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Molecular Engineering of the *Autographa californica* Nuclear Polyhedrosis Virus Genome: Deletion Mutations Within the Polyhedrin Gene

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We describe a method to introduce site-specific mutations into the genome of *Autographa californica* nuclear polyhedrosis virus. Specifically, the *A. californica* nuclear polyhedrosis virus gene for polyhedrin, the major protein that forms viral occlusions in infected cells, was mutagenized by introducing deletions into the cloned DNA fragment containing the gene. The mutagenized polyhedrin gene was transferred to the intact viral DNA by mixing fragment and viral DNAs, cotransfecting *Spodoptera frugiperda* cells, and screening for viral recombinants that had undergone allelic exchange. Recombinant viruses with mutant polyhedrin genes were obtained by selecting the progeny virus that did not produce viral occlusions in infected cells (occlusion-negative mutants). Analyses of occlusion-negative mutants demonstrated that the polyhedrin gene was not essential for the production of infectious virus and that deletion of certain sequences within the gene did not alter the control, or decrease the level of expression, of polyhedrin. An early viral protein of 25,000 molecular weight was apparently not essential for virus replication in vitro, as the synthesis of this protein was not detected in cells infected with a mutant virus.

Autographa californica nuclear polyhedrosis virus (AcMNPV) is a member of the family Baculoviridae and is pathogenic to several species of insects. In the nucleus of most AcMNPV-infected cells occlusions form in which many virus particles are embedded. A 33,000 (33K)-molecular-weight protein called polyhedrin is the major structural component of the occlusion bodies, and the gene for AcMNPV polyhedrin maps to a contiguous DNA sequence of about 1,200 base pairs near a position designated as the zero point of the DNA restriction map (33). The approximate location of about 25 other AcMNPV genes is also known (2, 28a, 32, 33), but little is known about their function or the sequences important in controlling their expression during infection.

Several laboratories have reported the isolation and characterization of temperature-sensitive AcMNPV mutants which exhibit a variety of phenotypes (3, 20). Although the approximate locations of several temperature sensitivity mutations have been mapped (27), a major limitation to their use is that they have not been assigned to specific viral genes. In addition, conditional mutations are usually confined to the amino acid coding sequences and are not generally found within control regions flanking structural sequences. AcMNPV mutants are also described that either produce fewer occlusions (FP mutants; 11, 16, 26, 27) or form aberrant occlusion bodies (4, 5). Examples of both types have recently been thoroughly characterized (5, 21a), but it is not yet possible to relate these mutant phenotypes to specific changes in the genome. FP mutants are of particular interest in that some are known to have acquired host DNA sequences (21a; M. J. Fraser, G. E. Smith, and M. D. Summers, submitted for publication), and the structure of one of these host sequences is similar to the *Drosophila* transposable element *copia* (21a).

A direct approach to elucidate the function and control of viral genes is to delete, insert, or transpose specific sequences in or around genes whose locations are known and then study the effects of these changes. Since site-directed mutagenesis is often done at or near unique restriction sites, DNA viruses with large genomes and many restriction sites are difficult to alter genetically by direct manipulation of the intact viral DNA. Mocarski et al. (23) developed an alternative procedure for the introduction of specific mutations into complex viral genomes. First, a cloned viral DNA fragment is mutagenized in vitro. The altered DNA fragment is then trans-

ferred to the intact viral genome by homologous recombination in cells cotransfected with the viral and fragment DNAs. Because only a small percentage of the viral progeny from cotransfected cells will be recombinants carrying the alterations, mutant viruses are usually obtained by selection for a particular phenotype, such as that conferred by the herpes simplex virus (23, 25) or the poxvirus (39) thymidine kinase genes. To use this method to construct mutants of AcMNPV it was necessary to develop a procedure to screen for recombinant viruses. We will demonstrate in this report that AcMNPV recombinant viruses with mutations in the polyhedrin gene can be obtained by selecting for progeny which did not produce occlusions in infected cells.

AcMNPV mutants with deletions in the polyhedrin gene were constructed and characterized. The polyhedrin gene was not essential for the production of infectious nonoccluded viruses. An analysis of proteins in mutant virus-infected cells demonstrated that the control and high level of expression of polyhedrin was not affected by the deletion of certain sequences within the polyhedrin gene. In addition, several AcMNPV mutants were defective in the production of one of the early viral proteins.

MATERIALS AND METHODS

Cells and virus. Virus stocks were prepared and titrated in *Spodoptera frugiperda* cells (IPLB-Sf21-AE) with TNM-FH medium (15) plus 10% fetal bovine serum. The procedures for the cultivation of viruses and cells are published (37, 38). The source of AcMNPV and description of the E2 isolate are as reported (29). Viral growth kinetics were determined as described by Volkman et al. (38), using *S. frugiperda* cells and a 1.5% agarose overlay.

Plasmid and viral DNAs. The characterization of AcMNPV *Eco*RI-I DNA cloned in the *Escherichia coli* plasmid pBR325 has been described (33).

