Mapping the Mutation Site of an *Autographa californica* Nuclear Polyhedrosis Virus Polyhedron Morphology Mutant

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Received 22 February 1982/Accepted 27 May 1982

A polyhedron morphology mutant of *Autographa californica* nuclear polyhedrosis virus, designated M5, was compared with wild-type virus by genotypic analysis with *EcoRI*, *BamHI*, *HindIII*, *SstI*, and *SmaI* restriction endonucleases. M5 DNA revealed several alterations relative to the wild-type pattern: (i) *EcoRI* fragment I was 400 base pairs larger; (ii) *BamHI* fragment F was missing; (iii) *HindIII* fragment F was 400 base pairs larger; (iv) an extra restriction fragment was obtained with both *HindIII* and *SmaI*; and (v) *SstI* fragment G was 400 base pairs larger. M5 virions contained two size classes of circular DNA, one of 100% of the wild type and one of about 58% of the wild-type molecule. A revertant of M5, designated M5R, was isolated on the basis of polyhedron morphology. The genome of M5R contained the insertion of DNA in *EcoRI* fragment I and in *HindIII* fragment F, but was similar to the wild type in its other restriction fragment patterns. M5-infected cell cultures synthesized a polyhedrin polypeptide smaller in size than the wild type or M5R.

Autographa californica nuclear polyhedrosis virus (AcMNPV) has become a model for the study of the molecular biology of baculoviruses. AcMNPV DNA is a double-stranded, supercoiled circular structure of approximately 128 kilobase pairs (kbp) (8, 11, 14). Restriction endonuclease fragment maps of the AcMNPV genome have been derived (4, 8, 12, 15) which indicate that the viral genome contains predominantly nonrepeated sequences. Plaque purification of wild isolates indicates that several genotypic variants may coexist in these populations (7, 11, 14), suggesting that genetic rearrangement may be common in baculoviruses.

We were interested in studying the functional organization of the baculovirus genome. Some of these studies take advantage of specific mutants of AcMNPV which are now available. A great deal of information from several labs has been generated on the polyhedrin protein synthesized in AcMNPV-infected cells. This protein makes up the matrix of the intranuclear crystal in which the virus particles are found occluded and is the major late translation product in infected cells (3). Polyhedron morphology mutants have been isolated and preliminarily characterized by electron microscopy and protein analysis (2). We have begun to analyze the genome of one of these mutants by physically mapping the mutation site.

This paper describes the DNA restriction endonuclease analysis of the morphology mutant designated M5 and of a spontaneous revertant of

M5, designated M5R. The M5 genome contains at least two regions affected by mutation. Purified extracellular virions of M5 contain two size classes of circular DNA, one of wild-type genome length and another smaller DNA molecule. The DNA sequences contained within these two populations will be described. They indicate that a specific region of the M5 genome has been deleted in some of the DNA molecules. The genome of M5R contains only some of the genetic changes of the parental mutant. Finally, it will be demonstrated that M5-infected cells synthesize a smaller polyhedrin polypeptide than wild-type- or M5R-infected cells.

MATERIALS AND METHODS

Virus and cells. AcMNPV and the morphology mutant M5 (2) were kindly supplied by Peter Faulkner (Queen's University). After plaque purification of all virus preparations, working stocks of virus were prepared by passing the virus three to four times on *Spodoptera frugiperda* cell cultures. The infectious tissue culture fluid was used directly as inoculum for the reported experiments. The cells were propagated as previously described (3).

Purification of AcMNPV and AcMNPV DNA. Non-occluded virus was purified from infected cell culture fluid by centrifugation as previously described (3). DNA was purified from the nonoccluded virus as previously described (14).

Restriction endonucleases. *BamHI*, *EcoRI*, *HindIII*, *SstI*, and *SmaI* were purchased from either Boehringer Mannheim Corp. (Dorval, Quebec) or Bethesda Research Laboratories (Rockville, Md.). Protocols sup-

plied with the restriction enzymes were followed for the cleavage of viral DNA. Enzyme incubations were carried out at 37°C for 2 to 3 h with excess enzyme to produce limit digestion. The reactions were stopped by the addition of 1/10 volume of a solution (7 M urea, 50% sucrose, 0.1 mM EDTA, 0.1% bromophenol blue). In some cases, the fragments were end labeled by "filling in" for 15 min, using 1 U of Klenow DNA polymerase (Boehringer Mannheim) and 0.5 μ Ci of each 32 P-nucleotide (Amersham Corp., Arlington Heights, Ill.) per μ g of total DNA before adding the stopping solution.

