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Isolation of Genotypic Variants of *Autographa californica* Nuclear Polyhedrosis Virus

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A nuclear polyhedrosis virus (MNPV) isolated from a lepidopteran (*Noctuidae*) insect, *Autographa californica,* was cloned by successive plaque purification using virions containing only one nucleocapsid per envelope as inoculum. The ability to clone the virus by this method was demonstrated by the isolation of nondefective, genotypic variants of the virus with similar but not identical restriction endonuclease fragment patterns. Five distinct variants were identified by genotypic analysis with *Hin*dIII, *Eco*RI, *Sal*I, and *Bam*HI restriction endonucleases. The characteristic genotype of each variant was maintained upon passage in insect larvae. The isolation of these virus variants demonstrates (i) the heterogeneity of the uncloned virus preparation and (ii) the ability to clone MNPVs by plaque purification of media-derived nonoccluded virions. The *A. californica* MNPV is being considered for commercial use as a pesticide in the United States, and the cloning of the virus, in view of the heterogeneity detected, may be advisable. The cloning and genotypic analyses are also significant with regard to understanding the genetic nature of multiply embedded NPVs (those NPVs containing more than one nucleocapsid per envelope in the occluded form of the virus) and indicate that further genetic analysis of these viruses is possible.

Nuclear polyhedrosis viruses (NPVs) are members of the family *Baculoviridae* and have been isolated from invertebrates primarily of the class *Insecta,* order *Lepidoptera* (4). NPVs are natural insect control agents; some NPVs cause lethal epizootic diseases in their host insect populations (3). Recently, serious attention has been given to the use of these viruses as commercially employed biological pesticides (21). The U.S. Environmental Protection Agency has registered three NPVs for commercial use as pesticides; the *Autographa californica* NPV is currently being considered for registration as a pesticide.

NPVs possess a double-stranded, circular, supercoiled DNA genome (19) with a molecular weight of approximately 85 million (1, 2, 5, 13, 16). The DNA genomes replicate within the nucleus and associate with capsid proteins to form rod-shaped nucleocapsids. The nucleocapsids acquire envelopes either by budding through cellular membranes (6, 11) or by an envelopment process within the nucleus itself (6, 18, 20). Enveloped virions in the nucleus may be occluded in a large crystalline protein matrix, the occlusion body, which results in an environmentally stable form of the virus. Within the occlusion bodies of many NPVs, including the NPV of *A. californica,* there are many virions with multiple nucleocapsids contained within a single envelope (17, 24). Viruses of this type are sometimes referred to as MNPV. Virions which bud through cellular membranes and are released from the cell without being occluded are apparently responsible for the spread of NPV infection via the hemolymph within insects and via the media in insect cell cultures (6, 7, 11, 26, 27). Occluded viruses, in contrast, are infectious in larvae by oral administration.

Although plaque assays have been developed for nonoccluded virions of *A. californica* MNPV (9, 10, 28), the possibility of multiple nucleocapsid envelopment poses the question of actually cloning MNPVs (i.e., the ability to develop a virus stock from a single viral DNA genome) by simple plaque purification. There is one example in the literature demonstrating the ability to isolate phenotypically different NPVs by plaque purification (14). These results were obtained with a plaque morphology variant of *Trichoplusia ni* MNPV. Similar attempts at isolating stable plaque morphology variants of *A. californica* MNPV have been unsuccessful to date (8). The *A. californica* MNPV has received considerable attention in relationship to pesticide use owing to the relatively broad host range. The ability to clone this virus is essential for ensuring a uniform virus for pesticide purposes and for the

genetic characterization of the virus.

MATERIALS AND METHODS

Virus source. The MNPV of *A. californica* was originally isolated by Vail et al. (24). In the third passage from the original isolation, M. R. Bell (U.S. Department of Agriculture, Agricultural Research Station, Phoenix, Ariz.) provided the virus in the form of infected *T. ni* (an alternate host) larvae. All passages were through *T. ni* larvae, and the virus had not been plaque purified. Hemolymph from the infected larvae was extracted, diluted in TC-100 basal media (Microbiological Associates), and stored by the method of Vaughn (25). The larval carcasses were frozen at −20°C.

Cell line. The *Spodoptera frugiperda* continuous cell line (IPLB-SF-21) was obtained from D. L. Knudson (Yale University, New Haven, Conn.). The cells were propagated at 27°C in TC-100 basal media supplemented with 0.26% tryptose broth (Difco), 8% fetal calf serum (Gibco), 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Virus inoculum. A stock of *A. californica* MNPV for tissue culture use (passage 1) was prepared by infecting a monolayer of *S. frugiperda* cells with hemolymph from *A. californica* MNPV-infected *T. ni* larvae provided by M. R. Bell. The TC-100 medium was removed from *S. frugiperda* cell monolayers in petri dishes (100 by 20 mm), and 0.2 ml of the hemolymph, diluted 1 to 5 in TC-100 basal media, was applied to cell monolayers. The inoculated cells were incubated at 27°C with intermittent tilting of the dishes for uniform virus distribution. After 1 h of incubation, 10 ml of supplemented TC-100 media was added, and the cells were incubated at 27°C for 40 h. The media were collected and centrifuged at 3,000 × *g* for 20 min, and the supernatant was sterilized by filtration through 0.45-μm HA filters. This passage 1 virus stock was used as inoculum in the first plaque purification step.

Plaque assay. A plaque assay was developed for *A. californica* MNPV on the *S. frugiperda* cell line; the method utilizes 0.5% agarose (Sea Kem) in supplemented TC-100 medium as an overlay. Approximately 3×10^6 *S. frugiperda* cells, in supplemented TC-100 medium, were transferred to petri dishes (60 by 15 mm) and allowed to attach in a monolayer overnight. The medium was removed and the virus inoculum, diluted as appropriate in TC-100 basal medium, was applied to the cell monolayer. The virus was allowed to adsorb to the cells at 27°C for 1 h with intermittent tilting for uniform virus distribution. The cells were then overlaid with 3.0 ml of supplemented TC-100 medium containing 0.5% agarose. Another 3 ml of agarose-containing overlay medium was added 24 to 48 h later for feeding purposes. The overlay medium was prepared by autoclaving 5.0 g of agarose in 95.0 ml of distilled water, cooling the agarose solution to 45°C, and diluting it 1:10 in TC-100 medium preheated to 45°C. The cells were incubated at 27°C in a saturated humidity for 3 to 4 days. The plaques were visualized by staining with a 0.01% solution of neutral red in TC-100 overlay medium for 12 h.

Cloning procedure. Passage 1 virus stock was diluted in basal TC-100 medium to provide 10 PFU or less in the inoculum for each 60- by-15-mm petri dish. The procedure for plaque assay was followed (see above). Twelve plaques were picked from a total of five petri dishes. Each plaque was delivered into 0.5 ml of TC-100 basal medium and pipetted gently to release virus from the agarose overlay. Each of the 12 virus solutions was used for inoculation of cell monolayers to develop a virus stock for the second plaque purification step. The purpose of preparing a virus stock between plaque purification steps is described in Results. The media from each of the 12 infected cultures were collected and centrifuged at 4,000 × *g* for 25 min. The supernatant was collected and centrifuged at 12,000 × *g* for 30 min. The clarified virus suspension was diluted in TC-100 appropriately (10 PFU per inoculum) and plaque purified again by the procedure described above. One plaque was picked from each plate for each of the 12 original virus isolates. Stocks of nonoccluded viruses were then developed for each of the "doubly plaqued" virus isolates. In total, there were six passages of the virus through the *S. frugiperda* cell line from the hemolymph extraction step before large-scale NPV production in suspension culture; two of these passages were plaque purification steps.

