Journal of General Virology (1999), **80**, 1367–1370. Printed in Great Britain SHORT COMMUNICATION

Persistent virus integration into the genome of its algal host, *Ectocarpus siliculosus* (Phaeophyceae)

Nicolas Delaroque, Ingo Maier, Rolf Knippers and Dieter G. Müller

Fakultät für Biologie, Universität Konstanz, D-78457 Konstanz, Germany

The brown alga *Ectocarpus siliculosus* frequently carries an endogenous virus, *E. siliculosus* virus (EsV-1), the genome of which is a circular, double-stranded DNA molecule of about 320 kbp. After infection, which occurs in the unicellular spores or gametes, the virus is present latently in all somatic cells of the host. Virus multiplication is restricted to cells of the reproductive organs. It has been an open question whether the latent viral DNA occurs as a free episome or becomes integrated into the host genome. PCR studies showed that viral DNA co-migrates with high molecular mass DNA in pulsed-field gel electrophoresis, which confirms that latent viral DNA is integrated into the host genome.

Marine filamentous brown algae of the order Ectocarpales frequently carry endogenous viruses with large double-stranded DNA genomes (Müller *et al.*, 1998). A well-studied example is *Ectocarpus siliculosus* virus-1 (EsV-1), the icosahedral capsid of which encloses a circular genome of about 320 kbp (Müller *et al.*, 1990; Lanka *et al.*, 1993). Like other brown-algal viruses, EsV-1 exclusively infects cell wall-free zoospores or gametes. Infected cells develop into mature thalli which can produce pathological symptoms in their sporangia or gametangia. These organs become densely packed with viral particles that are eventually released into the surrounding sea water (Fig. 1*a*). It is not unusual, however, for infected algae to appear normal and to produce viable spores (Fig. 1*d*) containing the viral genome (Fig. 1*e*, lane 4). In fact, genetic studies and PCR analyses have shown that the latent viral genome is transmitted vertically through meiosis as a Mendelian trait (Müller, 1991*a*; Bräutigam *et al.*, 1995).

These earlier reports could not distinguish between an episomal free state of the EsV-1 DNA and integration into the host genome. We report here the results of experiments that were designed to distinguish between these possibilities. We prepared high molecular mass DNA from zoospores and gametes of infected and uninfected algae by using a modification of the protocol of Liu & Whittier (1994). The following clonal isolates of *E. siliculosus* were used: strain PAr 10n (Fig. 1*b*), a healthy female gametophyte (Müller, 1979; Sengco *et al.*, 1996); NZVicZ14 (Fig. 1*a*, *c*), which is infected by EsV-1 and produces virions as well as zoospores carrying a latent viral genome (Müller *et al.*, 1996; Sengco *et al.*, 1996); and strain Nap R-B1 (Fig. 1*d*), which is a phenotypically normal male gametophyte that is latently infected by EsV-1 and does not produce virions (Sengco *et al.*, 1996). The cultures of PAr 10n and NZVicZ14 were axenic, Nap R-B1 was unialgal. EsV-1 DNA was prepared from virions released by strain NZVicZ14 as described by Lanka *et al.* (1993). Culture conditions were as described by Müller (1991*a*, *b*). Mature algae were stored in the dark at 2 °C overnight. Mass release of reproductive cells within 20 min was induced by rapid transfer into a small volume of fresh culture medium combined with a temperature increase to 18 °C and exposure to light.

The cells were collected by centrifugation at 1400 ***g*** for 15 min, washed and resuspended in culture medium and then gently mixed with an equal volume of low-melting-point agarose (Biozym) prepared in culture medium. Agarose plugs were treated twice with 2 mg/ml proteinase K (Boehringer Mannheim) in 10 mM Tris–HCl (pH 8·0), 50 mM EDTA and 1% SDS for 16–18 h at 50 °C. The agarose plugs were washed three times for 1 h each in 500 mM EDTA at 50 °C and finally stored at 4 °C. This procedure yielded large amounts of DNA that was suitable for restriction nuclease digestion (not shown) and pulsed-field gel electrophoresis.

Ethidium bromide staining showed a strong band of DNA at the limit of size separation of the gel system (approx. 600 kbp), although a considerable fraction of DNA was smeared over a region corresponding to lower molecular mass. Another part of the stainable DNA remained in the gel slots, probably due to incomplete proteolysis (Fig. 2). Ethidium bromide staining did not reveal the presence of free viral DNA (Fig. 2, lanes 3 and 4), except in experiments where spores of NZVicZ14 were accompanied by simultaneously released virions (Fig. 2, lane 5).

