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Genome Structure of a Virus Infecting the Marine Brown Alga *Ectocarpus siliculosus*

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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.

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 zoidangia 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-mediating 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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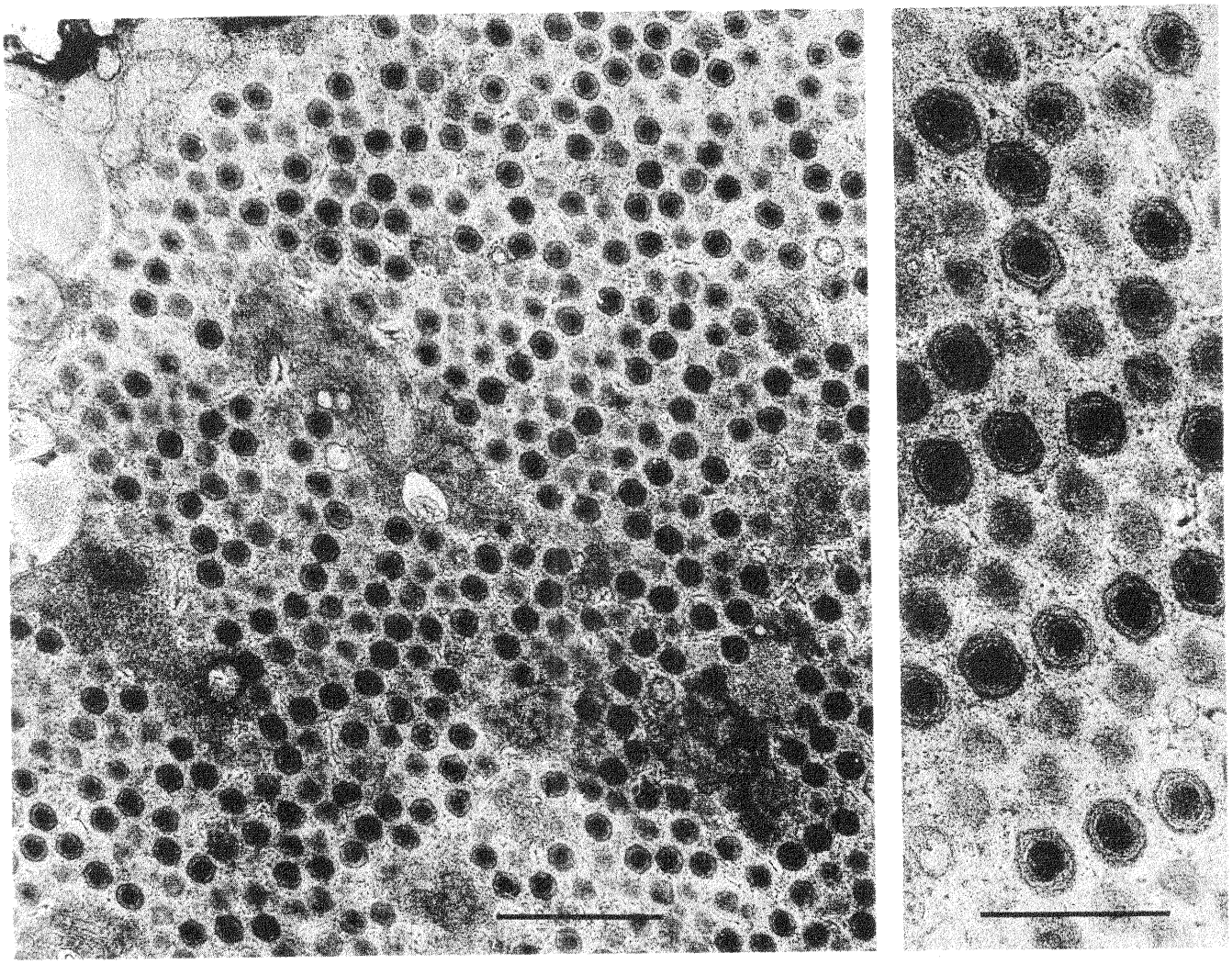

FIG. 1. Virus particles in infected gametophytes. (Left) Densely packed virus particles are located between residual components of the plant cell. Bar: 1 μm. (Right) A larger amplification demonstrating the two-layered viral shell. Bar: 0.5 μm.

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.