For transfection of cells, AcMNPV DNA was extracted from virions and purified by equilibrium centrifugation in cesium chloride density gradients (29). To analyze viral recombinants produced by transfection, DNA was labeled with $^{32}P_i$ and purified from the extracellular virus as described (30).

Construction of deletion mutant plasmids. Deletions progressing in both directions from the single *Kpn*I site in the cloned *Eco*RI-I fragment (33) were constructed by sequentially digesting the *Kpn*I-linearized plasmid DNA with exonuclease III (New England Biolabs) and S1 nuclease (Boehringer). The reaction conditions and experimental procedures were as described by McKnight and Gavis (21). The shortened DNA fragments were diluted to 1 μg/ml, circularized with 200 U of T4 DNA ligase (New England Biolabs) per ml at room temperature for 2 h, precipitated with ethanol, and introduced into *E. coli* RR1 (obtained from Savio Woo, Baylor College of Medicine, Houston, Tex.) by transfection (8). Ampicillin-resistant, tetracycline-resistant, chloramphenicol-sensitive colonies were isolated, and plasmid DNA was isolated (17). The extent of deletions was approximated by restriction enzyme mapping.

Labeling infected-cell proteins and gel electrophoresis. To pulse-label cell proteins with L-[4,5-^{3}H]leucine, cells were infected with 20 PFU of virus per cell, washed twice at various times postinfection (p.i.) with defined medium (29) minus L-leucine, and labeled for 3 h in defined medium containing 100 μCi of L-[^{3}H]leucine (60 Ci/mmol; ICN) per ml. The labeled cells were washed twice with ice-cold phosphate-buffered saline and disrupted in 2% sodium dodecyl sulfate–125 mM Tris (pH 6.8)–10% glycerol–5% 2-mercaptoethanol–0.01% bromophenol blue. Proteins were analyzed on polyacrylamide gels as described by Laemmli (19). The gels were impregnated with En^{3}Hance (New England Nuclear Corp.), dried, and exposed at −80°C to X-ray film.

Protein blot radioimmunoassay. Preparation and characterization of AcMNPV polyhedrin antiserum was as described (31). Infected-cell proteins were transferred from polyacrylamide gels to nitrocellulose filters and incubated with a 1/500 dilution of antiserum, and specifically bound antibody was detected with ^{125}I-labeled protein A (31).

Marker transfer of deletion mutant plasmids. The following procedure was used to transfer mutant polyhedrin gene sequences to the AcMNPV genome. Cells were transfected with a mixture of plasmid and viral DNAs by a modification of the procedure described by Graham and Van der Eb (13). Viral DNA (0.01 μg) was mixed with the amounts of plasmid DNA described in the text and brought to 950 μl in 1× HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (13) (pH 7.05) with 15 μg of carrier calf thymus DNA per ml. While the mixture was being stirred rapidly in a Vortex mixer, 50 μl of 2.5 M $CaCl_2$ was added dropwise, and a precipitate was allowed to form at room temperature for 30 min. One milliliter of precipitated DNA was added to 3×10^6 cells seeded in 2 ml of medium in 60-mm culture plates. After 4 h the cell monolayer was washed with medium and overlaid with 1.5% SeaPlaque agarose (Marine Colloids) made up in medium plus 10% fetal bovine serum. From 300 to 500 plaques were visible after 3 to 4 days. Plaques that had formed from infected cells without viral occlusion bodies were initially distinguished from wild-type plaques by their appearance under a low-power binocular microscope. Virus plaques of interest were marked and examined with the aid of an inverted-phase microscope, in which individual viral occlusions, if present, could be seen in the nucleus of infected cells. In certain experiments the transfected cells were not overlaid with agarose but were incubated with 2 ml of medium for 3 days.

Electron microscopy. *Trichoplusia ni* cells were infected and prepared for examination by electron microscopy (11, 24) at 24 and 36 h p.i.

RESULTS

The strategy used to construct deletion mutants of AcMNPV employed three major steps: (i) deletions were introduced into the cloned *Eco*RI-I fragment within the protein-coding se-

