Virology **302,** 123–131 (2002)
doi:10.1006/viro.2002.1572

Chitin Synthesis in Chlorovirus CVK2-Infected *Chlorella* Cells

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Received February 8, 2002; returned to author for revision March 25, 2002; accepted May 15, 2002

Hyaluronan synthesis in chlorovirus PBCV-1-infected *Chlorella* cells was previously reported (DeAngelis *et al.,* 1997). In contrast, we report here on the detection, characterization, and expression of a gene for chitin synthase (*chs*) encoded by chlorovirus CVK2 isolated in Kyoto, Japan. The CVK2 *chs* gene encoding an open reading frame of 516 aa was expressed as early as 10 min postinfection (p.i.), peaked at 20–40 min p.i., and disappeared at 120–180 min p.i. The chitin polysaccharide began to accumulate as chitinase-sensitive, hair-like fibers on the outside of the virus-infected *Chlorella* cell wall by 30 min p.i. All chloroviruses without the gene for hyaluronan synthase (*has*) alternatively contained the *chs* gene, suggesting the importance of polysaccharide production in the course of virus infection. A few chloroviruses possessed both the *chs* and *has* genes and produced chitin and hyaluronan simultaneously. Polysaccharide accumulation on the algal surface may protect virus-infected algae from uptake by other organisms, such as protozoa. Since CVK2 was reported to encode two chitinases and one chitosanase, CVK2 is a very peculiar virus that encodes enzymes required for both the synthesis and the degradation of chitin materials.

INTRODUCTION

Chitin, a β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant polymer in nature, after cellulose. Chitin is present in insects, crustaceans, and most fungi; plants, vertebrates, and prokaryotes do not contain chitin. In recent years, chitin and chitosan, partially or fully deacetylated chitin, have received a great deal of attention because of their many potential applications in the biochemical, medical, agricultural, and environmental science fields (Sanford, 1989; Shigemasa and Minami, 1996). In this report, we demonstrate for the first time the production of chitin by algae infected by chloroviruses. Chloroviruses or the *Chlorella* viruses are large icosahedral, double-stranded DNA-containing viruses that infect certain strains of the unicellular green alga *Chlorella* (Van Etten *et al.,* 1991; Van Etten and Meints, 1999). These viruses belong to the family Phycodnaviridae and are ubiquitous in natural environments (Van Etten *et al.,* 1991; Yamada *et al.,* 1991). Analysis of the 330,740-bp genomic DNA sequence of PBCV-1, the prototype member of Phycodnaviridae, revealed various unexpected genes (Kutish *et al.,* 1996; Li *et al.,* 1995, 1997; Lu *et al.,* 1995, 1996). One of these genes, *a98r,* encodes a functional hyaluronan synthase (HAS) (DeAngelis *et al.,* 1997) and is expressed early in viral infection to produce hyaluronan polysaccharide on the outside of the host *Chlorella* cell wall (Graves *et al.,* 1999). Hyaluronan is a simple linear polysaccharide chain composed of alternating β-1,4-glucuronic acid (GlcA) and β-1,3-GlcNAc moieties (Laurent and Fraser, 1992). HAS adds sugar residues from UDP-GlcA and UDP-GlcNAc. Landstein *et al.* (1998) revealed that PBCV-1 encodes two other enzymes, glutamine:fructose-6-phosphate amidotransferase and UDP-Glucose dehydrogenase (UDP-GlcDH), that produce sugar precursors (GlcN-6-P and UDP-GlcA, respectively) required for hyaluronan synthesis. The presence of multiple enzymes involved in the hyaluronan biosynthetic pathway suggests some importance of hyaluronan production in the PBCV-1 infection. However, the biological functions for PBCV-1 hyaluronan are largely unknown (DeAngelis *et al.,* 1997; Graves *et al.,* 1999; Landstein *et al.,* 1998). Graves *et al.* (1999) examined the occurrence of the *has* gene in other chloroviruses isolated from diverse geographical regions and found that the PBCV-1 *has* gene probe did not hybridize to 9 of 37 DNAs from viruses that infect *Chlorella* NC64A. This result indicates that the *has* gene is not always essential for chlorovirus replication. One possible explanation is that those *has*-lacking viruses encode an enzyme or enzymes to produce alternative polysaccharide on the external surface of the infected *Chlorella* cells.

In this study, we report that chlorovirus CVK2 has a gene for functional chitin synthase (CHS) instead of HAS and produces chitin fibers surrounding the external surface of virus-infected *Chlorella* cells. All chloroviruses

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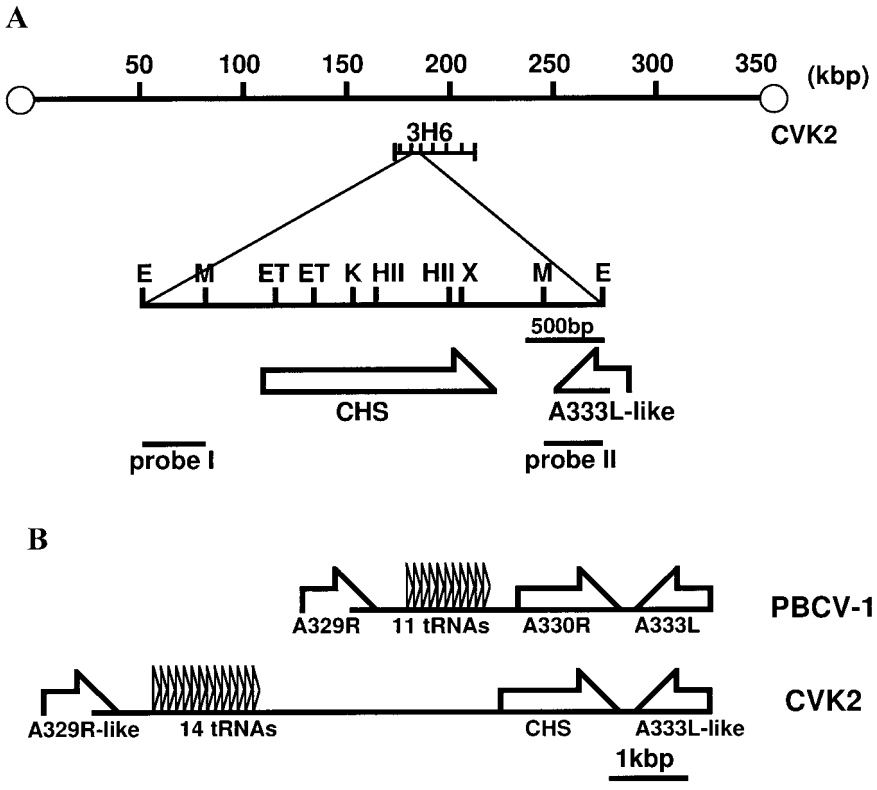

