Naturally Occurring Deletion Mutants Are Parasitic Genotypes in a Wild-Type Nucleopolyhedrovirus Population of *Spodoptera exigua*

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A wild-type nucleopolyhedrovirus (NPV) isolate from *Spodoptera exigua* from Florida (Se-US2) is a variant of the SeMNPV type strain since it has a unique DNA profile but is closely related to other known geographical isolates of SeMNPV. It consists of several genotypic variants, of which seven were identified in a Se-US2 virus stock by a modification of the in vivo cloning method developed by Smith and Crook (Virology 166:240–244, 1988). The US2A variant was the most prevalent genotype, and it was designated the prototype Se-US2 variant, while four of the variants (US2B, US2D, US2F, and US2H) were found at low frequency. US2C and US2E were also very abundant, and their diagnostic bands were easily observed in wild-type isolate restriction endonuclease patterns. The analysis of each variant, compared to the prototype US2A, showed that US2B and US2H presented minor differences, while US2D and US2F contained slightly larger insertions or deletions. Variants US2C and US2E contained major deletions of 21.1 and 14 kb, respectively, mapping at the same genomic region (between 14.5 and 30.2 map units [m.u.] and between 12.8 and 23 m.u., respectively). This is the first report of such deletion mutants in a natural baculovirus population. Variants US2A, US2B, US2D, US2F, and US2H were isolated as pure genotypes, but we failed to clone US2C and US2E in vivo. When these two variants appeared without apparent contamination with any other variant, they lost their pathogenicity for *Spodoptera exigua* larvae. A further biological characterization showed evidence that these two naturally occurring deletion mutants act as parasitic genotypes in the virus population. Bioassay data also demonstrated that pure US2A is significantly more pathogenic against second-instar *S. exigua* larvae than the wild-type isolate. The need for precise genotypic characterization of a baculovirus prior to its development as a bioinsecticide is discussed.

The beet armyworm, the larval stage of *Spodoptera exigua*, causes extensive economic losses for many cultivated plants throughout the temperate and subtropical geographical regions of the world (7). It is a difficult pest to control by common and biorational chemical insecticides (3, 36), and therefore alternative control methods have been extensively studied with the aim of developing more-effective control agents.

The usefulness of a multinucleocapsid nucleopolyhedrovirus (MNPV) in control of the beet armyworm (16, 35) has focused interest in selecting isolates with increased virulence and genetic stability. Naturally occurring NPVs have been isolated from beet armyworms collected in several geographical regions, including Egypt and The Netherlands (37), California (14), Spain (5), Florida (22), and Thailand and Japan (18, 23). In some of these regions, this virus causes epizootic infections almost every year (5, 15). The biochemical and biological properties of most of these NPV isolates have been investigated. On the basis of restriction endonuclease (REN) analysis of the viral genomes, all of the wild-type (wt) SeMNPV isolates are closely related to SeMNPV-US1 (Se-US1 [6, 15, 18, 23]), an isolate from California which has been proposed as the type strain of SeMNPV (6). Other NPVs isolated from field-infected *S. exigua* larvae were identified as variants of the *Autographa californica* MNPV (AcMNPV [23]). Comparative activity of some SeMNPV strains, expressed in terms of both dosage and time, showed differences in their insecticidal properties (6,

18) and showed that the SeMNPVs were more virulent than any other NPVs pathogenic to the beet armyworm (34). Use of SeMNPV preparations resulted in effective control of the beet armyworm in the field (16, 17, 22) as well as in greenhouses (35).

A wt SeMNPV isolate from Florida (which we have called SeMNPV-US2, or Se-US2 for short) is effective in field experiments (17, 22) and has been marketed with the trade name of Spod-X (22). Most of the current viral insecticides now in the marketplace, including Spod-X, are being produced in vivo, while AcMNPV and *Lymantria dispar* MNPV (LdMNPV) are the only viruses at this time that have been produced in vitro and extensively field tested (2). There have been recent advances in in vitro production with regard to the development of baculoviruses as bioinsecticides, but in vivo production still leads this technology. In vivo production has several advantages, particularly with regard to wt viruses. However, a precise genotypic characterization of the virus should be made to ensure an acceptable product and to facilitate the future identification of any genetic alterations which may occur either during commercial virus production or in the field.