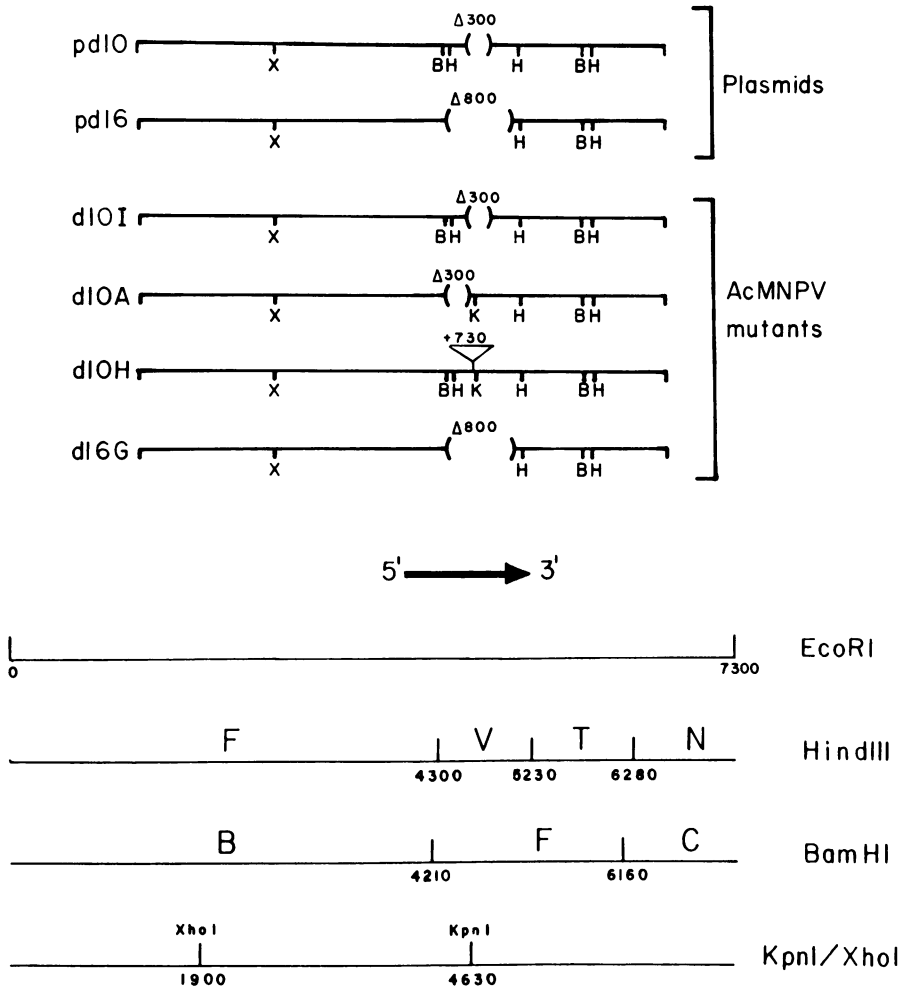

FIG. 1. Approximate location of mutations in the polyhedrin gene in mutant plasmid and AcMNPV O^- viral DNAs. At the bottom is a restriction map of AcMNPV *Eco*RI-I with the locations of *Eco*RI, *Hin*dIII, *Bam*HI, *Kpn*I, and *Xho*I fragments as described previously (35) and the restriction sites are indicated in base pairs from the origin of the AcMNPV physical map (7, 35). The arrow shows the position of the polyhedrin gene, and the 5′ and 3′ ends of the transcript are marked (33). The upper diagram shows the location of *Xho*I (X), *Bam*HI (B), *Hin*dIII (H), and *Kpn*I (K) in *Eco*RI-I fragments present in plasmid and viral mutants. The parentheses define the position of deleted sequences, and the size in base pairs is given for each. The approximate position of a 730-base pair insertion in the viral mutant d10H is shown.

quences of the AcMNPV polyhedrin gene; (ii) plasmid DNA containing the mutant gene was mixed with intact viral DNA and cotransfected into *S. frugiperda* cells; and (iii) recombinant viruses carrying the defective polyhedrin genes were selected by screening plaques that were formed from infected cells that did not produce occlusions. The success of this procedure relied on two assumptions. First, relatively large deletions within the polyhedrin gene would result in either no synthesis or the synthesis of truncated polyhedrin; second, viruses with such mutations would produce viral plaques, but the cells within these plaques would be devoid of occlusions. These assumptions proved correct, and the plaques formed from mutant virus could be distinguished easily from wild-type plaques.

Construction of AcMNPV deletion mutants. The AcMNPV polyhedrin gene is located on the *Eco*RI-I fragment between about 3,990 and 5,200 base pairs from the origin of the AcMNPV restriction map and has no detectable introns (33). A single *Kpn*I site located about 640 base pairs in the 3′ direction from the 5′ end of the polyhedrin gene is present on the cloned *Eco*RI-I fragment (see Fig. 1). Standard procedures

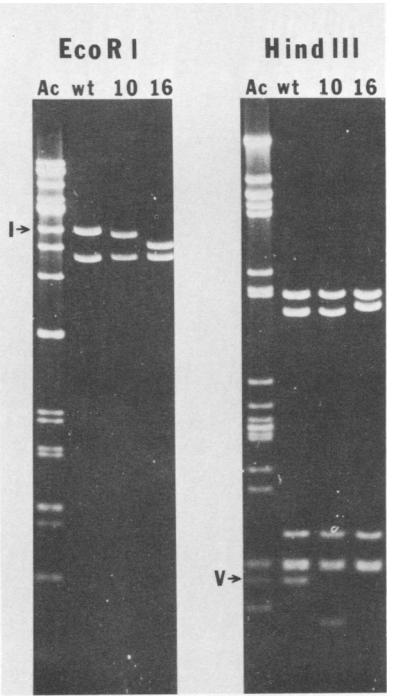

FIG. 2. Deletion mutants of the cloned *Eco*RI-I fragment of AcMNPV. Deletions were introduced into the polyhedrin gene around the unique *Kpn*I-I site in the AcMNPV *Eco*RI-I as described in the text. The mutagenized fragments were cloned in pBR325, and two mutant plasmids, pd10 and pd16, with 300- and 800-base pair deletions in *Eco*RI-I, respectively, were restricted with *Eco*RI and *Hin*dIII and electrophoresed in a 1% agarose gel. Total AcMNPV (Ac) and the cloned *Eco*RI-I fragment from wt AcMNPV (wt) were restricted with *Eco*RI and *Hin*dIII and electrophoresed in adjacent slots. The locations of *Eco*R-I (I) and *Hin*dIII-V (V) fragments in wt AcMNPV DNA are indicated.

