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An Early Gene of the Chlorella Virus PBCV-1 Encodes

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PBCV-1 belongs to a family of large viruses that replicate in the exsymbiont green algae *Chlorella* strain NC64A. The viral, 330-kb DNA genome encodes a relatively large number of functionally active proteins including restriction and modification enzymes, DNA polymerase, glycosylation, and cell wall degrading enzymes. Sequencing of the viral DNA, now in progress, revealed many major open reading frames (ORF), which resemble known genes in sequence data bases and which have not previously been found in viral genomes. Here we report on the identification and characterization of one such gene, aspartate transcarbamylase (ATCase), an enzyme that catalyzes the committing step in the *de novo* biosynthetic pathway of pyrimidines. The cloned gene is highly homologous to a variety of plant ATCases and includes the typical ATCase catalytic motif. When cloned into the pGEX-2T expression vector, a fusion protein with ATCase activity could be demonstrated and distinguished from the host ATCase activity. The viral enzyme is expressed early and transiently in the infection. To our knowledge, this is the first virus known to encode and express its own *de novo* nucleotide precursors' synthetic enzymes.

INTRODUCTION

PBCV-1, the first isolated and characterized virus of eucaryotic algae (Van Etten *et al.,* 1982), belongs to a family of large, polyhedral, dsDNA containing viruses that replicate in the exsymbiont, *Chlorella* strain NC64A. The viral genome is a ca 330-kbp linear nonpermuted DNA containing terminal identical inverted repeats that have covalently closed hairpin structures (Rohozinsky *et al.,* 1989). PBCV-1 infection is synchronous and brings about a reduction of host RNA synthesis. The exact location of its replication in the host cell is not clear. Viral DNA synthesis begins about 60 min postinfection, and the progeny viruses appear about 4 hr postinfection. The release of virus particles is completed within 6–8 hr by cell lysis and infectious virus can be plaque assayed (Van Etten *et al.,* 1983a, 1983b). Early viral transcripts are readily detected as early as 5 min postinfection and transcription is regulated in a temporal fashion (Schuster *et al.,* 1986). Viral proteins are synthesized on cytoplasmic ribosomes. At least some of the transcripts contain an intron with nuclear splicing sequences, suggesting nuclear processing (Grabherr *et al.,* 1992). Some of the early transcripts, but not the late ones, are polyadenylated (Van Etten *et al.,* 1988). Cytological examination revealed that capsid assembly and DNA packaging occur in distinct regions in the cytoplasm (Mients *et al.,* 1986).

An intriguing feature of the PBCV-1 (and related viruses) DNA is that it codes for a relatively large number of putative genes homologous to proteins and enzymes previously not shown to exist in viral genomes. These include genes for enzymes involved in the alteration of sugars and DNA (Lu *et al.,* 1995; Li *et al.,* 1995). PBCV-1-infected cells were shown to synthesize functionally active, virus-coded DNA restriction and modification enzymes, glycosylation and cell wall degrading enzymes, and structural proteins of the capsid (Van Etten *et al.,* 1991). The expression regulation of these genes varies. Adenine DNA methyltransferase, M.CviAI, is made between 30 and 60 min postinfection, and coincides with the onset of host DNA degradation (Xia and Van Etten, 1986). The elongation factor vEF3, which is probably involved in translation regulation, is expressed both early and late in the infectious cycle of the virus CVK2 (Yamada *et al.,* 1993).

The aim of our work was to identify genes that were transiently expressed early in the viral infection of PBCV-1. Identification of such genes is crucial for studying the regulation of PBCV-1 infection in *Chlorella* and its relationship with the host cell. Genes expressed very early in the infection are likely to be involved in the onset of the virus infectious cycle, as well as in the shutoff of the host macromolecular biosynthesis. Here we report on the identification of an early viral gene coding for aspartate transcarbamylase (ATCase), also known as aspartate carbamoyltransferase. This enzyme, which catalyzes the committing step in the *de novo* pyrimidines

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biosynthetic pathway, is expressed during the early stages of the infection, suggesting a role in the biosynthesis of viral DNA.

MATERIALS AND METHODS

Cells and virus

Chlorella NC64A, obtained from Dr. J. L. Van Etten (Lincoln, NE), was grown in a modified Bold's basal medium (MBBM) as described (Van Etten *et al.,* 1983a). The *Chlorella* virus PBCV-1 was purified from infected cultures and titered by plaque assay, as described (Van Etten *et al.,* 1983a).

