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Chlorella Viruses Isolated in China†

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Plaque-forming viruses of the unicellular, eucaryotic, exsymbiotic, *Chlorella*-like green algae strain NC64A, which are common in the United States, were also present in fresh water collected in the People's Republic of China. Seven of the Chinese viruses were examined in detail and compared with the *Chlorella* viruses previously isolated in the United States. Like the American viruses, the Chinese viruses were large polyhedra and sensitive to chloroform. They contained numerous structural proteins and large double-stranded DNA genomes of at least 300 kilobase pairs. Each of the DNAs from the Chinese viruses contained 5-methyldeoxycytosine, which varied from 12.6 to 46.7% of the deoxycytosine, and N^6-methyldeoxyadenosine, which varied from 2.2 to 28.3% of the deoxyadenosine. Four of the Chinese virus DNAs hybridized extensively with DNA from the American virus PBCV-1, and three hybridized poorly.

The discovery that viruses which formed plaques on lawns of a unicellular, eucaryotic, exsymbiotic *Chlorella*-like green alga, strain NC64A, were common in fresh water collected throughout the continental United States established that a previously unknown biotic interaction is present in aquatic environments (11, 14, 19). The concentration of these viruses is usually about 1 to 100 PFU/ml of fresh water in nature. Occasionally, however, their titer can be very high; e.g., a water sample collected in North Carolina contained 40,000 PFU/ml (14).

We have examined 30 of these large double-stranded DNA-containing viruses in some detail. Although the viruses were morphologically similar and had a common host, they could be grouped into 11 classes on the basis of plaque morphology, reaction with antibody, resistance of the virus DNAs to restriction endonucleases, and the nature and abundance of methylated bases in their genomic DNAs (11). Each of the viral DNAs contained 5-methyldeoxycytosine (m^5dC); the concentration of m^5dC as a percentage of deoxycytosine varied from 0.1 to 47.5%. In addition, 18 of the 30 virus DNAs also contained N^6-methyldeoxyadenosine (m^6dA); the concentration of m^6dA as a percentage of deoxyadenosine varied from 1.5 to 37% (11, 18). The finding that methylation was sequence specific led to the suprising discovery that at least some of these viruses code for DNA methytransferases and DNA site-specific (restriction) endonucleases (6, 20–24). In fact, these virus-infected algae were the first source of DNA restriction endonucleases from a nonprocaryotic system.

Because all of the *Chlorella* viruses isolated to date have been from water collected in the United States, we wondered whether these viruses were present in other parts of the world. In this report we demonstrate that plaque-forming viruses of *Chlorella* sp. strain NC64A are also common in fresh water collected in the People's Republic of China and that these viruses are similar to those isolated in the United States.

MATERIALS AND METHODS

Cultural conditions. Freshwater samples were collected in three cities in the People's Republic of China in March 1987 at the 13 sites listed in Table 1. The samples were filtered through 0.4-μm-pore-size filters (Nuclepore Corp., Pleasanton, Calif.), and 100-μl portions were assayed for plaque formation on lawns of *Chlorella* sp. strain NC64A grown on MBBM medium (13–15). Individual plaques were picked and plaque purified, and actively growing cultures of *Chlorella* sp. strain NC64A were inoculated. After incubation for 60 h, progeny viruses were purified from the lysates as described previously for virus PBCV-1 (15).

Analysis of virus DNAs. DNA was isolated from the purified viruses on CsCl equilibrium gradients as described previously (16). These DNAs were treated with restriction endonucleases by the protocols provided by the suppliers, and the resulting fragments were separated by electrophoresis on either 0.8 or 1.2% agarose gels in 0.08 M Tris phosphate–0.008 M EDTA (pH 8.5).

For hybridization studies DNA fragments were transferred to nitrocellulose filters and hybridized to ^{32}-P-labeled virus PBCV-1 DNA as described previously (11). DNAs were labeled with [α-^{32}P]dCTP (800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) by using a nick-translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Filters were exposed to X-ray film (XAR-5; Eastman Kodak Co., Rochester, N.Y.) at −80° by using an intensifying screen (Cronex Lightning Plus; E. I. duPont de Nemours & Co., Inc., Wilmington, Del.).

For base analyses about 50 μg of virion DNA was enzymatically digested to nucleosides with nuclease P1 and *Escherichia coli* alkaline phosphatase (3). The resulting deoxynucleosides were separated by high-performance liquid chromatography with an improved reversed-phase column (2).

