

Coat Protein of the *Ectocarpus siliculosus* Virus

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Ectocarpus siliculosus virus, EsV, multiplies in sporangia and gametangia of the marine brown alga *Ectocarpus siliculosus*. We describe an improved method for the isolation of morphologically intact and infectious virus from diseased plants. We show that treatment of virus particles with high concentrations of CsCl results in a substantial loss of structural proteins. One of the proteins which resists CsCl treatment is glycoprotein-1, the largest of the three viral glycoproteins. We have isolated an EsV genomic fragment with an open reading frame encoding glycoprotein-1. The predicted amino acid sequence is rich in hydrophilic amino acids, but contains hydrophobic regions close to the amino and carboxy termini. A discrepancy between the molecular weight predicted from the coding region and the molecular weight determined by gel electrophoresis suggests that proteolytic processing is required for the maturation of the protein. © 1995 Academic Press, Inc.

INTRODUCTION

Ectocarpus siliculosus is a cosmopolitan benthic filamentous marine brown alga. It inhabits coasts in temperate climate zones of both hemispheres. Representative plants from many localities carry symptoms of an infection caused by a latent virus, the *Ectocarpus siliculosus* Virus, EsV (Müller *et al.*, 1990; Müller and Stache, 1992).

Induction and multiplication of EsV occurs in the reproductive organs of the host. EsV genomes replicate and assemble as closely packed viral particles in prospective gametangia and sporangia and are eventually discharged into the surrounding seawater (Müller, 1991a). Free virus particles are infectious for a few days (Müller and Frenzer, 1993) and can invade swimming gametes or spores of healthy plants, but cannot infect somatic *Ectocarpus* cells which are protected by their solid cell wall (Müller *et al.*, 1990). Infected gametes or spores develop into infected plants, implying that all cells of the progeny plant carry the virus genome, but permit virus induction and multiplication only in the prospective reproductive cells. In genetic crosses between healthy and infected gametophytes, the EsV genome is transmitted like a Mendelian trait as meiosis produce equal numbers of healthy and infected spores (Müller, 1991b).

Thus, EsV in its natural environment remains infectious for short time periods, easily invades the host via its gametes or spores, and can be eliminated by meiosis. These are features of a nonlethal, well balanced host/pathogen system with interesting evolutionary and ecological implications.

Previously, we isolated EsV particles from infected plants and found that the viral genome is double stranded DNA of 320 kb which is isolated in linear and circular forms (Lanka *et al.*, 1993). Our isolation procedure involved a precipitation of virus particles from disrupted infected plants by polyethylene glycol followed by equilibrium centrifugation in CsCl gradients. EsV particles, purified by this procedure, were not infectious (in contrast to virus particles spontaneously released from infected cells into seawater; Müller *et al.*, 1990). They also differ in their electron microscopic appearance from those seen in infected plant cells (Lanka *et al.*, 1993).

In the present communication, we describe an improved isolation procedure replacing the CsCl step with Percoll gradient centrifugation. This procedure yields morphologically intact EsV particles that readily infect unicellular zoospores which then develop into mature plants showing pathological symptoms. As one explanation for the lack of infectivity of CsCl-purified EsV we found that CsCl induces the dissociation of several viral coat proteins. We have determined properties of one coat protein, glycoprotein 1, as a first step for investigating the complex architecture of EsV.

MATERIALS AND METHODS

Cells

Stocks of clonal sporophyte cultures of *E. siliculosus* were maintained bacteria-free on 1% agar in culture medium (autoclaved seawater with ES enrichment according to Starr and Zeikus, 1993). Mass cultures were initiated by inoculation of agar-grown fragments into liquid medium in Petri dishes which were later expanded to 0.5-liter cultures. Conditions for cultivation were as previously described (Lanka *et al.*, 1993).

The virus-free strain is a female parthenosporophyte derived from a native New Zealand gametophyte (code number NZ 4a3 f).

For virus production we used the previously described *E. siliculosus* clone NZ-Vic-Z14 which develops symptoms of viral infection when transferred from agar to liquid medium (Lanka *et al.*, 1993).

