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Variation in *Cydia pomonella* Granulosis Virus Isolates and Physical Maps of the DNA from Three Variants

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SUMMARY

Cydia pomonella granulosis viruses (CpGV) from seven different sources in Europe, America and New Zealand were compared by restriction enzyme analysis. Most samples were indistinguishable from the Mexican isolate (CpGV-M). Isolates from Russia (CpGV-R) and England (CpGV-E) showed small genotypic differences. CpGV-E was shown to be a mixture of two variants, E1 and E2. CpGV-E1 was indistinguishable from CpGV-M. A physical map of CpGV-M was constructed for the enzymes *Eco*RI, *Bam*HI, *Hin*dIII, *Sma*I and *Apa*I. A comparison of fragment profiles allowed construction of maps for CpGV-R and CpGV-E2. Relative to CpGV-M, CpGV-R had a single deletion of 2·4 kbp and CpGV-E2 was modified in one area resulting in an additional *Eco*RI site, a shift in a *Bam*HI site and in total about 1 kbp more DNA. The map was orientated by locating the granulin gene using the cloned granulin gene from *Trichoplusia ni* GV as a probe. There was no significant difference between the infectivities of the Mexican, Russian and English isolates for neonate larvae.

INTRODUCTION

Cydia pomonella granulosis virus (CpGV) has received considerable attention as an agent for controlling its host insect which is a pest in orchards throughout the world (for review, see Payne, 1982). Most field trials appear to have been carried out with a virus strain (CpGV-M) isolated in Mexico in 1963 (Tanada, 1964) although another isolate (CpGV-R) was obtained from a field-collected larva in 1974 in Russia (Harvey & Volkman, 1983). A third isolate (CpGV-E) was obtained in England from diseased larvae in a laboratory stock of *C. pomonella* at the University of Reading. Several other isolates of granulosis virus from *C. pomonella* have been made in various countries although it appears that in many cases the virus had been introduced at an earlier date and it was probable that the virus was not an endemic strain.

Two sources of the Mexican isolate, maintained in different laboratories, and the Russian isolate have previously been compared by restriction enzyme analysis of their DNA, polyacrylamide gel electrophoresis of virus particles and bioassay (Harvey & Volkman, 1983). These workers showed differences in one or two DNA fragments between the Russian and Mexican isolates with five of the six restriction enzymes they used. Together with hybridization studies, these results indicated a very close relationship between the two isolates. One enzyme, *Xho*I, showed a single fragment difference between the two viruses (CpGV-MB and CpGV-MD) derived from the Mexican isolate; this was interpreted as showing that a slight modification had occurred to the genome of one of the virus stocks. We have re-examined this difference and show evidence suggesting that the additional *Xho*I band was due to incomplete digestion and therefore that the two virus stocks are probably identical.

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In this paper, we also show physical maps of the CpGV-M, CpGV-R and CpGV-E genomes for *Eco*RI, *Bam*HI, *Sma*I, *Hin*dIII and *Apa*I restriction endonucleases. Although profiles for CpGV-M and CpGV-R DNA digests with *Eco*RI and *Bam*HI appeared to be essentially the same as those previously published (Harvey & Volkman, 1983), analysis of our results showed additional fragments in both digests and also significantly different fragment size estimates. The position of the granulin gene was located on the map by hybridization studies using the cloned granulin gene from *Trichoplusia ni* GV.

The conclusions from our bioassays also differed substantially from those of Harvey & Volkman (1983) who reported that CpGV-R was over 70 times less virulent than CpGV-MD for first instar larvae. We attempted to measure LD_{50} values with neonate larvae using the method of Hughes & Wood (1981) but were unable to induce more than a small proportion of larvae to imbibe virus suspension. Consequently, we adopted a LC_{50}-type assay (Payne, 1981) which, under standardized conditions, is very reproducible and allows accurate comparisons between viruses. Using this method we were unable to detect any significant difference in virulence between the three isolates.

