

Genome Structure of a Virus Infecting the Marine Brown Alga *Ectocarpus siliculosus*STEFAN T. J. LANKA, MICHAEL KLEIN, UWE RAMSPERGER, DIETER G. MÜLLER, AND ROLF KNIPPERS¹

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We describe a procedure for the isolation of virus particles from the marine brown alga *Ectocarpus siliculosus*. Virus particles are composed of at least 13 different polypeptides, including two glycoproteins, and double-stranded DNA. A typical virus DNA preparation contains three fractions, namely linear DNA and circular DNA, each composed of about 320 kilobase pairs, as well as DNA fragments, 10 to 60 kilobase pairs in size. The large linear and the circular DNA contain single-stranded regions (average length: 2.9 kilobases). We propose that the native *Ectocarpus* virus genome is a circular DNA molecule whose double strand is interrupted by single-stranded regions. During the preparation procedure, the DNA circles tend to break at the single-stranded sites producing large linear as well as fragmented DNA.

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INTRODUCTION

Viruses or virus-like particles have been detected in a large number of marine algae (reviewed by van Etten *et al.*, 1991). However, many of these viruses were observed in field-collected samples and remained unavailable for detailed investigations.

More recently, Müller *et al.* (1990) described a viral infection of the marine brown alga *Ectocarpus siliculosus*, a cosmopolitan plant growing at all ocean coasts of temperate climate zones. The *Ectocarpus* system appears to be suited for molecular and cell biological investigations because the organism has been extensively studied, and its life cycle is well known (Müller, 1967). Furthermore, the species can be grown under laboratory conditions, and the development of disease symptoms and the appearance of virus particles can be well studied in cultivated algae (Müller *et al.*, 1990).

Symptoms of virus infection become manifest in the reproductive organs, gametangia, and sporangia, but not in the vegetative cells of the infected host. Normally, multiple mitoses produce densely packed zooidangia with several hundred loculi. In virus-infected organisms, however, cell divisions come to an early halt, and the nuclei acquire a severalfold higher DNA content compared to that of normal cells. After nuclear breakdown, virus particles are formed in the cytoplasm which eventually becomes densely packed with these particles. Finally, the infected cells burst, and virions are released into the surrounding sea water. Mature

Ectocarpus plants are resistant against infection, but free zoospores or gametes are infected by the virus resulting in pathologically altered reproductive organs of the progeny plants (Müller *et al.*, 1990).

An analysis of the *Ectocarpus* virus is interesting for several reasons. The virus may be of considerable ecological importance as symptoms of viral infection were discovered in *Ectocarpus* isolates from the coasts of New Zealand, Australia, Northern Europe, the Americas and are most likely present in all *Ectocarpus* populations worldwide (Müller and Stache, 1992). Interestingly, virus multiplication is to some degree temperature-sensitive: the disease develops at 10–15°, but pathological symptoms are reduced at about 20°, suggesting that seasonal temperature changes may affect algal proliferation through the induction of viral multiplication (Müller, 1991a). In addition, the virus could serve as a vector-mediated interspecific gene transfer, since it was found to infect not only different isolates of the species *Ectocarpus siliculosus* but also the related genus *Kuckuckia* (Müller, 1992).

The *Ectocarpus* virus is also intriguing from a cell biological point of view. Its genome is transmitted like a Mendelian trait to progeny plants (Müller, 1991b) and is present in a latent form in all cells of an infected adult plant. However, virus multiplication specifically occurs in developing reproductive organs. It should be an interesting future task to more closely investigate the nature of the lysogenic state and the conditions for induction.

In this communication, we describe some molecular properties of the isolated virus and its genome, providing the basis for a more detailed exploration of the ge-

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netics and the biological behavior of the *Ectocarpus* virus.

MATERIALS AND METHODS

Cells and the preparation of virus particles

All experiments were performed with a clonal sporophyte culture of *E. siliculosus* (Dillw.) Lyngb. designated NZ-Vic-Z14. Its parents were a male virus-infected gametophyte from Kaikoura, New Zealand, and a healthy female plant from Flinders, Victoria, Australia. Stock cultures were maintained bacteria-free on culture medium in 1% agar. Mass cultures were started by inoculation of agar-grown fragments into petri dishes with sterile culture medium (autoclaved North Sea water, enriched according to the ES formula of Starr and Zeikus, 1987). The cultures were illuminated with a white fluorescent lamp for 16 hr/day. After 4 weeks the plants were fragmented and transferred to volumes of 0.5 liter of culture medium for an additional culture period of 3 weeks. Mature plants showed prolific virus expression and were harvested on nylon nets followed by drying on cellulose paper.