DNA isolation and analysis

DNA was isolated from PBCV-1 by incubating the virus at 65° for 60 min in the presence of 0.1 *M* NaCl, 0.01 *M* Tris (pH 7.4), 1 m*M* EDTA, 0.1% Sarkosyl, and 1 mg/ml proteinase K. DNA was purified by two extractions with phenol/chloroform followed by two extractions of chloroform and used for restriction enzymes and Southern blot analyses. Digested DNA was electrophoresed on an agarose gel, transferred to a nylon membrane (MSI) and hybridized with probes labeled with [^{32}P]dCTP, using a random priming labeling kit (USB). Hybridizations were performed at 42° for 24 hr as described (Davies *et al.,* 1986). The membranes were scanned by Phosphorimager (Molecular Dynamics) or exposed to Kodak X-ray films at −70°.

RNA analysis

Infected NC64A cells were frozen in liquid nitrogen, homogenized in a mortar, and suspended in 1 ml TRI-REAGENT (Molecular Research Center, Inc.). RNA was isolated following the manufacture's instructions. Total RNA was electrophoresed in 1.2% denaturing formaldehyde agarose gel and transferred to nylon membranes (MSI). The membranes were hybridized with ^{32}P-labeled PBCV-1 DNA fragments as indicated in each experiment. For stripping, the filters were incubated in 50% formamide, 2× SSPE at 65° for 1 hr.

Cloning and sequencing viral DNA fragments

Viral DNA was digested with *Hin*dIII. Several purified fragments were cloned into pGEM2 (Promega) in *Escherichia coli* HB101. For nucleotide sequencing, DNA fragments were subcloned into pBluescript (Stratagene) in *E. coli* JM109. Sequencing was performed by the dideoxy chain termination method using the Sequenase version II kit (USB) according to the manufacturer's instructions, with [^{35}S]dATP. The reaction was carried out in the presence of either universal primers or synthetic oligonucleotides. A few of the nucleotides sequences were determined in an automated fluorescent sequencer (Applied biosystems). Searching data bases for homologous sequences and alignment of sequences was done with the Sequence Analysis Software Package (Genetics Computer Group, Inc. (GCG)).

Construction of a gluthatione *S*-transferase (GST)-ORF-1 fusion protein

ORF-1 was amplified by polymerase chain reaction (PCR) using synthetic oligonucleotides as primers, each containing a *Bam*HI restriction site. The primers for the 3′ and 5′ ends were 5′-GCGGGATCCACACTCACCCCA-GGTG-3′ and 5′-GCGGGATCCAGATGATATGAGAGT-ATCG-3′, respectively. The amplification reaction was performed with vent R DNA polymerase (New England BioLabs). PCR-amplification products were purified from agarose gel, digested with *Bam*HI, and ligated in frame to *Bam*HI-cleaved pGEX-2T expression vector (Pharmacia). The resulting plasmid, designated pGE18, was cloned in *E. coli* HB101. To detect the GST–ORF-1 fusion protein, bacteria induced with 0.1 m*M* isopropylthio-*b*-D-galactoside (IPTG) were disrupted by sonication in the presence of 200 m*M* phenylmethyl-sulfonyl fluoride (PMSF), pelleted and gluthatione–Sepharose beads were added to the supernatant as described (Ausbel *et al.,* 1990). The washed beads and bacterial extracts were mixed with SDS-loading buffer, electrophoresed on 10% SDS–polyacrylamide minigel, and stained with Coomassie brilliant Blue.

Aspartate carbamoyltransferase activity in *Chlorella* and in bacterial extracts

ATCase activity in uninfected and PBCV-1-infected *Chlorella* cells was determined by the standard ATCase assay (Patterson and Carnright, 1977). Briefly, cell extracts were incubated with the ATCase substrates L-aspartate (1.6 m*M*), L-[U-^{14}C]aspartate (6.5 μCi) and carbamyl-phosphate (1 m*M*) in HEPES (*N*-2-hydroxyethypiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 8.5, for 30 min at 30°. The conversion of L-[U-^{14}C]aspartate to carbamyl [U-^{14}C]aspartate was quantitated by separating the reaction products by chromatography on cellulose plates which were air-dried and scanned in a phosporimager (General Dynamics).

