Selection and Characterization of *Autographa californica* Multiple Nucleopolyhedrovirus DNA Polymerase Mutations

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Autographa californica multiple nucleopolyhedrovirus (AcMNPV) DNA polymerase (DNApol) is essential for viral DNA replication. AcMNPV mutants resistant to aphidicolin, a selective inhibitor of viral DNA replication, and abacavir, an efficacious nucleoside analogue with inhibitory activity against reverse transcriptase, were selected by the serial passage of the parental AcMNPV in the presence of increasing concentrations of aphidicolin or abacavir. These drug-resistant mutants had either a single (C543R) (aphidicolin) or a double (C543R and S611T) (abacavir) point mutation within conserved regions II and III. To confirm the role of these point mutations in AcMNPV DNA polymerase, a *dnapol* knockout virus was first generated, and several repair viruses were constructed by transposing the *dnapol* wild-type gene or ones containing a single or double point mutation into the *polyhedrin* locus of the *dnapol* knockout bacmid. The single C543R or double C543R/S611T mutation showed increased resistance to both aphidicolin and abacavir and, even in the absence of drug, decreased levels of virus and viral DNA replication compared to the wild-type repair virus. Surprisingly, the *dnapol* mutant repair viruses led to the generation of occlusion-derived viruses with mostly single and only a few multiple nucleocapsids in the ring zone and within polyhedra. Thus, these point mutations in AcMNPV DNA polymerase increased drug resistance, slightly compromised virus and viral DNA replication, and influenced the viral morphogenesis of occlusion-derived virus.

The molecular mechanisms of baculovirus DNA replication have been studied in *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), the type species of the genus *Alphabaculovirus* of the family *Baculoviridae*. Baculoviruses are distinctive in that they have a biphasic replication cycle producing budded virions (BVs) early in infection, and later in infection, single or multiple nucleocapsids are enveloped in the ring zone to form occlusion-derived virus (ODV), which becomes embedded within polyhedra (14). AcMNPV replicates its large (134-kb), circular, double-stranded DNA genome (1) in the nucleus of infected cells following a molecular interaction between virus-encoded *trans*-acting factors and *cis*-acting DNA sequences. To date, the functional roles of only some *trans*-acting factors involved in DNA replication have been elucidated (24, 31).

The AcMNPV DNA polymerase (DNApol) plays an essential role in AcMNPV DNA replication (27, 48). The 3-kb DNApol open reading frame (ORF) encodes a polypeptide of 984 amino acids (aa) with a predicted molecular mass of 114.31 kDa (46). Previous biochemical studies suggested that baculovirus-encoded DNApol is most closely related to α - and δ -like DNApol of various DNA viruses, such as herpes simplex viruses (HSVs), adenoviruses, and poxviruses (18, 30). Moreover, the AcMNPV DNApol is sensitive to aphidicolin, a feature common to α -like DNApol (18, 37, 43). The δ -like (replicative-form) DNApol requires proliferating cell nuclear antigen (PCNA) (a processivity factor and sliding clamp) during the polymerization of the DNA molecule. PCNA orthologues are not found in most baculovirus genomes, but a homologue of the *pcna* gene in the AcMNPV genome has been reported (6, 33). The AcMNPV DNApol consists of several conserved domains, designated regions I through VII, which are in the order region IV-region II-region VI-region III-region I-region VII-region V and are shared among α -like DNApol of eukaryotes and various DNA viruses (5, 17, 23, 46).

These conserved regions were postulated to be functional domains involved in substrate recognition (23).

Most of the known inhibitors of viral DNA replication are nucleoside or nucleotide analogues. This class of compounds targets DNApol by mimicking and/or competing with the normal deoxy-nucleoside triphosphate (dNTP) substrates. Several inhibitors of baculovirus DNA replication have been documented, with the majority falling within this category, including, surprisingly, the antiretroviral drug abacavir (26, 43, 51). Of these, the effects of abacavir and aphidicolin on AcMNPV replication were reversible (43).

The high incidence of antiviral resistance in both clinical and laboratory settings suggested that baculovirus drug escape mutants could be selected following exposure to various antiviral compounds. Such escape mutants are expected to harbor a specific mutation(s) that would contribute to their resistance.

