Virology 273, 383–390 (2000)
doi:10.1006/viro.2000.0383, available online at http://www.idealibrary.com on IDEAL®

The Brown Algal Virus EsV-1 Particle Contains a Putative Hybrid Histidine Kinase

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Received April 4, 2000; returned to author for revision April 10, 2000; revised April 25, 2000

The *Ectocarpus siliculosus* virus, EsV-1, occurs worldwide in all populations of the filamentous marine brown alga *E. siliculosus.* We have screened an expression library of EsV-1 restriction fragments and identified a DNA clone with the potential to code for a 52-kDa histidine protein kinase. The derived amino acid sequence includes all homology boxes diagnostic for histidine protein kinases and, in addition, amino acid motifs that are commonly found in response regulators of bacterial two-component signal transduction proteins. Thus, the novel viral protein can be classified as a hybrid histidine protein kinase of a type that has previously been detected in fungi, slime molds, and plants. By using purified antibodies, we found that the protein with its potential kinase activity is located on the outer shell of viral particles. This is the first report on a two-component regulator-like protein in viruses and could provide the basis for speculations with regard to the evolution of EsV-1 and related viruses.

INTRODUCTION

The *Ectocarpus siliculosus* virus (EsV-1) is pandemic in populations of the marine brown alga *E. siliculosus* on all coasts of temperate climate zones. The genomes of EsV-1 and of other viruses infecting marine brown algae (phaeoviruses) are double-stranded DNA molecules ranging from 160 to 340 kilobase pairs (kb) in size with the potential to encode hundreds of proteins. A considerable number of these proteins may be necessary to orchestrate the peculiar infection cycle of these viruses (review: Müller *et al.,* 1998).

Studies under controlled laboratory conditions show that EsV-1 and other phaeoviruses only infect the walless, free-swimming spores or gametes of the algal host (Müller *et al.,* 1998). Strong evidence indicates that the viral DNA is incorporated into the genome of the infected cell and transmitted to all cells of the developing organism (Müller, 1991a; Bräutigam *et al.,* 1995; Delaroque *et al.,* 1999). The viral DNA remains dormant in the vegetative cells of the adult organism, but can be expressed in cells of the reproductive organs, sporangia, and gametangia. Virus multiplication begins with an extensive replication of viral DNA in the cell nuclei which increase in size and eventually break down (Müller *et al.,* 1998; Wolf *et al.,* 1998). Virions are assembled subsequent to nuclear disintegration until the host cell is filled with closely packed mature viral particles. Virus release is triggered by environmental cues such as changes of culture medium or temperature. The same stimuli cause the release of gametes or spores from uninfected brown algae (Müller, 1991b). Thus, free viruses have a short window of opportunity to meet their susceptive host cells before being dispersed in the surrounding sea water. The synchronous release of viruses and host cells is an intriguing ecological situation which may contribute to the widespread distribution of EsV-1 in the *Ectocarpus* populations at all ocean coasts (Sengco *et al.,* 1996).

Thus, the infection process, the host-stage-restricted multiplication, and the timely release of virions from the infected host are precisely coordinated to guarantee that virus and host meet under rather specific ecological conditions. This implies that the virus genome should encode factors that are able to receive and respond to signals from within and without the host.

As a first step to address this point, we report here that EsV-1 encodes a protein with high structural similarities to a histidine protein kinase, a constituent of the two-component signal transduction pathway that was originally discovered in bacteria (Hoch and Silhavy, 1995), but was later shown to occur also in a number of eukaryotes, including yeast, fungi, the slime mold *Dictyostelium,* and plants (Chang and Meyerowitz, 1994; Loomis *et al.,* 1997). We also show that the EsV-1-encoded putative histidine protein kinase is associated with the outer layer of the virion. This is the first report on a virus-encoded two-component signal transduction protein.

RESULTS

Screening of an EsV-1 expression library

We have screened an expression library of EsV-1 restriction fragments with 1000-fold diluted primary serum against detergent-disrupted purified virus particles. As