METHODS

CpGV production and bioassay. The Mexican strain of virus was obtained from Dr J. Huber, Institut für Biologische Schädlingsbekämpfung, Darmstadt, F.R.G., the Russian strain from Dr J. Harvey, University of California, Berkeley, U.S.A. and the English isolate from Dr F. Hunter, University of Reading, U.K. Samples of CpGV were also obtained from Dr R. Jaques, Canada Department of Agriculture, Harrow, Ontario, Canada, Dr J. Longworth, DSIR Entomology Division, Auckland, New Zealand, Dr K. Deseo, Centro di Studio di Fitofarmacia, Università di Bologna, Italy and Dr G. Benz, Entomologisches Institut der ETH, Zürich, Switzerland. Virus was propagated, purified and bioassayed as described by Payne (1981).

DNA purification. DNA was purified by dissolving virus capsules in 0·05 M-Na_2CO_3, 1% SDS, extracting the solution three times with buffered phenol and dialysing it extensively against 10 mM-Tris–HCl, 1 mM-EDTA, pH 8·0.

Restriction endonuclease analysis. DNA was digested with restriction endonucleases under the conditions recommended by the suppliers (Bethesda Research Laboratories and NBL Enzymes). Digests were electrophoresed at 60 V for 16 h in submerged 3 mm-thick 0·7% agarose gels in 40 mM-Tris–acetate, 20 mM-sodium acetate, 1 mM-disodium EDTA, pH 7·8. After staining in ethidium bromide (0·5 μg/ml), gels were photographed through a Kodak Wratten No. 4 (yellow) filter on Polaroid 544 film using a transilluminator at 300 nm. Fragment sizes were computed using a BASIC version of the program given by Schaffer & Sederoff (1981) and *Hin*dIII, *Xho*I or *Bgl*I fragments of λ DNA as standards.

Individual fragments were prepared by excising ethidium bromide-stained bands from 0·6% low gelling temperature agarose (Sigma, Type VII) gels. The DNA was concentrated by re-casting the gel fragments into tubes sealed at one end with dialysis membrane and electrophoresing the DNA towards the dialysis membrane. After brief reversal of the current, the end few millimetres of gel containing all the DNA were removed and equilibrated in 10 mM-Tris–HCl, 1 mM-EDTA, pH 8·0. Subsequent digestion was done in the presence of melted agarose and then fragments were electrophoresed, usually in vertical 0·7% agarose gels at 150 V for 3 to 4 h.

Location of granulin gene. A *Sal*I fragment of *T. ni* GV DNA containing the entire granulin gene (Akiyoshi *et al.*, 1985) was labelled with ^{32}P by nick translation (Maniatis *et al.*, 1982) and allowed to hybridize to blots of CpGV DNA fragments (Howley *et al.*, 1979).

RESULTS

Identification of isolates

The DNA from samples of CpGV obtained from Canada, New Zealand, Italy, Switzerland, England, Russia and Mexico was compared by digestion with *Eco*RI and *Bam*HI. The first four of these were all indistinguishable from the Mexican strain. Only the English and Russian isolates showed slightly different DNA fragment profiles and only these two together with the Mexican isolate were studied in more detail.

Electrophoresis of digests of CpGV-M and CpGV-R with *Eco*RI, *Bam*HI, *Xho*I, *Sma*I and *Hin*dIII gave profiles essentially similar to those shown by Harvey & Volkman (1983) although a number of additional fragments, *Eco*RI H and I, *Bam*HI E and M and *Xho*I M and N, were observed (Table 1). The CpGV *Eco*RI profile of Burgess (1983) also resembled our results but lacked the *Eco*RI H fragment. If the estimated total genome size is recalculated from the data of

Table 1. *Size of fragments produced by digestion of CpGV-M with restriction endonucleases*