Preparation of virus particles

Virus particles were isolated from infected plants and collected by precipitation in polyethylene glycol (PEG) as previously described (Lanka *et al.*, 1993). The PEG pellet was resuspended in 0.5 ml seawater/0.05 M Tris-HCl (pH 7.8) and mixed with a Percoll solution (Sigma) to give a final concentration of 80% Percoll (in seawater/0.05 M Tris-HCl, pH 7.8). The solution was centrifuged to equilibrium for 1 hr at 65,000 g and 4' in a fixed-angle rotor. The turbid virus band was collected by puncturing the tube with a hypodermic needle.

Electron microscopy

Isolated virus particles were mounted on carboncoated grids which had been exposed to glow discharge of tripropylamine vapor (Dubochet and Groom, 1982). The grids were then treated with uranyl acetate (Haschemeyer, 1970). Micrographs were taken in a Zeiss EM 900 electron microscope.

Molecular cloning

We have restricted EsV DNA with the endonucleases EcoR1 and Sall. The resulting fragments were cloned into plasmid vector pUC 9. Several of the cloned virus DNA fragments were sequenced by the chain termination method of Sanger *et al.* (1977). A 0.93-kb fragment contained an open reading frame, and computer analysis revealed some limited degree of similarity to known viral coat proteins (see below). The 0.93-kb fragment was used as a probe to isolate a 2.3-kb BamH1 fragment from an EsV genomic library (see below). Part of this DNA fragment was subcloned in plasmid pRSET (Invitrogen/ ITC Biotechnology) and induced for expression after transfection into bacteria as suggested by the supplier. The overexpressed protein was purified from bacterial extracts and used to immunize rabbits (Harlow and Lane, 1988).

For preparation of a genomic library, the EsV genome was partially digested with the restriction endonuclease Sau3A, and DNA fragments of 9-23 kb were cloned in the BamH 1 sites of vector X-G EM 12 (Promega) according to standard procedures (Sambrook *et al.*, 1989).

Protein analysis

Virus particles, obtained after Percoll centrifugation, were pelleted at 80,000 g and resuspended in loading buffer. Proteins were electrophoresed on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1974). The separated polypeptides were visualized by silver staining (Wray *et al.*, 1981) or transferred to a nylon membrane for the staining of glycoproteins (Gershoni *et al.*, 1985) or for immunostaining (Western blotting; Towbin *et al.*, 1979). Immunostaining of membrane-bound proteins was performed with rabbit antibodies specific for the bacterially expressed EsV coat protein.

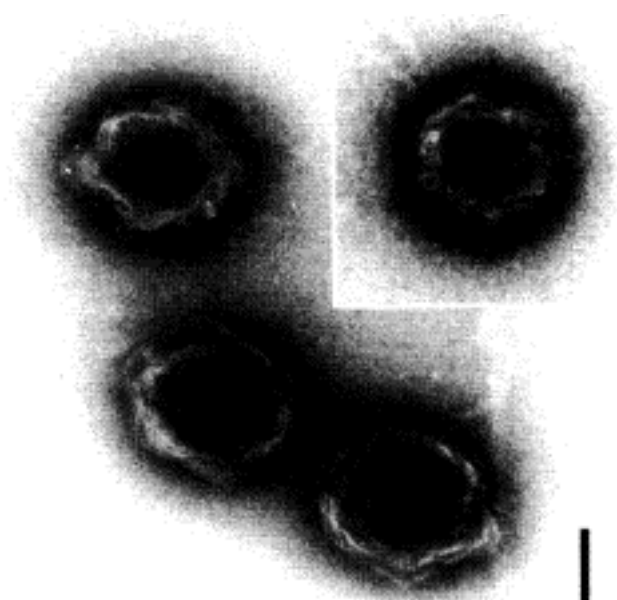


FIG. 1. Morphology of isolated EsV. The multilayered structure corresponds to that previously described for densely packed intracellular virus particles (see Müller *et al.*, 1990; Lanka *et al.*, 1993). Bar, 0.1 μ m.

RESULTS

Isolation of infective virus particles

Previously, we purified PEG-precipitated virus particles by CsCl gradient centrifugation (Lanka *et al.*, 1993). These virus preparations were not infectious in contrast to native virus particles released from infected plants (Müller *et al.*, 1990). We concluded that viral particles were damaged by the high ionic

strength of CsCl solutions and consequently tried Percoll gradients as an alternative final purification step. Indeed, electron microscopic examination revealed that EsV particles recovered after the Percoll step were morphologically similar (Fig. 1) to the viral structures seen in EM sections of plant cells (Müller *et al.*, 1990; Lanka *et al.*, 1993).