Bacterial extracts were prepared as described above. The fusion protein was bound to the gluthatione–Sepharose beads in phosphate-buffered saline (PBS), washed with PBS, and eluted with 5 m*M* reduced gluthatione as described (Ausbel *et al.,* 1990). Samples were electrophoresed in 5% polyacrylamide native minigels at 4°. Enzyme activity was determined as published (Ruiz and Wahl, 1986) except that enzyme activity was performed at 30°. Briefly, the gel is incubated in a solution of 1 m*M* carbamyl phosphate and 20 m*M* aspartate at pH 7.0 for 10 min. ATCase position in the gel is detected by determining the inorganic phosphate, Pi, generated in the course of the enzymatic reaction. Pi is precipitated in the

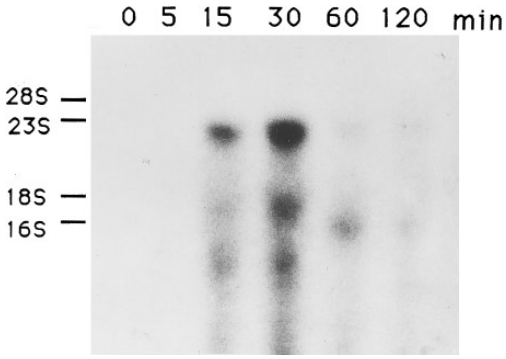

FIG. 1. RNA transcripts encoded by DNA clone HF7. Northern blot of RNA isolated from NC64A *Chlorella* cells at different times after PBCV-1 infection were hybridized with ^{32}P-labeled HF7 DNA (1.3-kb *Eco*RI–*Hin*dIII fragment. The size markers were a mixture of bacterial rRNA and algal rRNAs and the sedimentation values of 28S, 23S, 18S, and 16S correspond to 3.0, 2.9, 2.0, and 1.6 kb, respectively.

gel by incubation with 3 m*M* lead nitrate, washed several times in cold water, and incubated in 5% ammonium sulfide for 10 min. The brown band of lead sulfide precipitate generated is indicative of the ATCase position and its intensity is directly related to ATCase activity.

RESULTS

Identification of early viral transcripts

Some PBCV-1 transcripts appear as early as 10 min postinfection and are thus likely to be involved in the regulation of the viral infection. To identify early regulatory genes, RNA isolated at different times postinfection was electrophoresed in formaldehyde gels, blotted onto nylon filters, and hybridized with some of the smaller size *Hin*dIII PBCV-1 DNA, cloned into a pGEM2 vector, and labeled with ^{32}P. In this work we focused on a 4.4-kb *Hin*dIII fragment that hybridized to several RNA transcripts transiently expressed at 15 min p.i. This fragment, designated HF7, was eventually identified as fragment H19 at position 85–88 in the PBCV-1 physical map (Girton and Van Etten, 1987). Further cleavage of this fragment with *Eco*RI yielded a 1.3-kb *Eco*RI–*Hin*dIII fragment, designated HF7/3, that hybridized with several, transiently expressed early transcripts with approximate sizes of 2.5, 1.8, and 1.0 kb, all appeared around 15 min, peaked around 30 min, and were undetectable by 60 min postinfection, at which time a newly synthesized, 1.6-kb transcript appeared (Fig. 1). HF7/3 was ligated to pBluescript, cloned in JM109, and the sequence determination revealed two contiguous incomplete open reading frames (ORFs) (Fig. 2). Using the HF7 as template and a synthetic oligonucleotide as a primer, the full length of ORF-1 and its upstream sequence were determined. ORF-1 is 969 bp and codes for a 323 amino acids protein with the expected molecular weight of 37 kDa (Fig. 4). To verify the early nature of ORF-1 RNA, Northern blots were hybridized with a ^{32}P-labeled, 400-bp *Eco*RI–*Bgl*II fragment of HF7/3. This fragment, which is located entirely within ORF-1 (Fig. 2) and does not contain other ORFs, hybridized to the 2.5- and 1.0-kb early transcripts (Fig. 3).

Sequence and protein analysis

The sequence of ORF-1 has been published (Li *et al.,* 1995; GeneBank Accession No. U32570). The 70-nucleotide region preceding ORF-1 is rich in A + T (71%), as is the case with a variety of viral promoters (Weir and Moss, 1983; Schuster *et al.,* 1990). This region contains several candidates for the transcriptional start signal, the most likely to be a functional one is the sequence TATATA, 63 nucleotides upstream of the initiation codon AUG. Nucleotides at positions −3 and +4 relative to the first AUG codon are both purines, suggesting a functional translation start site (Kozak, 1984). A consensus sequence for polyadenylation (AATAAA) is found 31 bases downstream of the ORF-1 stop codon.

