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Locus-Specific Gene Expression Pattern Suggests a Unique Propagation Strategy for a Giant Algal Virus†

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Emiliania huxleyi virus strain 86 is the largest algal virus sequenced to date and is unique among the Phycodnaviridae since its genome is predicted to contain six RNA polymerase subunit genes. We have used a virus microarray to profile the temporal transcription strategy of this unusual virus during infection. There are two distinct transcription phases to the infection process. The primary phase is dominated by a group of coding sequences (CDSs) expressed by 1 h postinfection that are localized to a subregion of the genome. The CDSs of the primary group have no database homologues, and each is associated with a unique promoter element. The remainder of the CDSs are expressed in a secondary phase between 2 and 4 hours postinfection. Compartmentalized transcription of the two distinctive phases is discussed. We hypothesize that immediately after infection the nucleic acid of the virus targets the host nucleus, where primary-phase genes are transcribed by host RNA polymerase which recognizes the viral promoter. Secondary-phase transcription may then be conducted in the cytoplasm.

Emiliania huxleyi virus strain 86, EhV-86, is the type species of the genus Coccolithovirus within the family of algal viruses, the Phycodnaviridae (14). EhV-86 is a large, double-stranded DNA virus that infects the marine coccolithophorid Emiliania huxleyi (13), a unicellular alga known for its elegant calcium carbonate scales (coccoliths) that are produced intracellularly and sequestered over its outer cell surface (21). E. huxleyi is perhaps best known for its immense coastal and open-ocean blooms at temperate latitudes and is a key species for current studies on global biogeochemical cycles and climate modeling (6, 19, 20).

We have previously sequenced the 407,339-bp genome of EhV-86 and predicted that it encodes 472 protein coding sequences (CDSs) (22). This is the largest Phycodnaviridae genome sequenced to date. The functions of the 472 CDSs are largely unknown: only 66 have been annotated with functional product predictions on the basis of sequence similarity or protein domain matches (22). Genes of known function encode DNA polymerase subunits, RNA polymerase subunits, sphingolipid biosynthesis enzymes, and eight proteases. The Phycodnaviridae family was previously thought to lack RNA polymerase; thus, the detection of six RNA polymerase subunits in EhV-86 suggested a lifestyle distinct from those of other Phycodnaviridae (1–3, 22). A lytic-phase transcriptional profile generated from E. huxleyi infected by EhV–86, 33 h postinfection, detected the presence of 65% of the predicted virus CDS transcripts (22).

In many well-studied biological systems, viral transcripts are commonly assigned into a kinetic class by their dependence on protein synthesis or viral DNA replication (5). This is usually achieved by monitoring the sensitivity of virus gene transcript levels upon exposure to inhibitory drugs (5). Through the inhibition of translation or DNA replication with chemicals such as cycloheximide and phosphonoacetic acid, transcripts can be assigned into kinetic classes such as immediate-early (α), early (β), early-late (γ1), and late (γ2). Kinetic stratification strongly correlates with functional classification. Classically, immediate-early proteins perform regulatory functions; early proteins, metabolic roles; and late proteins, structural and morphogenic roles. The culturing conditions needed to sustain optimum growth in tandem with the number of cells needed to extract total RNA in useable quantities (experiments typically involve tens of liters of culture medium) limit what can be done presently with the E. huxleyi experimental system. The application of inhibiting drugs to E. huxleyi cultures is both impractical and costly. Furthermore, the molecular biology of E. huxleyi is almost as poorly understood as the molecular biology of its virus, EhV-86. The use of drugs may have unknown affects on both host and virus systems. To build on previous genome sequencing work and to begin the task of characterizing all the EhV-86 CDSs, the majority of which are of unknown function, an expression profile of the first 4 h of infection was determined. We know from experience that mature virions are released from infected cells 4 h postinfection (data not shown); hence, this initial period was profiled in detail. By exploiting a synchronous infection and sampling early on, we avoided the need for inhibitor drugs and profiled a natural infection cycle.

MATERIALS AND METHODS

Growth conditions and RNA extraction. Experimental methodology is described in depth in the MIAME compliant database entry E-MAXD-8, available at www.arrayexpress.com. Briefly, exponentially growing Emiliania huxleyi (1 ×
10^6 cells ml^-1 was infected with EHV-86 (fresh lysate, 5 x 10^8 virions ml^-1) in t/2 medium at 15°C in a Sanyo MLR-350 incubator with a ratio of 16:8 h of light-dark illumination.

