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MOLECULAR BIOLOGY OF *ANTICARSIA GEMMATALIS* BACULOVIRUS

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Anticarsia gemmatalis, the velvetbean caterpillar, is a lepidopterous pest capable of causing serious defoliation of soybeans. The *A. gemmatalis* nuclear polyhedrosis virus (AgNPV) was isolated the first time in Peru from dead insects collected in alfalfa (Steinhaus & Marsh, 1962). In Brazil a baculovirus from the same insect was found in dead larvae collected from soybean fields in the region of Campinas, SP (Allen & Knell, 1977) other isolates were found in other parts of the country (Carner & Turnipseed, 1977; Corso et al., 1977).

It is important in molecular biology research to be able to grow baculoviruses in insect cell lines. It is also important to have homologous cell lines to the insect host in which a given virus replicates for host range and virulence studies. For these reasons a cell line from *A. gemmatalis* was established to study AgNPV molecular biology.

Embryos from 2 and 3 day old eggs of *A. gemmatalis* were crushed through a 100 micron sieve to initiate cell lines in TC-100 medium with 10% fetal bovine serum. Although the first subculturing was done 9 days later it required about a year for the cells to divide rapidly for experiments. The cell lines were characterized by isozyme analysis and by restriction enzyme analysis of mitochondrial DNA to show that the cell lines were from the host insect (Sieburth & Maruniak, 1988a). The cells were designated UFL-AG-286.

We then determined how the UFL-AG-286 cells would replicate the AgNPV, *Autographa californica* NPV (AcNPV), and *Spodoptera frugiperda* NPV (SfNPV) baculoviruses. AgNPV and AcNPV replicated well in the UFL-AG-286 cells while SfNPV did not. AgNPV produced $10^{7.6}$ mean tissue culture doses ($TCID_{50}$) and AcNPV produced $10^{5.8}$ $TCID_{50}$ per ml at 48 hours post infection in UFL-AG-286 cells. However, the optimal time for harvesting infectious nonoccluded virus was at 72 hours post infection. The AgNPV was also shown to replicate well in IPLB-SF21AE *(Spodoptera frugiperda)* cells. It produced $10^{7.5}$ $TCID_{50}$ per ml at 48 hours post infection. AgNPV polyhedra were formed in significant quantities in both cell lines, too (Sieburth & Maruniak, 1988b).

Wild-type baculoviruses have been shown to consist of a population of genotypic variants by plaque purifying the virus in cell culture and then by analyzing the genomes with restriction enzymes (Lee & Miller, 1978; Smith & Summer, 1978). Analysis of plaque purified virus can indicate whether the virus is changing and if these changes result in changes in virulence of the virus. The genetic stability of the *Anticarsia gemmatalis* baculovirus is of interest because there are reports that other baculoviruses are subject to genetic changes that may affect the virulence. It has been shown that few polyhedra variants which are less virulent than many-polyhedra genotypes occur upon passage of baculoviruses either in larvae or cell culture (Fraser & Hink, 1982).

AgNPV was obtained originally from diseased velvetbean caterpillar larvae near Campinas, Brazil in 1972 (Allen & Knell, 1977) and used in Florida in 1979. Hemolymph from infected *A. gemmatalis* was serial plaqued three times in IPLB-SF-21AE cells (Maruniak and colleagues, 1984). Extracellular virus from these plaque purified isolates was inoculated into IPLB-SF-21AE cells in 24 cluster well dishes, and the DNA was labelled with $^{32}PO_4$. The viral DNAs were restricted and electrophoresed (Maruniak and colleagues, 1984). When the DNAs from the plaque purified isolates were compared with PstI and HindIII, six different genotypic profiles were detected and the sizes are given in Table I. The prototype isolate, AgNPV-2 comprised one-third of the 24 original plaques. The AgNPV 1-6 plaque isolates are different from each other by inser-