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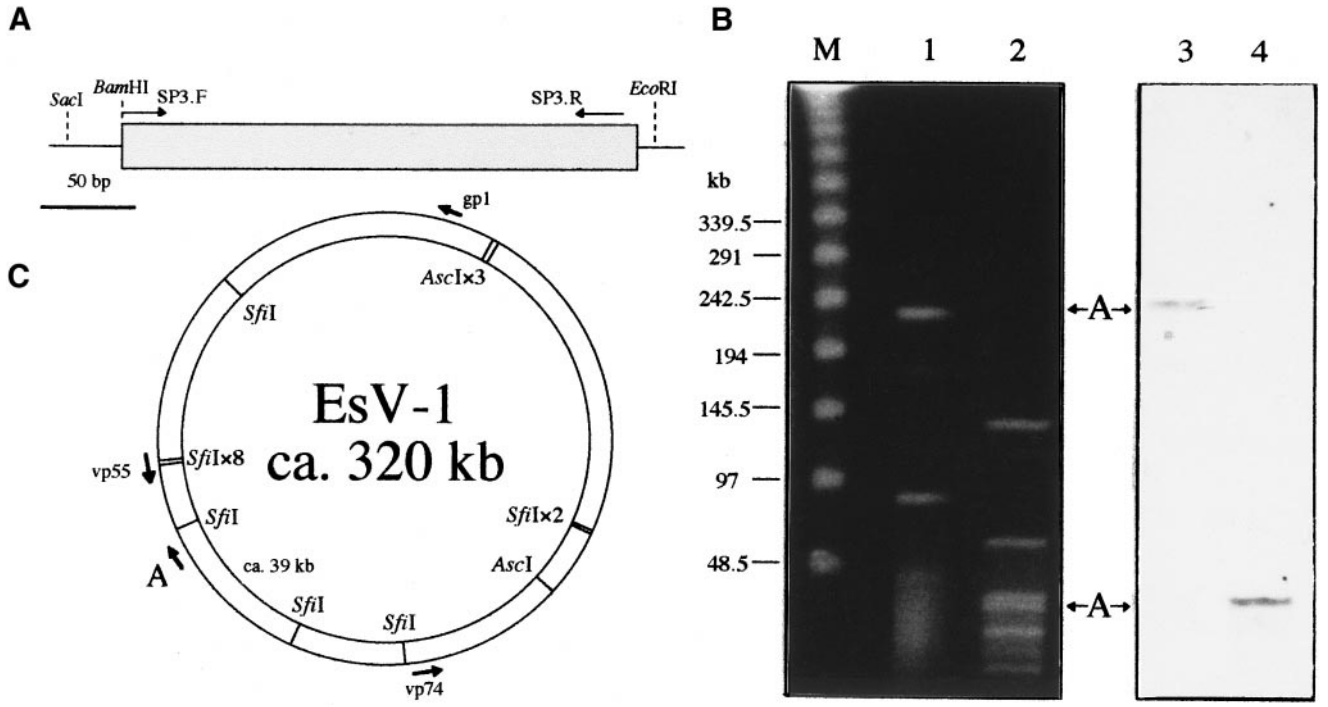

FIG. 1. A novel segment of the EsV-1 genome. (A) Fragment A was isolated by screening an EsV-1 expression library with antibodies raised against purified viral particles. SP3.F and SP3.R, forward and reverse primers for PCR amplification. (B) EsV-1 DNA was restricted with endonucleases *Asc*I (lane 1) and *Sfi*I (lane 2) and investigated by pulse-field agarose gel electrophoresis (Lanka *et al.,* 1993) using phage λ concatemers as DNA size markers (M; New England Biolabs, Beverly, MA). The gel was transferred to Nylon membranes (Hybond-N+, Amersham, Uppsala, Sweden) and probed with digoxigenin-labeled fragment A DNA (Roche, Mannheim, Germany; lane 3, *Asc*I-restricted DNA; lane 4, *Sfi*I-restricted DNA; Southern, 1975). (C) Restriction map of EsV-1 DNA. Locations of the fragment A sequence and the previously mapped viral structural proteins gp1 (Klein *et al.,* 1995), vp55, and vp74 (Delaroque *et al.,* 2000).

expected, we isolated several genomic EsV-1 fragments with the potential to code for major structural proteins (Delaroque *et al.,* 2000) and, in addition, a fragment of 283 base pairs (fragment A; Fig. 1A) with an open reading frame (ORF) encoding amino acid sequences known from bacterial and eukaryotic histidine protein kinases.

To confirm that the isolated fragment A-DNA was part of the viral genome, we restricted EsV-1 DNA with *Sfi*I and *Asc*I (Fig. 1B, lanes 1 and 2) and used fragment A as a probe in Southern blot experiments (Southern, 1975). The results obtained were consistent and showed that fragment A hybridized with a 227-kb *Asc*I fragment and a 39-kb *Sfi*I fragment (Fig. 1B, lanes 3 and 4). With additional sequence information (not shown) we were able to more precisely map the fragment A sequence as shown in Fig. 1C which also demonstrates that the fragment A locus is not linked to the previously mapped genes for the EsV-1 structural proteins gp1 (Klein *et al.,* 1995), vp55, and vp74 (Delaroque *et al.,* 2000).