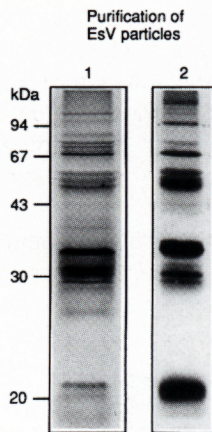


FIG. 2. Structural proteins of EsV purified by centrifugation in a CsCl gradient (1) and in a Percoll gradient (2). About 5 Mg protein each was electrophoresed on a 12% polyacrylamide gel and visualized by silver staining.

We have performed infection experiments to test whether Percoll-purified EsV retains its biological activity. As a recipient we used symptom-free parthenosporophytes with mature plurilocular sporangia. These plants are induced to release their spores by transfer to fresh culture medium and a temperature increase from 4 to 18°C (Müller, 1991a). Aliquots of 200-300 free-swimming spores are dispensed into 0.5 ml medium in 5-cm plastic dishes. Aliquots of the EsV preparation are then added and gently stirred. After 1 hr the zooids had settled at the bottom of the plastic dishes. Fresh medium (10 ml) was then added to each dish and incubation continued at 18°C for 2 weeks. Depending on the amounts of virus added, between 10 and 35% of the developing plants showed the symptoms characteristic for virus infection (Müller *et al.*, 1990). Mock infection experiments with parallel cultures never produced pathological symptoms.

These experiments fulfill Koch's postulates: the infectious agent as isolated from a diseased organism and used to infect a healthy recipient which thereafter developed the same symptoms originally observed in the field sample.

Infection experiments with CsCl-treated virus preparations were always negative, and one reason for this could be a loss of structural proteins during CsCl gradient centrifugation. Gel electrophoretic analyses revealed qualitative differences between the two virus preparations. As shown in Fig. 2, polypeptides of about 35 and 21 kDa molecular weight are the most prominent protein constituents of Percoll-purified EsV, but they are far less abundant in CsCl-treated virus preparations. In addition, CsCl-purified preparations almost entirely lack several polypeptides of molecular weight larger than 70 kDa (Fig. 2).

We initiated an analysis of the EsV coat and investigated some properties of a major viral structural protein.

The EsV glycoprotein-1

We have cloned and sequenced a 2.3-kb piece of EsV DNA. This DNA segment hybridizes with the 65-kb AscI

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SfiI fragment of the EsV genome (Lanka *et al.*, 1993) and is included in a 13.7-kb fragment of an EsV genomic library constructed in the lambda-derived vector XGEM12. These findings allow a tentative assignment of the 2.3-kb fragment to the EsV genome as shown in Fig. 3.

The 2.3-kb fragment does not hybridize to DNA from uninfected *E. siliculosus* cells or to DNA from a virus infectious for the marine brown alga *Feldmannia* (Henry and Meints, 1992; Friess-Klebi *et al.*, 1994, data not shown).

The fragment contains an open reading frame of 1983 bases. The region (50 bp) upstream of the initiation codon is rich in AT base pairs (not shown) with an AT content of 63% compared to 50% in the open reading frame. Interestingly, AT-rich upstream regions were also found in genes from *Chlorella* viruses and may have a function in gene expression (Schuster *et al.*, 1990; Graves and Meints, 1992).

In Fig. 4A, we show the amino acid sequence deduced from the open reading frame of the 2.3-kb EsV DNA fragment. The amino acid sequence corresponds to a polypeptide with a calculated molecular weight of about 72 kDa. The sequence includes four amino acid motifs frequently found at sites modified by N-glycosylation (boxed in Fig. 4A) (Hart *et al.*, 1979). The predicted polypeptide is rich in hydrophilic amino acids with a calculated pI of 4.2, but there are two hydrophobic domains close to the