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TABLE I

AgNPV 1-6 and Lambda DNA HindIII or PstI fragments sizes in kilobases

Band	HindIII						
	Ag1	Ag2	Ag3	Ag4	Ag5	Ag6	Lambda
A	16.12	16.12	16.12	16.12	16.12	16.12	
B	14.92	14.92	14.92	14.92	14.92	14.92	
B'			12.97				
C	10.35	10.35	10.35	10.35	10.35	10.35	
D	9.42	9.42	9.42	9.42	9.42	9.42	9.42
E	8.65	8.65	8.65	8.65	8.65	8.65	
F	8.64	8.64	8.64	8.64		8.64	
F'				8.31	8.31	8.31	
F"			7.94				
G	7.45	7.45	7.45	7.45	7.45	7.45	
G'			7.20	7.20			
G"				6.97			
H	6.19	6.19	6.19	6.19	6.19	6.19	6.56
I	5.46	5.46	5.46	5.46			
J	4.99	4.99	4.99	4.99	4.99	4.99	
J'			4.60	4.60	4.60	4.60	
K	4.14	4.14	4.14	4.14	4.14	4.14	4.37
L	3.67	3.67	3.67	3.67	3.67	3.67	
M		3.52		3.52			
N	3.43	3.43	3.43	3.43	3.43	3.43	
O	3.15	3.15	3.15	3.15	3.15	3.15	
P	2.90	2.90	2.90	2.90	2.90	2.90	
Q	2.81	2.81	2.81	2.81	2.81	2.81	
R	2.79	2.79	2.79	2.79	2.79	2.79	
S	2.71	2.71	2.71	2.71	2.71	2.71	
T	2.66	2.66	2.66	2.66	2.66	2.66	
U	2.66	2.66	2.66	2.66	2.66	2.66	
V	2.62	2.62	2.62	2.62	2.62	2.62	
W	2.01	2.01	2.01	2.01	2.01	2.01	
X	1.92	1.92	1.92	1.92	1.92	1.92	

Band	PstI					
	Ag1	Ag2	Ag3	Ag4	Ag5	Ag6
A	17.71	17.71	17.71	17.71	17.71	17.71
B	15.02	15.02	15.02	15.02	15.02	15.02
B'						13.95
C	10.38	10.38	10.38	10.38	10.38	10.38
D	8.02	8.02	8.02	8.02	8.02	8.02
D'				6.77		
E	6.57	6.57	6.57	6.57	6.57	6.57
F		6.38	6.38	6.38	6.38	6.38
G	5.88	5.88	5.88	5.88	5.88	5.88
H	5.33	5.33	5.33	5.33	5.33	5.33
I	4.99	4.99	4.99	4.99	4.99	4.99
J	4.89	4.89	4.89	4.89	4.89	4.89
K	4.21	4.21	4.21	4.21	4.21	4.21
L	3.72	3.72	3.72	3.72	3.72	3.72
L'				3.30		
M	3.08	2.08	3.08	3.08	3.08	3.08
N	3.05	3.05	3.05	3.05	3.05	3.05
O	2.90	2.90	2.90	2.90	2.90	2.90
P	2.81	2.81	2.81	2.81	2.81	2.81
Q	2.69	2.69	2.69	2.69	2.69	2.69
R		6.64		6.64	6.64	
S	2.55	2.55	2.55	2.55	2.55	2.55
T	2.44	2.44	2.44	2.44	2.44	2.44
T'				2.41	2.41	
U	2.31	2.31	2.31	2.31	2.31	2.31
V	1.91	1.91	1.91	1.91	1.91	1.91
W	1.64	1.64	1.64	1.64	1.64	1.64

The extracellular virus of the six plaque-purified genotypic variants of AgNPV from Campinas was titered in microtest wells (Knudson & Tinsley, 1974) using four cell lines: TN-368, IPLB-SF-21AE, and IAL-PiD *(Plodia interpunctella)*, (Table II).

tions or deletions of viral DNA. We have shown by hybridization of radioactive cellular DNAs to these viral isolates that no chromosomal DNA inserted into the viral genomes (Maruniak & Gowan, unpublished) as has been shown for few polyhedra mutants of AcNPV (Fraser et al., 1983).