Searches in peptide and protein databases revealed homology between the translation product of ORF-1 and ATCase from various organisms. As shown in Fig. 4, the highest degree of homology (about 70% similarity and 54% identity) was to ATCases from plants (tomato, arabidopsis, and pea). The identification of the gene as ATCase is also verified by the presence of a typical motif of the ATCase catalytic site, F-X-E-X-S-G/T-R-T, 64 amino acids downstream from the first methionine. Furthermore, dot plot alignment of three plant ATCase sequences with the viral ATCase (vATCase) revealed five major regions of conserved amino acids. No putative donor and acceptor consensus sequences for splicing were found, in agreement with previously published data (Li *et al.,* 1995).

Expression of ORF-1 product

To demonstrate that the gene product of ORF-1 has ATCase activity, a 1054-bp sequence was amplified by PCR and cloned into the expression vector pGEX-2T, as described under Materials and Methods (Fig. 5). The expected protein product is a 64.5-kDa fusion protein that would lack three amino acids at the amino terminus of the ATCase gene and a substitution of the fourth amino acid from proline to serine. The advantages of a fusion protein were twofold. It allowed distinguishing the viral from the cellular ATCase by two criteria, namely the size of the fusion protein and its inducibility with IPTG. In addition, it enabled the purification of the enzyme by affinity binding to a resin-bound gluthatione.

The production of a fusion protein with the expected size was confirmed by SDS–PAGE analysis of bacterial extracts and of affinity bound protein, before and after induction by IPTG (Fig. 6a). A protein with an expected size, which was barely detectable in untreated cultures,

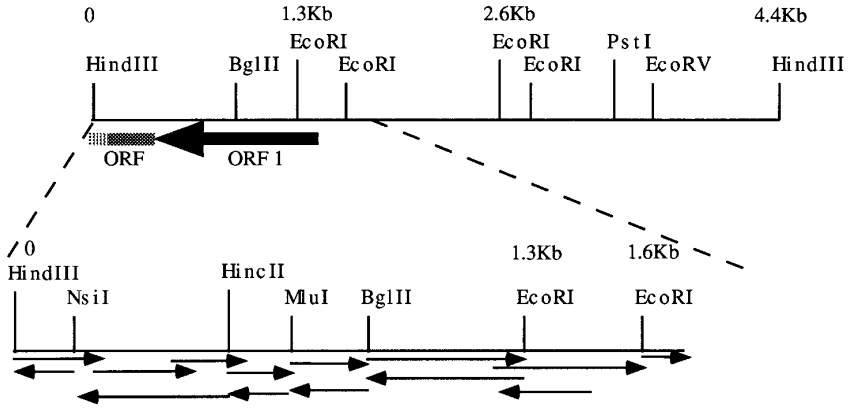

FIG. 2. Restriction map of 4.4-kb HF7 and sequencing strategy of HF7/3. The physical map was determined by cleavages with various restriction enzymes. The nucleotide sequence of 1728 nucleotides of HF7 was determined as described under Materials and Methods. The large arrows indicate the direction of ORFs.

became visible after IPTG induction and a protein of the same size was enriched over 20-fold after glutathione–Sepharose purification. Finally, untreated and IPTG-induced bacterial extracts, before and after glutathione–Sepharose purification, were electophoresed in a 5% acrylamide gel under nondenaturing conditions and assayed *in situ* for ATCase activity according to the method described by Ruiz and Wahl (1986). ATCase activity is visualized in the gel by precipitation of the inorganic phosphate formed in the ATCase reaction. This method, therefore, enabled us to distinguish between the bacterial enzyme and ATCase activity derived from the cloned gene. As shown in Fig. 6b, the native molecular weight of the enzyme extracted from *E. coli* HB101 cells was close to 200,000 (lane 5). In contrast, two activity bands were observed in extracts of HB101/pGE18, one of which comigrated with the bacterial enzyme, and the other had a molecular weight of at least 500,000 (lanes 1 and 2). The latter represents the basal expression level of the Ptac promoter, and is 2- to 5-fold lower than that of the bacterial enzyme. This ratio increased dramatically after IPTG induction (lane 4).

