JOURNAL OF VIROLOGY, Jan. 2010, p. 532–542 Vol. 84, No. 1
0022-538X/10/$12.00 doi:10.1128/JVI.01698-09

Microarray Analysis of *Paramecium bursaria* Chlorella Virus 1 Transcription▽†

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Received 12 August 2009/Accepted 7 October 2009

***Paramecium bursaria* chlorella virus 1 (PBCV-1), a member of the family *Phycodnaviridae*, is a large double-stranded DNA, plaque-forming virus that infects the unicellular green alga *Chlorella* sp. strain NC64A. The 330-kb PBCV-1 genome is predicted to encode 365 proteins and 11 tRNAs. To monitor global transcription during PBCV-1 replication, a microarray containing 50-mer probes to the PBCV-1 365 protein-encoding genes (CDSs) was constructed. Competitive hybridization experiments were conducted by using cDNAs from poly(A)-containing RNAs obtained from cells at seven time points after virus infection. The results led to the following conclusions: (i) the PBCV-1 replication cycle is temporally programmed and regulated; (ii) 360 (99%) of the arrayed PBCV-1 CDSs were expressed at some time in the virus life cycle in the laboratory; (iii) 227 (62%) of the CDSs were expressed before virus DNA synthesis begins; (iv) these 227 CDSs were grouped into two classes: 127 transcripts disappeared prior to initiation of virus DNA synthesis (considered early), and 100 transcripts were still detected after virus DNA synthesis begins (considered early/late); (v) 133 (36%) of the CDSs were expressed after virus DNA synthesis begins (considered late); and (vi) expression of most late CDSs is inhibited by adding the DNA replication inhibitor, aphidicolin, prior to virus infection. This study provides the first comprehensive evaluation of virus gene expression during the PBCV-1 life cycle.**

Paramecium bursaria chlorella virus 1 (PBCV-1), the prototype of the genus *Chlorovirus* (family *Phycodnaviridae*), is a large, icosahedral (190 nm in diameter), plaque-forming virus that infects the unicellular, eukaryotic green alga *Chlorella* sp. strain NC64A. The PBCV-1 virion has a lipid membrane located inside an outer glycoprotein capsid. The 330-kb genome is a linear, nonpermutated, double-stranded DNA (dsDNA) molecule with covalently closed hairpin ends that has approximately 365 protein encoding genes (CDSs), as well as 11 tRNA encoding genes (reviewed in references 34, 39, and 40). The CDSs are evenly distributed on both strands and intergenic space is minimal (typically fewer than 100 nucleotides); the exception is a 1,788-bp sequence in the middle of the genome that encodes the tRNA genes. Approximately 35% of the 365 PBCV-1 gene products resemble proteins in the public databases.

PBCV-1 initiates infection by attaching rapidly and specifically to the cell wall of its host (22), probably at a unique virus vertex (4, 26). Attachment is immediately followed by host cell wall degradation by a virus-packaged enzyme(s) at the point of contact. After wall degradation, the viral internal membrane presumably fuses with the host membrane, causing host membrane depolarization (9), potassium ion efflux (25), and an increase in the cytoplasm pH (2). These events are predicted to facilitate entry of the viral DNA and virion-associated proteins into the cell. PBCV-1 lacks a gene encoding a recognizable RNA polymerase or a subunit of it, and RNA polymerase activity is not detected in PBCV-1 virions. Therefore, viral DNA and virion-associated proteins are predicted to migrate to the nucleus, and early viral transcription is detected 5 to 10 min postinfection (p.i.), presumably by commandeering a host RNA polymerase(s) (possibly RNA polymerase II) (14, 29). Virus DNA synthesis begins 60 to 90 min p.i., followed by virus assembly at 3 to 5 h p.i. in localized regions of the cytoplasm, called virus assembly centers (21). At 6 to 8 h p.i., virus-induced host cell lysis occurs resulting in release of progeny virions (~1,000 viruses/cell, ~25% of which are infectious). These events are depicted in Fig. 1.

To initiate PBCV-1 transcription, the host RNA polymerase(s), possibly in combination with a virus transcription factor(s), must recognize virus DNA promoter sequences. Recently, three short nucleotide sequences were identified in putative virus promoter regions (150 bp upstream and 50 bp downstream of the ATG translation site) that are conserved in PBCV-1 and other *Chlorovirus* members (7). PBCV-1 CDSs are not spatially clustered on the genome by either temporal or functional class, suggesting that transcription regulation must occur via *cis*- and possible *trans*-acting regulatory elements.

To understand the dynamics of PBCV-1 global gene expression during virus replication, we constructed a microarray containing 50-mer probes to each of the 365 PBCV-1 CDSs.

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† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 14 October 2009.