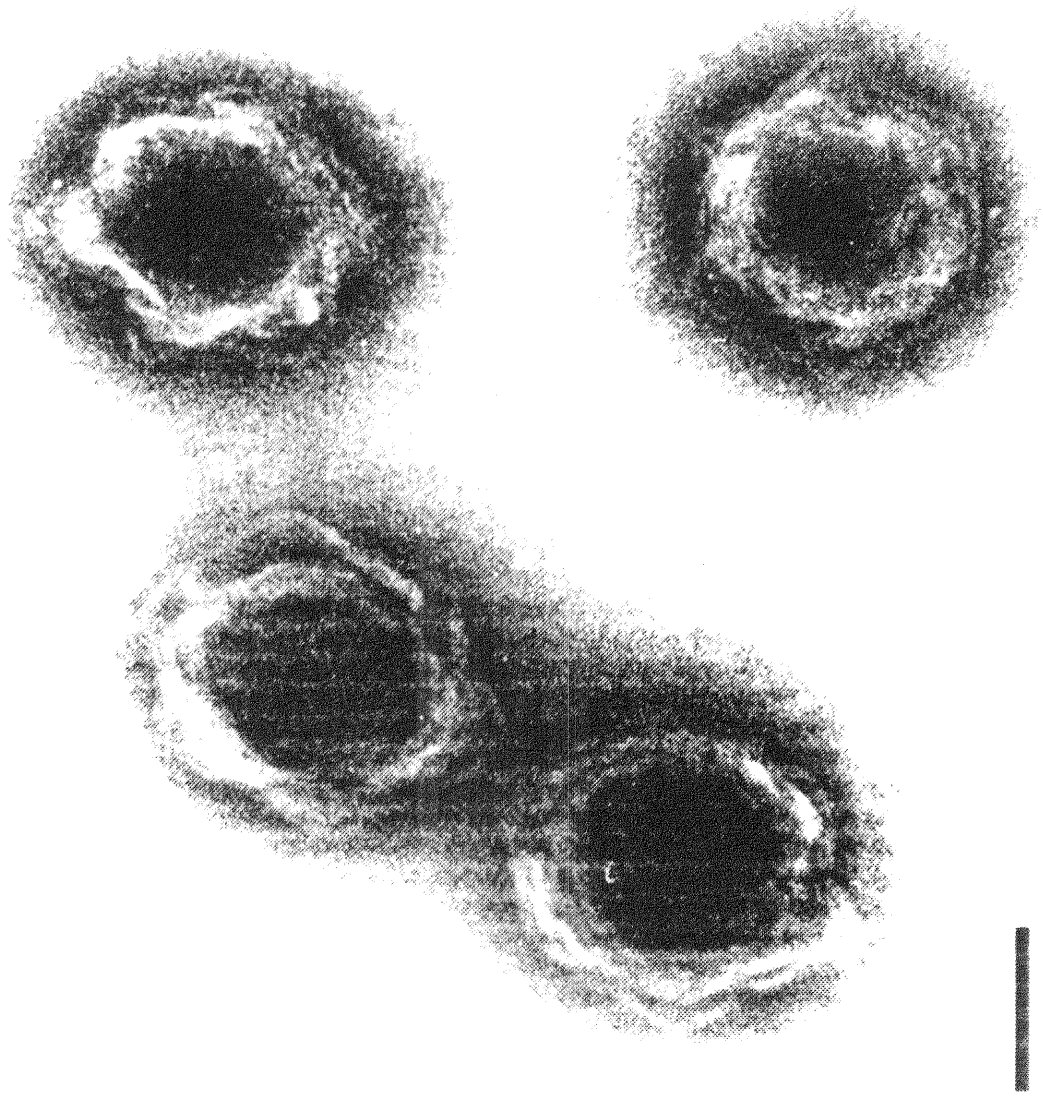

FIG. 1. Morphology of isolated LsV. The multilayered structure corresponds to that previously described for densely packed intracellular virus particles (see Muller *et al.*, 1990; Lanka *et al.*, 1993). Bar, 0.1 μm.

gels in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1974). The separated polypeptides were visu-