Agarose gel electrophoresis. DNA restriction fragments were routinely separated by electrophoresis through 0.8% agarose (type V; Sigma Chemical Co., St. Louis, Mo.) in 0.1 M Tris-hydrochloride (pH 9.0)–0.077 M H_3BO_3 –2.5 mM EDTA (TEB buffer) at 2 V/cm for 16 h and then stained with 1 μ g of ethidium bromide per ml. To purify individual fragments of DNA, long gel slices containing specific fragments were individually placed on the inside wall of a plastic test tube and frozen at -60°C for 2 h. When the tubes were then thawed, most of the liquid from the gel slices could easily be removed from the bottom of the tube. The DNA-containing buffer was brought to 0.2 M sodium acetate and then precipitated with 2 volumes of 100% ethanol. After precipitation at -20°C , the DNA was recovered by centrifugation. Recovery rates were usually above 60%. The purified DNA was then suspended in TEB and rerun through 0.6% low-melting-temperature agarose (Sea-Plaque agarose; Marine Colloids, Rockland, Maine). The ethidium bromide-stained fragments were then cut out of the gel and stored at -20°C . These fragments were digested with a second restriction enzyme by melting the DNA fragment-containing gel slice at 70°C for 3 min and then incubating liquid samples at 37°C with the appropriate enzyme buffer and restriction enzyme. To maximize the digestion of the DNA, 0.1% bovine serum albumin was included in all of the reaction mixtures (9). After 2 h, stopping buffer was added, and the samples were run on 0.8% agarose gels.

Mapping the DNA fragments. Purified whole viral DNA and purified restriction fragments were cleaved with various restriction enzymes. The resulting fragments were separated by electrophoresis through 0.8% agarose gels and were detected by blotting by the Southern technique (13), as previously described (14). Preincubation and hybridization of the nitrocellulose filters (Schleicher & Schuell, Keene, N.J.) were carried out by the rapid method of Wahl et al. (17). The dried nitrocellulose filters were placed in sealable plastic bags and preincubated for 4 h at 42°C in 50% formamide–5 $\times$ SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate)–5 $\times$ Denhardt reagent (5)–0.05 M sodium phosphate (pH 6.5)–300 μ g of heat-denatured salmon sperm DNA per ml. Subsequently, the filters were incubated at 42°C for 20 h in 50% formamide–5 $\times$ SSC–1 $\times$ Denhardt reagent–10% sodium dextran sulfate–100 μ g of heat-denatured salmon sperm DNA per ml–AcMNPV DNA which was 32 P labeled by nick translation (14) to a specific activity of 1×10^8 to 2×10^8 cpm/ μ g. After removing the 32 P probe, the filters were washed with three changes of 2 $\times$ SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature followed by three changes of 0.1 $\times$ SSC–0.1% SDS at 50°C . The filters were dried at 80°C and autoradio-

graphed with either Kodak XR-5, Kodak Ortho-G, or Kodak XAR-5 film.

Polyacrylamide gel electrophoresis. To analyze total intracellular polypeptides, infected *S. frugiperda* cells were pulse-labeled with 25 μ Ci of [35 S]methionine per ml at various times after infection. Samples were then prepared and electrophoresed in polyacrylamide gels, as previously described (3). The following methylated ^{14}C -protein mixture (Amersham) was used as molecular weight markers: myosin (200,000), phosphorylase *b* (100,000 and 92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

Electron microscopy. Purified virions and purified DNA were prepared for visualization by electron microscopy as previously described (3, 14).

RESULTS

The AcMNPV polyhedron morphology mutant M5 synthesizes single, large, cubic polyhedra in the nuclei of infected cells (2). These infections can therefore be easily distinguished from wild-type-infected cells which synthesize many small polyhedra per infected cell. Since virtually no virions are occluded into the M5 polyhedron, all of the viral DNA in this report was isolated from purified extracellular virus.