Total RNA was extracted 0, 1, 2, and 4 h postinfection, using a QIAGEN midiprep kit, and used for microarray analysis as described previously (22). Briefly, cell cultures (150 ml) were filtered (0.45-μm Supor-200 membrane filter; Pall Corporation), resuspended in 2 ml phosphate-buffered saline solution, pelleted by centrifugation (2,000 x g, 1 min), resuspended in 2 ml RNAlater (QIAGEN), and stored at −20°C until processed with the QIAGEN midiprep kit.

As a control, virus particles were checked for the presence of RNA. The virus particles were purified and concentrated as described previously (14). Briefly, virus lysate (5 l) was filtered (0.45-μm Supor-200 membrane filter; Pall Corporation), concentrated by a tangential flow ultrafiltration unit with a molecular weight cutoff of 50,000 (Vivaflow 50; Sartorius) to a final volume of 20 ml. Aliquots (3.5 ml) of the concentrate were adjusted with CsCl to densities of 1.1, 1.2, 1.3, and 1.4, and gradients from 1.1 and 1.4 were created by ultracentrifugation at 25,000 rpm at 22°C for 2 h in a SW40 Ti Beckman rotor. Virus bands were removed with a syringe and dialyzed against 4 x 1-liter volumes of 10 mM Tris-HCl, pH 7, at 4°C. To remove any contaminating RNA associated with the outside of the virus particles, the concentrate was incubated with RNaseA (final concentration, 50 μg ml^-1) for 1 h at 37°C and treated with Proteinase K (final concentration, 50 μg ml^-1) and sodium dodecyl sulfate (SDS) (final concentration, 0.5% SDS) for 1 h, then treated with SDS (final concentration, 0.5% SDS) for 1 h. Samples were then treated to extract total RNA as described previously. Purified total RNA was then treated with RNase free DNase (QIAGEN) in a 50 μl volume. Following DNase treatment, RNA was cleaned and purified, using the Roche Target Purification kit. The EhV-86 microarray has been generated on the cDNA for subsequent PCR amplification with a TAS-PCR primer. First- and second-strand cDNA synthesis was performed from 200 ng of template RNA and 10 pg of spike mRNA (Stratagene), and the product was purified using the Target Purification kit (Roche). Purified cDNA was then randomly amplified by 24 cycles of PCR with TAS primers. PCR products were purified using the Microarray Target Purification kit (Roche) and then concentrated using YM30 Microcon concentration columns (Millipore). The resulting PCR-amplified cDNA was then transcribed into Cy3-labeled cRNA, using the Microarray Target Synthesis kit (Roche). Labeled cRNA was then purified using the Microarray Target Purification kit. The EHV-86 microarray has been described previously (22). Briefly, 75-mer oligonucleotides are spotted in triplicate for 425 of the 472 predicted CDSs of EHV-86. For hybridization to microarrays, 7.5 μl 20 x SSC (Sigma), 1.0 μl 10% SDS, and the labeled cRNA sample were combined in a total volume of 50 μl. After being incubated at 100°C for 2 min (lid temperature, 110°C), the samples were cooled (room temperature, 5 min) and the volume was checked (and made up to 50 μl when necessary) prior to loading the samples onto the microarray slide covered by a LifterSlip (Erie Scientific Company). Microarray hybridization was performed in a microarray hybrid chamber (Camlab) at 65°C for 18 h. The microarray slides were given three posthybridization washes. The first wash was in 50 ml of 1 x SSC-0.1% SDS for 5 min with constant agitation, foloped by a second wash for another 5 min (with constant agitation) in 50 ml of 0.1 x SSC-0.1% SDS. A third wash was performed by plunging the slides 20 times into 50 ml of 0.1 x SSC. The microarray slides were immediately centrifuged to remove residual liquid (200 g, 1 min) and stored in the dark prior to being scanned. Three biologically independent samples for each of the four main study conditions (uninfected Emiliana huxleyi, postinfection at 0, 1, 2, 3, and 4 h) were hybridized to one array each. For validation purposes, three further arrays were hybridized with RNA extracted from purified EHV-86 virus particles, and one array was hybridized with just the Stratagene spike RNA.

**Microarray data processing and analysis.** (i) Scanning and image processing. Hybridized arrays were scanned with an Affymetrix 418 array scanner. Six scans at incremental settings (gains of 0, 5, 10, 15, 20, and 25) were performed for each array in order to determine and select the optimal scan setting, producing a high dynamic signal range without saturation (9). Images from all scans were quantified using GMS Scanner software, version 1.51.04.2, and ImaGene, version 5.6.1. All subsequent data processing and analysis steps were carried out with the R version 2.1.1 statistical programming environment (www.r-project.org) and Bioconductor version 1.6 microarray modules (www.bioconductor.org).

