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Studies on Baculoviruses as a Potential Agent for Pest Control

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- Manuscript: Kislev (1983b): Replication of nuclear polyhedrosis virus of S. littoralis in continuous insect cell-lines.

C. Abstract

A comprehensive survey and collection of Spodoptera littoralis larvae was done at 17 geographically distinct regions throughout Israel to determine the types of nuclear polyhedrosis viruses enzootic in the insect populations and to subsequently survey for more virulent and efficacious isolates with better potential for pest control. Two distinctly different strains of nuclear polyhedrosis viruses (MNPV) were isolated from Spodoptera littoralis larvae. The two viruses were analyzed by comparing restriction endonuclease cleavage patterns and their degree of genetic relatedness was determined by Southern blot hybridization. The two viruses have different restriction endonuclease cleavage patterns and no detectable sequence homology. A naturally occurring variant, homologous to the more common virus isolate, was also found. S. littoralis MNPV (SlMNPV) isolates were used successfully for initiation of infection of cultured insect cells.

DNA sequence homologies between S. littoralis NPV (SlMNPV) and five other lepidopteran NPVs usually associated with SlMNPV epizootics and pest populations were studied by hybridization of ^{32}P -labeled NPV DNAs to Southern blots of restriction endonuclease-digested NPV DNA. SlMNPV shows extensive DNA sequence homology with Spodoptera frugiperda NPV (SfMNPV) and Spodoptera exigua NPV (SeMNPV). Heliothis armiger NPV (HaMNPV) shared fewer regions of homology with SlMNPV. Using the cloned HindIII fragment-V of the Autographa californica MNPV, genome fragments with sequence homology to the polyhedrin gene of Autographa californica MNPV were identified.

Significant differences in plaque phenotype were found between the two SlMNPV strains, and between the wild isolates within each strain. Wild isolates of both strains were examined for genetic variation by comparison of REN patterns. Variation was evident in several of the wild isolates studied. Seventeen wild isolates were examined further by comparison of REN patterns

from 20 plaque-purified isolates of each wild type. Seven different wild isolates were found to have variants, and at least 10 distinct variant types were identified.

D. Scientific Report

I. Field surveys, collection and identification of virus isolates.

The genus Spodoptera includes some of the world's major agricultural insect pests and recent studies indicate that baculoviruses of certain pest species of Spodoptera may be useful for biological control (7, 12). Spodoptera littoralis (Egyptian cottonworm) is an important insect pest in the countries of the Mediterranean, Africa, and Asia (8). Several reports describing the nuclear polyhedrosis viruses (NPV) and viral genomes of four Spodoptera spp. have been published (3,7,8,12,14,16,18,20,25,33,39). To date there are no reports comparing the genomes of different geographic isolates of any given species of Spodoptera, although close homology has been reported for the four NPVs of Spodoptera sp. (12, 20).

Field surveys and collections of Spodoptera littoralis larvae and eggs were conducted in 22 locations (geographical areas) within 17 regions of Israel (Table I, Figs. 7 and 8).

Infectious blood from a few diseased larvae from each location or from a few larvae inoculated in the laboratory (Table 1: Kislev and Edelman, 1982) were collected in Tris buffer, and both the blood and cadavers were frozen at -70°C to be used later as a source of inoculum for infection of cultured S. littoralis cells (see Kislev 1983b, Section H). Some of these isolates were also used as sources of purified polyhedra and virus. Small samples of purified viruses were frozen at -20°C or -40°C to be used later as inocula for infection of S. littoralis cells (see Kislev 1983b, Section H).

In our initial study (17), viral DNA was extracted from six geographic S1MNPV isolates and subsequently from isolates obtained from laboratory colonies of infected insects. The viral DNA was purified by banding to equilibrium on ethidium bromide - cesium chloride (EtBr-CsCl) gradients. The

Table 1. List of surveys and sources of geographical isolates of Spodoptera littoralis nuclear polyhedrosis viruses collected, 1980.

Region No. and identity ^a	Isolate designation	Region No. and identity ^a	Isolate designation
3. Hula Basin	KS(1) ^b	17. Nahariya Region	- ^c
5. Hazor Region	- ^c	18. Akko Region	A(1) ^b
6. Kinerot	D(4)	23. Hadera Region	-
8. Bet Shean Basin	B(4)&AS(1)	27. Lod Region	KT(1)
9. Harod Valley	S(2)	28. Rehovot Region	R(1)
10. Kokhav Plateau	-	30. Tel Aviv Region	T(1)TA(1) RH(3)M(1)
11. Yizreel Basin	RD(1)	34. Ashquelon Region	Y(1)
16. Elon Region	EL(1)	35. Gerar Region	KA(1)
		38. Northern Arava	E(2)

a. "Statistical Abstract of Israel" 1978, vol. 29, pp. 38-39.

b. Number in parenthesis = numbers of collections made in each area.

c. No collection made (either very low infestation or larvae collected did not survive).

purified DNAs were analyzed with the restriction endonucleases (REN) EcoRI, XhoI, BamHI, HindIII, BglI, PvuII, KpnI, SalI and SmaI. Southern hybridizations with nick-repair labeled DNA were used to compare DNA homology (17).