DNA restriction endonuclease fragment patterns. DNA prepared from purified extracellular wild-type and morphology mutant viruses (M5) was cleaved with the restriction endonucleases *Eco*RI, *Bam*HI, *Hind*III, *Sma*I, and *Sst*I, and the resulting fragments were separated on agarose gels. The fragment patterns are shown in Fig. 1, where individual fragments are lettered sequentially by increased mobility through the gel. DNA fragments which comigrated were identified by their increased intensity of staining with ethidium bromide and their increased radioactivity as seen on autoradiograms of 32 P-end-labeled fragments (data not shown). End labeling of DNA restriction fragments also aided in identifying the total number of restriction fragments produced by each restriction enzyme. The molecular weights of the fragments were estimated by comparing their mobility in agarose gels to the *Hind*III fragments of λ DNA and the *Hae*III fragments of ϕ 174 DNA.

*Eco*RI produced 24 fragments (A to X), *Bam*HI produced 7 fragments (A to G), *Hind*III produced 24 fragments (A to X), *Sma*I produced 4 fragments (A to D), *Sst*I produced 9 fragments (A to I). Some of the smallest *Eco*RI and *Hind*III fragments migrated off the gel in Fig. 1.

The M5 restriction patterns, although very similar to those of the wild type, were characterized by several differences. (i) M5 *Eco*RI fragment I was larger by approximately 400 base pairs (bp) than wild-type *Eco*RI fragment I. (ii) M5 *Hind*III fragment F was larger than wild-type *Hind*III fragment F also by approximately

FIG. 1. Cleavage of AcMNPV DNA with restriction endonucleases. DNA was isolated from purified extracellular virus of (1) wild type, (2) M5, and (3) M5R and digested with the indicated restriction endonucleases. Samples (1 μ g) were separated on 0.8% agarose gels, as described in the text, and then stained with 1 μ g of ethidium bromide per ml. The various fragments are labeled alphabetically according to decreasing size. Small *EcoRI* and *HindIII* fragments have migrated off the gels.

400 bp. (iii) There was an additional *HindIII* fragment in M5 migrating slightly faster than the AB fragments. (iv) Wild-type *BamHI* fragment F was absent in M5. (v) Cleavage of M5 DNA with *SmaI* resulted in the appearance of five fragments, in contrast to the four fragments produced when the wild type was cleaved with this enzyme. (vi) M5 *SstI* fragment G was 400 bp larger than the wild type fragment G.

In addition to these very obvious changes in the genome of M5, several more subtle alterations were observed. Many *EcoRI* and *HindIII* fragments of M5 DNA appeared to be present in less than molar ratios. Specifically, *EcoRI* fragments I, J, K, M, N, O, T, U, V, and the *HindIII* fragments D/E, F, I, J, L, M, N, O, R, T, U, V, W/X were present in submolar ratios compared with the other fragments. M5 *SmaI*-A and *SmaI*-D also appeared to be present in submolar ratios when compared with fragments B and C as well as the extra *SmaI* fragment A1 migrating between A and B. In addition, M5 *SmaI* fragment D appeared to migrate slightly slower than did wild-type *SmaI* fragment D.

Obviously, many changes from the wild type have occurred in the genome of M5. Its very unusual phenotype and genotype implied that it might be a very useful mutant to aid in the study of the organization of the AcMNPV genome.

Mapping the mutation sites. The restriction fragment patterns of wild-type DNA closely

resembled those of the AcMNPV E2 DNA (12), for which physical maps have been derived. We have recently produced similar maps for our wild-type strain of AcMNPV (4). The physical mapping of the M5 mutation site was investigated to locate the region(s) on the genome potentially affecting the synthesis and morphogenesis of polyhedrin. As can be seen in Fig. 1, M5 *BamHI* fragment B appeared to be slightly larger than the wild type or M5R *BamHI* B fragments, implying that the *BamHI* site between *BamHI*-F and *BamHI*-B had been lost in M5 (see Fig. 7). This was confirmed by purifying the *EcoRI* I fragments of the wild type and M5 and redigesting them with another restriction enzyme. The purified *EcoRI* fragments were cut with either *BamHI* or *SmaI*, then blotted and probed with 32 P-labeled wild-type DNA. *EcoRI* fragment I was contaminated with other *EcoRI* fragments, particularly *Eco*-B, -C, and -D. However, this did not interfere with the subsequent analysis. When wild-type *EcoRI* fragment I was digested with *BamHI*, *BamHI* fragment F (labeled c) was generated along with fragments corresponding to part of *BamHI* fragment C (labeled d) and part of *BamHI* fragment B (labeled b) contained within *EcoRI* fragment I. However, with M5 *EcoRI* fragment I digested with *BamHI*, *BamHI* fragment F and partial *BamHI* fragment B were missing. Instead, there was only a larger fragment (Fig. 2, lane 6) which corresponded to a