Aliquots of about 3 g algae (fresh weight) were suspended in 50 ml seawater (with 0.05 M Tris-HCl, pH 7.8), and the reproductive cells were disrupted with 1-mm glass beads in a Braun homogenizer at 3000 rotations/min for 20 sec under cooling. Cellular debris was removed by centrifugation at 13,000 g for 10 min in a Sorvall swing-out rotor. Polyethylene glycol (PEG 6000; 8% final concentration) and 1 M NaCl (final) were added to the supernatant. After 1 hr on ice, the precipitate was collected by centrifugation at 4° and 11,000 g for 10 min. The pellet was resuspended in 0.5 ml seawater-0.05 M Tris-HCl (pH 7.8).

The resuspended sample was added onto a step gradient of CsCl solutions, 2 ml each, with densities of 1.45, 1.35, 1.30, 1.25, and 1.2 g/ml, made up in 0.01 M MgCl₂, 0.05 M Tris-HCl, pH 7.8. Centrifugation was performed in the Beckman SW40 rotor at 67,000 g for 90 min at 4°. A turbid band of viral material appeared at the interface between the 1.25-g/ml and the 1.3-g/ml layer. The virus band was collected by puncturing with a hypodermic needle. CsCl was removed by dialysis against seawater-0.05 M Tris-HCl, pH 7.8.

Extraction and investigation of DNA

Virus particles were embedded in blocks of 0.7% low-melting-point agarose (InCert agarose, FMC). For deproteinization, the agarose blocks were incubated 2 times for 12 hr at 50° with proteinase K (1 mg/ml in 0.5 M EDTA, 1% sodium laurylsarcosine, 10 mM Tris-HCl, pH 7.8). For inhibition of proteinase K, the agarose blocks were then kept for 2 cycles of 6 hr each in TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.8) with 2

mM phenylmethylsulfonyl fluoride (PMSF), followed by incubations in TE buffer without PMSF. The blocks are then stored at 4° in TE buffer until use.

For digestion with restriction enzymes, the agarose-embedded DNA was first dialyzed against the corresponding enzyme buffer (2 cycles of 1 hr dialysis time) and then incubated for several hours with at least 10 enzyme units/ μ g DNA.

Untreated or *Sfi*I- and *Asc*I-digested DNA was investigated in 0.8 to 1.2% agarose gels (Fast Lane, FMC) by rotating field gel electrophoresis (Ziegeler *et al.*, 1987) using the Biometra Rotaphor-5 apparatus. Electrophoresis conditions: 22 mM Tris-borate, 0.5 mM EDTA (pH 8.0); times of intervals: 3 to 40 sec; field angle: 120° to 130°; 140 to 200 Volts; 36 hr. All parameters increase in a linear manner.

DNA, restricted with more frequently cutting enzymes, was analyzed by conventional agarose gel electrophoresis (Sambrook *et al.*, 1989).

Electrophoretic size markers were phage λ DNA concatemers (Waterbury and Lane, 1987) or restricted λ DNA (Sambrook *et al.*, 1989).

If necessary, DNA was released from agarose by treatment with agarase as suggested by the manufacturer (Clontech).

Protein analysis

Virus particles were obtained after CsCl centrifugation, diluted threefold in seawater, and pelleted at 80,000 g for 1 hr at 4°. The pellet was resuspended in loading buffer (with sodium dodecylsulfate) and analyzed by denaturing gel electrophoresis (Laemmli, 1974). The separated polypeptides were visualized by staining with silver salts (Wray *et al.*, 1981) or transferred to a nitrocellulose membrane (Towbin *et al.*, 1979) for the determination of glycoproteins using the enzyme-hydrazide method of Gershoni *et al.* (1985).

Electron microscopy

Infected plant cells were fixed for 2 hr on ice with 3% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.7. After washing in cacodylate buffer and postfixation in 1% OsO₄, the material was washed in distilled water, dehydrated stepwise in acetone, and embedded in resin (Spurr, 1969; Plattner and Zingsheim, 1987). Sections were stained with 2% uranyl acetate followed by 0.5% lead citrate in 0.1 M NaOH. Isolated virus particles were negatively stained with uranyl acetate as described by Haschemeyer (1970). DNA was investigated using the BAC-spreading technique of Vollenweider *et al.* (1975). The resulting surface film was mounted on carbon-coated grids which had been exposed to glow discharge of tripropylamine vapor (Dubochet and Groom, 1982). The grids were then treated with 5 mM uranyl acetate and rotary shadowed by