Viral DNAs from five different geographic isolates (collected from fields B, E, S, T, A; see Table I) were indistinguishable when digested with either EcoRI, HindIII, BglI, PvuII, KpnI, BamHI, or XhoI. The REN pattern obtained from viral DNA of a sixth geographic isolate, D, was distinct from all other samples studied.

Homology among the viral DNAs was studied by Southern blot hybridization (44). Probe DNA from isolate T hybridized with all EcoRI and HindIII DNA restriction enzyme fragments of isolates T (homologous), B, and E. Complete hybridization with all REN fragments was obtained among the viral DNAs from isolate T and isolates A, B, E, and S following digestion of the DNAs with BglI, PvuII, KpnI, BamHI, and XhoI. Hybridization was not detected with any EcoRI fragments of viral DNA from isolate D or of bacteriophage lambda DNA. Virus DNA from T1 was isolated, digested by REN, and analyzed as described above. The REN pattern for isolate T1 indicated that it was a variant related to all isolates except D. S1MNPV-T1 was found to be a variant with DNA sequences homologous to S1MNPV-T DNA but smaller in size by approximately 0.2 to 0.3×10^6 daltons.

We identified two NPVs isolated from Spodoptera littoralis populations (designated S1MNPV-T and S1MNPV-D) with distinct REN patterns and with no detectable sequence homology. In this initial study, the S1MNPV-T isolate was found to be the most common type existing in the geographical isolates analyzed. A naturally occurring variant of the S1MNPV-T virus, designated S1MNPV-T1, was also identified. The restriction endonuclease cleavage patterns reported here are different from the restriction profiles reported

for other baculoviruses (5,10,18,23,24,25,28,30,33,39,40), indicating that both S1MNPV-T and S1MNPV-D are distinct from these other baculoviruses.

II. Genomic Relationships between Spodoptera littoralis MNPV and other Baculoviruses.

Several reports describing the NPVs and viral DNAs isolated from four Spodoptera sp. have been published (2,3,7,8,13,15,16,17,18,20,25,26,33,36,39, 41). DNA reassociation kinetics have shown close homology among the DNAs of a few NPVs of Spodoptera sp. (12, 20). Recently, genetic heterogeneity in cloned isolates of Spodoptera frugiperda MNPV and geographic isolates of Spodoptera littoralis MNPV was reported (17, 18). Although DNA hybridization studies cannot produce absolute values of relatedness, the Southern blot hybridization technique (44) provides a relative measure for comparing different NPV genomes for sequence homology (11, 17, 31, 32, 36, 39). In Israel, Spodoptera littoralis is a major pest for field and garden crops, often infesting together with Heliothis spp. and rarely with Spodoptera exigua populations. In the USA, S. frugiperda and S. exigua are important pests which may infest together with Heliothis spp. Because it might be desirable to have an efficacious virus preparation to be used for control of each pest, or one with broader host range effective against several pests species in the same crop complex, we decided to evaluate the genetic relationships of these viral species. As a first step we checked the DNA homology among NPVs of three Spodoptera spp. and two Heliothis spp. In addition, we compared S. littoralis NPV DNA to DNA of Autographa californica NPV, the prototype for all MNPVs. The present study assesses the relatedness between the genome of S. littoralis NPV and those of five other nuclear polyhedrosis viruses which are candidates for use as viral insect control agents.

Viral DNA was purified (33, 39) from the following baculoviruses:

Spodoptera frugiperda (SfMNPV), plaque purified isolate D7, (18); Spodoptera littoralis (SlMNPV) geographic isolate T (17); Spodoptera exigua (SeMNPV); Heliothis armiger (HaMNPV); Autographa californica (AcMNPV), plaque purified isolate E2, (33); and Heliothis zea (HzSNPV). Viral DNAs were digested with the restriction endonucleases XhoI, EcoRI, or BamHI, electrophoresed in 0.7% agarose gels and transferred to nitrocellulose filters as described previously (17, 33, 35, 44). SlMNPV DNA, SeMNPV DNA, and HindIII-V fragment of AcMNPV-E2 DNA (38; cloned into pBR322) were labeled with ^{32}P -dCTP (2000 ci/mole; Schwarz Mann) by nick-repair synthesis (29). Specific activities of 3.8×10^7 cpm/g, 4.3×10^7 cpm/ug and 3.0×10^8 cpm/ug were obtained for SlMNPV, SeMNPV and AcMNPV HindIII fragment DNAs, respectively. Labeled probes (10 or 1 ng/ml) were hybridized to Southern transfers of viral DNAs at 65°C for 24 hrs as described (17, 39).

