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Evidence for virus-encoded glycosylation specificity

(algal virus/chlorella/serotypes)

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Abstract: Four spontaneously derived serologically distinct classes of mutants of the *Paramecium bursaria* chlorella virus (PBCV-1) were isolated using polydonal antiserum prepared against either intact PBCV-1 or PBCV-1-derived serotypes. The oligosaccharide(s) of the viral major capsid protein and two minor glycoproteins determined virus serological specificity. Normally, viral glycoproteins arise from host-specific glycosylation of viral proteins; the glycan portion can be altered only by growing the virus on another host or by mutations in glycosylation sites of the viral protein. Neither mechanism explains the changes in the glycan(s) of the PBCV-1 major capsid protein because all of the viruses were grown in the same host alga and the predicted amino acid sequence of the major capsid protein was identical in the PBCV-1 serotypes. PBCV-1 antiserum resistance is best explained by viral mutations that block specific steps in glycosylation, possibly by inactivating glycosyltransferases.

Abbreviations: PBCV-1, *Paramecium bursaria* chlorella virus; TFMSA, trifluoromethanesulfonic acid.

Paramecium bursaria chlorella virus (PBCV-1) is a large (≈190 nm in diameter) polyhedral plaque-forming virus that replicates in certain unicellular eukaryotic exsymbiotic Chlorella-like green algae (1). The PBCV-1 genome is a linear nonpermuted 333-kb double-stranded DNA with covalently closed hairpin ends (2, 3). PBCV-1 contains at least 50 proteins and a lipid component located inside the capsid shell (4). The major capsid protein (Vp54) of PBCV-1 is one of four proteins located on the viral surface and is one of three glycosylated viral proteins. PBCV-1 and its related viruses have recently been assigned to a virus family, named Phycodnaviridae (5). Additional features of these viruses have been reviewed (6).

Chase et al. (7) showed that chlorella viruses exclude one another during dual inoculation of the host. For these studies we isolated a spontaneous mutant of PBCV-1, named EPA-1, which was resistant to PBCV-1 polyclonal antiserum (dilution of 1:4). This antiserum completely inhibited PBCV-1 infection even at a dilution of 1:1000. Polyclonal antiserum prepared against EPA-1 inhibited EPA-1 infection (dilution of 1:1000) but not PBCV-1 infection (dilution of 1:4). In this study we have addressed two questions: (i) Can additional PBCV-1 serotypes be isolated using polyclonal antisera to intact viruses? (ii) What virus component determines antiserum resistance? We have found four PBCV-1 serotypes and discovered that the antisera react primarily with oligosaccharide(s) attached to the major capsid protein plus two minor glycoproteins. More importantly, in contrast to normal virus protein glycosylation, the virus rather than the host dictates glycosylation specificity.

Materials And Methods

Culture Conditions. The procedures for producing, purifying, and “plaquing” the viruses and the growth of the host

alga (*Chlorella* NC64A) on MBBM medium have been described (1, 8). Virus glycoproteins were specifically labeled by adding 100 μ Ci of D-[6-³H]-galactose (1 Ci = 37 GBq) to 1 ml of cells (6×10^8 cells per ml) 30 min after virus infection. After lysis, the labeled virions were isolated by standard procedures.

Isolation of PBCV-1 Serotypes. Spontaneous serotype mutants of PBCV-1 or its relatives were isolated by incubating 1×10^9 plaque-forming units (pfu) of virus with virus polyclonal antiserum (1:10 dilution) for 30 min, removing agglutinated virus by centrifuging ($3000 \times g$) for 5 min, and titrating the supernatant. Typically 500–1,000 antiserum-resistant viruses were obtained from 10^9 pfu of parent virus and >95% of these plaques consisted of mutant viruses that were insensitive to the original virus antiserum; i.e., <5% of the plaques were from parent viruses that escaped antiserum precipitation.

Polyacrylamide Gel Electrophoresis of Virus Proteins and Western Blot Analysis. Purified viruses [$0.06 A_{260}$ unit in 10 μ l of 50 mM Tris HCl (pH 7.8)] were mixed with 10 μ l of 2 \times dissociation buffer [125 mM Tris HCl, pH 8.4/3% (wt/vol) SDS/20% (vol/vol) glycerol/0.1 M dithiothreitol/0.08% bromophenol blue] and heated to 65°C for 1 hr or 100°C for 5 min. Samples were electrophoresed on a linear 6–18% polyacrylamide gradient slab gel with a 4% stacking gel at 70 V for 16 hr in the buffer system of Laemmli (9). Proteins were visualized by silver staining (10).