Aphidicolin is an effective inhibitor of viral DNApol which has been used as a selective agent for the isolation of drug-resistant mutants of HSV (17) and vaccinia viruses (9, 42). Such aphidicolin-resistant viruses exhibit altered sensitivities to other substrate analogues (17). Sequence analysis and marker transfer experiments located point mutations within the conserved regions of both HSV and vaccinia virus DNApol, which conferred resistance to aphidicolin (10, 22, 47). Recombinant HSV harboring point mutations within conserved regions I and VI of HSV-1

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DNApol exhibit altered sensitivities to various nucleotide or nucleoside analogues (2, 23). Similarly, a single point mutation within conserved region VII of HSV-1 DNApol confers resistance to other nucleoside analogues, including acyclovir (22).

Most of the current antiretroviral drugs targeting human immunodeficiency virus (HIV) are nucleoside reverse transcriptase (RT) inhibitors (NRTIs) (8). Abacavir, also referred to as 1592U89 (44) or Ziagen, is a potent and selective NRTI of HIV and is currently included in the multiple regimen of highly active antiretroviral therapy (HAART) (40). Like other antiviral drugs, mutations in HIV RT following prolonged passage or treatment with abacavir can confer resistance to a magnitude of 11-fold (36). Abacavir-resistant mutants and those of other NRTIs such as lamivudine have been mapped to the nucleoside triphosphate binding site of the RT enzyme (4, 16, 35, 36, 38, 45).

The aim of this study was to select and characterize AcMNPV mutants resistant to antiviral compounds that target the viral DNApol enzyme. We hypothesized that “escape” mutants selected from the serial passage of the parental AcMNPV in the presence of increasing concentrations of these drugs would harbor mutation(s) in the AcMNPV genome, particularly within the DNA polymerase gene (*dnapol*). This assumption is reinforced by the fact that the majority of the already characterized drug-resistant viruses, such as those of the *Herpesviridae*, carry genomic changes within the substrate recognition domains of the DNApol. In accordance with this prediction, we have selected and characterized AcMNPV drug escape mutants that conferred resistance to both aphidicolin and abacavir in cell culture. While both mutants had a single or double point mutation in their *dnapol* genes, we wished to confirm that it was these specific mutations that were responsible for resistance. To that end, a *dnapol* knockout (KO) AcMNPV bacmid was first generated, and different repair bacmids were constructed by transposing the *dnapol* wild-type (WT) gene or ones containing the single or double mutations. Our results demonstrated that these single (aphidicolin) or double (abacavir) point mutations in the *dnapol* gene conferred drug resistance but also slightly compromised viral DNA replication and growth in the absence of the drug. Surprisingly, the mutations also altered the virion morphogenesis of single and multiple nucleocapsids of the ODV virions.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* clonal isolate 21 (Sf21) (49) cells were cultured in spinner flasks (Corning) containing Grace's insect medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen), penicillin (25 units/ml), and streptomycin (25 µg/ml). The spinner cultures were maintained at 28°C under constant stirring on a low-profile-multistir, 4-position magnetic stirrer (Bellco Biotechnology). Viral stocks of the parental E2 strain of AcMNPV, recombinant AcGFP (the AcMNPV bacmid bMON14272 with *polyhedrin* and *gfp*), and primary aphidicolin-resistant (APC^r) and abacavir-resistant (ABC^r) mutants were all amplified in Sf21 cells. *dnapol* “repair” viruses were constructed by repairing a *dnapol* knockout bacmid with *dnapol* from AcMNPV (AcMNPV^{rep}) and the abacavir-resistant (ABC^{rep}) and aphidicolin-resistant (APC^{rep}) viruses and including a green fluorescent protein (GFP) reporter gene in each virus.

Antiviral compounds. Aphidicolin was purchased from Sigma-Aldrich, while abacavir (1592U89) was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). Aphidicolin was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to a stock solution of 3 mM, while abacavir was dissolved in phosphate-buffered saline (PBS) to a stock solution of 14 mM. Solubilized stock solutions were

aliquoted into small amounts and stored at −20°C for future use. Working solutions of the antiviral compounds were prepared in complete Grace's insect medium to the desired concentration.