In this work, the genetic composition of Se-US2 was determined by an in vivo cloning method, since SeMNPV does not replicate adequately in tissue culture. This method used budded virions (BVs), which typically contain a single nucleocapsid, rather than occlusion-derived virions (ODVs), which may allow the replication of two or more different genotypes even if the infection starts from a single virion (33, 38). We demonstrated that the Se-US2 isolate consists of at least seven genotypic variants, which have been genetically characterized, and that two of them are deletion mutants. These two mutants

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were considered parasitic genotypes, since they were not capable of killing *S. exigua* larvae without a helper virus and, additionally, act as defective interfering particles when mixed with other variants.

MATERIALS AND METHODS

Insect rearing. *S. exigua* stocks were reared at 25°C with a 16:8-h photoperiod on a semisynthetic diet as described by Muñoz et al. (32).

Virus strains. Se-US2 of *S. exigua* was isolated in the mid-1980s from beet armyworm larvae in a commercial nursery in Alva, Fla. This strain was later selected to be developed as a bioinsecticide, and since 1994 formulated SeMNPV has been registered and sold in the United States, The Netherlands, and Thailand under the trade name Spod-X. The active ingredient was amplified in vivo by infecting larvae with a liquid formulation kindly provided by a private company (Du Pont Iberica, S.A.). The Se-US1 strain was received from M. D. Summers (Texas A and M University, College Station, Tex.). Se-SP2 was collected in 1990 from horticultural crops grown in greenhouses at El Ejido, Spain, and established as a new SeMNPV strain (6).

Isolation and REN analysis of viral DNA. Dead larvae with symptoms of nucleopolyhedrosis were individually transferred to a microcentrifuge tube and homogenized in 300 µl of bidistilled water. The homogenates were filtered first through a cheesecloth and secondly through 300 µl of a 30% sucrose cushion which was centrifuged at 8,000 × g for 5 min. The pellet of pure occlusion bodies (OBs) was resuspended in 350 µl of bidistilled water, and an aliquot was kept aside for further infections while the rest (ca. 300 µl) was used for viral DNA extraction and purification following the method described by Muñoz et al. (32).

For REN analysis, 1 µg of DNA was mixed with 10 U of enzyme (Promega), and the mixture was incubated for 4 to 12 h at 37°C. Digested DNA was electrophoresed in 0.8% TAE agarose gels with ethidium bromide (0.12 µg/ml) at low voltage (20 to 40 V) for 8 to 16 h. The Molecular Analyst 1.5 software (Bio-Rad) was used to photograph the gels and to analyze the intensity of the DNA fragments.

Cloning of genotypes. A modification of the method described by Smith and Crook (33) was used to in vivo clone the Se-US2 genotypic variants. A small number (10 to 15) of *S. exigua* L₄ larvae were starved for about 12 h and allowed to drink from an aqueous suspension containing 10% sucrose, 0.01% Fluorella blue dye, and a water suspension of the wt virus isolate at a concentration of 10⁷ OBs/ml. *S. exigua* larvae were calculated to drink a volume of 3.3 µl (34), so that each larva ingested approximately 3 × 10⁴ OBs. Forty-eight hours later and, in some cases, 72 h later, some of these larvae were bled and their BV-containing hemolymph was collected, diluted 1:50 in an aqueous suspension, prepared as indicated above, and used to infect a large number (100 to 500) of *S. exigua* L₄ larvae that had also previously been starved for 12 h. This dilution was expected to kill fewer than 20% of the infected insects. For cloning, even the less abundant genotypes present in the mixture, more than 1,000 larvae, were infected with hemolymph containing the wt virus mixture. For the second round of cloning, OBs from each dead larva were used to infect other batches of 15 to 25 larvae. Hemolymph from these larvae was collected, diluted, and fed to about 100 larvae to achieve approximately 20% mortality. DNA obtained from each larva was REN analyzed, and this process was repeated until DNA profiles indicated homogeneity in the genotypes.