The AgNPV variants replicated to the highest titer in IPLB-SF-21AE cells. They also replicated fairly in IAL-PiD and TN-368 cells. There were mostly minor changes in titers within a cell type for the AgNPV variants. However, there was up to 1.5 logs of infectious virus difference between AgNPV-1 and AgNPV-2 in IAL-PiD cells. These data indicate that cell culture can be used as a model for studying virulence.

The prototype AgNPV-2 DNA was mapped with the restriction enzymes BamHI, Bg1II, BstEII, EcoR1, and HindIII. The construction of the map and presentation of molecular weight values for each restriction fragment was published (Johnson & Maruniak, 1989). Mapping of the restriction sites was accomplished by double reciprocal digestion of fragments and by criss-cross hybridization. The DNA was 133,000 bases and circular doubled-stranded. The mapping of AgNPV restriction sites provides a solid foundation to allow the localization of viral gene products on the physical map.

The physical map indicates the order of restriction enzyme fragments as they appear sequentially on the viral genome. Once the order is known any changes in size of restriction fragments allows a precise location of the change on the genome. This in turn permits the analysis of this altered region to determine if it is nonessential or an essential coding region. Transcriptional mapping of RNA is facilitated by having the physical map.

The physical map of AgNPV showed that it is distinct from other baculoviruses by sizes and order of DNA fragments. At the same time it had some similarities to other baculoviruses. We used hybridization of an AcNPV DNA fragment containing the polyhedrin gene to several restriction enzyme digests of AgNPV to locate the homologous polyhedrin coding region in AgNPV. The AcNPV polyhedrin DNA hybridized primarily to AgNPV HindIII-G fragment (Johnson & Maruniak, 1989). The DNA sequence for the AgNPV polyhedrin is being determined in this cloned DNA fragment (Zanotto & Maruniak, unpublished).

In addition to locating genes on the AgNPV physical map, changes in DNA are being localized from baculovirus that has been applied over nine years on soybeans in Brazil. We are attempting to determine whether any changes occurring are due to cellular DNA transposing into the viral genome and whether these changes decrease the virulence of the virus to *A. gemmatalis* larvae. A 1985 preparation of AgNPV obtained from Dr. Flavio Moscardi, (EMBRAPA, Londrina), was plaque-purified as mentioned above. When the DNAs from these plaque isolates were analyzed by restriction enzyme digestion, many genomic changes had occurred in more of the samples than in the AgNPV 1-6 which is from a 1979 preparation. This indicates that the AgNPV is changing over the years as it is applied in a field situation. Again these changes appear to be insertions and deletions of DNA, but we do not known if it is viral DNA that is inserting or cellular DNA. Recombination events could also occur between the applied virus and existing virus in the field. These possibilities are being tested. Additionally, we are testing the virulence of these isolates in cell culture and larvae to try to correlate changes in DNA structure with changes in biological activity. By knowing which genes are responsible for virulence will allow us to modify these genes or similar genes from other baculoviruses.

An AgNPV baculovirus with an increased biological activity to an alternate host *Diatrea saccharalis* has been studied through collaboration with Dr. Octavio Pavan (UNICAMP, Campinas). Initially AgNPV had an LD_{50} of $> 10^5$ polyhedra per third instar larvae; after 20 passages in *D. saccharalis* the LD_{50} dropped to about 100 polyhedra per third instar larvae. Since there was the possibility of a contaminating virus in the initial inoculum that would increase in frequency upon passage in *D. saccharalis*, plaquing the virus to get a pure virulent isolate was necessary. Polyhedra from the AgNPV-F20 passed in *Diatrea* larvae were used to infect *A. gemmatalis* larvae. Hemolymph was collected from diseased larvae and plaqued in IPLB-SF-21AE cells three times. Viral DNAs from 34 isolates were screened by restriction enzyme analysis with HindIII and PstI to obtain unique isolates. Thirteen unique genotypes could be distinguished from the parental virus.