Fragment	Size* of fragments after digestion with					
	*Eco*RI	*Bam*HI	*Xho*I	*Sma*I	*Apa*I	*Hin*dIII
A	27·9	25·2	34·7	91·4†	108·6†	92·5†
B	22·2	25·2	23·7	32·4	17·1	33·5
C	17·5	15·4	13·0	2·2	0·3	
D	12·6	9·5	8·5			
E	10·4	9·5	8·1			
F	6·4	7·0	7·2			
G	5·0	6·4	6·7			
H	5·0	5·9	5·4			
I	4·9	5·7	5·1			
J	4·8	5·2	4·2			
K	3·9	3·9	3·6			
L	3·1	3·1	1·5			
M	1·7	3·1	1·3			
N	1·1	1·2	0·8			
Total	126·5	126·3	123·8	126	126	126

* Expressed in kbp.
† Size estimate based on total genome size of 126 kbp.

Harvey & Volkman (1983) and Burgess (1983) allowing for the undetected fragments, the values obtained are in close agreement with our estimate of 126 kbp.

Analysis of the CpGV-M DNA with *Xho*I usually gave a profile similar to that for CpGV-MB (Harvey & Volkman, 1983) although some additional small fragments were detected (Table 1). However, in one digest the additional 4·8 kbp band which had been found in CpGV-MD was observed. Further digestions with different amounts of enzyme (Fig. 1) showed that the 4·8 kbp band could be obtained as a result of incomplete digestion. Since this band appeared before any other partial digest bands were evident it seems that the *Xho*I site contained in this band is less readily digested than other *Xho*I sites.

Digestion of CpGV-E DNA with *Eco*RI and *Bam*HI produced all the fragments present in the CpGV-M digest plus two additional sub-molar fragments for both enzymes (Fig. 2). This indicated that the virus was probably a mixture of different variants and attempts were made to obtain clones of the individual components in the mixture. Neonate larvae were allowed to feed on a diet containing 2×10^3 CpGV-E capsules/ml for 24 h and then individually transferred to a fresh diet in microtitre plates and maintained for 10 days at 26 °C. At this concentration of virus only about 5% of larvae died and there is a high probability that these larvae had acquired only a single infectious unit (i.e. probably a single virus capsule). Each dead larva was used to infect about 100 fifth instar larvae from which virus was purified. Restriction enzyme analysis of the DNA from several clones (type CpGV-E1) obtained in this way gave profiles identical to CpGV-M but two clones (type CpGV-E2) yielded fragment profiles in which the submolar bands of CpGV-E were equimolar (Fig. 2). In these clones the first *Eco*RI band and the second and eighth *Bam*HI bands were greatly reduced. Repeated re-cloning of CpGV-E2 failed to decrease further the amount of CpGV-E1 virus and frequently resulted in CpGV-E1 becoming the major component again. Thus, it was not possible to obtain a clone of CpGV-E2 which remained stable on passage.

Mapping of CpGV DNA

A physical map of the CpGV-M genome was determined largely by reciprocal digestion of DNA fragments using *Eco*RI and *Bam*HI endonucleases. Fig. 3 shows a complete profile of the fragments produced by a double digestion with *Eco*RI and *Bam*HI. Table 2 indicates which double digestion fragments were obtained from each single digest fragment. Digestion of co-migrating fragments *Bam*HI D and E, *Bam*HI L and M, and *Eco*RI G and H gave bands which could only be grouped in one possible combination but the *Eco*RI digest of *Bam*HI A and B could