FIG. 2. Cleavage of purified *EcoRI* fragment I with *Bam*HI and *Sma*I. Analysis was by the Southern method. *EcoRI* fragment I of the wild type and M5 were purified and digested with either *Bam*HI or *Sma*I. The resulting fragments were separated on agarose gels, blotted onto nitrocellulose filters, and probed with 32 P-labeled wild-type DNA, as described in the text. Autoradiographs were obtained on Kodak Ortho-G film. (1) 0.25 μ g of wild-type DNA, cut with *Bam*HI; (2) 0.025 μ g of wild-type DNA cut with *Bam*HI; (3) purified wild-type *EcoRI*-I; (4) purified M5 *EcoRI*-I; (5) purified wild-type *EcoRI*-I, cut with *Bam*HI; (6) purified M5 *EcoRI*-I, cut with *Bam*HI; (7) purified wild-type *EcoRI*-I, cut with *Sma*I; (8) purified M5 *EcoRI*-I, cut with *Sma*I; (9) 0.025 μ g of wild-type DNA, cut with *EcoRI*; (10) 0.25 μ g of wild-type DNA, cut with *EcoRI*. (a) Partial product, *Bam*HI-B + *Bam*HI-F; (b) part of *Bam*HI-B; (c) *Bam*HI-F; (d) part of *Bam*HI-C.

partial digest product *Bam*HI-F plus part of *Bam*HI-B seen in the wild type (labeled a). In the case of M5, *Bam*HI-F plus *Bam*HI-B was 400 bp larger than the corresponding wild-type fragment. These results indicated that it was the *Bam*HI site at 3.0 between *Bam*HI-F and *Bam*HI-B which was missing in M5. In addition, the increase in size of M5 *EcoRI* fragment I corresponded to the increase in size of M5 *Bam*HI-F plus a partial *Bam*HI-B, indicating that the insertion was located between 0.0 and 4.5 map units.

Information could also be deduced from the *Sma*I digestions of *EcoRI* fragment I: (i) there was no cleavage with *Sma*I of *EcoRI* fragment I from either the wild type or M5; (ii) there was cleavage of the contaminating *EcoRI*-C and *EcoRI*-D in both the wild type and M5, but the generated fragment patterns were similar for the two viruses, implying that the extra *Sma*I site in M5 was not located within either *EcoRI*-C or *EcoRI*-D. These results satisfactorily explained the disappearance of *Bam*HI fragment F and the increase in size of *EcoRI* fragment I in M5.

The results of digesting M5 DNA with *Sst*I indicated that a second mutation site was located within *Sst*I fragment G (Fig. 1). This fragment showed an increase in size of about 400 bp. All of the other restriction enzymes used in this study produced either very large fragments from this region, or produced several fragments very similar in size so that it was not possible to see the effect of this second insertion with these enzymes. The only exception was *Sma*I, where *Sma*I fragment D of M5 was slightly larger than the wild-type fragment (Fig. 1).

These results revealed that there were two widely separated insertions of about 400 bp each in the M5 genome, but they did not explain other results, including the presence of extra *Sma*I and *Hind*III fragments.

Digestion of purified *Sma*I fragments. To determine the origin of the extra DNA apparently present in M5, all of the *Sma*I fragments from the wild type and M5 were isolated by two consecutive electrophoresis purification steps. All of the purified fragments appeared as single bands on blots of uncut material, indicating that they were not cross-contaminated with the other fragments. In addition, M5 *Sma*I fragment A1 did hybridize to the wild-type DNA probe, indicating that it did contain at least some viral DNA sequences. These purified fragments were then cut with either *EcoRI* or *Hind*III, the resulting fragments were blotted onto nitrocellulose filters, and the filters were probed with 32 P-labeled wild-type DNA.

Wild-type and M5 *Sma*I fragments B, C, and D produced all of the expected *EcoRI* and *Hind*III fragments (data not shown). These digestions also revealed the presence of a second, very small DNA fragment, *EcoRI*-X, identical in size to *EcoRI* fragment W. These two fragments have not been previously mapped, but one maps within *Sma*I fragment C, probably near 60.0, whereas the other maps somewhere within *Sma*I fragment A, probably near *EcoRI* fragment S at 87.8 (4). The insert in M5 *EcoRI* fragment I was observed in digestions of *Sma*I fragment A (Fig. 3), and the second insert within *EcoRI* fragment C was observed in digestions of *Sma*I fragment D (data not shown).