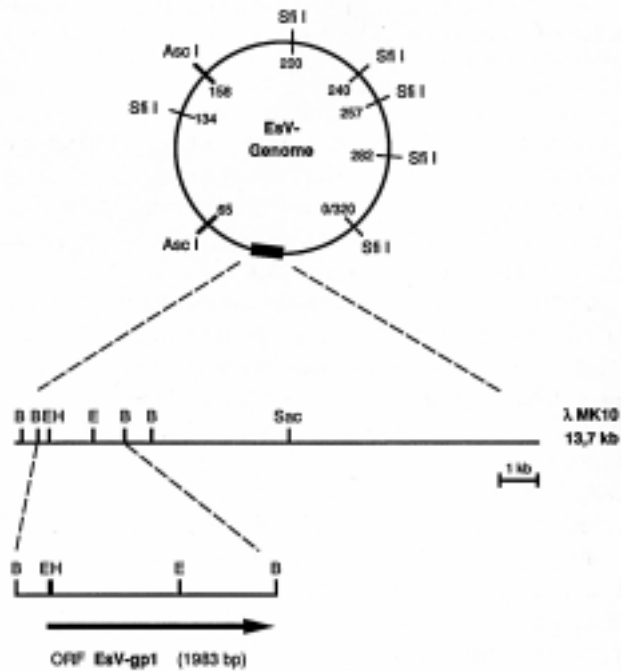


FIG. 3. Mapping of the EsV-gp1 gene. (Top) Restriction map of the EsV genome (Lanka *et al.*, 1993). (Bottom) Insert of phage clone λ MK10 (restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III) containing an open reading frame (ORF) of 1983 bp encoding the EsV glycoprotein-1 (gp1). Arrow, direction of transcription.

FIG. 4. Predicted amino acid sequence of EsV glycoprotein-1. (A) Amino acid sequence. Underlined regions are rich in hydrophobic (straight line) or hydrophilic (broken line) amino acids. Potential N-linked glycosylation sites are boxed. (B) Hydrophilicity plot. (C) Regions with limited similarities to known viral and cellular proteins (see text). The EsV nucleotide sequence has been deposited with the EMBL data library under Accession No. X76296.

aminoterminal end and the carboxyterminal end of the polypeptide (Fig. 4B). Application of the rules of Chou and Fassman (1978) predicts a high content of β strands as a major secondary structure element for the entire amino acid sequence (not shown).

Computer-assisted sequence comparisons revealed that the extreme hydrophobic amino terminal section (homology region 1 in Fig. 4C) of the predicted sequence has a limited similarity (29% identities in 24 amino acids) with the flavivirus encoded glycoprotein NS1 that is found on the surface of flavivirus-infected cells (Hahn *et al.*, 1988). The sequence of hydrophobic amino acids, included in homology region 2 of Fig. 4C, is similar (39% identity in 23 amino acids) to a hydrophobic aminoterminal part of an envelope protein of hepatitis C virus (Takamizawa *et al.*, 1991), and the more centrally located stretch of amino acids 140 to 230 (homology region 3, Fig. 4C) shares 20 of 90 amino acids with an external part of a mammalian membrane-associated surface glycoprotein (Brümmendorf *et al.*, 1989). Finally, computer analysis indicates that the extreme carboxyterminal hydrophobic region (Fig. 4B) of the predicted amino acid

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sequence is similar to the transmembrane domains of a number of membrane-associated proteins as diverse as apoprotein p680 of photosystem 11 from different plants (33% identity in an 18-amino-acid sequence) (Offermann-Steinhard and Herrmann, 1990) and neural cell adhesion glycoproteins (36% identity in 33 amino acids) (Miura *et al.*, 1991).

Even though the similarities to other viral and cellular membrane proteins are limited, they could indicate that the open reading frame of Fig. 4A may encode an EsV coat protein. We therefore expressed the major part of the open reading frame (the *Hind*III-*Bam*HI subfragment of Fig. 3) in bacteria and used the bacterially expressed and purified polypeptide as an antigen to raise antibodies in rabbits.

The specificity of the antibodies was tested with extracts from bacteria expressing the EsV sequence. The EsV antibodies but not preimmune antibodies specifically reacted in Western blots with a polypeptide expressed in bacteria induced to synthesize the EsV open reading frame (Fig. 5). The apparent molecular

weight of the immunoreactive polypeptide is close to 100 kDa, significantly higher than the molecular weight as calculated from the predicted amino acid sequence. This discrepancy may be due to high local concentrations of negatively charged amino acids (see Fig. 4B) which could prevent the binding of sufficient amounts of sodium dodecyl sulfate (Matagne *et al.*, 1991).