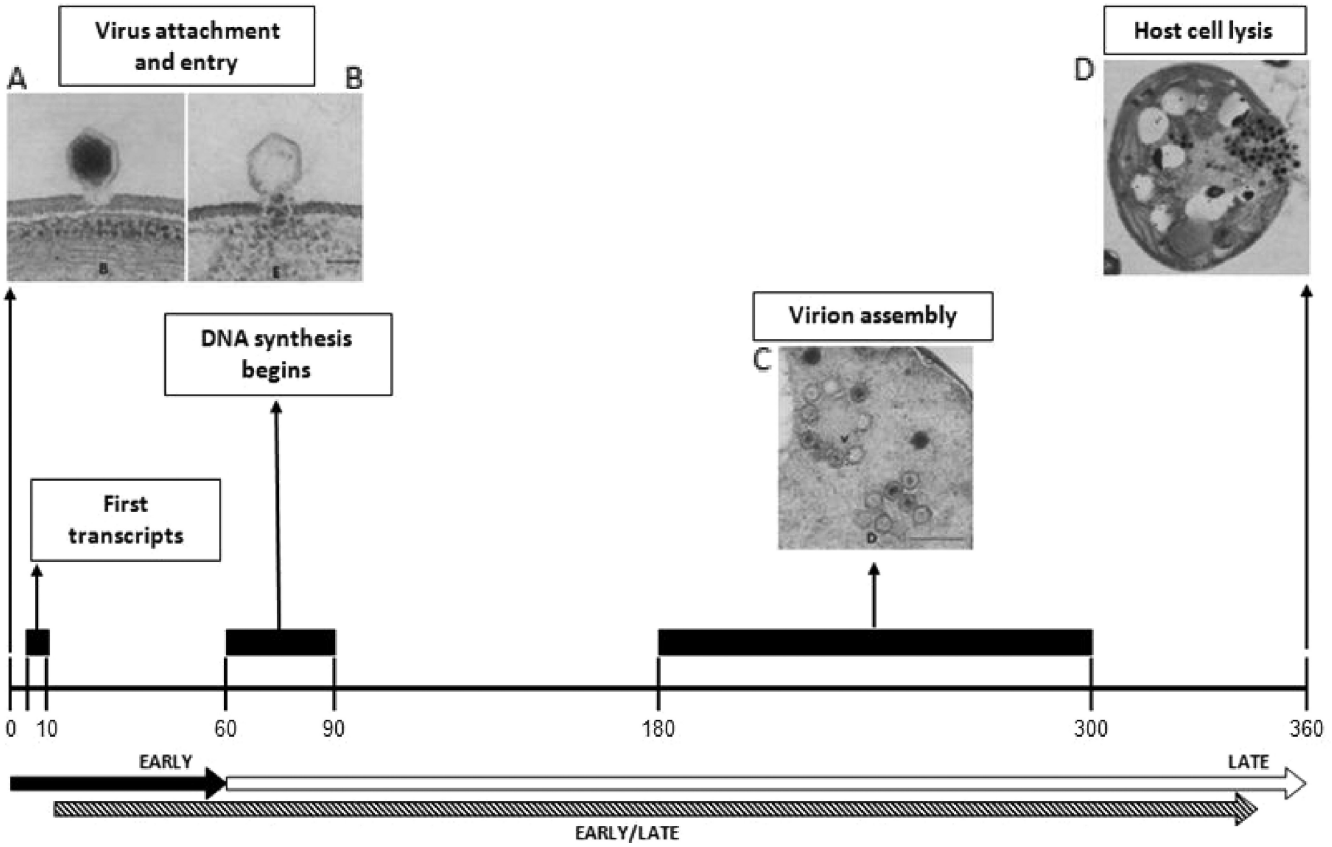

FIG. 1. Timeline representing the PBCV-1 life cycle in *Chlorella* strain NC64A. Numbers represent minutes after infection. CDSs expressed before viral DNA synthesis begins were classified as early (black arrow), CDSs expressed after DNA synthesis begins were classified as late (white arrow), and CDSs expressed before and after DNA synthesis begins were classified as early/late (arrow with diagonal lines). Electron micrographs A and B were reproduced with permission from Meints et al. (22), and micrographs C and D were reproduced with permission from Meints et al. (21).

cDNAs from poly(A)-containing RNAs isolated from cells at seven times after PBCV-1 infection were competitively hybridized against a reference sample on the microarray. To further delineate early and late gene expression, cells were treated with the DNA replication inhibitor, aphidicolin, prior to infection. The results provide the first comprehensive transcriptional map of the virus genome, conferring insights about the characterization of each PBCV-1 CDS, the majority of which have unknown functions. In addition, the microarray data suggest that viral DNA replication plays a significant role in the temporal regulation of gene expression.

MATERIALS AND METHODS

RNA isolation and drug treatment. *Chlorella* strain NC64A cells (10^8 cells/ml) were infected with PBCV-1 at a multiplicity of infection of 5 to ensure synchronous infection. Uninfected cells and cells at 20, 40, 60, 90, 120, 240, and 360 min p.i. were harvested by centrifugation (4,000 rpm) for 5 min at 4°C and disrupted with glass beads (0.25 to 0.30 mm in diameter) by using a bead beater (Disruptor Genie; Scientific Industries, Bohemia, NY) in the presence of TRIzol (Invitrogen, Carlsbad, CA). RNAs were isolated by using the Absolutely RNA miniprep kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. RNA integrity was verified in denaturing 1% agarose gels by monitoring host cytoplasmic and chloroplast rRNAs. Total RNA was quantified with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

To determine the effect of virus DNA synthesis on virus gene expression, aphidicolin (20 μg/ml) was added to the cells 15 min prior to infection, and samples were collected at the same times after infection as described above. Control samples were obtained from infected nontreated cells at the same times. Preliminary experiments to determine optimal drug dosage and time of application indicated that 20 μg of aphidicolin/ml completely inhibits DNA synthesis in 15 min.

Microarray construction and hybridization. The PBCV-1 genome is predicted to have 365 CDSs and 11 tRNA encoding genes. The tRNAs sequences were not included in the microarrays. Fifty-mer probes representing each CDS in the PBCV-1 genome were designed and synthesized by MWG Biotech (Ebersberg, Germany) (20 to 80% GC, with a melting temperature of 60 to 80°C). A table with the probes' sequences is in Supplement S1 in the supplemental material. Probes were spotted onto CMT-GAPS silane-coated slides (Corning, Lowell, MA) by using Omnigrid 100 (Genomic Solutions, Ann Arbor, MI) according to the manufacturer's instructions. Probes were printed in quadruplicates on every slide. For each time point, 20 μg of total RNA was reverse transcribed by using oligo(dT) as a primer, and cDNAs were labeled with either Cy3- or Cy5-dUTP (GE Healthcare, Piscataway, NJ) with the SuperScript indirect cDNA labeling system (Invitrogen) according to the supplier's directions. The reference sample, for the time course experiments, consisted of a pool of transcripts obtained by mixing equal amounts of total RNA from each time point. Competitive hybridization experiments were conducted for each sample against the reference sample (15, 18, 41). For the aphidicolin experiments, a direct comparison was carried out with each treated sample versus the corresponding untreated infected control.

Labeled cDNAs were resuspended in 40 μl of preheated (68°C) Ambion hybridization buffer 2 (Ambion, Austin, TX). Arrays were hybridized (42°C) for 16 h in a Corning hybridization chamber (Corning, Lowell, MA). The slides were washed twice (42°C) in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) for 15 min, followed by two

washes in 0.5× SSC–0.5% SDS for 15 min. Slides were then dried by low-speed centrifugation and subjected to fluorescence detection with an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA).

Microarray analysis. Results from three independent experiments were analyzed by using the GenePix Pro v.6.0 software (Molecular Devices) and TIGR microarray software suite (TM4) (28). Several transformations were performed to eliminate low-quality data, to normalize the measured intensities using the Lowess algorithm, and to regulate the standard deviation of the intensity of the Cy5/Cy3 ratio across blocks. CDSs that displayed statistically significant modulation were identified by a one-way analysis of variance, using P values of <0.01 as a cutoff. For the aphidicolin experiments, significant analysis of microarray (33) was used to identify CDSs with statistically significant changes in expression compared to an untreated infected sample (false discovery rate, $\leq 5\%$). CDSs with similar expression profiles were grouped into different clusters with a K-means algorithm by using Euclidean distance and 50 maximum iterations. PBCV-1 microarray data sets were deposited at NCBI's Gene Omnibus Express (GEO) under the accession number GSE18421.