FIG. 3. Cleavage of purified *Sma*I fragments with *Eco*RI. Analysis was by the Southern method. The *Sma*I fragments of the wild type, M5, and M5R were purified and digested with *Eco*RI. The resulting fragments were separated on agarose gels, blotted onto nitrocellulose filters, and probed with 32 P-labeled wild-type DNA, as described in the text. (1) Wild type; (2) M5; (3) M5R; (4) *Sma*I fragment A1 of M5. (A) purified *Sma*-A and *Sma*-A1 digested with *Eco*RI; (B) shorter exposure of (A); (M) 0.05 μ g of wild-type DNA cut with *Eco*RI. Terminal fragments are indicated by <.

When *Sma*I fragment A1 from M5 was cut with *Eco*RI, it was found to contain a few of the same *Eco*RI fragments found only in *Sma*I fragment A. Specifically, those *Eco*RI fragments mapping at the left-hand end of *Sma*I fragment A (84.1 to 100) were seen, the *Eco*RI part of H, S, X, P, and B. However, all of the *Eco*RI fragments mapping to the right of *Eco*RI fragment B (0.0 to 38.1) were not observed. A single fragment of 10.1 kb (labeled IC) appeared to form the right end of this A1 fragment. The *Eco*RI fragments which were missing from the right end of the *Sma*I fragment A region were exactly those which appeared to be underrepresented in the *Eco*RI digest of whole M5 DNA.

Double digests of total M5 DNA with *Sma*I and *Eco*RI produced very similar patterns to similarly digested wild-type DNA, excepting one extra fragment of 10.1 kb in M5 (Fig. 4). In these digests, M5 *Eco*RI fragments U, V, N, M,

T, K, J, and A were all left intact, indicating that none of these fragments contained an extra *Sma*I restriction site.

Since fragments U, V, and F were also underrepresented in *Eco*RI digests of whole M5 DNA, these results suggested that a continuous stretch of DNA containing the *Eco*RI fragments part of C, U, V, F, N, M, T, K, J, A, O, R, and part of I had been deleted in some of the M5 genomes. This deletion would remove the *Sma*I site at 39.1 and create a new *Sma*I fragment (*Sma*I-A1), consisting of sequences from the 84.1 to the 0.0 to 3.0 region of *Sma*I-A joined to sequences from the 43.4 to 49.4 region of *Sma*I fragment D. This mutated DNA molecule would also contain an aberrant *Hind*III fragment (*Hind*III-B1), consisting of sequences from 96.3 to 0.0 to 3.0 of *Hind*III fragment F joined to sequences from

FIG. 4. Cleavage of AcMNPV DNA with *Eco*RI and *Sma*I. DNA purified from extracellular virus was doubly digested with restriction endonucleases *Eco*RI and *Sma*I and analyzed as described in the legend to Fig. 1. (1) Wild type, (2) M5, and (3) M5R. The *Eco*RI fragments not cut by *Sma*I have been labeled alphabetically, whereas the extra M5 fragment has been labeled 10.1.

FIG. 5. Neutral sucrose gradient analysis of viral DNA. Purified virions were treated with 0.5 μ g of proteinase K–0.1% SDS–0.1% sarcosine for 1 h at 37°C. The total extract was then layered on top of linear 5 to 20% sucrose gradients in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–0.1 M NaCl and centrifuged for 2.5 h at 45,000 rpm in an SW60 rotor. The gradients were then fractionated, and each fraction was ethanol precipitated. A sample of each precipitate was digested with *Hind*III, electrophoresed, blotted, and then probed with 32 P-labeled wild-type DNA. Autoradiograms of the wild-type fractions 7, 8, and 9 and the M5 fractions 6, 7, and 8 are shown. Dots to the right of the M5 fraction 8 track indicate the *Hind*III fragments which are missing in this fraction.

43.4 to 53.3 of *Hind*III fragment A. Similar types of hybrid fragments would, of course, be seen with all restriction enzymes. However, the mobilities of these fragments were similar to those of normal fragments, and their appearance was masked in the digestions of total M5 DNA with these restriction enzymes.