Southern blot hybridization. Agarose gels were transferred to Hybond N⁺ nylon membranes (Amersham) and were hybridized with probes labeled with the digoxigenin (DIG) system (Boehringer Mannheim). The blots were washed under stringent conditions, twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) for 5 min at 65°C and twice with 0.1× SSC–0.5% SDS for 1 h at 65°C. The membranes were later treated first with anti-DIG antibodies and then with substrate following the protocol recommended by the supplier. Autoradiography was with Hyperfilm-ECL (Amersham) and an intensifying screen at room temperature for 2 to 24 h.

Bioassays. The 50% lethal doses (LD₅₀s) and 50% lethal times (LT₅₀s) of the wt isolate and several isolated genotypic variants were determined in a bioassay by the droplet-feeding method of Hughes and Wood (20). *S. exigua* L₂ larvae were starved for 16 to 20 h at 25°C and then allowed to drink from an aqueous suspension containing 10% (wt/vol) sucrose, 0.001% (wt/vol) Fluorella blue, and OBs at five different concentrations. The concentration range used for each viral preparation was determined in preliminary bioassays. The first 75 larvae that drank from the solution within 10 min were transferred to individual wells of 25-well tissue culture plates with a semisynthetic formaldehyde-free diet plug. A batch of 25 larvae was allowed to drink from a polyhedron-free suspension and served as a control. The whole bioassay was repeated three times, and the results were combined in the probit analysis. Larvae were reared at 25°C, and mortality was recorded every 12 h until they had either died or pupated.

The dose mortality data were analyzed with POLO-PC software (10), which is based on the probit analysis method described by Finney (11). LT₅₀s were calculated by the mathematical equation of Biever and Hostetter (1).

FIG. 1. REN profiles with *Bam*HI, *Pst*I, and *Bgl*II of Se-US1, Se-US2, and Se-SP2 DNAs. Asterisks indicate submolar bands. The lettered 25.1-kb *Bgl*II (a) and 3.8-kb *Pst*I (m) fragments are two of the Se-US2 diagnostic RFLP bands. Molecular size markers are on the left.

RESULTS

REN analysis of Se-US1, Se-US2, and Se-SP2. Viral DNAs of three geographical strains isolated from the United States (Se-US1 and Se-US2) and Spain (Se-SP2) were analyzed with *Bam*HI, *Bgl*II, *Pst*I (Fig. 1), and *Xba*I. Digestion with *Bgl*II and *Pst*I resulted in unique and characteristic DNA profiles for the three strains, as indicated by the presence or absence of one or more fragments. The Se-US2 25.1-kb *Bgl*II fragment and the 3.8-kb *Pst*I fragment are unique to Se-US2 and can be used as restriction fragment length polymorphism (RFLP) markers to distinguish it from Se-US1 and Se-SP2. Se-US2 *Bam*HI, *Pst*I, and *Xba*I DNA profiles revealed the presence of fragments in submolar frequency, indicating the heterogeneity of the viral population.

Description of the Se-US2 genotypic variants. We isolated seven individual genotypes, also called genotypic variants, from the wt Se-US2 strain following a modification of the in vivo method described by Smith and Crook (33). For simplicity, the seven variants are hereafter referred to as US2A, US2B, US2C, US2D, US2E, US2F, and US2H. REN analysis with *Bam*HI, *Bgl*II, *Pst*I (Fig. 2a), and *Xba*I allowed us to differentiate each variant at the molecular level. The physical maps of the seven genotypes are summarized in Fig. 2b, which shows the presence of additions, deletions, and other differences relative to the US2A genotype. These variants were placed into three groups according to similarities in their genomes. The first group comprised genotypes US2A, US2B, and US2H, which were characterized because their genome sizes were similar to that of the type strain Se-US1 (19). Although closely related, these three genotypic variants could be distinguished by the *Bgl*II, *Pst*I (Fig. 2a), and *Xba*I profiles. US2B showed two small differences with respect to US2A. A 3.6-kb *Pst*I-M fragment was 100 bp smaller than the equivalent in US2A. The *Xba*I-R and *Xba*I-O fragments present in US2A were fused as a 4.0-kb *Xba*I-K fragment in US2B. US2H differed from US2A only in a 0.2-kb shorter *Bgl*II-J and a 0.2-kb larger *Bgl*II-K.