(ii) Background correction. Assessment of global noise and signal distributions showed evidence for a background noise gradient across some arrays. The effect was only noticeable at very low fluorescence levels and had no corresponding visible effect on signal levels. A log-linear interpolation method (Edwards) was used to correct lower-intensity values for any background carryover effects (8).

(iii) Normalization. A targeted viral array is likely to violate the assumptions that are made before applying global normalization methods; in this case it can be expected that 10% or more of the probes on the array will change expression levels between different biological conditions or time points. In anticipation of this, a large set of positive-control probes were included on the array (10 Stratagene alien probes, printed in triplicate on each of the 16 subgrids). The chosen normalization method was scaling of subset (n = 480 probes) medians to a common reference value, after an initial log 2 transformation of all data values.

(iv) Expression determination. Expression and/or not expressed calls for each gene and time point were generated on the basis of a detection threshold determined by the ninetieth percentile value of negative-control probes. A probe with an intensity value above this threshold was considered to be “on.”

(v) Differential expression and hypothesis testing. Prior to further analysis, a non-specific filter was applied to remove biologically irrelevant genes (control probes). This process reduces problems with statistical testing on multiple variables simultaneously. Of a total of 2,496 probes, 1,440 remained in the analysis such that a threshold for the detection of a change was set in this case based on the slightly less stringent eightieth percentile of negative-control probe intensities. Differential expression was determined in two ways: (i) from an uninfected baseline sample to each of the postinfection time points and (ii) between postinfection time points. A statistical test with relative robustness for small sample sizes (simple linear model enhanced by empirical Bayes) was used to test the null hypothesis of nondifferential expression for each individual gene. Simultaneous testing on large numbers of variables (genes) leads to an increased number of potentially false-positive results; a Benjamini and Hochberg P-value correction was therefore applied.

(vi) Exploratory analysis. Prior to explorative analysis, a further non-specific filter was applied to remove genes which were not “on” in at least 3 out of 18 arrays, reducing the number of probes to 1,288. Absolute expression data were grouped and visualized using a hierarchical biclustering algorithm on expression profiles of genes and samples, combined with a heat map. The clustering parameters were “1 minus correlation” as the distance measure and “average” as the linkage method. The gene clusters were standardized to have means and standard deviations of 0 and 1, respectively, across arrays.

**Microarray data accession number.** Microarray data (including microarray design, hybridization, and analysis) were stored and curated in moadLoad2 prior to submission in MAGE-ML format to the EBI ArrayExpress database (http://www.ebi.ac.uk/arrayexpress). Accession number E-MAXD-8 (10). This data is also available at the EGTD C data catalog (http://envgen.nox.ac.uk/) under accession number ega:cm000100.

**RESULTS AND DISCUSSION**

Characterization of temporal expression profile. By random amplification of mRNA extracted from infected cells and using microarrays, the expression profile of the EHV-85 infection of *E. huxleyi* was determined over the first 4 h of infection (see Table S1 in the supplemental material). RNA samples were extracted at 0, 1, 2, and 4 h postinfection. CDSs were categorized into group T1, T2, or T4, based upon whether their expression was first detected at 1, 2, or 4 h postinfection, respectively (Fig. 1). Thirty-nine CDSs were assigned to group T1, 194 CDSs to group T2, and 7 CDSs to group T4. Transcripts for a further 115 were not detected, including 51 that have previously been detected in the 33-h-postinfection lytic-phase transcriptional profile (22), suggesting that the virus infection cycle may not be fully completed at 4 h postinfection. However, the transcript for the major capsid protein gene, ehv085, was detected at 4 h postinfection, suggesting that, at
this point, the virus is in the latter stages of the infection process.

**Early transcripts.** During the preliminary annotation of the EhV-86 genome, CDSs were numbered sequentially, starting with ehv001 (bases 276 to 1,022) through to ehv468 (bases 406,039 to 406,896). The subsequent addition of any extra CDSs was achieved by naming according to the nearest CDS and adding an “A” suffix (e.g., ehv185A). Hence, the CDS number indicates the genomic location of a CDS relative to the neighboring CDSs. The 39 T1 virus transcripts are all localized to a specific region of the EhV-86 genome and have CDS numbers ranging between 218 and 366 (Fig. 1). These CDSs are found in a 104-kb section of the genome that has previously been identified as containing unique putative promoter elements known as family A repeats (3). The 151 CDSs in this region have few database homologues, and their origin and function are completely unknown (2, 3). Family A repeats are characterized by the presence of the conserved nonamer GTTCCC(T/C)AA that is found directly upstream of the predicted start of translation methionine codon (ATG) for 87 of the CDSs in this region. Expression of 39 CDSs from this region (P values < 0.01) at only 1 h postinfection, in combination with no expression from CDSs outside of this region, is a significant finding (Fig. 1; see also Table S2 in the supplemental material for P values).