FIG. 1. Physical map of the CVK2 *chs* gene and its location on the CVK2 genome. (A) CVK2 *chs* is located on a 3.0-kb *Eco*RI fragment of cosmid 3H6 mapped on the central region of the 350-kb CVK2 genome (Nishida *et al.,* 1999). Arrow indicates the position and orientation of the gene. Probes used for Northern blot analysis are indicated by bars: probe I, a 460-bp *Eco*RI/*Mlu*I fragment of 3H6 and probe II, a 400-bp *Mlu*I/*Eco*RI fragment of 3H6. Restriction sites: E, *Eco*RI; ET, *Eco*T22I; HII, *Hin*cII; K, *Kpn*I; M, *Mlu*I; X, *Xba*I. (B) Comparison of the gene arrangement between the CVK2 and PBCV-1 genomes. Arrows indicate each gene and its orientation.

isolated in Japan that lacked the *has* gene contained the *chs* gene. A few viruses contained both the *has* and *chs* genes and formed both hyaluronan and chitin on the surface of the infected cells. The biological significance of these polysaccharides in virus infection is discussed.

RESULTS

Detection, cloning, and characterization of the CVK2 chitin synthase gene

In our previous work, we compared the genomic gene arrangement between CVK2, a chlorovirus isolated in Kyoto, Japan (Yamada *et al.,* 1991), and PBCV-1 and found that there is extensive colinearity along both of the genomes. Exceptions are (i) an insertion of a 20-kb region around the left terminus of CVK2 DNA, (ii) a duplication of the gene for a major capsid protein in CVK2 DNA, (iii) deletion/insertion of some ORFs (open reading frames), and (iv) divergence in the terminal inverted repeat sequences (Nishida *et al.,* 1999). After determining the nucleotide sequence of the 20-kb extra CVK2 region, we found that the *has* gene corresponding to PBCV-1 A98R (ORF), encoding hyaluronan synthase, was absent from the CVK2 genome; the approximately 2.0-kb *has* region (position 50,860–52,708 in PBCV-1) was replaced in CVK2 DNA with a different sequence containing fragmented ORFs, some of which showed homology to chitin synthase genes of various organisms (data not shown). From this region, no transcript was formed throughout the viral infection cycle based on Northern blot analysis (data not shown). Southern blot analysis of CVK2 DNA probed with PBCV-1 *has* DNA did not show any hybridizing signals, indicating no *has* gene on the CVK2 genome. Therefore, we are interested in the possibility of another polysaccharide that is produced early in infection by virus-encoded enzyme(s) replacing hyaluronan.

To seek such polysaccharide-producing enzymes, a cDNA library was prepared with poly(A)$^+$ RNA isolated from CVK2-infected host cells at 20 min postinfection (p.i.). At least 30 different cDNA clones were obtained that specifically hybridized to CVK2 DNA and that showed significant sequence homology with PBCV-1 ORFs and/or genes in the databases (Kawasaki *et al.,* in preparation). Among them, there was a clone (K2E8) showing significant homology with the chitin synthase gene (*chs*) of various organisms. This clone hybridized to a 3.0-kb *Eco*RI fragment of cosmid 3H6 (Nishida *et al.,* 1999) that corresponds to a position around the central part of the 350-kb CVK2 genome (Fig. 1). The nucleotide sequence around this region (DDBJ Accession No.

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CVK2 CHS            1  MRLSWPLTLGLLSLVIIFHEYWYAFAPILVLGAASSLWYIAWVLMHRVYLGFKGKPVLTAPKEPMMFLVTAYRETKEELDRTVESVTMQKID
E.dermatitidis    659  VGFIHENVVPQPPPEWQPFGYPLAHVICLVTAYSEGEDGIRTTLDSIATTDYPNSHKAILVVCDGMI--KGKGEAQSTPDIVLGMMGDFVIA
T.magnatum        657  AGFIHEAVVPQPPPEYQPFGFPLAHALCLITCYSEGEQGIRTTLDSIATTDYPNSHKLMLIVCDGMI--KGHGETKTTPEIILSMLKDHAVH
C.albicans        600  PDIIHPDVVPQPPVEYQPFGYPLAHTINLVTCYSEDEEGIRITLDSIATTDYPNSHKLILVICDGII--KGSGNDETTPDIVLDMMSDLTVP
                            :              *    *:         *  :*   :   : ::   :      ::      : :      :       :