The results of hybridization of ^{32}P -DNAs of SlMNPV, SeMNPV, and AcMNPV fragment HindIII-V to Southern blots of viral DNAs are shown in Figs. 1 and 2. Ethidium bromide stained EcoRI or XhoI-digested viral DNA are on the left panels, and autoradiographs of each ^{32}P -DNA are on the right panels (Fig. 1A-SlMNPV; Fig. 1B-SeMNPV; Fig. 1C-E2 HindIII-V). Under the stringent conditions employed, both labeled SlMNPV and SeMNPV DNA probes hybridized to DNAs of SlMNPV, SeMNPV and SfMNPV throughout most of their genomes (Figs. 1A, 1B; Figs. 2A, B; Tables 2-4). The fragments are designated as suggested for baculoviruses (42). Hybridizations to DNAs of HaMNPV, HzSNPV and AcMNPV were limited and occurred to fewer fragments (Fig. 1A, 1B; 2A, 2B; Tables 2-4). Results of the hybridizations show that there is more sequence homology between the DNAs of Spodoptera sp. MNPVs (SlMNPV, SeMNPV, and SfMNPV), than with DNAs of the other baculoviruses. Some viral DNA fragments hybridized with ^{32}P -DNA more intensely than hybridizations of DNA fragments of similar sizes, for example: SlMNPV-DNA EcoRI fragments M and D; SfMNPV-DNA EcoRI

fragments P and S (Fig. 1A, 1B; and Table 2). However, these are only relative measures of DNA sequence homology and more detailed analysis is needed in order to confirm the specific nature of the DNA sequences apparently shared between the viruses (14).

A majority of the mRNA for AcMNPV polyhedrin is encoded by the HindIII-V fragment (1, 37, 38). To identify the EcoRI digested fragment containing polyhedrin sequences in these viruses, the cloned HindIII-V fragment (38) was labeled and hybridization was conducted as described above. For each viral DNA compared, one or two restriction endonuclease fragments hybridized with the probe (Figs. 1C, 2C; Tables 2-4), indicating that these fragments of SeMNPV, HaMNPV, HzSNPV (See Fig. 1C) contain sequence homology to the AcMNPV polyhedrin gene.

Legends to Figures

Figure 1: Left panels A, B, and C represent ethidium bromide stained bands of agarose gels after electrophoresis of EcoRI digested viral DNAs. Electrophoresis was in a 0.7% agarose-ethidium bromide slab gel. Migration was from top (cathode) to bottom (anode). Restriction fragments of the DNAs from S1MNPV (S1), AcMNPV (Ac), SfMNPV (Sf), HaMNPV (Ha), HzSNPV (Hz), and SeMNPV (Se) are shown. Phage lambda DNA (λ) digested with HindIII was used as a molecular weight standard and as a control for the hybridizations. The right panels in the Figures are Southern blot hybridizations of viral DNAs which were transferred to nitrocellulose filters from the corresponding agarose gels, to the following probes: Figure 1A-S. littoralis MNPV-³²P-DNA; Figure 1B-S. exigua MNPV-³²P-DNA; Figure 1C-A. californica MNPV ³²P-DNA fragment HindIII-V; hybridization was carried out at 65°C as described (ref). Figure 2 A-C: Digestion with XhoI restriction endonuclease. Details as described for Figure 1.