were used to create deletions around this *Kpn*I site (21), and individual mutant plasmids were cloned and purified. Two deletion mutant plasmids, pd10 and pd16, which had about 300- and 800-base pair deletions, respectively, centered approximately around the *Kpn*I site (Fig. 2), were selected for further studies. The *Hin*dIII fragment V in the plasmid pd10 was reduced in size about 300 base pairs (Fig. 2). The deletion in pd16 extended past the *Hin*dIII site between fragments V and F. Therefore, *Hin*dIII-V was missing, and the remaining sequences were fused to the *Eco*RI-*Hin*dIII fragment F (Fig. 1 and 2). Restriction analysis with additional enzymes mapped the location of the deleted sequences (Fig. 1).

S. frugiperda cells were transfected with a mixture of 0.01 μg of AcMNPV DNA and 0.1 μg of either pd10 or pd16 plasmid DNA per 3×10^6 cells and then overlaid with agarose. After 4 days the 300 to 500 plaques that formed were inspected visually for the presence of occlusions in the infected cells as described above. In three separate experiments, 1.1, 1.7, and 2.3% (mean = 1.7%) of the plaques were formed from cells that were not producing nuclear occlusions (Table 1). Approximately 10,000 plaques from cells transfected with AcMNPV DNA alone were also inspected, and no mutant plaques were detected. We refer to mutants that are defective in producing occlusions as occlusion negative (O^-), rescued or revertant viruses that produce occlusions as occlusion positive (O^+), and the parental AcMNPV E2 virus as wild type (wt). At 4 days after transfection the O^- plaques were similar in size to the wt plaques but were distinctly less refractile than wt plaques (Fig. 3b and c). In the nucleus of O^--infected cells the virogenic stroma was condensed, and some of the cells appeared to be undergoing lysis (Fig. 3c). After 5 to 6 days, lysis of O^--infected cells was extensive (Fig. 3d), and some of the mutant virus plaques appeared as clear areas within the cell monolayer.

Restriction analysis of mutant viral DNAs. Of interest was whether the O^- viral genomes had deletions within the polyhedrin gene as a result of recombination between the wt viral and mutant plasmid DNAs. O^- mutant viruses derived from cells cotransfected with the plasmid pd10 were designated d10A, B, C, etc., and those derived from the plasmid pd16 were designated d16A, B, C, etc. The DNA from each mutant virus was purified and analyzed with several

TABLE 1. Production of recombinant virus in cells cotransfected with intact viral and cloned fragment DNAs

DNA		Molar ratio (virus/plasmid)	Recombinants[a] (%)	Phenotype[b]
Viral (μg)	Plasmid (μg)			
wt (0.01)	pd10 (0.1)	1:100	1.7	O^-
wt (0.01)			0	
d10A (0.01)	*Eco*RI-I (0.1)	1:100	12.0	O^+
d10A (0.01)	*Eco*RI-I (0.05)	1:50	7.3	O^+
d10A (0.01)	*Eco*RI-I (0.025)	1:25	5.7	O^+
d10A (0.01)	*Eco*RI-I (0.010)	1:10	3.3	O^+
d10A (0.01)	*Eco*RI-I (0.003)	1:3	0.3	O^+
d10A (0.01)			0	

[a] The percentage of recombinants was determined from an analysis of 300 viral plaques produced in 3×10^6 *S. frugiperda* cells cotransfected with viral and plasmid DNAs.

[b] Phenotype of plaques produced by the viral recombinants. Plaques formed from viruses that produce occlusions in the nucleus of infected cells are O^+, and those formed from viruses that do not produce occlusions are O^-.