NPV production in suspension culture. Occluded NPVs of each of the 12 virus isolates were prepared in suspension cultures of *S. frugiperda* cells. Suspension cultures were prepared by transferring 1.1×10^7 cells from petri dishes into 200 ml of TC-100 in a 500-ml bottle containing a magnetic stirring bar. The cells were incubated at 27°C with continuous gentle stirring action provided by magnetic stirring motors. Cell growth proceeded exponentially under these conditions with a doubling time of 24 h. When the cell density reached 1.7×10^6 cells per ml, the cells were inoculated with virus at a multiplicity of infection of 0.1. Adsorption of the virus was aided by stopping the action of the magnetic stirring bar and intermittently swirling the media for a 1-h period. Infection was allowed to proceed with stirring for 2 to 3 days. The cell suspension was centrifuged at 4,000 × *g* for 25 min, and occluded NPVs were purified from the pellet.

Occluded MNPV purification. Occluded NPVs produced in cell culture were purified in the following manner. The 4,000-×-*g* pellet from cell suspension cultures (see above) was suspended in phosphate-saline buffer (pH 6.2, osmolarity 340 mosM, made by dissolving 0.268 g of $Na_2HPO_4 \cdot 7H_2O$, 1.425 g of KH_2PO_4, 8.2 g of NaCl, and 3.0 g of KCl in 1 liter of distilled water) and recentrifuged at 4,000 × *g* for 25 min. This pellet was suspended in distilled water, and a 2% solution of sodium dodecyl sulfate was added to achieve a final concentration of 0.4% sodium dodecyl sulfate and left overnight at room temperature. The suspension was layered onto 25 to 65% linear sucrose gradients (buffered by phosphate-saline buffer) and centrifuged in an SW27 rotor at 96,000 × *g* for 3 h. The band of occlusion bodies was collected, diluted threefold with distilled water, and centrifuged at 12,000 × *g* for 30 min. The pellet was suspended in 0.25 M NaCl and pelleted again. The pellet was suspended in distilled water. The occlusion bodies were disrupted

by bringing the suspension to 0.05 M Na_2CO_3 and incubating at 27°C for 20 min.

Occlusion bodies were purified in some cases from infected larval carcasses and alkali disrupted as previously described (13).

DNA isolation. The viral DNA was purified, after disruption of the occlusion body with 0.05 M Na_2CO_3, by sodium dodecyl sulfate treatment and phenol extraction as described previously (13). The DNA was dialyzed extensively in 0.01 M Tris-hydrochloride–0.001 M EDTA (pH 7.6) (TE buffer) before restriction endonuclease digestion. The DNA used to establish the fragment patterns of each of the twelve variants (see Fig. 2 through 5) was isolated from occluded viruses produced in cell suspension cultures.

Restriction endonuclease digestion. Digestions of viral DNAs with *Bam*HI, *Sal*I, and *Hin*dIII (13) were carried out in 0.01 M Tris-hydrochloride (pH 7.6), 0.01 M $MgCl_2$, 0.05 M NaCl, and 1 mM dithiothreitol at 37°C. *Eco*RI digestion conditions included 0.1 M NaCl in the reaction mixture rather than 0.05 M NaCl. Sufficient enzyme was added for complete digestion within 3 h. Digestion of λ DNA (isolated from *Escherichia coli* CI 857 S7) was used as a control to ensure proper digestion procedure and molecular weight standards. For estimations of the molecular weights of the viral DNA fragments, additional standards were used including T4 DNA and *Hpa*II fragments of polyoma virus DNA, depending on the size of the fragment analyzed. Estimations of large DNA fragment molecular weights utilized 0.2% horizontal agarose slab gels. Details of gel electrophoresis and visualization of the DNA fragments have been described previously (13).

Tests for infectivity of occlusion bodies. The infectivity of occlusion bodies was tested in *T. ni* larvae during their third instar development stage. *T. ni* were reared by the general method of Treat and Halfhill (23). The larvae were contained in 6-ounce (ca. 177-ml) paraffin-coated paper cups. Approximately 50 g of food in the cups was contaminated with 0.5 ml of 10^8 occlusion bodies per ml obtained from infected suspension cultures. Between 10 and 20 larvae were contained in each cup with the contaminated food, and the larvae were examined for virus disease symptoms. At least 100 larvae were used for each test. Occlusion bodies were isolated from dead larvae as described above, and the virions were released by alkali disruption (0.05 M Na_2CO_3 for 20 min at 27°C). The virions were layered on 25 to 60% sucrose gradients (buffered with phosphate-saline buffer) and centrifuged for 3 h at 96,000 × *g* to test for multiple nucleocapsid envelopment characteristics. The viral DNA was isolated directly from alkali-disrupted occlusion bodies (13) and analyzed by restriction endonuclease digestion as described above.

Electron microscopic visualization of nonoccluded viruses. A drop of viral solution was placed on Formvar-coated grids and air dried. A drop of a 0.2% solution of the nonionic detergent Nonidet P-40 was applied to the grids. The grids were then washed in distilled water for 1 to 2 min, dried, and stained in a 1% phosphotungstic acid solution (pH 6.6) for 1 or 2 min. After staining, the grids were gently rinsed in distilled water. The viruses were examined with a Zeiss E-10 transmission electron microscope.

RESULTS

Development of a plaque assay. When this research was initiated, the generally employed method for plaque purification of NPVs utilized a 0.6% methyl cellulose overlay medium developed by Hink and Vail (10) for titrating *A. californica* MNPV on TN-368 cells. The following limitations of this method were noted in our laboratory: (i) the 0.6% methyl cellulose overlay medium was more fluid than semisolid; (ii) the attachment of TN-368 cells to the surface of petri dishes was loose and easily disturbed in the overlay procedure; (iii) the plaques were microscopic; and (iv) the original procedure suggested the removal of the methyl cellulose overlay medium before plaque visualization. Therefore, we developed a plaque assay procedure utilizing a 0.5% agarose overlay medium on a *S. frugiperda* cell line which attaches more firmly to petri dishes (see Materials and Methods). The cells react well to the overlay medium and survival extends to 10 days, although by 4 days, *A. californica* MNPV plaques are large enough for visualization. Staining with neutral red is achieved without removal of the agarose overlay medium, and plaques are easily picked with a pipette.

Cloning of *A. californica* MNPV. The greatest concern in developing a cloning method for MNPVs was using a virus inoculum that contained virions with primarily only one nucleocapsid per virion. The virions released from *A. californica* MNPV polyhedral occlusion bodies isolated from infected larvae primarily have more than one nucleocapsid per envelope (see Fig. 1B). A small proportion of virions contain singly enveloped nucleocapsids as analyzed by centerifugation in sucrose density gradients and electron microscopy.

Virions released from occlusion bodies in this manner show low infectivity (a low PFU-to-particle ratio) compared to nonoccluded virions (NOV) isolated from the media of infected cell cultures (27). Summers and Volkman reported that virions released into the media of MNPV-infected cell cultures contain primarily one nucleocapsid per envelope (22). Media-derived NOVs were therefore the preferred virus inoculum for cloning purposes.