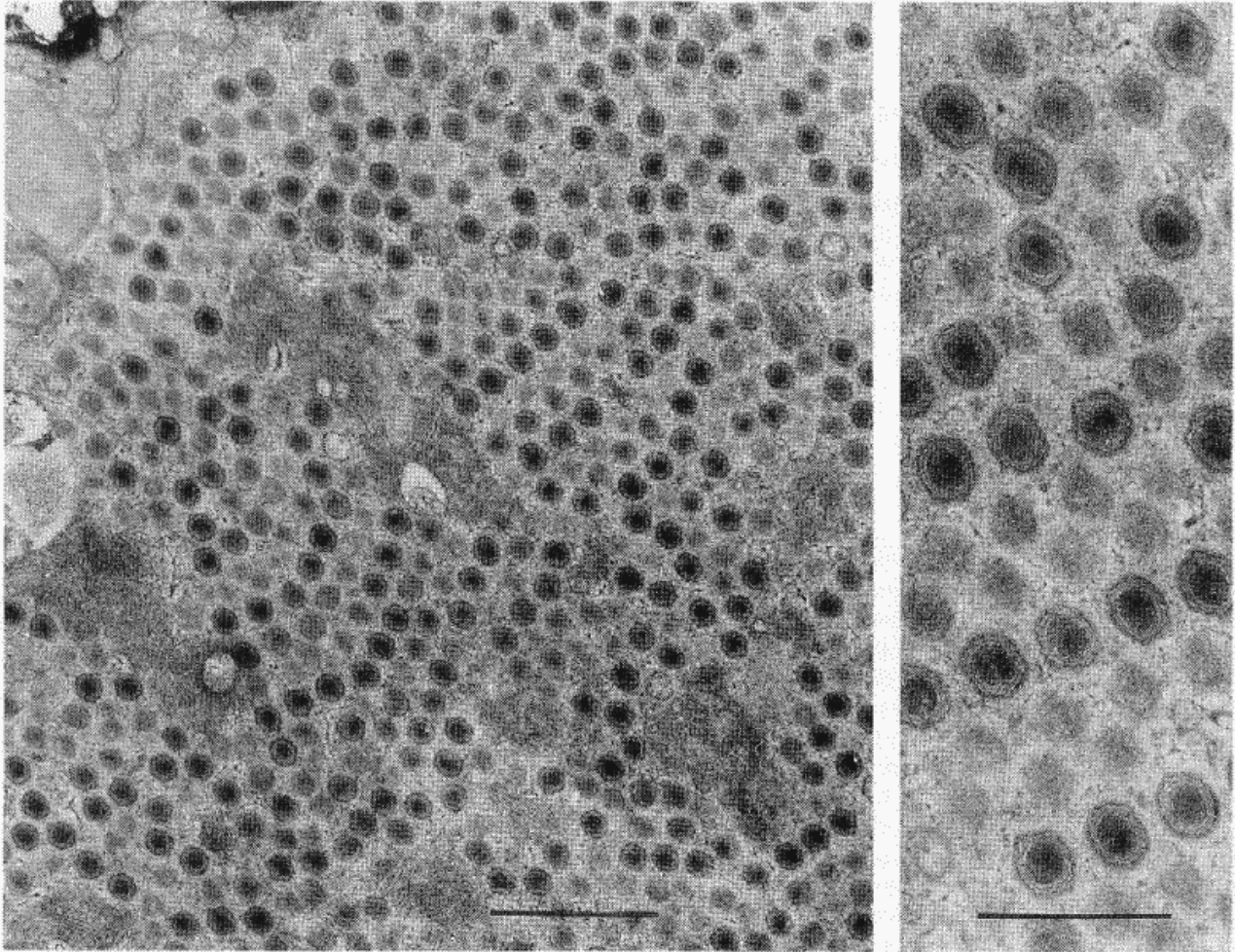


Fig. 1. Virus particles in infected gametophytes. (Left) Densely packed virus particles are located between residual components of the plant cell. Bar: 1 μm . (Right) A larger amplification demonstrating the two-layered viral shell. Bar: 0.5 μm .

evaporation of tungsten as detailed in Wessel *et al.* (1990). To visualize single strands, DNA was incubated with the bacterial single-strand-specific binding (SSB) protein (Pharmacia) at a ratio of 10 μg protein/ μg DNA, fixed in 0.1% glutaraldehyde, and processed for BAC spreading.

Micrographs were taken in a Zeiss EM 900 electron microscope.

Length measurements of DNA relative to internal length standards were performed using magnified positive prints.

RESULTS

Virus particles

A cross-section through an infected gametophyte, grown at 12°, shows densely packed isometrical virus-like particles. These particles have apparent diameters of 130–150 nm and consist of a shell with 2 layers surrounding an electron-dense core (Fig. 1).

The particles were prepared from disrupted cells and purified by a procedure involving differential centrifugation, followed by PEG precipitation and centrifugation through a cesium chloride step gradient. Essentially all cellular debris, including mitochondria and chloroplasts, were removed by differential centrifugation. The virus particles appeared as a turbid band at the border between the CsCl density steps 1.25 and 1.3 g/ml. The turbid band did not appear when the extraction procedure was performed with healthy plants (not shown).

In Fig. 2, we show negatively stained virus particles, demonstrating that the viral particles remained morphologically intact after the standard preparation procedure.

A buoyant density of approximately 1.3 g/ml is in a range characteristic for viruses composed of nucleic acid and protein without a substantial lipid component. However, further experiments are needed to specifically investigate the presence of lipids in the *Ectocarpus* virus.

