

Related RNAs in Lepidopteran Cells after *in Vitro* Infection with *Hyposoter didymator* Virus Define a New Polydnavirus Gene Family

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Received March 4, 1999; returned to author for revision March 29, 1999; accepted July 27, 1999

In the present study, we describe the isolation and the characterization of three different *Hyposoter didymator* virus (HdV) lepidopteran host-expressed genes, the products of which might interfere with the host physiology during parasitism. In this report, we study the expression of HdV genes in Sf9 cells infected with HdV since results indicate that the Sf9 model mimics to some extent the *in vivo* model and may be utilized to study expression of HdV genes in lepidopteran host cells. This system allowed us to isolate three HdV-specific cDNAs, termed M24, M27, and M40. cDNA nucleotide sequence analysis demonstrated significant regions of homology. The three cDNAs displayed repeated sequences arranged in tandem array that might have evolved through domain duplication. Similar to other previously described polydnavirus host-expressed genes, two intron positions have been found in the M24 leader region. The cDNAs corresponded to RNAs of 1.5, 1.6, and 2.3 kb that are also detected in parasitized *Spodoptera littoralis* larvae. They are encoded by different genes likely located on different HdV DNA molecules. Corresponding RNAs are detected early postinfection and remain detectable for at least 10 days postinfection. They encode secreted glycine- and proline-rich proteins. An antiserum raised against a baculovirus recombinant M24-encoded protein detected similar proteins in the culture medium of infected lepidopteran cells and in parasitized host hemolymph. We propose that the three cloned genes belong to an HdV gene family specifically expressed in parasitized lepidopteran hosts. © 1999 Academic Press

INTRODUCTION

Polydnaviruses (PDVs) are unusual viruses observed in some hymenopteran parasitoids from the ichneumonid and braconid families. Up to now, PDVs have been described only from parasitic wasps whose preimaginal development occurs inside lepidopteran hosts. PDVs are characterized by polydisperse, double-stranded, superhelical circular DNA genomes (Stoltz *et al.*, 1995) with fewer than 10 and up to more than 25 molecules (Fleming, 1992). PDV DNA is integrated into the wasp genome and PDVs are transmitted vertically as proviruses (Fleming and Summers, 1991; Gruber *et al.*, 1996; Savary *et al.*, 1997; Xu and Stoltz, 1991). After replication in the reproductive tract of infected female wasps, PDVs are inoculated into lepidopteran host larva during oviposition along with the proteins produced in the calyx. Once inside the lepidopteran host larva, the viruses do not replicate but specific viral genes are expressed (Summers and Dib-Hajj, 1995).

The PDV genome has been shown to be functionally multipartite (Theilmann and Summers, 1987,1988). Some viral genes are transcribed solely in the hymenopteran

parasitoid or in the lepidopteran host, whereas others are transcribed in both (Webb, 1998). Genes expressed specifically in the lepidopteran host, or “host-expressed genes,” have been described from several PDVs associated with braconids (Asgari *et al.*, 1996; Harwood and Beckage, 1994; Hayakawa *et al.*, 1994; Strand *et al.*, 1997) and also from the virus associated with the ichneumonid *Campoletis sonorensis* (Blissard *et al.*, 1987,1989; Theilmann and Summers, 1988; Dib-Hajj *et al.*, 1993; Cui and Webb, 1996).

PDVs associated with parasitic wasps are known to play an essential role in the success of the endoparasitic part of the parasitoid life cycle in their lepidopteran hosts. Indeed, parasitic wasps evade some immune responses of the host insect larvae through expression of PDV genes (Asgari *et al.*, 1996,1997; Harwood and Beckage, 1994; Lavine and Beckage, 1995; Shelby *et al.*, 1998; Strand *et al.*, 1997; Yamanaka *et al.*, 1996). In addition, expression of PDV genes causes some cellular and physiological effects in the parasitized host, among them hormonal and development changes (Beckage *et al.*, 1994; Lawrence and Lanzrein, 1993; Tanaka *et al.*, 1994). Regarding infection, several tissues of the lepidopteran host are known to be infected. So far, infection has been described in hemocytes (Beckage, 1998; Strand, 1994; Strand and Pech, 1995) and few reports concern other tissues like the fat body (Shelby and Webb, 1994,1997; Strand *et al.*, 1992). Two modes of action

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have been suggested for PDVs: PDV-associated proteins interact directly with host cells and/or virus DNA transcribed within the host cell exerts cellular changes through viral gene expression (Asgari *et al.*, 1996; Webb, 1998). The first case is illustrated by the proteins encoded by the Cys-motif genes of the *C. sonorensis* PDV. Viral proteins bind to and enter host hemocytes, presumably by receptor-mediated endocytosis. This results in disruption of the cytoskeleton, altering the adhesive properties of the hemocytes and thereby inhibiting encapsulation of the wasp egg (Cui *et al.*, 1997; Li and Webb, 1994; Webb, 1998).

Other than these few reports, little is known about the function of viral gene products within the parasitized host. Further analysis of viral gene expression is necessary to elucidate the mechanisms by which PDV genes alter lepidopteran host physiology.

In the present study, we describe the isolation and characterization of three different *Hyposoter didymator* virus (HdV) host-expressed genes the products of which might interfere with the host physiology. This PDV is carried by the ichneumonid *H. didymator*, a polyphagous parasitoid able to develop in several noctuids, particularly in species of *Spodoptera* (Caballero *et al.*, 1990; Harrington *et al.*, 1993; Tillman and Powell, 1991). We show in this work that Sf9 (*Spodoptera frugiperda*) cell line is suitable for study of HdV expression in lepidopteran cells, since HdV expression in Sf9 cells was comparable to HdV expression in the larva's hemocytes. Three viral cDNAs generated from HdV-infected Sf9 cells were cloned. Their corresponding RNAs were detected early postinfection and for at least 10 days postinfection. The cDNA nucleotide sequence analysis demonstrated that the RNAs have significant regions of homology. They are encoded by different genes likely located on different HdV DNA molecules. They encode secreted glycine- and proline-rich proteins presenting repeated sequences arranged in a tandem array. We propose that the three cloned genes belong to an HdV gene family specifically expressed in parasitized lepidopteran hosts.

RESULTS

HdV expression in lepidopteran cells: are Sf9 cells suitable for studying viral expression in lepidopteran host?

The same proteins were detected whether cell lines or hemocytes were infected with HdV. In lepidopteran cell lines infected with HdV, [³⁵S]methionine incorporation revealed four proteins of approximately 40, 42, 50, and 70 kDa synthesized *de novo* in the culture medium (Fig. 1). They were detected 6 h postinfection (p.i.) and continued to be secreted for at least 1 week p.i. (data not shown). These four secreted proteins were observed in the medium after infection of all the cell types tested, freshly collected *S. littoralis* hemocytes (Fig. 1, lane 2), the newly established *S. littoralis* cells derived from hemocytes

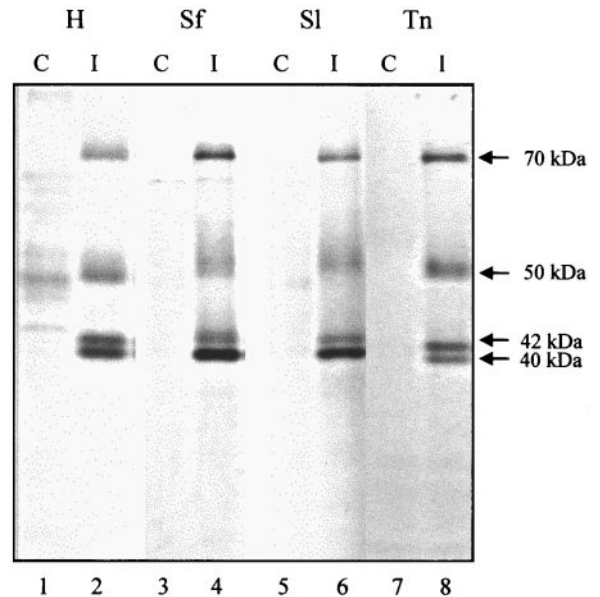


FIG. 1. Proteins secreted by HdV-infected lepidopteran cells. Proteins were detected by autoradiography after [³⁵S]methionine labeling (4-h pulse) followed by 12.5% SDS-PAGE. 30 μ l of culture medium was loaded per lane. Four cell types were analyzed: *Spodoptera littoralis* hemocytes (lanes 1 and 2 [H]), Sf9 cell line (lanes 3 and 4 [Sf]), *S. littoralis* cell line (lanes 5 and 6 [Sl]), and *Trichoplusia ni* Hi5 cell line (lanes 7 and 8 [Tn]). Lanes 1, 3, 5, and 7, uninfected cells (C); lanes 2, 4, 6, and 8, HdV-infected cells, 24 h postinfection (I). Molecular masses in kDa are given for the four proteins detected.

(Fig. 1, lane 6), Sf9 cells (Fig. 1, lane 4), and Hi5 cells (Fig. 1, lane 8), but not in the medium of uninfected cells (Fig. 1, lanes 1, 3, 5, and 7). Furthermore, Sf9 cells infected with HdV that had been previously UV-irradiated did not produce any of the proteins (data not shown), thus suggesting they might be encoded by the viral genome. The intracellular protein patterns contained a high number of cellular proteins, and no significant difference was observed between infected and uninfected cells whether [³⁵S]methionine labeling or Coomassie blue staining was used (data not shown).