Since the Summers and Volkman work involved *A. californica* MNPV infections of a *T. ni* cell line, we extended their results concerning single nucleocapsid envelopment of *A. califor-*

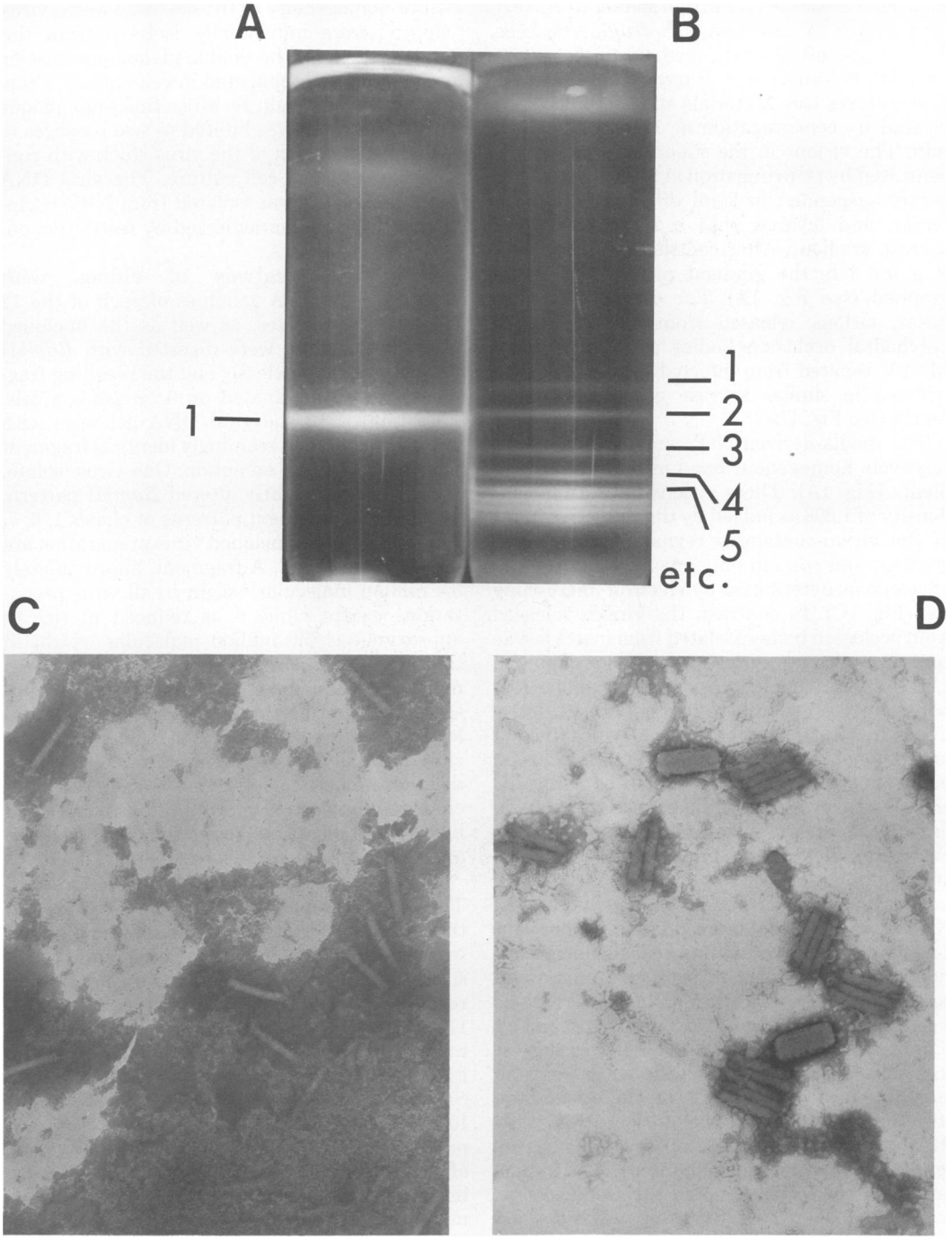

FIG. 1. *Sucrose density gradient analysis and electron microscopic observation of variant forms of A. californica MNPV virions. Virions of A. californica MNPV isolated from media of infected S. frugiperda cell culture (A) are compared to virions of A. californica MNPV released from occlusion bodies (B) on parallel 20 to 60% linear sucrose gradients. The virions were also examined in the electron microscope after mild detergent treatment of virions attached to Formvar-coated grids; A. californica MNPV media-derived nonoccluded viruses (C); and A. californica MNPV virions released from occlusion bodies (D). The larger virus particles in (D) are virions containing multiple nucleocapsids per envelope; the mild detergent treatment did not totally disrupt the envelopes of these virus particles. Magnification is approximately ×40,000.*

nica NPV NOV derived from media to *A. californica* MNPV infection of *S. frugiperda* cells. At 40 h postinfection, the media from *A. californica* MNPV-infected *S. frugiperda* cell suspension cultures (see Materials and Methods) were cleared by centrifugation at 12,000 × *g* for 20 min. The virions in the supernatant were concentrated by centrifugation at 96,000 × *g* for 1 h, gently suspended in 2 ml of phosphate-saline buffer, and layered onto a 25 to 60% linear sucrose gradient. After centrifugation at 96,000 × *g* for 3 h, the gradient profile was photographed (see Fig. 1A). For comparative purposes, virions released from alkali-disrupted polyhedral occlusion bodies of *A. californica* MNPV isolated from infected larvae were centrifuged in similar sucrose gradients concurrently (see Fig. 1B).

The media-derived NOV appear as a single, relatively homogenous band in the sucrose gradients (Fig. 1A). These virions have a buoyant density of 1.208 as judged by the refractive index of the virion-containing region of the sucrose gradient and contain only one nucleocapsid per envelope as determined by electron microscopy (see Fig. 1C). In contrast, the virions released from occlusion bodies isolated from insect larvae contain a discrete series of bands containing 1, 2, 3, 4, 5, etc., nucleocapsids per envelope (denoted by these numbers in Fig. 1B) as determined by electron microscopy. A typical field of unfractionated virions from disrupted occlusion bodies is presented in Fig. 1D. In both Fig. 1C and 1D, the envelopes of the viruses are partially or fully disintegrated by mild detergent treatment after fixation on the grid (see Materials and Methods).

The buoyant density of the media-derived NOV (1.208) does not correspond to the buoyant density of virions containing a single nucleocapsid per envelope released from alkali-disrupted occlusion bodies (1.194). Similar observations were made by Knudson and Tinsley (12) and by Summers and Volkman (22). The latter suggest that the buoyant density differences may be largely due to differences in the membrane structures surrounding the nucleocapsid. Further work to clarify these observations is currently underway in our laboratory. The important point in relationship to the cloning of *A. californica* MNPV is that the media-derived NOV primarily contain only a single nucleocapsid per envelope.

The procedure developed for cloning *A. californica* MNPV, described in Materials and Methods, employs media-derived NOV as an inoculum for plaque purification. The basic procedure of isolating media-derived NOV followed by plaque purification was repeated twice to ensure homogeneity of the clones. Twelve virus "clones" were individually isolated from the original stock by the double plaque purification procedure and propagated in cell culture. Propagation in cell culture after the final plaque purification step was limited to two passages to avoid degeneration of the virus stock with continued passage in cell culture. The viral DNA from each clone was isolated from NPV occlusion bodies and characterized by restriction endonuclease analysis.