CVK2 CHS           93  PE-V-SKTVVVIVDGEKETAHELRKYNQYDETFVIKDAYEDWHNKPKDVTIFKKIHNGI-DVVYLIKSENAGKRDS-VVLARTLAYGNLFEH
E.dermatitidis   649  PEDVQAFSYVAVSSGAKR--HNMAKV--YAGFYDYGPKSRIDPTKQQRVPMMVVVKCGTPDEATKSKPGNRGKRDSQIILMSFLQKVMFDER
T.magnatum       647  PDAVQGFSYVAVASGAKR--HNMAKV--YAGFYDYGENSQIPPEKQQRVPMLVVVKCGTPDEEGGGKAGNRGKRDSQIILMSFLQKVMFDER
C.albicans       690  RDEVEAYSYVAVAQGSKR--HNMAKV--YAGFYKYN-DETVPPEKQQRIPMITIVKCGTPEEASAPKPGNRGKRDSQIILMSFLQKVVFDER
                      :  *     *:: :* *:  *:: *   *               * : : ::  :: *        *  * ***** ::*   *      *

CVK2 CHS          181  SENRHAMKISGELDLIW---SRLVPKVTRMI-GIDADT-VFHEDCSQALLEEM-NYPGDRPVDGVVGYIDIEMGKGKSPYQKAWI-WFQGIG
E.dermatitidis   737  -MTELEYEM---FNGLWKVTG-MSPDFYEMVLMVDADTKVFPDSLTHMVSA-MVKDPE---IMGLCGETKIAN-KNA-----SWVSRIQVFE
T.magnatum       735  -MTELEFEM---FNGIWKITG-ISPDYYEVVLMVDADTKVFPDSLTHMVAA-MVNDPD---IMGLCGETKIAN-KTG-----SWVSMIQVFE
C.albicans       778  -MTSLEYEM---LQSIWRITG-LMAEFYEIVLMVDADTKVFPDSLTHMVAE-MVKDPT---IMGLCGETKISN-KAQ-----TWVTAIQVFE
                        :::   :  :*   : :     :::  :**** ** :: :: :   * : *    : *: *   * : *        *:  :* :
                                 I                                                                  II
CVK2 CHS          266  YIIGQHVMRVYQSRITEKVSCLSGACYGIY----------VPTMCEPELLKEFNTPPPPNAGLFLSILGYASEDRRSVVLSLCRDRNVRF
E.dermatitidis   814  YFISHHLSKSFES-VFGGVTCLPG-CFCMYRIKSPKGGQNYWVPILANPDIVEHYSENVVDT--LHKKNLLLLGEDRYLSTLML-KTFPKR-
T.magnatum       812  YFISHHLTKAFES-VFGGVTCLPG-CFCMYRIKTPKGGQNYWVPILANPDVVEHYSENVVDT--LHKKNLLLLGEDRYLSTLML-KTFPKR-
C.albicans       855  YYISHHQAKAFES-IFGGVTCLPG-CFCMYRIKAPKGSDGYWVPILANPDIVERYSDNVVDT--LHRKNLLLLGEDRYLSSLML-RTFPTR-
                      * * :*  : ::* :   *:** * *: :*           ** :  *:::: ::     :  *    *  ::***    * * :    *
                                 III
CVK2 CHS          346  RQALDSRAVAYTVPPDNFTVFISQRRRWSLGTVCNNLWLFLYGK---NLYISERIIALVQVIGFLFSPLYLMVNVYLIYILVSRFDIKLIYI
E.dermatitidis   900  KQVFVPQAVCKTTVPDEFKVLLSQRRRWINSTVHNLMELVLVRDLCGTFCFSMQFVVFIELIGTLVLP--AAISFTFYLIILS-IVKKPVPV
T.magnatum       898  KQVFVPQAVCKTIVPDEFKVLLSQGRRWINSTVHNLMELVLVRDLCGTFCFSMQFVVFIELIGTLVLP--AAITFTFYLIIIS-FVAKPVPV
C.albicans       941  KQVFVPKAACKTVVPDKFKVLLSQRRRWINSTVHNLFELVLVKDLCGTFCFSMQFVIFIELIGTLVLP--AAITFTIYVIIVA-IVSKPTPV
                      :*::   *:  *: **:  *::** ***  :** * : *:*          *  :: :: :** *  *  :  : *:* :  : :  *   :

CVK2 CHS          435  SIPMFLVYLNNLCIPVWSPCMGSLRNRLSYYPKLIMAFFYSP-WVSVIIQANSVIKSFSVSWGKTVVKTTSETTKITQTNTLV
E.dermatitidis   989  -IPLVLLALI-LGLPAILIVLTA--HRWSYILWMGIYLLSLPIW-NFVLPAYAFWKFDDFSWGETR-KTAGEKTKKAGLEYEGEFDSSKIT
T.magnatum       987  -IPLVLLALI-LGLPAVLIVLTA--HRWSYVFWMLIYLISLPVW-NLILPSYAFWKFDDFSWGDTR-KVAGETTKKAGIEYEGEFDSSKIV
C.albicans      1030  -MSLVLLAVI-FGLPGCLIVITV--SSLSYLVYFVIYLFALPIW-NFVLPSYAYWKFDDFSWGETR-TVAGGD-KGDHSAVEGKFDSSKIA
                       : : *: :  : :*     :       **   : : ::  * * : ::   :  *    *** *   :::   *
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FIG. 2. Comparison of the predicted amino acid sequence of CVK2 *chs* with those of chitin synthase from various organisms. *T. magnatum, Tuber magnatum* (Garnero *et al.,* 1999); *C. albicans, Candida albicans* (Sudoh *et al.,* 1993); *E. dermatitidis, Exophiala dermatitidis* (Wang *et al.,* 1999). Numbers indicate amino acid positions. Amino acids shaded are those within subdomains I, II, and III that are conserved among fungal chitin synthases (Nagahashi *et al.,* 1995). The invariant amino acid residues are indicated by asterisks. The equivalent residues are shown by colons.