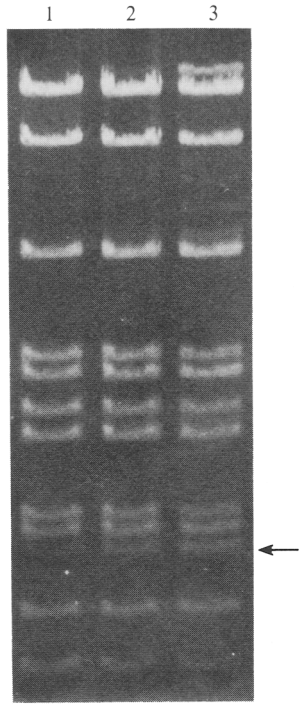

Fig. 1

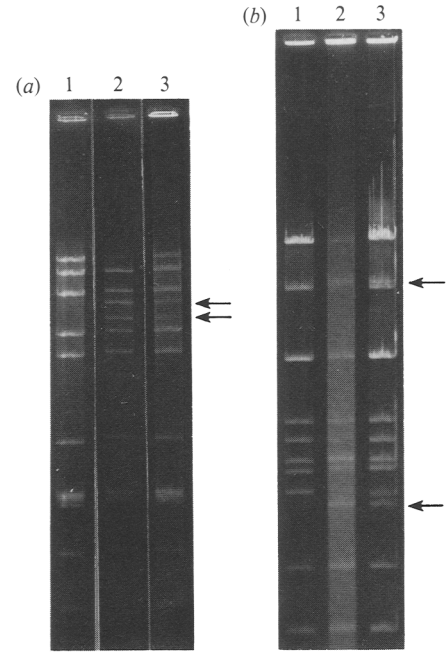

Fig. 2

Fig. 1. Agarose gel electrophoresis of CpGV-M DNA fragments generated by digestion for 1 h of 1 μg DNA with 1 (lane 1), 0·5 (lane 2) or 0·25 (lane 3) units *Xho*I. The 4·8 kbp partial digest band is arrowed.

Fig. 2. Agarose gel electrophoresis of DNA of CpGV-E1 (lanes 1), CpGV-E2 (lanes 2) and CpGV-E (lanes 3) digested with *Eco*RI (*a*) or *Bam*HI (*b*). Arrows indicate sub-molar bands in CpGV-E which were found in CpGV-E2 but not CpGV-E1.

Table 2. *Fragments resulting from digestion of* Eco*RI or* Bam*HI fragments of CpGV-M DNA with* Bam*HI or* Eco*RI*

*Eco*RI fragment	Resultant double digest fragments	*Bam*HI fragment	Resultant double digest fragments
A	a, g, l, q, w	A	f, c, j
B	d, e, p	B	b, d
C	b, r	C	a
D	c	D	h, r, v, x
E	f, s	E	e
F	j, t	F	l, n
G	h	G	i, t
H	i, α	H	m, q
I	k, y, β	I	o, u, z, β
J	m, v	J	g
K	n, z	K	k
L	o	L	p, α
M	u	M	s, y
N	x	N	w

not be interpreted unambiguously. This was resolved by isolating *Bam*HI A from a *Bam*HI–*Hin*dIII double digest, in which *Bam*HI B is cut by *Hin*dIII, and then digesting the single fragment with *Eco*RI.

From these results it was possible to map the positions of most *Eco*RI and *Bam*HI fragments. The order of *Eco*RI G and N, and L and M, and *Bam*HI J, C and N was determined by partial