Genotypic analysis of clones with *Bam*HI. The DNA genomes of each of the 12 cloned virus isolates, as well as the uncloned virus preparation, were digested with *Bam*HI restriction endonuclease, and the resulting fragments were fractionated on 0.7% agarose gels. Digestion of all the viral DNA genomes with *Bam*HI resulted in seemingly identical fragment patterns, with one exception. One virus isolate, clone 6, had a slightly altered *Bam*HI pattern. The *Bam*HI fragment patterns of clones 1, 6, 7, 9, and 10 and the uncloned virus preparation are presented in Fig. 2. A fragment, approximately 5.4 million molecular weight in all virus preparations except clone 6, is reduced in size to approximately 5.0 million molecular weight in the clone 6 fragment pattern. No additional fragment of 0.4 million molecular weight was observed, suggesting that a small 0.4 million molecular weight region has been deleted from the clone 6 genome. To determine if clone 6 has a deletion and also to determine if any of the other clones contain genotypic alterations undetected by *Bam*HI digestion, restriction endonuclease analysis with other enzymes was performed.

Genotypic analysis of clones with *Sal*I. The DNA genomes of the 12 cloned viruses and the uncloned virus preparation were digested with *Sal*I restriction endonuclease, and the resulting fragments were fractionated on 0.7% agarose gels. Nine of the 12 clones (1, 2, 3, 4, 5, 8, 9, 11, and 12) produced seemingly identical fragment patterns. Alterations were detected in the fragment patterns of clones 6, 7, and 10. The *Sal*I fragment patterns of clones 1, 6, 7, 9, and 10, as well as the uncloned virus preparation, are presented in Fig. 3. Clone 6 is missing a fragment of approximately 1.8 million molecular weight, but has a fragment of approximately 1.4 million molecular weight which is not present in the other *Sal*I fragment patterns. This result is in accord with the *Bam*HI data, which suggested that a 0.4 million molecular weight region has been deleted in clone 6.

Clones 7 and 10 are both missing two fragments of approximately 3.8 million and 2.4 million molecular weight present in the other clones. The absence of the 2.4 million molecular

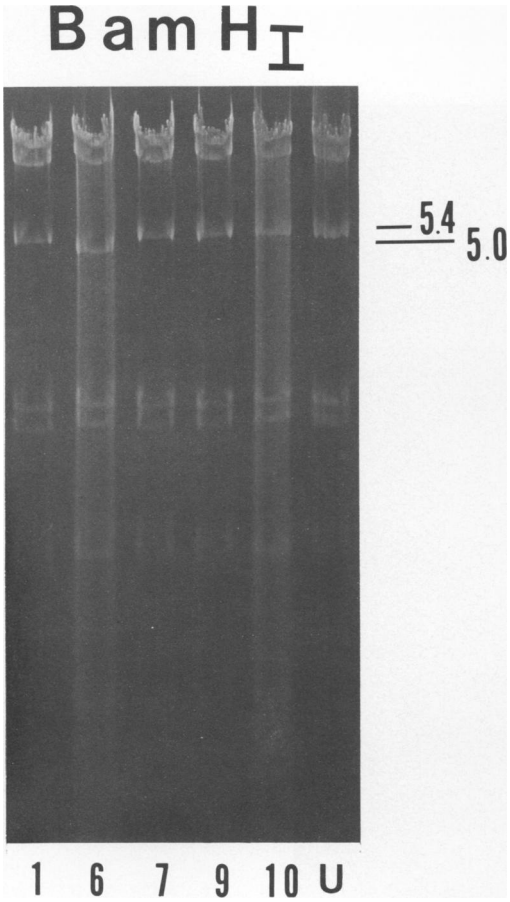

FIG. 2. *BamHI restriction endonuclease fragment patterns of viral DNAs. The viral DNAs of clones 1, 6, 7, 9, and 10, as well as uncloned (U) A. californica MNPV, were purified from occlusion bodies and digested with BamHI restriction endonuclease. The digested DNA samples, containing approximately 2 μg of DNA per 50 μl, were loaded on 0.7% agarose slab gels. Electrophoretic resolution of the DNA fragments was visualized by ethidium bromide staining (13). The numbers at the bottom of the lanes indicate the clone number; the numbers at the side of the gel indicate approximate molecular weights (in millions) of the fragments.*

weight fragment is difficult to observe since it is one of three fragments which comigrate in 0.7% gels; a reduction of staining intensity in that region of the patterns of clones 7 and 10 (Fig. 3) can be observed. The absence of this fragment is more noticeable in acrylamide gels. Clones 7 and 9 also appear to be missing a fragment, approximately 1.2 million molecular weight, which essentially comigrates with one or two other fragments of that size. This is difficult to observe owing to the complexity of the pattern in this region and the low staining intensity of these small fragments. Clones 7 and 10 both have a fragment not present in the fragment patterns of the other clones. The two bands are approximately 6.9 million and 6.7 million molecular weight for clones 7 and 10, respectively. The fragment pattern of the uncloned virus preparation shows two faint bands in the region of the bands unique to clones 7 and 10. The weak staining intensity of these bands in the uncloned virus preparation suggests that clones 7 and 10 are present in nonstoichiometric quantities in the uncloned virus preparation. Extended digestion of the uncloned virus preparation clearly indicates that these faint bands are not simply partial digestion products. One hypothesis to account for these results is that three fragments of 3.8 million, 2.4 million, and 1.2 million molecular weight are linked in the physical map of the *A. californica* MNPV. Several alterations in this region, including a deletion of approximately 0.5 million or 0.7 million molecular weight for clones 7 and 10, respectively, would result in a fusion product of 6.9 million and 6.7 million molecular weight, respectively, with the concomitant loss of individual fragments of 3.8 million, 2.4 million, and 1.2 million molecular weight. One prediction of this hypothesis would be the observation of deletions in clone 7 and clone 10 in other restriction endonuclease patterns. The fact that deletions were not detectable in the *Bam*HI pattern is accounted for by the presence of a very large fragment of approximately 59 million molecular weight in the *Bam*HI digests. A change in size of up to 4 million molecular weight in this *Bam*HI fragment would be virtually undetectable by gel electrophoresis. To further investigate the clones, additional restriction endonucleases were employed.

Genotypic analysis of clones 7 and 10 with *Eco*RI. Digestion of the DNA of the 12 clones and the uncloned virus preparation with *Eco*RI restriction endonuclease resulted in four distinct fragment patterns. The patterns of clones 1, 2, 3, 4, 5, 8, 9, 11, and 12 appeared to be identical. The *Eco*RI fragment patterns of clones 1, 6, 7, 9, and 10 and the uncloned virus preparation are presented in Fig. 4. In the clone 6 pattern, a fragment of approximately 9.5 million molecular weight was altered in its mobility to a size of approximately 9.1 million molecular weight; this agrees with the *Bam*HI and *Sal*I data suggesting a 0.4 million molecular weight deletion in clone 6.