AB071039) revealed that the location of the K2E8 gene (K2 *has*) corresponded to a position between ORFs A333L and A329R, very close to the tRNA cluster on the PBCV-1 genome (Li *et al.,* 1995), as indicated in Fig. 1B. The orientation of the CVK2 *chs* is from left to right. The nucleotide sequence of the CVK2 *chs* coding region showed an ORF for 516 amino acids, whose sequence is similar to the CHS sequences of *Tuber magnatum* (FASTA score 283) (Garnero *et al.,* 1999), *Candida albicans* (FASTA score 277) (Sudoh *et al.,* 1993), and *Exophiala dermatitidis* (FASTA score 270) (Wang *et al.,* 1999). These sequences are compared in Fig. 2. All of these fungal chitin synthases are classified in Class IV or V, highly similar to the CHS3 of *Saccharomyces cerevisiae* (Valdivieso *et al.,* 1999). The conserved domains I–III of fungal chitin synthases (Nagahashi *et al.,* 1995) are also shared by these enzymes (Fig. 2).

A 100-bp nucleotide sequence immediately upstream of the *chs* coding region is AT-rich (70% AT), showing the consensus feature of the chlorovirus promoter (Schuster *et al.,* 1990). Within this region, there is also a possible −10 sequence (5′-TATTTG) and a −35 sequence (5′-TTGACT) with 16-bp spacing, resembling *Escherichia coli* promoter elements. This suggests that the CVK2 *chs* gene may be expressed in bacterial cells like several other CVK2 genes (Yamada *et al.,* 1999). As for the downstream sequence, a poly(A) was added 28 bp downstream of the termination codon UGA, and a putative poly(A) addition signal 5′-AAATAT was found 10 bp preceding the poly(A). Flanking this terminal region, there appeared to be a PBCV-1-homologous region (position ~168,050 in PBCV-1) containing highly repetitive sequences and an ORF A333L.

Expression of the CVK2 *chs* gene in CVK2 infection

To determine if the CVK2 *chs* gene is transcribed in the course of viral infection, total RNA was extracted at various times after CVK2 infection and analyzed by Northern blotting using cDNA K2E8 as a probe. The result, shown in Fig. 3, indicates that a single transcript of approximately 1.6 knt first appeared at 10 min p.i., peaked at 20–40 min p.i., and disappeared at 120–180 min p.i.; that is, CVK2 *chs* is an early gene. As demon-

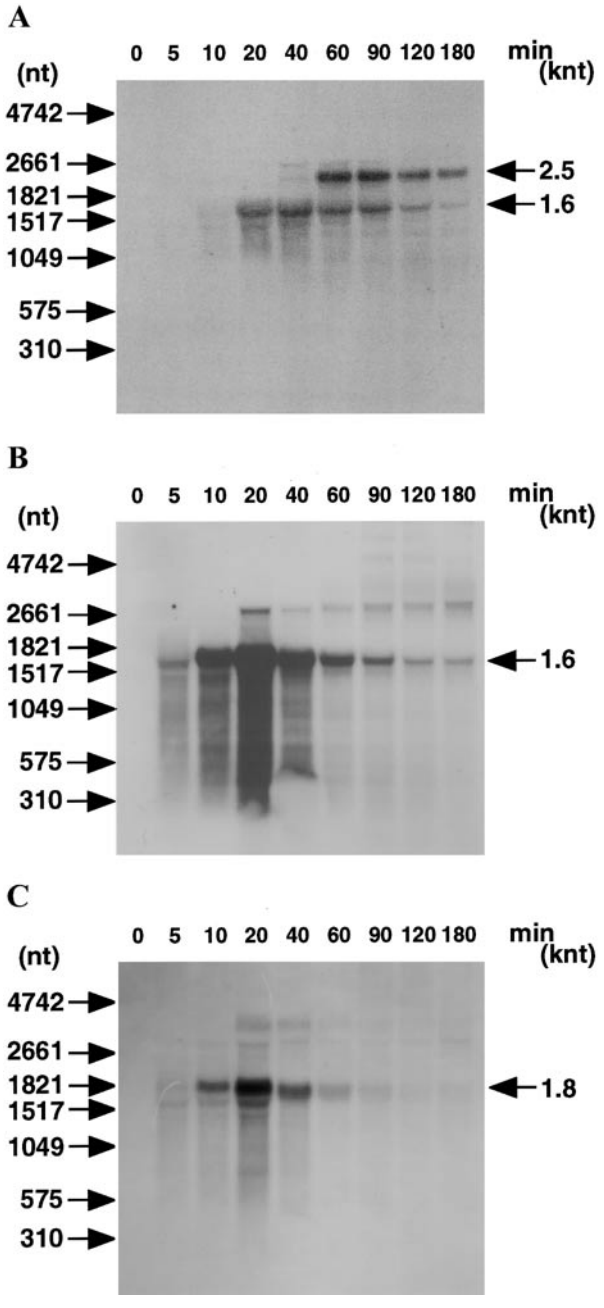

FIG. 3. Northern blot analysis of RNAs isolated from virus-infected *Chlorella* cells at various times p.i. (A) Total RNA isolated from CVK2-infected *Chlorella* NC64A cells was hybridized with ^{32}P-labeled cDNA (K2E8) as a probe. (B) Total RNA isolated from CVIK1-infected cells was hybridized with the same probe as in A. (C) Total RNA isolated from CVIK1-infected cells was hybridized with the PBCV-1 *has* gene as a probe. Positions of RNA size markers (Boehringer–Mannheim) are indicated on the left side.

strated by the nucleotide sequence of cDNA K2E8, this transcript was polyadenylated and was a slightly larger size than the ORF. In addition to this major transcript, an additional transcript of 2.5 knt appeared from 60 min p.i. and disappeared at 180 min p.i. This transcript was also detected by a probe of an upstream sequence (probe I in Fig. 1A) and not by a downstream probe (probe II in Fig. 1A). Therefore, this larger transcript should have resulted from readthrough transcription from an upstream gene into the adjacent *chs* gene. Since the two transcripts appeared at different times in the viral infection, a sophisticated mechanism must be involved in early gene expression.