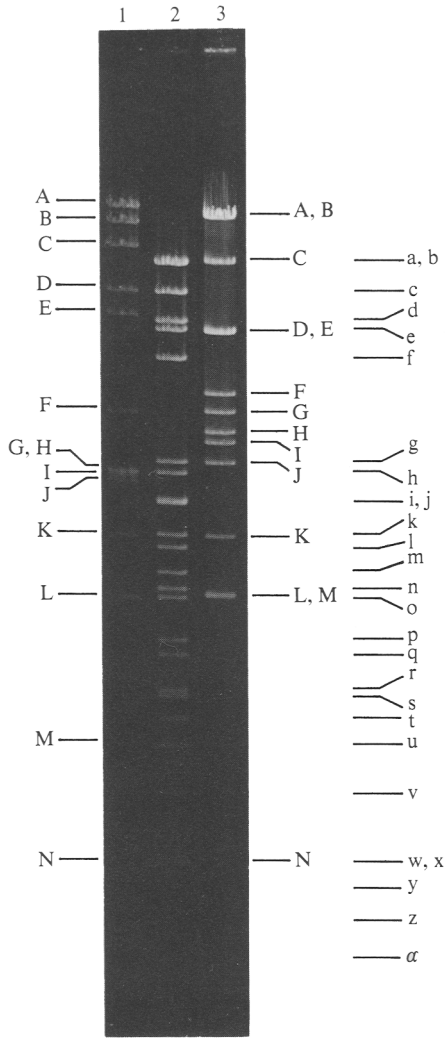

Fig. 3

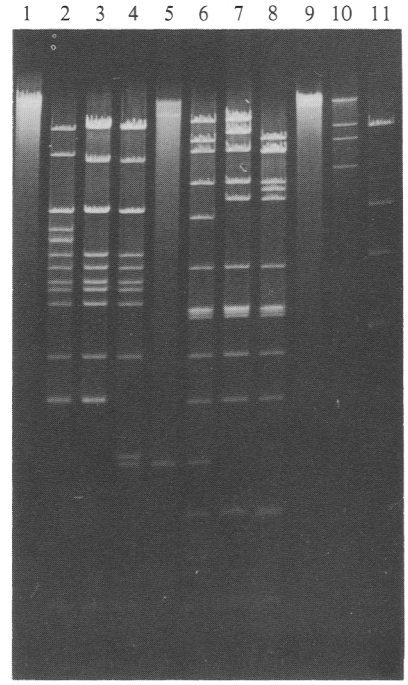

Fig. 4.

Fig. 3. Agarose gel electrophoresis of CpGV-M DNA digested with *Eco*RI (lane 1), *Eco*RI + *Bam*HI (lane 2) and *Bam*HI (lane 3). Fragments from single enzyme digests are designated using upper case letters; lower case and Greek letters are used for the double digest (Table 2).

Fig. 4. Agarose gel electrophoresis of single and double digests of CpGV-M. Lane 1, *Hin*dIII: lane 2, *Hin*dIII + *Bam*HI; lane 3, *Bam*HI; lane 4, *Sma*I + *Bam*HI; lane 5, *Sma*I; lane 6, *Sma*I + *Eco*RI; lane 7, *Eco*RI; lane 8, *Hin*dIII + *Eco*RI; lane 9, *Hin*dIII. Size standards: lane 10, *Sma*I digest of *A. californica* NPV DNA; lane 11, *Hin*dIII digest of λ DNA.

digestion of *Bam*HI D and I and *Eco*RI A respectively. The order of *Bam*HI J, C and N was confirmed by comparing the position of *Apa*I and *Hin*dIII sites on these fragments with their position on *Eco*RI A.

Since *Hin*dIII cuts CpGV DNA at only two sites and *Apa*I and *Sma*I each cut at only three sites, it was possible to map these sites by analysing double digests using each of these enzymes with *Eco*RI and with *Bam*HI (Fig. 4).

Restriction enzyme analysis of CpGV-R and CpGV-E2 DNAs showed that the genomes are very closely related to CpGV-M and it may be assumed that fragments of these two genomes will

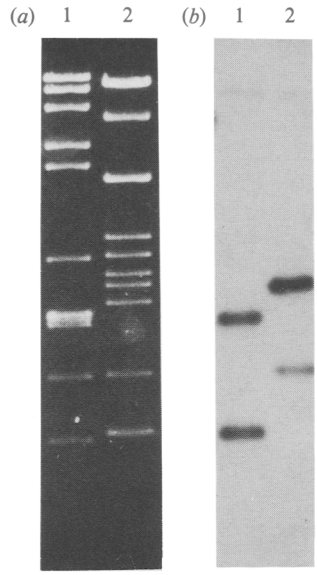

Fig. 5. (*a*) Agarose gel electrophoresis of CpGV-M DNA digested with *Eco*RI (lane 1) or *Bam*HI (lane 2). (*b*) Autoradiogram of blot of same gel after hybridization to ^{32}P-labelled *T. ni* GV granulin gene fragment.