Proteins were transferred to nitrocellulose sheets at a constant current flux of 1 mA/cm² for 5 hr at room temperature in the buffer system described by Towbin et al. (11) with 20% (vol/vol) methanol. The sheets were washed for 5 min in 1 \times TBST buffer (10 mM Tris-HCl, pH 8.0/150 mM NaCl/0.05% Tween 20) and incubated for 1 hr in TBST with 5% (wt/vol) nonfat dry milk (blocking solution) containing primary antiserum diluted 1:750 to 1:1000. The sheets were washed three times in TBST, incubated for 1 hr at room temperature with goat anti-rabbit IgG-conjugated alkaline phosphatase (1:2000 dilution) in blocking solution, washed three times in TBST, and incubated in alkaline phosphate (AP) buffer (100 mM Tris-HCl, pH 9.5/100 mM NaCl/5 mM MgCl₂) for 5 min. Color was developed by incubating the sheets in 15 ml of AP buffer containing 40 μ l of nitroblue tetrazolium (112 mg dissolved in 1 ml of *N,N*-dimethyl formamide and 0.5 ml of water) and 32 μ l of 5-bromo-4-chloro-3-indolyl phosphate (100 mg dissolved in 2 ml of *N,N*-dimethyl formamide) (12). Color development was stopped by placing the sheets in distilled water.

Isolation of Virus Major Capsid Protein. Viral glycoproteins, including the major capsid protein Vp54, were dissociated from virions by incubating purified virus in 50 mM Tris HCl (pH 7.8) at 65°C for 1 hr and then centrifuging at $10,000 \times g$ for 10 min. At least 80% of the supernatant protein consisted of Vp54. The

protein was dried, resuspended in dissociation buffer, and electrophoresed on preparative polyacrylamide gels as described above. After electrophoresis the major capsid protein was visualized by soaking the gel in 4 M sodium acetate (13). The major capsid protein region was excised with a razor blade and cut into small blocks, and protein was electroeluted and concentrated using an ISCO electrophoretic concentrator. The concentrator contained 10 mM NH_4HCO_3 /0.1% SDS in the sample cup and 50 mM NH_4HCO_3 /0.1% SDS in the buffer chambers. After elution, protein was precipitated with 5 vol of acetone.

Deglycosylation. The major capsid proteins were deglycosylated with trifluoromethanesulfonic acid (TFMSA) by a modification of the procedure of Edge et al. (14). Virus or purified major capsid proteins were suspended in 50 μl of TFMSA and 25 μl of anisole and incubated on ice for 1-3 hr. The reaction was stopped by adding 125 μl of *N*-ethylmorpholine (15). Protein was recovered by acetone precipitation and immediately analyzed on SDS/polyacrylamide gels.

Sugar Analysis. Equal volumes of 65°C solubilized viral proteins (see above) were mixed with equal volumes of 4 M trifluoroacetic acid and hydrolyzed for 4 hr at 100°C. The samples were cooled and the acid was evaporated. The samples were suspended in deionized water and passed through an OnGuard A cartridge (Dionex). Monosaccharides were bound to a Dionex Carbpac PA1 anion-exchange column and eluted for 35 min with isocratic 2.5 mM NaOH (16) by procedures recommended by Dionex. Peaks were integrated and plotted on a Dionex 4400 integrater. The internal standard was 2-deoxyglucose. Retention times and peak areas were compared to standards including fucose, galactosamine, galactose, glucosamine, glucose, xylose, arabinose, rhamnose, and mannose.

Other Procedures. Nitrocellulose sheets containing virus proteins were mildly oxidized with periodic acid as described by Woodward et al. (17). Polyclonal antiserum to the viruses was raised against purified viruses as described (18). DNA was isolated from the purified viruses as described (19) and treated with restriction endonucleases according to suppliers' protocols. DNA was sequenced in both directions by the procedure of Sanger et al. (20), as modified by Tabor and Richardson (21), using a Sequenase (Version 2.0) kit from United States Biochemical.

