JOURNAL OF VIROLOGY, Jan. 1986, p. 318–327
0022-538X/86/010318-10$02.00/0

Vol. 57, No. 1

Identification, Mapping, and In Vitro Translation of *Campoletis sonorensis* Virus mRNAs from Parasitized *Heliothis virescens* Larvae

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Received 8 July 1985/Accepted 23 September 1985

Expression of *Campoletis sonorensis* virus (CsV) in parasitized *Heliothis virescens* larvae was investigated by Northern blot analysis of poly(A)$^+$ mRNAs isolated from *H. virescens* larvae at various times after parasitization by *C. sonorensis*. At least 12 CsV mRNAs were detected in parasitized *H. virescens* larvae. Injection of nonparasitized *H. virescens* larvae with purified CsV resulted in a pattern of viral mRNAs similar to that observed in naturally parasitized larvae. With CsV DNA restriction fragments which contained expressed sequences, individual CsV mRNAs were mapped to the superhelical DNAs of the viral genome. Two gene-specific probes, which consisted of cloned S1 nuclease-protected restriction fragments, each hybridized to several CsV superhelical DNAs, suggesting that some CsV genes may be shared on several superhelical DNAs. Cloned restriction fragments containing sequences which flank the expressed sequences also hybridized to numerous CsV superhelical DNAs. Some CsV proteins were identified by in vitro translation of hybrid-selected CsV mRNAs.

Campoletis sonorensis virus (CsV) is a double-stranded DNA virus which is associated with both a parasitic wasp, *C. sonorensis*, and its host, *Heliothis virescens*. The viral genome appears to consist of at least 28 molecules of double-stranded DNA which are polydisperse in size (6 to 20 kilobase pairs), covalently closed, and superhelically coiled and appear to be nonequimolar in distribution (8; G. W. Blissard, J. G. W. Fleming, S. B. Vinson, and M. D. Summers, J. Insect Physiol., in press). In a preliminary study of the complexity of the CsV genome, Krell et al. (8) isolated eight of the CsV superhelical (SH) DNAs and individually hybridized these SH DNAs to the entire CsV genome. Because little cross-hybridization was detected between individual CsV SH DNAs, they hypothesized that the SH DNAs of the CsV genome were composed mostly of unique sequences and that the aggregate size of the CsV genome may be as large as 190 to 240 kilobase pairs.

CsV replicates in the calyx epithelial cells of the oviduct of the wasp, and virions are secreted into the lumen of the oviduct where they accumulate in a dense fluid known as the calyx fluid (15). During oviposition, this fluid is injected into the host larva along with the parasite egg. Parasitism of *H. virescens* by *C. sonorensis* produces a number of dramatic changes in host physiology, and the virus is believed to be involved in some of these changes. One effect of CsV in the host (*H. virescens*) larva is an alteration of the response of the host to the parasite egg (2). In the absence of virus, the parasite egg is encapsulated by host hemocytes, preventing development of the egg. However, when purified virus and *C. sonorensis* eggs (washed free of calyx fluid) are coinjected, the parasite eggs are not encapsulated. UV irradiation of the virus prior to injection results in encapsulation of the parasite eggs, suggesting that viable CsV is required for protection from cellular encapsulation. Another dramatic effect of parasitism by *C. sonorensis* is the retardation of growth of the parasitized host. Parasitized *H. virescens* larvae feed less vigorously and show significant reductions in weight gain when compared with nonparasitized larvae. Vinson et al. (16) reported that injection of purified CsV into *H. virescens* larvae causes a retardation in host weight gain similar to that observed in parasitized larvae and suggested that the virus may be responsible for at least some of the physiological and behavioral changes that occur in parasitized larvae.

Since several studies (2, 16) suggest that viable virus is required to produce the observed effects of CsV on the host larva, some recent studies have analyzed the expression of CsV in the parasitized host. With cDNA probes, CsV mRNAs were detected in parasitized *H. virescens* larvae as early as 2 h postparasitization (p.p.) and throughout the course of parasite development (4). Expression from only a portion of the viral SH DNAs was detected in parasitized *H. virescens* larvae. Recently, we identified at least 10 CsV mRNAs that are present in parasitized *H. virescens* larvae shortly after parasitization (4 h p.p.) (G. W. Blissard et al., in press). Also, cDNA probes were used to identify several cloned CsV DNA fragments containing viral genes expressed in parasitized larvae, and the transcribed regions on three of these DNA fragments were mapped.

In the present study, we examined CsV mRNAs at various times postparasitization and used cloned CsV DNA fragments to map seven CsV mRNAs to the SH DNAs of the viral genome. Mapping of CsV mRNAs reveals that the CsV genome is transcriptionally multipartite. Contrary to the findings of a previous study (8), we show that homology exists between a number of CsV SH DNAs. We also show that at least two of the CsV genes appear to each be present on more than one SH DNA of the CsV genome. Putative protein products of specific CsV mRNAs were identified by hybridization selection and in vitro translation.

MATERIALS AND METHODS

Insects and virus. The insects *C. sonorensis* and *H. virescens* were reared as described previously (4, 8). Virus

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was isolated from the oviducts of adult female *C. sonorensis* wasps by the method of Krell et al. (8). For injections of *H. virescens* with purified CsV, the purified virus isolated from 300 *C. sonorensis* females was suspended in 600 μl of cold phosphate-buffered saline (1 mM $CaCl_2$, 2.5 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 137 mM NaCl).

Isolation of total poly(A)$^+$ mRNA and viral DNA. To prepare total poly(A)$^+$ mRNA, third-instar *H. virescens* larvae were naturally parasitized by *C. sonorensis*, injected with 0.5 μl of the purified virus solution, or mock injected with 0.5 μl of 1× phosphate-buffered saline. Total nucleic acid was isolated from parasitized, nonparasitized, or injected *H. virescens* larvae by homogenization of 200 to 400 larvae in guanidine hydrochloride by the procedure of Fuchs and Green (5). For the natural parasitization and injection of *H. virescens*, larvae were homogenized at various times p.p. or postinjection. Poly(A)$^+$ mRNA was isolated by oligo(dT) cellulose chromatography as described by Aviv and Leder (1). Unless otherwise stated, poly(A)$^+$ mRNA refers to the poly(A)$^+$ mRNA isolated from larvae which were naturally parasitized by *C. sonorensis*. Viral DNA was isolated as described previously (8) and stored at 4°C.

Northern and Southern hybridizations. To study the temporal pattern of CsV mRNAs in parasitized *H. virescens* larvae, 10 μg of poly(A)$^+$ mRNA from each time point p.p. or postinjection was electrophoresed on 1.25% agarose, 6% formaldehyde gels and transferred to nitrocellulose. For hybridizations of cloned CsV DNA restriction fragments to Northern blots, each lane on Northern blots contained 8 μg of poly(A)$^+$ mRNA isolated from *H. virescens* larvae at 48 h p.p. Formaldehyde gels, Northern transfers, and hybridizations of DNA probes to Northern blots were performed as described previously (11; G. W. Blissard et al., in press). For Southern hybridizations, undigested CsV DNA (1.3 μg per lane) was electrophoresed on 0.8% agarose gels and transferred to nitrocellulose by the method of Southern (14) as modified by Smith and Summers (13). Procedures for hybridization of labeled DNA to Southern blots were described previously (4). Undigested viral CsV DNA and cloned CsV DNA restriction fragments for hybridization to Northern and Southern blots were labeled with [^{32}P]dATP to high specific activity (2×10^8 cpm/μg) by nick translation (11).