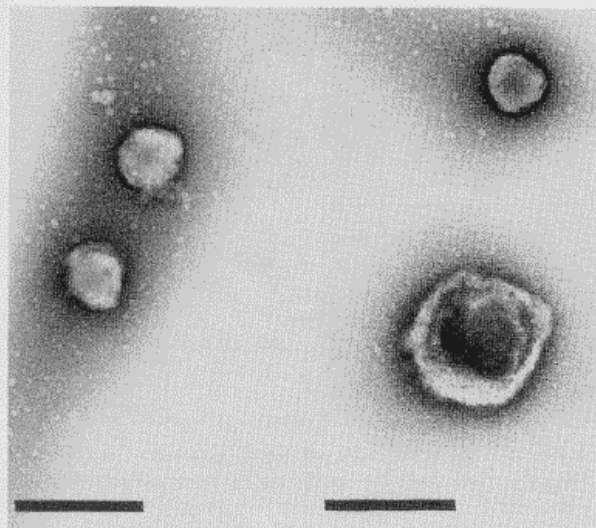


FIG. 2. Isolated virus particles. A negative stain technique was used to demonstrate that the virus structure remained largely intact after PEG precipitation and repeated centrifugations. Bars: 0.4 μm (left); 0.2 μm (right).

Structural proteins

Purified virus particles were disrupted in sodium dodecylsulfate and investigated by denaturing polyacrylamide gel electrophoresis (Laemmli, 1974). Staining of the gel with silver salts reproducibly revealed 13 major and several additional minor polypeptide bands in a molecular weight range of 20 to 150 kDa (Fig. 3, lane 2). The large number of detectable polypeptides suggests a complex virus structure as might be expected from its electron microscopical appearance (Fig. 1).

The gel was blotted onto nitrocellulose membranes and stained using the enzyme-hydrazide method of Gershoni *et al.* (1985) to investigate the possible presence of glycoproteins. As shown in Fig. 3 (lane 3), two stainable bands could be detected suggesting that at least two of the major structural proteins (apparent molecular weights: 56 and 60 kDa) are glycosylated.

Ectocarpus virus DNA

Previous experiments had shown that DAPI-stainable material accumulates in the reproductive organs of infected plants (Müller *et al.*, 1990). This finding suggested that the genetic material of the *Ectocarpus* virus most likely consists of DNA. To investigate its nucleotide composition, DNA was prepared from purified virus particles, hydrolyzed, and processed for chromatography as described by Flatau *et al.* (1984). We determined a content of about 50% GC and 50% AT base pairs. In addition, *Ectocarpus* virus DNA contains a low, but significant amount of methylated bases: about 1% of the cytosine as well as 3% of the adenine residues chromatographed as 5-methylcytosine and

6-methyladenine, respectively. The presence of methylated adenine residues is characteristic for bacterial DNA but quite unusual for DNA of eukaryotic origin. However, 6-methyl-adenine appears to be a typical component of the DNA obtained from other viruses infecting algae (van Etten *et al.*, 1991).

When we first started to investigate the nature of the virus DNA we used standard phenol-chloroform extraction protocols but always obtained heavily fragmented DNA. In order to avoid breakage, we embedded aliquots of the virus preparation in low-melting-point agarose before deproteinization and agarose gel electrophoresis (see Materials and Methods).

As a typical result of pulse-field electrophoresis, we observed ethidium-bromide-stainable DNA bands at three locations in the agarose gel (Fig. 4): (i) one prominent band, migrating as linear DNA of about 320 kbp (estimated relative to a series of concatemeric λ DNA markers); (ii) a second prominent band remaining at or close to the start of the gel; and (iii) a spectrum of DNA fragments in a size range of 10 to 60 kbp. This distribution was found in each one of the many independent DNA preparations investigated but the relative amounts of the three fractions varied from experiment to experiment.

When agarose-embedded, deproteinized *Ectocarpus* virus DNA was treated with restriction endonucleases we detected distinct patterns of DNA bands.

As examples, we show the digestion products after treatment with restriction nucleases *Sfi*I and *Asc*I (Fig.

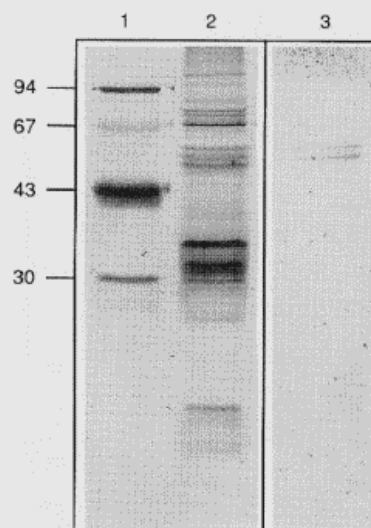


FIG. 3. Structural viral polypeptides as investigated by denaturing polyacrylamide gel electrophoresis. Isolated virus particles (Fig. 2) were concentrated by centrifugation. A sample, containing a total of 5 μg protein, was denatured with sodium dodecylsulfate in loading buffer (Laemmli, 1974) and investigated using a 12% polyacrylamide gel. Lane 1, marker proteins; lane 2, viral polypeptides as visualized by silver staining; lane 3, a parallel lane, blotted onto nitrocellulose sheets for the identification of glycoproteins (Gershoni *et al.*, 1985).

