12-5-1997

Hyaluronan Synthase of Chlorella Virus PBCV-1

Paul L. DeAngelis
*University of Oklahoma Health Sciences Center, 940 Stanton L. Young Boulevard, Oklahoma City, OK*

Wei Jing
*University of Oklahoma Health Sciences Center, 940 Stanton L. Young Boulevard, Oklahoma City, OK*

Michael V. Graves
*University of Nebraska - Lincoln*

Dwight E. Burbank
*University of Nebraska - Lincoln*

James L. Van Etten
*University of Nebraska - Lincoln, jvanetten1@unl.edu*

Follow this and additional works at: [http://digitalcommons.unl.edu/plantpathpapers](http://digitalcommons.unl.edu/plantpathpapers)

Part of the [Plant Pathology Commons](http://digitalcommons.unl.edu/plantpathpapers)


[http://digitalcommons.unl.edu/plantpathpapers/157](http://digitalcommons.unl.edu/plantpathpapers/157)

This Article is brought to you for free and open access by the Plant Pathology Department at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Plant Pathology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Hyaluronan Synthase of Chlorella Virus PBCV-1

Paul L. DeAngelis,1 Wei Jing,1 Michael V. Graves,2 Dwight E. Burbank,2 James L. Van Etten 2

1 Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, 940 Stanton L. Young Boulevard, Oklahoma City, OK 73104, USA.
2 Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583–0722, USA.

Corresponding author — P. L. DeAngelis, email paul-deangelis@OUHSC.edu

Abstract
Sequence analysis of the 330-kilobase genome of the virus PBCV-1 that infects a chlorella-like green algae revealed an open reading frame, A98R, with similarity to several hyaluronan synthases. Hyaluronan is an essential polysaccharide found in higher animals as well as in a few pathogenic bacteria. Expression of the A98R gene product in Escherichia coli indicated that the recombinant protein is an authentic hyaluronan synthase. A98R is expressed early in PBCV-1 infection and hyaluronan is produced in infected algae. These results demonstrate that a virus can encode an enzyme capable of synthesizing a carbohydrate polymer and that hyaluronan exists outside of animals and their pathogens.

Hyaluronan or hyaluronic acid (HA), a member of the glycosaminoglycan family that also includes heparin and chondroitan, is a linear polysaccharide composed of alternating β1,4-glucuronic acid (β1,4-GlcA) and β1,3-N-acetylglucosamine (β1,3-GlcNAc) groups. Typically the full-length polymer chains are composed of $10^3$ to $10^4$ monosaccharides ($10^6$ to $10^7$ daltons). HA is an important structural element in the vitreous humor of eye, synovial fluid, and skin of vertebrates (1). Furthermore, HA interacts with proteins such as CD44, RHAMM, and fibrinogen, thereby influencing many natural processes such as angiogenesis, cancer, cell motility, wound healing, and cell adhesion (2). HA also constitutes the extracellular capsules of certain bacterial pathogens such as group A and C Streptococcus and Pasteurella multocida type A (3, 4). These capsules act as virulence factors that protect the microbes from phagocytosis and complement during infection (5, 6). Because HA, a component of the host tissues, is not normally immunogenic, the capsule serves as molecular camouflage (7).

HA synthases (HASs) are integral membrane proteins that polymerize the HA molecule using activated uridine...
diphosphate (UDP)-sugar nucleotides as substrates. Amino acid sequences for some HASs have been deduced from gene sequencing (8); their sizes range from 419 to 588 residues. The vertebrate enzymes (DG42, HAS1, HAS2, and HAS3) and streptococcal HasA have several regions of sequence similarity. Recently, while sequencing the doublestranded DNA genome of virus PBCV-1 (Paramecium bursaria chlorella virus), we unexpectedly discovered an open reading frame (ORF), A98R (GenBank accession number U42580), encoding a 568-residue protein with similarity to the known HASs (28 to 33% amino acid identity in pairwise comparisons by FASTA) (Figure 1).

PBCV-1 is the prototype of a family (Phycodnaviridae) of large (175 to 190 nm in diameter) polyhedral, plaque-forming viruses that replicate in certain unicellular, eukaryotic chlorella-like green algae (9). PBCV-1 virions contain at least 50 different proteins and a lipid component located inside the outer glycoprotein capsid (10). The PBCV-1 genome is a linear, nonpermutated 330-kb double-stranded DNA molecule with covalently closed hairpins (11).