Mapping CsV mRNAs to CsV SH DNAs. In a previous study (G. W. Blissard et al., in press), cloned CsV DNA restriction fragments p2H-5300, p3H-2200, p4H-8460, p5H-6330, and pQB-7200 were reported to contain sequences expressed in *H. virescens* larvae, and expressed sequences were mapped to regions on the physical maps of p2H-5300, p3H-2200, and pQB-7200. These five cloned CsV DNA fragments were used for mapping individual CsV mRNAs to regions of the CsV genome and will be referred to as cDNA-positive fragments. Plasmid DNAs for mapping experiments were prepared by the procedure of Holmes and Quigley (6), and the inserted CsV DNA fragments were further isolated by electroelution (12). For mapping of CsV mRNAs to SH DNAs of the CsV genome, cloned cDNA-positive CsV DNA restriction fragments were labeled with [^{32}P]dATP by nick translation and hybridized to Northern blots of poly(A)$^+$ mRNA from parasitized *H. virescens* larvae (48 h p.p.) and to Southern blots of undigested CsV DNA.

S1 nuclease analysis. To generate specific probes which contained only mRNA coding sequences (gene-specific probes), we identified S1 nuclease-protected regions on two of the cDNA-positive CsV DNA fragments (p2H-5300 and p3H-2200) and subcloned restriction fragments from within these regions. Plasmid DNAs were digested with restriction enzymes, 5′ or 3′ end labeled, and used for S1 nuclease analysis by the procedure of Favaloro et al. (3). Plasmids containing CsV restriction fragments in pUC8 or pUC19 vectors were labeled at a single 5′ or 3′ end of the CsV restriction fragment by cleaving the plasmid (p3HB-1350 or p2HS-2000) at one of the junctions between CsV sequences and pUC vector sequences. The resulting linearized plasmid, which contained one end from the vector and one end from the CsV fragment, was end labeled. For 5′ end labeling, DNAs were dephosphorylated at the 5′ termini with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals) and labeled with T4 polynucleotide kinase (Bethesda Research Laboratories, Inc.) and [γ-^{32}P]ATP (5,000 Ci/mmol; ICN Pharmaceuticals, Inc.) as described by Maniatis et al. (10). Specific activities of 5′-end-labeled probes ranged from 0.5×10^6 to 1.0×10^6 cpm/pmol of ends. DNAs were 3′ end labeled with [α-^{32}P]dATP (800 Ci/mmol; New England Nuclear Corp.) and T4 DNA polymerase (Boehringer Mannheim). Specific activities for the 3′-end-labeled probes ranged from 1×10^6 to 5×10^6 cpm/pmol of ends. For each S1 nuclease protection experiment, approximately 10 μg of poly(A)$^+$ mRNA (from parasitized *H. virescens* larvae [48 h p.p.]) and 30 to 100 ng of the DNA probe were suspended in 20 μl of S1 hybridization buffer (80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 1 mM EDTA, 0.4 M NaCl). The DNA-RNA solution was heated to 65°C for 15 min and then hybridized at 52°C for 3 h. After hybridization, 300 μl of S1 nuclease digestion buffer (0.28 M NaCl, 50 mM sodium acetate [pH 4.4], 4.5 mM $ZnCl_2$, 20 μg of denatured calf thymus DNA per ml, 0.6 U of S1 nuclease [P-L Biochemicals, Inc.] per μl) was added, and the solution was incubated at 37°C for 30 min. S1 nuclease digestion was terminated by the addition of 75 μl of termination buffer (2.5 M ammonium acetate, 50 mM EDTA), followed by precipitation in 2 volumes of ethanol. S1 nuclease-protected DNA fragments were electrophoresed on 1.2% alkaline agarose gels (10), dried under vacuum, and autoradiographed for 12 to 48 h.

Hybridization selection and in vitro translations. For hybridization selection of CsV mRNAs, 15 μg of plasmid DNA from p2HS-2000, p3HB-1350, p4H-8460, p5H-6330, and pQBS-4800 was denatured and blotted onto nitrocellulose filters by the procedure of Kafatos et al. (7). p2HS-2000 and p3HB-1350 were subcloned restriction fragments from p2H-5300 and p3H-2200, respectively (see Fig. 3 and 4). pQBS-4800 (see Fig. 5), a subcloned *Bam*HI-*Sal*I fragment from pQB-7200, was supplied by J. G. W. Fleming. Hybridization selection was performed essentially as described by Maniatis et al. (10). The DNA filters were prehybridized in hybridization selection buffer (65% formamide, 20 mM PIPES [pH 6.4], 0.4 M NaCl, 0.2% sodium dodecyl sulfate (SDS), 100 μg of poly(A) per ml, 100 μg of wheat germ tRNA per ml) for 4 h at 50°C. Total poly(A)$^+$ mRNA (80 μg) from parasitized *H. virescens* larvae (48 h p.p.) was resuspended in 220 μl of hybridization selection buffer, denatured by heating to 70°C for 10 min, added to the DNA filters, and hybridized for 12 to 24 h at 50°C. The filters were washed 10 times in 1 ml of a solution containing 10 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, and 0.5% SDS at 60°C, followed by five 1-ml washes with a solution consisting of 10 mM Tris (pH 7.6), 150 mM NaCl, and 1 mM EDTA at 60°C. CsV mRNAs were eluted from the DNA filters by the addition of 200 μl of H_2O and 30 μg of tRNA, followed by heating to 100°C for 1 min and freezing at −80°C. The

mRNAs were precipitated by the addition of 1/10 volume of 2 M potassium acetate and 2 volumes of ethanol.

Hybrid-selected mRNAs were suspended in 5 μl of H_2O and translated in a rabbit reticulocyte lysate in vitro translation system (Promega Biotec) by the directions of the manufacturer. Each 30-μl reaction contained 50 μCi of [^{35}S]methionine (1,100 Ci/mmol; New England Nuclear). In vitro translation reactions were incubated at 30°C for 60 min, then 3 μl of an RNase solution (1 mg of RNase per ml in 300 mM EDTA) was added, and the reaction was incubated at 37°C for 15 min. The translation products were precipitated by the addition of 0.5 ml of 80% acetone, suspended in 50 μl of disruption buffer (125 mM Tris [pH 6.8], 2% SDS, 1% glycerol, 4 mM 2-mercaptoethanol, 0.001% bromophenol blue), and incubated at 100°C for 3 min. In vitro translation products were analyzed by electrophoresis on 12% SDS polyacrylamide gels by the method of Laemmli (9). For comparison of in vitro translation products to CsV structural proteins, purified CsV was disrupted and electrophoresed in an adjacent lane. Gels were stained with Coomassie blue, treated with En^{3}Hance (New England Nuclear) by the instructions of the manufacturer, dried under vacuum, and exposed to Kodak X-Omat AR X-ray film at −80°C.