Electrophoretic analysis of viral proteins. Purified viruses (1 A_{260} unit per sample) were suspended in 62.5 mM Tris hydrochloride (pH 6.8)–3% (wt/vol) sodium dodecyl sulfate–0.1 M dithiothreitol–20% (wt/vol) glycerol–0.02% (wt/vol) bromophenol blue and either heated at 100°C for 5 min or heated at 60°C for 10 min. The samples were layered onto a linear 7.5 to 15% acrylamide gradient slab gel stabilized by a 4 to 8 M urea gradient with a 4.5% acrylamide–4 M urea stacking gel and electrophoresed with the buffers described by Laemmli (5); proteins were visualized by staining with Coomassie brilliant blue R-250. Molecular weight markers were myosin, β-galactosidase, phosphorylase *b*, bovine se-

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TABLE 1. Source of Chinese viruses and viruses selected for detailed characterization[a]

City water was collected	Water source	Code no. of water sample	No. of unique DNA restriction patterns	Viruses selected for study
Beijing	Pond	BJ-2	9	BJ-2C
Xuzhou	Stream	XZ-1	7	None
	Stream	XZ-2	8	None
	Lake	XZ-3	7	XZ-3A
	Lake	XZ-4	9	XZ-4A
				XZ-4C
	Pond	XZ-5	10	XZ-5C
	Pond	XZ-6	9	XZ-6E
Shanghai	Stream	SH-1	10	None
	Stream	SH-2	9	None
	Lake	SH-3	10	None
	Lake	SH-4	7	None
	Pond	SH-5	9	None
	Pond	SH-6	10	SH-6A

[a] There were 10 viruses screened from each water sample.

rum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, and lysozyme.

Other procedures. Antisera were raised against purified viruses and assayed by the microprecipitin test as described previously (1, 17). Purified viruses were negatively stained with 1.5% uranyl acetate before they were viewed with an electron microscope. The sensitivity of the viruses to chloroform was assayed as described previously (12).

RESULTS

Initial screening. Each of the 13 water samples collected in the People's Republic of China contained plaque-forming viruses on *Chlorella* sp. strain NC64A. The titers of these samples ranged from about 10 to 1,000 PFU/ml. The viruses formed sharply defined clear plaques which ranged from 1 to 3 mm in diameter. Ten of the smaller plaque-forming viruses randomly picked from each of the 13 samples were purified, and their DNAs were treated with *Mbo*I and *Hin*fI restriction endonucleases. DNAs from 114 of the 130 viruses produced unique restriction patterns; however, some of the virus DNAs differed from one another by only a few bands. DNAs from 21 of these viruses were treated with the 13 DNA restriction endonucleases previously used in grouping the *Chlorella* viruses isolated in the United States (11). The sensitivity or resistance of the DNAs to these 13 endonucleases revealed that 14 of these 21 viruses could be included in one of the 11 classes of American *Chlorella* viruses (11). The remaining seven viruses listed in Table 1, which might represent new classes of viruses, were selected for further examination, and their properties were compared with those of the prototype American virus PBCV-1.

General properties. The seven Chinese viruses sedimented as single bands and at the same rate (ca. 2,300 S) as PBCV-1 on sucrose density gradients. All of the Chinese viruses were disrupted by CsCl equilibrium gradient centrifugation. The viruses had a specific infectivity of 1×10^{10} to 3×10^{10} PFU per A_{260} unit of virus, and chloroform treatment reduced the specific infectivity of each virus by several orders of magnitude. Negative staining and electron microscopy revealed that all of the particles were polyhedra ranging from 150 to 190 nm in diameter. The sensitivities of the Chinese viruses to antisera prepared against four American viruses are reported in Table 2. The Chinese viruses reacted with either PBCV-1 antiserum or antisera to viruses NY-2A and NYs-1. None of the Chinese viruses reacted with antiserum to virus NY-2C.

TABLE 2. Reaction of Chinese viruses to antisera to American *Chlorella* viruses

Virus	Reacts with antiserum to[a]:			
	PBCV-1	NY-2C	NY-2A	NYs-1
XZ-3A	Yes	No	No	No
SH-6A	Yes	No	No	No
BJ-2C	Yes	No	No	No
XZ-6E	Yes	No	No	No
XZ-4C	No	No	Yes	Yes
XZ-5C	No	No	Yes	Yes
XZ-4A	No	No	Yes	Yes
PBCV-1	Yes	No	No	No

[a] Purified virus (5 μg) was mixed with twofold dilutions of antiserum in a total volume of 40 μl, and the precipitate was monitored 2 h later (1). Yes means that a reaction was obtained at an antibody dilution of 1:64 or greater, and no means a dilution of 0 to 1:4 did not produce a precipitate.