Author for correspondence: Nicolas Delaroque.
Fax +49 7531 88 2966. e-mail Nicolas.Delaroque@uni-konstanz.de

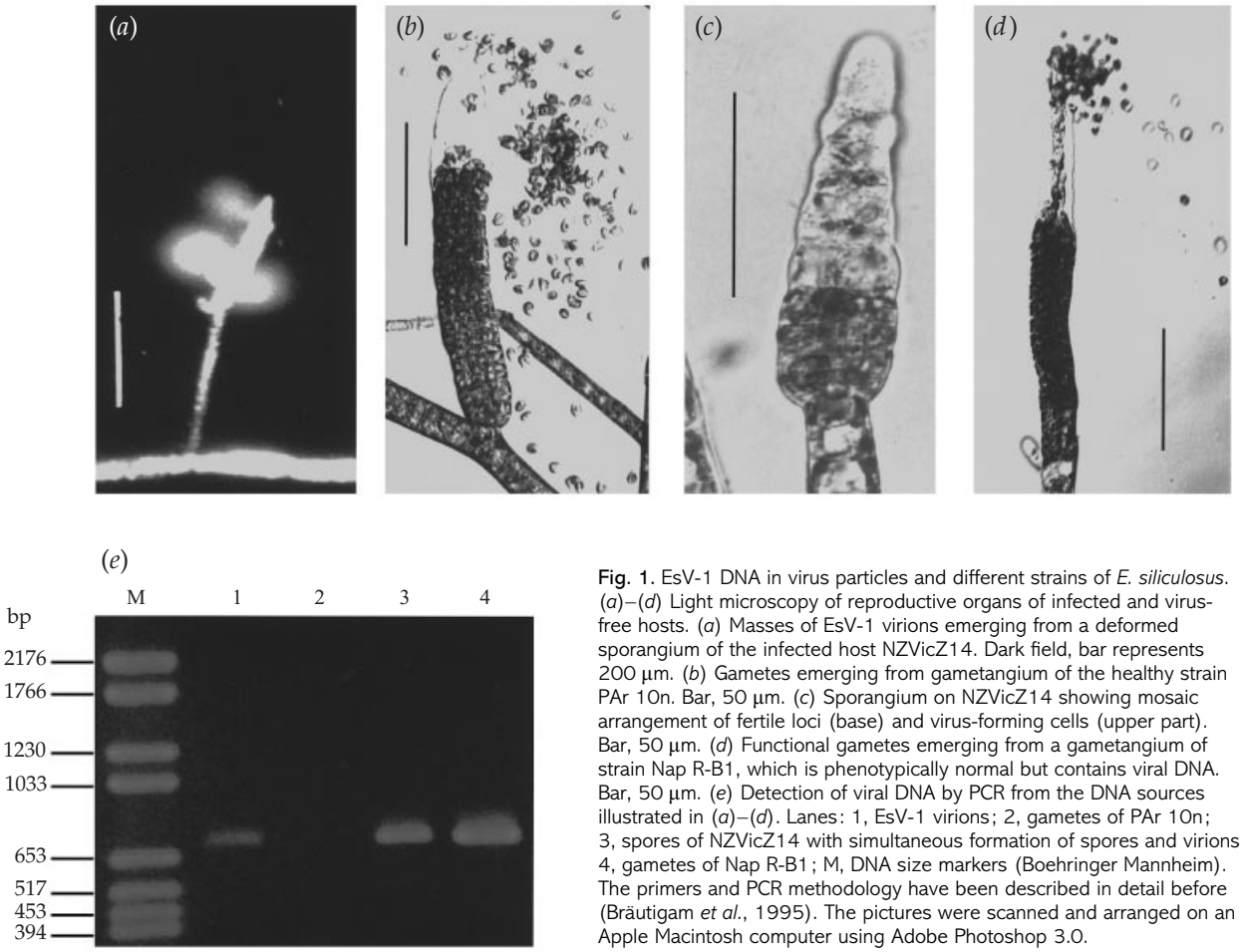

Fig. 1. EsV-1 DNA in virus particles and different strains of *E. siliculosus*. (*a*)–(*d*) Light microscopy of reproductive organs of infected and virus-free hosts. (*a*) Masses of EsV-1 virions emerging from a deformed sporangium of the infected host NZVicZ14. Dark field, bar represents 200 μm. (*b*) Gametes emerging from gametangium of the healthy strain PAr 10n. Bar, 50 μm. (*c*) Sporangium on NZVicZ14 showing mosaic arrangement of fertile loci (base) and virus-forming cells (upper part). Bar, 50 μm. (*d*) Functional gametes emerging from a gametangium of strain Nap R-B1, which is phenotypically normal but contains viral DNA. Bar, 50 μm. (*e*) Detection of viral DNA by PCR from the DNA sources illustrated in (*a*)–(*d*). Lanes: 1, EsV-1 virions; 2, gametes of PAr 10n; 3, spores of NZVicZ14 with simultaneous formation of spores and virions; 4, gametes of Nap R-B1; M, DNA size markers (Boehringer Mannheim). The primers and PCR methodology have been described in detail before (Bräutigam *et al.*, 1995). The pictures were scanned and arranged on an Apple Macintosh computer using Adobe Photoshop 3.0.

In order to detect virus sequences in the DNA preparations shown in Fig. 2, we first performed Southern blotting experiments. However, the hybridization signals were very weak and unreliable (data not shown), except where viral DNA could also be identified by ethidium bromide staining as in lane 5 of Fig. 2.

As an alternative, we used a PCR approach. Lanes were excised from gels like that shown in Fig. 2 and then cut into 0·5 cm slices. DNA was extracted from individual slices with β-agarase I (New England Biolabs). Samples of 1–3 μl were analysed by PCR by using EsV-1-specific primers and protocols from Bräutigam *et al.* (1995).