NPV
E c o R I

Sl λ Ac Sf Ha Hz λ Se Sl λ Ac Sf Ha Hz λ Se

³²P-labelled: Sl MNPV-DNA

Figure 1A

^{32}P -labelled: Se MNPV-DNA

Figure 1B

^{32}P -labelled : Ac Hind III V-DNA

Figure 1C

^{32}P -labelled: SL MNPV-DNA

Figure 2A

³²P-labelled : Se MNPV-DNA

Figure 2 B

^{32}P -labelled : Ac Hind III V-DNA

Figure 2C

Table 2. Hybridization of Viral EcoRI DNA Fragments^a

DNA Fragment Designation	Viral DNA on Blot											
	S. littoralis			S. frugiperda			H. armiger			H. zea		
	S. littoralis lit. exlg. Hind III-V	S. littoralis lit. exlg. Hind	S. littoralis lit. exlg. Hind	S. frugiperda lit.	S. frugiperda lit. exlg. Hind	S. frugiperda lit. exlg. Hind III-V	H. armiger lit. exlg. Hind	H. armiger lit. exlg. Hind III-V	H. armiger lit. exlg. Hind	H. zea lit. exlg. Hind	H. zea lit. exlg. Hind III-V	A. californica lit. exlg. Hind III-V
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	+	+	+	+	+	+	+	+	+	+	+	+
H	+	+	+	+	+	+	+	+	+	+	+	+
I	+	+	+	+	+	+	+	+	+	+	+	+
J	+	+	+	+	+	+	+	+	+	+	+	+
K	+	+	+	+	+	+	+	+	+	+	+	+
L	+	+	+	+	+	+	+	+	+	+	+	+
M	+	+	+	+	+	+	+	+	+	+	+	+
N	+	+	+	+	+	+	+	+	+	+	+	+
O	+	+	+	+	+	+	+	+	+	+	+	+
P	+	+	+	+	+	+	+	+	+	+	+	+
Q	+	+	+	+	+	+	+	+	+	+	+	+
R	+	+	+	+	+	+	+	+	+	+	+	+
S	+	+	+	+	+	+	+	+	+	+	+	+
T	+	+	+	+	+	+	+	+	+	+	+	+
U	+	+	+	+	+	+	+	+	+	+	+	+
V	+	+	+	+	+	+	+	+	+	+	+	+
W	+	+	+	+	+	+	+	+	+	+	+	+
X	+	+	+	+	+	+	+	+	+	+	+	+
Y	+	+	+	+	+	+	+	+	+	+	+	+
Z	+	+	+	+	+	+	+	+	+	+	+	+
a	+	+	+	+	+	+	+	+	+	+	+	+
b	+	+	+	+	+	+	+	+	+	+	+	+
c	+	+	+	+	+	+	+	+	+	+	+	+
d	+	+	+	+	+	+	+	+	+	+	+	+
e	+	+	+	+	+	+	+	+	+	+	+	+
f	+	+	+	+	+	+	+	+	+	+	+	+

^a Overexposed radioautographs were used for the detection of weak homologies.

+ Positive hybridization.

- Hybridization note detected.

* Positive hybridization in more than 1:1 ration.

• Two bands not well separated.

? Preliminary identification.

Table 3. Hybridization of Viral XhoI DNA Fragments.^a

DNA Fragment Designation	Viral DNA on Blot											
	S. littoralis			S. exigua			S. frugiperda			H. armiger		
	S. litt. lit.	S. litt. E2 Hind III-V	S. litt. exig. Hind III-V	S. exig. lit.	S. exig. E2 Hind III-V	S. exig. Hind III-V	S. frug. lit.	S. frug. lit.	S. frug. exig. Hind III-V	H. arm. lit.	H. arm. exig. Hind III-V	H. arm. exig. Hind III-V
32P-DNA Probes												
	S. littoralis			S. exigua			S. frugiperda			H. armiger		
	S. litt. lit.	S. litt. E2 Hind III-V	S. litt. exig. Hind III-V	S. exig. lit.	S. exig. E2 Hind III-V	S. exig. Hind III-V	S. frug. lit.	S. frug. lit.	S. frug. exig. Hind III-V	H. arm. lit.	H. arm. exig. Hind III-V	H. arm. exig. Hind III-V
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	+	+	+	+	+	+	+	+	+	+	+	+
H	+	+	+	+	+	+	+	+	+	+	+	+
I	+	+	+	+	+	+	+	+	+	+	+	+
J	+	+	+	+	+	+	+	+	+	+	+	+
K	+	+	+	+	+	+	+	+	+	+	+	+
L	+	+	+	+	+	+	+	+	+	+	+	+
M	+	+	+	+	+	+	+	+	+	+	+	+
N	+	+	+	+	+	+	+	+	+	+	+	+
O	+	+	+	+	+	+	+	+	+	+	+	+
P	+	+	+	+	+	+	+	+	+	+	+	+
Q	+	+	+	+	+	+	+	+	+	+	+	+
R	+	+	+	+	+	+	+	+	+	+	+	+
S	+	+	+	+	+	+	+	+	+	+	+	+
T	+	+	+	+	+	+	+	+	+	+	+	+
U	+	+	+	+	+	+	+	+	+	+	+	+
V	+	+	+	+	+	+	+	+	+	+	+	+
W	+	+	+	+	+	+	+	+	+	+	+	+
X	+	+	+	+	+	+	+	+	+	+	+	+
Y	+	+	+	+	+	+	+	+	+	+	+	+
Z	+	+	+	+	+	+	+	+	+	+	+	+
a	+	+	+	+	+	+	+	+	+	+	+	+
b	+	+	+	+	+	+	+	+	+	+	+	+
c	+	+	+	+	+	+	+	+	+	+	+	+

^a Symbol designation on Table 1.

III. Replication of S. littoralis NPV in Continuous Cell Lines in Culture.

Two continuous cell lines of Spodoptera littoralis were established in the Department of Entomology, Texas A&M University and at the Weizmann Institute of Science, Israel. Several different inocula (see Table 1) of S1MNPV, representing viruses from different geographical regions were used successfully to initiate infection of these cells. Inocula included purified virus preparations, infectious hemolymph and homogenates of diseased larvae.