RESULTS

Temporal expression of CsV mRNAs in *H. virescens*. ^{32}P-labeled CsV DNA hybridized to 12 different size classes of mRNAs on Northern blots of poly(A)$^+$ mRNA isolated from parasitized *H. virescens* larvae from 2 h to 9 days p.p. (Fig. 1). ^{32}P-labeled CsV DNA did not hybridize to poly(A)$^+$ mRNA from nonparasitized *H. virescens* larvae (data not shown). CsV mRNAs appeared to be most abundant from 12 to 48 h p.p. and ranged in size from approximately 0.6 to 3.2 kilobases (kb). The number of CsV mRNAs detected varied at the different time points. At 2 h p.p. at least 10 CsV mRNAs were detected, and a 1.6-kb mRNA was the predominant transcript. At later times also (6 h to 9 days p.p.), the 1.6-kb mRNA appeared to be abundant. During the times examined (2 h to 9 days p.p.), most viral mRNAs appeared to be present in the same relative quantities except the 3.2-kb mRNA, which decreased in intensity at later times (48 h to 9 days p.p., Fig. 1). The sizes and relative abundance of CsV mRNAs detected from larvae injected with purified CsV were similar to those observed from parasitized larvae (Fig. 1, lanes I and P).

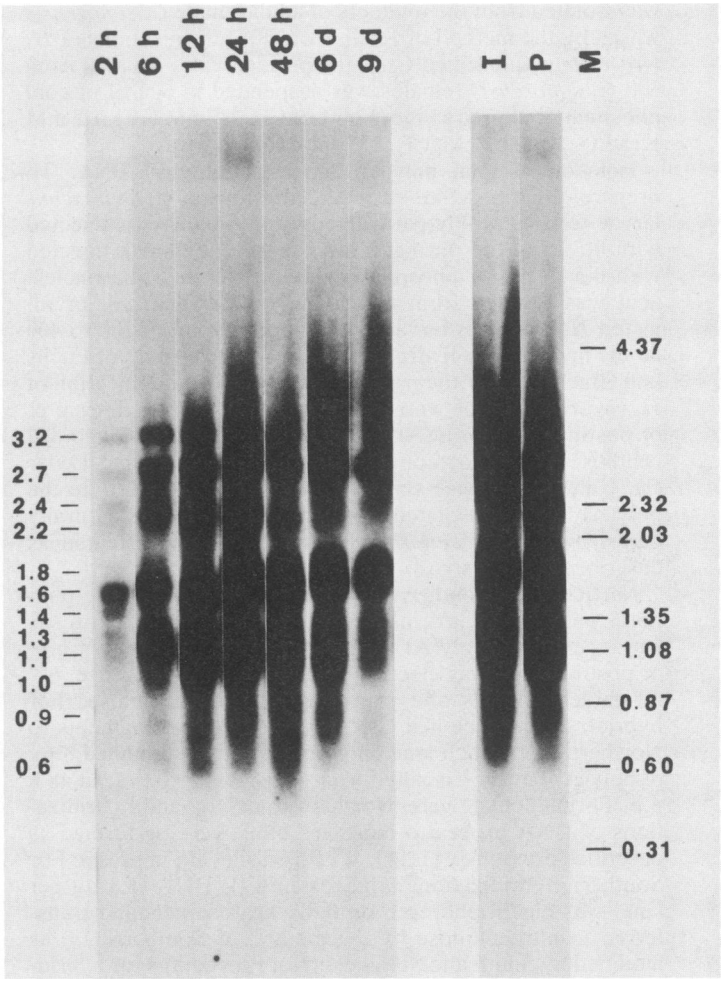

FIG. 1. CsV mRNAs detected in *H. virescens* larvae at various times after parasitization by *C. sonorensis* or after injection with purified CsV. ^{32}P-labeled CsV DNA was hybridized to a Northern blot of poly(A)$^+$ mRNAs isolated from parasitized *H. virescens* larvae at 2, 6, 12, 24, and 48 h and 6 and 9 days (d) p.p. Lanes I and P show hybridization of ^{32}P-labeled CsV DNA to poly(A)$^+$ mRNA isolated from *H. virescens* larvae either 24 h after injection with purified CsV (lane I) or 24 h after parasitization by *C. sonorensis* (lane P). The numbers to the right of the marker lane (M) show the sizes (in kilobases) of the ϕX174 *Hae*III and lambda *Hin*dIII DNA fragments which were used as markers. The numbers to the left of lane 2 h represent the sizes (in kilobases) of CsV mRNAs.

Identification and mapping of CsV mRNAs. The five cloned cDNA-positive restriction fragments which were used as ^{32}P-labeled probes (p2H-5300, p3H-2200, p4H-8460, p5H-6330, and pQB-7200) hybridized to seven CsV mRNAs from parasitized *H. virescens* larvae (Fig. 2). Three of these clones (p4H-8460, p5H-6330, and pQB-7200) each hybridized to a single mRNA. The p4H-8460 probe hybridized to a 3.2-kb mRNA on Northern blots. On Southern blots, this probe hybridized strongly to CsV SH DNAs H and X and lightly to N, Q, and T (Fig. 2, Table 1). The p5H-6330 probe hybridized to a 2.7-kb mRNA and to CsV SH DNAs E, H, L^1, M, and Q (Fig. 2, Table 1). p5H-6330 hybridized more intensely to SH DNAs L^1 and Q than to E, H, and M. The pQB-7200 probe hybridized to a 0.9-kb mRNA and to CsV SH DNAs A^2, J, L^1, and Q (Fig. 2, Table 1). The intensities of the first three bands were very light compared with that of Q.

The p2H-5300 and p3H-2200 probes each hybridized to two CsV mRNAs. The p2H-5300 probe hybridized to mRNAs of 1.4 and 1.1 kb and to eleven CsV SH DNAs with differing intensities (Fig. 2, p2H-5300). p2H-5300 hybridized strongly to CsV SH DNAs V and K, moderately to CsV SH DNAs C^1, H, I, L^2, M, P, Q, and R, and lightly to CsV SH DNA A^1 (Fig. 2). p3H-2200 hybridized to CsV mRNAs of 1.6 and 1.0 kb and to CsV SH DNAs C^2, H, M, R, and W. p3H-2200 hybridized more intensely to CsV SH DNAs C^2, M, R, and W and less intensely to CsV SH DNA H (Fig. 2, p3H-2200).

In Southern hybridizations of the cDNA-positive probes to CsV SH DNAs, two probes (p3H-2200 and pQB-7200) hybridized to mutually exclusive sets of SH DNAs (Fig. 2, Table 1), indicating that sequences from these two probes were not shared on a common SH DNA. Similarly, probe DNAs p4H-8460 and pQB-7200 also appeared to hybridize to different sets of SH DNAs except for the very weak hybridization of p4H-8460 to SH Q. Also, except for very weak hybridization of p3H-2200 to SH H, this probe and its