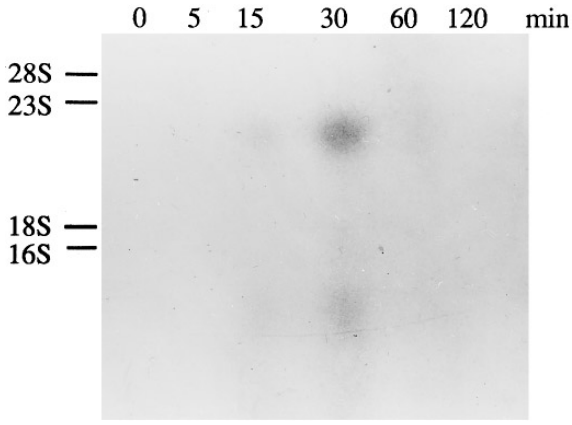

FIG. 3. RNA transcripte encoded by HF7/3 DNA. Northern blot of total RNA isolated from NC64A *Chlorella* cells at different times after PBCV-1 infection was hybridized with ^{32}P-labeled, HF7/3 DNA (400-bp *Eco*RI–*Bgl*II fragment).

ATCase activity in PBCV-1-infected *Chlorella* cells

The results above confirmed the presence of a gene coding for ATCase in the viral genome. To find out if this gene is indeed expressed in viral infection, NC64A *Chlorella* cells were infected with PBCV-1 and ATCase levels in the infected cells were assayed throughout the infection. Figure 7 shows that at 60 min postinfection ATCase levels increased over twofold beyond the basal level of the enzyme in uninfected cells and remained constant over the test period of 180 min.

DISCUSSION

The large dsDNA *Chlorella* virus PBCV-1 has already been reported to code for several enzymes involved in nucleic acids synthesis. Van Etten *et al.* (1991) reported the existence of virus-encoded DNA polymerase and DNA methyltransferase. Bornemann and Follmann (1993) reported a protein with ribonucleotide reductase activity that is expressed as a consequence of viral infection of *Chlorella* Pbi. Here we report that aspartate transcarbamylase, the committing enzyme of *de novo* pyrimidine biosynthesis, is expressed by the PBCV-1 genome. The reaction catalyzed by ATCase involves the formation of aspartate-carbamyl and P_i from aspartate and carbamylphosphate. The ATCase sequence is located at map position 87 and is identical to ORF A169R in the recently published physical map (Li *et al.,* 1995). The expression pattern of ATCase mRNA and protein in infected cells conforms with its function as a synthetic enzyme responsible for the synthesis of pyrimidine precursors for DNA biosynthesis. Viral ATCase mRNA is synthesized early in the infection and declines around 60 min postinfection (Figs. 1 and 3), at the onset of viral DNA synthesis (Van Etten *et al.,* 1984). A concomitant two- to threefold in-

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Arabidopsis  R N V G P V R C H A M Q A G T R E L K K F E L S D V I E G K Q F D R E M L S A I  182
     Tomato  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  0
        Pea  G A L Q V E S A P L F S V G . . . . Q K F Q L D D V I E A Q Q F D R E T L S A I  155
     Pbcv-1  M I I P T L T P G V E N F N A P V W F R D P V K H I V K S Q Q F S K Q T V Q E L  40

Arabidopsis  F D V A R E M E K I E K S S S Q S E I L K G Y L M A T L F Y E P S T R T R L S F  222
     Tomato  . . . . . . . . . . . . . . . . . . . . G Y L M A T L F Y E P S T R T R L S F  19
        Pea  F E V A R S M E N I R G N S S G S Q M L K G Y L M A T L F Y E P S T R T R L S F  195
     Pbcv-1  C I L A S E F K N V R N D S . . . . . L R G K K M L T Y F E E P S T R T R L S F  75

Arabidopsis  E S A M K R L G G E V L T T E N A R E F S S A A K G E T L E D T I R T V E G Y S  262
     Tomato  E S S M K R L G G E V L T T E N A R E F S S A A K G E T L E D T I R T V E G Y S  59
        Pea  E S A M K R L G G D V L T T E N A R E F S S A A K G E T L E D T I R T V E G Y S  235
     Pbcv-1  E S A M Y D L G G Y V M S V E N A V N . S S K A K G E T I E D T V R T I E R Y A  114