map in positions corresponding to co-migrating CpGV-M fragments. This assumption is strongly supported by the very high degree of hybridization between all fragments of CpGV-M DNA and CpGV-R DNA (Harvey & Volkman, 1983). Maps of CpGV-R and CpGV-E2 were therefore derived from the CpGV-M map by comparing single and double digests of the two related DNAs with those of CpGV-M DNA. CpGV-R DNA contained all the restriction sites, for the five enzymes, found in CpGV-M DNA but there was a 2·4 kbp deletion in the overlapping region of *Eco*RI C and *Bam*HI B. The differences between CpGV-M and CpGV-E2 also occurred in a single region of the genome contained within the CpGV-M *Eco*RI A fragment and resulted in an additional *Eco*RI site, a shift in the position of a *Bam*HI site, and in total about 1 kbp more DNA.

Orientation of CpGV map

Since the proposal of Vlak & Smith (1982) for orientation of the *Autographa californica* nuclear polyhedrosis virus (NPV) genome, most NPV genome maps have used a restriction site close to the polyhedrin gene as a zero point. The most appropriate zero point for maps of GV DNAs is therefore the position of the granulin gene. This was located on the CpGV map using a cloned *T. ni* GV DNA *Sal*I fragment (Akiyoshi *et al.*, 1985), containing the entire 742 bp granulin gene, as a probe. With an *Eco*RI digest of CpGV-M DNA, the ^{32}P-labelled probe hybridized to fragments L and also one or more of G, H and I; with a *Bam*HI digest, the probe hybridized to fragments K and I (Fig. 5). This indicated that the CpGV granulin gene contains the restriction sites between *Eco*RI I and L and *Bam*HI K and I and occupies an area of a few hundred bp at the adjacent ends of these four fragments. The *Bam*HI site between fragments K and I was therefore chosen as the zero point of the linearized circular map (Fig. 6).

Dosage–mortality responses

There was no significant difference in the virulence of the three CpGV isolates for *C. pomonella*. LC_{50} values (and 95% confidence limits) were 2·6 (1·6–4·3) $\times 10^3$, 3·3 (1·9–6·3) $\times 10^3$ and 2·5 (1·0–6·0) $\times 10^3$ capsules respectively for CpGV-M, CpGV-R and CpGV-E with slopes of 1·21, 1·03 and 0·90. Although it was not possible to calculate accurate LD_{50} values from these