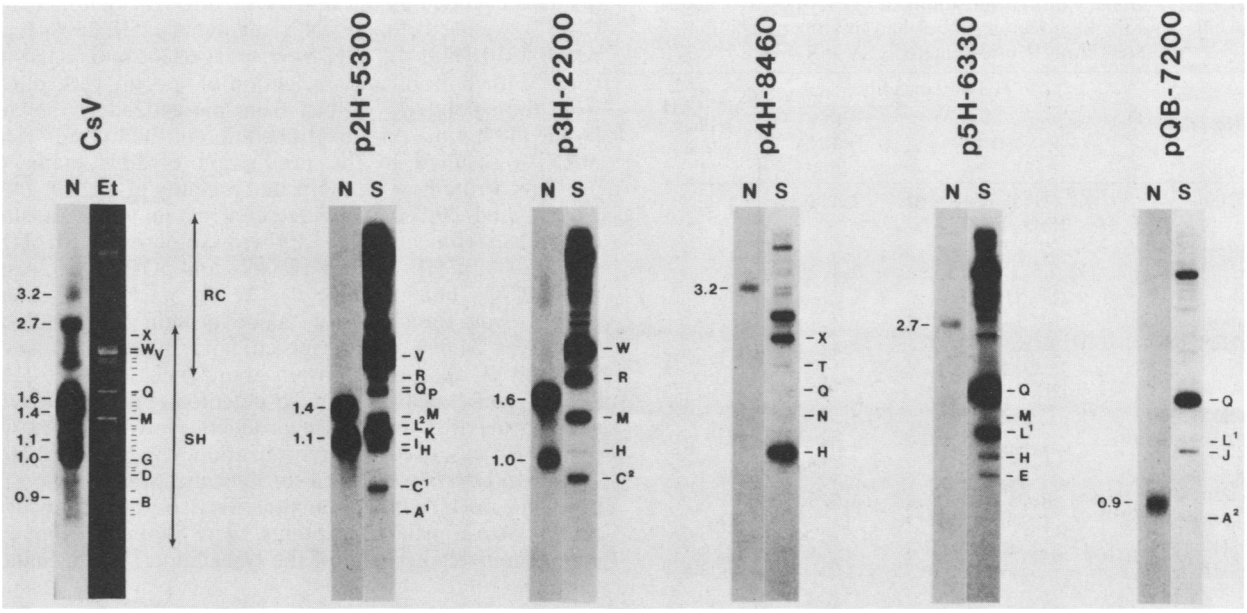

FIG. 2. Identification of mRNAs and viral SH DNAs which hybridize to cDNA-positive CsV DNA fragments. ^{32}P-labeled CsV DNA fragments (p2H-5300, p3H-2200, p4H-8460, p5H-6330, and pQB-7200) were hybridized to Northern blots (N) of poly(A)$^+$ mRNA from parasitized *H. virescens* larvae (48 h p.p.) and to Southern blots (S) of undigested CsV DNA. ^{32}P-labeled CsV DNA was also hybridized to a Northern blot (probe CsV, lane N) of poly(A)$^+$ mRNA from parasitized *H. virescens* larvae (48 h p.p.). The ethidium bromide-stained gel from which the Southern blots were made is shown (lane Et). The region indicated as RC contains the nicked forms (relaxed circular and linear) of the viral SH DNAs. The region indicated as SH contains the SH CsV DNAs. Letters to the right of lanes Et and S identify the CsV SH DNAs by the established nomenclature (8). Numbers to the left of the N lanes show the sizes (in kilobases) of CsV mRNAs.

derivatives (see Fig. 4, p3CB-100 and p3HPs-400) hybridized to a different set of SH DNAs than did the p4H-8460 probe.

Gene-specific and flanking region probes. To determine whether hybridization of the p2H-5300 probe to such a large number of SH DNAs resulted from homology of the mRNA coding sequences or flanking sequences, or both, we generated hybridization probes containing either coding sequences or flanking sequences. Restriction fragments containing only sequences from within mRNA coding regions are referred to as gene-specific probes, and restriction fragments containing only flanking sequences (CsV sequences not expressed in *H. virescens*) are termed flanking region probes. To generate gene-specific probes, we used S1 nuclease analysis to map a portion of the transcribed region on p2H-5300 and subsequently subcloned a small restriction fragment from within the S1 nuclease-protected region. p2HS-2000, a recombinant pUC8 plasmid containing the 2,000-base pair (bp) *Sal*I-*Hin*dIII fragment of p2H-5300 (Fig. 3), was cleaved at the insert-vector junction with *Sal*I and separately 3′ and 5′ end labeled for use in S1 nuclease analysis. Hybridization of 3′-end-labeled plasmid DNA with poly(A)$^+$ mRNA (48 h p.p.) and subsequent S1 nuclease digestion resulted in an S1 nuclease-resistant fragment of approximately 350 nucleotides (Fig. 3, p2HS-2000 plus *Sal*I, lane 3′). No S1 nuclease-resistant fragments were detected with the 5′-end-labeled plasmid DNA (Fig. 3, p2HS-2000 plus *Sal*I, lane 5′). This indicated that an mRNA coding region extended from the *Sal*I site at map position 3.30 on p2H-5300, approximately 350 bp (5′ to 3′ on the mRNA) toward the *Xba*I site (Fig. 3). Excision of the 340-bp *Sal*I-*Hin*cII fragment from p2HS-2000 (Fig. 3), followed by 3′ end labeling, hybridization to poly(A)$^+$ mRNA (48 h p.p.), and S1 nuclease digestion resulted in protection of the entire fragment from S1 nuclease (data not shown). This provided further evidence that all the sequences within the 340-bp *Sal*I-*Hin*cII fragment were contained within the mRNA coding region of the CsV gene. This gene-specific 340-bp *Sal*I-*Hin*cII fragment was subcloned into pUC8, labeled by nick translation, and hybridized to Northern blots of poly(A)$^+$ mRNA from *H. virescens* larvae (at 48 h p.p.) and to Southern blots of undigested CsV DNA. This cloned 340-bp gene-specific probe (Fig. 3, p2SHc-340) hybridized to both the 1.4- and 1.1-kb mRNAs and to CsV SH DNAs C^1, H, I, K, L^2, Q, R, and V (Fig. 3, Table 1). Except for SH DNAs P, M, and A^1, the gene-specific probe (p2SHc-340) hybridized to the same SH DNAs as did the parental fragment (Fig. 2, p2H-5300; Fig. 3, p2SHc-340; and Table 1). Hybridization of the gene-specific probe to different CsV SH DNAs varied in intensity, with SH DNAs V and K hybridizing most intensely, followed by decreasing intensities for Q and L^2, H and C^1, R and I (Fig. 3). The relative hybridization intensities were similar to those observed with the parental fragment (Fig. 2, p2H-5300). A restriction fragment from a region which does not contain sequences expressed in *H. virescens* (at 48 h p.p.) was excised from p2H-5300, subcloned into pUC8, and used for Northern and Southern hybridizations. This cloned restriction fragment, which is referred to as a flanking region probe, consisted of a 760-bp *Hin*dIII-*Sal*I fragment of p2H-5300 and was labeled p2HS-700 (Fig. 3). The flanking region probe does not hybridize to CsV mRNAs, but the pattern of hybridization to undigested CsV DNA was similar to the pattern produced by the gene-specific probe (p2SHc-340) derived from the same parental CsV DNA restriction fragment (Fig. 3). Two CsV SH DNAs (P and B) hybridized to the flanking region probe (p2HS-700) but not to the gene-specific probe (p2SHc-350, Fig. 3).

To generate a gene-specific probe for the gene on p3H-2200, a portion of the region encoding the CsV mRNA(s) was identified by S1 nuclease protection.