Arabidopsis  D I I V M R H F E S G A A R K A A A T A N I P V I N A G D G P G E H P T Q A L L  302
     Tomato  D I I V M R H F E S G A A R R A A L S A S I P I I N A G D G P G Q H P T Q A L L  99
        Pea  D I I V L R H F E S G A A R R A A A T A N I P V I N A G D G P G Q H P S Q A L L  275
     Pbcv-1  D V F V I R S N T A G T A E K A A R V S G I P V I N A G D G A G Q H P T Q A L L  154

Arabidopsis  D V Y T I Q S E I G K L D G I S V A L V G D L A N G R T V R S L A Y L L A K F K  342
     Tomato  D V Y T I G R E I G K L D G I N I A L V G D L A Y G R T V R S L A H L L A L Y K  139
        Pea  D V Y T I E R E I G K L D G I K V G L V G D L A N G R T V R S L A Y L L A K Y R  315
     Pbcv-1  D V Y T I Y D K F G D L N D L T I V V V G D L L Y S R T V H S L V Y M L S L F K  194

Arabidopsis  D V K I Y F V S P E I V K M K D D I K D Y L T S S G V E W E E S S D L M E V A S  382
     Tomato  D V K I Y F V S P D V V K M K D D I K D Y L T S M G V R W E E S A D L I E V A S  179
        Pea  D V K L Y F V S P N V V K M K D D I K E Y L T S K G V E W E E S S D L M E V A S  355
     Pbcv-1  . V R M I F V A P T E C Q M K Y D L K N Y L Q D V G V V Y E E S N D L E S V S K  233

Arabidopsis  K C D V V Y Q T R I Q R E R F G E R L D L Y E A A R G K F I V D K D L L G V M Q  422
     Tomato  K C D V V Y Q T R I Q R E R F G E R V A L Y E E A R G K Y I V D M S A V N A M Q  219
        Pea  K C D V V Y Q T R I Q K E R F G E K L N L Y E E A R G K Y I V N Q D V L K V M Q  395
     Pbcv-1  I A D V V Y M T R I Q K E R F T D R P D D Y D K C V G K Y I M S K M I V E Q M K  273

Arabidopsis  K K A I I M H P L P R L D E I T A D V D A D P R A A Y F R Q A K N G L F I R M A  462
     Tomato  K H A V V M H P L P R L D E I T V D V D G D P R A A Y F R Q A K N G L Y I R M A  259
        Pea  N H A V V M H P L P K L D E I E A D V D N D P R A A Y F R Q A K N G L Y I R M A  435
     Pbcv-1  Q N S I I M H P L P R V D E I S P D V D T N H R A V Y F D Q V E R G L E M R K A  313

Arabidopsis  L L . K L L L V G W * 471
     Tomato  L L . K L L L L G W * 268
        Pea  L L . K V L L L G W * 444
     Pbcv-1  L L F S I F Y N A F * 323
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FIG. 4. Alignment of amino acid sequence of ATCase from various plants with PBCV-1 ATCase (vATCase). Alignment was achieved by the pile-up program of the GCG.

crease in total ATCase activity is observed (Fig. 7), and these high levels persist throughout the infection (Mincberg and Tal, unpublished).

This is the first report of a functionally active ATCase in a virus genome. Its function is not yet understood. Some of the possible explanations are (i) The short growth cycle of the virus requires a rapid buildup of nucleotide precursors for viral DNA synthesis (Van Etten *et al.,* 1984), a requirement that may not be satisfied by the host cell's metabolic machinery. (ii) The activity of viral ATCase may differ from that of the host enzyme in the infected cell's environment. Differences in activity may include sensitivity to possible inhibitors like CTP or activators like ATP (Kantrowitz and Lipscomb, 1988), pH optimum, K_m etc. As an example, altered enzymatic properties (i.e., response to metal ions and the optimal temperature) were found in ribonucleotide reductase of infected *Chlorella* Pbi (Bornemann and Folmann, 1993). (iii) Plant ATCase and most enzymes of this pathway are located in the chloroplast (Doremus and Jagendorf, 1985). The first 55 bases of pea ATCase code for a leader sequence (Williamson and Slocum, 1993, and our sequencing data). Although organelle arrangement was not affected cytologically by infection, it might be that due to nonvisible changes occurring during the infection, pyrimidines are no longer available in the nucleus or cytoplasm. This possibility is supported by the findings that PBCV-1 infection leads to immediate cessation of CO_2 fixation