The main HdV RNA class size detected in HdV-infected Sf9 cells was also detected in parasitized S. littoralis larvae. To analyze HdV-specific RNAs, Northern blot studies were conducted with RNAs isolated from HdV-infected Sf9 cells and parasitized larvae (Fig. 2). ³²P-labeled total genomic HdV DNA used as a probe hybridized with at least seven RNA class sizes isolated from Sf9 cells infected with HdV. The RNA class sizes ranged from 0.1 to 5.0 kb, the most abundant corresponding to the 1.5- to 1.8-kb band (Fig. 2, lane 1). This band was detected as early as 2 h p.i., thus suggesting that some viral RNAs were transcribed early in infection, and it was observed up to 1 month p.i. (data not shown). The 1.5- to 1.8-kb RNA band was the only band easily observed in samples isolated from parasitized larvae (Fig. 2, lane 2). However, the intensity of the hybridization signal was weak with crude extracts from parasitized larvae sam-

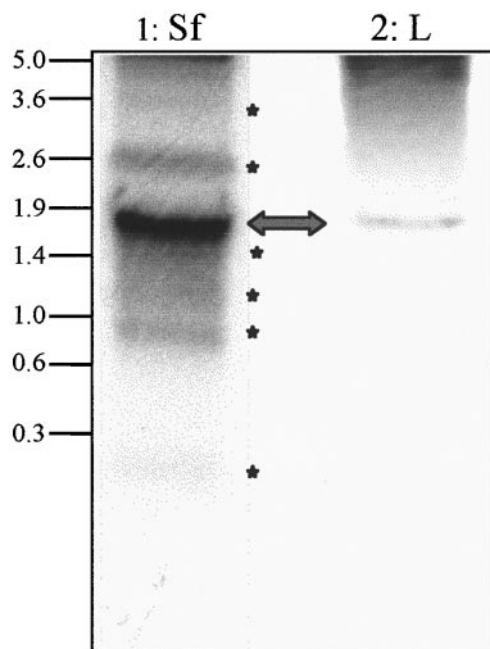


FIG. 2. Northern blot analysis of HdV RNAs. HdV-specific RNAs were detected with ^{32}P -labeled *Bam*HI–*Hind*III-digested genomic viral DNA in total RNA extracted from Sf9 cells 44 h postinfection (lane 1 [Sf]) and in *S. littoralis* second-instar larvae, 20 h postparasitism (lane 2 [L]). For infected Sf9 cells, detected RNA class sizes are indicated by asterisks or the arrow for the major one. About 20 μg total RNA was loaded per lane, exposure was for 24 h. Molecular size markers (kb) are on the left.

ples compared to that of Sf9 samples. This could probably account for the absence of the other RNA species in the larvae samples. In naturally parasitized *S. littoralis*, HdV RNAs were detected from 4 h postparasitism (p.p.) to the end of parasitoid development, i.e., 9–10 days p.p. (data not shown). No hybridization was observed with RNA isolated from nonparasitized larvae or uninfected Sf9 cells (data not shown).

Isolation of HdV genes expressed in lepidopteran cells: generation of virus-specific cDNAs from infected Sf9 cells

Based on the previous results, Sf9 cells were infected for 24 h with HdV to generate a cDNA library. From 720 analyzed clones, 49 were HdV-specific cDNAs, with sizes ranging from 0.8 to 2.5 kb. Thirteen of them were randomly chosen to probe all the HdV-specific cDNAs. This allowed a classification into eight different groups, each representing one or more cross-hybridizing HdV-specific cDNAs (Table 1). Total RNAs from HdV-infected Sf9 cells (24 h p.i.) were then probed with the longest *Eco*RI-digested fragment of each group. Northern blot analysis showed that each probe hybridized with different RNAs, ranging from 0.8 to more than 3.0 kb (Table 1). In this study, we focused on the group that we referred to as the M group (with 26 cDNA clones; Table 1), which contained 1.5- and 2.3-kb RNAs that hybridized intensely with its specific probe.

The M-group cDNAs were sequenced on both their 5' and their 3' ends. Their alignment (Fragment Assembly; Genetics Computer Group) revealed that most of these cDNAs overlapped with three distinct sequences of 1481, 1636, and 2327 bp, respectively termed M24, M27, and M40.

Characterization of three putative HdV genes expressed in lepidopteran cells: nucleotide and peptide sequence analysis indicated high similarity between the M-group cDNAs M24, M27, and M40 (Fig. 3).

Homologies in untranslated regions. In the 5' untranslated region (UTR), M40 was almost identical to M27 (99% identity, compared to 86% with M24); conversely, in the 3'UTR, M40 was more related to M24 (98% identity, compared to 79% with M27) (Fig. 3A).

TABLE 1

Number and Size of the RNAs Detected by Hybridization with a Specific Probe from Each Characterized HdV cDNA Group

Group	Number of clones	cDNA probe size (<i>Eco</i> RI fragment)	Number of RNAs detected	RNAs with strong hybridization signal
S	2	S6: 0.8 kb	3	1.0 kb
D	5	D8: 0.9 kb	3	0.8 kb 3.0 kb
C	1	C28: 1.6 kb	3	1.0 kb 1.8 kb
K	5	K29: 1.7 kb	1	2.0 kb
P	6	P30: 1.8 kb	2	1.8 kb 3.6 kb
B	2	B36: 1.3 kb	2	1.0 kb 2.0 kb
X	1	X41: 1.1 kb	2	1.5 kb
M	26	M24: 1.5 kb	4	1.5 kb 2.3 kb

A

5'UTR and N-terminal coding region

M24	gccccgcaggaacgcaggcgtcaaacacctaaccacttgg-acacaaagcttttgaattcctctaata-----atactctgtcagaaa	79
M40	-----c.....a.....t.....c.....g.....c.....ataagta.....	85
M27	-----t.....a.....t.....c.....	68
M24	ataccgctacacaatg aag att ctg ctg ccc ttg atg gtc gca gtg aca ctg gtc cag gca agg cca gga	148
P40	M K I L L P L M V A V T L V Q A R P G	19
M40g.....t..... att c.t tg. .tg .gg .a. --- ---	147
P69	M I . W K	17
M27	-.g.....t..... at. c.t tg. .tg .g .ag --- ---	130
P45	M I . W	17
M24	gga gga gga gaa gaa gcc gta aag gac gct gag ttc gga ttc gca tgg aac gat cca gtt ctt ctg	217
P40	G G G E E A V K D A E F G F A W N D P V L L	41
M40	--- --- --- --- ac. .ta aac g.a agg cag a.a .t ttt ga. tgg .t. g..	201
P69	T V N E R Q K F D W L D	35
M27	--- --- --- --- ac. .ta agc g.a .t. cag a.a .at ttg gaa tgg .t.a	184
P45	T V S E V Q K Y L E W L . E	35
M24	ccg ctc gga cct aca gtc cca gcg gac gac cgt aag ccc gat tca gga ccg atg gcc ccc gga ttt	280
P40	P L G P T V P A D D R K P D S G P M A P G F	63
M40a. .	267
P69	L E .	57
M27c. .a.gt .	250
P45	P E G .	57
M24	gtc gag cca gaa tcg aga gtc gcc gtc gcc gcc tct agt gca gct cca gtt gga tgg aaa gac aat	346
P40	V E P E S R V A V A G S S A A P V G W K D N	85
M40	. .	333
P69	. .	79
M27g.g. .g. .a .	316
P45	G G G .	79
M24	gaa atg ggt gag gga agc gct act cca ccg gat gac aga aag acc ggt cca ttg atg gcc tct	412..1087
P40	E M G E G S A T P P D D R K T G P L M A S	107..332
M40	. .	399..1950
P69	. .	101..618
M27	.g. .g.g. .	382..1240
P45	G R A .	101..387