TABLE 1. Summary of hybridizations of cDNA-positive, gene-specific, and flanking region probes and in vitro translation products from hybrid-selected CsV mRNAs

DNA probe	Hybridization to:		Mol wt of protein (10^3)[b]
	CsV SH DNAs[a]	CsV mRNA (kb)	
p2H-5300	C^1, H, I, K, L^2, M, P, Q, R, V (A^1)	1.4, 1.1	
Gene-specific p2SHc-340	K, L^2, Q, V (C^1, H, I, R)	1.4, 1.1	
Flanking region p2HS-700	H, I, K, L^2, P, Q, R, V (B, C^1)		
p2HS-2000			45
p3H-2200	C^2, M, R, W (H)	1.6, 1.0	
Gene-specific p3CB-100	M, R, W	1.6, 1.0	
Flanking region p3HPs-400	C^2, M, R, W (H)		
p3HB-1350			24 (27, 21, 20, 19)
p4H-8460	H, X (N, Q, T)	3.2	24
p5H-6330	E, H, L^1, Q (M)	2.7	
pQB-7200	J, Q (A^2, L^1)	0.9	
pQBS-4800			20

[a] Letters in parentheses indicate those SH DNAs which hybridized with very low intensity.
[b] Numbers in parentheses indicate minor proteins.

p3H-2200 was digested with *Hin*dIII and *Bam*HI, and the 1,350-bp *Hin*dIII-*Bam*HI fragment was subcloned into pUC8 (Fig. 4, p3HB-1350) for subsequent S1 nuclease mapping. p3HB-1350 was cleaved at the insert-vector junction with *Bam*HI, separately 5′ and 3′ end labeled, hybridized to poly(A)$^+$ mRNA (48 h p.p.), and digested with S1 nuclease. When the cleaved and 5′-end-labeled DNA from p3HB-1350 was used for S1 nuclease analysis, a 420-nucleotide S1 nuclease-resistant fragment was detected by alkaline gel electrophoresis (Fig. 4, p3HB-1350 plus *Bam*HI, lane 5′). No S1 nuclease-resistant fragments were detected with 3′-end-labeled DNA (Fig. 4, p3HB-1350 plus *Bam*HI, lane 3′). A 100-bp *Cla*I-*Bam*HI fragment from within the S1-protected region of p3HB-1350 was subcloned into pUC19, labeled by nick translation, and hybridized to Northern blots of poly(A)$^+$ mRNA (48 h p.p.) and Southern blots of undigested CsV DNA. This gene-specific probe (Fig. 4, p3CB-100) hybridized to both the 1.6- and 1.0-kb mRNAs and to CsV SH DNAs M, R, and W. To determine whether an unexpressed flanking region was also homologous to the same CsV SH DNAs as this gene-specific probe, the 400-bp terminal *Hin*dIII-*Pst*I fragment of p3H-2200 (Fig. 4) was subcloned into pUC8, labeled with [^{32}P]dATP by nick translation, and hybridized to Northern blots of poly(A)$^+$ mRNA (48 h p.p.) and to Southern blots of undigested CsV DNA. This flanking region probe (Fig. 4, p3HPs-400) did not hybridize to CsV mRNAs but did hybridize to CsV SH DNAs C^2, H, M, R, and W. Thus, while the flanking region probe (p3HPs-400) hybridized to the same SH DNAs as did the parental DNA fragment (p3H-2200), the gene-specific probe (p3CB-100) hybridized only to SH DNAs M, R, and W (Fig. 2 and 4, Table 1).

Hybridization selection and in vitro translation. To identify proteins encoded by the seven CsV mRNAs identified above (Fig. 2), we used cloned DNA fragments p2HS-2000 (Fig. 3), p3HB-1350 (Fig. 4), p4H-8460, p5H-6330, and pQBS-4800 (Fig. 5) for hybridization selection of specific CsV mRNAs from total poly(A)$^+$ mRNA from parasitized *H. virescens* larvae (48 h p.p.). In vitro translation of the hybrid-selected mRNAs resulted in the production of four major CsV proteins. Proteins with molecular weights of 45,000, 24,000, 24,000, and 20,000 were detected as in vitro translation products from mRNAs hybrid selected with DNAs p2HS-2000, p3HB-1350, p4H-8460, and pQBS-4800, respectively (Fig. 6 and Table 1). When p3HB-1350-selected mRNA was translated, a major protein with molecular weight of 24,000 was observed, and in addition, several proteins of apparently lower abundance (27,000, 21,000, 20,000, and 19,000) were also detected (Fig. 6). No virus-specific in vitro translation products were detected when p5H-6330 was used for hybridization selection. When the same five DNAs were used for hybridization selection from poly(A)$^+$ mRNA from nonparasitized *H. virescens* larvae, no in vitro-translated proteins were detected above the endogenous background of the translation system (data not shown).

DISCUSSION

We detected at least 12 CsV mRNAs in *H. virescens* larvae after parasitization by *C. sonorensis*. In a previous study, CsV mRNAs were examined at only one time point (4 h p.p.), and only 10 CsV mRNAs were detected (G. W. Blissard et al., in press). Fleming et al. (4) originally demonstrated that CsV expression is detectable in parasitized *H. virescens* larvae as early as 2 h p.p., and in the present study, we show that at least 10 CsV mRNAs are detectable at 2 h p.p. While most mRNAs were present in relatively low quantities at 2 h p.p., the 1.6-kb mRNA appears to be the most abundant species present at this time. It is possible that the early and continued presence of this mRNA at what appears to be relatively high levels may suggest a function which is required both immediately and continuously. Because published reports (2, 16) indicate that CsV is involved in some of the physiological changes associated with parasitism, we could hypothesize that this gene product might interact with or affect the immune system, nervous system, or endocrine system of the host. Since the relative intensities of the CsV mRNAs detected in Northern hybridizations represent only steady-state levels of CsV mRNAs, the apparently large quantities of CsV mRNAs observed at 12 to 48 h p.p. (Fig. 1) may result from either more rapid transcription rates or the accumulation of stable transcripts. Because constant amounts of poly(A)$^+$ mRNA were analyzed, we cannot eliminate the possibility that the apparent increase in the abundance of viral mRNAs at 12 to 48 h p.p. may result from a reduction in the level of *H. virescens* transcripts and therefore only represents an increase in the proportion of viral transcripts.

To detect possible differences in the temporal regulation of the CsV genes expressed in *H. virescens*, we examined CsV mRNAs at various times p.p. Some CsV mRNAs (0.6 and 0.9 kb) were detected only as very faint bands or not at all at times 2 h and 9 days p.p. (Fig. 1). Because most CsV mRNAs appear to be present in relatively low quantities at 2 h p.p. the apparent absence of the 0.6- and 0.9-kb mRNAs at 2 h p.p. may indicate only our inability to detect the less abundant mRNAs at this time. However, at 9 days p.p. the 0.6- and 0.9-kb mRNAs were not detected even though other mRNAs (2.7, 1.8, and 1.6 kb) appear to be present at