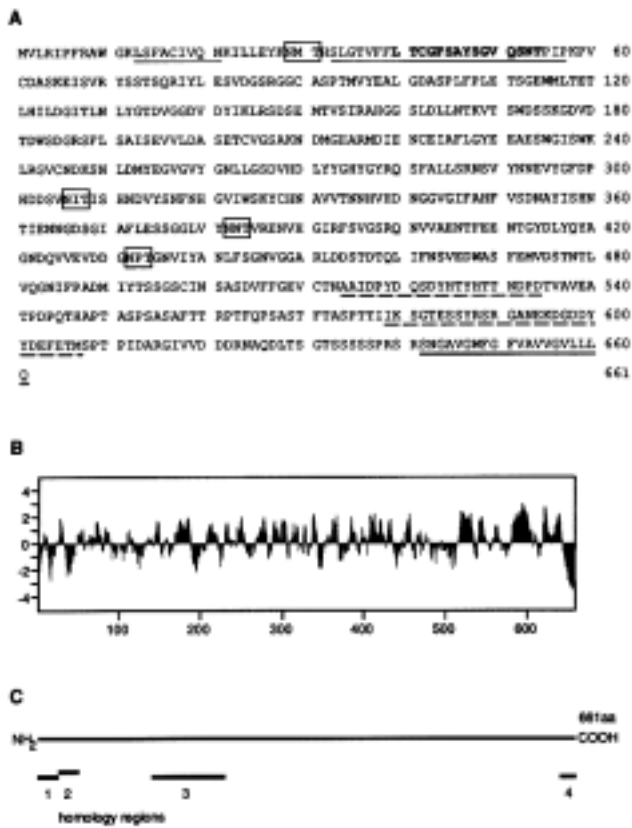


FIG. 5. Bacterial expression of the EsV gpl-sequence. (Left) The open reading frame between the *HindIII* and BamH1 restriction sites as shown at the bottom of Fig. 3 was linked to the multiple cloning site of expression vector pRSET. We show a silver-stained polyacrylamide gel (8%) with equal amounts of proteins from uninduced and induced bacteria. (Right) The bacterially expressed EsV-protein was used as an antigen to raise antibodies in rabbits. Proteins from induced bacteria were electrophoretically separated on polyacrylamide gels (10%), blotted onto nylon membranes, and probed with EsV-protein antibodies or with preimmune control antibodies as indicated (Towbin *et al.*, 1979).

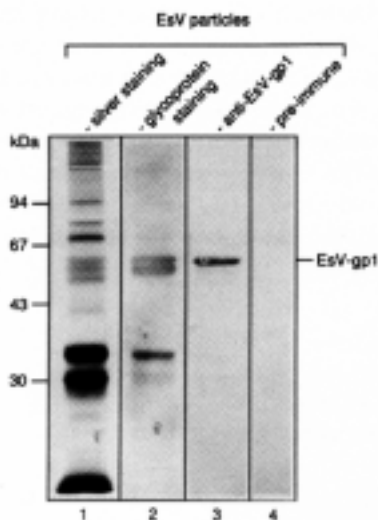


FIG. 6. Identification of glycoprotein-1 as a viral structural protein. Percoll-purified EsV was disrupted in SDS and investigated by denaturing polyacrylamide gel (10%) electrophoresis (Laemmli, 1974). Lane 1, silver staining; lane 2, blotting on nylon membrane and staining according to Gershoni *et al.* (1985) for the identification of glycoproteins; lane 3, blotting and probing with EsV protein-specific antibodies; lane 4, probing with preimmune control antibodies (see Fig. 5).