FIG. 2. (a) REN profiles of the Se-SP2 genotypic variants. *PstI* profiles are in pairs, with US2A (A) on the left and US2B (B), US2C (C), US2D (D), US2E (E), or US2F (F) on the right. *BamHI* profiles for US2A, US2C, US2D, and US2F are shown. Lettered fragments are RFLP bands, and asterisks indicate RFLP bands unique for one particular genotype. Underlined numbers 1, 2, and 3 represent molecular size markers of 17.5, 6.8, and 3.9 kb, respectively. (b) Restriction enzyme maps with *BamHI* (B), *BglII* (Bg), *PstI* (P), and *XbaI* (X) of the Se-US2 genotypic variants. The complete map for the US2A variant is shown, and at the bottom are the fragments characteristic of US2B (only with *PstI* and *XbaI*), US2C, US2D, US2E, US2F, and US2H (only with *BglII* and *XbaI*). Dotted lines, deletion in the genome; triangles, insertion; numbers, positions (in map units) in relation to the scale at the bottom. The values in parentheses are differences in size with respect to the equivalent fragments in US2A.

Variants US2C and US2E, whose genomes contained major deletions, were placed in the second group. Genotype US2C showed a 21.1-kb deletion, mapping between 14.5 and 30.2 map units (m.u.) with respect to US2A (Fig. 2b). Due to this deletion, some of the US2A fragments were no longer present in US2C (i.e., *BamHI*-F), some were shortened (i.e., *XbaI*-A), and some were shortened and fused to adjacent ones (i.e., *PstI*-J). Finally, as for US2B, US2C showed the presence of a 4.0-kb *XbaI*-K fragment.

The US2E variant was characterized by a deletion of 11.5 kb, between 13 and 21 m.u., with respect to US2A (Fig. 2b).

Variants US2D and US2F, whose genomes contained minor insertions or deletions, were placed in the third group. By analysis with *SpeI*, *PstI*, and *BamHI*, genotype US2D, relative to US2A, appeared to be the result of a 2.5-kb insertion between 22 and 24.4 m.u. (Fig. 2). The genotypic variant US2F showed a minor deletion of 2.5 kb located between 16 and 20.4 m.u. (Fig. 2b). These two diagnostic fragments were unique to US2F and were detectable in the wt profiles as submolar bands.

Relative abundance of the genotypic variants. The relative proportion of each variant in the wt virus population was estimated by the number of times that the variant profile was observed after the first infection and also by the relative intensity of variant RFLPs in the wt profiles. Variant US2A, which was easily cloned after only one passage and was recovered at the highest frequency, was designated the prototype virus. Of all of the larvae that died of NPV after the first passage (larvae that were infected with the Se-US2 inoculum), 33% showed a

homogeneous profile of US2A. Virus from the other larvae killed by NPV after one passage showed different profiles consisting of a mixture of two or more genotypes, most of which included US2A. For example, US2C was identified in 30% of the larvae and, except for one profile which was exclusively US2C, was always contaminated with various amounts of US2A. US2E, which was observed in approximately 21% of the NPV-killed larvae, was also contaminated mainly with US2A, but the amplified progeny OBs in one of these larvae consisted predominantly of US2E. The variant US2D could be distinguished in 5% of the NPV-killed larvae but was always mixed with other genotypes and became dominant after the second passage. US2F, although only barely detectable in the wt profiles, was not the predominant genotype until after the third passage and was cloned after the fourth passage. The US2B RFLP was not visible in the wt profiles but was detected as a clean profile in 2% of larvae that died after the first passage. US2H, which was also not detectable in the wt profiles, could not be detected among that of contaminating variants until after the third passage. US2H was not completely purified until passage 5. The remaining 8% of NPV-killed larvae could not be analyzed.