Initially, the replication of three geographic isolates of S. littoralis NPV (17) was compared in three insect cell lines.

The continuous cell lines of S. littoralis, UIV-SL-573 and HPB-SL-26, were obtained from Dr. D.L. Knudson (Yale University, New Haven, CT., USA) and from Prof. H.G. Miltenburger (Darmstadt, Germany), respectively. The cells were maintained as described previously (9, 22). The cell line of S. frugiperda, IPLB-SF-21, was obtained and maintained as described (18).

S1MNPV obtained from diseased larvae collected in Bet-Shean basin (isolate-B); Harod Valley (isolate-S) and Kinerot (isolate-D), were used as inocula (17). Using restriction endonuclease analyses and Southern blot hybridization (17), the viral DNAs of S1MNPV-B and S1MNPV-S were shown to be identical to the viral DNA of S1MNPV-T the more commonly occurring virus in Israel. From S1MNPV-B, virus isolated from polyhedra by alkali treatment was used as the inoculum; from S1MNPV-S, frozen hemolymph was obtained from diseased larvae. As stated previously, S1MNPV-D shows little sequence homology with the other isolates (17). Log phase S. littoralis UIV-SL-573 cells ($1-2.5 \times 10^5$) in 24 well microtiter plates (Costar) were infected with S1MNPV at 28°C as described (18). Cells were observed with an inverted light microscope. Polyhedra were detected in infected cells after 20-24 hrs. postinfection (p.i.) with maximum polyhedra production observed on the 4th

day. Infection of cells with either S1MNPV -B or -S, resulted in an appearance of many polyhedra per cell (Fig. 3A). After infection with S1MNPV-D, few cells were infected and most of the infected cells contained fewer than 10 polyhedra (Fig. 3B). The supernatant fluids containing the NOV were clarified by centrifugation (6000 xg for 5 min.) and the supernatants were (a) used as inocula for S. littoralis cells; (b) used for identification of the viruses produced in cells in vitro by ^{32}P labeling viral DNA (18); and (c) used for comparative studies of infection of different cell lines by S1MNPV.

Replication of S1MNPV was compared in 2 cell lines of the host insect S. littoralis (UIV-SL-573; HPB-SL-26) and a cell line from S. frugiperda (IPLB-SF-21). Cells of each line were seeded in wells (1.5 to 2×10^5 cells per well), infected with the non-occluded virus form (NOV) of S1MNPV isolates -B, -S at a M.O.I. of 1 to 5 PFU (plaque forming unit) per cell under the same conditions as described. Infection resulted in production of many polyhedra in most infected cells. Levels of infection were high (90% of cells contained polyhedra) in S. littoralis UIV-SL-573 cells (Fig. 3A) but relatively low in the other cell lines tested. Small polyhedra-like bodies were observed only in about ten percent of S. littoralis HPB-SL-26 cells. The most noticeable change in HPB-SL-26 cell line after infection (2-7 days p.i.) was hypertrophy of many of the cells (Fig. 4. B,D). Such changes were not observed in either control or mock-infected control cells (Fig. 4. A,C). Infection of S. frugiperda IPLB-SF-21 cells under the same conditions, with S1MNPV -B or -S, resulted in much lower number of infected cells (less than three percent). In contrast to the formation of many polyhedra in cells infected with S1MNPV -B or -S, infection with S1MNPV-D (NOV from passages 1 to 5) resulted in the production of mostly few polyhedra per cell, and the percentage of cells containing polyhedra was much lower in all of the cell lines tested (Figs. 3.

B,C; 4D). In cells which contained only few polyhedra, several large cuboidal polyhedra were often detected (Fig. 3C).

The observations reported herein confirm a previous report on replication in vitro of SLMNPV in the continuous cell line UIV-SL-573 obtained from ovary tissue of its host S. littoralis (21). High yield of polyhedra and NOV (10^6 - 10^7 P.F.U./ml) were obtained after infection of this cell line with SLMNPV -B or -S. Low levels of SLMNPV production in the S. littoralis HPB-Sl-26 cell line were confirmed by the production of polyhedra after infection with each of the three SLMNPV isolates and by the infection of S. littoralis cell line UIV-SL-573 with NOV harvested from infected HPB-SL-26 cells. These results differ from those reported by Roder (30).

Legends to Figures

Figure 3: a, b - S. littoralis UIV-SL-573 cells infected with: a. SLMNPV isolate-B (3d p.i.). Most cells contain many polyhedra per nucleus (MP), a small percentage of cells contain less than 10 polyhedra (LP). Figure 1b. Figure 2b SLMNPV isolate-D (6d p.i.). A small percentage of cells are infected and contain few polyhedra (LP).

c. S. frugiperda IPBL-SF-21 cells infected with SLMNPV isolate-D (6d p.i.). A small percentage of cells contain polyhedra. Of these, most contain few polyhedra (LP) while some contain many polyhedra per cell (MP). Bar=40 um.