In a control experiment with purified EsV-1 DNA (Fig. 3*a*), we obtained positive PCR signals from slices containing intact viral DNA as well as from slices with smaller viral DNA fragments, which arise because EsV-1 DNA tends to break at one or more of its many single-stranded regions (Klein *et al.*, 1994). A second control experiment was performed with PAr 10n algal DNA, which confirmed the absence of virus DNA as expected (Fig. 3*b*). In contrast, all slices containing DNA of infected *Ectocarpus* cells contained viral DNA sequences, regardless of whether the DNA had been prepared from spores of NZVicZ14 (Fig. 3*c*) or gametes of Nap R-B1 (Fig. 3*d*). The important point here is that positive virus signals were found not only at the positions where intact or fragmented viral DNA could be expected, but also at positions corresponding to high molecular mass host DNA (compare Fig. 3*d* with Fig. 3*a*).

Concatemers of viral DNA can be excluded, since virus replication does not occur in the unicellular gametes or spores of the hosts that we used for the isolation of DNA (Müller *et al.*, 1990). All known examples of concatemer formation are linked to viral DNA replication (Mosig *et al.*, 1995; Boehmer & Lehman, 1997).

In order to exclude the possibility that viral DNA was bound, trapped or mechanically retained by cellular DNA aggregates, the 600 kbp complexes were excised from a gel similar to that shown in Fig. 2 and again subjected to electrophoresis and PCR analysis (Zimmer & Verrinder Gibbins, 1997). The results (not shown) were similar to those shown in Fig. 3. Thus, our data indicate a covalent association of viral and cellular DNA. Our conclusion is that EsV-1 DNA occurs as an integrated provirus in latently infected cells. Recently, Lee *et al.* (1998) claimed, without presenting