Results

Isolation of PBCV-1 Serotypes. We have isolated (7) a mutant of PBCV-1, EPA-1, that was resistant to PBCV-1 polyclonal antiserum. Polyclonal antiserum prepared against EPA-1 inhibited EPA-1 infection (dilution of 1:1000) but not PBCV-1 infection even at high antiserum concentrations (7). Additional spontaneously derived PBCV-1 serotypes were selected with polyclonal antisera prepared against either PBCV-1 or serotypes derived from PBCV-1. Four serologically distinct mutant classes (summarized in Table 1) of PBCV-1 were obtained: P91; P31 and EPA-1; P41 and E11; and P100. Virus E11 arose from EPA-1 and virus P100 arose from P31; presumably, these two viruses contain two independent mutations. Each of these viruses was stable and polyclonal antiserum made to one of the serotypes completely inhibited infection by members of the same serotype (at a dilution of 1:1000) but had no effect at a 1:4 dilution on the other serotypes.

The polyclonal antisera inhibit homologous virus infection by agglutination. Antisera treated with papain, which produces monovalent Fab fragments (22), did not inhibit homologous virus infection. However, adding goat anti-rabbit IgG to Fab-treated virus inhibited virus infection.

Table 1. Virus PBCV-1 serotypes

Virus	Source	Antiserum agglutination				
		PBCV-1	P91	EPA-1	P41	P100
PBCV-1	—	Yes	No	No	No	No
P91	PBCV-1	No	Yes	No	No	No
EPA-1	PBCV-1	No	No	Yes	No	No
P31	PBCV-1	No	No	Yes	No	No
P41	PBCV-1	No	No	No	Yes	No
E11	EPA-1	No	No	No	Yes	No
P100	P31	No	No	No	No	Yes

Polyclonal antiserum was prepared against PBCV-1, P91, EPA-1, P41, and P100 viruses. **Yes** = a 1:1000 dilution of the indicated antiserum indicated agglutinated the virus; **No** = a 1:4 dilution of the antiserum indicated did not agglutinate the virus.

Virus DNA. DNA from each of the viruses was digested with several DNA restriction endonucleases and analyzed on agarose gels. Identical restriction fragments were observed with DNA from PBCV-1, P91, P31, P41, and P100 with each of the restriction endonucleases. A single common *Bam*HI fragment and a single *Hha*I fragment (Figure 1) were altered in DNA from EPA-1 and E11. The altered *Bam*HI fragment (B8a) is located at positions 55-66 on the physical map of PBCV-1 (6). This suggests that EPA-1 arose from a deletion and that P91, P31, P41, and P100 arose from either point mutations or very small deletions in separate PBCV-1 genes. E11 likely arose from a point mutation in EPA-1, possibly in the same gene that produced P41.

Protein Patterns of the PBCV-1 Serotypes. Each of the viruses was heated either to 65°C for 1 hr or 100°C for 5 min in dissociation buffer and the proteins were electrophoresed on SDS/polyacrylamide gels. The PBCV-1 major capsid protein (Vp54), which makes up $\approx 40\%$ of the total virus protein, migrates as a dimer when heated to 65°C and as a monomer when heated to 100°C (4, 23). As shown in Figure 2A, both the dimer and monomer forms of the major capsid protein of each

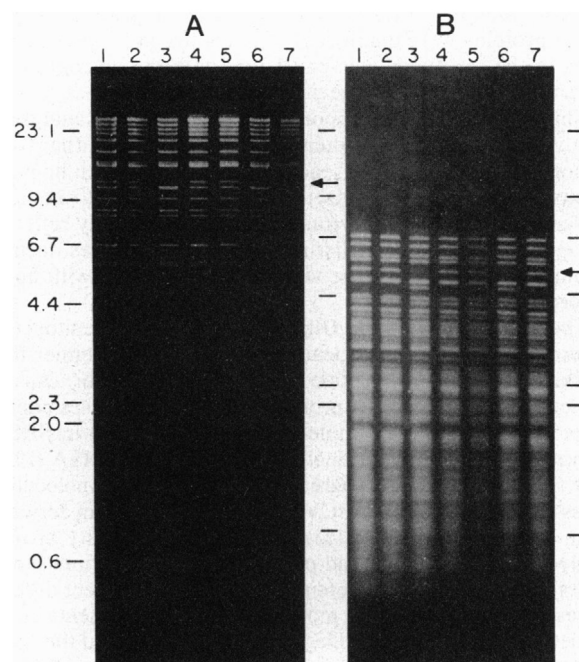


Figure 1. Digestion patterns of PBCV-1 (lane 1), P91 (lane 2), EPA-1 (lane 3), P31 (lane 4), P41 (lane 5), E11 (lane 6), and P100 (lane 7) viral DNA. The DNAs were digested with *Bam*HI (A) or *Hha*I (B). A single *Bam*HI fragment and a single *Hha*I fragment (arrow) were altered in the DNAs from EPA-1 and E11.