Chitin accumulation on the surface of CVK2-infected cells

Graves *et al.* (1999) reported that the exterior surface of the PBCV-1-infected *Chlorella* cell wall takes on a "hairy" appearance and by 240 min p.i., the infected cell is covered with a highly developed, dense fibrous network. This "hairy" material is removed by treatment with hyaluronan lyase, indicating that the fibrous network is composed of hyaluronan. When we examined *Chlorella* strain NC64A cells infected with CVK2 by electron microscopy, similar hair-like fibers accumulated on the outside of the host cell wall by 60 min p.i. (Fig. 4B). By 240 min p.i., the infected cells were covered with a highly developed fibrous network (0.2–0.3 μm thick). The network was loosely associated with the cell wall. Treatment of the virus-infected cells (90 min p.i.) with chitinase for 60 min removed the fibrous material from the cell wall (data not shown). These results suggested that the hyaluronan-nonproducing virus CVK2 can form another polysaccharide (probably chitin) on the surface of host cells, giving them a "hairy" appearance. Chitin accumulation on the surface of CVK2-infected *Chlorella* cells was monitored by fluorescent microscopy using a biotinylated chitin-binding protein, chitin-binding protein CB-1 from *Bacillus licheniformis* (Oita *et al.,* 1996) (bt–CBP), in conjunction with an avidin–Cy3 conjugate. For comparison, the presence of hyaluronan was also tested using a biotinylated hyaluronan-binding protein (bt–HABP) in conjunction with an avidin–Cy3 conjugate. As shown in Fig. 4, most CVK2-infected cells developed uniform yellow fluorescence over the entire cell surface by 60 min p.i. when bt–CBP was used for detection (Fig. 4E); uninfected and PBCV-1-infected cells did not show any discernible yellow fluorescence (Figs. 4C and 4G). Treatment of the CVK2-infected cells at 120 min p.i. with chitinase for 60 min abolished most of the yellow fluorescence (Fig. 4D). In contrast to this, bt–HABP did not detect hyaluronan on the CVK2-infected *Chlorella* cells (Fig. 4F), but did detect it on the PBCV-1-infected cells (Fig. 4H). These results confirmed that CVK2 does not form hyaluronan but another polysaccharide, chitin, on the infected cell surface. Typically, chitin synthase is an integral membrane-bound protein, and the newly synthesized chitin is secreted across the membrane to the extracellular matrix (Bulawa, 1993). Determination of chitin synthase activity associated with the membrane fraction of CVK2-infected *Chlorella* cells has been unsuccessful, so far.

Occurrence of the *chs* gene in chloroviruses

As demonstrated above, CVK2 encodes the *chs* gene and lacks the *has* gene, while PBCV-1 lacks the *chs* gene but has the *has* gene. Therefore, we were interested in the differential distribution of the two genes among the chloroviruses. The *chs* and *has* gene probes were hybridized to *Eco*RI fragments of genomic DNAs from 27 randomly isolated viruses from diverse geographical regions of Japan. Among them, the *chs* probe hybridized to 10 viral DNAs and the *has* probe hybridized to 19 viral DNAs. Some examples of the Southern blot analysis are shown in Fig. 5. As seen here, *chs*-hybridizing viruses in general did not hybridize to the *has* probe (like CVK2, lane 1) and vice versa (like PBCV-1, lane 15). The exceptions were CVHA1 (lane 2) and CVIK1 (lane 9), which hybridized to both the *chs* and *has* probes. With the *chs* probe, both CVHA1 and CVIK1 gave a single hybridizing band of 3.0 kb that was the same as CVK2 and other hybridizing viruses (Fig. 5). However, the *has* probe gave two bands of 3.5 and 0.4 kb for all hybridizing viruses including CVHA1 and CVIK1. These two bands were produced by *Eco*RI digestion at a site within the *has*-coding region. These results indicate that the nucleotide sequences of these genes and their flanking regions, if present, are highly conserved among the viruses. The frequency of the *has* gene was two times larger than that of the *chs* gene in Japanese viruses, and among 37 American and Chinese viruses, 28 isolates hybridized to the *has* gene (Graves *et al.,* 1999). This frequency may reflect some functional superiority of hyaluronan. However, there was no significant difference in the expression levels between the *chs* and *has* genes in CVIK1-infected cells; almost the same levels of mRNA were detected for both genes examined by Northern blot analysis (Figs. 3B and 3C). Fluoromicroscopy also demonstrated that both chitin and hyaluronan were accumulated on the surface of CVKI1-infected *Chlorella* cells (Figs. 4I and 4J).

DISCUSSION

In this work, we demonstrated that chlorovirus CVK2 encodes a chitin synthase gene, *chs,* and produces hairy chitin polysaccharide on the infected cell surface. Several other chloroviruses also produce chitin instead of hyaluronan, contrasting with PBCV-1. Chitin is a polymer of β-1,4-linked GlcNAc that occurs in the exoskeleton of arthropods, in the cell wall of fungi, and in various components of diverse invertebrates. In addition to the structural facets of chitin polymers, chitin oligosaccharides function as important signals in the developmental processes of plants; for example, as elicitors for defense responses and as nodulation inducers for leguminous plants. Interestingly, several genes sharing homology with the chitin synthase gene from *Rhizobium* have been found in animals, birds, fish, and amphibians (Bakkers *et al.,* 1999). Therefore, like hyaluronan, chitin may act both as a structural element and as a signaling molecule.