5). Both enzymes recognize octameric nucleotide sequences, and are therefore expected to produce relatively few DNA fragments. In fact, *Sfi*I degraded the virus DNA to give six bands, and *Asc*I digestion resulted in two restriction fragments.

In many digestion experiments, we detected the products of partial digests, visible as more faintly stained bands as indicated in Fig. 5. These bands were also obtained at a 10-fold higher enzyme concentration and at longer incubation times. It is thus possible that a fraction of viral DNA lacks one or two restriction sites.

The sum of the sizes of unique *Sfi*I or *Asc*I restriction fragments gave a total of about 320 kb. A similar result was achieved by adding up the sizes of the 50 or more bands, obtained after complete digestion of viral DNA by restriction enzymes *Hind*III, *Bam*HI, or *Sal*I (data not shown). Thus, the restriction enzyme data are in good agreement with the electrophoretic analysis of undigested *Ectocarpus* virus DNA (Fig. 4).

Some additional points concerning the experiment shown in Fig. 5 must be considered. First, the fact that restriction enzyme digestions resulted in reproducible patterns of DNA bands excludes the possibility that *Ectocarpus* virus DNA may be circularly permuted. Second, it should be noted that restriction enzymes degraded not only the linear 320-kb DNA but also the DNA species which remained at the start of the gel. It is therefore unlikely that this fraction of viral DNA failed to enter the gel because of insufficient deproteinization. This could rather be a consequence of some special structural feature.

One possibility is that this DNA fraction may be circularly closed. It is well known that high electric fields

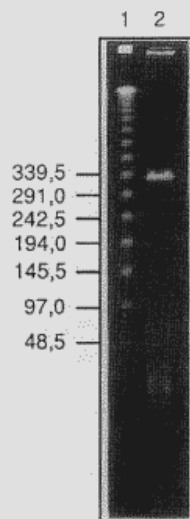


FIG. 4. Viral DNA as investigated by rotating field agarose gel electrophoresis. The conditions for electrophoresis are described under Materials and Methods. Lane 1, λ DNA concatemers as length marker (their sizes in kbp are noted on the left); lane 2, *Ectocarpus* virus DNA.

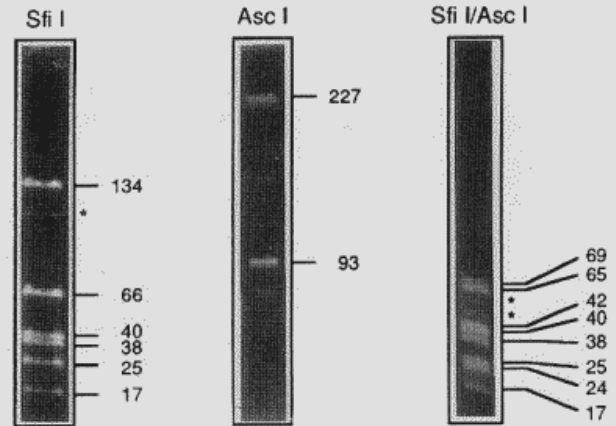


FIG. 5. Restriction fragment pattern. *Ectocarpus* virus DNA was prepared and restricted as described under Materials and Methods. The resulting restriction fragments were investigated by rotating field agarose gel electrophoresis using λ DNA concatemers and λ DNA *Hind*III restriction fragments as lengths markers. We show the restriction fragment pattern as obtained after *Sfi*I and *Asc*I digestion as well as after double digestion with both enzymes. Asterisks denote incomplete digestion products.

have drastically different effects on the mobilities in agarose of linear and of open circular DNA. Sufficiently large circular DNA forms are prevented from migrating into the gel, whereas linear DNA molecules migrate normally (Levene and Zimm, 1987; Louie and Serwer, 1989).

Circular DNA

The possibility of circularity is consistent with our restriction enzyme analyses. In Fig. 5, we present the result of an *Asc*I/*Sfi*I double digest. The number of fragments obtained was eight. The sum of all eight bands amounts to a total of 320 kb, the size of unrestricted *Ectocarpus* virus DNA. Thus, the number of fragments, obtained by *Asc*I/*Sfi*I double digests corresponds to the sum of the fragments after two single digestions (six *Sfi*I fragments plus two *Asc*I fragments; Fig. 5).

A restriction map was constructed by using the double digestion data of Fig. 5 as well as partially digested fragments obtained from a series of experiments with very low and increasing amounts of *Sfi*I (not shown). The fragments could be arranged to form a circular map as shown in Fig. 6.