The fragment-A-encoded polypeptide occurs in viral particles

We used purified antibodies against the fragment-A-encoded polypeptide as probes in immunoblot experiments. To demonstrate the specificity of the antibodies, we analyzed an extract from bacteria overexpressing the fragment A polypeptide and found that the monospecific antibodies reacted well with the an-

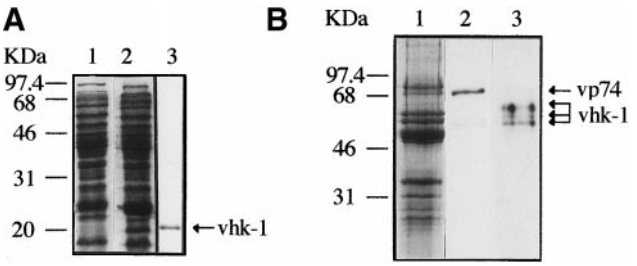

FIG. 2. The fragment-A-encoded polypeptide as a constituent of virions. (A) Characterization of antibodies. Fragment A was cloned in a plasmid vector and expressed in bacteria (lane 1, bacterial extract before induction; lane 2, bacterial extract after induction; Coomassie stain of a polyacrylamide gel). The expressed polypeptide was used as antigen to raise antibodies in rabbits. The purified antibodies (diluted 1:5000) detect in Western blots of induced bacterial extracts only the fragment-A-encoded vhk-1 polypeptide (lane 3). (B) Purified EsV-1 particles were analyzed by denaturing polyacrylamide gel electrophoresis (lane 1, Coomassie stain; lane 2, Western blot with 5000-fold diluted vp74-specific antibodies; lane 3, Western blot with 1000-fold diluted fragment-A-specific antibodies). Left margins, electrophoretic size markers; right margins, vhk-1, the fragment-A-encoded polypeptide; vp74, viral protein 74 (Delaroque *et al.,* 2000).