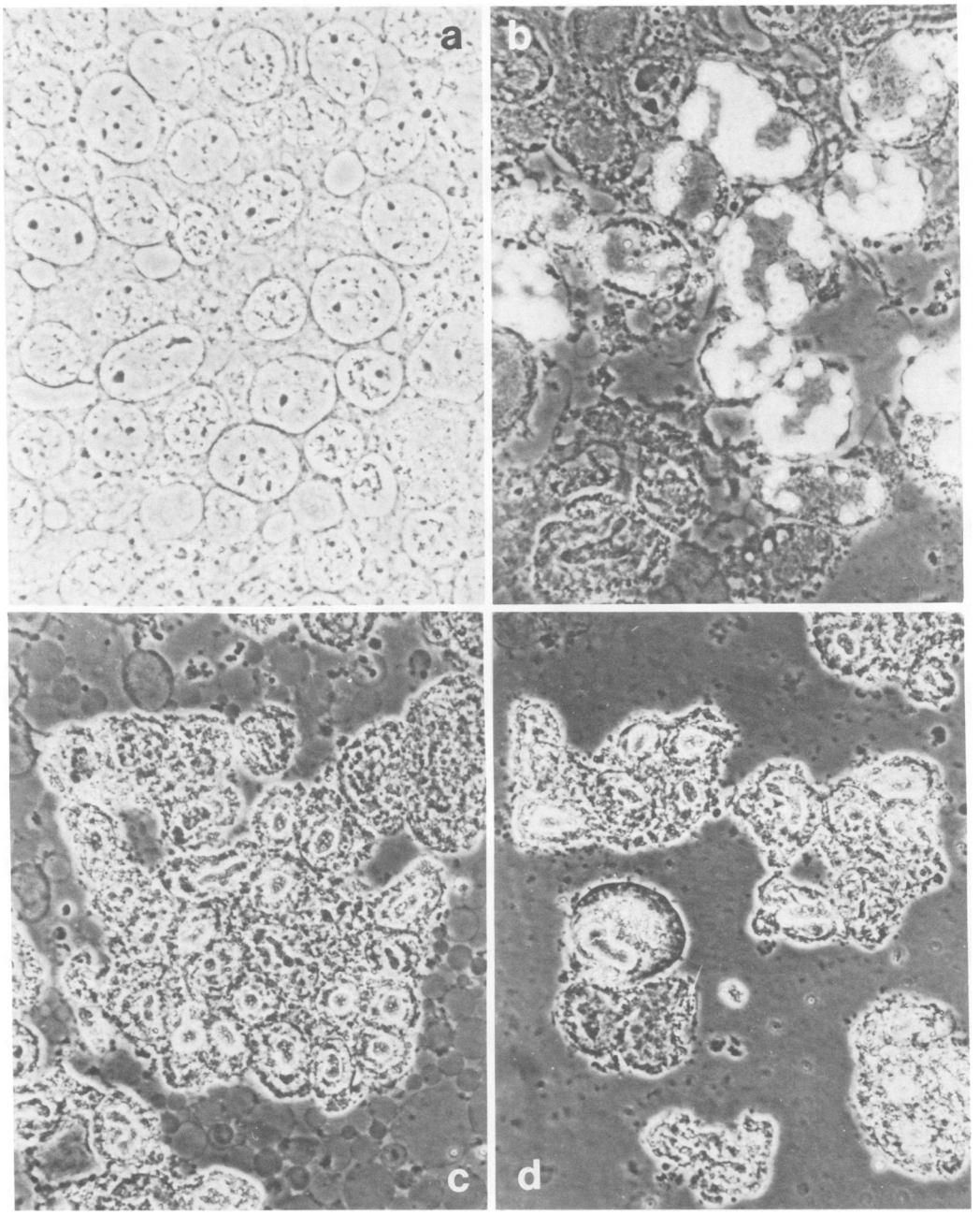

FIG. 3. *S. frugiperda* cells infected with the occlusion-negative mutant virus d10A or wt AcMNPV. (a) Uninfected cells; (b) virus plaque formed from wt AcMNPV with occlusions present in the nucleus of infected cells; (c) virus plaque at 4 days p.i., formed from the O^- mutant virus d10A; and (d) O^- mutant plaque at 6 days p.i.

restriction enzymes. All but 1 of 15 d10 viral DNAs analyzed had a 300-base pair deletion in *Eco*RI-I (e.g., d10A and d10I; Fig. 4). In addition, deletions in 13 of the d10 mutant viruses, for example, d10I, mapped to the same location as was originally introduced into the plasmid (Fig. 1). One of the mutants, d10A, had a 300-base pair deletion located about 300 bases to the left relative to the deletion in the mutant plasmid (Fig. 1). This shift may have resulted from

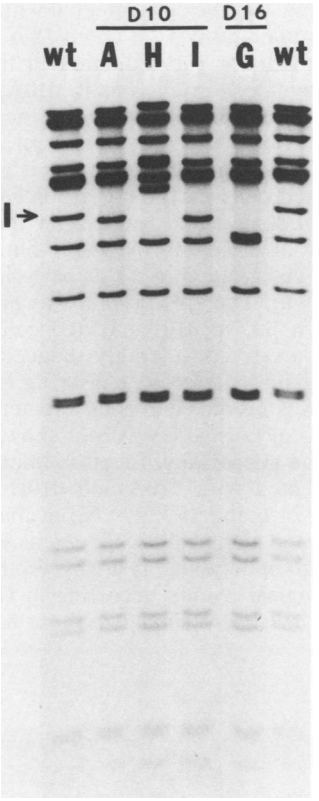

FIG. 4. Restriction analysis of AcMNPV O⁻ mutant viral DNAs. AcMNPV O⁻ mutants were obtained from cells transfected with a mixture of wt and plasmid DNAs. The O⁻ viruses d10A, H, and I were isolated from cells cotransfected with wt AcMNPV viral and pd10 plasmid DNAs. The d16G O⁻ virus was isolated from cells cotransfected with wt viral and pd16 plasmid DNAs. Viral DNAs were labeled with $^{32}P_i$, purified, restricted with *Eco*RI, and electrophoresed in a 0.7% agarose gel. The gel was dried and exposed to X-ray film at −80°C overnight. The location of the *Eco*RI-I fragment from wt AcMNPV DNA is marked.