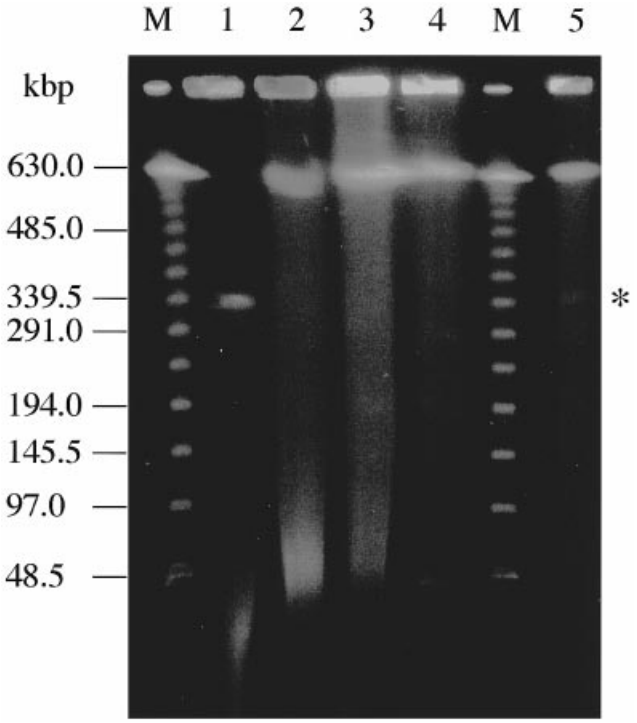

Fig. 2. Pulsed-field gel electrophoresis. DNA was isolated from EsV-1 virions (lane 1), PAr 10n (2), NZVicZ14 (3 and 5) and Nap R-B1 (4). The DNA (2–3 μg) was processed as described in the text. Electrophoresis was carried out in a 1% agarose gel (Seakem; FMC) with 45 mM Tris–borate–EDTA buffer (pH 8·0) by using a CHEF mapper system (Bio-Rad) at 14 °C for 22 h. Running conditions: voltage gradient 6 V/cm; included angle 120 °; linear switch time ramp 0·7–45 s. Lane M, DNA size markers (concatemeric lambda DNA; New England Biolabs). Asterisk, position of full-length EsV-1 DNA. The gel was imaged as described previously.

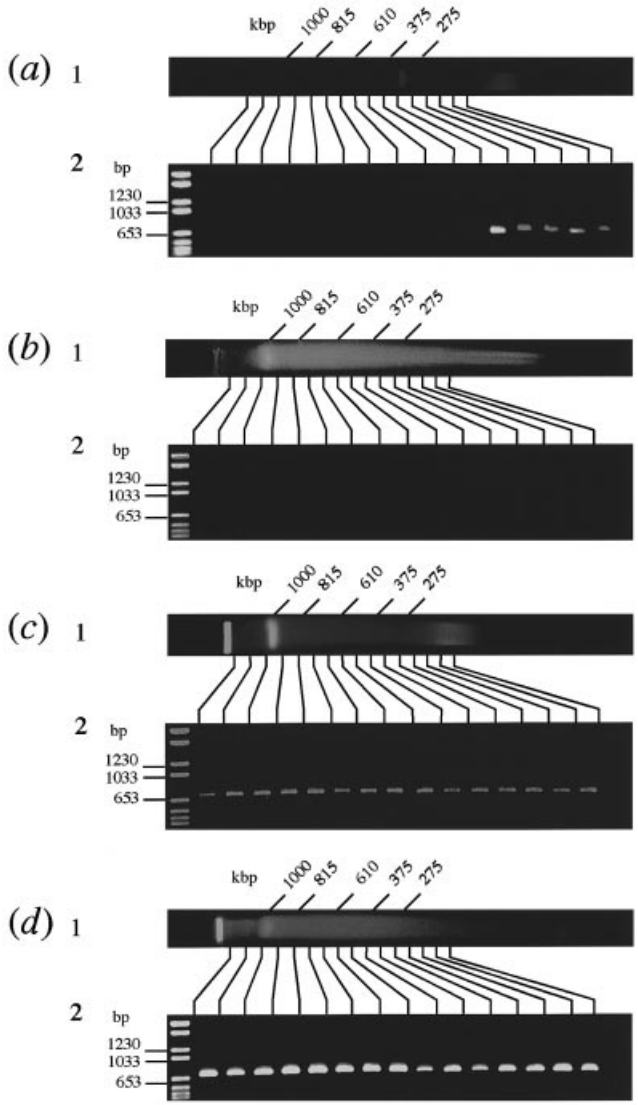

Fig. 3. Identification of virus sequences in algal DNA isolated from the sources illustrated in Fig. 1. Samples of EsV-1 DNA (*a*) and cellular DNA from PAr 10n (*b*), NZVicZ14 (*c*) and Nap R-B1 (*d*) were subjected to pulsed-field gel electrophoresis. The 1% low-melting-point agarose gel in 45 mM Tris–borate–EDTA buffer was run under the following conditions: voltage gradient 6 V/cm; included angle 120 °; linear switch time ramp 12–83 s; temperature 14 °C; running time 23 h. Ethidium bromide-stained DNA lanes (1) were cut into 0·5 cm slices for PCR analysis (2) as described in the text. The results were digitized (see legend to Fig. 1).

experimental evidence, that the genome of the brown-algal virus *Feldmannia* sp. virus may also be integrated into the host genome.