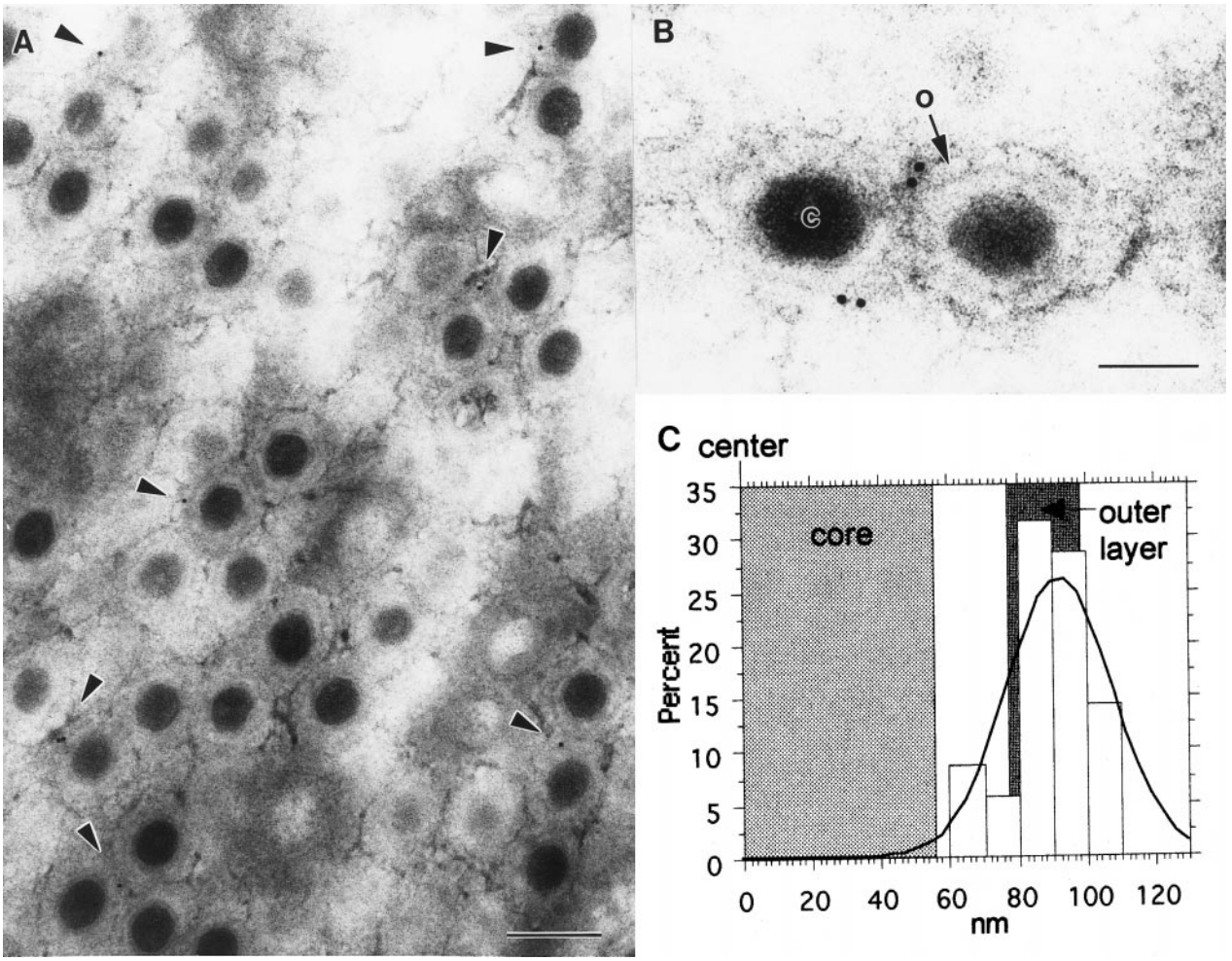

FIG. 3. Location of vhk-1. (A) Electron micrograph: section through a virus-producing host cell. Arrowheads, gold particles. Scale bar, 0.2 μm. (B) Virions with the central core (c) and outer layer (o) labeled by gold particles. Scale bar, 0.1 μm. (C) Distribution of gold particles. Absissa, distance from the center of the viral core. Shaded areas correspond to nucleoprotein core (center to surface) and outer viral layer. Ordinate, percentage of gold particles. The total number of gold particles determined was 35.

tigen, but not with any one of the many bacterial proteins on the blot (Fig. 2A).

Next, we investigated purified EsV-1 particles by immunoblotting using the fragment-A-specific antibody as probe. As a control, we used antibodies specific for the vp74 structural protein (Delaroque *et al.,* 2000; Fig. 2B, lane 2). The anti-fragment-A-specific antibodies gave a comparatively weak staining of three closely spaced bands with apparent molecular weights between 52 and 58 kDa (Fig. 2B, lane 3). Increasing the concentration of fragment A antibodies did not result in a stronger immunological response (not shown) suggesting that the fragment-A-encoded polypeptide may be a minor constituent of the viral particle, at least in comparison to vp74.

To determine the location of the fragment A polypeptide in virus particles, we investigated thin sections of virus-producing cells by electron microscopy. As demonstrated before, EsV-1 particles appear pentagonal or hexagonal in sections and are composed of a nucleoprotein core surrounded by several electron-dense layers (Müller *et al.,* 1998; Wolf *et al.,* 1998). We used the purified antibodies against the fragment A polypeptide to localize the corresponding protein in virus particles with immunogold electron microscopy.

The average labeling was 0.2 gold particles/virion which is considerably less than the 1.0 gold particles/virion when using vp74-specific antibodies under identical conditions (not shown; Delaroque *et al.,* 2000). This difference is consistent with the immunoblot experiments of Fig. 2 and shows again that the fragment A polypeptide is a minor constituent of the virion.

To demonstrate the specificity of the gold labeling in Fig. 3, we compared virus-producing cells and nonproducing somatic filament cells on the same section. The average density of gold particles on sections of virus-producing cells was 2 particles/μm^2, compared to only 0.2 particles/μm^2 on sections of healthy cells. An average density of 0.2 gold particles/μm^2 was also determined in control experiments when only secondary an-