Genes in the 104-kb central region not associated with the putative promoter show a different absolute expression profile compared to that of uninfected samples to be statistically significant for all three print replicates of a CDS.
apparently non-promoter-associated CDSs can be seen to cluster with the promoter-associated CDSs; these correspond to ehv349, which encodes a putative protease. This CDS is associated with the promoter element but was grouped with the proteases for the purpose of this analysis. The three replicate probes for ehv247 (which is not directly associated with the promoter) can be seen to cluster with the promoter-associated CDSs. It is likely that this CDS is cotranscribed with the immediately adjacent ehv248, which does have the promoter.

Those associated with the putative promoter have the highest expression levels at 1 h postinfection, whereas the CDSs in this region not associated with the putative promoter show similar expression profiles to those from the remainder of the genome. This early expression pattern provides important new evidence that family A repeats do function as promoter elements. Indeed, it has previously been suggested that family A repeats may function as immediate-early promoters (2, 3, 22). Expression driven by these promoter elements is likely to play an integral role during the early stages of the virus infection process. However, the lack of database homologues for the CDSs putatively driven by these promoters reveals no obvious insights into the function of this group of genes. Their early expression suggests that they could perform a regulatory role akin to the immediate-early genes identified in other viruses (4, 7, 16). Future functional analysis of this region will provide vital clues to the life cycle of this virus and the unique role of this 104-kb region.
and T4 is represented by the linear distribution observed in Fig. 2A and D. Viral transcripts are expressed at increasing levels for the first 2 h of infection (Fig. 2B and C), after which the expression levels of the majority of CDSs are maintained at a more consistent level.

When a target was labeled from a total RNA extraction performed on purified EhV-86 virions as a control, the only transcript detected above threshold levels on the microarray was that of ehv315 (P value < 0.01) (data not shown). CDS ehv315 is a proline-rich putative membrane protein of unknown function (22). Proline-rich proteins have previously been implicated in calcium binding (22), which is of particular interest since the host, _E. huxleyi_, is well known for the sequestration of calcium carbonate onto its surface (in the form of coccoliths) during active growth (21). Whether the protein EHV315 plays a role in disrupting this pathway is yet to be determined but clearly warrants further investigation.

Virion message. When a target was labeled from a total RNA extraction performed on purified EhV-86 virions as a control, the only transcript detected above threshold levels on the microarray was that of ehv315 (P value < 0.01) (data not shown). CDS ehv315 is a proline-rich putative membrane protein of unknown function (22). Proline-rich proteins have previously been implicated in calcium binding (22), which is of particular interest since the host, _E. huxleyi_, is well known for the sequestration of calcium carbonate onto its surface (in the form of coccoliths) during active growth (21). Whether the protein EHV315 plays a role in disrupting this pathway is yet to be determined but clearly warrants further investigation.

**CDSs not expressed.** Many of the EhV-86 CDSs where no transcript was detected at 4 h postinfection have been assigned a putative function (22) (Fig. 4). Noteworthy is the lack of detection of transcripts for five of the eight proteases predicted to be encoded in the EhV-86 genome (ehv021, ehv109, ehv160, ehv349, and ehv361) (22). Virally encoded proteases have previously been shown to be involved in the maturation of infectious virions, suggesting further that at 4 h postinfection the full virus replication cycle may not yet have been completed (15). At 4 h postinfection, the cell has only just started releasing virus particles and we know it continues to release viruses for up to 2 days while remaining intact (data not shown). This leaves scope for a range of new transcripts to be up-regulated...
at specific time points after this initial 4-h period. Moreover, the EhV-86 genome also encodes key components of sphingolipid biosynthesis, a pathway that leads to the production of ceramide (22), and these genes are expressed at 2 h postinfection (Fig. 1). It is known that there is a connection between protease activation and ceramide-induced apoptosis (17). A virus-controlled apoptotic mechanism is unlikely to induce cell death in the early stages of infection; consequently, protease expression will most likely be up-regulated closer to the onset of apoptosis and cell disintegration, up to 2 days later.