uneven crossing-over. No other differences could be detected between these 14 d10 mutant viral DNAs and wt AcMNPV DNA. The genome of one O⁻ mutant, d10H, was unusual in that it had a 730-base pair insertion near the *Kpn*I site in the polyhedrin gene, a 500-base pair insertion in *Eco*RI-A, and a 600-base pair insertion in *Eco*RI-F (Fig. 4). Hybridization experiments (not shown) have suggested that the insertion fragments in d10H were host DNA sequences and that this mutant virus may have resulted from spontaneous mutations. Of four d16 O⁻ mutants analyzed, each had an 800-base pair deletion which mapped in the same location in *Eco*RI-I as in the mutant plasmid pd16 (e.g., d16G; Fig. 1 and 4). No other differences could be detected between the d16 mutants and wt viral DNAs.

Protein synthesis in cells infected with occlusion-negative virus. To measure what effect mutations in the polyhedrin gene had on protein synthesis in O⁻ virus-infected cells, *S. frugiperda* cells were infected with d10A, d10I, d10H, d16G, or wt viruses, and the infected cells were pulse-labeled with L-[^{3}H]leucine at 3-h intervals from 0 to 48 h p.i. The appearance of d10I and d16G infected-cell-specific proteins was similar to what was observed in wt-infected cells except that these O⁻ mutants produced no 33K polyhedrin (not shown). A 26K protein that was first detected at 48 h p.i. was produced in d16G- but not in wt-infected cells. This protein was made at a relatively low level and may have been a truncated or aberrant form of polyhedrin. An abundant polypeptide of 23K was made in d10A but not in wt AcMNPV-infected cells (Fig. 5). The 23K protein was first detected between 15 and 18 h p.i. and was one of the major polypeptides in d10A-infected cells labeled at 21 to 24 h and 33 to 36 h p.i. (Fig. 5). The 23K protein also accumulated by 48 h p.i. to a level comparable to that of polyhedrin in wt-infected

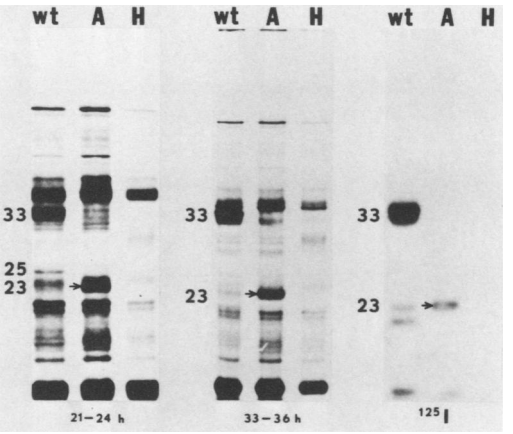

FIG. 5. Pulse-labeled proteins in *S. frugiperda* cells infected with AcMNPV wt (wt), d10A (A), and d10H (H) viruses and a protein blot radioimmunoassay for AcMNPV polyhedrin. Infected cells were labeled with L-[^{3}H]leucine at 21 to 24 h p.i. (left panel) or 33 to 36 h p.i. (middle panel), disrupted, and electrophoresed in a 10% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate. The gel was impregnated with En^{3}Hance (New England Nuclear Corp.) and exposed to X-ray film at −80°C for 2 days. The molecular weights ($\times 10^3$) of certain polypeptides identified in the text are indicated, and the truncated 23K polyhedrin made in d10A-infected cells is marked (→). At 36 h p.i., infected cells were transferred to nitrocellulose and incubated with a 1:500 dilution of AcMNPV polyhedrin antiserum, and specifically bound antibody was detected with ^{125}I-labeled protein A (right panel).

cells, i.e., about 25% of the total mass of protein in the cell (not shown). In a protein blot radioimmunoassay (31) using antiserum specific for AcMNPV polyhedrin, the 23K polypeptide reacted with polyhedrin antibody (Fig. 5). Since the 300-base pair deletion in the d10A polyhedrin gene has sufficient coding capacity to specify about 10,000 daltons of protein, the 23K protein (approximately 10K less than wt AcMNPV polyhedrin) was most likely a truncated polyhedrin. The reduced immunological reaction of 23K as compared to the wt 33K polyhedrin might have been due to the loss of some of the antigenic determinants on the truncated 23K polyhedrin. Polypeptides of 22K, 21K, and 10K which may have resulted from posttranslational modification of wt polyhedrin also reacted with polyhedrin antiserum (Fig. 5). These polypeptides were not detected in wt-infected cells until very late in infection (36 h p.i. or later).