We have directly determined the presence of the protein in EsV particles as prepared by the Percoll procedure. Viral particles were disrupted in the presence of β -mercaptoethanol and SDS and investigated by denaturing gel electrophoresis (Laemmli, 1974). We added identical aliquots of the SDS-treated virus preparation to several adjacent lanes of the polyacrylamide gel and stained one lane with silver salts (lane 1, Fig. 6). The proteins in other lanes were transferred to nylon membranes for the identification of glycoproteins and for immunoreactions with specific and preimmune control antibodies. Using the enzyme-hydrazide method of Gershoni *et al.* (1985) we could identify three glycoproteins with apparent

molecular weights of about 60, 56, and 35 kDa (lane 2, Fig. 6). The specific antibodies strongly reacted with the largest of the three EsV glycoproteins (lane 3, Fig. 6). We refer to this protein as EsV glycoprotein-1 or EsV-gpl.

The relationship between glycoprotein-1 and the bacterially expressed protein was supported by biochemical means using endoproteinase Lys-C. Proteolytic fragments of identical electrophoretic properties were obtained after Lys-C treatment of glycoprotein 1 (eluted from a polyacrylamide gel; see Fig. 6) and of the bacterially expressed polypeptide (Figs. 5, 7).

We conclude that the open reading frame of 1983 bases (Fig. 4) corresponds to the EsV gene-encoding viral glycoprotein-1.

There is, however, an important discrepancy between the apparent molecular weight of 60 kDa, electrophoretically determined for the mature glycoprotein-1, and the molecular weight of 72 kDa, predicted from the open

reading frame. Presently, we cannot explain this difference, but processing of a precursor translation product may be a realistic possibility (Kräusslich and Wimmer, 1988). In fact, we could identify in the aminoterminal part of the predicted gpl sequence a signal sequence cleavage site, characteristic for proteins which are processed during the passage of membranes (Fig. 4) (Heijne, 1986). However, further experiments must show whether this site is used for the processing of glycoprotein 1, or, more generally, how the mature viral structural protein is related to its larger precursor protein.

We have incubated Percoll-purified EsV particles with the gpl-specific antibodies to determine possible effects on viral infectivity. The results obtained were inconclusive: we observed an antibody-specific reduction of infected plants with some viral preparations, but no effects of the antibody on the infectivity of other preparations. A possible explanation may be that the presence of viral glycoprotein-1 (or its antigenic part) on the surface of viral particles varies with different preparations, depending, for example, on the status of virus assembly or maturation at the time when plants are disrupted for the isolation of virus. This possibility is supported by a comparison of the structural proteins in the virus preparation used for the electrophoretic analysis in Fig. 2 (lane 2) with those in the virus preparation in Fig. 6 (lane 1): the two independent virus preparations contain identical protein components, but in different relative amounts.

DISCUSSION

We have described an improved method for isolating EsV from infected and diseased algae. Using this method, we avoid an exposure of PEG-precipitated virus to the high ionic strength of CsCl solutions and use

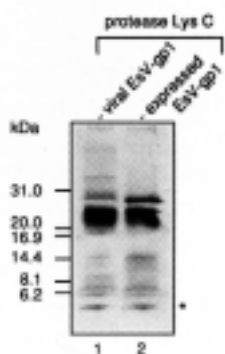


FIG. 7. Proteolysis. Glycoprotein 1 was identified on a Laemmli gel as shown in Fig. 6 (lane 1). The corresponding gel slice was excised and added to a slot of a Tricine-SDS gel (Schägger and von Jagow, 1987). A parallel lane received 1.5 Mg of the bacterially expressed protein. Proteins in both lanes were then treated with 1 Mg protease Lys C (Boehringer Mannheim) for 30 min. Electrophoresis was performed as described (Schägger and von Jagow, 1987). Peptides were visualized by silver staining. Asterisk, polypeptide from the proteinase preparation used.

instead Percoll-centrifugation as the final purification step. The improved method yields viral particles with higher protein/DNA ratios than those of CsCl-purified viruses. It appears therefore that high CsCl concentrations disrupt the viral protein shell. We found that treatment with CsCl interferes with the biological activity of EsV.

Percoll-purified viruses are composed of at least 16 electrophoretically detectable structural proteins. Some of these proteins are rather intensely stained by silver salts and are therefore present in many copies per particle (Figs. 2, 6). These abundant proteins are most likely the major building blocks forming the characteristic twolayered shell of densely packed virus particles in the infected cell (Müller *et al.*, 1990) and of isolated infective viruses. But we have no information yet concerning the distribution of these proteins between the thin outer layer and the broader, more diffuse inner layer of an EsV particle (Fig. 1).