Other transcripts notable for their lack of detectable transcription include those coding for a putative lectin protein (ehv060), DNA-binding proteins (ehv072 and ehv152), phosphate permease (ehv117), thioredoxin (ehv358), protein kinase (ehv402), esterase (ehv363), fatty acid desaturase (ehv415), and a topoisomerase (ehv444). Indeed, for a number of CDSs transcripts have not been detected in the previous lytic-phase transcriptional profile (generated 33 h postinfection) or during the work described here (Fig. 4) (22). Four of these are “core genes” (ehv072, ehv128, ehv166, and ehv444) previously identified as being conserved in the nuclear-cytoplasmic large DNA virus (NCLDV) family. Their high degree of conservation in this family would suggest a role crucial to the virus, and with such a role they would be expected to be expressed. The lack of detection of these transcripts could be caused by their lack of expression or their expression at levels too low to be detected. Future work involving real-time PCR may determine whether these CDSs are expressed and at what levels.

Closing discussion. The infection cycle of EhV-86 can be divided into two broad stages: a primary stage in which a distinctive subgroup of localized CDSs associated with a putative promoter element are transcribed and a secondary stage during which CDSs are transcribed regardless of their genomic location. The function of the primary stage is difficult to ascertain, since the vast majority of the CDSs expressed have little or no database homologues. CDSs from this region have been shown to have some of the highest levels of expression during the infection process (22), presumably due to their early and then constant high levels of expression, suggesting that they are of vital importance to the infection strategy.

The presence of RNA polymerase genes in the EhV-86 genome implies that the virus has the capacity to transcribe its own genes from within the cytoplasm during infection. It is not implausible to suggest that the biphasic stages of expression that occur during EhV-86 infection of *E. huxleyi* are compartmentalized. There is no evidence that transcription of virus RNA polymerase genes occurs until at least 1 h into the infection; therefore, there are two main possibilities that could account for the expression of the primary genes: (i) a functional viral RNA polymerase that recognizes family A promoters is packaged into the mature virion and causes expression of primary CDSs in the cytoplasm, or (ii) following infection, viral DNA targets the host nucleus where host RNA polymerase recognizes family A promoters, leading to transcription of primary genes. Targeting of virus genomic DNA to the nucleus has previously been suggested to occur during the infection of

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Chlorella by PBCV-1 (18). The change from nuclear to cyttoplasmic intracellular compartments (or vice versa) could account for the distinctive change in transcriptional profile observed in these experiments. If promoters similar to the family A repeats are identified during the genomic sequencing of E. huxleyi (currently under way), the second option above should certainly gain greater credence. If this is the case, subsequent breakdown of host genomic DNA (ehv041 encodes an endonuclease and is expressed between 1 and 2 h postinfection) and the nuclear envelope (a viral protease, encoded by ehv349, is expressed during the first hour of infection) could initiate the secondary stage of infection, where the remaining virus genes are expressed.

The presence of RNA polymerase in the EhV-86 genome, so far unique among the Phycodnaviridae, suggested a unique replication cycle for this giant virus. The virally encoded RNA polymerase may be intrinsically linked to the virus-encoded sphingolipid biosynthesis pathway (22). Sphingolipids are well documented as playing a crucial role in controlling cell death (12). If degradation of host DNA does occur during infection, then the ability to make new host-encoded RNA polymerase would be lost. Furthermore, if cell death is delayed extensively by the manipulation of sphingolipid biosynthesis, the active production of a virus-encoded RNA polymerase during the prolonged infection would prove vital to the replication strategy of the virus. Consequently, this could account for the retention of RNA polymerase function in coccolithoviruses (2).

The NCLDV group is composed of the Asfarviridae, Poxviridae, Mimiviridae, Iridoviridae, and Phycodnaviridae families (2). These diverse families are likely to have shared a common ancestor which was likely to have had both nuclear and cyttoplasmic phases in its life cycle (11). Lineage-specific gene loss and gain within the NCLDV families is thought to contribute to the highly diverse characteristics of present-day forms. Poxviruses, asfarviruses, and iridoviruses encode their own transcription and replication machinery and undergo their replication cycles entirely in the cytoplasm (poxviruses) or start it in the nucleus and complete it in the cytoplasm (asfarviruses and iridoviruses) (11). Prior to the sequencing of EhV-86, the phycodnaviruses were thought to be characterized by their nuclear-dependent replication cycles. This interesting coccolithovirus appears to have a replication cycle more similar to that of the ancestral virus, a cycle distinct from all currently known Phycodnaviridae.

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