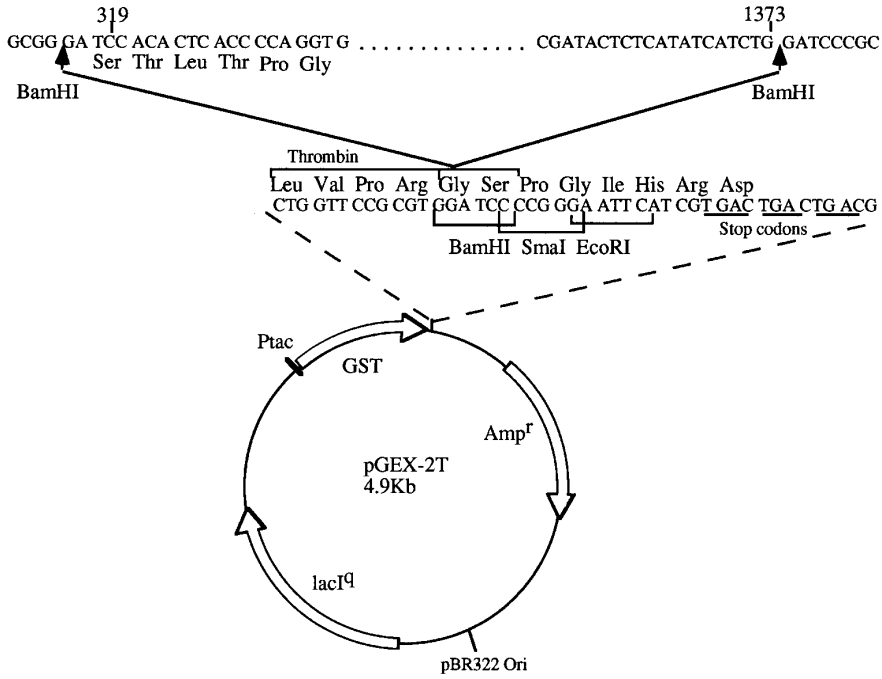

FIG. 5. Construction of pGE18. The PCR amplification product of ORF-1 (see Materials and Methods) was ligated to the *Bam*HI site of pGEX-2T, resulting in *in-frame* insertion. The numbers of the first and last nucleotides, taken from the nucleotide sequence in Fig. 4, are shown.

and photosynthesis and to degradation of chloroplast DNA and ribosomal RNA (J. Van Etten, personal communication).

The multiple transcripts detected by the 1.3-kb *Eco*RI–*Hin*dIII fragment and their overall cumulative size (which exceeds that expected from this size fragment) suggest overlapping of mRNA transcripts and/or transcription from both strands. These results are in agreement with those of Schuster *et al.* (1986, 1990) who found complex patterns of RNA and the total length of transcribed RNA exceeded that of the ORFs identified in these DNA fragments.

The Northern hybridization analysis, together with the sequence data suggest that the primary transcript of ATCase may undergo some posttranscriptional modifications. The 2.5-kb transcript size is roughly the combined sizes of ORF-1 (about 1 kb) and its downstream ORF (about 1.2 kb, not shown; J. Van Etten, personal communication). The 2.5- and 1-kb transcripts both hybridize to the *Eco*RI–*Bgl*II, 400-bp fragment (Fig. 3), suggesting a precursor–product relationship. Since these two transcripts did not hybridize the 1000-bp *Eco*RI–*Eco*RI fragment of HF7, located at the 5′ end of ORF-1 (data not shown), any extension of the 1-kb transcript must be in