RESULTS AND DISCUSSION

Microarray quality check. To evaluate our probes, equal amounts of PBCV-1 genomic DNA (2 μg) were labeled in two independent reactions with either Cy3-dCTP or Cy5-dCTP (GE Healthcare) by using random primers (Invitrogen). The two reactions were then competitively hybridized with the probes in the microarray. No difference was detected in hybridization of the two DNA samples on all spots (results not shown), indicating the probes were specific and also excluding any preferential hybridization to one of the dyes. In addition, to check for host cross-hybridization, PBCV-1 DNA (2 μg) was labeled with Cy3-dCTP and host *Chlorella* strain NC64A DNA (2 μg) with Cy5-dCTP. Only two PBCV-1 CDSs (A260R and A625R) hybridized with host DNA; however, these two CDS are false positives since recently available *Chlorella* strain NC64A genome sequence did not have detectable homologous sequences.

PBCV-1 transcription program. RNAs were isolated from infected cells at 20, 40, 60, 90, 120, 240, and 360 min p.i. Competitive hybridization results from each time point against the RNA reference pool revealed that transcripts of 360 (99%) of the 365 PBCV-1 CDSs display statistically significant variation in at least one of the experimental time points (Fig. 2). CDSs A60L, A328L, A482R, and A646L did not pass statistical tests in the time course experiment. CDS A689L was not spotted onto the array because it was accidentally omitted during the probe synthesis. The gene expression analysis was based on relative levels, rather than absolute levels of expression. Mapping the PBCV-1 transcription pattern to the genome revealed no large regions that were biased as to time of expression, indicating that gene expression in PBCV-1 is mostly controlled by multiple initiation sites (Fig. 3). However, a few early or late CDSs clustered in small regions of the genome, as can be seen in shaded areas in Fig. 3. Interestingly, these small regions are also clustered and conserved in another sequenced chlorovirus (NY-2A) that infects the same host chlorella (results not shown). Unlike the phage T4 genome, where most late CDSs are located in contiguous regions on the same DNA strand (18), PBCV-1 expression did not show a strong strand-specific bias. In addition, there is no relationship between time of expression and G+C content of the genome (Fig. 3).

Classification of PBCV-1 CDSs was based on when the transcript was detected by the microarray. Globally, the 360 statistically significant CDSs were grouped into three classes (Fig. 4): (i) 227 (62%) of the CDSs were expressed before viral DNA synthesis begins at 60 to 90 min p.i.; (ii) these 227 CDSs were divided into two classes: transcripts of 127 CDSs disappeared prior to initiation of virus DNA synthesis (considered early), while transcripts of 100 CDSs were still detected after virus DNA synthesis begins (considered early/late); (iii) transcripts of 133 (37%) CDSs were detected after virus DNA synthesis begins (considered late). Functional categorization of PBCV-1 CDSs was reported elsewhere (8), and the functional distribution compared to each transcriptional category is summarized in Fig. 4 and 5. Forty-four of the PBCV-1 encoded proteins have been expressed, and recombinant proteins were shown to be functional enzymes. These are indicated with an asterisk in Fig. 5. The functions of the remaining CDSs are either putative or unknown.

A previously described putative promoter sequence (AAT GACA) and a similar sequence (ATGACAA) (7, 14) were detected in 50 early or early/late PBCV-1 CDSs. However, promoter sequences for most early, early/late, and late CDSs remain unidentified.

Early CDSs. A total of 127 (35%) of the 360 PBCV-1 CDSs were expressed early, 20 to 60 min p.i. (see Supplement S2 in the supplemental material). Sixty-one percent of the early CDSs have no known function. Many of the early CDSs are predicted to encode the machinery for the virus to begin DNA replication. In fact, PBCV-1 encodes seven proteins involved in DNA replication, recombination and repair that were expressed early including: δ DNA polymerase (A185R), superfamily III helicase (A456L), DNA topoisomerase II (A583L), RNase H (A399R), and PCNA (A574L). A pyrimidine dimer-specific glycosylase (A50L), a well-characterized DNA repair enzyme involved in pyrimidine photodimer excision (20), was also expressed early. Additional PBCV-1 encoded proteins involved in virus DNA synthesis and DNA recombination were in the early/late class including, DNA primase (A468R), a 5′-3′ exonuclease (A166R) and a second PCNA (A193L). The PBCV-1 genome contains methylated nucleotides, both N^6-methyl adenine and 5-methylcytosine (35). Therefore, it is not surprising that the virus encodes three functional DNA methyltransferases that were transcribed early: two enzymes that form N6-methyladenine (A251R and A581R) and one that forms 5-methylcytosine (A517L).

PBCV-1 DNA synthesis also requires large quantities of deoxynucleotide triphosphates (dNTPs) that cannot be accounted for simply by recycling deoxynucleotides from host DNA. By 4 h p.i., the total amount of DNA in the cell increases fourfold due to viral DNA synthesis (37). To guarantee a supply of dNTPs in nonproliferating host cells, large DNA viruses, including PBCV-1, encode proteins involved in dNTP biosynthesis: dUTP pyrophosphatase (A551L), thioredoxin (A427L), thymidylate synthase X (A674R), and cytosine deaminase (A200R) CDSs were transcribed early. Additional dNTP synthesizing CDSs in the early/late class included aspartate transcarbamylase (A169R), both subunits of ribonucleotide reductase (A476R and A629R), glutaredoxin (A438L), and dCMP deaminase (A596R).

Several PBCV-1 CDSs predicted to encode proteins involved in transcription were also expressed early. These proteins included three putative transcription factors (TFIIB [A107L], TFIID [A552R], and TFIIS [A125L]), two helicases