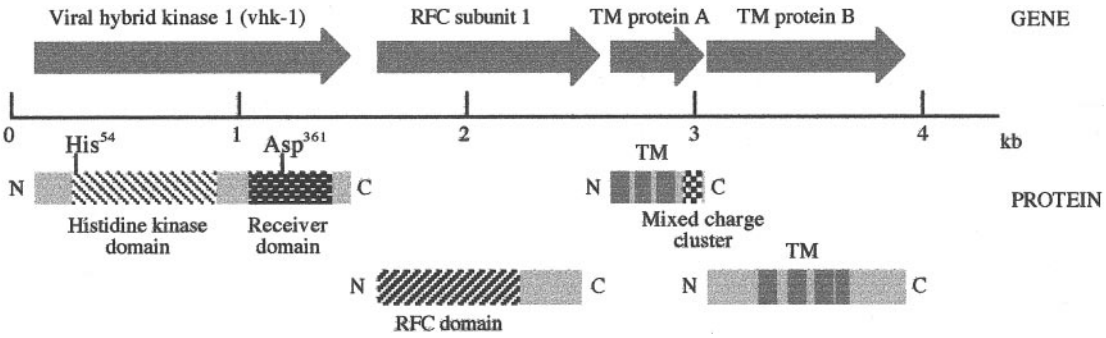

FIG. 4. Section of the EsV-1 genome including the fragment-A-containing gene. A ca. 4-kb segment of the EsV-1 genome was isolated and sequenced (Accession Number AF210454). We identified four open reading frames (arrows) and compared the encoded amino acid sequences with data banks. The results of the data searches are noted in the protein symbols: N, amino terminal ends; C, carboxy terminal ends; TM, hydrophobic transmembrane regions; RFC, replication factor C. The deduced viral hybrid histidine kinase (vhk-1) contains the homology boxes, diagnostic for histidine protein kinases (hatched box: His^{54}, the putative phospho-accepting histidine residue) and for the receiver domain of the response regulator (stippled box: Asp^{361}, the putative phospho-accepting aspartate residue).

tibodies were used and therefore defines the background in these experiments.

The specificity of the reaction is further demonstrated by the nonrandom distribution of gold particles, which are almost exclusively found on the outer layer of viral particles. The mean radius of gold particle distribution was 92.5 ± 15.3 nm (n = 35). The outer layer was determined to lay between 77.3 ± 3.2 and 97.8 ± 3.4 nm from the center of the viral nucleoprotein core (Fig. 3C).

Thus, we can conclude that the protein encoded by the fragment A DNA is a constituent of the outer layer of EsV-1 particles.

DNA sequences

We used fragment A as a hybridization probe to isolate a ca. 4-kb section of the EsV-1 genome. Sequencing revealed that this section includes four ORFs (Fig. 4). BLAST program-assisted searches (Altschul *et al.,* 1990) of the EMBL GenBank and the Swissprot databases suggested that one ORF can encode a protein with similarities to the small subunits of the eukaryotic replication factor C (RFC; review: Mossi and Hubscher, 1998) while two additional ORFs could encode proteins related to several of the transmembrane proteins in the databases.

The fourth ORF, which includes the fragment A sequence, has the potential to code for a protein of 461 amino acids with a calculated molecular weight of 52,464 Da. This value is in agreement with the apparent molecular weight of 52 kDa as determined for the faster migrating form of the three immunologically detectable bands in the Western blot of Fig. 2B (lane 3). The nature of the slower migrating forms cannot yet be accounted for. We note, however, that the predicted sequence contains close to its amino terminal end the N-X-T motif (Fig. 5A) as a putative asparagine-linked glycosylation site. Thus, a fraction of the protein could be glycosylated which would be in agreement with its location at the surface of the viral particle where other EsV-1 glycoproteins may also reside (Klein *et al.,* 1995).

Computer-assisted comparisons with data banks revealed that the deduced amino acid sequence contains regions that are closely related to the conserved elements in two-component regulator proteins (Fig. 5). Alignments with other members of this large protein family demonstrate the presence of variations of the highly conserved homology boxes of the histidine protein kinase domain including homology box H with the phospho-accepting histidine residue (Fig. 5B). In addition, the EsV-1 protein also contains signature sequences of the receiver domain with its phospho-accepting aspartate residue (Fig. 5B). Thus, the EsV-1-encoded protein belongs to the class of "hybrid histidine protein kinases" that combine on one polypeptide chain a histidine protein kinase with elements of the response regulator. We designate this novel EsV-1 protein as vhk-1 for *viral hybrid kinase* 1.

To support this conclusion we used the PSIPRED program for secondary structure predictions (Jones, 1999) and determined that the histidine protein kinase part of the vhk-1 could form an alternating α/β structure of four α-helices and seven β-sheets (Fig. 5C), very similar to the catalytic core of the classic bacterial EnvZ histidine kinase domain whose three-dimensional structure has been determined by X ray crystallography (Tanaka *et al.,* 1998). Furthermore, the carboxyterminal response regulator domain of vhk-1 could fold into a series of secondary structure elements similar to those that characterize the crystallized bacterial CheY response regulator (Stock *et al.,* 1989).