Selection and characterization of AcMNPV primary drug escape mutants. The AcMNPV primary drug-resistant (escape) APC^r and ABC^r mutants were selected by the sequential passage of the parental AcMNPV in increasing concentrations of aphidicolin and abacavir, respectively. Briefly, 500 µl of Sf21 cells (1×10^6 cells/ml) from spinner cultures were seeded into each well of 24-well Costar plates (Corning) and allowed to attach for 1 h at 28°C. The cells were infected with AcMNPV at a multiplicity of infection (MOI) of 10 and rocked gently for 1 h at 28°C. The monolayer was washed twice with PBS and replenished with 500 µl of complete medium containing aphidicolin or abacavir at concentrations close to the 50% effective concentrations (EC₅₀s) of 0.2 µM and 3 µM, respectively (43). Treated cells were incubated at 28°C, and infections were allowed to proceed in the presence of inhibitors until a cytopathic effect (CPE) was evident in 90% of the cells at 72 h postinfection (hpi). At this point, the medium from each well was harvested and centrifuged at $1,000 \times g$ for 5 min at 4°C, and 0.1 ml of the clarified supernatant containing progeny virus was used to infect cells for the next passage in the presence of drugs. This procedure was repeated 9 times in the presence of the drugs or in a series of 2-fold increases in concentrations thereof up to 12.5 µM aphidicolin and 200 µM abacavir. AcMNPV mutants resistant to aphidicolin (APC^r) and abacavir (ABC^r) were plaque purified through three cycles in Sf21 cells in the presence of the inhibitors at concentrations of 6.25 µM aphidicolin and 100 µM abacavir.

Viral growth curves. For virus growth curve analysis, Sf21 cells in T-75 flasks (~70% confluent) were infected or mock infected with parental AcMNPV, the APC^r and ABC^r primary drug-resistant viruses, and the corresponding repair viruses with the same mutation, APC^{rep} and ABC^{rep}, respectively, at an MOI of 0.1. Infected cells were gently rocked for 1 h at 28°C. After 1 h of virus adsorption, infected cells were rinsed once with fresh medium and then incubated at 28°C. At different times postinfection, viral yields were determined by a 50% tissue culture infective dose (TCID₅₀) endpoint dilution assay on Sf21 cells and calculated according to the Reed-Muench method (31). For all the infections, time zero (0 hpi) was considered to be the time when the monolayer was replenished with fresh medium after the unadsorbed virus had been aspirated from the wells. Each single-step growth curve experiment was replicated three times.

To assess whether DNApol is essential for virus production, a viral growth curve analysis was performed following transfection with a *DNApol* KO bacmid and other bacmids. Sf21 cells (2×10^6) were transfected in triplicate with 5.0 µg of the appropriate bacmid DNA by using Cellfectin liposome reagent (Invitrogen). The cell monolayers were incubated for 5 h posttransfection (hpt), rinsed twice with Grace's medium, and replenished with 2 ml of fresh Grace's medium supplemented with 10% FBS. Virus supernatants were collected from the extracellular medium at the indicated time points. The titers of budded virus (BV) were determined by a TCID₅₀ assay.

Comparison of virus-induced CPE and quantification of OBs. The CPE and structural morphologies of occlusion bodies (OBs) from mutant viruses were compared with those of the parental virus by examining infected cells with an inverted phase-contrast light microscope (Zeiss) at different times postinfection. Photographs of cells exhibiting CPE were taken from a monolayer of cells grown on coverslips by using a Leica DM 4000 B microscope (Leica Microsystems).

The purification and quantification of OBs from cells infected with parental AcMNPV or mutants were done by using a method similar to that described previously by Harrison and Lynn (19). Briefly, Sf21 cells maintained in a spinner culture were seeded at a density of 1×10^6 cells/well into six-well plates containing glass coverslips. Cells were infected with either parental AcMNPV or the APC^r or ABC^r virus at an MOI of 10 and incubated at 28°C. At 96 hpi, OBs were purified from infected cells (3 wells/virus), as previously described (32), and enumerated by using a hemocytometer.