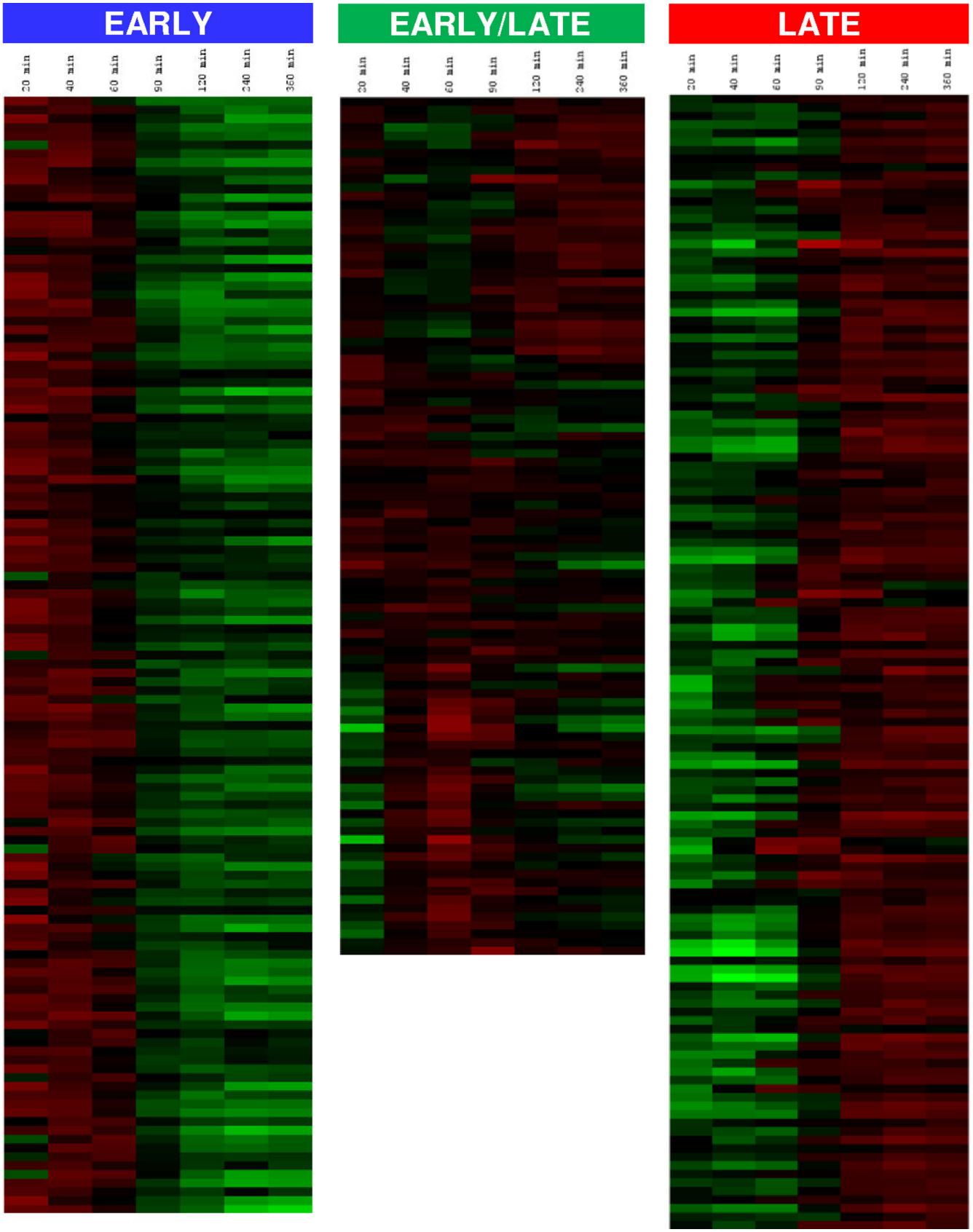

FIG. 2. Heat map illustrating the expression of 360 PBCV-1 CDSs during the infection cycle. cDNAs from each time point were labeled with Cy5, and the reference control was labeled with Cy3. Color code represents the $\log_2$ (Cy5/Cy3) ratio for each time point. CDSs with similar expression profiles were grouped into three classes representing early, early/late, and late by using a K-means algorithm. Each column corresponds to the time point when total RNA was collected (numbers represent minutes after infection). Each row represents a different CDS in PBCV-1. A list of all of the CDSs is available in Supplement S2 in the supplemental material.

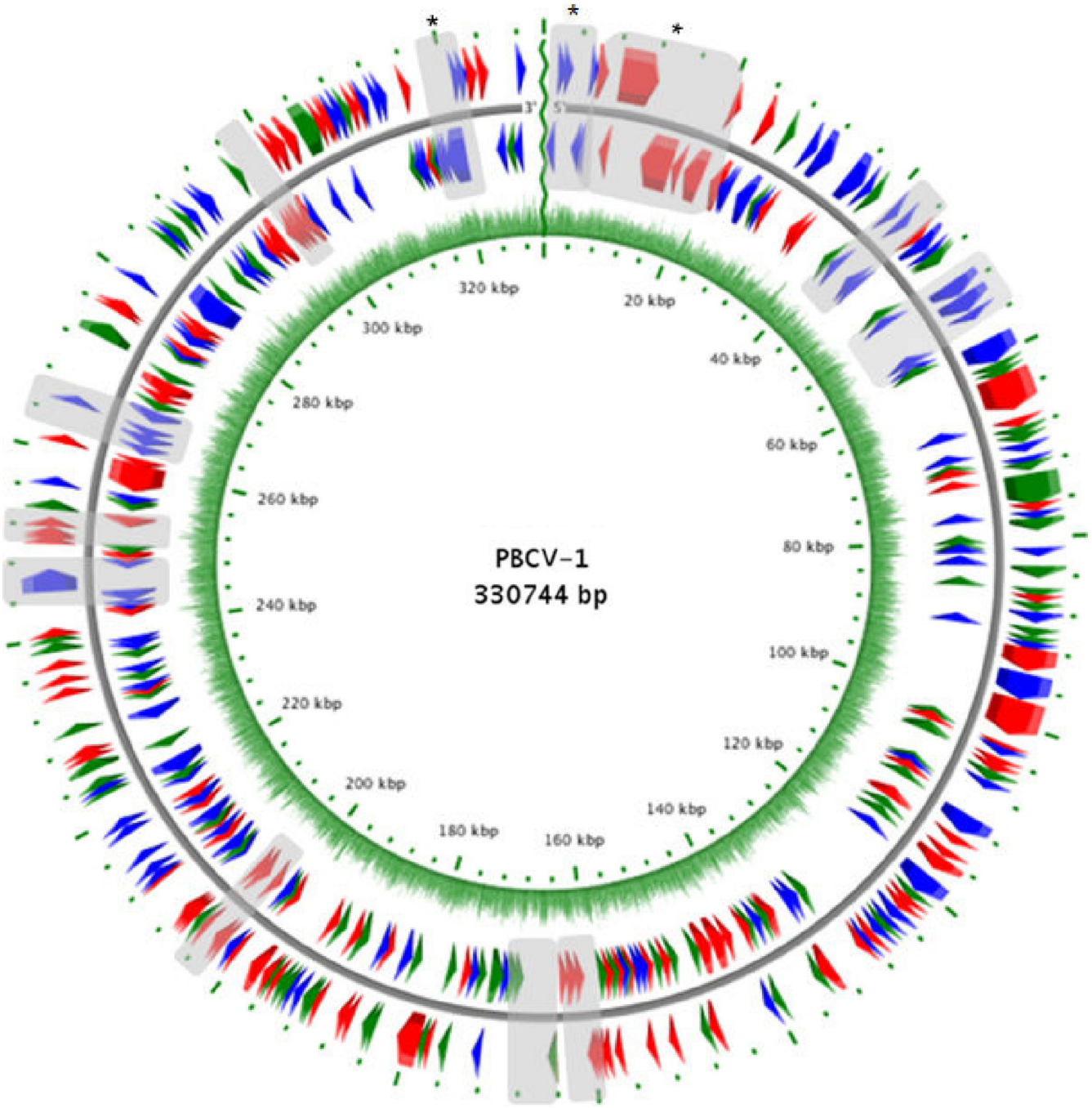

FIG. 3. Mapping of the PBCV-1 transcriptome. Blue arrows, early CDSs; green arrows, early/late CDSs; red arrows, late CDSs. Arrow points indicate the transcription direction. Shaded areas indicate small transcription CDS clusters that are also conserved in another chlorovirus (NY-2A), except for the areas marked with asterisks. The middle circle shows the G+C content of the genome. Note that the PBCV-1 genome is linear, and a circular map was generated for illustration purposes only.