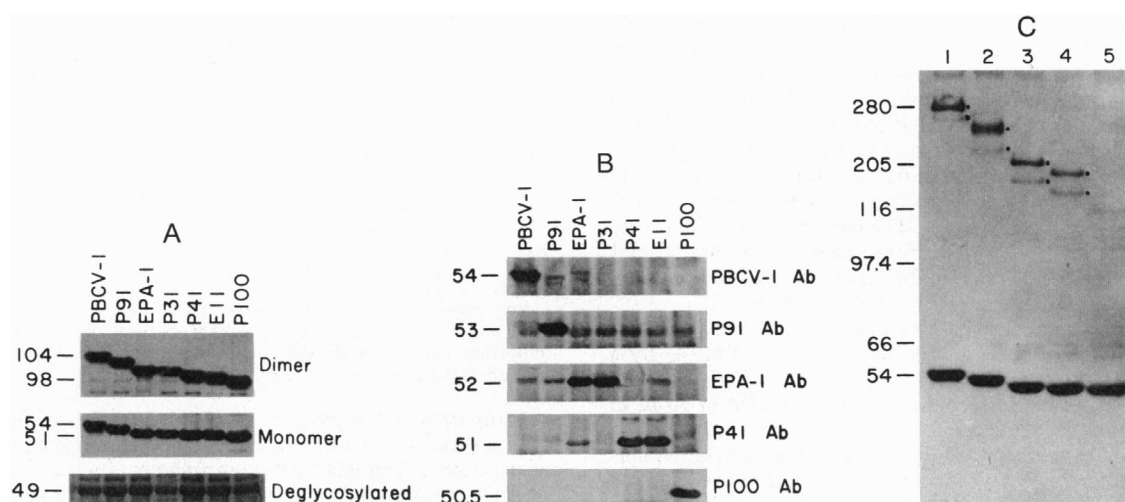


Figure 2. (A) SDS/PAGE of the major capsid protein from PBCV-1, P91, EPA-1, P31, P41, E11, and P100 viruses. The samples were heated to 65°C for 1 hr (dimer), 100°C for 5 min (monomer), or deglycosylated with TFMSA prior to electrophoresis. (B) Immunoblot of the major capsid protein from PBCV-1, P91, EPA-1, P31, P41, E11, and P100. The blots were treated with antiserum (Ab) against PBCV-1, P91, EPA-1, P41, or P100 as indicated. Note: the major capsid proteins reacted primarily with homologous antiserum. (C) Migration of [3 H] galactose-labeled glycoproteins from PBCV-1, P91, P31, P41, and P100 viruses. The samples were heated at 100°C for 5 min prior to electrophoresis. Note: the virus mutations that affect the migration of the major capsid protein also affect the migration of the two minor viral glycoproteins. Molecular masses in kDa are shown.

mutant migrated faster than the major capsid protein of its parent. Furthermore, the major capsid proteins from the serotypes migrated differently from one another, whereas the major capsid protein from viruses in the same serotypes migrated at identical rates. The viruses PBCV-1, P91, EPA-1 and P31, P41 and E11, and P100 are arranged in Figure 2A by decreasing size of the major capsid protein. The virus mutations that affected the migration of the major capsid protein also affected the migration of the other two viral glycoproteins (Figure 2C). Like the major capsid protein, these two glycoproteins, each being 1–2% of the total virus protein, migrated faster than the parental glycoproteins. Other proteins were unaffected. The change in the glycoproteins in the serotypes was consistent with the glycoprotein determining serological specificity of the viruses.

This possibility was supported by Western blot analysis. The virus major capsid proteins (Figure 2B) and the other two minor virus glycoproteins reacted strongly only with homologous antiserum. Each antiserum also reacted, but to a much lesser extent, with other virus proteins, presumably reflecting epitopes exposed by disruption of virus particles during immunization. None of the virus antisera reacted with host *Chlorella* proteins.