Infectivity, genomic stability, and predominance of the Se-US2 genotypic variants. Cloned variants US2A, US2B, US2D, US2F, and US2H were able to kill *S. exigua* larvae independently. Moreover, the REN profiles, which are diagnostic for each of these variants, were stable through 10 consecutive passages of each variant in insects. However, when suspensions of

FIG. 3. REN profiles of viral DNAs of US2A (lane 1), inoculum MAC1 (lane 2), and inoculum C15 showing only US2C (lane 3) with *Pst*I. The hatched and open arrowheads indicate the characteristic fragments of US2A and US2C, respectively. Fragment size markers (kilobases) are on the left.

these genotypes were mixed with small amounts of US2A and used to inoculate *S. exigua* at doses approximating the LD₅₀. US2A emerged as the predominant genotype. In contrast, US2C and US2E, which lacked 15.7 and 8.6%, respectively, of their viral genomes compared to US2A, could not be cloned in vivo. This suggested that they acted as parasitic genotypes, since by themselves they were not capable of killing *S. exigua* larvae. No mortality resulted when L₄ larvae were infected with a low dose (10² OBs/L₄) of C15 (an inoculum which apparently contained only US2C [Fig. 3]). At a very high dose (10⁵ OBs/L₄), mortality was low (2 to 5%), and only US2A or mixtures of US2A and US2C were obtained from these larvae. Similar results were achieved with an OB suspension containing predominantly US2E. When this inoculum was supplied to L₄ larvae at high doses, only US2A or mixtures of US2A and US2E were recovered from the few larvae which died.

Biological activity. The insecticidal activities of the wt Se-US2, the purified predominant variant US2A, and samples C15 and E15R of variants US2C and US2E, respectively (apparently uncontaminated with any other genotypic variants), were determined for second-instar larvae in terms of LD₅₀ and LT₅₀. Doses supplied were 365, 81, 18, 4, and 0.9 OBs/larva for the four virus inocula tested. Mortality increased directly with the dosage for wt Se-US2 and US2A, while US2C and US2E were not infectious at all even at the highest dose. Second-instar *S. exigua* larvae were significantly more susceptible to US2A than to wt Se-US2 based on the lack of overlap of LD₅₀s at the 95% fiducial limits (Table 1). The LD₅₀ of US2A (14 OBs/L₂) was 3.5 times lower than the LD₅₀ of wt Se-US2, while both had similar LT₅₀s (ca. 93 h for 81 OBs and ca. 78 h for 365 OBs). A second experiment, carried out in parallel, involved determining the response of L₂ larvae to mixtures of US2A and US2C. The inocula for this experiment were constructed with an OB sample (C15; REN profiles in Fig. 3, lane 3) containing

TABLE 2. Comparative dosage-mortality responses of second-instar *S. exigua* larvae to infection with inocula MAC1 and MAC2

Virus dose (OBs/ml)	Virus inoculum	
	MAC1 ^a	MAC2 ^b
0.9	6	ND ^c
4	12	ND
18	24	21
81	64	28
365	88	38

^a The dose-mortality data for inoculum MAC1 (a mixture of US2C and US2A containing a fixed 20% OBs of US2A [see Fig. 3]) were fitted to the probit regression line $y = 1.04x + 3.2$, which was statistically equal to that obtained for wt Se-US2 (see Table 1).

^b No regression line could be calculated for data for inoculum MAC2 (a mixture of US2C and US2A containing a fixed final amount of 4 OBs of US2A per larva), since the mortality was too low at the highest dose. Data are percent mortality values.