DISCUSSION

We show here for the first time that a viral genome encodes a protein with homologies to enzymes of the two-component signal transduction pathway. The classic two-component pathway has originally been described

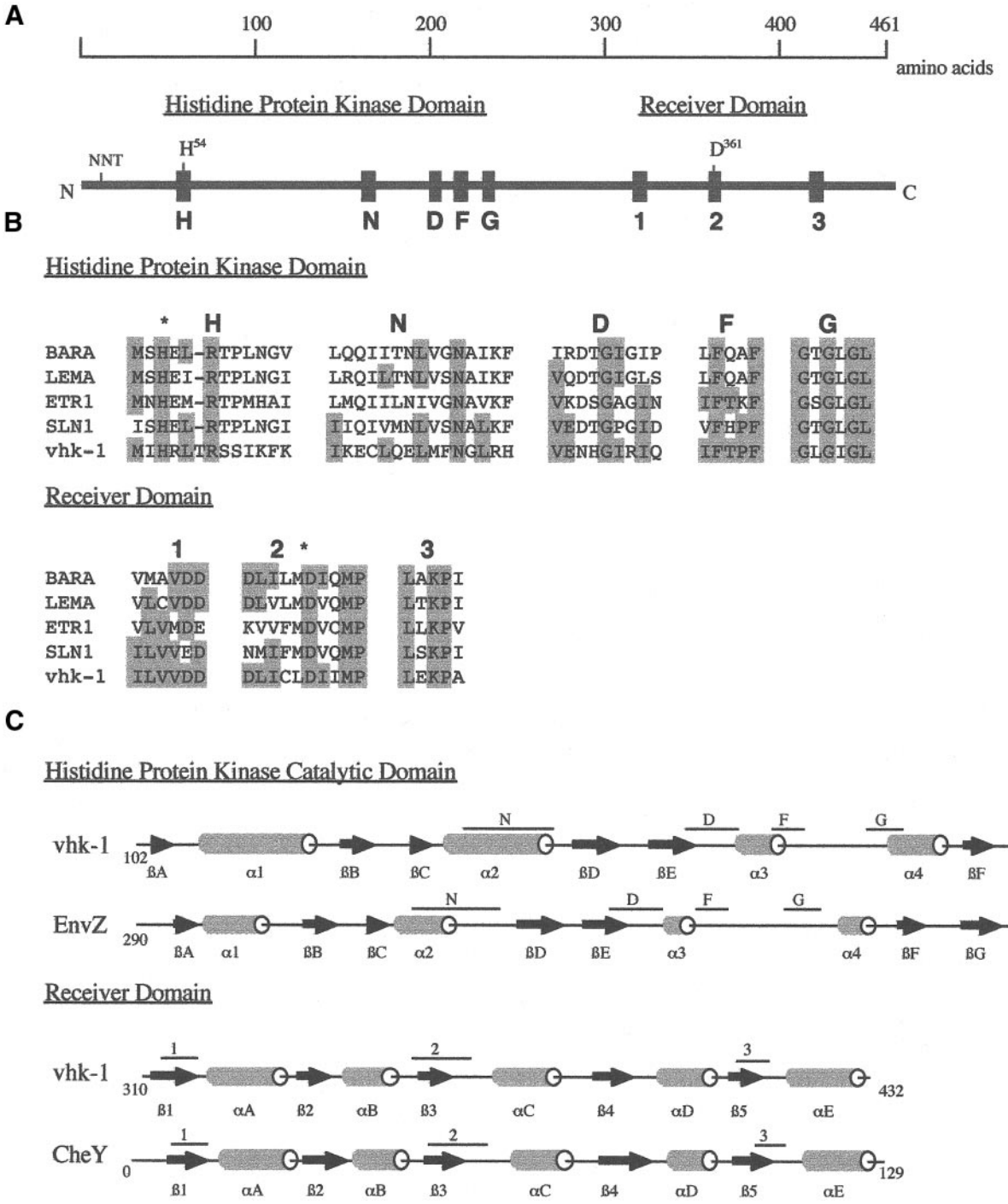

FIG. 5. Structural characterization of the viral hybrid histidine kinase. (A) The encoded protein is schematically drawn as a horizontal line with the diagnostic homology boxes of a histidine protein kinase (H, N, D, F, and G) and of a response regulator (1, 2, and 3). The presumed phospho-accepting residues (H^{54} and D^{361}) and the single glycosylation site (NNT) are indicated. (B) The homology boxes of the EsV-1 protein (vhk-1) in comparison with other hybrid kinases: *BARA* (*Escherichia coli* BarA; Nagasawa *et al.,* 1992); *LEMA* (*Pseudomonas syringae*; Hrabak *et al.,* 1992); *ETR1* (*Arabidopsis thaliana*; Chang *et al.,* 1993); *SLN1* (*Saccharomyces cerevisiae*; Ota and Varshavsky, 1993). Asterisks, phospho-accepting amino acids. (C) Prediction of secondary structure elements. The PSIPRED program was used to predict α-helices (cylinders) and β-strands (arrows) in the sequence of the viral vhk-1 protein 1 (Jones, 1999). Numbers refer to the amino acid positions defined under (A). The secondary structure elements were labeled (βA, α1, etc.) as in the crystal structures of the bacterial proteins EnvZ (Tanaka *et al.,* 1998) and CheY (Stock *et al.,* 1989). The position of the homology boxes as shown under (B) are indicated by horizontal lines.