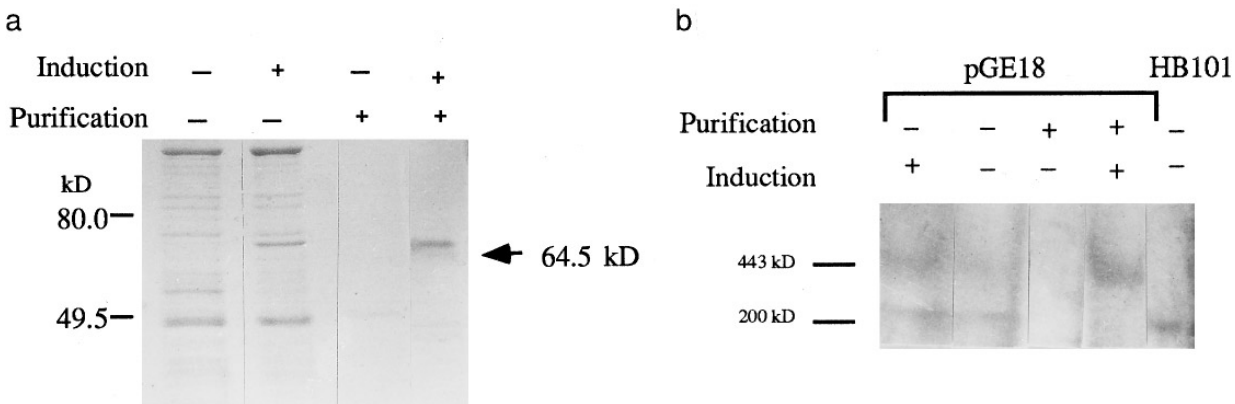

FIG. 6. Demonstration of IPTG-induced ATCase activity. (a) SDS–PAGE of extracts from pGE18/HB101 before and after induction with IPTG. Extracts were electrophoresed on a 10% gel with and without affinity purification on gluthatione–Sepharose beads. The gel was stained with Coomassie briliant blue. (b) ATCase activity in native gel. Extracts of HB101 and pGE18/HB101 were electrophoresed on a 5% native gel and tested for ATCase activities described under Materials and Methods. Extracts from pGE18/HB101 were used before and after induction with IPTG and with or without affinity purification on gluthatione–Sepharose beads.

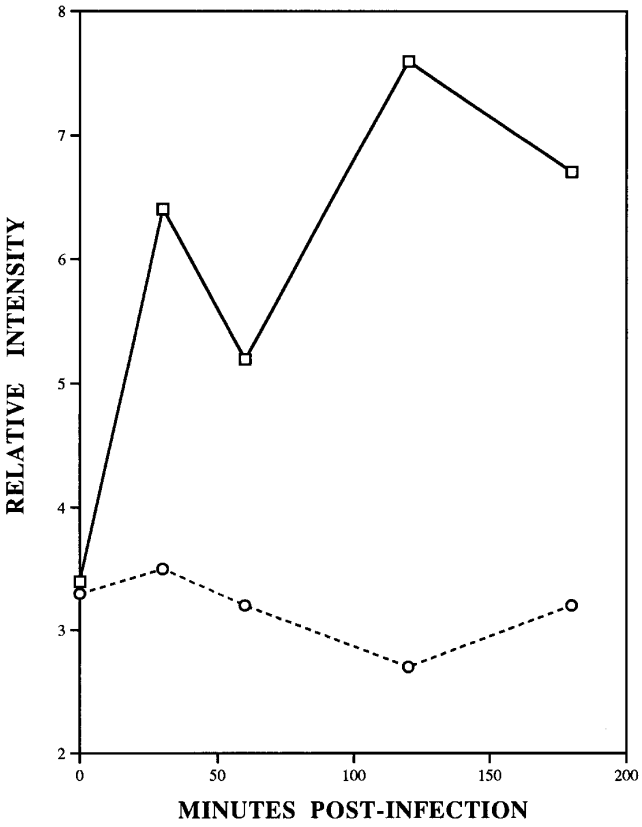

FIG. 7. Expression of ATCase in PBCV-1-infected NC64A cells. Lysates of infected cells were assayed for ATCase as described under Materials and Methods. Solid line, infected cells; dashed line, uninfected cells.

the downstream direction. The transcription of ORF-1 most probably extends beyond its 3′ end into the downstream ORF, but the present data is not sufficient to determine whether the 2.5-kb RNA is a joint primary transcript encompassing the two open reading frames, or it terminates within the downstream ORF. The polyadenylation signal downstream of ORF-1 is a further indication that this RNA is cleaved and at least the ATCase mRNA undergoes polyadenylation, in agreement with the observation that some of the early genes of PBCV-1 are polyadenylated (Van Etten *et al.,* 1991).

Regulation of transcription/translation of the pyrimidine biosynthetic pathway enzymes is controlled in different ways in various organisms. In bacteria, enzymes of the metabolic pathway form an operon (O'Donovan and Neuhard, 1970). In mammalians three different activities of the pathway are combined in the multifunctional CAD protein (Faure *et al.,* 1989). The protein from fungi possesses the first two committing activities of the pathway (Williams *et al.,* 1970). vATCase, like ATCases from plants (Williamson and Slocum, 1993; Overduin *et al.,* 1993), is also a single function enzyme. The question of whether ATCase is the only virus-encoded enzyme of the pyrimidine biosynthetic pathway remains open.

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