^c ND, no determined.

mostly US2C and with OBs from a pure clone of US2A (Fig. 3, lane 1). Larvae were tested with a series of inocula at final doses of 365, 81, 18, 4, and 0.9 OBs/larva, each containing a fixed 20% concentration of US2A OBs (MAC1; DNA profile shown in Fig. 3, lane 2), and with another series at final doses of 365, 81, and 18 OBs/larva, each containing a fixed final amount of 4 OBs of US2A (MAC2) per larva. For example, at the highest dose, the 20% US2A mixture contained 73 US2A OBs and 292 US2C OBs, while the other mixture contained 4 US2A OBs and 361 US2C OBs. The mortality from the mixture containing 20% US2A was not significantly different from a straight-line response between the log of dose and percent mortality. The LD₅₀s (44 OBs/larva) and LT₅₀s (78 h for the 365-OBs/L₂ dose and 96 h for the 81-OBs/L₂ dose) calculated from the MAC1 mixtures were similar to those of the wt Se-US2. However, infection of L₂ larvae with the MAC2 mixtures resulted in significantly lower mortality, at comparable doses, than that for the MAC1 sample (Table 2).

DISCUSSION

Viral DNA from the SeMNPV Florida isolate (Se-US2) presented REN patterns very closely related to those of other SeMNPV isolates but with several unique fragments which distinguished it from the other strains described to date, including the Californian Se-US1 and the Spanish Se-SP2 from Almería (Fig. 1). Therefore, isolate Se-US2 should be considered a variant of the SeMNPV type strain. Furthermore, submolar bands were detected in *Bam*HI, *Bgl*II, *Pst*I, and *Xba*I DNA profiles, showing that the population consisted of a mixture of several distinct genotypes. This genotypic heterogeneity is common in other wt strains of SeMNPV (6) as well as in many other NPVs (13, 27) and in GVs (33). Because of the lack of suitable cell lines for SeMNPV replication, we used a modification of the in vivo method developed by Smith and Crook (33) to isolate the different variants present in Se-US2. Since in MNPVs different genotypes can be co-occluded in one OB or even coenveloped in a single virion, several different variants could replicate in a larva which ingests only a single OB, even if the infection starts from a single virion. The method that we developed for in vivo purification of genotypic variants used virus-containing hemolymph, which is also used for plaque purification. These BVs, which remain infectious per os (39), were diluted and fed to larvae so that only one or very few singly enveloped nucleocapsids would be responsible for initiating the infection, thereby allowing for a faster purification of,

TABLE 1. LD₅₀s and relative potencies of wt Se-US2 and the purified predominant variant US2A for second-instar *S. exigua* larvae

Virus	LD ₅₀ (OBs/ larva)	Fiducial limit (95%)		Regression line	Potency	Fiducial limit (95%)	
		Low	High			Low	High
wt Se-US2	49	33	75	$y = 1.04x + 3.1$	1.00		
Se-US2A	14	10	20	$y = 1.04x + 3.9$	3.46	2.07	5.84

at least, the most abundant variants within the population. By introducing this modification, we could purify seven Se-US2 genotypic variants, which could be differentiated by REN digestion.

Genotypic variants US2A, US2B, and US2H were similar to each other, and DNA bands from them were shared with REN fragments from the wt. The few differences observed could be due to duplication of viral sequences and point mutations in areas like the hypervariable *Pst*I-M fragment (Fig. 2a), as was already observed for the separated genotypes of Se-SP2 (31) and SfMNPV (13). Other genotypic variants consisted of genotypes carrying larger genomic deletions (US2C and US2E) or containing minor insertions or deletions (US2D and US2F). All of these alterations mapped to the same region of the SeMNPV genome, between 19 and 34 m.u. (Fig. 2b), suggesting that this part of the genome was hypervariable and may play an important role in the generation of genotypes and genetic evolution. Genotypic and phenotypic variants were isolated from several baculoviruses (4, 8, 9, 29, 30, 33, 40), and the generation of variant genomes has involved separate mechanisms. Baculovirus mutants carrying insertions have been clearly associated with host transposable elements that are integrated into the virus genome during the passage of the virus in vitro (12) as well as in vivo (21). Likewise, the serial passage of baculoviruses in cell culture also generates defective variants containing large deletions within a particular region of the genome (24, 26). Apparently, these defective variants tend to retain only those regions of the genome absolutely essential for their replication (24). While the occurrence of this phenomenon in vitro has been well documented (28), this is the first report of naturally occurring deletion mutants in a wt population of a baculovirus. The deletion mutants US2C and US2E that we have identified were already present in the wt population and appeared as an abundant genotype in some of the larvae after the first passage in vivo. This might also be the case for a deletion variant obtained in vitro from the wt population of Se-US1 (19). This plaque-purified variant of Se-US1 was described as having a large DNA deletion of 25 kb after only one passage in vitro (19) in the very same genomic area in which the US2C 21-kb deletion occurred. Likewise, the US2E variant shows a *Pst*I profile similar to that of I1, a plaque-purified variant of the Japanese wt isolate SeMNPV-IW (18). These two variants might have been selected for during replication from a mixed infection in cell culture rather than generated after only one passage in vitro.