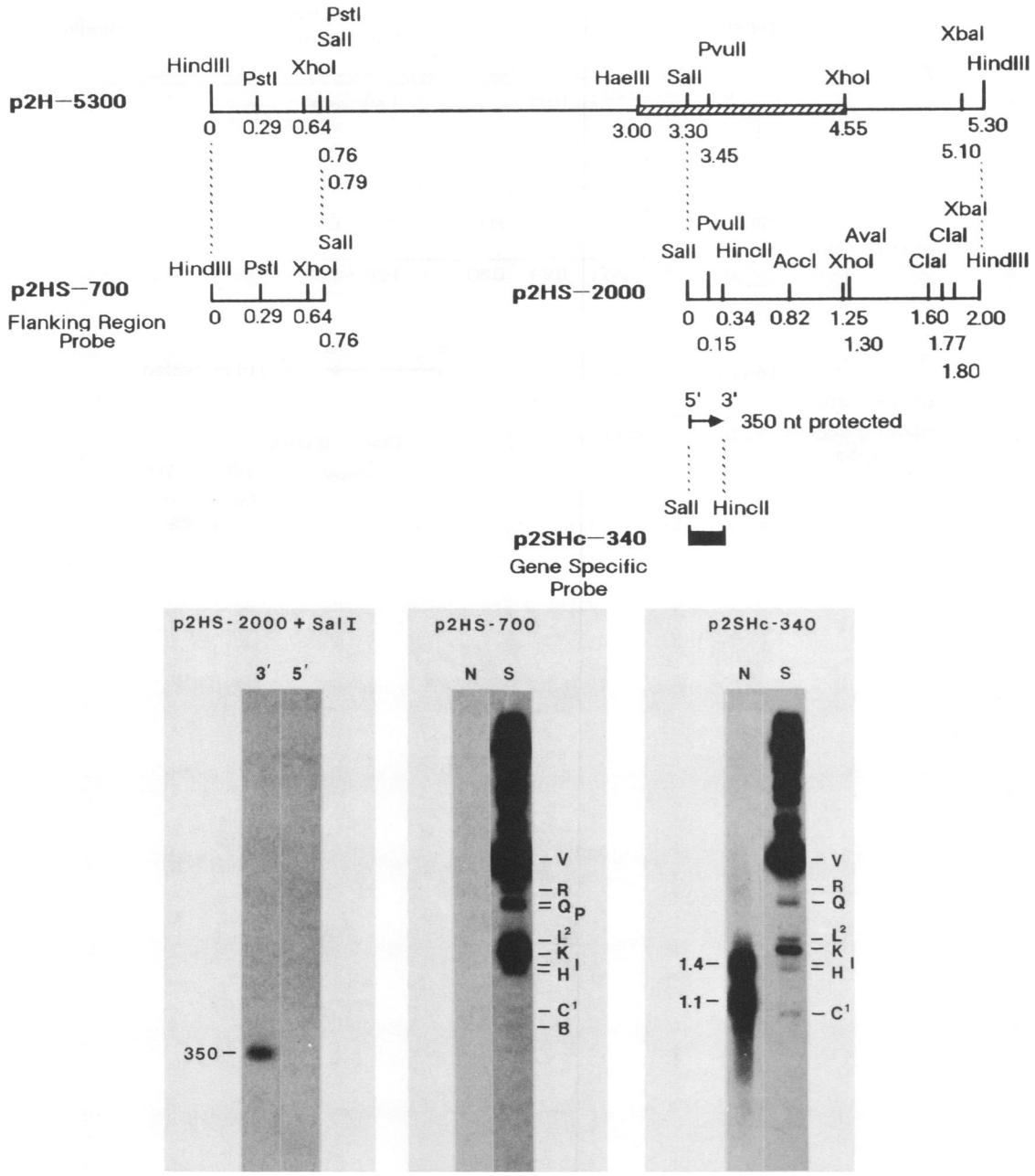

FIG. 3. Mapping with subclones of p2H-5300. The schematic illustrates the strategy for subcloning and S1 nuclease mapping portions of p2H-5300. For p2HS-2000 plus *Sal*I, lanes 3′ and 5′ show products of an S1 nuclease protection experiment in which recombinant plasmid p2HS-2000 was cleaved with *Sal*I, either 3′ or 5′ end labeled, hybridized to poly(A)$^+$ mRNA (48 h p.p.), digested with S1 nuclease, and fractionated on a 1.2% alkaline agarose gel (3′, 3′-end-labeled plasmid; 5′, 5′-end-labeled plasmid). The number beside lane 3′ shows the size (in nucleotides) of the S1 nuclease-protected fragment. The arrow in the schematic represents the S1 nuclease-protected region and the direction of transcription. Both the gene-specific probe (p2SHc-340) and the flanking region probe (p2HS-700) were hybridized to Northern blots (N) of poly(A)$^+$ mRNA (48 h p.p.) and to Southern blots (S) of undigested CsV DNA. Numbers beside Northern blots represent the size of CsV mRNAs (in kilobases). Letters beside Southern blots identify CsV SH DNAs. The hatched area on the schematic of p2H-5300 represents the restriction fragments to which transcripts were mapped in a previous study (G. W. Blissard et al., in press). Numbers below diagrams of restriction fragments indicate distances (in kilobase pairs) from the leftward terminal restriction enzyme site.

relatively high levels. Also, when compared with other mRNAs at time points late after parasitization (48 h to 9 days p.p.), the relative level of the 3.2-kb mRNA appeared to decrease (Fig. 1). These apparent temporal changes in the relative levels of CsV mRNAs may suggest alterations in the transcription rates or differences in the stabilities of individual CsV mRNAs.

All previous studies of CsV expression in *H. virescens* (4; G. W. Blissard et al., in press) have used poly(A)$^+$ mRNA isolated from naturally parasitized *H. virescens* larvae. Analysis of CsV mRNAs after injection of *H. virescens* larvae with purified CsV showed a pattern of expression similar to that observed in naturally parasitized larvae. This indicates that CsV expression in *H. virescens* is independent of the