for bacteria and consists of two proteins, a stimulus-dependent histidine protein kinase and its substrate, the response regulator. The first component frequently has two functional domains: a variable amino terminal receptor domain, which receives the external stimulus, and a conserved carboxy terminal kinase domain that auto-

phosphorylates at a conserved histidine residue. The phosphoryl group is transferred from the histidine in the first component to an aspartate residue in the amino terminal domain (receiver domain) of the second component, the response regulator. This protein typically possesses a carboxy terminal output domain with the ability for specific DNA binding or other activities. The two-component system evolved in bacteria to monitor external conditions such as fluctuations in many chemical and physical conditions (Parkinson and Kofoid, 1992). Both the histidine protein kinase and the response regulator form large families of bacterial proteins. For example, *Escherichia coli* alone can express more than 40 histidine kinase–response regulator pairs (Blattner *et al.,* 1997) with as many signaling pathways.

Enzymes, similar to the bacterial two-component proteins, have been discovered over the past years in yeast, fungi, slime molds, and plants. Many of the eukaryotic enzymes and several bacterial enzymes show a characteristic variation of the classic molecular architecture. These forms combine on one polypeptide chain the histidine protein kinase domain and a response regulator domain. They are referred as to hybrid kinases. The phosphate group on the histidine of the kinase domain is transferred to an aspartate residue in the response receiver domain which then phosphorylates the true substrate (Alex and Simon, 1994). Examples are the hybrid kinase encoded by the SLN1 gene of the yeast *Saccharomyces cerevisiae,* which is responsible for osmoregulation (Ota and Varshavsky, 1993), and the hybrid kinase, encoded by the ETR1 gene of *Arabidopsis thaliana,* which controls the ethylene response (Chang *et al.,* 1993). Clearly, vhk-1 belongs to this class of eukaryotic hybrid kinases, although it lacks the long amino terminal extension that usually precedes the histidine protein kinase domain, and its kinase activity must be demonstrated. The classification of the viral protein as a hybrid kinase is based on the presence of several conserved amino acid sequence motifs (Fig. 5).

Grebe and Stock (1999) have recently compared the primary structures of more than 300 known histidine protein kinases and showed that members of this superfamily of proteins can dramatically differ in size, amino acid sequence, and general architecture, but all share conserved sequence elements, termed homology boxes. The structure of these sequence elements allows a classification of the many known histidine kinases into 11 subfamilies. The EsV-1 protein cannot easily be associated with one of these subfamilies.

For example, the putative site for histidine phosphorylation in the H-box element of vhk-1 is preceded by an isoleucine residue rather than the usual serine and followed by an arginine instead of the usual glutamate (see Fig. 5B). A similar configuration occurs in the methanobacterial group of kinases. But another variation of the classic structure is typically found in histidine kinases from *Rhizobia* and mycobacteria, namely the replacement of the first asparagine by glutamate in the N-box of the EsV-1 enzyme (Grebe and Stock, 1999).

The replacement of the conserved proline in the H-box has been observed for histidine kinases from a variety of sources, but a major difference from the conserved elements in most histidine kinases is that the phosphoaccepting histidine in the H-box is separated by three, rather than the usual two, residues from a conserved arginine residue. It will certainly be interesting to determine how this variation affects the structure and the function of vhk-1.

The homology regions in the response regulator domain of the predicted EsV-1 protein are closely related to the corresponding regions of other hybrid kinases (Fig. 5B) and, together with the histidine kinase homologies, justify the conclusion that vhk-1 performs functions regulating steps in the EsV-1 infection cycle.

What could these functions be ? It is well known that two-component systems control the virulence genes in several bacterial pathogens. This allows the bacteria to discriminate between extra- and intracellular locations and to fully adapt to the changing conditions after invasion of the host cells before they initiate a program of growth and gene expression (Mekalanos, 1992; Charles *et al.,* 1992).