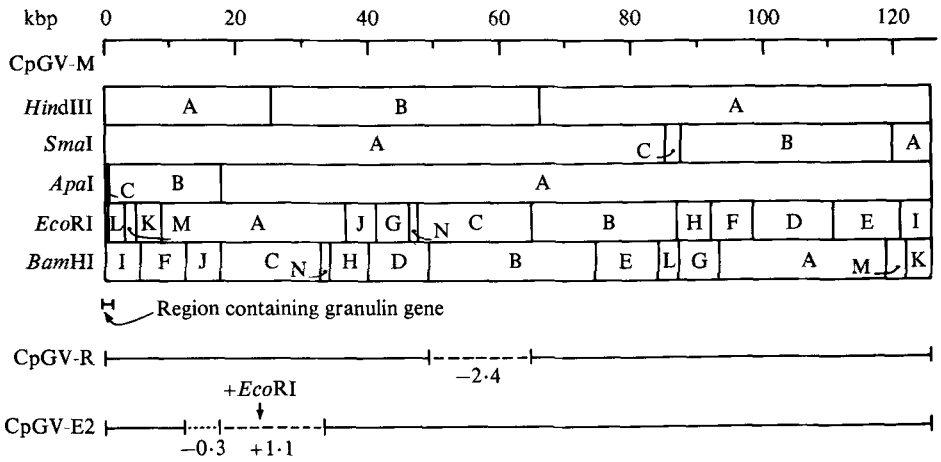

Fig. 6. Linearized physical map of *Hin*dIII, *Sma*I, *Apa*I, *Eco*RI and *Bam*HI restriction sites on the CpGV-M genome. The zero point has been chosen as the *Bam*HI site between *Bam*HI K and I. The approximate position of the granulin gene, within which this site occurs, is indicated. Differences between this map and maps of CpGV-R and CpGV-E2 are indicated. Deletions or insertions occur within the dashed areas and are indicated by their size in kbp. The additional *Eco*RI site in CpGV-E2 is arrowed.

figures, an approximate upper limit can be calculated based on two assumptions. Firstly, only virus acquired during the first 72 h of the assay is capable of causing mortality by the 6th day, since Payne (1981) has shown that even when very high virus concentrations are used no deaths occur within 4 days. Secondly, larvae do not consume more than their own body weight of diet in a 24 h period. These assumptions lead to an estimated maximum LD_{50} value of three capsules for neonate larvae.

DISCUSSION

Although *C. pomonella* is a widely distributed pest which is highly susceptible to infection with GV, there appears to be very little genotypic variation between virus isolates that have so far been obtained, unlike the situation with some other baculoviruses where many variants have been isolated, e.g. *A. californica* multiple nucleocapsid NPV (Smith & Summers, 1979), *Heliothis* single and multiple nucleocapsid NPVs (Gettig & McCarthy, 1982; Williams & Payne, 1984), *Spodoptera frugiperda* multiple nucleocapsid NPV (Maruniak *et al.*, 1984) and *Pieris* GV (Crook, 1981*a*, *b*). This may indicate that CpGV has a particularly stable genome but it seems more likely that most of the virus in laboratories today has originated knowingly or unknowingly from a single isolation made in Mexico in 1963. Because of the life-cycle of the insect, few virus-infected larvae are ever found, even in orchards which have been sprayed with the virus. The three isolates that are distinct differ only slightly and our results suggest that one of these variants (CpGV-E2) may not be capable of independent replication in larvae fed low doses of virus. Although our observations on the digestion of CpGV-M DNA with *Xho*I do not prove that CpGV-MB and CpGV-MD (Harvey & Volkman, 1983) are identical, it certainly appears to be a strong possibility. If this is so, there is no evidence of mutation in virus stocks maintained in different laboratories.

Previous findings (Harvey & Volkman, 1983) indicated that CpGV-R was much less infectious than CpGV-M for *C. pomonella* larvae, especially at the first instar, although it was not clear how LD_{50} values were obtained. Only first instar larvae were used in this work because (due to the life-cycle of the insect) only this stage is likely to be susceptible to virus in the field and also because this instar was reported to show the greatest difference between infectivity of CpGV-M and CpGV-R (Harvey & Volkman, 1983). Because of the small size and feeding habits of first instar larvae it is difficult to feed them known doses. The method of Hughes &

Wood (1981) would have been suitable but neonate *C. pomonella* could not be persuaded to drink from droplets of virus suspension. An LC_{50} method was therefore adopted and this showed no significant differences between the infectivities of the three isolates. The loss of 2·4 kbp of DNA in the CpGV-R genome appeared to have no effect on the replication of the virus.

Although several NPVs have now been mapped, this is the first map of a GV. This virus is particularly interesting not only because of the wide interest in it as a biocontrol agent but also because it is the only GV which has been shown to replicate in cell culture (Naser *et al.*, 1984). This should allow more detailed studies of GV replication and genetics than has previously been possible. A physical map of the virus genome will form the basis for further genetic studies.

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