On the basis of its deduced amino acid sequence, the A98R gene product should be an integral membrane protein. To test this hypothesis, we produced recombinant A98R protein in Escherichia coli and assayed the membrane fraction for HAS activity (12, 13). UDP-GlcA and UDP-GlcNAc were incorporated into polysaccharide by the membrane fraction derived from cells containing the A98R ORF on a plasmid, pCVHAS, (average specific activity of 2.5 pmol of GlcA transferred per minute per microgram of protein), but not by samples from control cells (< 0.001 pmol of GlcA transferred per minute per microgram of protein). No activity was detected in the soluble fraction of cells transformed with pCVHAS. UDGPglA and UDP-GlcNAc were simultaneously required for polymerization. The activity was optimal in Hepes buffer at pH 7.2 in the presence of 15 mM MnCl₂, whereas no activity was detected if the metal ion was omitted. The ions Mg²⁺ and Co²⁺ were ~20% as effective as Mn²⁺ at similar concentrations. The P. multocida HAS (14) has a similar metal requirement, but other HASs prefer Mg²⁺.

We also tested the specificity of recombinant A98R for UDP-sugars (15). Only the two authentic HA precursors were incorporated into polysaccharide; neither UDP-galacturonic acid (UDP-GalA) nor UDP-N-acetylgalactosamine (UDP-GalNAc), the C4 epimers of UDP-GlcA or UDP-GlcNAc, respectively, were incorporated. Likewise, UDP-glucose (UDP-Glc) was not polymerized in place of either HA precursor. This strong substrate specificity

Figure 1. Sequence similarity of HASs. The Multalin program (26) was used to align the amino acid sequences of HASs Xenopus laevis DG42, human HAS2, PBCV-1 A98R, and Streptococcus pyogenes HasA (red, 90% consensus; green, 50% consensus, as calculated by Multalin) (8). In the consensus sequence, the symbols are: !, any one of I or V; $, any one of L or M; %, any one of F or Y; #, any one of N, D, E, or Q. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Figure 2. Size exclusion chromatography of polymer product of recombinant A98R HAS. Membranes derived from E. coli cells transformed with pCVHAS were incubated with both radiolabeled HA precursors diluted to the same specific activity (27). After deproteinization and removal of unincorporated precursors, samples were injected onto a Sephacryl S-500HR size exclusion column, and the radioactivity in the fractions was measured (³H, solid squares; ¹⁴C, solid circles). A duplicate sample was treated with HA lyase before deproteinization and chromatography (³H, open squares; ¹⁴C, open circles); no polymer remains after digestion. Size standards: V₀ arrow, void volume, HA derived from recombinant streptococcal HasA (17 ml; ≥ 2 × 10⁷ daltons) (13); crosshatched box, blue dextran (29 to 32 ml; average molecular size 2 × 10⁸ daltons; Pharmacia); V₅ arrow, totally included volume, UDP-sugars (37 ml).
for UDP-GlcA and UDP-GlcNAc is a general feature of the HASs HasA (13) and DG42 (16).

The recombinant A98R enzyme synthesized a polysaccharide with an average molecular size of 3 × 10^6 to 6 × 10^6 daltons (Figure 2), which is smaller than that of the HA synthesized by recombinant HasA or DG42 in vitro (~10^7 daltons and ~5 × 10^6 to 8 × 10^6 daltons, respectively) (13, 16). The polysaccharide was completely degraded by Streptomyces hyalurolyticus HA lyase, an enzyme that depolymerizes HA but not structurally related glycosaminoglycans such as heparin and chondroitan (17).

We examined PBCV-1-infected chlorella cells for A98R gene expression. A ~1700-nucleotide A98R transcript appeared about 15 min after infection and disappeared by 60 min after infection (18), indicating that A98R is an early gene. Consequently, we assayed membrane fractions from uninfected and PBCV-1-infected chlorella cells at 50 and 90 min after infection for HAS activity. Infected cells, but not uninfected cells, had activity (Table 1). Like the bacterially derived recombinant A98R enzyme, radioactive label incorporation from UDP-[14C]GlcA into polysaccharide depended on both Mn^2+ and UDPGlcNAc. This labeled product was also degraded by HA lyase. Disrupted PBCV-1 virions had no HAS activity.

PBCV-1-infected chlorella cells were analyzed for HA polysaccharide by means of a highly specific 125I-labeled HA-binding protein (19, 20). Extracts from cells at 50 and 90 min after infection contained substantial amounts of HA (0.7 and 1400 ng per microgram of protein, respectively), but not extracts from uninfected algae (<0.04 ng per microgram of protein) or disrupted PBCV-1 virions (<0.04 ng per microgram of dry weight). The labeled HA-binding protein also interacted with intact infected cells at 50 and 90 min after infection, but not with healthy cells (21). Therefore, a considerable portion of the newly synthesized HA polysaccharide was immobilized at the outer cell surface of the infected algae. The extracellular HA does not play any obvious role in the interaction between the virus and its algal host because neither plaque size nor plaque number was altered by including either testicular hyaluronidase (465 units/ml) or free HA polysaccharide (100 μg/ml) in the top agar of the PBCV-1 plaque assay (9).