TABLE II

$TCID_{50}$ titers of AgMNPV 1-6 in three cells lines

Cell line	Clones					
	Ag1	Ag2	Ag3	Ag4	Ag5	Ag6
IPLB-SF-21AE	5.43	5.47	6.17	5.73	5.93	5.87
TN-368	4.73	4.63	4.80	4.97	4.73	4.73
IAL-PiD	4.47	5.20	5.33	4.67	4.97	5.07

The differences were limited to insertions or deletions within 1 to 3 different DNA fragments. The 13 AgNPV-*Diatrea* plaque isolates with unique genotypes, AgNPV-2D (1979, Florida from Campinas) plaque isolate and the AgNPV-F20 in *Diatrea* were tested for replication and virulence in cell cultures. Infectious extracellular virus was titered and used to infect IPLB-SF-21AE cells at a multiplicity of infection (MOI) of 1. At 48 hours the cell culture supernatant was collected and titered in IPLB-SF-21AE or UFL-AG-286 cells by end point dilution microtitrations (Danyluk & Maruniak, 1987; Sieburth & Maruniak, 1988b). Three replications were done and the mean $TCID_{50}$ calculated. Serial passage in *Diatrea* of AgNPV did not reduce the virulence of the plaque isolates to either *Anticarsia gemmatalis* cells (UFL-AG-286) or to *Spodoptera frugiperda* cells (IPLB-SF-21AE). In fact, the AgNPV-*Diatrea* plaque isolates generally replicate better in cells than the non-selected AgNPV.

Dr Octavio Pavan, UNICAMP, showed AgNPV-*Diatrea*-7 plaque isolate caused 69% mortality compared to 55% mortality for the unplaqued AgNPV-F20 in *Diatrea.* All other plaque isolates and the AgNPV-2D prototype gave no mortality at this dose (about an LD_{50}). At the same time the isolates virulent to *D. saccharalis* remained virulent to *A. gemmatalis* larvae at 10^2 polyhedra per third instar larva. AgNPV-*Diatrea*-7 caused 75% mortality which was comparable to AgNPV-2D prototype. The production of polyhedra per gram of infected larva showed good amounts of polyhedra were produced in *Diatrea* larvae.

AgNPV-*Diatrea*-7 had a change in HindIII-F DNA fragment that was different from the other plaque isolates. This is the only change we can currently identify in the genome that can correlate with an increased virulence to *Diatrea.* We propose to sequence this fragment of viral DNA and the AgNPV-2D prototype to determine how the change in structure could result in this increase in virulence. Additionally, we propose to transfer the changed DNA fragment to the AgNPV prototype to determine if we can increase its virulence to *Diatrea.* Finally, we will attempt to decrease the virulence of AgNPV-*Diatrea* by replacing the DNA fragment corresponding to increased virulence with the corresponding AgNPV prototype DNA fragment.

In summary, the *Anticarsia gemmatalis* nuclear polyhedrosis virus and its host insect are a good system for studying the molecular biology and ecology of baculoviruses. An *in vitro* system is available for plaque purifying the virus and for analyzing events at the molecular level. Since the virus has been applied in the field for many years, the evolution of changes in a baculovirus genome can be studied. The physical map permits localization of these changes for subsequent cloning, sequencing and transcriptional analysis. The availability of an *Anticarsia* baculovirus with an altered virulence is a breakthrough in the biology of baculoviruses. The identification of these changes will allow the genetic manipulation and improvements of other baculoviruses used throughout the world, thereby enhancing their usefulness.

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