The Primary Epitope Is Oligosaccharide(s). The epitope(s) responsible for the serological specificity could be either the carbohydrate or polypeptide portion of the major capsid protein. The major capsid proteins from all the viruses comigrated with an apparent molecular mass of 49 kDa in SDS/polyacrylamide gels after deglycosylation with TFMSA (Figure 2A). The value of 49 kDa agrees with the predicted molecular mass of 48.2 kDa for the PBCV-1 major capsid protein derived from sequencing its gene (23). The apparent identity of the deglycosylated major capsid proteins from all the virus serotypes indicates that the protein size differences reflect differences in the carbohydrate moieties. Three experiments confirmed this conclusion. (i) Deglycosylation abolished the specific reaction of the major capsid proteins with homologous antiserum. (ii) Periodic acid treatment of virus proteins after transfer to nitrocellulose, which destroys carbohydrate epitopes of glycoproteins (17), reduced the reaction of the antisera with the major capsid proteins, but not with the other virus proteins. (iii) The ratios of six and/or seven sugars (fucose, galactose, glucose, xylose,

mannose, and arabinose and/or rhamnose) associated with the viral glycoproteins of PBCV-1 and the mutants varied in a predictable manner that was related to their serotypes and the apparent size of the glycoproteins (Table 2). Thus we conclude that the oligosaccharide(s) attached to the major capsid proteins determines the serological differences among the viruses.

Sequence of the Major Capsid Protein Gene. The major capsid protein gene from the parent virus PBCV-1 was recently isolated and sequenced; the Vp54 gene is located at positions 209–211 on the PBCV-1 physical map (23). An amino acid change(s) in the major capsid protein could alter a glycosylation site(s) in the protein leading to oligosaccharide(s) differences among the PBCV-1 serotypes. To examine this possibility, we cloned and sequenced the major capsid protein gene from viruses EPA-1 and P100. The base sequence and, hence, the predicted amino acid sequence for the major capsid proteins from both mutants was identical to that of PBCV-1.

Discussion

Four spontaneously derived serologically distinct classes of mutants of the *Chlorella* virus PBCV-1 were isolated using polyclonal antiserum made against either PBCV-1 or the

Table 2. Sugar composition of the glycoproteins from PBCV-1 serotypes

Sugar	Relative sugar content						
	PBCV-1	P91	EPA-1	P31	P41	E11	P100
Fucose	0.69	0.73	0.88	0.81	0.78	0.83	0.61
Ara/Rham	1.57	1.24	0.64	0.65	0.65	0.65	0.19
Galactose	1.02	0.97	1.02	1.03	0.99	0.97	0.91
Glucose	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Xylose	1.58	1.47	1.59	1.57	0.88	0.77	0.60
Mannose	1.02	0.70	0.14	0.17	0.24	0.15	0.12

Values are normalized to that of glucose. Note: fucose and galactose also do not change. The mutants are arranged so that the major capsid protein decreases in size from PBCV-1 to P100. Viruses EPA-1 and P31 and viruses P41 and E11 are serologically similar even though they are separate isolates. Viruses P91, EPA-1, P31, and P41 are derived from PBCV-1; virus E11 is derived from EPA-1; and virus P100 is from P31. Ara/Rham = arabinose/rhamnose.

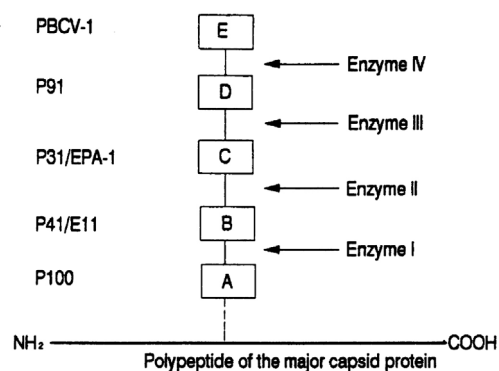


Figure 3. Proposed model for the structure of the oligosaccharide moieties attached to the viral major capsid protein. In this model, sugars B-E may represent monosaccharides, oligosaccharides, or different types of linkages. Enzymes I-IV are responsible for the transfer of sugars B-E to preexisting substrates. Presumably, the sugars can only be added in the order presented so that, e.g., a defect in enzyme I prevents the addition of any sugars onto sugar A. PBCV-1 serotypes that could be missing an enzyme activity are listed on the left.

serotypes. Virus major capsid protein and two minor viral glycoproteins (apparent molecular masses of 280 and 270 kDa from PBCV-1), from the serotypes migrated in a distinctive but predictable fashion on SDS/polyacrylamide gels (Figure 2C), whereas the remaining viral proteins comigrated. The polyclonal antisera recognize oligosaccharide epitopes on the major capsid protein. The two other viral glycoproteins share these epitopes and are affected in parallel by antiserum-resistance mutations. By these criteria the oligosaccharides of the three glycoproteins are indistinguishable.