(SWI/SNF helicase [A548L] and superfamily II helicase [A241R]), and RNase III (A464R). The genes for two enzymes involved in mRNA capping, an RNA triphosphatase (A449R) and a guanylyltransferase (A103R), were also transcribed early. The products of at least some of these early CDSs are undoubtedly involved in the switching of virus early gene transcription to late gene transcription.

A few PBCV-1 enzymes involved in protein synthesis and protein degradation were transcribed early, including translation elongation factor-3 (A666L), ubiquitin C-terminal hydrolase (A105L), Skp1 protein (A39L), SCF-E3 ubiquitin ligase (A481L), a zinc metallopeptidase (A604L), and an ATPase (AAA + class) (A44L).

Unlike other viruses, PBCV-1 encodes at least part, if not all, of the machinery required to glycosylate its major capsid protein (19), including five glycosyltransferases (11, 38, 43). Further-

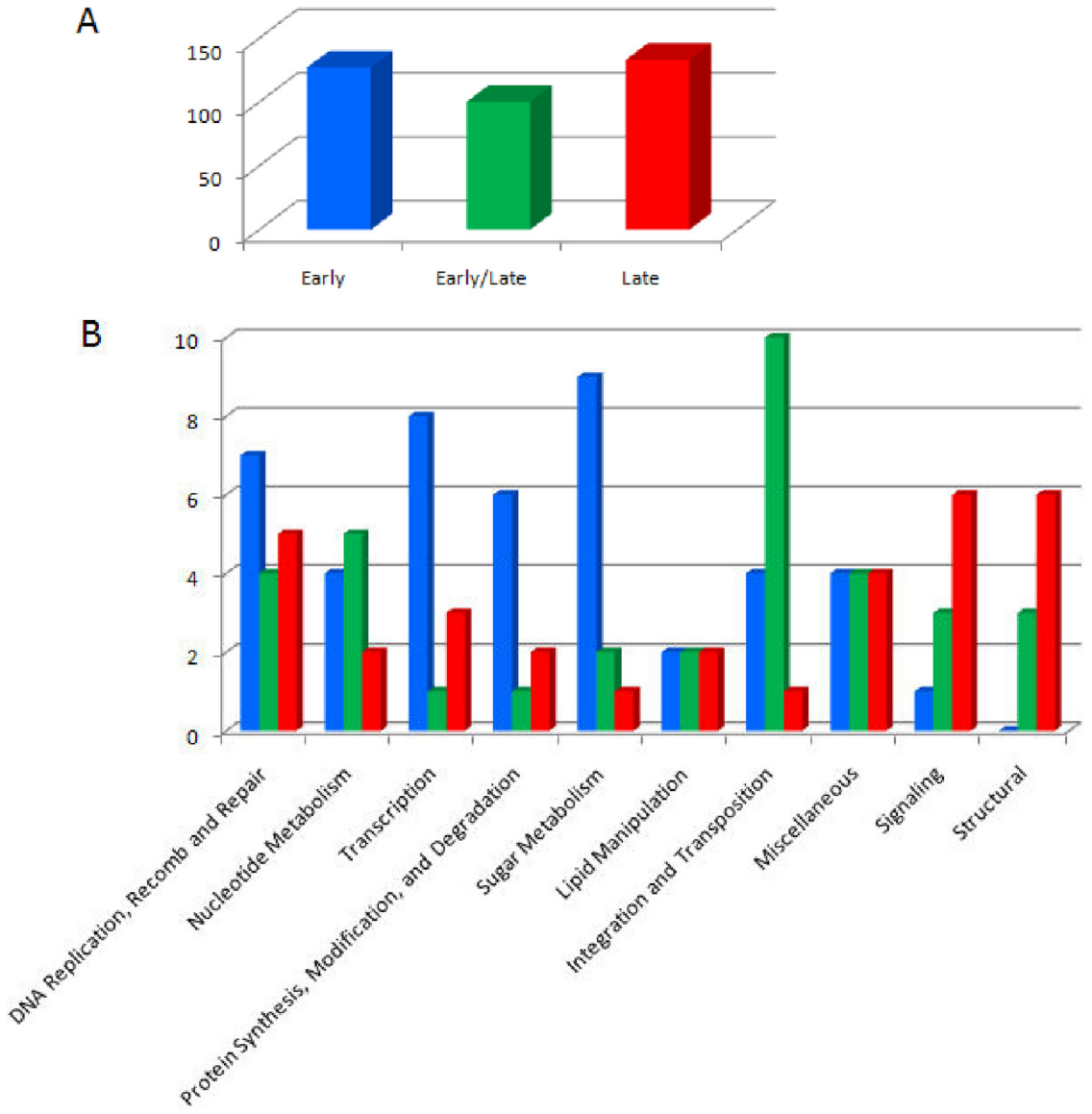

FIG. 4. (A) PBCV-1 global expression pattern. (B) Distribution of CDSs according to their putative function and time of expression. CDS products with unknown function are not listed in this graph.

more, glycosylation of the virus major capsid protein probably occurs independently of the host endoplasmic reticulum-Golgi system (19). All of the glycosyltransferase CDSs were expressed early (A64R, A111/114R, A219/222/226R, A473L, and A546L).

The chlorella viruses are also unusual because they encode enzymes involved in sugar metabolism. Two PBCV-1-encoded enzymes synthesize GDP-L-fucose from GDP-D-mannose, GDP-D-mannose dehydratase (A118R), and fucose synthase (A295L) (10, 32) and three enzymes, glucosamine synthetase (A100R), UDP-glucose dehydrogenase (A609L), and hyaluronan synthase (A98R), contribute to the synthesis of hyaluronan, a linear polysaccharide composed of alternating β-1,4-glucuronic acid and β-1,3-*N*-acetylglucosamine residues (6, 16). The CDSs for these five enzymes were expressed early.