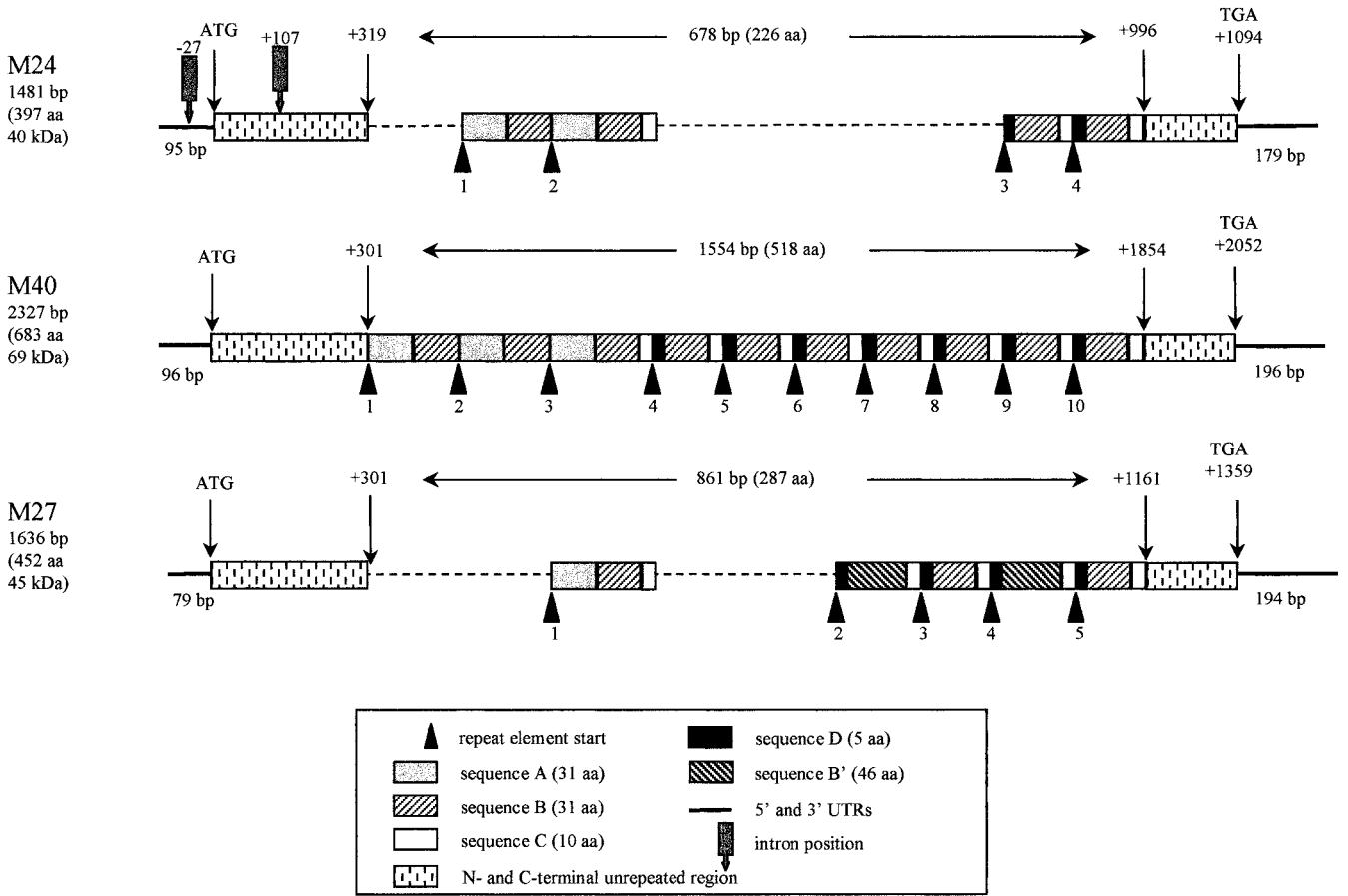
C-terminal coding region and 3'UTR

M24	ggc ggt gta gca gga ccc ccc gaa aaa ccg tgt gtg ttg gta ttg aaa cca gta tct gga aaa aga	1153
P40	G G V A G P P E K P C V L V L K P V S G K R	354
M40	. .	2016
P69	G .	640
M27aagg .	1306
P45	K .	409
M24	aac cta gca gag ttg gga gca aat ttg att ctg gag gac gtc ata atc gaa gta aaa att aaa caa	1219
P40	N L A E L G A N L I L E D V I I E V K I K Q	376
M40	. .	2082
P69	. .	662
M27gc.aa. .a.t.t.aca. .g.g.a..	1372
P45	. A K E I Q G M K	431
M24	gcg aag att tta ctg ggt act gga aat ggt gct atc acc atg gca gga atc gat gtg tct tcc tga	1285
P40	A K I L L G T G N G A I T M A G I D V S S *	397
M40	. .	2148
P69	. .	683
M27c. .gc.t.ccct.c.a. .g. .t. .g. .c. .gt. .	1438
P45	. L A S P S F I E M V G P V *	452
M24	aagttatgactttcttctgagggaaacacacattttcttagacttaaggattgttctaacattcgttttaccataaattgtatcttg	1372
M40	. .	2235
M27a.a.c.a.t.c.t.t.t.t.	1525
M24	ctttgtatctgcctcactaactattcttctgtatgattttttttataaatgatttctttttggaatttttttcaacttttcaaaata	1459
M40	. .	2321
M27c.c.tg.c.atgaaa.t.ttc.tgc.t.gtaa.c.g. . . .	1612
M24	aaaattataacataataaaaaa	1481
M40	-----	2327
M27t.a.	1633

B

	D20 genomic clone	gen	aagcttttgaattcctctaata gtaagta ttccggtgtcaggattctc	176
	M24 cDNA clone	M24	AAGCTTTTGAATTCCTCTAAT-----	64
gen	tgaagattctcacttcagacctgctctgatggttctactcaagaatgtagtagctgtattcttactaaaaatgtattttctatttctg			267
M24	-----		----->	
gen	ttctattacagatactctgtcagaaaaatccgtaacaca atg agattctgtgcctctgatggctgcagtgacactcgtgcaggcaagg			356
M24	-----			
M24	-----	ATACTCTGTCAGAAAATACCGTACACA ATG AAGATTCTGCTGCCCTTGATGGTCGCAGTGACACTCGTGAGGCAAGG		142
			-----<	
gen	ccaggaggaggaggagaagaagccgtaaaaggacgctgagttcggatccgatggaacgtgagtacatcattccgaactctttatacatgt			445
M24	-----			
M24	CCAGGAGGAGGAGGAGAAGCCGTA AAGGACGCTGAGTTCGGATTCCGATGGAAAC-----			199

C



D

A. SEQUENCE		B. SEQUENCE		C. SEQUENCE		D. SEQUENCE	
<p>P40 A1 GSVVPRERRDITFARMDEVVFVRPDDDGFWAYIRG</p> <p>A2 GSVVPRERRENFARMDGVFVRPDDDGFWAYIRG</p>		<p>AB B1 ERFVVMPAAGAV</p> <p>B2 GRFVVMPAAGAV</p>		<p>** * * * *</p> <p>SPRSGGGASMNPGAGSV</p> <p>PPRLGGGDSLGPTARV</p>		<p>**** *</p> <p>C2 QLEDGSWAYA</p>	
		<p>DBC B3 QETVVMPAAPTV</p> <p>B4 QETVVAMPFPEA</p>		<p>**** * * *</p> <p>SPRLGGGDSMPGTGRV</p> <p>SPRLGGGDSMPGTGRV</p>		<p>C3 QLEDGSWAYA</p> <p>C4 RLEDGFWAYG</p>	
		<p>**** * * *</p> <p>B5 QETVVMPAAGAV</p> <p>B6 QETVVMPAAPTV</p> <p>B7 QETVVMPAAPTV</p> <p>B8 QETVVMPAAPTV</p> <p>B9 QETVVMPAAPTV</p> <p>B10 QETVVAMPFPEA</p>		<p>**** * * *</p> <p>FPKLGGGDSMPVTIGID</p> <p>SPRLGGGDSMPGTGRV</p> <p>SKLGGGDSMPVTALID</p> <p>SPRLGGGDSMPGTGRV</p> <p>SKLGGGDSMPVTALID</p> <p>SPRLGGGDSMPGTGRV</p> <p>SPRLGGGDSMPGTGRV</p>		<p>C4 ELEDGSWAYA</p> <p>C5 QLEDGSWAYA</p> <p>C6 QLEDGSWAYA</p> <p>C7 QLEDGSWAYA</p> <p>C8 QLEDGSWAYA</p> <p>C9 QLEDGSWAYA</p> <p>C10 RLEDGFWAYG</p>	
		<p>**** * * *</p> <p>B1 AGFVVAMPPTATT</p> <p>B3 AGFVVAMPPTATT</p> <p>B5 AGFVVAMPPTVTA</p>		<p>** * * *</p> <p>AQRSEGVVAMGGETGPV</p> <p>APRSEGVVLGETGPV</p> <p>LPRSGGVVRMDGTDPV</p>		<p>**** *</p> <p>C1 ESEDGSWAYA</p> <p>C3 VSEVGSNAYV</p> <p>C5 GSEDGSWACG</p>	
<p>P45 A1 GSVVPRERDTVASIFGGADPVTSGDNSWYRTV</p>		<p>**** * * *</p> <p>B'2 QGEVVARAPTGGF</p> <p>B'4 QGEVVARAPTVGA</p>		<p>**** * * *</p> <p>AARWGDAMPPAPTA</p> <p>TPRWGDTTAPAPTA</p>		<p>**** *</p> <p>C2 GSEDNSFRAYD</p> <p>C4 GSEDNSFRAND</p>	
		<p>**** * * *</p> <p>D3 IDGKI</p> <p>D5 IDGKI</p> <p>D6 IDGKI</p> <p>D7 IDGKI</p> <p>D8 IDGKI</p> <p>D9 IDGKI</p> <p>D10 IDGKI</p>		<p>**** *</p> <p>D3 IDGKI</p> <p>D5 IDGKI</p>			

FIG. 3. M-group nucleotide and peptide sequence analysis. (A) Alignment of the nucleotide and predicted peptide sequences of the cDNAs M24, M40, and M27 for the 5'UTR, N-terminal and C-terminal coding regions, and 3'UTR. Dots indicate identical nucleotides and gaps (–) are introduced to optimize alignment. (B) Positions and sequences of the two introns observed when 5' *Hind*III-digested ends of M24 and D20 are aligned. Selected restriction site *Hind*III is in italics and start codon ATG in bold. The region homologous to *C. sonorensis* cysteine-motif genes is bounded by arrows, and the intron region homologous to M40 is boxed (see text). (C) Schematic representation and alignment of the M-group cDNAs M24, M40, and M27, showing their respective regions of repeated elements, A, B, B', C, and D. The A of the first ATG is considered +1. (D) Alignments of the repeated sequences A, B and B', C, and D found in P40 (M24-encoded), P69 (M40-encoded), and P45 (M27-encoded). B sequences from (AB) and (DBC) repeats are analyzed separately. Numeration of repeated sequences indicates the number of the repeat (for example, P40 is A₁B₁A₂B₂C₂D₃B₃C₃D₄B₄C₄, see C). Asterisks above a group of sequences indicate identical amino acids. Conserved amino acids for the three proteins are in bold.

In the 3'UTR, a putative polyadenylation signal was identified downstream of the TGA stop codon (170 bp for M24 and M27; 169 bp for M40).

Presence of two introns in the M24-related gene. Comparison of M24 cDNA with the *HindIII*–*XbaI* fragment generated from the D20 genomic clone, which specifically hybridized with M24, indicated the presence of two introns. These introns were localized 27 bp upstream and 107 bp downstream of the ATG and were 124 and 196 bp long, respectively (Fig. 3B). Flanking exon sequences were in agreement with eukaryotic consensus splicing sites.

The open reading frames displayed repeated elements arranged in tandem array. Each of the three cDNAs encoded a single open reading frame (ORF). The sequences preceding the first ATG were in agreement with the Kozak consensus sequence. The M24, M27, and M40 ORFs were 1094, 1359, and 2052 bp, respectively (Fig. 3C). Corresponding proteins, named P40, P69, and P45, had a predicted molecular mass of 40, 69, and 45 kDa with 397, 452, and 683 amino acids, respectively.