Glycoproteins are important structural components of many viruses (24). Usually the carbohydrate moieties of virus glycoproteins are added in the rough endoplasmic reticulum and Golgi by host-encoded glycosyltransferases (24). Virus glycoproteins are then transported to the cell membrane and viruses acquire their glycoprotein(s) by budding through the host membrane as they are released from the cell. Consequently, changes in the glycan portion of virus glycoproteins can occur (i) by varying the host [e.g., herpesvirus (25, 26) and influenza virus (27, 28)] or (ii) by altering an amino acid sequence to create or destroy a glycosylation site [e.g., vesicular stomatitis virus (29)]. However, neither of these mechanisms explain the altered glycan(s) in the major capsid protein of PBCV-1: (i) All viruses are grown in the same host, and (ii) the predicted amino acid sequences of the major capsid protein from PBCV-1 and two of its serotypes, EPA-1 and P100, are identical. Furthermore, the PBCV-1 major capsid protein and the other two virus glycoproteins are added to the virus inside the cell since intact infectious PBCV-1 particles accumulate within the cell prior to virus release (1).

The pattern of antiserum-resistant mutants suggests that PBCV-1 influences at least four steps of a common glycosylation pathway. Because mutant glycoproteins migrate faster on SDS/polyacrylamide gels than those of the parents, it is unlikely that the mutation affects a glycosidase cleavage pathway since this would produce a larger oligosaccharide. Three other observations are consistent with the glycosylation hypothesis: (i) Each of the four virus serotypes appear to arise from separate single mutations in the PBCV-1 genome. Although more sophisticated genetic analyses are required to determine mutation frequencies, the serotypic variants are present at ≈ 1 per 10^6 virus particles. This number is consistent with a single mu-

tation. (ii) The major capsid protein of PBCV-1 and the serotypes contains six and/or seven sugars (fucose, galactose, glucose, xylose, mannose, and arabinose and/or rhamnose) that vary in a predictable manner that is related to their serotype and the migration of the major capsid protein (Table 2). (iii) The viral antisera did not react with host proteins.

A model consistent with the data is presented in Figure 3. The PBCV-1 major capsid protein has one or more glycan components containing sugars in sequential order: sugar A-sugar B-sugar C-sugar D-sugar E. Sugar A is attached to the amino acid and the terminal sugar E serves as the PBCV-1 epitope. Virus P91 contains a mutation that prevents the addition of sugar E; consequently, terminal sugar D serves as the P91 epitope. The other virus serotypes result from mutations that eliminate additional terminal sugars; in each case the terminal sugar serves as the epitope. Two variations of the model are also consistent with the data. (i) The linkage of the terminal sugar differs with each serotype or (ii) the sugars represented as A, B, C, D, and E consist of more than one sugar.

This simple testable model explains the specificity of each antiserum, the different carbohydrate contents of the major capsid proteins of each serotype, and the mobility differences of the major capsid proteins on SDS/polyacrylamide gels. The model also predicts that the gene for each enzyme can be independently mutated and that genetic crosses between appropriate serotypes should produce wild-type viruses. It will also be important to determine the intracellular location of PBCV-1 protein glycosylation. The proteins could be glycosylated via the conventional rough endoplasmic reticulum and Golgi pathway, possibly using virus-encoded glycosyltransferases. If so, the virus glycoproteins need a signal for targeting them to the virus cytoplasmic assembly centers. Alternatively, viral glycosylation could be independent of the traditional rough endoplasmic reticulum and Golgi system. In this regard, evidence is accumulating for nuclear and cytoplasmic glycosylation of some cell proteins (for review, see reference 30).

In summary, chlorella virus PBCV-1 has an interesting and apparently unusual method for glycosylating its glycoproteins. Regardless of whether PBCV-1 encodes its own glycosylating enzymes or somehow manipulates host enzymes, the mechanism adds still another function encoded by PBCV-1. The chlorella viruses are unusual in that they code for DNA site-specific (restriction) endonucleases and DNA methyltransferases (6).

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