viruses or virus-like particles in eucaryotic algae have been detected during ultrastructural studies of the algae, and only a few attempts have been made to characterize these particles, primarily because they could not be obtained in sufficient quantities. Several factors account for this problem. (i) Usually only a few algal cells contain particles; (ii) usually the cells only contain particles in one stage of the life cycle; (iii) the cells that have particles may not lyse; and (iv) the particles may not be infectious. All of these factors contribute to the lack of a sensitive biological assay for viruses infecting eucaryotic algae; consequently, it is not known how common such viruses are in freshwater environments.

Thus, it is interesting that we have identified, isolated, and partially characterized a large (negatively stained particles are ca. 190 nm in diameter) polyhedral virus, PBCV-1, from a eucaryotic chlorella-like green alga symbiotic with the protozoan *Paramecium bursaria* (11). Subsequent experiments established that PBCV-1 infects and replicates in a culturable chlorella-like green alga (strain NC64A) also originally isolated from paramecia (8, 10). PBCV-1 is a complex virus that contains a lipid component, at least 50 structural proteins, and a large (at least 300 kilobase pairs), double-stranded DNA genome (7, 11). Most importantly, the virus can be assayed by plaque formation, which provides a sensitive biological assay for the virus (8).

We recently looked for additional plaque-forming *Chlorella* viruses from natural sources. Twenty freshwater samples were collected from ponds and streams in North and South Carolina, Florida, Nebraska, and Illinois and assayed for viruses. To our surprise, 4 of the 20 samples produced plaques on *Chlorella* strain NC64A lawns (9). The virus titer in these water samples varied from 60 to 4×10^4 PFU/ml. A few of the plaques were picked from each of the four samples and partially characterized. All of the viruses were similar to PBCV-1 with regard to host range, morphology, and sensitivity to organic solvents. Furthermore, the viruses

reacted with PBCV-1 antiserum and the viral DNAs hybridized extensively with PBCV-1 DNA. However, the viruses could be distinguished from PBCV-1 and from each other by plaque morphology, DNA restriction patterns, resistance to certain DNA restriction endonucleases (9), and the percentage of 5-methylcytosine or *N*⁶-methyladenine or both in their genomic DNA (J. L. Van Etten, A. M. Schuster, L. Girton, D. E. Burbank, D. Swinton, and S. Hattman, manuscript in preparation).

The ease with which these new lytic viruses were found suggests that viruses which infect this particular strain of

TABLE 1. Presence of plaque-forming viruses on *Chlorella* strain NC64A^a

Location	Source	Code no.	Date collected	PFU/10 ml	Plaque size (diam, mm) ^b
Nebraska	Stream	NE-8	September 1984	800	3 (800)
Nebraska	Stream	NE-9	September 1984	1	3 (1)
Nebraska	Lake	NE-14	April 1984	100	3 (100)
Alabama	Pond	AL-1	October 1984	100	3 (100)
Alabama	Pond	AL-2	October 1984	150	3 (100) 1 (50)
Alabama	Pond	AL-3	October 1984	550	3 (400) 1 (150)
Alabama	Stream	AL-4	October 1984	4	3 (4)
New York	Pond	NY-1	July 1984	6	3 (6)
New York	River	NY-2	August 1984	200	3 (100) 1 (100)
Massachusetts	River	MA-1	August 1984	800	3 (800)
California	Stream	CA-1	November 1984	170	3 (170)
California	Stream	CA-2	November 1984	65	3 (65)
California	Drainage ditch	CA-4	November 1984	65	3 (65)

^a Only water samples containing virus are listed in the table. Samples that tested negative (i.e., less than 1 PFU/10 ml) are listed as follows: one from California, one from Iowa, three from Minnesota, four from Nebraska, two from New Hampshire, one from New York, eight from North Dakota, and two from Wyoming.

^b The numbers in parentheses refer to the number of plaques that were 3 mm in diameter or 1 mm in diameter.

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TABLE 2. Titer of plaque-forming viruses on *Chlorella* strain NC64A in fresh water collected at periodic intervals during 1984^a

Illinois county	Source	Code no.	PFU/ml at the following sampling time ^b :					
			April	May	June	August	September	November
Tazewell	Farm pond	IL-3	57	— ^c	110	5	0	5
Tazewell	Farm pond	IL-4	185	243 (3 mm, 168; 1 mm, 75)	5	15	75 (3 mm, 50; 1 mm, 25)	35
Tazewell	Farm pond	IL-5	0	570 (3 mm, 270; 1 mm, 300)	5	90 (3 mm, 45; 1 mm, 45)	0	5
Mason	Pump Lake	IL-7	15	0	0	0	5	0
Mason	Drainage ditch	IL-9	—	3,200	10 (3 mm, 5; 1 mm, 5)	60 (3 mm, 45; 1 mm, 15)	20	10
Mason	Farm pond	IL-10	—	0	0	0	0	0
Mason	Crane Creek	IL-11	—	14 (3 mm, 13; 1 mm, 1)	35 (3 mm, 15; 1 mm, 20)	0	90 (3 mm, 72; 1 mm, 18)	35

^a All of the water samples were collected from central Illinois at the indicated times and then sent to Lincoln, Neb., for assay.