Analysis of virus DNAs. DNAs from the seven Chinese viruses were treated with 36 DNA restriction endonucleases. The DNAs varied widely in sensitivity to the enzymes (Table 3). In the extreme cases DNA from virus XZ-3A was cleaved by 28 of the restriction endonucleases, while DNA from XZ-4A was only cleaved by 10 endonucleases. *Bgl*II, *Dpn*I, *Sau*3A, *Dde*I, *Hpa*I, *Ava*I, *Xba*I, *Pvu*I, *Xho*I, and *Acc*I were the only enzymes to cleave each of the Chinese virus DNAs. The inability of *Mbo*I and *Bcl*I to cleave any of the DNAs from the Chinese viruses suggests that each of these DNAs contained G^{m}ATC sequences. Each of the Chinese virus DNAs were also resistant to *Hin*dIII, *Sst*I, and *Pvu*II. These three restriction endonucleases recognize a common sequence AGCT, and each is inhibited by cytosine methylation (7). Thus, each of the virus DNAs presumably contains AG^{m}CT sequences. All of the Chinese virus DNAs were also resistant to *Hae*III, which does not cleave GG^{m}CC sequences. Finally, four of the virus DNAs (XZ-3A, SH-6A, BJ-2C, and XZ-6E) were cleaved by both *Msp*I and *Hpa*II, and three (XZ-4C, XZ-5C, and XZ-4A) were not cleaved by either enzyme. Presumably, the first four virus DNAs do not contain m^{5}dC in CCGG sequences, whereas the last three contain ^{m}CCGG or ^{m}C^{m}CGG sequences.

The size of the viral DNAs estimated by summing *Xba*I (Fig. 1A) or *Bgl*II (data not shown) DNA restriction fragments was at least 300 kilobase pairs (10). To compare the sequence homology of the Chinese viral DNAs with PBCV-1 DNA, the DNA fragments in Fig. 1A were transferred to a nitrocellulose filter and hybridized with ^{32}P-labeled PBCV-1 DNA. DNA from four Chinese viruses hybridized reasonably well with PBCV-1 DNA and three hybridized poorly (Fig. 1B).

Base composition of virus DNAs. The resistance of the Chinese virus DNAs to certain restriction endonucleases suggested that they contained modified bases. This expectation was confirmed by base analyses of the viral DNAs (Table 4). Each of the viral DNAs contained m^{5}dC, which varied from 12.6 to 46.7% of the deoxycytosine. In addition each of the DNAs contained m^{6}dA, which varied from 2.2 to 28.3% of the deoxyadenosine. The overall deoxyguanosine plus deoxycytosine content (including m^{5}dC) for the Chinese virus DNAs ranged from 40.4 to 42.6 mol%. No other modified bases, including N^4-methyldeoxycytosine, were detected in the virus DNAs.