No polyhedrin-like polypeptide could be detected in cells infected with the insertion mutant d10H, and there was an overall reduction in the recovery of labeled cell proteins from about 18 h p.i. on (Fig. 5). The cells infected with d10H also began to undergo lysis at about 18 h p.i., which may account for the reduction in the level of protein synthesis. Cell lysis in d10A, d10I, and d16G mutant-infected cells also occurred but was not evident until about 48 h p.i.

A 25K infected-cell protein that is produced in wt-infected cells early and late in infection (6, 32) was not detected (even after much longer exposure of the gel to the X-ray film) in d10H-infected cells late (Fig. 5) or early in infection (not shown). The 25K protein was not detected until 36 h p.i. in d10A-infected cells and was made at a rate considerably reduced from that observed in wt-infected cells (Fig. 5). A 25K protein was produced in cells infected with d10I and d16G at normal levels (not shown).

Electron microscopy. Electron micrographs of cells infected with d10A and d10H were prepared at 24 h and 36 h p.i. No occlusions were present, and many cells were extensively vacuolated and undergoing lysis by 36 h p.i. The morphological events occurring in O^- mutant-infected cells were similar to those reported for

FIG. 6. Electron micrograph of an *S. frugiperda* cell infected with the AcMNPV O^- mutant d10H at 24 h. p.i. Fibrous material (F) and bundles of nucleocapsids (B), some of which are associated with viral envelopes, were present in the nucleus of mutant-infected cells. No occlusions were observed in O^- infected cells, but the membrane-like structures that normally surround the occlusion bodies were being assembled in association with the fibrous material. Bar, 0.6 μm.

wt AcMNPV in vitro (1, 10, 14). Significantly, although cells infected with d10A and d10H did not produce occlusions, enveloped virus particles with one or more nucleocapsids common to one envelope were assembled in the nucleus (Fig. 6). The fibrous material and the membrane-like structures typically associated with fibrous material (1, 10, 14) in wt-infected cells were also present in d10A- and d10H-infected cells (Fig. 6).

Replication of O^- mutant viruses in vitro. The kinetics of infectious extracellular virus (ECV) release in O^- and wt virus-infected *S. frugiperda* cells were compared. In three separate experiments no significant differences in the time and rate of release of ECV were detected (data not shown). The media from *S. frugiperda* cells infected with AcMNPV wt, d10A, and d10H viruses had titers of 2×10^8 to 3×10^8 PFU/cell at 48 h p.i.

Recombination between cloned fragments and viral DNAs. The utility of the approach described in this report to alter the AcMNPV genome is dependent upon (i) the frequency of recombination between cloned fragments and intact viral DNA, and (ii) the ease of identifying recombinants. These factors were investigated in the following experiments. Our initial experience indicated that it was easier to screen for O^+ than O^- viral plaques. Therefore, to measure for the number of recombinants produced between viral and cloned fragment DNAs, d10A O^- mutant DNA was mixed with cloned AcMNPV *Eco*RI-I DNA at varying ratios, and cells were transfected. Rescued or revertant viruses had the O^+ phenotype and developed plaques readily distinguishable from the parental d10A viral plaques. When 0.1 to 0.003 μg of the cloned *Eco*RI-I plasmid DNA was mixed with 0.01 μg of viral DNA and 3×10^6 cells were cotransfected, the number of O^+ plaques detected decreased from about 12% to 0.3% (Table 1). Each of the O^+ plaques contained a focus of infected cells that produced occlusions and therefore probably resulted from the presence of recombinant viruses rather than from complementation. To test this, 20 O^+ plaques were isolated and replated. Each of them produced plaques that were predominantly O^+. Thus, most, if not all, of the O^+ plaques observed were formed from recombinant viruses. Five of these O^+ viruses were further plaque purified, and their DNAs were found to have restriction patterns identical to that of wt DNA (not shown).

Recombinant O^- and O^+ viruses have also been obtained by plaque purifying the progeny ECV from cells cotransfected with plasmid and viral DNAs. The number of O^+ or O^- viruses detected by using this indirect approach was similar to that described above.

Stability of AcMNPV deletion and insertion mutants. The O^- mutants with deletions in the polyhedrin gene were very stable. We did not detect any reversions of d10A or d16G mutant viruses to the O^+ phenotype among more than 10,000 plaques examined for each mutant. The insertion O^- mutant d10H was equally stable. In addition, d10A and d10H were passed five times in *S. frugiperda* cells, using about 10 PFU/cell, and no occlusions were detected in cells infected with fifth-passage virus.