US2A was the most prevalent genotype in wt Se-US2, since the DNA bands diagnostic for US2A were among the predominant bands of the uncloned wt virus and US2A was recovered from most larvae which died after only one passage of the wt virus. Variants US2C and US2E were also abundant genotypes, since bands diagnostic for US2C and US2E were easily observed in the wt REN patterns. However, they were always contaminated with US2A. A further biological characterization of these two deletion mutant genotypes by bioassays in second- and fourth-instar larvae demonstrated that they are noninfectious in vivo by themselves, either per os (administered as OBs) or when injected into the hemolymph as BVs. The previously mentioned plaque-purified defective variant of Se-US1 (19) was also not infectious for *S. exigua* larvae but was able to kill in vitro-cultured cells of the same host. The lack of pathogenicity of US2C and US2E in vivo may be attributed to the lack of genes important for viral DNA replication, as was demonstrated for defective genomes produced in vitro (28).

Probit analysis with second-instar larvae demonstrated that treatments containing pure US2A resulted in significantly higher mortality than treatments containing the wt mixture (Se-US2) or two different US2A-US2C mixtures. The observation that

the LD₅₀ of the wt virus was 3.5 times higher than that of pure US2A could be explained because in the wt virus population, US2A is diluted by less pathogenic or nonpathogenic genotypic variants (or ones that are defective for replication on their own). When this dilution effect was tested with US2C, a deletion variant defective in independent replication, that was mixed with US2A at 20% on second-instar *S. exigua* larvae, the effect on larval mortality was not significantly different from that of the wt mixture. Nevertheless these mortality levels were higher than expected from the small relative amounts of US2A in the mixture, suggesting that US2C becomes pathogenic in the presence of US2A. These findings suggest that US2A has a helper effect on US2C, allowing the propagation of this deletion mutant genotype along with the helper virus. Furthermore, the lower mortality produced by the MAC2 mixture indicated that a minimum amount of genotype US2A is required to allow the replication of the deletion mutant US2C. Such a helper effect can be realized only if deletion mutant and helper virus infect the same cell. This is similar to the requirement of helper virus to support replication of defective interfering particles (DIPs) of baculoviruses grown in bioreactors or tissue culture flasks (24, 25). The requirement of US2A as a helper for US2C is also supported by the previous observation that the US2C inoculum C15, apparently uncontaminated with other genotypic variants (Fig. 2a and 3), is not pathogenic even at a high dose. While the presence of parasitic and defective genotypes is quite common during the production of bioinsecticides or recombinant baculoviruses in bioreactors (25), this is the first report on the presence and prevalence of such parasitic genotypes in the wt population of a baculovirus.

These studies are also of great practical value with respect to the crop protection industry, as well as from an ecological point of view. Biological studies of the different variants will help select the most competitive variants for biopesticide design, since those affected by large deletions or insertions, which might disrupt the expression of essential genes, may actually interfere in the biological activity of regular variants. Our results clearly show that US2A should be considered for biocontrol use over the wt Se-US2. In addition, it is desirable to work with a single genotype for large-scale production of bioinsecticides to monitor possible changes that are likely to appear during the virus amplification. Mass production of baculovirus for insecticidal purposes has been done in the insect host with wt virus isolates, which most frequently contain heterogeneous viral populations. While the coexistence of such a range of genotypic variants may be important for virus survival under field conditions, a quality control of commercial virus products would prevent the spread of undesirable parasitic viral variants.

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