Chitin synthesis is directly governed by chitin synthase activity. In fungi, the chitin synthase enzyme occurs as an inactive zymogen in vesicles called chitosome, whereas in arthropods, this enzyme is membrane-bound (Palli and Retnakaran, 1999). CVK2 CHS showed a high similarity to the CHS3-type enzymes of yeasts and fungi (Fig. 2); however, its size (516 aa) is significantly smaller than fungal enzymes (1000–1300 aa), and the sequence homology is restricted to a carboxy-terminal region of those enzymes where the conserved catalytic site exists (Nagahashi *et al.,* 1995). The N-terminal regions extended in fungal enzymes are suggested to be involved in the processing and regulation of enzyme activities (Nagahashi *et al.,* 1995). The smaller size of the CVK2 CHS protein may reflect its simpler regulatory and processing mechanism as well as different localization processes in the cell. Newly synthesized chitin was efficiently secreted across the *Chlorella* membrane and cell wall to the extracellular matrix (Fig. 4), suggesting that the CVK2 CHS protein may be integrated into the membrane and cell wall, where it synthesizes chitin molecules by the addition of UDP-GlcNAc and transports the insoluble material to the extracellular space (Cabib, 1987). All chloroviruses along with the CVK2 studied so far contained a functional gene for GFAT that produced the sugar precursor GlcNAc-6P required for chitin synthesis (Landstein *et al.,* 1998, and data not shown). This makes CVK2 chitin production more efficient and abundant.

Although the gene for UDP-GlcDH that forms GlcA required for hyaluronan synthesis is also conserved in all the chloroviruses studied (data not shown), the alternative occurrence of the *chs* or *has* gene in chloroviruses is very interesting. In a few exceptional cases where the two genes are coexisting, represented by CVIK1 and CVHA1, both *chs* and *has* are almost equally expressed during infection (Fig. 3), and both chitin and hyaluronan are simultaneously accumulated on the cell wall of a single virus-infected cell (Figs. 4H and 4I). These results suggest that there is no functional incompatibility between the two genes or the two gene products. It is noteworthy that the mouse HAS1 protein has both hyaluronan synthase activity and chito-oligosaccharide synthase activity *in vitro* (Yoshida *et al.,* 2000). Two of the three conserved aspartate residues and the short motif QXXRW found in HAS1 are also conserved in all Class I hyaluronan synthases, cellulose synthases, and chitin synthases and are essential for glycosyltransferase activity (Yoshida *et al.,* 2000). From this information, Lee and Spicer (2000) proposed that the higher eukaryotic HAS have evolved from chitin or cellulose synthase through the addition of the β-1-3 glycosyltransferase activity to a preexisting β-1-4 glycosyltransferase enzyme, and that the ability to synthesize hyaluronan is a comparatively recent innovation in the evolution of metazoan organ-