The three ORFs contained a region of imperfectly repeated elements arranged in tandem array and flanked by nonrepeated regions (Fig. 3C). The coding sequences of these ORFs could be divided into N-terminal nonrepeated, "repeat elements," and C-terminal nonrepeated regions (Fig. 3A). In the N-terminal nonrepeated region, peptide sequences were 84% identical between P40 and P69, 46% between P40 and P45, and 81% between P69 and P45. In the C-terminal nonrepeated region, sequences were 95% identical between P40 and P69, 54% between P40 and P45, and 56% between P69 and P45.

Within the repeat-element region, the elements were successively named A, B or B', C, and D in the order they appeared in the sequence. The repeated region began at amino acid 107 in P40 and was 226 amino acids long. In P45 and P69, the repeated regions began at amino acid 101 and were 287 and 518 amino acids long, respectively. A, B, C, and D sequences were present in all three ORFs, while B' sequence, related to B sequence, was specific to P45 ORF (Figs. 3C and 3D). A, B, B', C, and D sequences were 31, 31, 46, 10, and 5 amino acids long, respectively. A, C, and D amino acid sequences were highly conserved within a same protein, and between the proteins, particularly between P40 and P69 (Fig. 3D). The B element was more variable and, regarding P40 and P69, could be differentiated whether it was part of an (AB) repeat or a (DBC) repeat (Fig. 3D). The P40 repeat region was symbolized by (AB)₂-C-(DBC)₂, corresponding to two (AB) repeats, followed by one C repeat and two (DBC) repeats. In the same way, the P69 repeat region was symbolized by (AB)₃-C-(DBC)₇, and that of P45 by AB-C-(DB'C-DBC)₂. Therefore, the main difference between P40 and P69 was found in the length of their repeat-element regions, while P45 presented less identity with the other two.

Putative genes related to M-group cDNAs encode related glycine- and proline-rich HdV proteins. All the predicted proteins P40, P69, and P45 started with a putative signal peptide of 16 amino acids (PSORT analysis; Nakai, 1991). A hydrophobic domain was also present at the C-termini of the proteins (HCA analysis; Caillebaut *et al.*, 1997). These three proteins were glycine (15–16%) and proline (13–14%) rich, particularly in the repeat-element region. No homology was found with any known potential N-glycosylation consensus site. However, the presence of multiple serine and threonine residues might predict some O-glycosylation sites. Prediction of subcellular localization of proteins by PSORT analysis (Nakai, 1991) indicated that these proteins have 67% probability of being extracellular. Therefore, the three related glycine- and proline-rich HdV proteins are probably secreted by the host cells.

Comparisons with sequences in GenBank/EMBL and SwissProt databases revealed no significant relationship with previously described polydnavirus gene products or with other proteins. Searches in the available databases did not show significant overall similarity between the repeated sequences A, B, C, and D and other proteins.

Localization of M24-, M27-, and M40-related genes on the HdV genome

The HdV genome consists of at least 20 DNA molecules ranging from 2 to over 20 kb in size (Fig. 4, lane 1; Volkoff *et al.*, 1995). However, in electrophoretically resolved undigested DNA, several bands may represent different DNAs or conformational differences in the same DNA. Moreover, the pattern may vary from one DNA preparation to the other. Therefore, Southern blot results from undigested genomic HdV DNA should be analyzed with care. To localize M24-, M27-, and M40-related genes on the HdV genome, specific 45-mer oligonucleotides were used: one specific for the M24 cDNA (corresponding to nucleotides 149 to 193 in the M24 N-terminal coding region) and one specific for the M27 cDNA (corresponding to nucleotides 653 to 697 in the M27 B' sequence). Due to the homologies between the sequences, no M40-specific probe could be designed.

M24-specific probe hybridized with the same intensity to two bands, a and g, from undigested HdV DNA (Fig. 4, lane 2), while M24 cDNA hybridized with six bands, namely a, b, c, g, h, and i. M24 also hybridized intensely with a 4.7-kb *Bam*HI–*Hind*III fragment of HdV DNA (Fig. 4, lane 4). The *Bam*HI restriction fragment of the D20 genomic clone (aligning with M24) was about 5.2 kb and presented a *Hind*III site. The D20 *Bam*HI–*Hind*III fragment was 4.7 kb size and matched with the 4.7-kb *Bam*HI–*Hind*III fragment of HdV DNA observed in the Southern blot. Taken together, this indicated that the D20 *Bam*HI restriction fragment might represent an almost complete HdV molecule of about 5.2 kb containing the

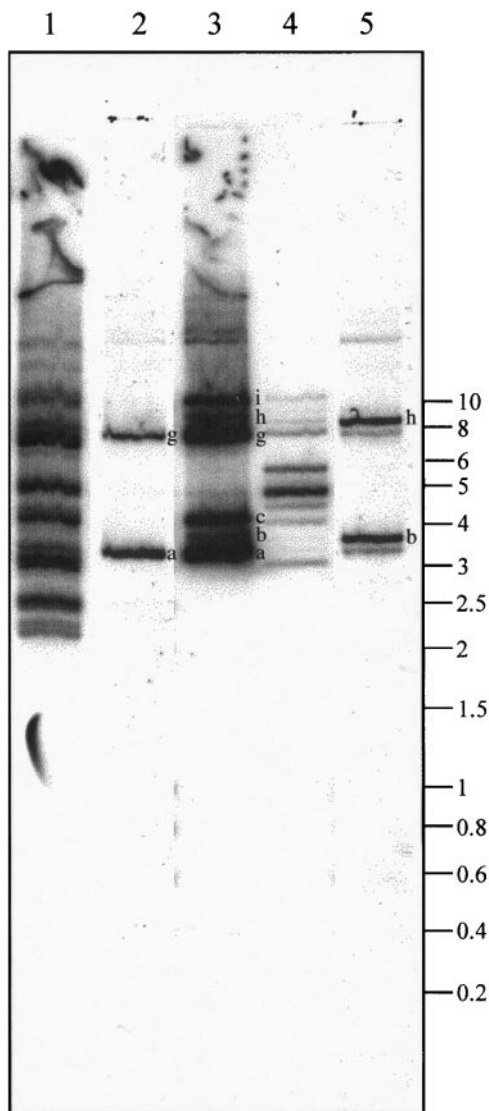


FIG. 4. Hybridization pattern of genomic HdV DNA with the M-group cDNAs. Lane 1 shows the pattern of total genomic HdV DNA, observed after hybridization of *Bam*HI-*Hind*III-digested ³²P-labeled viral DNA with undigested viral DNA (2 μg per lane, 10 h exposure). Lanes 2 and 5 represent hybridization of M24- and M27-specific probes, respectively, with undigested viral DNA (1 μg per lane, 48 h exposure). Lanes 3 and 4 represent hybridization of *Eco*RI-digested ³²P-labeled M24 with undigested viral DNA and *Bam*HI-*Hind*III-digested viral DNA, respectively (1 μg per lane, 48 h exposure). DNA molecular size markers (kb) are indicated at the right. Names of detectable bands (a, b, ...) are indicated on their right.

M24-related gene. These results showed that the M24-related gene was more likely present in one HdV molecule, bands a and g representing different conformational forms of the same molecule. On the other hand, the M27-specific probe hybridized to the bands termed b and h (Fig. 4, lane 5). Observation of a slight hybridization with bands a and g might be explained by the slight similarity existing between the B' sequence (corresponding to the M27-specific probe) and the M24 B sequence. These results indicated that the genes encoding each of

these two cDNAs were on two different HdV molecules. As with M40, this probe was the only one to hybridize significantly with bands c and i (data not shown), suggesting that the M40-related gene was localized on a third molecule.