Early/late CDSs. A total of 100 (27%) CDSs were classified as early/late, 67 (67%) of which have unknown function (see Supplement S2 in the supplemental material). This class contains CDSs that were expressed before 60 min p.i., but whose transcripts were also present after PBCV-1 DNA synthesis begins. At least three mechanisms can lead to classification of CDSs into the early/late class: (i) the CDSs were transcribed both before and after viral DNA replication begins; (ii) the CDSs were only transcribed prior to initiation of virus DNA synthesis, but complete degradation of their transcripts only occurred after DNA synthesis begins; and (iii) the CDSs encode polycistronic mRNAs, e.g., one could have a dicistronic mRNA in which one CDS is required for an early function and the other CDS is required for a late function. To add to the complexity, ~30 transcripts were detected early, disappeared,

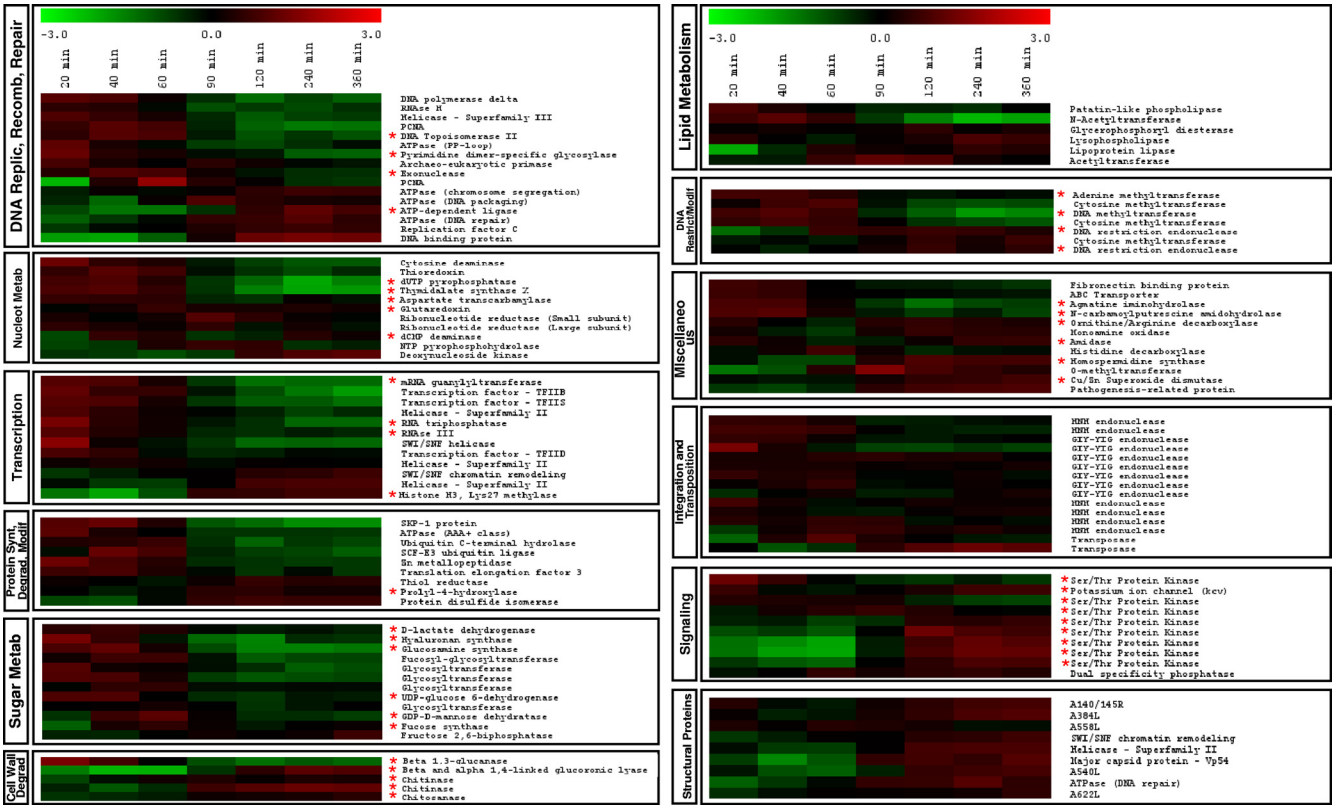

FIG. 5. Heat map showing distribution of selected CDSs according to their putative functional class. cDNAs from each time point were labeled with Cy5, and the reference control was labeled with Cy3. The color code represents the $\log_2$ (Cy5/Cy3) ratio for each time point. Each column corresponds to the time point when total RNA was collected (numbers represent minutes after infection). Each row represents a different PBCV-1 CDS. Recombinant proteins have been characterized for the CDSs marked with an asterisk.

and then reappeared as late transcripts (these CDSs are marked in Supplement S2 in the supplemental material). A similar phenomenon has been reported in transcriptional studies with the bacteriophage T4 (18) and Red Sea bream iridovirus (17).

In addition to the early/late CDSs described in the preceding section, the most striking feature of this early/late class was the presence of many genes encoding proteins associated with genome integration, including five GIY-YIG endonucleases (A134L, A287R, A315L, A495R, and A651L) and four HNH endonucleases (A354R, A422R, A478L, and A490L). Also, one of the two transposases (A366L) coded by PBCV-1 was expressed early/late. The functions of these proteins in the PBCV-1 life cycle are unknown.

Late CDSs. A total of 133 (37%) of the 360 CDSs were classified as late CDSs (see Supplement S2 in the supplemental material), 74% of which have no match in the public databases. The expectation is that most late CDS products are involved in either virus capsid assembly, DNA packaging, or virus release or are packaged in the virus particles. Indeed, an SDS-polyacrylamide gel electrophoresis mass spectrometry proteomic analysis of purified virions indicated that 118 PBCV-1 gene products were detected in the virions (D. D. Dunigan et al., unpublished results). Of these 118 proteins, 83 had their corresponding CDS transcribed late, 29 were transcribed early/late, while only 6 virion-associated proteins were expressed early.

At least nine late CDSs are predicted to have their gene products serving a purely structural role in the virion including the major capsid protein A430L (12) and A140/145R, which is associated with the unique vertex of the virus (26). Other putative structural proteins are A189/192R, A363R, A384L, A540L, A558L, A561L, and A622L. Homologs of all of these proteins are present in all of the chloroviruses (Dunigan et al., unpublished). In addition to structural proteins, a DNA-binding protein (A437L), predicted to aid in neutralization of the virus dsDNA, was in the virion, and its gene was transcribed late.

Several CDSs encoding proteins with putative enzyme functions were expressed late, and their gene products were packaged in the PBCV-1 virion. Presumably, these enzymes are released into the cell during virus infection and aid in establishing infection. These proteins include an ATPase (A561L), a superfamily II helicase (A363R), a SWI/SNF chromatin remodeling complex subunit (A189/192R), and a SET domain-containing histone 3, Lys27 methyltransferase (named vSET) (A612L). The vSET protein is predicted to aid in the rapid inhibition of host transcription during virus infection (24). Two site-specific (restriction) endonucleases (A252 and A579L) were also expressed as late CDSs, and their proteins are pack-

aged in the virion. The restriction endonucleases are involved in host chromosome degradation, which begins within a few minutes after virus infection (1). Thus, PBCV-1 has at least two avenues to inhibit host transcription and subvert the host RNA polymerase (presumably RNA polymerase II) for virus transcription: virus packaged restriction endonucleases and a vSET histone 3, Lys27 methyltransferase.