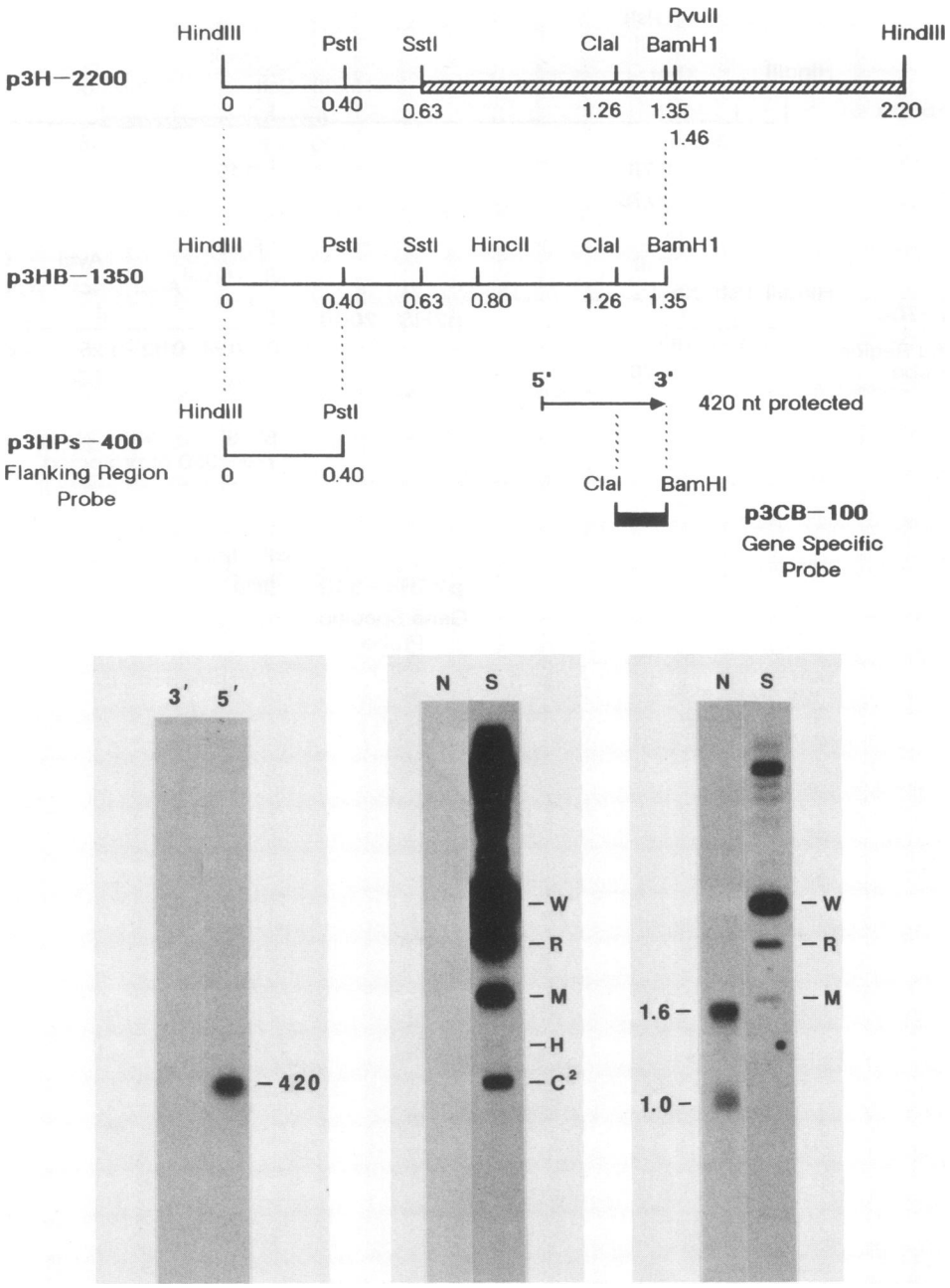

FIG. 4. Mapping with subclones of p3H-2200. The schematic illustrates the strategy used for subcloning and S1 nuclease-mapping portions of p3H-2200. For p3HB-1350 plus *Bam*HI, lanes 3′ and 5′ show products of an S1 nuclease protection experiment in which recombinant plasmid p3HB-1350 was cleaved with *Bam*HI, either 3′ or 5′ end labeled, hybridized to poly(A)$^+$ mRNA (48 h p.p.), digested with S1 nuclease, and fractionated on a 1.2% alkaline agarose gel (3′, 3′-end-labeled plasmid; 5′, 5′-end-labeled plasmid). The number beside lane 5′ shows the size (in nucleotides) of the S1 nuclease-protected fragment. The arrow in the schematic represents the S1 nuclease-protected region and the direction of transcription. Both the gene-specific probe (p3CB-100) and the flanking region probe (p3HPs-400) were hybridized to Northern blots (N) of poly(A)$^+$ mRNA (48 h p.p.) and to Southern blots (S) of undigested CsV DNA. Numbers beside Northern blots show the size of CsV mRNAs in kilobases. Letters beside Southern blots identify CsV SH DNAs. The hatched area on the schematic of p3H-2200 represents the restriction fragments to which transcripts were mapped in a previous study (G. W. Blissard et al., in press). Numbers below diagrams of restriction fragments indicate distances (in kilobase pairs) from the leftward terminal restriction enzyme site.

presence of the parasite egg, the developing parasite, or other components of the calyx fluid.

Major goals of this study were to identify CsV mRNAs and proteins that are expressed in parasitized *H. virescens* larvae and to map the respective CsV genes to the SH DNAs of the CsV genome. Table 1 summarizes these results. Our data showing that the two gene-specific probes were completely protected from S1 nuclease by CsV mRNAs and that each gene-specific probe hybridized to two mRNA size classes demonstrate that each gene-specific probe contains sequences which are shared on two mRNAs. Although we detected transcription from only one strand on each gene-

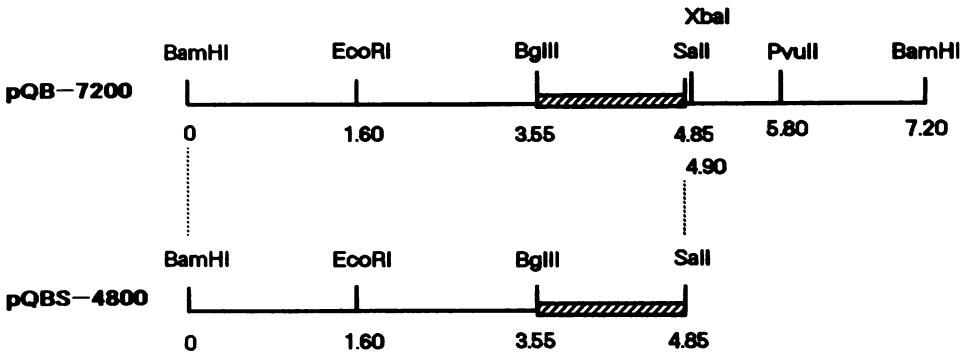

FIG. 5. Schematic showing the derivation of subclone pQBS-4800, which was used for hybridization selection. The hatched area on both fragments represents the transcribed region which was previously mapped to pQB-7200 (G. W. Blissard et al., in press). Numbers below diagrams indicate distances (in kilobase pairs) from the leftward terminal restriction enzyme site.

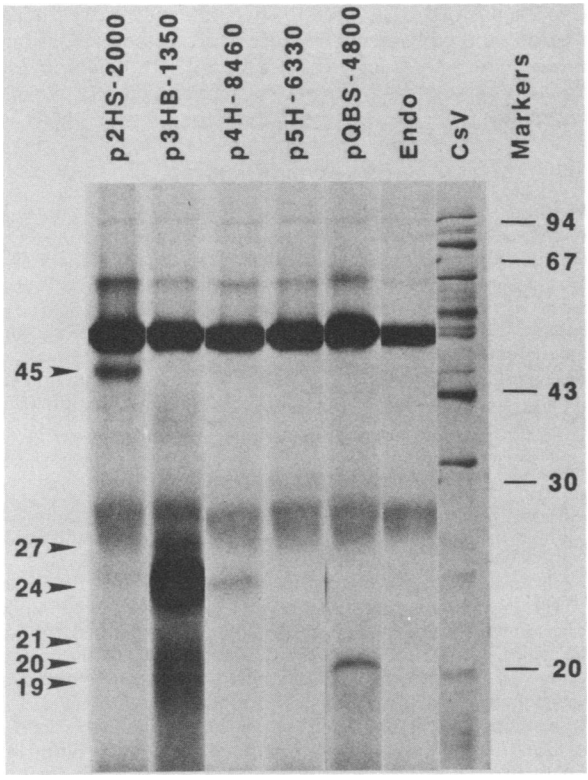

FIG. 6. In vitro translation of hybrid-selected CsV mRNAs. CsV mRNAs were individually hybrid selected from poly(A)$^+$ mRNA (48 h p.p.) with cloned CsV DNAs p2HS-2000, p3HB-1350, p4H-8460, p5H-6330, and pQBS-4800. Hybrid-selected CsV mRNAs were translated in a rabbit reticulocyte lysate cell-free translation system in the presence of [^{35}S]methionine, and the translation products were analyzed on a 12% SDS-polyacrylamide gel. Lanes are labeled with the cloned DNAs used to hybrid select viral mRNAs. The lane marked endo shows endogenous protein synthesis in the in vitro translation system (no mRNA added), and lane CsV shows CsV structural proteins stained with Coomassie blue. Lines and numbers to the right of lane CsV represent the relative migrations and molecular weights (in thousands) of marker proteins (rabbit phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor [Pharmacia, Inc.]). Numbers and arrows to the left of lane p2HS-2000 indicate the molecular weights (in thousands) of proteins translated from hybrid-selected mRNAs.

specific probe (Fig. 3 and 4), we cannot exclude the possibility that the two mRNAs detected by each gene-specific probe may be transcribed from opposite strands. To investigate the origin of the two mRNAs which hybridize to p3CB-100, we are presently mapping the ends of the gene(s) with S1 nuclease and sequencing the expressed region on p3H-2200.