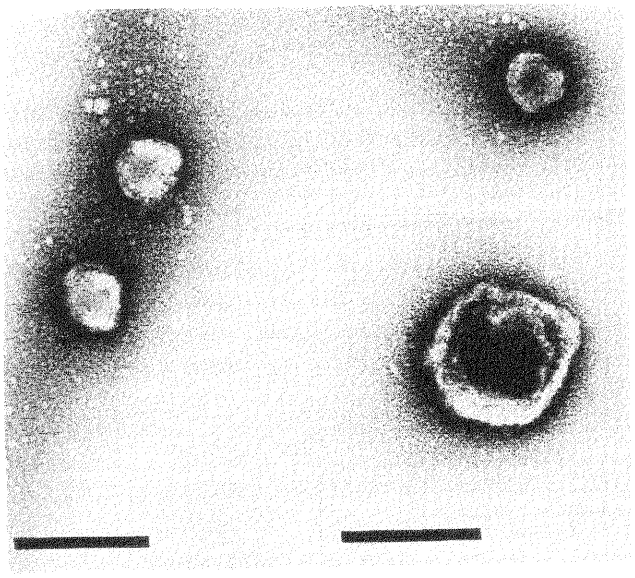

FIG. 2. Isolated virus particles. A negative stain technique was used to demonstrate that the virus structure remained largely intact after PEG precipitation and repeated centrifugations. Bars: 0.4 μm (left); 0.2 μm (right).

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.

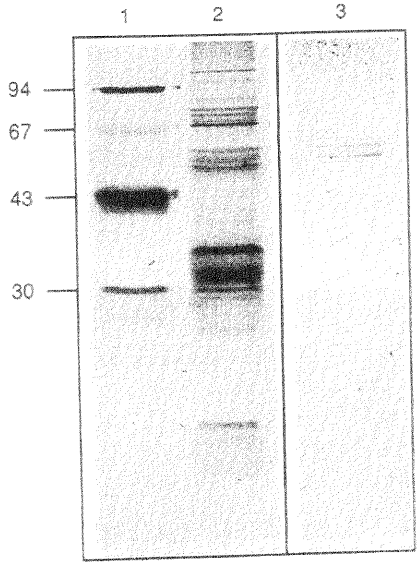

FIG. 3. Structural viral polypeptides as investigated by denaturing polyacrylamide gel electrophoresis. Isolated virus particles (Fig. 2) were concentrated by centrifugation. A sample, containing a total of 5 μg protein, was denatured with sodium dodecylsulfate in loading buffer (Laemmli, 1974) and investigated 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 *Hin*dIII, *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 circularily 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 circularily closed. It is well known that high electric fields

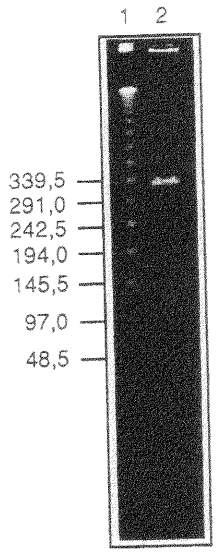

FIG. 4. Viral DNA as investigated by rotating field agarose gel electrophoresis. The conditions for electrophoresis are described under Materials and Methods. Lane 1, λ DNA concatemers as length marker (their sizes in kbp are noted on the left); lane 2, *Ectocarpus* virus DNA.

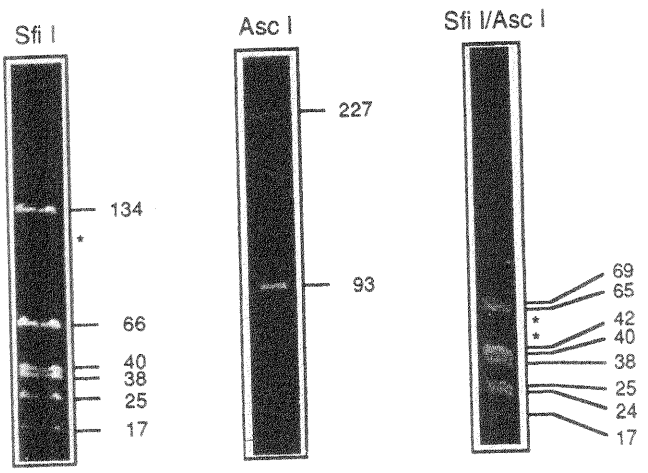

FIG. 5. Restriction fragment pattern. *Ectocarpus* virus DNA was prepared and restricted as described under Materials and Methods. The resulting restriction fragments were investigated by rotating field agarose gel electrophoresis using λ DNA concatemers and λ DNA *Hin*dIII restriction fragments as lengths 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.