Clone 7 has a clearly visible fragment of approximately 5.3 million molecular weight which is not present in the other clones. Clone 7 is missing a fragment of approximately 5.8 million molecular weight. The absence of this fragment is difficult to detect owing to the comigration of

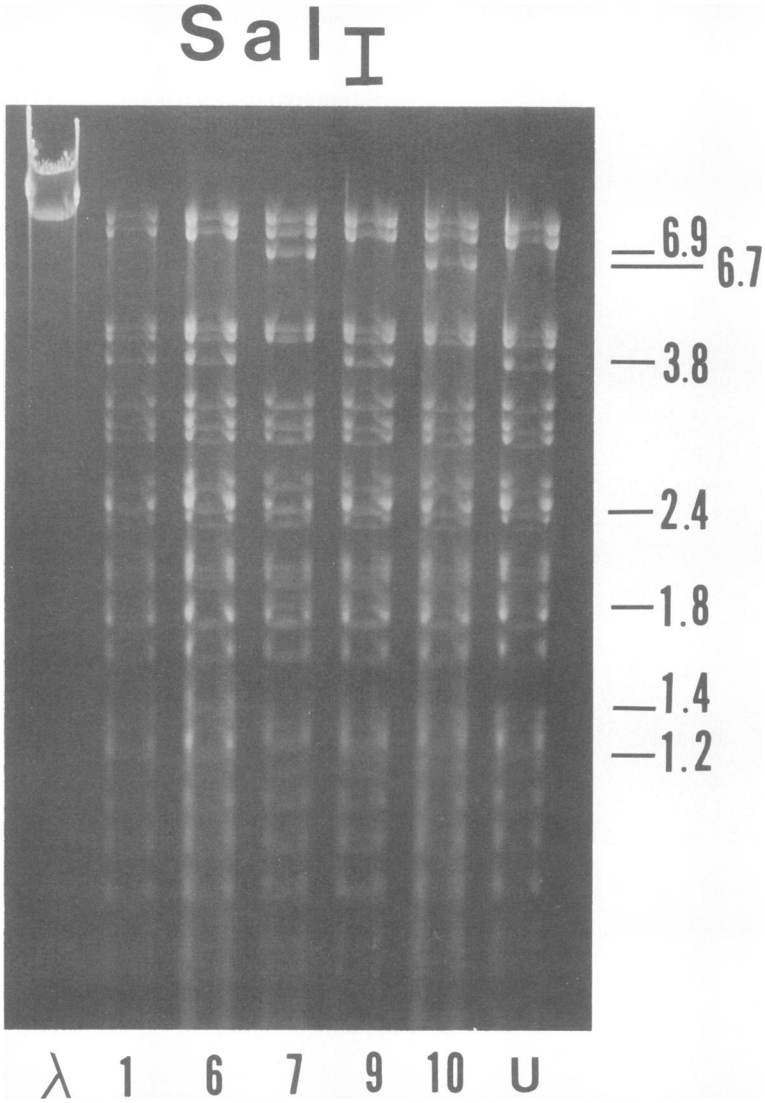

FIG. 3. *SalI restriction endonuclease fragment patterns of viral DNAs. The viral DNAs of clones 1, 6, 7, 9, and 10, as well as uncloned (U) A. californica MNPV, were purified from occlusion bodies and digested with SalI restriction endonuclease. A sample of lambda bacteriophage DNA* (λ) *was also digested with SalI and included as a standard on the 0.7% agarose gel. Electrophoresis was performed in a similar fashion as in Fig. 2, and details were described previously (9). Numbers at the side of the gel refer to the approximate molecular weight of DNA fragments in millions. The numbers or letters at the bottom of the lanes refer to clone numbers, lambda DNA* (λ), *or uncloned virus (U).*

two other fragments of approximately 5.6 and 5.9 million molecular weight in this region. However, the absence can be discerned by the clearer resolution of the 5.6 million and 5.9 million molecular weight fragments. The difference can be accounted for by a 0.5 million molecular weight deletion in the 5.8 million molecular weight fragment, giving rise to a 5.3 million molecular weight fragment in clone 7. This observation supports the deletion hypothesis suggested by the *Sal*I data. The loss of the 5.8 million molecular weight fragment is also observed in clone 10, as expected. Owing to the larger deletion in clone 10, the molecular weight of the 5.8 million molecular weight fragment is shifted to 5.1 million molecular weight and thus comigrates with a 5.1 million molecular weight fragment present in all the clones. The presence of this extra 5.1 million molecular weight fragment is detected by an increased staining intensity in the 5.1

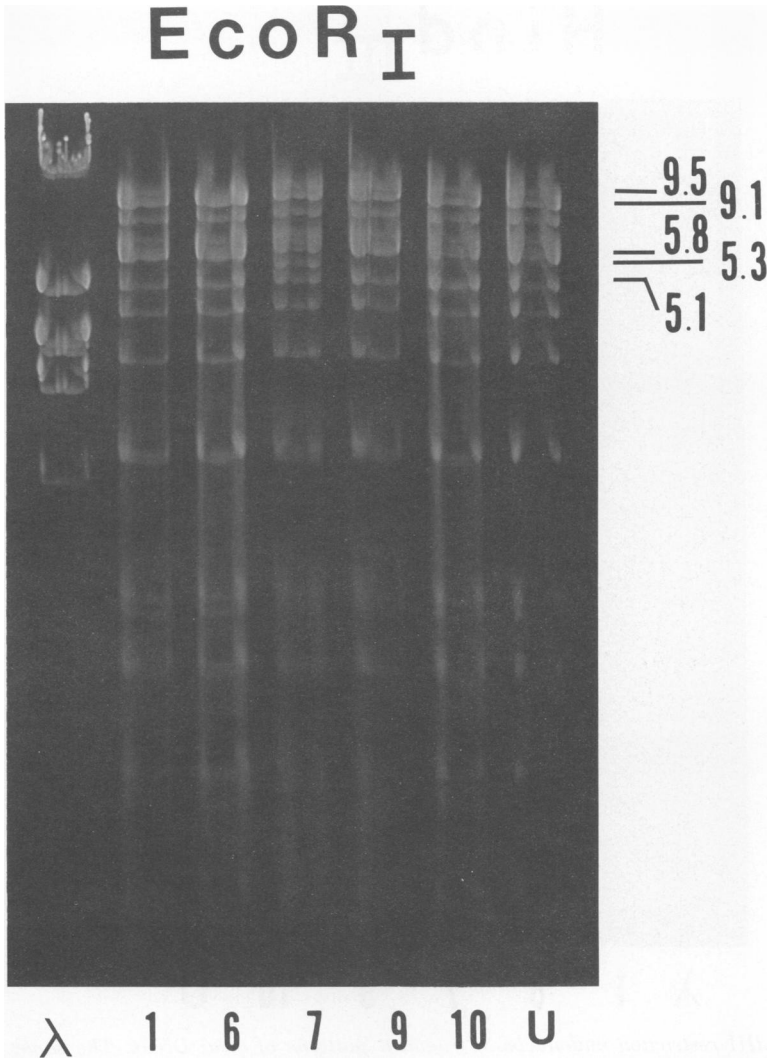

FIG. 4. *EcoRI restriction endonuclease fragment patterns of viral DNAs. The same procedures and designations as in Fig. 3 apply to this figure except that EcoRI restriction endonuclease was used to fragment the viral DNAs.*

million molecular weight region.