Separation of two sizes of M5 DNA. The restriction enzyme analysis of DNA prepared from purified extracellular M5 virions suggested that they contained two populations of DNA. Attempts were made to separate these two DNA populations. Purified wild-type and M5 virus particles were treated with proteinase K and SDS. The total extracts were then sedimented through 5 to 20% neutral sucrose gradients. Each fraction was precipitated with ethanol;

then a portion of each precipitated fraction was digested with *Hind*III. The resulting restriction fragments were separated on agarose gels, blotted onto nitrocellulose paper, and then probed with 32 P-labeled wild-type DNA. Figure 5 shows the results obtained with the peak fractions of wild-type and M5 DNA. The peak of wild-type DNA was found in fraction 7, exhibiting a normal *Hind*III fragment pattern. The peak of M5 DNA, found in fraction 6, also exhibited a normal M5 *Hind*III fragment pattern. However, M5 DNA in fractions 7 and 8, sedimenting slower than normal, appeared to be missing certain *Hind*III fragments. Specifically, *Hind*III fragments A, I, U, O, E, R, M, L, J, X, D, N, T, V, and F were missing. These fragments come from a continuous stretch of DNA mapping from 96.3 through 0.0 to 53.3. This DNA population was approximately 60% of the size of the wild-type DNA.

The presence of two size classes of circular M5 DNA, one of 100% and one of about 58% of the wild-type length, was confirmed by electron microscopy (Fig. 6).

Revertant of M5. A single spontaneous revertant of M5, designated M5R, was isolated as a plaque from the stock of M5 on the basis of polyhedron morphology. Instead of a single, large polyhedron, M5R produced polyhedra closely resembling the wild-type phenotype. The frequency of reversion is not known. The frequency must be very low since only one plaque showing wild-type polyhedra has been observed during the experiments with M5 over the past 3 years. Based on endpoint 50% tissue culture infective dose titrations, the reversion frequency was below the detectable limit of 3.3×10^{-5} (2).

DNA was purified from M5R extracellular virus and analyzed with a series of restriction enzymes (Fig. 1). Some of the fragment patterns of M5R were similar to that of M5. For example, *Eco*RI fragment I and *Hind*III fragment F were the same in M5 and M5R. However, the extra *Hind*III-B1 and *Sma*I-A1 of M5 were absent. The *Bam*HI and *Sma*I fragment patterns of M5R were similar to the wild-type pattern. Apparently, M5R had lost the small insertion near 45.6 to 48.6 and had regained the *Bam*HI site at 3.0.

These data suggested that the mutation causing the increase in length of *Eco*RI fragment I and the mutation causing the loss of the *Bam*HI site in M5 could be differentiated and were probably affecting separate sequences within *Eco*RI fragment I.

Polypeptides in cells infected with wild type, M5, and M5R. The polyhedrin polypeptide can be detected in wild-type-infected cells by 10 to 12 h postinfection (3), and its synthesis continues at a very high rate until very late times after infection. The synthesis of this polypeptide in

FIG. 6. Electron micrograph of purified M5 DNA. DNA was purified from extracellular M5 virions and spread for examination in the electron microscope. The circular DNA molecules of different lengths are shown. Measurements of 20 such molecules indicated that the smaller circles were 58% of the length of the larger circles.

M5- and M5R-infected cells was studied by pulse-labeling infected cells for 1 h at various times after infection and then analyzing the whole cell extracts on SDS-polyacrylamide gels (Fig. 8).

The polypeptide synthesis patterns of the wild type, M5, and M5R were all very similar. The only obvious difference among them was the altered mobility of the M5 polyhedrin polypeptide (2). The polyhedrin polypeptide of both the wild type and M5R had a molecular weight of 34,000, whereas that of M5 had a molecular weight of 33,000.

DISCUSSION

A morphology mutant of AcMNPV, designated M5 (2), which synthesizes a single, large, cubic crystal in infected cells, was investigated by restriction endonuclease analysis of the purified viral DNA. A complicated set of alterations has occurred in the M5 genome, resulting in DNA fragment patterns which, although very similar to the wild-type patterns, can easily be distinguished from them. These alterations have been physically mapped on the M5 genome.

Digesting purified *EcoRI* fragment I with *BamHI* indicated that the *BamHI* site at 3.0 was altered by mutation. In addition, the data revealed that an insertion of DNA of about 400 base pairs had occurred within *EcoRI* fragment I between 0.0 and 5.9 on the map. This insert was

also observed in *HindIII* fragment F. Since the right end of *HindIII* fragment F maps at 3.3 (4), this places the insertion site between 0.0 and 3.3. However, this site did not correlate with the increase in size of *SmaI* fragment D of M5, which maps between 38.1 and 49.4. Digestion of M5 DNA with *SstI* confirmed that there was an insertion in *SstI* fragment G, again of approximately 400 base pairs, mapping between 45.6 and 48.6.