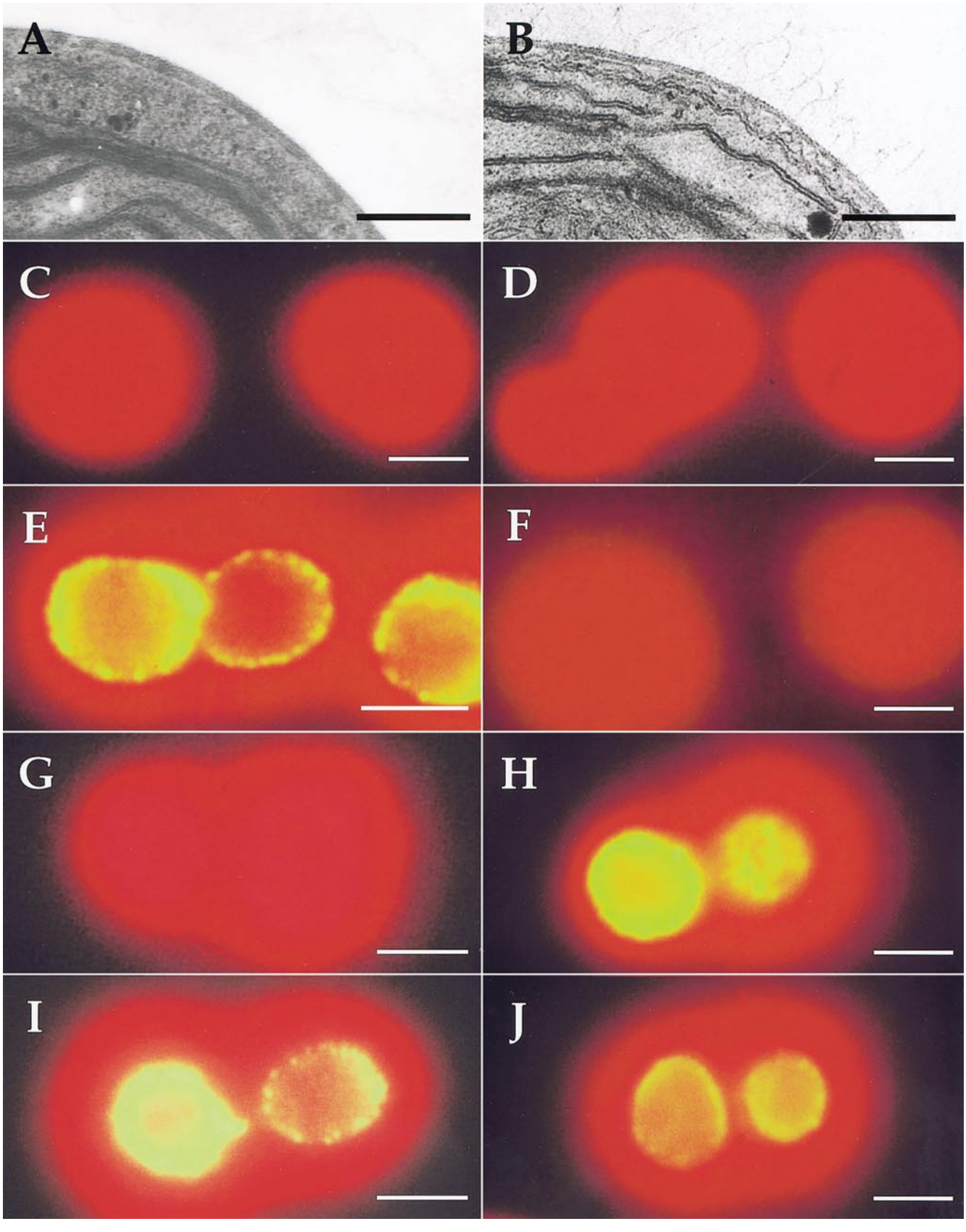

FIG. 4. Ultrastructural changes in the algal cell wall after viral infection and fluorescent visualization of chitin and hyaluronan on the surface of infected cells. Electron micrographs of uninfected *Chlorella* NC64A cells (A) and of CVK2-infected cells at 90 min p.i. (B). Chitin accumulation was detected by bt–CBP and avidin–Cy3 conjugates on the surface of uninfected cells (C), CVK2-infected cells at 90 min p.i. (E), PBCV-1-infected cells at

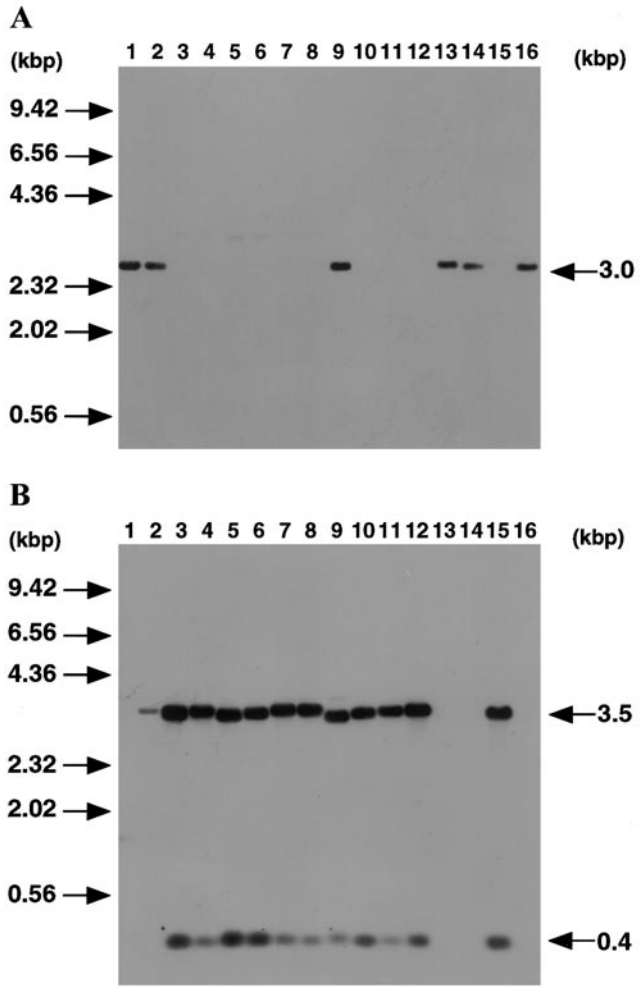

FIG. 5. Southern blot analysis of genomic DNAs isolated from 15 different chloroviruses. (A) *Eco*RI fragments of genomic DNAs were hybridized with K2E8 DNA as a probe. A single hybridizing band of 3.0 kb was seen (right arrow). (B) The same blot as in (A) was hybridized with the PBCV-1 *has* gene as a probe. Two hybridizing bands of 3.5 and 0.4 kb are indicated by arrows on the right. Positions of size markers are shown on the left side. Lanes: 1, CVK2; 2, CVHA1; 3, CVHI1; 4, CVKA1; 5, CVBR4; 6, CVA1; 7, CVNI1; 8, CVNA1; 9, CVIK1; 10, CVTS1; 11, CVSE1; 12, CVO1; 13, CVNA2; 14, CVKU2; 15, PBCV-1; 16, CVK2.

isms. In this regard, we compared the chlorovirus-encoded *chs* and *has* genes and found no significant similarity in either the amino acid sequence or the nucleotide sequence between the CVK2 *chs* and the PBCV-1 *has,* except for the important aspartate residues and the QXXRW motif (Yoshida *et al.,* 2000). The G + C contents of the CVK2 CHS and PBCV-1 HAS coding regions are 41 and 47%, respectively. The codon usage of these genes is also similar (data not shown). Although the promoter sequences of the two genes do not share significant similarity, they are both rich in A + T (70–80%). From these results, we could not conclude a direct evolution of the chloroviral *has* gene from the chloroviral *chs* gene exists. However, the CVK2 genomic region corresponding to the PBCV-1 *has* gene (A98R) contained fragmented ORFs, some of which showed homology to chitin synthase, suggesting some direct interactions between the *chs* and *has* genes on the viral genome.

The finding that CVK2 produces chitin on the host cell surface supports the possibility that polysaccharide accumulation on the algal surface protects virus-infected algae from uptake by other organisms such as protozoa (Graves *et al.,* 1999). Two chitinases (Hiramatsu *et al.,* 1999) and one chitosanase (Yamada *et al.,* 1997) encoded by CVK2 can be understood to have a role in digestion of such a "protector or cocoon" before cell lysis and before viral progenies are released into the safe medium. Chlorovirus CVK2 is a very peculiar virus that encodes enzymes required for both the synthesis and the degradation of chitin materials.