Only the fraction of the viral DNA remaining at the start of the gel (Fig. 4) is likely to be in the form of open circular DNA. However, our restriction enzyme analyses were performed with total *Ectocarpus* virus DNA and reproducibly gave the results shown above in Fig. 5. In fact, circular (i.e., nonmigrating) and linear DNA gave an identical restriction fragment pattern (not shown). This indicates that the DNA circles were broken at random sites.

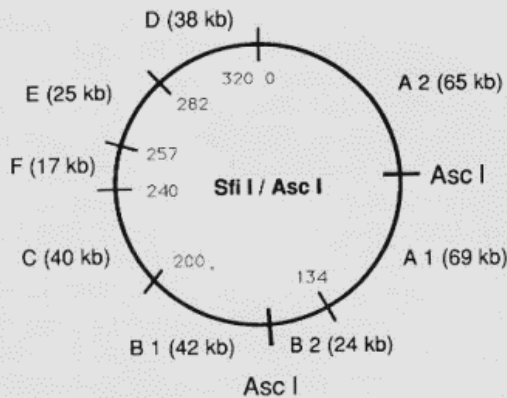


FIG. 6. Restriction map of *Ectocarpus* virus DNA.

We have also tried to specifically degrade the ends of the *Ectocarpus* virus DNA using a procedure which has been successfully employed for the identification of the ends of a *Chlorella* virus genome (Rohozinski *et al.*, 1989). However, we obtained no indication for a specific degradation of one of the *Sfi*I restriction fragments (not shown).

Even though our data were consistent with the possibility that the *Ectocarpus* virus DNA was circularly closed, we looked for additional evidence. This was achieved by electron microscopy.

A fresh preparation of *Ectocarpus* virus DNA was spread on electron microscopy grids using the procedure of Vollenweider *et al.* (1975). Among linear DNA strands of various lengths we detected a significant number of circular DNA molecules. One of these is shown in Fig. 7.

The lengths of 10 circular molecules were determined using as an internal length marker a circular cosmid DNA clone of known size (45 kbp) which was added to the viral DNA preparation before spreading for electron microscopy. The estimated size of circular *Ectocarpus* virus DNA corresponded to 320 kbp (± 10 kbp) which agrees well with the electrophoretic data and the restriction fragment analyses.

To summarize this section, we conclude that a considerable fraction of the extracted *Ectocarpus* virus DNA may be circularly closed. It remains to be shown whether all virus particles contain circular DNA and whether linear DNA is produced as an artifact during preparation.

Single-stranded regions

As described above, a typical preparation of *Ectocarpus* virus DNA contains the two high-molecular-weight DNA species as just discussed and, in addition, a class of DNA fragments of 10 to 60 kbp (Fig. 4). This fraction of fragmented DNA varied from experiment to experiment, but it cannot be caused by unspecific nu-

cleolytic breakdown as this would result in a broad smear ranging from very small DNA fragments to unit length 320-kb DNA. A more likely possibility is that the

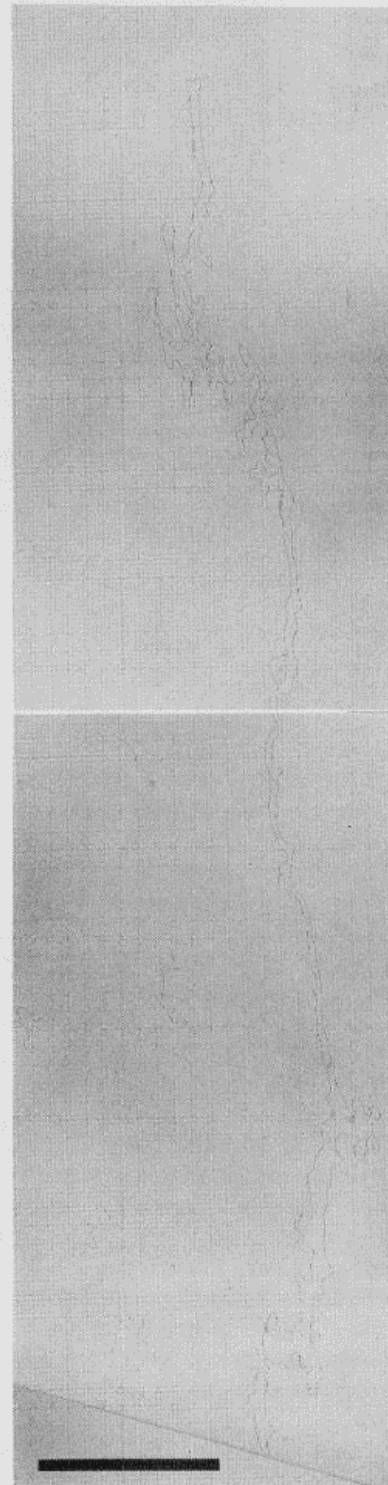


FIG. 7. Electron micrograph of circular *Ectocarpus* virus DNA. Bar: 5.5 μ m.