The presence of these two insertion sites in the M5 genome did not explain the presence of submolar amounts of some DNA restriction fragments, easily seen with *EcoRI* and *HindIII*, or the presence of extra DNA restriction fragments seen with *HindIII* and *SmaI*. By correlating the intensity of ethidium bromide staining to the relative quantity of a particular DNA fragment, it was determined that a continuous sequence of DNA, including *EcoRI* fragments N, M, T, K, J, A, O, R, and I was present in a submolar ratio compared with sequences represented by the other *EcoRI* fragments. The same region of the genome was found to be underrepresented, as well, in *HindIII* fragments I, U, O, E, R, M, L, J, X, D, N, T, V, and F. An obvious explanation of these results would be that the stock of M5 contained two or more variants. It has already been shown that uncloned polyhedrosis virus preparations may contain several different genotypic variants which can be isolated by plaque purification (7, 11, 14). However,

FIG. 7. Physical map of AcNPV HR3 DNA. The diagram shows the map positions of various restriction endonuclease fragments of HR3 DNA, the parental wild-type strain of M5 and M5R (4).

extensive plaque purifications of M5 have failed to remove the submolar bands from the M5 DNA preparations.

Another explanation would be that more than one nucleocapsid was enveloped as the virus particles budded out through the cytoplasmic membrane, generating a heterogeneous population of extracellular virions, some of which were singly enveloped, whereas others were multiply enveloped. The multiply enveloped particles could contain two or more virions with differing genomes. However, electron microscopic examination of M5 extracellular virus preparations revealed only singly enveloped nucleocapsids (data not shown).

A third explanation would be specific heterogeneity in the population of DNA molecules isolated from purified M5 virions. The presence of extra DNA fragments generated by cutting M5 DNA with *SmaI* or *HindIII* suggested that perhaps the M5 genome was much larger than the wild type. However, because the DNA fragment patterns generated by cutting M5 DNA with *EcoRI* or *SstI* were extremely similar qualitatively to the wild type, it was likely that the extra *SmaI* and *HindIII* fragments resulted from the deletion of a specific region of the M5 genome in some DNA molecules. This deletion would result in two phenomena. The DNA restriction fragments in the genome within the deletion region would be present in relatively

fewer copy numbers than DNA restriction fragments outside the deletion region since only a portion of the genomes would carry a complete set of DNA sequences. Secondly, sequences which overlapped the two ends of the deletion would be fused, generating new restriction fragments not seen in normal length DNA. Thus, there could be two types of DNA molecules in M5; one would consist of genome-length circular DNA, the second would consist of a shorter, circular DNA, having a large deletion of viral sequences, spanning the region between the two previously mapped insertion sites at 0.0 to 3.0 and 46.5 to 48.6.

Evidence for this model was derived by cleaving individual *SmaI* fragments purified from wild-type and M5 DNA with either *EcoRI* or *HindIII*. *SmaI* fragments A, B, C, and D of the wild type generated the expected *EcoRI* fragments and also indicated that *EcoRI*-W mapped within *SmaI* fragment C, whereas a previously undetected *EcoRI* fragment X mapped within *SmaI* fragment A. These fragments have now been precisely mapped (4). *SmaI* fragments A, B, C, and D of M5 generated the same *EcoRI* fragments as did the wild type, except for the increase in length of *EcoRI*-I and *EcoRI*-C.

The extra M5 *SmaI* fragment, A1, generated fragments the size of the *EcoRI* part of H, S, X, P, B, and a new fragment of about 10.1 kb. The *EcoRI* part of fragment H, S, X, P, and B forms

FIG. 8. Autoradiogram of SDS-polyacrylamide gel electrophoresis of infected cell extracts. *S. frugiperda* cells, infected with the wild type (WT), M5, and M5R were pulse-labeled for 1 h with [32 S]methionine at the indicated times after infection. The whole cell extracts were analyzed by gel electrophoresis. The polyhedrin polypeptide was apparent by 16 h postinfection and continued to be synthesized up to 32 h postinfection. The M5 polyhedrin polypeptide had an apparent molecular weight of 33,000, whereas the wild-type and M5R polyhedrin polypeptides had molecular weights of 34,000.