Among chlorella viruses, HA biosynthesis during infection is not limited to the PBCV-1 prototype strain. Thirty-three independently isolated and plaque-purified viruses from the United States, South America, Asia, and Australia were tested for the presence of an A98R-like gene and for the ability to direct production of HA polysaccharide in Chlorella NC64A. Dot blot hybridization analyses of the individual viral genomes with the PBCV-1 A98R probe indicated that 19 isolates (58%) had a similar gene; the algal host DNA did not cross-react with the probe (21). Chlorella cells infected with each of these 19 viruses produced cell surface HA as measured by interaction with the 125I-HA-binding protein (21).

Surprisingly, the PBCV-1 genome also has additional genes, named A609L and A100R, that encode for a UDP-Glc dehydrogenase (UDP-Glc DH) and a glutamine:fructose-6-phosphate amidotransferase (GFAT), respectively. UDP-DH converts UDP-Glc into UDP-GlcA, a required precursor for HA biosynthesis. GFAT converts fructose-6-phosphate into glucosamine-6-phosphate, an intermediate in the UDP-GlcNAc metabolic pathway. Both of these PBCV-1 genes, like the A98R HAS, are expressed early in infection and encode enzymatically active proteins (22); however, these three genes do not function as an operon. Although two of these genes, A98R and A100R, are near one another in the viral genome (bases 50,901 to 52,607 and 52,706 to 54,493, respectively), A609L is located ~240 kb away and is transcribed in the opposite orientation (bases 292,916 to 291,747). The presence of multiple enzymes in the HA biosynthesis pathway indicates that HA production must serve an important function in the life cycle of these chlorella viruses.

The details of the natural history of the phycodnaviruses are unknown. These viruses are ubiquitous in freshwater collected worldwide, and titers as high as 4 × 10^8 infectious viruses per milliliter of native water have been reported (23). The only known hosts for these viruses are chlorella-like green algae, which normally live as hereditary endosymbionts in some isolates of the ciliate, P. bursaria. In the symbiotic unit, algae are enclosed individually in perigal vacuoles and are surrounded by a host-derived membrane (24). The endosymbiotic chlorella are resistant to virus infection and are only infected when they are outside the paramecium (9). We hypothesize that HA synthesis and its accumulation on the algal surface may block the uptake of virus-infected algae by the paramecium. Alternatively, the chlorella viruses might have another host in nature (such as an aquatic animal); perhaps the virus is transmitted because this other host is attracted to or binds to the HA polysaccharide on virus-infected algae.

As depicted in Figure 1, HASs of Streptococcus, vertebrates, and PBCV-1 have many motifs of two to four residues that occur in the same relative order. These conserved motifs probably reflect domains crucial for HA biosynthesis. Regions of similarity between HASs and other enzymes that synthesize β-linked polysaccharides from UDP-sugar precursors are also being discovered as more glycosyltransferases are sequenced (25). The significance of these similar structural motifs will become more apparent as the three-dimensional structures of glycosyltransferases are determined.

The fact that Chlorella virus PBCV-1 encodes a functional glycosyltransferase that can synthesize HA is contrary to the general observation that viruses either (i) use host cell glycosyltransferases to create new carbohydrate structures, or (ii) accumulate host cell glycosconjugates during virion maturation.

---

Table 1. HAS activity of membranes derived from Chlorella cells infected with PBCV-1. The membrane fractions (370 μg of protein) from uninfected cells or cells at 50 and 90 min after infection (a.i.) were assayed with UDP-[14C]GlcA (60 μM, 0.02 μCi) in parallel reactions containing the following components as indicated (300 μM UDP-GlcNAc or 15 mM MnCl2 or both) for 1 hour at 30°C (28). HAS specific activity (presented as pico moles of [14C]GlcA transferred per hour per milligram of protein) was detected in the algal membranes after infection with PBCV-1, but not in uninfected cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>UDP-GlcNAc</th>
<th>Mn^2+</th>
<th>HAS specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>+</td>
<td>+</td>
<td>≤ 6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>≤ 6</td>
</tr>
<tr>
<td>50 min a.i.</td>
<td>+</td>
<td>+</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>≤ 6</td>
</tr>
<tr>
<td>90 min a.i.</td>
<td>+</td>
<td>–</td>
<td>≤ 6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>+</td>
<td>≤ 6</td>
</tr>
</tbody>
</table>

DeAngelis et al. in SCIENCE 278 (1997)
Furthermore, HA has been generally regarded as restricted to animals and a few of their virulent bacterial pathogens. Though many plant carbohydrates have been characterized, to our knowledge, neither HA nor a related polysaccharide has previously been detected in cells of plants or protists.