Figure 4: S. littoralis HPB-SL-26 cells: A. uninfected control cells; B. cells infected with SLMNPV isolate-B (5d p.i.); c. "mock infected" with media harvested from uninfected cells; d. cells infected with SLMNPV isolate-D (7d p.i.). After infection enlarged cells (arrows) are seen. Infection with SLMNPV isolate-B resulted in a small percentage of cells containing many small polyhedra-like bodies (MP), while infection with SLMNPV isolate-D resulted in cells containing mainly few polyhedra (LP). Bar=40 um.

Figure 3A

Figure 3C

Figure 3B

Figure 4A

Figure 4B

Figure 4C

Figure 4D

IV. Analysis of genotypic variation in geographic isolates of Spodoptera littoralis MNPV.

There are now several reports in which restriction enzyme fragment patterns of baculovirus DNAs have been compared (36). The restriction patterns of viruses from subgroups A, B, and C are not similar, which suggests that there may not be extensive DNA homology between them; in contrast, there are several examples of baculoviruses from the same subgroup that have similar, but not identical, restriction fragment patterns. The best characterized of these are the MNPVs of Autographa californica and Rachoplusia ou; about 50% of the restriction enzyme fragments are of the same size and they share more than 95% DNA homology under stringent conditions. Another example is the NPVs of Heliothis zea (6, 19); studies have shown that H₂SNPV restriction fragment patterns are similar to the closely related SNPVs of Heliothis virescens, Heliothis armiger, and Heliothis punctata. These studies demonstrate that although certain baculovirus strains can be closely related, there can be distinct genetic markers differing among wild isolates of the closely related baculoviruses, thus representing genetically similar strains. Another approach has been to analyze genotypic variation existing within a given wild isolate: for example, by plaque purification of a wild isolate of A. californica NPV, a variety of genotypic variants and mutants have been identified from several laboratories (36 and references contained therein). Several variants have been mapped, and serial passage mutants as well as a variety of insertion and deletion mutants have been isolated from A. californica, and Spodoptera frugiperda (18, 33).

Any discernable patterns of genetic variation in the baculovirus genome of a given wild isolate continue to be of interest as a means of elucidating the stability of the genome to mutation and recombination and as indicators of

naturally occurring biological variation existent within a baculovirus wild isolate. In addition, there is a continued search for specific kinds of biological variation that may be considered as reliable phenotypic markers for any genetic differences in closely related strains from plaque purified isolates. In these studies, we attempted to evaluate the genetic variation existing in wild isolates recovered from different geographical regions in Israel, and decided to analyze several of those isolates for genotypic variation existent within them. Of the many isolates collected from differing geographical areas (Table V), about 95% were induced to replicate in cell culture. The following data is representative of those viral isolates for which we were successful in producing viral infection in vitro and from which we were able to obtain viral plaques under an agarose overlay (43).

Extracellular virus was produced from each wild type isolate by infection and subsequent serial passage in S. littoralis cell cultures. The resultant virus preparation was then diluted and plaque purified using standard plaque assay methods (43). Twenty plaques were picked from each wild type, and each plaque was subsequently purified through two additional plaque assays, then propagated in cell cultures for 1-4 passages before infecting cells for restriction analysis of resulting viral DNAs. A total of 22 wild isolates and 340 plaque purified isolates were examined in this study; viral DNA was successfully purified from 361 of these and compared by restriction analysis.

Viral DNAs were purified by a modification of the method reported by Smith and Summers (36). Cell monolayers were established by seeding 25 cm² Corning tissue culture flasks with 1.2×10^7 SLC cells and incubating 1 hour for attachment. The cells were then inoculated with extracellular virus at a multiplicity of infection of 5 to 10 PFU per cell in 0.5 ml medium. After gently rocking the flasks for 1 hour, the inoculum was removed and replaced by 4 ml fresh medium with 10% FBS.

At 4 to 7 days post infection, cells were resuspended in the medium, then centrifuged at 300 g for 10 min. The cell pellets were rinsed twice with cold PBS, then resuspended in 0.03M Tris, pH 7.5, 0.01M magnesium acetate, 1% Nonidet P-40, and vortexed at 10 min. intervals for 30 min. to disrupt cells. Nuclei were pelleted at 1000g for 10 min., then resuspended in 0.01M Tris, pH 7.5, 0.01M EDTA, 0.2M KCl, and made 2% with respect to sodium lauryl sarcosinate. After incubation for 15 min. at 65°C, proteinase K was added to a final concentration of 50 ug/ml and the samples were incubated at 45°C for 4 hours. Samples were then extracted with equal volumes of buffer-saturated phenol and chloroform:isoamyl alcohol (24:1). DNA was ethanol precipitated from the aqueous layer, pelleted by centrifugation, rinsed with 90% ethanol and resuspended in sterile distilled water.