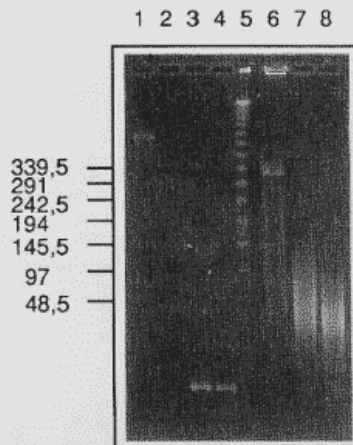


FIG. 8. Treatment with single-strand-specific mung bean nuclease. Incubations were at 37° and as described in Sambrook *et al.* (1989). Investigation of DNA by rotating field agarose gel electrophoresis. The virus DNA was agarose-embedded as described under Materials and Methods. Lane 1, single-stranded phage M13 DNA, incubated in the absence of enzyme; lane 2, M13 DNA, incubated for 120 min with 1 enzyme unit/ μ g DNA (degradation to fast-migrating small products); lane 3, linearized double-stranded plasmid pBR322 DNA, incubation without enzyme; lane 4, linearized plasmid DNA, incubated for 120 min with 1 enzyme unit/ μ g DNA; lane 5, concatemeric λ marker DNA (lengths as indicated on the left); lane 6, *Ectocarpus* virus DNA, untreated control; lanes 7 and 8, *Ectocarpus* virus DNA, treated with 1 enzyme unit/ μ g for 60 and 120 min, respectively.

10- to 60-kbp fragments observed arose as a consequence of discontinuities in the *Ectocarpus* virus DNA.

These discontinuities could be single-stranded breaks or single-stranded gaps. To investigate this possibility we treated agarose-embedded virus DNA with single-strand-specific nucleases. We found that incubations with mung bean nuclease for various lengths of time converted an increasing fraction of circular and linear unit length virus DNA into 10- to 70-kbp fragments (Fig. 8). Identical results were obtained using the single-strand-specific S1 nuclease (data not shown).

These results indicated that the *Ectocarpus* virus DNA contains single-stranded regions. We used electron microscopy to independently confirm this conclusion and to obtain an information concerning the lengths and the structure of the single-stranded regions. To better visualize these regions, we treated the viral DNA with the bacterial single-strand-specific binding (SSB) protein before spreading for electron microscopy. Single-stranded phage M13 DNA was added to the preparation and served as an internal control and a length marker.

In a significant number of DNA-molecules, we observed SSB-coated single-stranded regions. These detectable DNA regions consisted of one continuous strand connecting two double-stranded sections and

one free-ended single strand (Fig. 9). In many cases, the continuous single strand and the broken strand were found to be of similar lengths.

The single-stranded DNA sections are most likely the preferred sites for DNA breakage as DNA fragments frequently ended in a single strand (Fig. 9).

We determined the lengths of the single stranded sections by measuring SSB-coated continuous single strands relative to the M13 marker (Fig. 9). We obtained values ranging from 1.0 to 8.5 kb with an average of 2.9 kb (number of single-stranded regions measured: 60). It is important to note, though, that single-stranded regions <100–200 bp cannot be detected by the electron microscopic technique but can be degraded by endonucleases as shown in the experiment of Fig. 8.

In addition, we measured the lengths of 40 double-stranded sections. Most of them were determined to be about 10 to 60 kbp in length, in agreement with the biochemical data of Fig. 8. However, some double-stranded sections were smaller than 10 kbp. These smaller double-stranded sections are probably too few and too dispersed to be detected by ethidium-staining of gels like those shown in Figs. 4 and 8.

In summary, the mung bean nuclease digestion data and electron microscopy unambiguously show that *Ectocarpus* virus DNA contains single-stranded gaps at distances of about 10 to 60 kbp. These single-stranded regions are most probably preferred breakage sites and could be responsible for the production of the fragmented DNA commonly found in viral DNA preparations.

DISCUSSION

We described some structural properties of a newly discovered virus which is endemic in populations of the cosmopolitan marine brown alga *E. siliculosus*. The most interesting finding so far concerns the viral genome, a large DNA molecule of about 320 kbp.

DNA molecules of this size are characteristic for algal viruses (van Etten *et al.*, 1991) but it is not known yet whether the entire genomes are composed of single-copy genes or whether they contain noncoding regions or multiple genetic elements.

From the composition of the viral shell with its 13 or more polypeptides (Fig. 3), it can be estimated that less than 20 kbp of the genome may be reserved as coding regions for structural proteins.