It could be assumed that the three viral glycoproteins, identified by the specific staining procedure of Gershoni *et al.* (1985), may be components of the outer shell because glycoproteins are frequently found as constituents of viral surfaces. However, viral glycoprotein-1 as well as glycoprotein-2, the second largest EsV glycoprotein, were also detected in CsCl-purified viral structures (Fig. 2; Lanka *et al.*, 1993) and may therefore be located in an inner protected part of the viral particle. In contrast, the third glycoprotein (apparent molecular weight: 35 kDa), which is much more abundant than glycoproteins-1 and -2 in native virus preparations (Fig. 6), was not detected in CsCl-purified EsV (Lanka *et al.*, 1993) and could therefore be located at the viral surface.

As a first step in analyzing the genetics of EsV, we identified an open reading frame that could encode glycoprotein-1, the largest of the three EsV glycoproteins. Our identification relies on two pieces of evidence: first, antibodies against the bacterially expressed polypeptide strongly and specifically react with glycoprotein-1; second, *in vitro* proteolysis produces identical peptides after treating the bacterially expressed polypeptide and glycoprotein-1 with endoprotease Lys-C. In addition, the predicted amino acid sequence contains several motifs known to be sites for N-glycosylation. The sequence has also several features commonly found in membrane-associated cellular and viral glycoproteins. This includes a hydrophobic carboxyterminal domain characteristic for proteins integrated in a lipid bilayer. Presently, however, we do not know whether the protein is anchored to a lipid membrane.

Determination of the nucleotide sequence in and around the *gpl*-gene gives a first glimpse at an EsV gene structure. The open reading frame is not interrupted by introns, and the upstream region of the *gpl*-gene is unusually rich in AT base pairs quite similar to the corresponding region of a *Chlorella* virus coat protein gene.

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(Graves and Meints, 1992) but the implications for gene expression and regulation have yet to be worked out.

From a more practical point, the known sequence of a piece of the EsV genome offers the opportunity to use PCR assays, which will be of great value in investigating the distribution of EsV in algal populations from natural habitats and in obtaining important new information concerning the ecological consequences of EsV induction and infection.

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REFERENCES

- BRÜMMENDORF, T., WOLFF, J. M., FRANK, R., and RATHJEN, F. G. (1989). Neural cell recognition molecule Fl 1: Homology with fibronectin type III and immunoglobulin type C domains. *Neuron* 2, 1351-1361.
- CHOU, P. Y., and FASMAN, G. D. (1978). Empirical predictions of protein conformation. *Annu. Rev. Biochem.* 47, 251-276.
- DUBOCHET, J., and GROOM, M. (1982). The mounting of macromolecules for electron microscopy with particular reference to surface phenomena and the treatment of support films by glow discharge. *Adv. Opt. Electron Microsc.* 8, 107-135.
- FRIESS-KLEBL, A. K., KNIPPERS, R., and MÜLLER, D. G. (1994). Isolation and characterization of a DNA virus infecting *Feldmannia simplex* (Phaeophyceae). *J. Phycol.* 30, 653-658.
- GERSHONI, J. M., BAYER, E. A. and WILCHEK, M. (1985). Blot analyses of glycoconjugates: Enzyme-hydrazide, a novel reagent for the detection of aldehydes. *Anal. Biochem.* 146, 59-63.
- GRAVES, M. V., and MEINTS, R. H. (1992). Characterization of the major capsid protein and cloning of its gene from algal virus PBCV-1. *Virology* 188, 198-207.
- HAHN, Y. S., GALLER, R., HUNKAPILLAR, T., DALRYMPLE, J. M., STRAUSS, J. H., and STRAUSS, E. G. (1988). Nucleotide sequence of Dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. *Virology* 162, 167-180.
- HARLOW, E., and LANE, D. (1988). "Antibodies. A Laboratory Handbook." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- HART, G. W., BREW, K., GRANT, G. A., BRADSHAW, R. A., and LENNARZ, W. J. (1979). Primary structural requirements for the enzymatic formation of the N-glycosidic bond in glycoproteins: Studies with natural and synthetic peptides. *J. Biol. Chem.* 254, 9747-9753.
- HASCHEMEYER, R. H. (1970). Electron microscopy of enzymes. *Adv. Enzymol.* 33, 71-118.
- HEJNE, V. G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14, 4683-4690.
- HENRY, E. C., and MEINTS, R. H. (1992). A persistent virus infection in *Feldmannia* (Phaeophyceae). *J. Phycol.* 28, 517-528.
- KRÄUSSLICH, H. G., and WIMMER, E. (1988). Viral proteinases. *Annu. Rev. Biochem.* 57, 701-754.
- L-AEMMLI, U. K. (1974). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- LANKA, S. T. J., KLEIN, M., RAMSPERGER, U., MÜLLER, D. G., and KNIPPERS, R. (1993). Genome structure of a virus infecting the marine brown alga *Ectocarpus siliculosus*. *Virology* 193, 802-811.