DISCUSSION

This report describes a procedure to introduce site-specific modifications into the AcMNPV genome. Specifically, a cloned viral DNA fragment containing the polyhedrin gene was mutagenized in vitro, the mutant gene was exchanged for the normal polyhedrin gene by recombination in cotransfected cells, and the formation of viral occlusions was used as a phenotypic marker to identify recombinant viruses. For this procedure to be of general application it is important that allelic exchange occur without further modification of the genome. To test for this we isolated mutant and rescued viruses directly from transfected cells to insure that each was a result of a separate genetic event. Of the 24 recombinants analyzed in this study, 22 had the expected genotype. These results demonstrated that recombination in cells cotransfected with intact viral and fragment DNAs was an effective means of introducing site-directed mutations into the AcMNPV genone.

To screen for recombinant viruses on the basis of plaque morphology presents a special problem not generally encountered when viral recombinants are selected for by their ability to replicate in the presence of specific drugs (12). Because both parental and recombinant viruses can replicate, viral plaques formed in cotransfected cells were often a mixture of recombinant and nonrecombinant viruses. For example, O^- plaques produced from a mixture of wt and deletion mutant plasmid DNAs usually had a few cells with occlusions, and additional plaque purification was necessary to obtain ultimately a homogeneous O^- viral mutant. O^- mutant plaques contaminated with a few wt-infected cells were especially difficult to locate. This may have been the reason why we detected fewer O^- mutants in cells cotransfected with wt viral and mutant plasmid DNAs than we did O^+ viruses in cells cotransfected with O^- viral and the wt fragment DNAs. Plaques formed from both recombinant and nonrecombinant viruses were not observed when mutant or rescued viruses were obtained by plaque purifying the ECV produced from cotransfected cells. Therefore, this indirect approach has been the method of

choice when the objective was to obtain a single mutant or rescued viral recombinant.

Studies have shown that AcMNPV ECV is produced in the absence of occlusion body formation (1, 3, 14, 20) and in the absence of polyhedrin protein synthesis (34). From the results presented in this study we can add that the polyhedrin gene is not essential for the production of infectious extracellular virus. The early AcMNPV 25K protein is also nonessential, or required in only minute quantities, in vitro, as it was not detected in d10H-infected cells and was dramatically reduced in d10A-infected cells. This was the only infected-cell-specific protein other than polyhedrin whose synthesis was significantly affected in O^- mutant viruses. The 25K gene maps to a region of the AcMNPV genome that includes *Eco*RI-A (32); therefore, the 500 base pairs of extra DNA in the *Eco*RI-A segment of d10H might have been responsible for the inactivation of the 25K gene. There may also be a mutation, not detected in DNA restriction analysis, in d10A that resulted in the nearly complete loss of expression of the 25K gene in d10A-infected cells.

Another difference between wt and O^- infections was the integrity of the cells late in infection. Normally, cells will remain intact at least 4 to 5 days after infection with wt AcMNPV. In contrast, many O^- mutant-infected cells were lysed from 1 to 3 days p.i. These results suggest that mutations in the polyhedrin gene may have had an effect on the stability of the plasma membrane late in infection. The simplest explanation is that more virions that acquire their envelopes by budding from the plasma membrane were produced in O^- mutant infections leading to cell lysis. However, we have no conclusive evidence that this occurred, as the titer produced by mutant viruses was not significantly different from that produced by wt AcMNPV. It is pertinent that a baculovirus from *Heliothis zea*, the Hz1 virus, which does not form occlusions in infected cells, also infects cells lytically (28).

Analyses of O^- mutants, in particular d10H and d16G, which produced no detectable polyhedrin, indicated that certain functions are apparently not dependent upon the synthesis of polyhedrin: (i) the decrease in the rate of ECV production from about 20 to 36 h p.i. (38); (ii) de novo assembly and acquisition of viral envelopes in the nucleus (1, 10, 14); (iii) formation of fibrous material in the nucleus (1, 10, 14); and (iv) the appearance and rate of synthesis of AcMNPV-infected cell specific proteins (6, 9, 18, 32, 36, 40). All of these processes occur in O^- mutant-infected cells in a manner similar to that observed in wt AcMNPV-infected cells.

In this report O^- mutants were constructed by deleting polyhedrin gene sequences; however, similar mutants could be constructed by inserting foreign DNA into the polyhedrin gene. If the structural sequences of a foreign gene were fused to the polyhedrin promotor, we would expect a high level of expression of the cloned gene late in infection when host RNA and protein synthesis are supressed. Experiments are currently in progress to test this hypothesis. In a recent review on the potential of using baculoviruses as vectors (22) it was emphasized that the use of O^- mutants should add an extra margin of safety in containing recombinant-DNA baculoviruses because it would decrease their chance of survival in nature. Preliminary experiments with the O^- AcMNPV mutants described in this study indicated that they will not form occlusions in infected insects and, therefore, might be more suitable than the wt AcMNPV as recombinant DNA vectors.

We have demonstrated that the polyhedrin gene can be used as a selectable marker to obtain recombinant viruses. The construction and analyses of recombinant baculoviruses with site-specific mutations or with insertions of foreign DNA sequences can be used to increase our understanding of the function and control of viral genes, to improve these viruses as biological control agents, and to facilitate the development of baculoviruses as cloning vectors.

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