^b The number of plaques that were 3 mm in diameter or 1 mm in diameter is indicated in the parentheses. If not indicated, all of the plaques were 3 mm in diameter.

^c —, Not assayed.

Chlorella and possibly other eucaryotic algae are common in freshwater environments. Consequently, we assayed a number of other freshwater samples collected from various geographical regions in the United States for ability to form plaques on *Chlorella* strain NC64A. In addition, five ponds, one stream, and one drainage ditch in central Illinois were assayed periodically over an 8-month period to determine whether the viral titer changed seasonally.

The source and growth of *Chlorella* strain NC64A in liquid shake culture on MBBM medium has been described previously (10). Water samples were collected in 11 states at various times in 1984 as indicated in the two tables. The samples were stored at 4°C before being assayed (usually for less than 2 weeks). Separate experiments established that the virus titer usually remained constant for at least 2 months when the water samples were stored at 4°C. Each water sample was passed through a 0.4- μ m-pore-size Nucleopore filter (Millipore filters disrupt PBCV-1), and either duplicate 100- μ l samples were assayed directly for plaque formation on lawns of *Chlorella* strain NC64A (8) or 10-ml samples were centrifuged at 20,000 rpm in a Beckman no. 30 rotor for 2 h at 4°C. The pellets were suspended in 400 μ l of 0.05 M Tris (pH 7.8), and duplicate 200- μ l samples were assayed for plaque formation. Thus, we were able to detect virus in water samples which contained at least 1 PFU/10 ml of water.

Thirty-five water samples collected in 10 states were assayed for plaque-forming ability on *Chlorella* strain NC64A (Table 1). Thirteen of the samples from five states contained at least 1 PFU/10 ml of fresh water. All of the samples produced sharply defined, clear plaques. Water samples which tested negative for virus are listed at the bottom of Table 1. The viruses were not limited to one geographical region of the United States, since viruses were present in at least one sample collected from California, Nebraska, Alabama, New York, and Massachusetts. All of the plaques produced by 10 of these 13 samples were about the same size as those produced by PBCV-1 (ca. 3 mm in diameter), whereas the other three samples contained viruses which produced both small (ca. 1 mm in diameter) and PBCV-1-sized plaques. We selected a few representatives from each of the samples and have begun to characterize them. All of the viruses sediment at the same rate as PBCV-1 in sucrose density gradients, and they all contain large double-stranded DNA genomes. However, the viruses can be distinguished

from one another and from our previously described viruses (9) by DNA restriction endonuclease digestion (data not shown).

To determine whether the virus titer remained constant or fluctuated with time in the environment, water samples from five ponds, one stream, and one drainage ditch in central Illinois were collected and assayed at approximately 1-month intervals. (One of these sources, IL-3, contained virus when assayed in the fall of 1983 [9].) The results of this experiment are reported in Table 2. Plaque-forming viruses were detected at least twice in water samples from all of the locations with the exception of IL-10 where none were obtained. Furthermore, the virus titer fluctuated dramatically in some of the locations. For example, the sample taken from a drainage ditch (IL-9) in the second sampling period had a very high titer (i.e., 3.2×10^3 PFU/ml) which then decreased to 60 PFU/ml or less during the subsequent sampling periods. Four of these samples contained viruses that produced both small (1 mm) and PBCV-1-sized (3 mm) plaques in at least one sampling period. Plaque size was a stable genetic characteristic of the viruses since plaque-purified viruses always produced the same size plaque as the parent.

The development of a plaque assay for these eucaryotic algal viruses allowed us to establish that large, double-stranded-DNA-containing viruses are common in aquatic environments. Also, the virus population fluctuates both qualitatively and quantitatively with time in these environments. The role that these viruses play in their aquatic environments is completely unknown. However, these viruses would severely limit the growth of *Chlorella* strain NC64A in fresh water if, in fact, this *Chlorella* strain exists free in the environment. Of course, it is possible that the viruses that infect *Chlorella* strain NC64A replicate in another host in nature and by chance happen to infect this particular *Chlorella* strain. From these results one might expect that additional viruses which infect and replicate in other eucaryotic algae are more common in the aquatic environment than is now suspected. However, attempts to find plaque-forming viruses for 10 other *Chlorella* strains (9) and two *Chlamydomonas* species (unpublished data) have so far been unsuccessful.

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