- MATAGNE, A., JORIS, B., and FRI-RE, J. M. (1991). Anomalous behaviour of a protein during SDS/PAGE corrected by chemical modification of carboxylic groups. *Biochem. J.* 280, 553-556.
- MIURA, M., KoBAYASHI, M., Asou, H., and UYEMURA, K. (1991). Molecular cloning of cDNA encoding the rat neural cell adhesion molecule LI. *FEBS Lett.* 289, 91-95.
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- MÜLLER, D. G. (1991 a). Marine viroplankton produced by infected *Ectocarpus siliculosus* (Phaeophyceae). *Marine Ecol. Progr. Series* 76, 101-102.
- MÜLLER, D. G. (1991 b). Mendelian segregation of a virus genome during host meiosis in the marine brown alga *Ectocarpus siliculosus*. *J. Plant Physiol.* 137, 739-743.
- MÜLLER, D. G., KAWAI, H., STACHE, B., and L-ANKA, S. (1990). A virus infection in the marine brown alga *Ectocarpus siliculosus* (Phaeophyceae). *Botanica Acta* 103, 72-82.
- MÜLLER, D. G., and STACHE, B. (1992). Worldwide occurrence of virusinfections in filamentous marine brown algae. *Helgoländer Meeresuntersuchungen* 46, 1-8.
- MÜLLER, D. G., and FRENZER, K. (1993). Virus infections in three marine brown algae: *Feldmannia irregularis*, *F. simplex*, and *Ectocarpus siliculosus*. *Hydrobiologia* 260/261, 37-44.
- OFFERMANN-STEINHARD, K., and HERRMANN, R. G. (1990). Nucleotide sequences of psbB and psbH, the plastid encoded genes for CP47 and the 10kDa phosphoprotein of photosystem 11 in *Oenothera hookeri* and *argillicola*. *Nucleic Acids Res.* 18, 6452.
- SAMBROOK, J., FRITSCH, E. F., and MANIATIS, T. (1989). "Molecular Cloning: A Laboratory Handbook." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- KLEIN ET AL.
- SANGER, F., NICKLEN, S., and COULSEN, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74,5463-5467.
- SCHAGGER, H., and VON JAGOW, G. (1987). Tricine-sodium dodecylsulfatepolyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166, 368-379.
- SCHUSTER, A. M., GRAVES, M., KORTH, K., ZIEGELBEIN, M., BRUMBAUGH, J., GRONE, D., and MEINTS, R. H. (1990). Transcription and sequence studies of a 4.3 kbp fragment from a dsDNA eukaryotic algal virus. *Virology* 176, 515-523.
- STARR, R. C., and ZEIKUS, J. A. (1993). UTEX-The culture collection of algae at the University of Texas at Austin. *J. Phycol.* 29 (Suppl.), 1 - 106.
- TAKAMIZAWA, A., MORI, C., FUKE, I., MANABE, S., MURAKAMI, S., FUJITA, J., ONISHI, E., ANDOH, T., YOSHIDA, I., and OKAYAMA, H. (1991). Structure and organization of the hepatitis C virus genome isolated from human carriers. *J. Virol.* 65, 1105-1113.
- TOWBIN, H. T., STAHELIN, T., and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350-4354.
- WRAY, VV., BOULIKAS, T., WRAY, V., and HANCOCK, R. (1981). Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118, 197-203.