EcoRI restriction enzyme was purchased from Bethesda Research Labs (Bethesda, MD) and all digests were incubated for 3 hours at 37°C in the recommended buffer. Enzyme activity was stopped by addition of 2 ul of 0.1M EDTA, 50% glycerol, 0.5% bromphenol blue. Samples were then electrophoresed in a 0.75% agarose horizontal slab gel for 18 hours at 50V. Gels were photographed under long wave UV illumination using Kodak Plus X Pan film.

V. Results and Discussion

Kislev and Edelman (17) describe two different strains of Spodoptera littoralis NPVs in their survey of six isolates from various geographical regions in Israel. These two different polyhedrosis viruses do not show any significant degree of homology by DNA hybridization. We expanded these studies to the analysis of 21 isolates of NPVs infecting Spodoptera littoralis populations of a more diverse geographical distribution in Israel (Table V). From the survey, we were able to confirm the presence of two different NPVs [which we describe as S1MNPV-A (Kislev and Edelman's isolate T) and S1MNPV-B (Kislev and Edelman's isolate D)] and demonstrate that, of the wild isolates, there was no apparent correlation of geographical distribution with either virus strains (see Table V and Figs. 7 and 8).

When comparing infectivity and plaque phenotype characteristics between types A and B, distinct differences were noted between the two strains (Table V). However, it is known that observed polyhedral plaque phenotype can be influenced by a variety of factors, including: degree of virus replication, amount of polyhedrin synthesis, the number of polyhedra produced per cell, the size and shape of polyhedra and the percentage of cell population observed to produce polyhedra after infection. Comparisons of isolates from types A and B show that with some isolates more than 95% of the cells contained polyhedra, whereas with others less than 2% of the S. littoralis cell population was obviously infected. Not only were those observations made comparing the two different NPVs, but similar plaque phenotype variation could be observed when comparing only the wild isolates within strain A or within strain B. This considerable variation of plaque phenotype within each strain is a good indication of genetic differences existent among the different isolates of each strain, although few distinct and reliable genetic markers (i.e.

restriction endonuclease fragment patterns) could be detected (see REN patterns, wild isolates Fig. 5).

Each of the isolates induced to propagate in cell culture was observed for up to seven days, and differences in infected cell morphology, polyhedra production and apparent rate of infection were noted (Table V). With few exceptions, the wild isolates classified by REN patterns as type A yielded faster rates of infection, (90-100% infected at 4 days) more polyhedra per cell, and more cells of the culture (75-95%) showing polyhedra. Infection with type B isolates, in contrast, generally produced slower infection rates (up to 7 days to reach 90-100% infected), fewer polyhedra per cell and fewer cells containing any polyhedra (0-2%). Because some of these isolates produced few to no polyhedra, they were at first believed to be uninfectious (See Table V). However, careful observation of some cells inoculated with those isolates did show the characteristic CPE of infected insect cells which do not produce polyhedra, and when the DNA from these infected cells was analyzed, characteristic restriction enzyme fragment patterns were routinely observed.

The presence of submolar fragments was not obvious in a majority of wild isolates, as has been noted for Autographa californica and Spodoptera frugiperda in previous studies. However, an analysis of plaque purified isolates from each of the wild type strains did detect a number of genotypic variants present within the virus populations (Tables VI and VII, Fig. 6). Genotypic variants were identified in 7 of the 17 wild isolates which were plaque-purified; three of these variants were found in the A strain and the remainder in the B strain. All of the variants were different from each other and from the wild type strains. Four different variants were obtained from isolate 9, a type B sample collected in Rehovot, which was the only isolate showing a mixture of genotypic variants. These variants comprised from 5-75%

of all the plaques examined for these wild isolates, indicating a large population of naturally occurring mutants of S1MNPV. One or more of these mutants, upon further study, may provide some advantage over the wild type strains for use in biological control.

Figure Legends

Figure 5: EcoRI restriction digest patterns of the 21 wild type isolates of S. littoralis virus examined. Isolates are divided into groups (A and B) based on general restriction pattern similarities, and into subgroups (+ and -) based on growth characteristics (+ types exhibited rapid infection rates with many polyhedra produced; - types exhibited slow infection rates with few to no polyhedra produced). Wild isolates 17, 18, 19 and 21 (not shown) and wild isolate 20 (not shown), gave digest patterns identical to isolates 13 and 4, respectively.

Figure 6: EcoRI restriction digest patterns of the 7 isolates which yielded one or more variants upon plaque purification, along with the variants found. Groups correspond to those in Figure 5.

Figures 7 and 8: Map of Israel indicating sites from which infected larvae were collected. Circles represent type A isolates and squares represent type B isolates. All numbers correspond to the isolate numbers used in the previous figures and text. Locations shown are approximate; see Table V for more accurate description of collection sites.