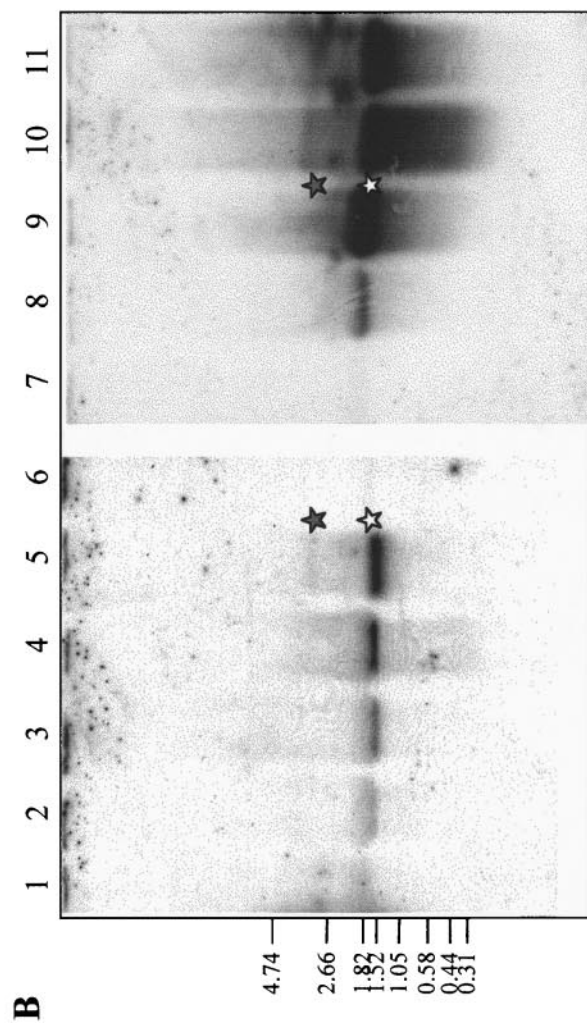
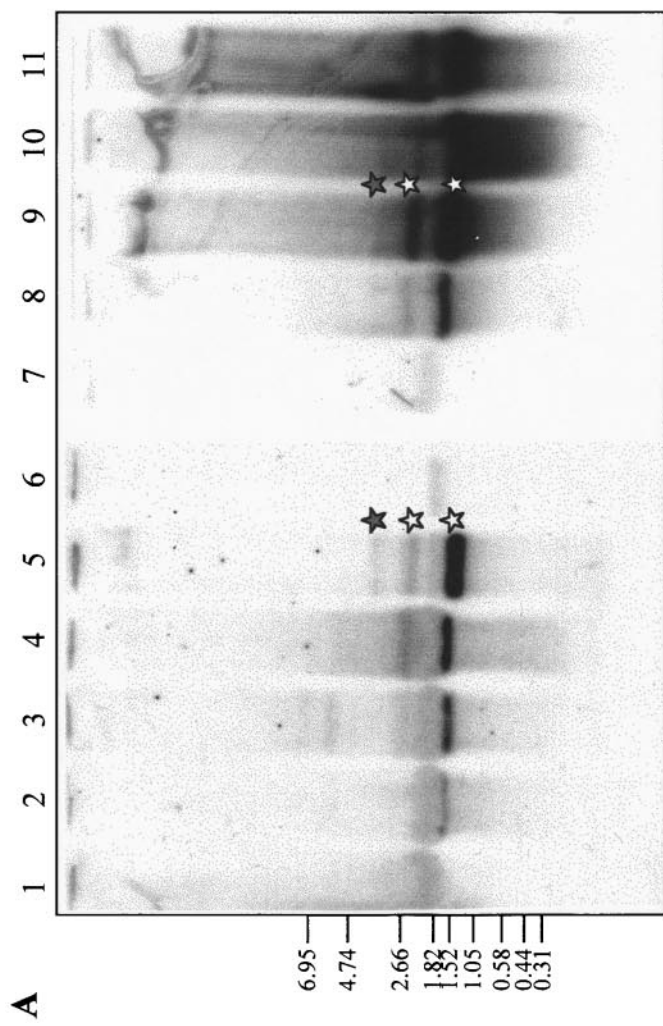
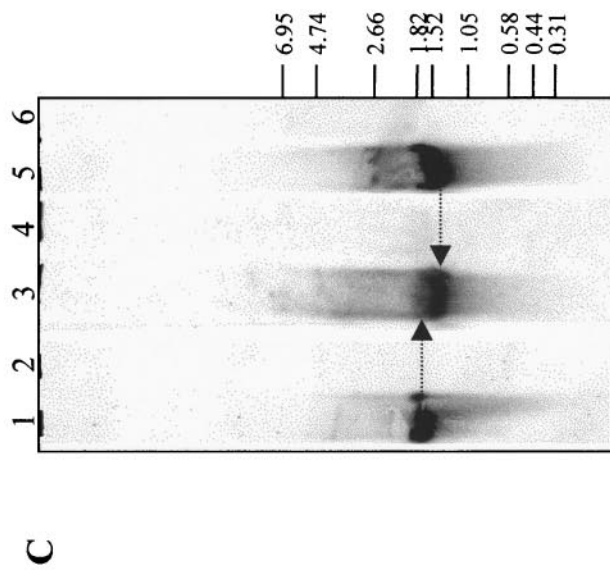
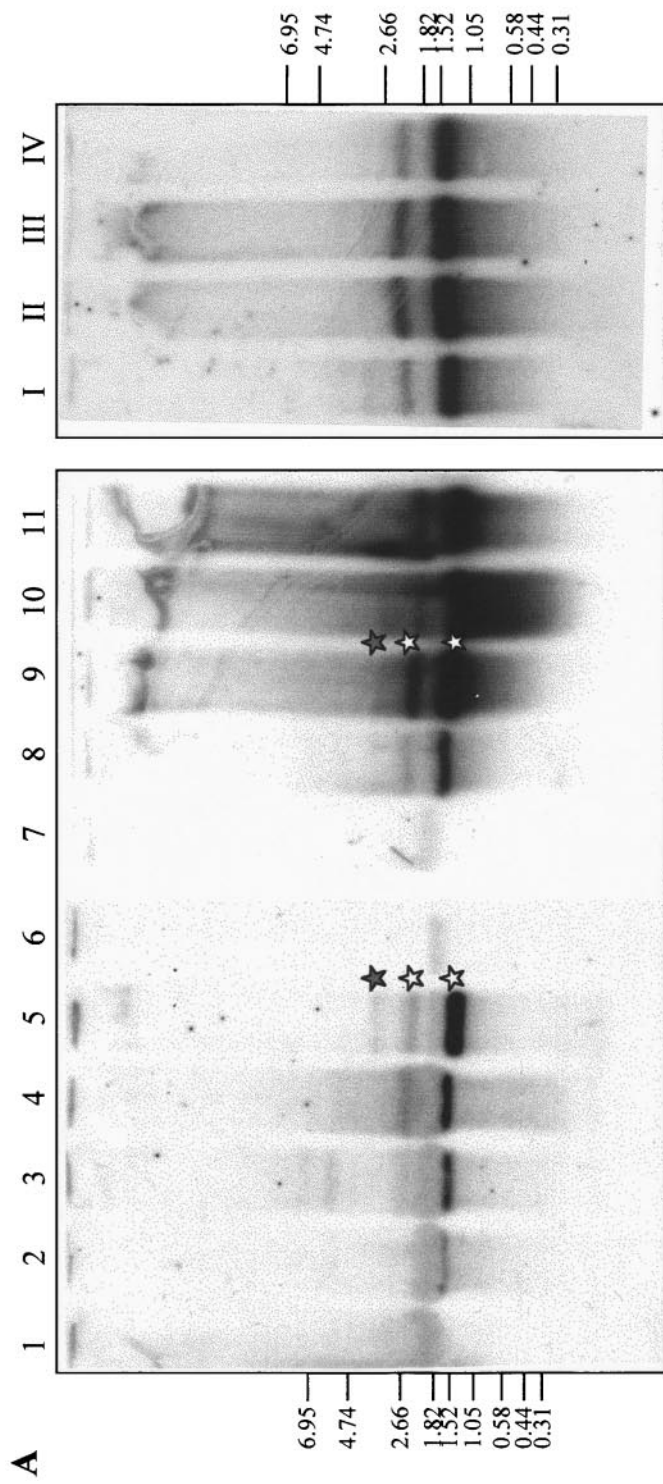
Synthesis of M24-, M27-, and M40-specific mRNAs during HdV infection of Sf9 cells and during parasitism

Northern blot analysis showed that the M-group cDNAs hybridized to two or three RNAs isolated from HdV-infected Sf9 cells and from parasitized larvae (Fig. 5), whereas no signal was detected in uninfected cells (Figs. 5A and 5B, lane 7) and in nonparasitized larvae (Figs. 5A and 5B, lanes 1 and 6). In infected Sf9 cells, in parasitized larvae crude extracts, and in parasitized larvae hemocytes, M24 hybridized essentially with RNAs of 1.5 and 2.3 kb (Figs. 5A and 3C, lane 5, for M24) while M27 hybridized with a 1.6-kb RNA (Figs. 5B, lanes 7 to 11, and 3C, lane 1). This result was confirmed when highly specific oligonucleotide probes were used (Fig. 5C, lane 5). All cDNAs also slightly hybridized to an approximately 3.0-kb RNA. For M40, results similar to those of M24 were obtained (data not shown), as was expected considering their similarity. Based on the respective sizes of M24 and M40, the 2.3-kb RNA observed on Northern blots might correspond to M40 while the 1.5-kb RNA might correspond to M24.

In HdV-infected Sf9 cells, M-group-specific RNAs were detected from 2 h p.i. (Figs. 5A and 5B, lanes 8) up to 10 days (Fig. 5A, lane IV). The present study indicated that high levels of expression of both transcripts occurred 24 h p.i. in Sf9 cells infected with HdV. No significant decline in their transcription was detectable for at least the first 10 days of infection. In parasitized larvae, transcripts were present 2 h p.p. and still detected 5 days p.p. (Figs. 5A and 5B, lanes 8 to 11). RNAs were detected until the end of the parasitoid development (data not shown).

Synthesis of P40 protein in HdV-infected Sf9 cells and in parasitized larvae

An antibody against HdV M24 cDNA-related protein was produced after expression of a histidine-tagged M24 recombinant protein (M24-His) in the baculovirus insect cell system. Protein samples prepared from Sf9 and SII cells or from *S. littoralis* larvae hemolymph were analyzed by Western blot using a polyclonal antiserum raised against this M24-His recombinant protein (Fig. 6). As predicted from sequence analysis, P40 was efficiently secreted in HdV-infected Sf9 cells (Fig. 6A, compare lanes 8 and 11, cell sample containing 2.5-fold more protein). P40 could also be detected in both cells and culture medium from HdV-infected cells and from parasitized larvae (Figs. 6B and 6C). In