Genotypic analysis of the clones with *Hin*dIII. Digestion of the DNA of the 12 clones and the uncloned virus preparation with *Hin*dIII gave additional information on the nature of the cloned viruses. Clones 1, 2, 3, 4, 5, 8, 11, and 12 had seemingly identical *Hin*dIII fragment patterns. The *Hin*dIII fragment patterns of clones 1, 6, 7, 9, and 10, as well as the uncloned virus preparation, are presented in Fig. 5. Clone 6 differs from clone 1 in a manner which can again be accounted for by a 0.4 million molecular weight deletion in clone 6. A 6.6 million molecular weight fragment present in clone 1 has shifted slightly in its mobility in the clone 6 pattern to a position corresponding to 6.2 million molecular weight.

In the fragment patterns of both clones 7 and 10, a fragment of approximately 8 million molecular weight is missing, and increased intensity of staining in the region of the gel corresponding to approximately 7 million molecular weight is observed. In the clone 1 pattern, the intensity in this region suggests that a doublet is present, so that three fragments comigrate in this region in the clone 7 and 10 patterns. The difference in molecular weight is larger than a 0.5 million molecular weight shift expected for the deletion suggested in the *Sal*I and *Eco*RI data. At the present time, the larger shift (equivalent to 1.0

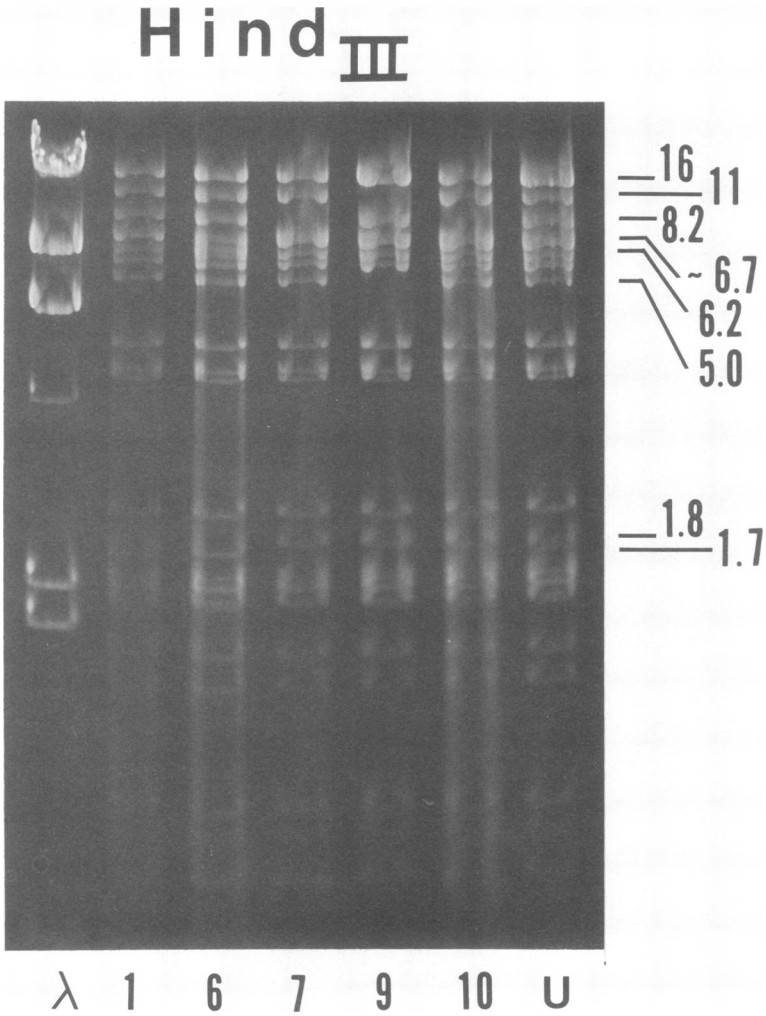

FIG. 5. *HindIII restriction endonuclease fragment patterns of viral DNAs. The same procedures and designations as in Fig. 3 apply to this figure except that HindIII restriction endonuclease was used to fragment the viral DNAs.*

or 1.4 million molecular weight) cannot be easily explained. Molecular weight calculations in this area of the gel are approximate, and the larger apparent shift may be actually due to inexact molecular weight estimates. Although it is also possible that there is an additional *Hin*dIII site near the end of this fragment, a new fragment of approximately 0.5 million to 0.9 million molecular weight is not observed in the fragment pattern. The *Hin*dIII fragment patterns of clones 7 and 10 are indistinguishable. The small 0.2 million molecular weight difference between clone 7 and clone 10 would be indistinguishable in the 7 million molecular weight region of the gel owing to comigration of other fragments.

Another difference between clone 1 and clones 7 and 10 can be detected in the lower position of the gel. Clones 7, 9, and 10 have a slightly larger fragment (1.8 million molecular weight) than the corresponding 1.7 million molecular weight fragment present in the patterns of the other clones. Skepticism concerning the real difference in mobility of the fragments is eliminated by the presence of two distinct fragments (1.8 million and 1.7 million molecular weight) in the uncloned virus patterns. The two fragments are present in nonstoichiometric quantities in the uncloned pattern but present in apparently stoichiometric quantities in each of the cloned virus patterns. It is possible that there is a small (0.1 million) addition in clones 7, 9, and 10. An addition of 0.1 million molecular weight is so small that it would

easily go undetected in the other fragment patterns. Alternatively, it is possible that a 0.1 million molecular weight *Hin*dIII fragment, which would be undetectable in clone 1 patterns, has fused with the 1.7 million molecular weight fragment, resulting in a 1.8 million molecular weight fragment in the patterns of clones 7, 9, and 10. Such a fusion could occur by a simple point mutation within a *Hin*dIII recognition site.

The *Hin*dIII fragment pattern of clone 9 is clearly different than all the other cloned virus patterns. In clone 9, two fragments of approximately 11 million and 5.0 million molecular weight are missing. A fragment of approximately 16 million molecular weight is unique to the clone 9 pattern. This 16 million molecular weight fragment virtually comigrates in the 0.7% agarose gels with the largest fragment of the clone 1 pattern (approximately 15 million molecular weight). The loss of a *Hin*dIII recognition site in clone 9 can account for the disappearance of the 11 million and 5 million molecular weight fragments in the clone 1 pattern and the appearance of the 16 million molecular weight fragment.

Overall, genotypic analysis of the clones demonstrates the isolation of at least five variants of the *A. californica* MNPV. Clone 1 is representative of clones 1, 2, 3, 4, 5, 8, 11, and 12, whereas clones 6, 7, 9, and 10 possess distinctive genotypes.

Nondefective nature of the variants. Although the NOV of the variants were apparently nondefective in their ability to infect the *S. frugiperda* cell line (IPLB-SF-21), it was of interest to determine if the occlusion bodies of the variants were also nondefective in their ability to infect *T. ni* larvae by per os administration. Occlusion bodies of clones 1, 6, 7, 9, and 10, produced in *S. frugiperda* cell cultures, were used to infect *T. ni* larvae by contaminating their food supply (see Materials and Methods). Each clone tested produced lethal infections in the larvae tested. Replication of the variant in the insect larvae was demonstrated by the increased quantity of occlusion bodies isolated from the larvae and subsequent genotypic analysis of the DNA isolated from occluded viruses. In addition, we determined that the variants were truly "M"NPV by centrifuging virions released from alkali-disrupted occlusion bodies through 25 to 60% sucrose gradients. Gradient profiles similar to Fig. 1B demonstrated the presence of virions primarily containing more than one nucleocapsid per envelope.