The PCR method used in our study does not give quantitative results and therefore we cannot determine the number of integrated virus genomes per host nucleus. Likewise, it is presently not possible to determine whether EsV-1 DNA is integrated randomly or at specific sites of the cellular genome. This question can be investigated once the complete nucleotide sequence of EsV-1 DNA is known. Another open question concerns the mechanism that keeps the integrated viral genome silent. Biological and physiological studies show that latent viral DNA does not reduce the growth of the host organism (Del Campo *et al.*, 1997). Thus, our finding that EsV-1 is integrated into the host genome suggests that it could potentially be useful as a transformation vector for various brown-algal hosts such as kelps and other commercially interesting species.

We thank Martina Baack and Eva Kessler for their suggestions and discussions. This work was supported by the Deutsche Forschungsgemeinschaft (Mu 196/18-3).

References

Boehmer, P. E. & Lehman, I. R. (1997). Herpes simplex virus DNA replication. *Annual Review of Biochemistry* **66**, 347–384.

Bräutigam, M., Klein, M., Knippers, R. & Müller, D. G. (1995). Inheritance and meiotic elimination of a virus genome in the host *Ectocarpus siliculosus* (Phaeophyceae). *Journal of Phycology* **31**, 823–827.

Del Campo, E., Ramazanov, Z., Garcia-Reina, G. & Müller, D. G. (1997). Photosynthetic responses and growth performance of virus-infected and non-infected *Ectocarpus siliculosus* (Phaeophyceae). *Phycologia* **36**, 186–189.

Klein, M., Lanka, S., Müller, D. & Knippers, R. (1994). Single-stranded regions in the genome of the *Ectocarpus siliculosus* virus. *Virology* **202**, 1076–1078.

Lanka, S. T. J., Klein, M., Ramsperger, U., Müller, D. G. & Knippers, R. (1993). Genome structure of a virus infecting the marine brown alga *Ectocarpus siliculosus*. *Virology* **193**, 802–811.

Lee, A. M., Ivey, R. G. & Meints, R. H. (1998). Repetitive DNA insertion in a protein kinase ORF of a latent FSV (*Feldmannia* sp. virus) genome. *Virology* **248**, 35–45.

Liu, Y. G. & Whittier, R. F. (1994). Rapid preparation of megabase plant DNA from nuclei in agarose plugs and microbeads. *Nucleic Acids Research* **22**, 2168–2169.

Mosig, G., Colowick, N., Gruidl, M. E., Chang, A. & Harvey, A. J. (1995). Multiple initiation mechanisms adapt phage T4 DNA replication to physiological changes during T4's development. *FEMS Microbiology Reviews* **17**, 83–98.

Müller, D. G. (1979). Genetic affinity of *Ectocarpus siliculosus* (Dillw.) Lyngb. from the Mediterranean, North Atlantic and Australia. *Phycologia* **18**, 312–318.

Müller, D. G. (1991 *a*). Mendelian segregation of a virus genome during host meiosis in the marine brown alga *Ectocarpus siliculosus*. *Journal of Plant Physiology* **137**, 739–743.

Müller, D. G. (1991 *b*). Marine virioplankton produced by infected *Ectocarpus siliculosus* (Phaeophyceae). *Marine Ecology Progress Series* **76**, 101–102.

Müller, D. G., Kawai, H., Stache, B. & Lanka, S. (1990). A virus infection in the marine brown alga *Ectocarpus siliculosus* (Phaeophyceae). *Botanica Acta* **103**, 72–82.

Müller, D. G., Sengco, M., Wolf, S., Bräutigam, M., Schmid, C. E., Kapp, M. & Knippers, R. (1996). Comparison of two DNA viruses infecting the marine brown algae *Ectocarpus siliculosus* and *E. fasciculatus*. *Journal of General Virology* **77**, 2329–2333.

Müller, D. G., Kapp, M. & Knippers, R. (1998). Viruses in marine brown algae. *Advances in Virus Research* **50**, 49–67.

Sengco, M. R., Bräutigam, M., Kapp, M. & Müller, D. G. (1996). Detection of virus DNA in *Ectocarpus siliculosus* and *E. fasciculatus* (Phaeophyceae) from various geographic areas. *European Journal of Phycology* **31**, 73–78.

Zimmer, R. & Verrinder Gibbins, A. M. (1997). Construction and characterization of a large-fragment chicken bacterial artificial chromosome library. *Genomics* **42**, 217–226.

Received 14 December 1998; Accepted 21 January 1999