Five of the eight PBCV-1-encoded Ser/Thr protein kinase CDSs were expressed late (A34R, A277L, A278L, A282L, and A614L). With the exception of A277L, the other four kinases were packaged in the virion (Dunigan et al., unpublished). A dual specific phosphatase (A305L) was also expressed late, and the protein is present in the virion. These results indicate that PBCV-1 has the potential to release several protein kinase/phosphatase proteins into the cell during infection; these enzymes are probably involved in regulatory mechanisms.

PBCV-1 encodes five proteins that degrade polysaccharides, and presumably some of these encoded proteins are involved in host cell wall degradation either during virus entry or in aiding lysis of the cell wall during virus release (30). With the exception of β 1,3-glucanase (A94L) that was expressed early, the remaining four CDSs were transcribed late. The late CDSs encode a β- and α-1,4-linked glucuronic lyase (A215L), two chitinases (A260R and A181/182R), and one chitosanase (A292L).

Finally, 49 late gene products were not detected in the virion. Fifteen of these proteins have a putative function including, for example, a DNA packaging ATPase (A392R) that is predicted to be involved in packaging DNA into the virion. Interestingly, some CDSs that were expected to be transcribed early because of their putative involvement in the DNA replication were transcribed late. These CDSs encode a replication factor C protein (A417L), which is similar to one of the two replication factor C proteins in *Archaea*, an ATP-dependent DNA ligase (A544R), and a deoxynucleoside kinase (A416R). However, none of these proteins were detected in the virion (Dunigan et al., unpublished).

Aphidicolin treatment. To confirm the early and late classification of PBCV-1 CDSs, aphidicolin was used to block viral DNA synthesis. The drug was added to the culture 15 min prior to the addition of PBCV-1, and cells were harvested at the same times after infection used in the previous experiments (20, 40, 60, 90, 120, 240, and 360 min p.i.). Each sample was analyzed by using the corresponding infected nontreated sample as a control.

Expression of 179 (49%) CDSs was inhibited by aphidicolin (see Supplement S2 in the supplemental material). Of these 179, 14 were expressed early, 57 were expressed early/late, and 108 were expressed late. This experiment established that transcription of most late CDSs relies on the synthesis of viral DNA. In contrast, expression of 181 CDSs was not affected by aphidicolin. Of these 181, 113 were early, 43 were early/late, and 25 were late. Three CDSs (A328R, A482R, and A464L) were not previously classified as early, early/late, or late because they did not pass statistical tests. However, they were expressed in the presence of aphidicolin, which indicates that these CDSs might be early. Collectively, expression of most early CDSs was not affected by aphidicolin and expression of late CDSs was inhibited by the drug. We have no explanation for why 14 early CDSs and 57 early/late CDSs were affected by aphidicolin, nor do we know why 25 late CDSs were not affected by aphidicolin.

Verification of the microarray results. Three independent sets of experiments support the microarray results. (i) Recombinant proteins from 19 PBCV-1 CDSs have been biochemically characterized previously, and their genes were Northern blotted to determine when they were expressed. Sixteen of these results completely agree with the microarray experiments. The other three Northern analyses were similar, but not identical, to the microarray analyses. One is a glycosyltransferase (A64R), which Northern analysis indicated is expressed from 45 to 360 min p.i. (11), whereas the microarray analysis indicated that the CDS was expressed only between 40 and 60 min p.i. The second CDS is a chitinase (A181/182R), which Northern results indicate transcription occurs between 30 and 360 min p.i. (30), whereas the microarray analysis showed the transcript was present from 60 to 360 min p.i. The third CDS is a dCMP deaminase (A596R), which Northern analysis indicated expression occurs from 30 to 120 min p.i. (42); the microarray experiments detected the transcript at 40 to 60 min p.i. and then again from 240 to 360 min p.i. This discontinuity in expression has also been observed with a few other virus CDSs, and it has been observed in both Northern blots and in the microarray results (e.g., ornithine/arginine decarboxylase [A207R] [23]). It is important to note that the microarrays measured relative levels of the transcript, and this fact could explain the few differences mentioned above.

(ii) The 46.2-Mb genome of the PBCV-1 host, *Chlorella* NC64A, was recently sequenced to ninefold coverage (http://genome.jgipsf.org/ChlNC64A_1/ChlNC64A_1.info.html). This prompted us to initiate a transcriptome analysis of *Chlorella* NC64A and PBCV-1-infected cells by shotgun sequencing cDNA derived from poly(A)$^+$ RNA using a new high-throughput sequencing instrument. To date, we have two sets of sequences, one from uninfected *Chlorella* NC64A and one from cells at 20 min p.i. We compared the 20-min p.i. viral microarray results with the infected 20-min p.i. viral transcriptome sequencing results. Of the 172 CDSs detected by the microarray analysis at 20 min p.i., expression of 159 CDSs (92%) were detected in the transcriptome study using a 200-fold coverage cutoff (G. M. Yanai-Balser et al., unpublished results).

(iii) Kawasaki et al. (14) identified 22 immediate-early PBCV-1 CDSs (expressed at 5 to 10 min p.i.). Our microarray results indicated that 20 of these 22 CDSs were expressed early. CDS A689L was not present in our array, and CDS A312L was classified as early/late and was not detected at 20 min p.i., which is the earliest time point in the present study. In the later case, the difference could be due to the relative measurement of the transcript.

Do late PBCV-1 mRNAs have a poly(A) tail? Both the microarray results and the preliminary transcriptome sequencing of PBCV-1 depended on cDNA synthesis using an oligo(dT) primer. We mention this issue for two reasons. First, about 20 years ago we conducted a set of pulse-labeling experiments wherein PBCV-1-infected cells were incubated with [H^3]adenine for 30-min periods (36). Poly(A)$^+$ and poly(A)$^-$ RNAs were separated on an oligo(dT)-cellulose column, and the radioactivity was determined. The results indicated that 22 to 26% of the radioactivity eluted in the poly(A)$^+$ fraction from cells infected from 0 to 30 min and from 30 to 60 min p.i.

In contrast, 6.1, 4.5, and 2.5% of the radioactivity eluted in the poly(A)$^+$ fractions at 60 to 90, 90 to 120, and 120 to 240 min p.i., respectively. Therefore, we suggested that PBCV-1 early mRNAs probably contain poly(A) tails and late mRNAs might lack them. However, there are other explanations for these earlier results, e.g., the pool size of unlabeled adenine certainly increases dramatically during infection; consequently, there could be a large dilution effect on the added [H^3]adenine compared to the controls. In addition, we now know that PBCV-1 infection leads to rapid depolarization of the host plasma membrane, which causes an immediate decrease in adenine transport into the infected cell (2). Both of these issues undoubtedly influenced our previous results.