TABLE 3. Sensitivity of Chinese virus DNAs to DNA restriction endonucleases

Restriction endonuclease	Sensitivity of DNAs of the following viruses[a]:							
	XZ-3A	SH-6A	BJ-2C	XZ-6E	XZ-4C	XZ-5C	XZ-4A	PBCV-1
*Bgl*II	+	+	+	+	+	+	+	+
*Eco*RI	+	+	+	+	+	−	−	+
*Bam*HI	+	+	+	+	−	−	−	+
*Sal*I	+	+	+	−	−	−	−	+
*Xho*I	+	+	+	−	−	−	−	+
*Bcl*I	−	−	−	−	−	−	−	−
*Pst*I	+	−	−	−	−	−	−	+
*Hin*dIII	−	−	−	−	−	−	−	+
*Sst*I	−	−	−	−	−	−	−	+
*Mbo*I	−	−	−	−	−	−	−	−
*Dpn*I	+	+	+	+	+	+	+	+
*Msp*I	+	+	+	+	−	−	−	+
*Hpa*II	+	+	+	+	−	−	−	+
*Sau*3A	+	+	+	+	+	+	+	+
*Hin*fI	+	+	−	−	−	−	−	+
*Hae*III	−	−	−	−	−	−	−	+
*Rsa*I	+	+	+	−	−	−	−	+
*Cla*I	+	+	+	−	−	−	−	+
*Pvu*II	−	−	−	−	−	−	−	NT
*Sst*II	+	+	+	+	−	−	−	+
*Taq*I	+	+	+	−	−	−	−	+
*Sau*96	+	+	+	+	−	−	−	+
*Dde*I	+	+	+	+	+	+	+	+
*Hha*I	+	+	+	+	−	−	−	+
*Hpa*I	+	+	+	+	+	+	+	+
*Kpn*I	+	+	+	−	−	−	−	+
*Ban*II	−	−	−	−	−	−	−	+
*Hph*I	+	+	+	+	−	+	−	NT
*Ava*I	+	+	+	+	+	+	+	+
*Xba*I	+	+	+	+	+	+	+	+
*Pvu*I	+	+	+	+	+	+	+	+
*Xho*I	+	+	+	+	+	+	+	NT
*Bgl*I	−	−	−	−	−	−	−	+
*Xmn*I	+	+	+	−	−	−	−	NT
*Mbo*II	+	−	+	+	+	−	−	NT
*Acc*I	+	+	+	+	+	+	+	NT

[a] A plus sign indicates that the DNA was digested by the endonuclease, a minus sign indicates that the DNA was resistant to the enzyme, and NT indicates not tested.

Analysis of viral proteins. The Chinese viruses, like PBCV-1, contained numerous structural proteins (Fig. 2). With one exception, the Chinese viruses contained a major protein which migrated with an approximate M_r of 52,000 after it was heated at 100°C (Fig. 2A). The major protein in virus XZ-6E migrated slightly faster than the others, with an M_r of about 48,000 (Fig. 2A, lane 4). If the viruses were heated in denaturing buffer at 60°C for 10 min, each of the major proteins, like the major protein in PBCV-1 (12), migrated as a dimer with an apparent M_r of about 100,000; the apparent M_r of the XZ-6E protein dimer was about 90,000 (Fig. 2B). Although some proteins migrated differently between the viruses, the majority of the proteins migrated at rates similar to those of PBCV-1 proteins.

DISCUSSION

Prior to this report all of the plaque-forming viruses of *Chlorella* sp. strain NC64A were isolated from fresh water collected in the continental United States (11, 14, 19). The host for these viruses, *Chlorella* sp. strain NC64A, was originally a hereditary endosymbiont of a *Paramecium bursaria* protozoan collected in the United States (4). The

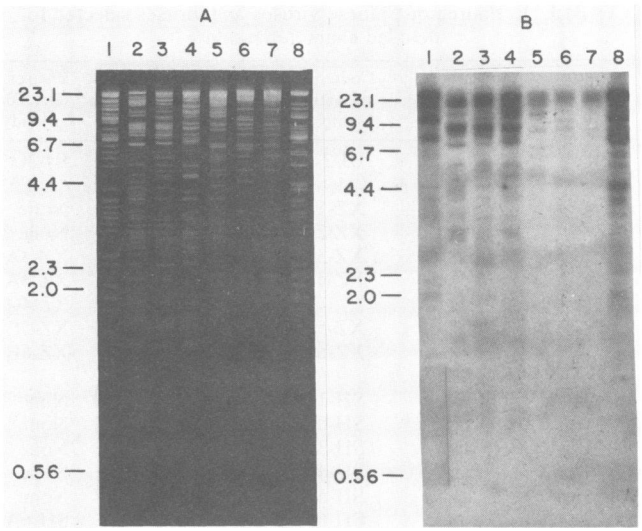

FIG. 1. Electrophoresis of Chinese virus DNAs after digestion with *Xba*I. (A) The DNAs were isolated from viruses XZ-3A, SH-6A, BJ-2C, XZ-6E, XZ-4C, XZ-5C, XZ-4A, and PBCV-1 (lanes 1 to 8, respectively). (B) The DNAs in panel A were transferred to nitrocellulose and hybridized with ^{32}P-labeled PBCV-1 DNA. The numbers to the left of each panel indicate kilobase pairs.