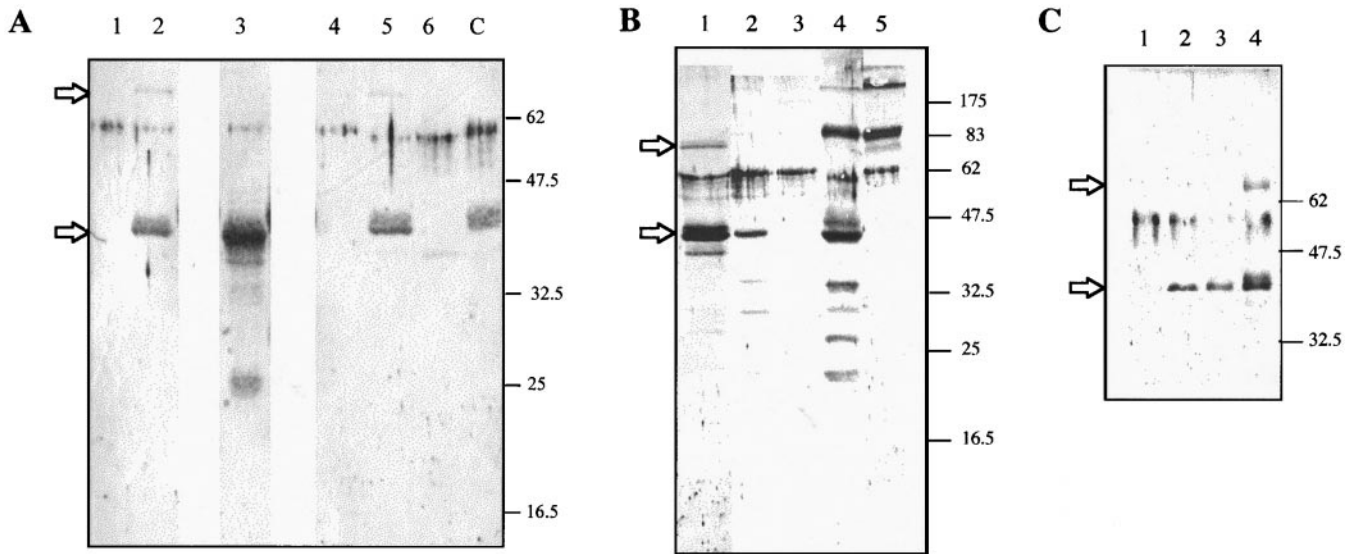


FIG. 6. Expression of M-group proteins in HdV-infected cells and parasitized larvae. All cell lines were infected for 72 h and larvae parasitized for 5 days at 27°C. Proteins were detected by immunoblotting with an antibody directed against the recombinant protein NhisP40. (A) Expression in infected Sf9 cells. Total proteins from whole-cell extracts (lanes 1 to 3; 15 μ l per lane) and culture medium (lanes 4 to 6; 30 μ l per lane) were separated on 12.5% SDS-PAGE, transferred onto a nitrocellulose membrane, and reacted with the NhisP40 antibody. Lanes 1 and 4, uninfected cells; lanes 2 and 5, HdV-infected cells; lanes 3 and 6, cells infected with the recombinant baculovirus M24Nhis. As a positive control, approximately 2.5 μ g of column partially purified recombinant NhisP40 protein was loaded in lane C. (B) Expression in *S. littoralis* larvae. Proteins were separated on 12.5% SDS-PAGE. Lane 1 represents a positive control with medium from HdV-infected Sf9 cells. P40-related proteins were analyzed in hemocytes (15 μ l) from parasitized (lane 2) and nonparasitized (lane 3) larvae and in the hemocyte-free hemolymph (30 μ l) from parasitized (lane 4) and nonparasitized (lane 5) larvae. (C) Expression in *S. littoralis* cell lines. Proteins were separated on 10% SDS-PAGE. Culture medium (30 μ l) from uninfected Sf9 cells as a positive control is in lane 1. P40-related proteins were analyzed in the culture medium (30 μ l) of HdV-infected SIJ2a (lane 2), SIJ2b (lane 3), and Sf9 cells (lane 4). Molecular weights in kDa are indicated on the right. Arrows indicate the immunoreactive bands corresponding to P40 and P69.

infected cell lines, an immunoreactive band was also detected around 70 kDa. As predicted by amino acid sequence identity between P40 and P69, this band might correspond to the P69 protein. Note that for *S. littoralis* larvae, an immunoreactive band was detected around 80 kDa for both parasitized and nonparasitized plasma (Fig. 6C, lanes 4 and 5) that might correspond to a host protein presenting a common epitope with P40. In addition to Western blot analysis, the [³⁵S]methionine incorporation experiments indicated that the radiolabeled prominent 40- and 70-kDa proteins secreted by several HdV-infected lepidopteran cell lines (Fig. 1) might correspond to P40 and P69.

DISCUSSION

In this paper, expression of some *H. didymator* polydnavirus genes was followed in Sf9 cells infected with HdV. Results indicated that the Sf9 model mimics to some extent the *in vivo* model and may be used to study expression of HdV genes in lepidopteran host cells. This system allowed us to identify three HdV-specific cDNAs, named M24, M27, and M40, corresponding to 1.5-, 1.6-, and 2.3-kb RNAs that were also detected in parasitized *S. littoralis* larvae.

The exact number of HdV genes expressed in the lepidopteran host is unknown. It has been described that more PDV genes were specifically host-expressed in

FIG. 5. Northern blot analysis of HdV RNAs related to the M-group cDNAs. (A) Time course of M24 transcription. RNAs were detected with the EcoRI-digested ³²P-labeled M24. Lanes 1 to 4 correspond to larvae samples (30 μ g per lane, 48 h exposure) with lane 1, nonparasitized larvae; lanes 2–4, larvae 2, 24, and 48 h postparasitism, respectively; lanes 5 and 6, hemocytes from fifth-instar larvae (8 μ g per lane, 48 h exposure) parasitized (lane 5) or not parasitized (lane 6); and lanes 7 to 11, Sf9 cell samples (15 μ g per lane, 20 h exposure), uninfected cells (lane 7) and cells 2 h (lane 8), 24 h (lane 9), and 24 h treated with RQ1 DNase (lane 10) and 48 h (lane 11) postinfection. Lanes I, II, III, and IV correspond to Sf9 cells 48 h, 6 days, 7 days, and 10 days postinfection, respectively (3 μ g in lanes I and IV, 4 μ g in lanes II and III, 20 h exposure). White asterisks indicate the major RNA species of 1.5 and 2.3 kb in size. The gray asterisks mark the approximately 3.0-kb RNA. (B) Time course of M27 transcription. RNAs detected with the EcoRI-digested ³²P-labeled M27. Lanes 1 to 11 are the same as in A. White asterisks indicate the major 1.6-kb RNAs. The gray asterisks mark the approximately 3.0-kb RNA. (C) RNAs detected with ³²P-labeled M24- and M27-specific oligonucleotides. Total RNA from Sf9 cells (30 μ g per lane, 20 h exposure) were hybridized with M27-specific probe (lanes 1 and 2), M24- and M27-specific probes (lanes 3 and 4), and M24-specific probe (lanes 5 and 6). Lanes 1, 3, and 5 correspond to cells 48 h postinfection and lanes 2, 4, and 6 to uninfected Sf9 cells. Molecular size markers are shown on the right.

ichneumonid than in braconid viruses (Webb, 1998). Two genes were described from the braconid *Cotesia rubecula* PDV (Asgari *et al.*, 1996), while 12 have been detected in the ichneumonid *C. sonorensis* PDV (Blissard *et al.*, 1987; Theilmann and Summers, 1988). For the ichneumonid-carried HdV, we identified at least eight groups of cross-hybridizing cDNAs expressed in the lepidopteran host. Using different probes from each cDNA group, at least 13 HdV-specific RNAs were detected in HdV-infected Sf9 cells. On Northern blots, total genomic HdV DNA hybridized with at least 5 bands. The major band was about 1.5–1.8 kb and probably represented several comigrating RNAs since several cDNAs from the different groups hybridized to RNAs of this size. This intense band was the only one readily detected in crude extracts of parasitized larvae probably due to the relative smaller amount of viral RNAs in crude extracts from whole insects, not all parasitized, compared to cell extracts.

When cDNAs were used as probes, the hybridization signal for larvae was weaker than for Sf9 samples despite the fact that two times as much total RNA was loaded for parasitized larvae samples. However, results from hybridization with RNA from host hemocyte isolates were similar to those obtained with Sf9 cells considering that one-third the amount of material was analyzed. This probably indicates that M-group-related genes are preferentially expressed in hemocyte tissue. These results also indicated that the M40-related gene is apparently less transcribed than M24- and M27-related genes.

In addition to the 1.5-, 1.6-, and 2.3-kb RNAs, M-group cDNAs hybridized also with a 3.0-kb RNA that might correspond to other related RNAs or to pre-RNAs. The presence of this RNA of greater length suggests that transcription regulation of this gene family should be considered.

The RNAs corresponding to the M-group cDNAs were detected in the lepidopteran host early in infection throughout endoparasite development, as has been reported for *C. sonorensis* PDV Cys-motif genes (Blissard *et al.*, 1987; Cui and Webb, 1996; Li and Webb, 1994). In infected Sf9 cells as in parasitized larvae, they were present from 2 h p.i. until at least 10 days p.i. During this period, no significant decline in M-group RNA transcription was detected. This indicates, together with the fact that Sf9 cells continue to divide, that most of the cells were infected and that viral DNA was maintained and transcribed. In Sf9 cells infected with HdV, M-group-related RNAs are no longer detectable after 1 month p.i.; however, other viral RNAs are still detected (Fig. 2) and are even detected several years after infection (Volkoff *et al.*, in preparation). This suggests that the viral DNA corresponding to these RNAs is maintained in infected cells in either an episomal form or, as suggested by Gundersen-Rindal *et al.* (1998), as an integrated form inside the host genomic DNA.

Results from Southern blots and nucleotide sequence

analysis indicated that RNAs related to the three M-group cDNAs are likely to be encoded by different genes located on at least three distinct viral segments. The M24-related gene is most likely present in one HdV molecule of about 5.2 kb. PDV genome molecules are present in nonequimolar ratio and display restriction length polymorphism (Fleming and Krell, 1993) and the status of the DNA molecules may vary from one species to another (Albrecht *et al.*, 1994; Krell *et al.*, 1982). Moreover, PDV genomes have complex homology relationships both within and between different DNA molecules (Cui and Webb, 1997; Webb, 1998; Xu and Stoltz, 1993) since members of gene families may be located on multiple segments but also in multiple copies on a single segment (Webb, 1998). In the preparation we analyzed, supercoiled, circular, and linear forms of viral DNA are difficult to differentiate. cDNA bands positive for one probe, a and g (Fig. 4), for example, may represent different DNAs or different conformations of the same DNA. It was therefore difficult to accurately estimate the number and the size of the HdV molecules which were related to the M-group cDNAs. Nonetheless, hybridization results indicated that genes related to M-group cDNAs might be located on at least three viral DNA molecules. It will now be necessary to determine whether these genes are really present in a single genomic molecule or are distributed on several molecules.