Another virus-specific gene may encode the enzyme(s), responsible for the generation of methylated nucleotides, in particular of methylated adenine. This modified base is present in the genomes of other viruses infecting eukaryotic algae (van Etten *et al.*, 1991) but does not normally occur in eukaryotic cellular DNA. In fact, it has been shown in the case of *Chlorella* vi-



FIG. 9. Electron micrograph of SBB-treated *Ectocarpus* virus DNA. The arrowheads indicate the single-stranded DNA regions. (Left) Internal single-stranded regions; (right) single-stranded ends. Circular single-stranded M13 DNA was added as an internal size marker. Bar: 0.8 μm .

ruses that a virus-specific methyltransferase (Xia and van Etten, 1986) may be a component of a virus-specific modification-restriction system (Xia *et al.*, 1987, 1988). It is likely that modification and restriction enzymes are also encoded by the *Ectocarpus* virus, but this has yet to be shown. Nevertheless, we estimate

that not more than a few percent of the viral genome are reserved to code for these enzymes.

This leaves a large part of the viral genome to code for functions involved in viral multiplication; and viral multiplication may well need a large number of specific gene products given the unusual architecture of the

Ectocarpus virus genome whose replication should be a complex series of reactions.

We have presented three lines of evidence suggesting that a typical preparation of *Ectocarpus* virus DNA contains circularly closed double-stranded DNA. First, during pulse-field electrophoresis, part of the DNA remains at the start of the agarose gel, a well-known feature of open circular DNA (Levene and Zimm, 1987); second, double digestion with two different restriction endonucleases gives the same number of fragments as two independent digestions with either restriction enzyme, and these fragments can be composed to give a circular restriction map; and third, and most compelling, electron microscopic investigations reveal that a considerable fraction of isolated virus DNA exists in the form of DNA rings of about 320 kbp. This explains why we failed to identify specific ends of the *Ectocarpus* virus DNA by an experimental procedure that had been used for a mapping of the genomic ends of a *Chlorella* virus (Rohozinski *et al.*, 1989).

A second fraction of isolated virus DNA migrates during gel electrophoresis as linear molecules of 320 kbp. A likely interpretation of our data is that the native DNA, present in intact virus particles, normally occurs in a circular form, and that a substantial fraction becomes linearized during preparation. Since a digestion of linear DNA gives the same pattern of restriction fragments as circular DNA, it can be concluded that linearization does not occur by breakage at one specific site but at many sites of the circular molecule.

Breakage is most probably facilitated as a consequence of another interesting feature of *Ectocarpus* virus DNA, its partial single-strandedness. We have demonstrated that sections of 10 to 60 kbp of viral duplex DNA strand are interrupted by single-stranded regions. These single strands are preferred break points as many of the DNA fragments, seen in our electron micrographs, possess frayed single-stranded ends. It is not known yet whether the extensive breakages observed are the sole consequence of disruptive shear forces or whether virus particles include or are associated with a single-strand-specific endonuclease that attacks the released DNA. However, we can presently not exclude the interesting possibility that DNA fragments are products of intracellular replication or processing events, and become enclosed in protein shells before maturation.

In any case, as demonstrated above, DNA fragments of 10 to 60 kbp are quite common in viral DNA preparations; and the most likely reason for their occurrence are breaks at single-stranded sites.

Thus, the most probable structure of native *Ectocarpus* virus DNA is that of a circular DNA molecule consisting of double-stranded sections connected by single-stranded regions. Presently, the molecular structure of the single-stranded regions is not entirely clear.

In most of our electron micrographs we detect one broken single strand and one continuous single strand connecting two double strands. However, we occasionally detect two continuous single strands in the form of a single-stranded bubble between double-stranded DNA sections (not shown). It is possible that most or all single-stranded regions were originally present as "bubbles" in native DNA but became disrupted during virus preparation due to their extreme vulnerability.

Another interesting question concerns the nucleotide sequences of the single-stranded DNA regions. They could be rich in adenine and thymine residues which may facilitate strand separation during manipulations. However, we also detect single strands when the DNA had been kept at 37° during the entire extraction procedure. Presently, we can therefore not exclude the possibility that the single strands are non-complementary. In this case, it will be necessary and interesting to determine whether they contain coding regions or whether they are just random pieces of DNA separating the genetically more important double-stranded sections. One possibility is that the single strands are acquired from the cellular genome as a consequence of a multiplication step. A precedent may be bacteriophage Mu which is integrated in the host genome and replicates *in situ* to produce progeny DNA with some 100 bp of host DNA at its right end and up to 3000 bp of host DNA at its left end (Symonds *et al.*, 1987). However, Mu DNA is linear and completely double-stranded; and its mode of replication therefore cannot serve as a model for the *Ectocarpus* virus.

Presently, we know neither the molecular structure of the latent viral DNA in infected somatic host cells nor the structure of replicating DNA in diseased reproductive cells. It has been suggested that the *Ectocarpus* virus DNA may be integrated as a provirus in the plant cell genome (Müller, 1991b), but this has yet to be directly demonstrated. An episomal state of the viral DNA can presently not be excluded.

In either case, the viral mode of DNA replication is likely to be quite bizarre. For example, the single strand complements could be the results of independent replication events and could later be joined via base pairing forming the partially double-stranded molecules that we observe.

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