results of this study indicate that viruses that infect this host are also common in fresh water collected in the People's Republic of China. We have tried 4 times, unsuccessfully, to find viruses of *Chlorella* sp. strain NC64A in water collected in various parts of Europe (J. Van Etten and W. Reisser, unpublished data). It is interesting, however, that plaque-forming double-stranded DNA-containing viruses of another *Chlorella* strain (isolate Pbi) have been detected recently in water in the Federal Republic of Germany (9). *Chlorella* sp. strain Pbi was isolated from a culture of *Paramecium bursaria* in the Federal Republic of Germany (8). No plaque-forming viruses on *Chlorella* sp. strain Pbi were detected in the Chinese water samples used in this study (unpublished data). Incidentally, *Chlorella* sp. strain Pbi viruses do not infect *Chlorella* sp. strain NC64A, and *Chlorella* sp. strain NC64A viruses do not infect *Chlorella* sp. strain Pbi because they do not attach (W. Reisser, D. E. Burbank, R. H. Meints, and J. Van Etten, manuscript in preparation).

All of the viruses that infected *Chlorella* sp. strain NC64A, including the Chinese viruses, have several common properties, including polyhedral morphology, sedimentation

TABLE 4. Concentration of methylated bases in the Chinese virus DNAs

Virus	Deoxyguanosine + deoxycytosine (mole%)	% Methylated bases in:	
		m^5dC[a]	m^6dA[b]
XZ-3A	40.4	12.8	2.2
SH-6A	41.1	12.6	10.3
BJ-2C	40.4	12.8	11.5
XZ-6E	41.7	21.2	15.2
XZ-4C	41.8	46.7	20.8
XZ-5C	42.6	42.7	27.9
XZ-4A	42.2	44.1	28.3
PBCV-1[c]	40.0	1.9	1.5

[a] Percent m^5dC per deoxycytosine + m^5dC.
[b] Percent m^6dA per deoxyadenosine + m^6dA.
[c] Data were from a previous report (18).

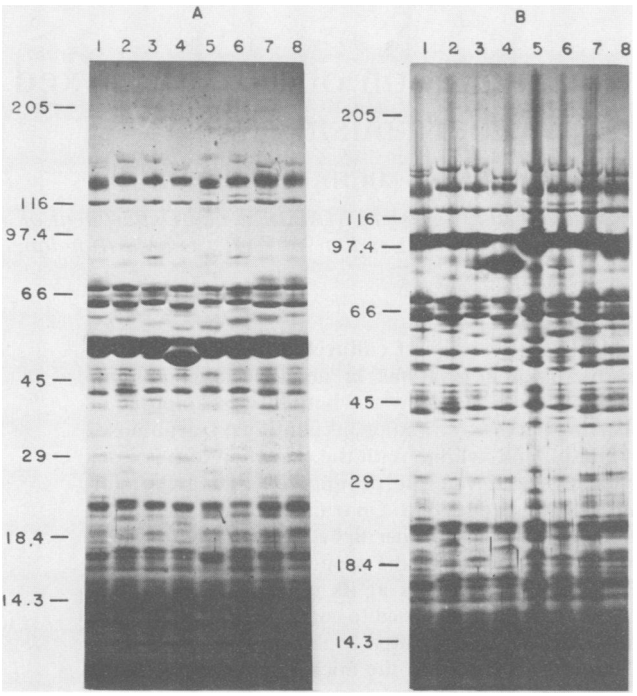

FIG. 2. Polyacrylamide gel electrophoresis of Chinese virus structural proteins. The proteins were isolated from viruses XZ-3A, SH-6A, BJ-2C, XZ-6E, XZ-4C, XZ-5C, XZ-4A, and PBCV-1 (lanes 1 to 8, respectively). The samples were heated at 100°C for 5 min (A) or at 60°C for 10 min (B) before they were layered onto the gel. Molecular masses (in kilodaltons) are indicated next to the gels.

coefficient, sensitivity to chloroform, and large double-stranded DNA genomes of at least 300 kilobase pairs. However, the Chinese viruses can be distinguished from PBCV-1 and from each other by at least one of the following properties: plaque morphology, reaction with antisera, protein patterns, the sensitivity or resistance of their genomic DNAs to DNA restriction endonucleases, and most importantly, the nature and abundance of methylated bases in their genomic DNAs. The presence of m^5dC and m^6dA in specific base sequences explains why Chinese viral DNAs differ in their susceptibilities to DNA restriction endonucleases. The finding that methylation of the Chinese virus DNAs is sequence specific suggests that the Chinese viruses, like the American viruses, encode for specific DNA methyltransferases. Furthermore, some may produce DNA restriction endonucleases with specificities that are not found in American viruses.

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