Comparison of M24 cDNA with its related genomic sequence indicated the presence of two introns. Splicing of polydnavirus genes was described in *C. sonorensis* virus (Blissard *et al.*, 1987; Cui and Webb, 1996; Dib-Hajj *et al.*, 1993) and in *Microplitis demolitor* virus (Strand *et al.*, 1997). In M24, the first intron was localized in the 5' untranslated region, which has also been described for genes belonging to the *C. sonorensis* virus host-expressed Cys-motif gene family (Cui and Webb, 1996). Moreover, these *C. sonorensis* virus genes showed some identity (BLAST analysis) with the M24-related genomic sequence in an intron–exon junction close to the 3' end. This shared gene feature suggests that these genes might be evolutionarily related and that specific regulation might occur through this consensus intron site. Compared to M24, the 5' untranslated region of M40 contained 7 additional nucleotides (Fig. 3B, +64 to +70). Alignment of M24-related genomic clone and M40 showed that this extra 7-nucleotide sequence matches almost perfectly the 5' end of the first intron (6 bp identical), suggesting an alternative splicing in this gene's family.

Further isolation and sequencing of genomic clones containing genes encoding M27- and M40-related RNAs would allow a better characterization of this gene family.

Homologies and the presence of imperfect repeated elements arranged in tandem array in the M-group cDNAs suggested that they belong to the same gene

family. The presence of a putative gene family in HdV is consistent with observations done in *C. sonorensis* PDV (Dib-Hajj *et al.*, 1993; Theilmann and Summers, 1988) and *M. demolitor* PDV (Strand *et al.*, 1997). Summers and Dib-Hajj (1995) suggested that polydnavirus genome segmentation and the existence of gene families in PDVs promote recombination and generation of novel genes. We described in this report three putative genes that belong to a "glycine- and proline-rich" gene family. All these genes encode secreted proteins with similar predicted peptide sequences, suggestive of similar structures and similar function. Their putative ORF contained repeated elements that were symbolized by (AB)₂-C-(DBC)₂ for P40, AB-C-(DB'C-DBC)₂ for P45, and (AB)₃-C-(DBC-DBC)₃-DBC for P69. Repeats in the nucleotide sequence can increase the likelihood of genetic changes such as mutation or recombination (Gordenin and Resnick, 1998). The presence of related genes with a different number of repeated sequences has been reported for *M. demolitor* PDV (Strand *et al.*, 1997). Variation in the number of repeat units or changes in individual sequences might result from recombination processes or polymerase inadequacy as described for prokaryotic genomes (van Belkum *et al.*, 1998). The similarity between amino acids of the C sequence and the last 14 amino acids of the A sequence was noteworthy. In both P40 and P69, the last B sequence started with a glycine (G) residue (Fig. 8, see P40-B₂ and P69-B₃). In P69 DBC repeats, similarity was high between B₄C₄, B₆C₆, and B₈C₈ but also between B₅C₅, B₇C₇, and B₉C₉ (Fig. 8). This high degree of identity suggests that, in P69, a duplication of two linked repeat elements (DBC-DBC) might have occurred. The described putative HdV genes might have a common ancestry and possibly be evolved from gene duplication, homologous recombination, or deletion events.

The presence of a putative signal peptide suggests that these HdV glycine- and proline-rich M-group encoded proteins are secreted through cellular membranes. Indeed, after infection with HdV particles, the protein P40 is efficiently secreted by *S. littoralis* hemocytes, SIJ cells, and Sf9 cells. Coomassie blue staining allowed its visualization after 72 h p.i., resulting from the accumulation in the culture medium (data not shown). Estimated production level is comparable to that observed for recombinant proteins expressed in baculovirus expression system. Most of the polydnaviral proteins described in the literature are glycosylated proteins with several N-glycosylation sites (Beckage, 1993; Harwood *et al.*, 1994; Yamanaka *et al.*, 1996). The HdV gene products described here do not present any potential N-glycosylation site and molecular masses of the observed proteins were close to the predicted molecular masses, suggesting little or no glycosylation.

The M group constitutes a family of related polydnavirus host-expressed genes whose products are se-

creted into the host hemolymph, similar to the Cys-motif gene products described in *C. sonorensis* PDV (Dib-Hajj *et al.*, 1993). The M-related proteins, produced in Sf9 and in hemocytes, were detected from 6 h p.i. until at least 1 week p.i., consistent with the Northern blot results. These results suggest that P40 and P69 are major HdV proteins secreted by lepidopteran cells and in particular by hemocytes. Therefore, they might play an important role in the parasitoid development within the lepidopteran host. PDVs disrupt several host physiological functions (Webb, 1998). The host development (Dover *et al.*, 1988; Strand and Dover, 1991) or the host protein titers (Beckage *et al.*, 1994) might be altered; however, no cloned viral genes have yet been identified as possessing one of these functions (Webb, 1998). On the other hand, the host immune system is the known or suspected target for several isolated viral genes expressed in the lepidoptera (Shelby *et al.*, 1998). Concerning the M-group HdV proteins, their physiological function remained to be elucidated. They are glycine and proline rich but no definite motif could be assigned. The fact that they were secreted by the host hemocytes into the medium and that possibly an 80-kDa host protein shares an epitope with the M-group proteins might suggest a role for these proteins in modulating host cellular functions such as interfering with the host immune system.

As only few genes have so far been characterized, it is not known whether all PDVs use similar or unrelated mechanisms to disrupt host defenses or alter host physiology. Thus it would be interesting to investigate the interactions between PDV gene products and cellular proteins to identify possible cellular targets for the viral proteins. Functional relationships between P40 and *S. littoralis* hemocytes are now being investigated to analyze the physiological and molecular mechanisms by which the glycine- and proline-rich HdV proteins act on lepidopteran cells and in the parasitized caterpillar.

MATERIALS AND METHODS

Insect rearing and cell cultures

H. didymator and its rearing host *S. littoralis* were reared as previously reported (Volkoff *et al.*, 1995). Usually *S. littoralis* larvae were parasitized upon second larval instar for 24 h. Parasitoid larvae hatched 3 days after oviposition, in third-instar host larvae, and reached pupal stage in about 10 days.

The culture medium used to maintain freshly collected hemocytes and cell lines was a TC100 modified medium (Gibco) supplemented with 5% fetal calf serum (Gibco). Hemocytes were collected by bleeding last-instar larvae from a proleg into 500 μ l of ice-cold anticoagulant buffer (Pech *et al.*, 1994). The hemocytes were pelleted by centrifugation at 250 g, 4°C. Hemocytes were washed twice with phosphate-buffered saline (PBS) and once with cell culture medium, and then were resuspended in

500 μ l of medium and placed in culture plates. The cell lines used in the different experiments originated from *S. littoralis* (SIJ2a, SIJ2b), *S. frugiperda* (Sf9, ATCC CRL1711) and *Trichoplusia ni* (High Five, Invitrogen). The continuous *S. littoralis* cell lines were established from freshly collected hemocytes from one *S. littoralis* larva (as described above). After 20 passages, cell population was cloned. Two cloned cell lines were obtained, named SIJ2a and SIJ2b, that had been through more than 40 passages before being considered continuous cell lines. SIJ2a is a clone composed of large cells while SIJ2b is composed essentially of twice smaller cells.

Virus preparation, viral DNA extraction, and infections

Polydnavirus were extracted from *H. didymator* females as described by Beckage *et al.* (1994). Ovaries were dissected in PBS, placed in 1.5-ml microfuge tubes, and disrupted by several passages through a 23-gauge needle. The resulting suspension was passed through a 0.45- μ m filter (cellulose acetate). Filtrate was used either for viral DNA extraction or for cell infection.

For viral DNA extraction, virions were pelleted from the filtrate at 30,000 *g* for 30 min, 4°C. The pellets were resuspended in extraction buffer (0.1 M Tris–0.1 M Na₂EDTA–0.2 M KCl; proteinase K (0.2 mg/ml); Sarcosyl (0.5%)) and incubated overnight at 50°C. After RNase A treatment (0.3 mg/ml, 2 h at 37°C), DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1; v/v) and precipitated with ethanol.

To construct a HdV genomic library, virus was purified from homogenized ovaries on a 20–50% (w/v in PBS) sucrose gradient. Purified viral particles were pelleted and DNA was extracted as described above. Viral DNA was digested with *Bam*HI and fragments generated were cloned into the *Bam*HI site of pUC 19 plasmid (Cérutti *et al.*, in preparation). The library was screened with the ³²P-labeled M24. The D20 genomic clone specifically hybridized with M24 and contained a fragment about 5.2 kb. The *Hind*III–*Xba*I fragment of 1737 bp generated from D20 was sequenced and aligned identically to M24.

Infectious medium (IM) was prepared by resuspending a filtrate obtained from 35–50 female ovaries into 12 ml of culture medium. For infections, cells were seeded and infected at the ratio of 1 ml of freshly prepared IM per 10⁶ cells. IM was incubated in the presence of the cells during the time of the experiment. Cells were diluted after 4 days (1:2 dilution), then weekly (1:6 dilution).