Second, a report by Kawasaki et al. (14) also suggests that there may be a shift in poly(A)$^+$ RNAs during chlorella virus replication. Twenty-two PBCV-1 immediate-early CDSs were analyzed, and the transcripts gradually decreased in size after 20 min p.i., suggesting weakening or cessation of poly(A) polymerase activity.

The current manuscript provides evidence that most, if not all, PBCV-1 mRNAs have a poly(A) tail because expression of 99% of the PBCV-1 CDSs was detected in our microarray experiments. Furthermore, many of the CDSs we identified were clearly expressed late and packaged in the virion.

PBCV-1 transcription is complex. As mentioned above, we and others have characterized several individual PBCV-1 gene products. In most of these reports, a Northern analysis was conducted when studying a specific CDS. The transcription patterns of ~50% of these CDSs are more complicated than just obtaining a single hybridizing RNA band of the predicted size at specific times. That is, full-length gene DNA probes often hybridize to mRNA transcripts that are 40 to 60% larger than the CDS itself, suggesting that PBCV-1 might have polycistronic mRNAs. In addition, some probes not only hybridize to mRNAs of the expected size, but they also hybridize to larger mRNAs at other times in the virus replication cycle. These complex patterns occur even with single-stranded DNA probes (e.g., the potassium ion channel CDS, *kcv*) (13). One difference between the previous Northern analyses and the microarray results is that hybridization to total RNA was used in the Northern analyses and in the research described in the present study the cDNAs were synthesized from poly(A)$^+$ RNAs.

We examined four of the CDSs that produced larger transcripts than expected to determine whether expression of their flanking CDSs were identical in the microarray experiments to the target CDS, possibly suggesting a polycistronic mRNA. A common expression pattern was obtained for two of the four CDSs. The RNase III (A464R) mRNA has a predicted size of 825 nucleotides (nt). However, in a Northern blot the RNase III hybridizing band is ~1,300 nt. Its two adjacent CDSs are 204 nt (A462R) and 354 nt (A465R) and, like A464R, both of them are expressed early. The combined sizes of them with the RNase III CDS are compatible with either a dicistronic or even a tricistronic mRNA. The other example is the potassium ion channel CDS (*kcv*, A250R) that Northern analysis indicated has a complex expression pattern, at early times A250R is expressed as a large message and at late stages as a monocistronic mRNA (13). However, when the early transcript for this CDS was mapped, the start and the stop sites were within the adjacent CDSs, so A250R is clearly not a polycistronic mRNA. The CDSs surrounding the other two CDSs with larger transcripts than expected, fucose synthase (A295L) and dCMP deaminase (A596R), were expressed at different times than the target CDS.

It is clear that a detailed transcription analysis using a high-throughput sequencing system of PBCV-1-infected cells will be required to precisely determine promoter and terminator sites, as well as splicing regions of PBCV-1 transcription. This analysis should include the use of random primers in addition to oligo(dT) primers and also allow the detection of PBCV-1 encoded small RNAs.

Microarray results with other large dsDNA viruses. PBCV-1 expressed 99% of its CDSs at some point in the virus life cycle during infection in laboratory conditions. Similar results were obtained for other large DNA viruses such as vaccinia virus and monkeypox viruses in which ~95% of their CDSs are expressed in cell cultures (3, 27). Transcription analysis using microarrays was also performed with two marine fish iridoviruses: (i) Singapore grouper iridovirus expressed 97% of its CDSs in cell culture (31), and (ii) Red Sea bream iridovirus expressed 96% of its CDSs during in vitro infection (17). Therefore, it appears that most CDSs are expressed in these large dsDNA viruses, even in laboratory conditions.

The temporal categorization of the transcripts in large viruses varies. Different studies classify virus transcripts in different ways, usually subdividing early CDSs into more specific categories. For example, Red Sea bream iridoviruses has ~9% of its CDSs classified as immediate-early, ~43% classified as early, and ~41% classified as late (17). T4 bacteriophage transcripts are divided into immediate-early (42%), delayed early (~12%), middle early (21%), and late (~22%) (18). Poxvirus transcription occurs in the cytoplasm of the infected cell and is programmed by a virus-encoded RNA polymerase and time-specific transcription factors to generate three transcription categories: early, intermediate, and late (5). Using tiling array technology, a recent study reveals immediate-early transcripts in vaccinia virus (3). An additional microarray-based classification of vaccinia virus and monkeypox virus gene expression divided the virus CDSs into early and late categories only (~50% of the CDSs in each class). The method used in this later study categorized CDSs according to the time the transcript is first detected, so the intermediate class (expressed early and also late) was not distinguished, implying that this class was a subgroup of the early CDSs (27). For PBCV-1, we classified a portion of the early CDSs as early/late when the transcripts were detected before and after the onset of viral DNA synthesis.

Conclusion. For the first time, a global mRNA transcription profile was conducted for a chlorella virus. The PBCV-1 life cycle is temporally programmed. This regulation is controlled by a precise gene expression pattern, where the time of transcription is dictated by initiation of viral DNA replication, which begins 60 to 90 min p.i. Early CDSs were transcribed between 20 and 60 min p.i., and products of the majority of the early CDSs are responsible for providing the machinery for viral DNA replication. Late CDSs were transcribed after 60 min p.i., and most of them were dependent on initiation of viral DNA synthesis, since aphidicolin blocked the expression of most late CDSs. Products of many late CDSs serve either a

structural role or were packaged in the virion, presumably to aid in virus infection. A total of 46% of the early transcripts were still detected after DNA synthesis begins and were called early/late CDSs, indicating a complex mechanism for mRNA maturation and degradation. Some of these early/late CDSs may only be synthesized early, but their transcripts were not degraded until after DNA synthesis begins. However, this scenario probably does not apply to all early/late CDSs because several early-late CDS products were packaged in the virion. These CDSs may also be transcribed as late CDSs.

The present study reveals that PBCV-1 gene expression time is independent of GC content, as well as the transcription direction and CDS location in the genome. It is anticipated that the temporal transcription map will provide clues to the functions of the large category of viral proteins whose functions are not yet known and to the events that must be orchestrated for a successful viral infection.

ACKNOWLEDGMENTS

We thank James Gurnon for technical help and maintaining the culture conditions and Tong Yin, Justin Jackson, and other members of the Microarray Core Facility for technical support. We also thank the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, for generously providing aphidicolin.

This research was supported in part by Public Health Service grant GM32441 (J.L.V.E.) and NIH grant P21RR15635 from the COBRE program of the National center for Research Resources (J.L.V.E.). The UNMC Microarray Core Facility receives partial support from NIH grant P20 RR016469 from the INBRE program (G.A.D. and J.D.E.).

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