Genotypic stability of the cloned variants. Although the occurrence of several of the variants in the original uncloned virus preparation was detected by the presence of non-stoichiometric bands in the restriction endonuclease fragment patterns of the uncloned virus, it was of interest to determine if the genotype of the cloned variants altered rapidly upon passage through insect larvae. To determine this, we serially passaged clones 1, 6, 7, 9, and 10 through *T. ni* larvae. The viral DNA was isolated from occluded virus purified from infected larval carcasses (see Materials and Methods) and analyzed with restriction endonucleases. The genotypes of each of the variants were unaltered by a few (two to four) serial passages. Clone 6, serially passaged three times through *T. ni* larvae, is genotypically compared with clone 6, passed twice in *S. frugiperda* cell cultures from the plaque purification step, in Fig. 6.

DISCUSSION

The cloning of *A. californica* MNPV has been achieved by using media-derived NOV from infected *S. frugiperda* cell cultures as inoculum for plaque purification in tissue culture. The media-derived NOV, isolated 40 h postinfection, contain primarily virions with only one nucleocapsid per envelope, thus avoiding the problem of multiple nucleocapsid envelopment characteristic of occluded virions of "M"NPVs. The actual ability to clone by this method was demonstrated by genotypic analyses of the DNA of the virus isolates using restriction endonucleases. The analyses revealed the existence of at least five variants of the *A. californica* MNPV, and the results are summarized in Table 1. For simplicity, the five variant types are heretofore referred to as L1, L6, L7, L9, and L10.

Of the 12 clones isolated from a previously uncloned *A. californica* MNPV preparation, eight clones (1, 2, 3, 4, 5, 8, 11, and 12) were similar in genotype to L1 as demonstrated by *Bam*HI, *Eco*RI, *Hin*dIII, and *Sal*I restriction endonuclease analyses. Finer structural analysis of the clones using additional restriction endonucleases may reveal more genotypic variations. However, alterations such as additions, deletions, or long stretches of nonhomologous regions should have been revealed by the restriction endonuclease analyses already performed.

The L6 variant is characterized by a deletion of 0.4 million molecular weight, which affects the mobility of one fragment in each of the four restriction endonuclease fragment patterns observed. The L9 variant is distinguishable from the other variants by *Hin*dIII analysis, but not by *Eco*RI, *Sal*I, or *Bam*HI analysis. The L7 and L10 variants are related to each other in the respect that a similar region of the genome has been altered. The alteration common to both L7 and L10 appears to involve a deletion, although

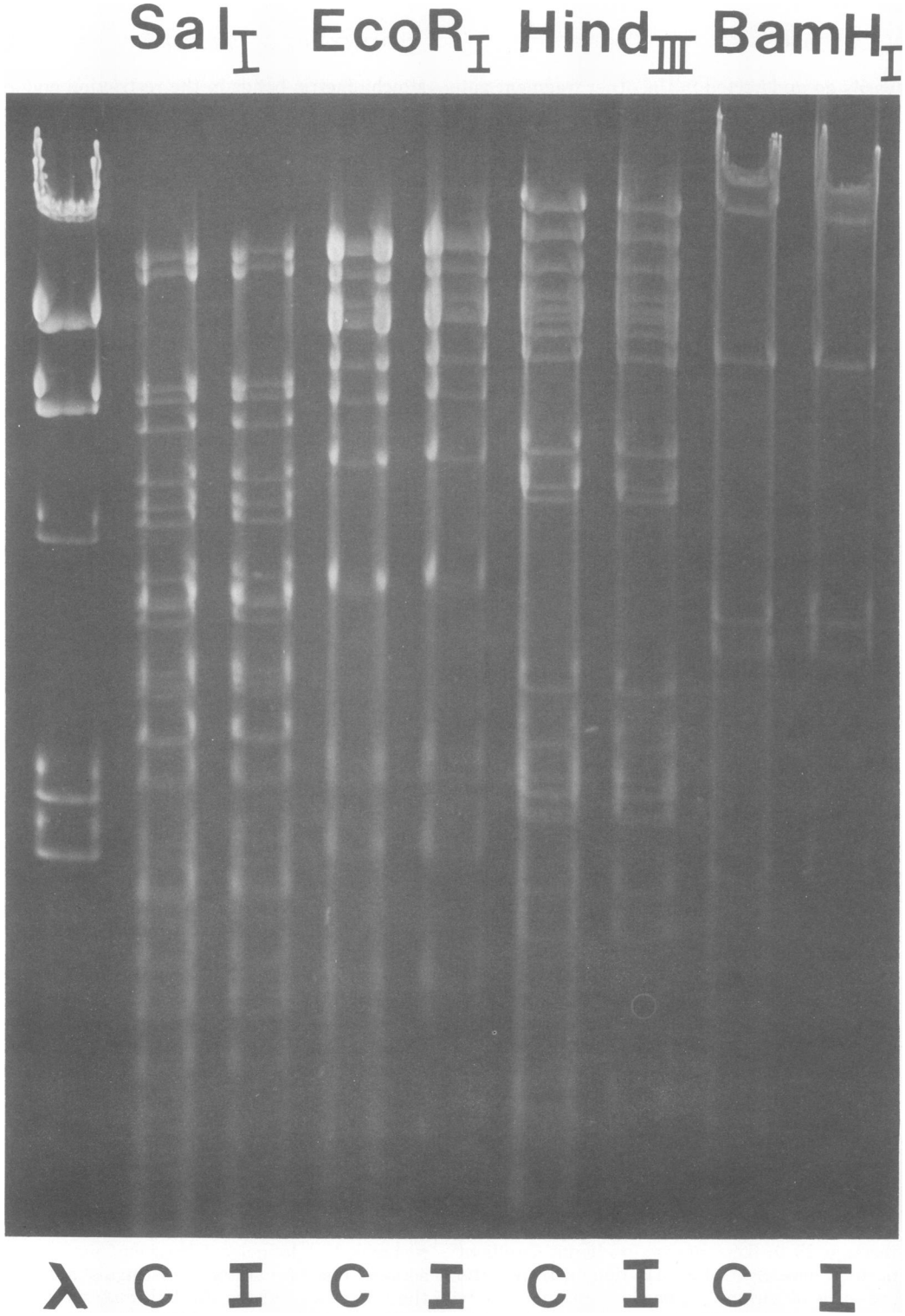

FIG. 6. *Comparison of clone 6 viral DNA isolated from occlusion bodies produced in cell culture or in insect larvae. Clone 6 A. californica MNPV was serially passaged through T. ni larvae by per os administration. Occlusion bodies were isolated from insects after the third passage. Viral DNA was extracted from the occlusion bodies produced in insects and digested with a series of restriction endonucleases, SalI, HindIII, EcoRI, and BamHI. The fragment patterns were compared with fragment patterns of clone 6 viral DNA isolated from occlusion bodies produced in S. frugiperda cell suspension cultures. The viral DNA from insect-derived occlusion bodies is denoted by I; viral DNA from occlusion bodies produced in cell culture is denoted by C.*

TABLE 1. *Summary of differences observed in restriction endonuclease fragment patterns of A. californica MNPV variants*[a]