**REFERENCES AND NOTES**

12. The A98R ORF was cloned after amplification of genomic viral DNA with 22 cycles of polymerase chain reaction (PCR) with Taq polymerase [F. M. Ausubel et al., in Short Protocols in Molecular Biology (Wiley, New York, ed. 3, 1995)]. The oligonucleotide primers [sense 5’- gagacctggttgatctaatggatccat-3’; antisense 5’-gcggcgcgctccagc-3’; Great American Gene Company] contained Nco I or Sal I restriction sites (underlined, respectively) flanking the ORF encoding 568 amino acids (uppercase letters). Codons 4 and 5 were altered to optimize bacterial expression. The PCR product was purified, digested with Nco I and Sal I, and ligated into a modified version of the plasmid pET-8C [B. A. Moffatt and F. W. Studier, J. Mol. Biol. 189, 113 (1986)] (it has an extra Sal I site in the polylinker) cleaved with Nco I and partially digested with Sal I. This construct placed the A98R ORF under the control of a T7 phage promoter. The resulting plasmid, pCVHAS, was transformed into the expression host, E. coli BL21 (DE3). The A98R protein was expressed by induction with 1 mM isopropyl β-D-galactoside. After 3 to 5 hours of further growth, the membrane fraction was isolated (3). Control membrane preparations were made from cultures with the same vector containing an irrelevant gene (a protein kinase). Total protein was measured according to M. M. Bradford [Anal. Biochem. 72, 248 (1976)]. The paper chromatography method was used to assay for HAS activity (13).
15. Assays with E. coli−derived A98R were incubated either with 120 μM UDP-[14C]GlcA or with 150 μM UDP-[3H]GlcNAc. The reactions also contained one unlabelled sugar nucleotide (an authentic precursor or UDP-Glc, UDP-GaA, or UDP-GaINAc) at 300 μM. Less than 5% of the maximal incorporation (assay with UDP-GlcA and UDP-GlcNAc present) was detected if an unnatural UDP-sugar was substituted for UDP-GlcA or UDP-GlcNAc, or if only a single precursor was present.
18. M. V. Graves, D. Landstein, J. L. Van Etten, unpublished results.
20. A competitive radiometric assay based on a [125I]-labeled HA-binding protein (19) (Pharmacia HA Test) was used to measure the amount of HA in disrupted (freeze-thawed) virus particles or the cultures of NC64A cells. The cells were disrupted by vigorous agitation with glass beads (1-mm beads, agitated for 3 min four times; Biospec Mini-Beadbeater-8). The cell lysate was clarified by centrifugation (15,000g, 5 min) before assay (average of two determinations).
27. Membranes (860 μg of protein) were incubated with 120 μM UDP-[14C]GlcA (0.36 μCi) and 840 μM UDP-[3H]GlcNAc (2.6 μCi) in 300 μl of 50 mM Hepes, pH 7.2, with 15 mM MnCl₂, for 3 hours at 37°C. EDTA (18 mM final concentration) was then added to stop the HAS activity. Half of the reaction was deproteinized by treatment with 0.5% SDS (w/v) and Pronase (final concentration of 200 μg/ml, 5 hours at 37°C; Boehringer Mannheim). Unincorporated precursors and other small molecules were removed by ultrafiltration (Microcon 10, 10,000 daltons cutoff; Amicon). Half of this semipurified sample was injected onto a Sepharoc S-500HR column (1 cm by 50 cm; Pharmacia) equilibrated in 0.2 M NaCl, 5 mM tris, pH 8.0 (0.5 ml/min, 1-ml fractions). To verify that the identity of the labeled polysaccharide was HA, we treated the other half of the original reaction with HA lyase (30 units at 37°C overnight; Sigma) before the deproteinization step. This treatment degraded the radioactive polymer to small oligosaccharides (tetramers and hexamers) that were removed by ultrafiltration before gel filtration chromatography.
28. Two cultures of NC64A cells (0.9 liter; 1.9 × 10⁷ cells) were infected with PBCV-1 (multiplicity of infection of 5) and incubated for 50 or 90 min after infection. Another culture served as an uninfected control. The cells were harvested, and the membrane fraction (yield ~3 mg of protein) was prepared as described [P. L. DeAngelis and A. M. Achyuthan, J. Biol. Chem. 271, 23657 (1996)], except that 1 mM mercaptoethanol was substituted for diithiothreitol. The paper chromatography method was used to assay for HAS activity (13).
29. We thank A. M. Achyuthan, G. M. Air, M. K. Brakke, R. D. Cummings, L. C. Lane, M. Nelson, and P. H. Weigel for helpful discussions. R. A. Steinberg provided the plasmids and host strain for T7 expression system. Supported by a NIH grant (R01-GM56497) and a University of Oklahoma Medical Alumni Scholarship to P.L.D. and a NIH grant (R01-GM32441) to J.V.E. Submitted September 15, 1997; accepted October 30, 1997.