MATERIALS AND METHODS

Chlorella and viruses

Cells from the *Chlorella* strain NC64A (Muscatine *et al.,* 1967) were cultured in a modified Bold's basal medium as described previously (Van Etten *et al.,* 1983). Chlorovirus CVK2 was a large plaque-forming virus isolated in Kyoto, Japan (Yamada *et al.,* 1991), and chlorovirus PBCV-1 was a gift from Dr. J. L. Van Etten. Other chloroviruses were those described previously (Yamada *et al.,* 1993). Procedures for producing and purifying these chloroviruses have been described (Yamada *et al.,* 1991). The cosmid contig clones for CVK2 DNA were described previously (Nishida *et al.,* 1999).

Preparation of DNA and RNA

DNA was isolated from purified virus particles by phenol extraction, as described previously (Yamada *et al.,* 1991). DNA restriction fragments separated by agarose gel electrophoresis were transferred to nylon filters (Biodyne, Pall Biosupport), hybridized with probes labeled with fluorescein (Gene Images kit, Amersham Pharmacia Biotech), and detected with a CDP-Star detection module (Amersham Pharmacia Biotech). The total RNA was isolated from uninfected and virus-infected *Chlorella* NC64A

90 min p.i. (G), CVK2-infected cells at 60 min p.i. treated with chitinase (D), and CVIK1-infected cells at 90 min p.i. (I). The yellow fluorescence surrounded by the red autofluorescence from chlorophylls indicates the presence of chitin on the cell surface. Detection of hyaluronan using a bt–HABP in combination with an avidin–Cy3 conjugate was likewise performed for CVK2-infected cells at 90 min p.i. (F), PBCV-1-infected cells at 90 min p.i. (H), and CVIK1-infected cells at 90 min p.i. (J). Note that the surface of the CVIK1-infected cells was accumulated with both chitin and hyaluronan. Bars, 1 μm in A and B, 5 μm in C–J.

cells at various times postinfection. The RNA was separated by 1.5% agarose gel with formamide, blotted onto nylon filters, and hybridized with ^{32}P-labeled probes under standard conditions. For the upstream (probe I) and downstream (probe II) probes, a 460-bp *Eco*RI/*Mlu*I fragment and a 400-bp *Mlu*I/*Eco*RI fragment of cosmid 3H6 (Nishida *et al.,* 1999) were used, respectively.

Construction of a cDNA library

A cDNA library was constructed using a Time-Saver cDNA synthesis kit (Amersham Pharmacia Biotech) with reverse-transcribed poly(A)$^+$ RNA isolated from CVK2-infected *Chlorella* cells at 20 min p.i. cDNA was ligated to a λ ZAPII vector and packaged with a Giga-pack Gold packaging kit (Stratagene), according to the manufacturer's instructions. Oligo(dT) primers and *Eco*RI/*Not*I adapters were used in the construction of the library.

Detection of chitin and hyaluronan on the surface of infected cells

Accumulation of chitin and hyaluronan on the surface of virus-infected *Chlorella* cells was detected by specific binding proteins that were biotinylated to interact with avidin–Cy3 conjugate (Amersham Pharmacia Biotech). For a chitin-binding protein, CB-1 from *Bacillus licheniformis* (Oita *et al.,* 1996) was biotinylated (bt–CBP conjugate) with a labeling kit (Seikagaku Corp.). A hyaluronan-binding protein was also labeled with biotin (Seikagaku Corp.). Afterwards, 1.5 μg of the biotinylated aggrecan was added to 2.0×10^8 cells in 100 μl and incubated on ice for 60 min. The cells were washed three times in PBS, resuspended in 100 μl of avidin–Cy3 conjugate, and incubated for 60 min on ice before being examined under green light illumination with an Olympus BX60 fluorescence microscope. In some experiments, duplicated samples were treated with chitinase for 30 min before the addition of aggrecans. CVK2-infected cells at 60 min p.i. (10^7 cells/ml) were washed in phosphate buffer (pH 6.8), treated with 1 mU of chitinase RS (Seikagaku Corp.) for 30 min at room temperature, and fixed with 4% paraformaldehyde before chitin detection. For electron microscopic observation, CVK2-infected cells at various times p.i. were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) and postfixed in 2% O_sO_4 in the same buffer. The dehydrated cells were embedded in Spurr resin, and the thin sections were cut on a Sorvall MT-1 ultramicrotome. After being stained with uranyl acetate and lead citrate, the sections were examined with a Hitachi H600A electron microscope (Yamada and Sakaguchi, 1982).

Other procedures

The CVK2 *chs* gene was obtained as a 3.0-kb *Eco*RI fragment of cosmid 3H6 (Nishida *et al.,* 1999). Subcloning for DNA sequencing was performed using a pBluescript II SK$^+$ vector to make overlapping clones. The PBCV-1 *has* gene was amplified by PCR using synthetic oligonucleotide primers: 5′-CTACGTACGACTTCTTGAAAGTTACAATGTGG (forward primer) and 5′-GTCCTGCAGGAAAAATCACACAGAC (reverse primer). The PCR product was cloned into the *Eco*RV site of the pBluescript II SK$^+$ vector. Single-stranded DNA was sequenced by the dideoxy method in an Automated Laser Fluorescent DNA sequencer (Amersham Pharmacia Biotech). The EMBL/GenBank/DDBJ databases were searched for homologous sequences.

ACKNOWLEDGMENTS

We thank Shigeru Oita, National Agricultural Research Organization, for supplying the chitin binding protein CB-1. This work was supported in part by a grant-in-aid for scientific research (13660094) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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