To determine the location of several CsV genes on the CsV genome, we hybridized the cDNA-positive probes, two gene-specific probes, and flanking region probes to undigested CsV DNA (Table 1). Because pQB-7200 was originally cloned from SH DNA Q and the transcribed region was mapped on pQB-7200 with a cDNA probe (G. W. Blissard et al., in press), the Northern and Southern hybridizations shown in Fig. 2 indicate that the 0.9-kb mRNA maps to the 1.3-kb *Bgl*II-*Sal*I fragment of SH DNA Q (Fig. 5). We do not know whether the homology between SH DNAs Q, L[1], J, and A[2] includes the gene for the 0.9-kb mRNA (Fig. 2, pQB-7200). Each of the other cDNA-positive probes (p2H-5300, p3H-2200, p4H-8460, and p5H-6330) hybridized to five or more CsV SH DNAs (Fig. 2, Table 1). These data indicate that the genes for the respective mRNAs are located on at least one and possibly all of the CsV SH DNAs which hybridized to each probe. To define the location and distribution of CsV genes within the viral genome more precisely, we identified and cloned restriction fragments from within S1 nuclease-protected sequences (gene-specific probes) and from sequences adjacent to these transcribed regions (flanking region probes) and hybridized these cloned probes to undigested CsV DNAs. The results (Fig. 3 and 4, Table 1) show that sequences from these CsV genes are shared on a number of CsV SH DNAs and also indicate that the entire parental DNA fragments (p2H-5300 and p3H-2200) may be shared on a number of CsV SH DNAs. Both the gene-specific probe, p3CB-100, and the flanking region probe, p3HPs-400, hybridized to CsV SH DNAs W, R, and M. The flanking region probe also hybridized to two additional SH DNAs, C[2] and H (Fig. 4). The significance of this apparent additional hybridization by the flanking region probe is unknown. With the exception of CsV SH DNA H, the parental, gene-specific, and flanking region probes from p3H-2200 all hybridized to larger CsV SH DNAs with increasing intensity (Fig. 2 and 4). Because the probe DNAs in these hybridizations were in molar excess, these results indicate an increasing number of copies of sequences homologous to the probe. This could be explained by either (i) different numbers of copies of the parental p3H-2200 sequence on SH DNAs W, R, and M, with larger SH DNAs

having greater numbers of copies or (ii) higher molar quantities of the larger SH DNAs and only one copy per SH DNA. We cannot exclude either possibility. A similarly consistent but somewhat more complex pattern of hybridization intensities was observed with the probes derived from parental DNA fragment p2H-5300 (Fig. 2 and 3). We might also speculate that if some CsV genes are present in a higher total copy number, the relatively high levels of some CsV mRNAs could result from a gene dosage effect.

Recently, we identified three CsV SH DNAs which had not been previously detected on ethidium bromide-stained gels (G. W. Blissard et al., in press). In the present study, another previously unidentified CsV SH DNA (C^2, Fig. 2) was detected by hybridization to probe p3H-2200. Because this SH DNA was not detected on an ethidium bromide-stained gel yet hybridized to the p3H-2200 probe (Fig. 2), caution must be exercised when interpreting cross-hybridizations between CsV SH DNAs. Indeed, it is possible that some of the hybridizations of different DNA probes to the same CsV SH DNAs could represent the presence of two or more similarly sized comigrating but unique SH DNAs in the CsV genome. To exclude such possibilities, it will be necessary to perform analyses of cross-hybridization with cloned SH DNAs.

Preliminary S1 analyses revealed no explanation for the two CsV mRNAs which hybridized to each gene-specific probe. Besides the possibilities mentioned earlier, the observation that these genes appear to be repeated on different SH DNAs also allows the speculation that different CsV genes which share partial homology may encode the two CsV mRNAs detected by each of the gene-specific probes p2SHc-340 and p3CB-100. Other possibilities include alternative transcription initiation or termination sites and alternative RNA processing. An explanation for the origin and identity of the two overlapping mRNAs will require more detailed S1 mapping and perhaps analysis of these genes on several cloned CsV SH DNAs.

The CsV proteins produced by in vitro translation ranged in molecular weight from 19,000 to 45,000, and each was within the estimated coding capacity of the specific CsV mRNA(s) which was identified on Northern blots (Fig. 2, 3, and 4, Table 1). The 24,000-molecular-weight protein translated from p3HB-1350-selected mRNA appeared to be the most abundant CsV protein of all those detected from in vitro translations. Since the 1.6-kb mRNA is one of the more abundant mRNAs at 48 h p.p., the observed relative intensities of the mRNA and this protein correlate. The origin of the minor protein bands (molecular weights, 27,000, 21,000, 20,000, 19,000) which were observed from the translation of p3HB-1350-selected mRNA is not known but may be related to the two mRNA size classes which are homologous to p3HB-1350. The failure to detect a labeled protein from translation of p5H-6330-selected mRNA may have resulted from comigration of a CsV-specific protein with an endogenous band from the reticulocyte lysate, inefficient hybrid selection of the 2.7-kb mRNA, or a lack of methionine residues in the translated protein. Some of the proteins encoded by the hybrid-selected mRNAs from parasitized *H. virescens* larvae comigrate with viral structural proteins (Fig. 5), suggesting the possibility that these proteins may have a structural rather than a regulatory role. However, CsV is not known to replicate in *H. virescens*.

The data presented in this report confirm the hypothesis that the CsV genome is multipartite. We have shown that several CsV mRNAs examined in this study are transcribed from different SH DNAs or different sets of SH DNAs. Although some hybridization probes hybridized to numerous CsV SH DNAs, all probes did not hybridize to a single large SH DNA, indicating that no one large SH DNA contains all the CsV sequences, as would be expected if the CsV genome were composed of a series of deletions of one large master molecule. However, our detection of cross-hybridization between as many as 11 SH DNAs contradicts the findings of an earlier study (8) which suggested that the CsV genome was composed largely of unique sequences. Also, we demonstrated that two of the CsV genes appear to each be present on a number of CsV SH DNAs. The rather extensive cross-hybridization between certain groups of SH DNAs could also suggest the possibility that the viral genome is composed of families of SH DNAs with several master SH DNAs and their subfamilies. Alternatively, larger SH DNAs may be tandem duplications of smaller SH DNAs. Cloning and physical mapping of the individual CsV SH DNAs will be necessary to clarify the relationships between the CsV SH DNAs which share homologous sequences. Because the products of CsV expression may play an important role in the parasite-host relationship, we are currently focusing attention on the physical structures and genomic locations of the CsV genes that are expressed early after parasitization and at relatively high levels in parasitized *H. virescens* larvae.

ACKNOWLEDGMENTS

We thank J. G. W. Fleming for providing clone pQBS-4800 and for assistance in the mapping with this clone. We are grateful to David Theilmann for valuable discussions during the course of this work and critical review of the manuscript. We thank Gale Smith, Linda Guarino, Brad Ericson, and John Brusca for helpful technical advice. We thank Diane Giusti, Rafael Avila-Jimenez, and Kimberly Russel for maintaining insect colonies.

This work was supported by National Science Foundation grant PCM8021992 and Texas Agricultural Experiment Station Project 6316.

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