RNA isolation

Total RNA was isolated from uninfected or HdV-infected Sf9 cells, and from nonparasitized or parasitized *S. littoralis* larvae, at various times postinfection or post-parasitism. Cells (10 to 12 \times 10⁶) were seeded in 75-cm² flasks (Falcon): 12 \times 10⁶ cells were seeded for 2-h-p.i. extraction, whereas 10⁷ cells were seeded for 48-h-p.i.

extraction. They were then infected with freshly prepared IM (ratio of 1 ml IM/10⁶ cells). Parasitism could be controlled only by larvae dissection. Until 3 days p.p., larvae were too small to be dissected, so parasitism rate was estimated as being approximately 50–70% and RNA samples were collected from pooled larvae (25–35 mg of larvae for the 2- and 24-h p.p. samples, 60–65 mg of larvae for the 4- to 5-days p.p. samples). Four to five days p.p., hemolymph and hemocytes were collected from 8–10 larvae, which were then dissected to control the parasitism. As a control, RNA samples were also collected from nonparasitized larvae of corresponding age. RNA was extracted with Trizol (Gibco) according to the manufacturer's protocol. Sf9 cells were washed in PBS and then resuspended in Trizol. Trizol was directly added to hemolymph and hemocytes, and pooled larvae were crushed directly in Trizol. RNA samples were resuspended in distilled RNase-free water and stored at –80°C. Total RNA concentration was estimated by spectrophotometry. As a control, some RNA samples were treated with RQ1 DNase (1 U/5 μ g RNA; Promega).

Southern and Northern blot hybridization

Total RNA samples, loaded in a formamide–formaldehyde buffer (Promega, TB256), and DNA samples were separated by electrophoresis in 1% agarose gels using a Tris–acetate buffer system (TAE). Gels were stained with ethidium bromide before transfer to confirm equal loading. They were transferred to charged nylon membranes (Boehringer Mannheim) by capillary blotting using 20 \times SSC and then fixed by UV irradiation for 3 min. Membranes were incubated at 42°C for 3 to 4 h in a prehybridization buffer (FPH) containing 50% formamide, 5 \times Dehnard's, 5 \times SSC, and 100 μ g/ml calf thymus DNA (Sigma).

DNA probes (*Eco*RI-digested cDNA inserts and *Hind*III–*Bam*HI-digested HdV DNA) were random prime (Boehringer Mannheim) labeled with [α -³²P]dCTP (3000 Ci/mmol; NEN–Dupont) and hybridized to blots (>2 \times 10⁶ cpm/ml). Hybridization was conducted overnight at 42°C in FPH buffer. Membranes were washed twice for 10 min in 2 \times SSC, 0.1% SDS at room temperature, twice for 15 min in 0.2 \times SSC, 0.1% SDS at 42°C, and twice for 15 min in 0.1 \times SSC, 0.1% SDS at 68°C and autoradiographed (Kodak Biomax MR).

Two HdV cDNA-specific 45-mer oligonucleotides were synthesized (Eurogentec): one specific for the M24 cDNA (nucleotides 149 to 193 in the M24 N-terminal coding region, 5'TGCGAATCCGAACTCAGCGTCCTTTACGGCT-TCTTCTCCTCCTCC3') and one specific for the M27 cDNA (nucleotides 653 to 697 in the M27 B'1 sequence, 5'TGGGGCGGGCGGCATTGCATCGCCTCCCCATCTT-GCTGCTGGACC3'). Oligonucleotides were phosphorylated with T4 polynucleotide kinase (Biolabs) in presence of [γ -³²P]ATP (6000 Ci/mmol; NEN–Dupont) and hybrid-

ized to Southern and Northern blots ($>10^6$ cpm/ml). Pre-hybridization and hybridization were conducted for 3 h in FPH buffer at 42°C. Membranes were washed twice for 5 min in $2\times$ SSC, 0.1% SDS at room temperature, then twice for 10 min in $0.1\times$ SSC, 0.1% SDS at 42°C and autoradiographed (Kodak Biomax MR).

cDNA cloning and analysis

cDNAs were generated from HdV-infected Sf9 cells 24 h p.i. Total RNA was extracted following Chirgwin's method (Sambrook *et al.*, 1989). Poly(A)⁺ mRNA was isolated with Straight A's mRNA Isolation System (Novagen), according to the manufacturer's protocol. cDNAs were generated using the Universal Riboclone cDNA Synthesis System (Promega). They were ligated to *Eco*RI adaptors and cloned into *Eco*RI-digested dephosphorylated pUC18 plasmid vectors. cDNA clones were then transformed on *Escherichia coli* strain XL1-Blue. To isolate specific HdV clones, randomly chosen *E. coli* clones were screened by plaque hybridization (Sambrook *et al.*, 1989) with nick-translated α -³²P-labeled HdV genomic DNA. By Southern blot analysis, cross-hybridization was performed to identify homologous cDNAs. For this purpose, 13 cDNAs were randomly chosen and corresponding *Eco*RI inserts were used individually as probes against the entire subset of HdV-specific cDNA clones. The plasmids carrying the cDNAs and derived constructs were sequenced with T7 Sequencing kit (Pharmacia) or with Abi Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) or by Eurogentec, Belgium. Overlapping nucleotide sequence fragments were assembled by Fragment Assembly programs, based on the method of Staden (1980), with the Wisconsin Sequence Analysis Package (Version 8 for Unix Server, Genetics Computer Group). Nucleotide and predicted peptide sequences were aligned manually. Percentages of identity between sequences were calculated excluding the gaps.

The sequence data reported here appear in the GenBank database under Accession Nos. AF131648, AF132023, AF132024, and AF156933.

Radiolabeling of proteins

Proteins produced in cells infected with HdV were analyzed by metabolic radiolabeling as reported (Chaabihi *et al.*, 1993). Cells were seeded in 24-well plates (10^6 cells per well) and infected with 1 ml of freshly prepared IM. At various times postinfection, medium was removed and cells were pulse-labeled with 0.5 ml of medium supplemented with 10 μ Ci of L-[³⁵S]methionine (Amersham). After 4 h exposure, medium was recovered and cells were lysed in 100 μ l 1% SDS. Labeled proteins from medium and cell lysate were analyzed by SDS-PAGE in a 12.5% acrylamide gel using the method described by Laemmli (1970). After electrophoresis, pro-

teins were stained with Coomassie brilliant blue and dried gels were autoradiographed.

Generation of an antiserum against M24-encoded protein

Generation of recombinant baculovirus. A 270-bp fragment encoding the N-terminal part of the gene (from the first ATG to the *Eco*47III site) was amplified by PCR with the plasmid carrying M24 as template. With the sense primer 5'GACTGGATCCTACGTAATGAAGATCTTGCTGC-CCTTGATGGTCGC3', two restriction sites, *Bam*HI and *Sna*BI, were added upstream of the ATG and a *Bg*III site was added downstream of the ATG. The antisense primer 5'CATGAAGCTTCTAGAAGTAGCGCTTCCCTCAC-CCATTTTCATTGTC3' allowed us to add *Xba*I and *Hind*III sites downstream of the *Eco*47III site. The 296-bp *Bam*HI-*Hind*III fragment was cloned into the *Bg*III-*Hind*III sites downstream of the P10 promoter in the baculovirus transfer vector p119 (M. Ogliastro, unpublished data) giving the p119-M24 construct. The 962-bp *Eco*47III-*Xba*I fragment encoding the C-terminal part of the gene was isolated from the pM24 and introduced into the *Eco*47III and *Xba*I sites of the p119-M24 construct. A 26-bp *Sna*BI-*Bg*III fragment encoding a 6-histidine tag was constructed with two overlapping oligonucleotides, 5'GTAATGCATCATCATCACCACCATCATAA3' and 5'CAT-TACGTAGTAGTGGTGGTAGTAGTATTCTAG3', and cloned into the *Sna*BI-*Bg*III site of the p119-M24 (6-his tag introduced between the first two amino acids of coding sequence), giving the p119-M24-Nhis construct. Sf9 cells were cotransfected with viral DNA purified from the modified baculovirus AcSLP10 (Chaabihi *et al.*, 1993) and DNA from recombinant p119-M24-Nhis transfer vector. Recombinant baculoviruses were plaque purified by standard methods (Summers and Smith, 1987).

Purification of the M24-encoded protein. Recombinant protein was produced by infecting Sf9 cells with the recombinant virus M24-Nhis at a multiplicity of infection of 10 PFU per cell. Cells were collected 4 days postinfection and purified by NTA-(Ni²⁺) resin-affinity column chromatography under denaturing conditions according to the manufacturer's protocol (Qiagen). Fractions containing the column-purified protein were concentrated using a Centricon-30K device (Amicon) and proteins were separated by 12.5% SDS-PAGE. M24Nhis protein was purified in acrylamide gel. The gel slice corresponding to the protein was used to generate a rabbit polyclonal antiserum (Eurogentec).

Immunoblotting with the antiserum. To detect the M24 cDNA product among the HdV-encoded proteins, Sf9 and *S. littoralis* cells were HdV-infected for 72 h. For the control M24-Nhis recombinant baculovirus, Sf9 cells were infected as described above for 72 h. Hemolymph and hemocyte samples were collected from nonparasitized and parasitized *S. littoralis* larvae 4–5 days p.p.

Proteins were resolved on 10 or 12.5% SDS-PAGE and electrotransferred to nitrocellulose membranes using a semidry blotting apparatus. Blots were processed with the polyclonal M24Nhis antiserum (1/100). The protein was detected with anti-rabbit peroxidase-conjugated goat IgG (Sigma) as a secondary antibody (1/1000) followed by detection with the peroxidase substrate (3-amino-9-ethyl carbazol; Sigma).

ACKNOWLEDGMENTS

We thank R. Bros and B. Limier for kindly providing *Spodoptera littoralis* larvae and Professor Santiago-Alvarez and Dr. Vargas for the *Hyposoter didymator* colony. Thanks to S. Berger for preparing the buffers. Thanks to L. Croizier for help with sequencing, to F. Cousserans and M. Lopez for help with GCG software. We are very grateful to S. Blanc, P. Krell, P. Fournier, and K. Shelby for their constructive comments on the manuscript. In addition, special thanks to M. Drucker, M. Ogliastro, and H. Volkoff for the English corrections.

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