The conclusion that the putative viral histidine protein kinase is a constituent of EsV-1 particles is supported by three lines of evidence: (i) the protein was discovered in an EsV-1 expression library by antibodies against highly purified virus particles; (ii) specific antibodies recognize distinct polypeptide bands in Western blots of virion proteins; (iii) immunogold labeling of electron microscopic sections locates the protein to the outer layer of EsV-1. The location of vhk-1 may give a clue as to its role in viral infection. Vhk-1 may respond to signals received after binding to the host cell or after entering the cytoplasm as a first step to shed its protein layers and to integrate its DNA into the genome of the host. It is also possible that vhk-1 phosphorylates key proteins of the infected cell to change its metabolic properties or the organization of its intracellular structures, thereby creating a milieu suitable for the initiation of an infection process. These and other possibilities will be the subject of future work on this interesting and ecologically important group of viruses.

In summary, we note that the large and widespread superfamily of two-component proteins has a new member which is encoded by a viral genome. DNA sequences encoding the potential viral hybrid kinase are not a specialty of EsV-1, but were also detected by PCR technology in the genomes of other phaeoviruses that infect marine brown algae (unpublished results). They are, however, clearly absent in *Chlorella* viruses, which share many structural properties with phaeoviruses, but have a fundamentally different infection cycle (Müller *et*

al., 1998; Van Etten and Meints, 1999). This invites interesting speculations on the origin of the vhk-1 gene in phaeoviruses. One possibility is that the vhk-1 gene was originally part of the host genome from where it was transferred to the evolving viral genome.

MATERIALS AND METHODS

Preparation of virus

The cultivation of algal cells and the production of viruses have been described (Lanka *et al.,* 1993; Kapp *et al.,* 1997). The purification of virus particles (Klein *et al.,* 1995) and their investigation by denaturing polyacrylamide gel electrophoresis (Laemmli, 1970) and immuno ("Western")-blotting (Towbin *et al.,* 1979) were performed according to published procedures. The immunopositive proteins were detected using the ECL Western blotting system (Amersham, Uppsala, Sweden).

Viral DNA and cloning

Viral DNA was prepared by deproteinization of viral particles in agarose blocks (Lanka *et al.,* 1993) and investigated by digestion with restriction endonucleases and pulse-field agarose gel electrophoresis (Lanka *et al.,* 1993). A genomic library was constructed by ligating partially *Sau*3A-digested EsV-1 DNA to λ-ZAP DNA and packaging using the Stratagene Gigapack kit (Stratagene, La Jolla, CA). The λ-expression library was screened with antibodies raised in rabbits against purified virus particles disrupted in 0.1% SDS (Harlow and Lane, 1988). We isolated several positive phage clones whose inserts were recovered by *in vivo* excision in the form of the plasmid pBK-CMV SK vector (Stratagene). Inserts were sequenced using the standard chain termination technology (Sanger *et al.,* 1977).

Antibodies

One of the inserts, termed fragment A-DNA, was amplified by PCR and ligated into the the pRSET plasmid vector (Invitrogen, Carlsbad, CA) for a preparation of bacterially expressed polypeptides (Studier and Moffatt, 1986). Antibodies against the purified fragment A polypeptide were raised in rabbits (Harlow and Lane, 1988) and were purified by immunoaffinity with sulfolink coupling gel according to the supplier's instructions (Pierce, Rockford, IL).

Electron microscopy

To prepare samples for electron microscopy, EsV-1-infected algal thalli were fixed (0.5% glutaraldehyde and 3% paraformaldehyde in cacodylate buffer with 0.2% caffeine), dehydrated, and slowly infiltrated with Lowicryl K4M (Polysciences, Warrington, PA). The samples were then flat-embedded between Aclar embedding film (Plano, Wetzlar, Germany) and polymerized by indirect UV irradiation at −20°C. Ultrathin sections (Reichert OM U3 ultramicrotome) were blocked in phosphate-buffered saline (PBS) containing 2% bovine serum albumin and then incubated with primary antibodies (1:200 dilution) for 2 h. After being washed with PBS, the sections were incubated with secondary gold-labeled antibodies (goat anti-rabbit IgG polygold 10 nm; Polysciences), washed with PBS, and fixed with 2.5% glutaraldehyde. In control experiments, the primary antibodies were omitted. Sections were stained by lead citrate (Venable and Coggeshall, 1965) and examined with a Zeiss EM 900 electron microscope. We determined on electron micrographs the radii of viral nucleoprotein cores and of the outer shells as well as the distances of gold particles to the core center. The data were statistically evaluated using the StatView program (Abacus Concepts, Berkeley, CA). Length standard was a crossgrating replica with 2160 lines/mm (Plano).

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (Mu 196/18-3). We thank Martina Baack and Ingo Maier for their suggestions and discussions and Christine Dittrich (AG Rathmayer, University of Konstanz) for sharing the EM facilities.

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