Figure 5

Fig re 6

FIGURE 7

Figure 8

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Table V

Isolate Number	Collection Site	Region Number	REN Pattern	Infection Type
①	Bet She'an Basin	8	A	+
②	Harod Valley	9	A	+
③	Hula Basin	3	A	+
④	Ashkelon Region	34	A	+
⑤	AKKO Region	18	A	+
⑥	Bet She'an Basin	8	A	+
⑦	Rehovot	28	A	-
⑧	Harod Valley	9	A	-
⑨	Rehovot	28	B	+
⑩	Kinerot	6	B	+
⑪	Rehovot	28	B	-
⑫	Kinerot	6	B	-
⑬	Kinerot	6	B	-
⑭	AKKO Region	18	B	-
⑮	Kinerot	6	B	-
⑯	AKKO Region	18	B	-
⑰	AKKO Region	18	B	-
⑱	Bet She'an Basin	8	B	-
⑲	Tel Aviv	30	B	-
⑳	Lod Region	27	A	+
㉑	Kinerot	6	B	-
22	Northern Region	38	N	N

"+" Indicates many cells with polyhedra

"-" Indicates few cells with polyhedra

"N" Indicates not done; Isolate could not be propagated in cell cultures.

Table VI

Isolate Number	REN Pattern	Number of Plaques Examined	Number of Variants	Percent Variants
2	A	20	5	25%
3	A	20	1	5%
8	A	20	1	5%
9	B	20	15	75%
12	B	16	2	12.5%
14	B	20	1	5%
15	B	20	3	15%

Table VII

Only isolate 9 had more than one type of variant;
the four types found and their frequencies were:

<u>Type</u>	<u>Number</u>	<u>% of variants</u>	<u>% of all plaques</u>
1	4	26%	20%
2	2	13%	10%
3	5	33%	25%
4	4	26%	20%

E. Evaluation of the Research Achievement

1. Field surveys and collection of virus isolates were carried out at 22 locations in 16 regions. In addition, virus isolates were collected from insect colonies spontaneously infected in the laboratory.
2. Insect cell cultures for many of the isolates were set up and carried through several generations, infected by the same virus isolate and the viruses were purified.
3. In our initial studies, identification of viral DNAs from S. littoralis larvae from 6 locations was made using restriction digest patterns and DNA blot hybridization. Two viruses, and a naturally occurring variant of the more common virus isolate, were found.
4. Repeated samples of S. littoralis larvae were collected from several locations and the viral DNA was identified. It was found that in the same field different MNPVs may exist in S. littoralis larval populations.
5. The DNA homology between S. littoralis NPV and five other baculovirus was studied. S. littoralis showed significant DNA hybridization with S. frugiperda and S. exigua NPVs, but very little with the other NPVs.
6. Two continuous cell lines from S. littoralis were obtained and established in our laboratories, and a plaque assay was developed.
7. An insect tissue culture laboratory was established in the Weizmann Institute of Science, Israel.
8. Infection and propagation in cultured S. littoralis cells was successful with most of the wild type SLMNPV isolates. The geographical distribution and the properties of infection in cultured cells were compared for the two viruses.
9. By plaque purification of several wild isolates, additional cloned genotypic variants were identified.

F. Description and Results of Cooperation

1. Virus isolates of S. littoralis MNPV collected in Israel were transferred to Texas A&M University.
2. Two Spodoptera littoralis cell lines were obtained from other laboratories and established at Texas A&M and the Weizmann Institute of Science, Israel.
3. Dr. Kislev received training in methodology of insect tissue culture and molecular biology of baculovirus DNAs in Texas, and later established an insect tissue culture laboratory in the Weizmann Institute.
4. Genomic relationships between S. littoralis MNPV-DNA and five baculoviruses collected in the USA were studied by DNA blot hybridization.
5. DNA fragments with sequence homology to the polyhedrin gene of Autographa californica MNPV were identified for the viruses isolated in Israel and the USA.
6. Host range studies of replication of S. littoralis viruses in a few cell lines were carried out in both countries.
7. Replication of the S. littoralis viruses in tissue culture were induced from several wild type viral isolates. A plaque assay was developed and 17 wild isolates were evaluated for the presence of genotypic variants.

G. Benefit to Agriculture

1. The isolation and identification of two distinctly different nuclear polyhedrosis viruses from Spodoptera littoralis larvae, collected from several areas in Israel, has documented the presence of these two viruses in S. littoralis insect populations. A significant number of genotypic variants were shown to exist in the wild type virus populations; thus, it may be possible to isolate and select more efficient strains of NPV for S. littoralis control from these which exist in nature. It is also possible that biological differences related to virulence may exist within this population of plaque purified genotypic variants. Alternatively, additional variants can be induced using recombinant DNA techniques. The replication of S. littoralis NPV in cell culture makes any of these approaches feasible. The studies presented herein are only the first step in this direction.

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