Clone no.	Restriction endonucleases—fragment changes			
	*Bam*HI	*Sal*I	*Eco*RI	*Hin*dIII
1, 2, 3, 4, 5, 8, 11, and 12				
6	5.4A; 5.0N	1.8A; 1.4N	~9.5A; ~9.1N	6.6A; 6.2N
7		3.8A, 2.4A, 1.2A; 6.9N	5.8A; 5.3N	8.2A; 6.9N 1.7A; 1.8N
9				~11A, 5.0A; ~16N 1.7A; 1.8N
10		3.8A, 2.4A, 1.2A; 6.7N	5.8A; 5.1N	8.2A; 6.7N 1.7A; 1.8N

[a] Since clones 1, 2, 3, 4, 5, 8, 11, and 12 have identical *Bam*HI, *Sal*I, *Eco*RI, and *Hin*dIII restriction endonuclease fragment patterns, these patterns are considered the standard pattern. Differences noted in clones 6, 7, 9, and 10 are noted relative to the standard (most common) fragment pattern. Fragments that are missing in a given fragment pattern are referred to as "A" (absent). Those fragments that do not appear in the standard fragment pattern, but appear in another clone, are referred to as "N" (new). The fragments are referred to using their approximate molecular weight in millions. Fragments which are apparently related to each other are listed in a single line with missing fragments and new "replacement" fragments separated by a semicolon.

the size of the deletion is slightly larger in L10 than in L7. The L7, L9, and L10 variants also have one alteration in common which may involve a small addition of approximately 0.1 million molecular weight of DNA. The deletion alterations noted in L7 and L10 are apparently unrelated to the 1.7 to 1.8 million molecular weight change since L9 also contains the 1.7 to 1.8 million molecular weight alteration. The fact that L7, L9, and L10 have a common alteration may be a result of recombination between the parents of L7 or L10 and L9 at some point in the past. However, it is not possible at this point in time to prove such a relationship.

Two lines of evidence demonstrate that the major virus in the uncloned virus preparation is the L1 variant. The predominant bands in the restriction endonuclease fragment patterns of the uncloned virus preparation are the same as the fragment patterns of L1. Furthermore, 8 of the 12 cloned virus isolates had the L1 genotype.

The presence of the L7 and L10 variants in the uncloned virus preparation is demonstrated by close inspection of the *Sal*I fragment patterns of the uncloned virus preparation. The 6.9 million and 6.7 million molecular weight fragments, unique to the L7 and L10 *Sal*I patterns, respectively, appear as faint bands in the *Sal*I pattern of the uncloned virus. The faint staining, relative to other bands in this region of the DNA, indicates that the 6.7 million and 6.9 million molecular weight fragments are present in nonstoichiometric quantity; roughly, 20% of the uncloned virus is L7 and L10 variants. This corresponds with the frequency with which L7 and L10 were isolated (1 in 12 isolates each). Since it was possible that L7 and L10 evolved from L1 due to passage in the alternate host, *T. ni* (the uncloned virus had been passaged three times in *T. ni* since its original isolation from *A. californica* before being cloned), we examined the *Sal*I fragment patterns of the uncloned *A. californica* MNPV that had been passaged exclusively in *A. californica* larvae after the original isolation by Vail et al. (24). This pattern was previously published (13). The predominant pattern is that of L1, but close inspection reveals the presence of L7 and L10. Therefore, L7 and L10 were present in the original isolate and not generated by passage through *T. ni* larvae or *S. frugiperda* cell culture.

From the data presented herein, it is not yet possible to determine with certainty whether the L9 variant was present in the original uncloned virus preparation. L9 differs only in the *Hin*dIII fragment pattern, and the 16 million molecular weight fragment unique to L9 is masked by comigration with another large *Hin*dIII fragment. However, the 1.8 million molecular weight fragment, present in L7, L9, and L10 *Hin*dIII fragment patterns, is present in the uncloned virus preparation, and the intensity of staining of the fragment is sufficiently strong to suggest that approximately 30 to 50% of the viruses in the uncloned virus preparation have the 1.8 million molecular weight fragment. It is also not possible to determine with certainty whether L6 was present in the original uncloned virus preparation or merely generated by passage through *S. frugiperda* cells. The best pattern to detect L6 presence is the *Bam*HI pattern. However, a 10% contamination of L6 in the uncloned virus preparation would require visualization in the 5.0 million molecular weight region of a fragment with staining intensity equivalent to a 0.5 million molecular weight DNA. Detection of such a faint

band is difficult. Nevertheless, we have carefully examined the *Bam*HI fragment pattern of the uncloned virus and have not found evidence of L6. Since L6 was isolated with a frequency of 1 in 12, it may be present in the uncloned virus preparation at the 5% level or possibly even less. In view of these considerations, it is not possible to determine with certainty if L6 was generated by passage through an alternate host.

All the variants, including L6, are nondefective in infection of *T. ni* larvae, and the genotype of each variant is stably maintained for at least a limited number (two to four) of passages. It is possible, and even probable, that evolution will occur with extensive serial passage. A long-term evolutionary study is now feasible owing to the ability to clone the viruses and detect relatively minor alterations in the genotype of the viruses detectable by restriction endonuclease analysis.

Although it is not clear why MNPVs primarily package more than one nucleocapsid per envelope for occlusion, a few reasons can be eliminated by the research reported herein. Our results indicate that a singly enveloped nucleocapsid (virions containing a single DNA molecule) gives rise to normal MNPVs. Furthermore, no significant genotypic heterogeneity is detected after several serial passages of MNPVs as demonstrated by restriction endonuclease analysis. The multiple nucleocapsid envelopment is therefore not a matter of multipartite genomes with different DNA molecules contained in each nucleocapsid. In uncloned virus preparations, it seems probable that different variant DNAs could be contained within the same enveloped structure if the variant DNAs have replicated in the same cell. However, in cloned virus preparations, all nucleocapsids, whether singly or multiply enveloped, apparently contain virtually identical DNA molecules.

The isolation of nondefective variants of the *A. californica* MNPV is significant in several respects. It demonstrates that the *A. californica* MNPV can be cloned and thus the genetics of the virus can be investigated through the isolation of mutants. The presence of variants in the uncloned virus preparation also demonstrates the heterogeneity of such virus preparations. In view of this heterogeneity, it may be advisable to clone MNPVs for use as pesticides. It would be useful to use a single clone so that future evolutionary changes in the virus can be more easily detected and investigated. It may also be possible that one of the variants is more pathogenic for a particular pest insect species than the others. We have demonstrated that all the variants are infectious by per os administration to *T. ni* larvae, but 50% lethal dose determinations could reveal variations in virulence. The *A. californica* MNPV has a relatively broad host range, infecting a variety of species in the family *Noctuidae*. The different variants may have differing virulence depending on the host tested, and this may also be important in terms of pesticide use.

The U.S. Environmental Protection Agency has been rather lenient in terms of permissible levels of the *Orgyia pseudotsugata* SNPV contamination in *O. pseudotsugata* MNPV pesticides. These two viruses have less than 1% sequence homology (15). The variants of *A. californica* MNPV described herein are extremely closely related, as evidenced in the similarity of their restriction endonuclease fragment patterns. In this context, insistence on cloning the *A. californica* MNPV would be difficult to justify. However, in view of the possible problems encountered in the long-term use of viruses as pesticides, cloning of the viruses may be ultimately advantageous from a safety perspective, as well as an efficacy perspective.

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