a continuous stretch of DNA from 84.1 to 100.0/0.0 of *Sma*I fragment A. Since *Eco*RI-I and *Bam*HI-C purified from M5 did not contain a *Sma*I site, the right-hand end of the M5 *Sma*I fragment A1 must be located farther to the right than 11.3 on the physical map. There are many *Eco*RI sites to the right of the *Eco*RI site at 0.0. However, only one other *Eco*RI fragment was seen in *Sma*I fragment A1, the 10.1-kb fragment. These results suggested that a portion of the sequences, most likely including the *Sma*I site at 38.1 and the *Eco*RI site at 5.9, had been deleted in M5 DNA to produce the A1 fragment. Since the 10.1-kb fragment was the only new fragment seen in *Sma*I plus *Eco*RI double digests of M5 DNA, the extra *Sma*I fragment was not generated by a new *Sma*I recognition site, but rather by a rearrangement in the viral DNA, resulting in sequences contained within the region of 0.0 to 3.0 being joined to sequences within the 45.6 to 48.6 region.

Two different size classes of M5 virion DNA could be separated by neutral sucrose gradient centrifugation. One population of M5 DNA sedimented like wild-type DNA, whereas a second smaller population sedimented slower than wild-type DNA. When the *Hind*III restriction fragments of these two DNA populations were separated on agarose gels, it was apparent that the

smaller DNA was generated by deleting a stretch of DNA from 0.0 to 3.0 to 45.6 to 48.6. This deletion resulted in a genome which was approximately 58% of the size of the wild-type genome. These results were confirmed by electron microscopy of purified M5 virion DNA where two size classes of circular DNA were seen.

This kind of genetic rearrangement is similar to the formation of defective particles observed with other DNA viruses (1, 10). However, in the case of M5, the mutant was not derived by serial passage but rather by mutagenization of the wild-type stock. Nitrosoguanidine seems to induce mutations at the growing fork of replicating DNA, resulting in gaps which may be filled by error-prone repair (6). Of course, it is possible that the mutation did occur before mutagenesis although we have never seen this phenotype in nonmutagenized virus stocks. It is possible that the synthesis of two closed circular DNA molecules of different sizes is a result of aberrant M5 DNA replication, which generates these two forms during every round of DNA replication. This is supported by evidence which indicates that the DNA restriction fragment patterns of serially passaged M5 (up to 16 passages to date) remain qualitatively and quantitatively similar to the fragment patterns of the original plaqued

stock. The small insertions may also function as transposable elements and, in this way, generate the two size classes of circular DNA at a high frequency. Virtually nothing is known about the mechanism of DNA replication in baculovirus, but M5 may prove to be useful in studying this aspect of AcMNPV.

The revertant of M5, M5R, appears to have resulted from a very rare mutational event where an alteration in the M5 genome has generated a new genome capable of coding for a full-length polyhedrin polypeptide. This wild-type-length polypeptide is able to assemble into structures phenotypically identical to the wild-type polyhedron, and in this case, the normal polyhedron of M5R acted as a suitable visual marker. The fact that the revertant genome retains the 400-bp DNA insertion in the 0.0 to 3.0 region indicates that M5R is a true revertant.

The M5R genome differs in two respects from M5. First, it has lost the 400-bp DNA insert in the 45.6 to 48.6 region. Secondly, it has regained the *Bam*HI site at 3.0. Either of these regions may be critical for the phenotypic expression of polyhedra in infected cells, but presumably there is only one site corresponding to the polyhedrin gene which could affect the size of the polyhedrin polypeptide. It is possible that the polyhedrin mRNA results from a splicing event where the region between 3.0 and 45.6 represents a very large intron. However, recent data indicate that the polyhedrin gene maps within *Bam*HI fragment F (16; Rohel, Cochran, and Faulkner, personal communication). These data suggest that the alterations in the 0.0 to 3.0 region of the M5 genome affect the size of the polypeptide translated from this region. However, the mutation affecting the synthesis of polyhedrin may not be related to the phenotypic expression of the polyhedron morphology since at least two distinct regions of M5 are mutated. Other regions of the M5 genome possibly affecting polyhedron morphology may also be mutated, but these were not detected by the present restriction endonuclease analysis. Experiments are currently under way to determine the origin of the inserted sequences in M5 and to study the transcription products of these mutated genome sequences.

ACKNOWLEDGMENTS

I thank Nancy Butterill and Margaret Hough for technical assistance, Dorothy Agnew for preparation of media, and P. Faulkner for gifts of cells and viruses.

This work was supported by a grant from the Medical Research Council of Canada.

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