Quantitative real-time PCR. Total intracellular DNA of the parental AcMNPV and the escape mutant viruses was extracted at different times postinfection from $\sim 5 \times 10^5$ infected Sf21 cells maintained in a 24-well plate by using the QIAamp DNA blood minikit (Qiagen) according to the manufacturer's recommendations. Final volumes of the total intracellular DNA were brought to 200 μ l. The concentration of DNA was determined at an A_{260} by using a NanoDrop ND-1000 UV-visible (UV-Vis) spectrophotometer (NanoDrop Technologies). Quantitative real-time PCR of the virus DNA from WT AcMNPV and escape mutants was performed with a Rotor-Gene 6000 series instrument (Corbett Robotics), using SYBR green 1 dye chemistry. The amplification of the target AcMNPV DNA was done with 20- μ l final volumes in 0.2-ml PCR tubes. The primers used for quantitative PCR (qPCR) analysis were designed as previously described (43).

The reaction mixture in each tube consisted of 10 μ l of 2 $\times$ QuantiTect SYBR green PCR master mix (Roche), 0.5 μ M each forward and reverse primer, and 2 μ l of template viral DNA. The amplification profile consisted of a 15-min initial activation of HotStarTaq DNA polymerase at 95°C, 45 cycles of 15 s of denaturation at 95°C, 12 s of annealing at 53°C, and 25-s extensions at 72°C. The viral DNA copies from total intracellular DNA isolated at different times postinfection were quantified by using an external calibration curve generated from serial 10-fold dilutions of a plasmid DNA harboring one copy of the AcMNPV *ie-1* insert (43).

Sequence analysis. To map mutations to the viral DNA replication-associated proteins (replicators), the target gene sequences were amplified from the viral DNA by PCR. In brief, total intracellular DNA was isolated from Sf21 cells infected with parental AcMNPV or the APC^r or ABC^r virus at 24 hpt, as described above. Purified intracellular DNA was used as a template to PCR amplify the entire *dnapol* gene using high-fidelity *Pfu* polymerase and overlapping primers spanning the entire gene. Amplicons coding for other *trans*-acting factors involved in AcMNPV DNA replication, P143 (helicase), LEF-1 (primase), LEF-2 (primase accessory factor), LEF-3 (single-stranded DNA binding protein), IE-1 (immediate early protein), and PCNA, were generated and sequenced by using gene-specific primers. All the primers used in the sequencing reactions are listed in Table S1 in the supplemental material and were designed based on the same melting temperature (T_m) by using Gene Runner software, version 3.05. The expected PCR products were confirmed on 1% agarose gels, purified by using a Biotech Inc. lambda PCR purification kit, and sequenced by the dideoxynucleotide chain termination method using an Applied Biosystems 377 Prism sequencer. Sequences were obtained for both DNA strands. Parental AcMNPV DNA sequences were compared with those of the mutant viruses to locate the nucleotide change(s) in the virus genome, if any.

Generation of the *dnapol* knockout from the AcMNPV bacmid. To substitute the parental AcMNPV *dnapol* gene with the mutant form, a *dnapol* knockout virus was first generated. The construction of a *dnapol* knockout in a bacmid, bMON14272, containing the AcMNPV genome was accomplished via a PCR-based protocol involving the λ Red homologous recombination system in *Escherichia coli* (BW2113), as previously described (7, 48). To delete the *dnapol* gene and provide a selectable marker, a chloramphenicol acetyltransferase (CAT) gene (*cat*) with flanking sequences corresponding to those flanking the parental *dnapol* gene was designed to replace the WT bacmid *dnapol* gene. The deletion was designed to functionally inactivate *dnapol* but to retain the promoter region of the adjacent, essential ORFAC66. Briefly, a linear CAT cassette was PCR amplified from template plasmid pKD3 (7) with primers (DNA54683 and DNA52934) containing a sequence of 50 nucleotides (nt) homologous to each of the 5' and 3' regions of the AcMNPV *dnapol* gene (see Table S1 in the supplemental material). The resultant 1,112-bp fragment was gel purified by using a Biotech Inc. lambda gel extraction kit, digested overnight with DpnI to eliminate contamination by the template plasmid, phenol-chloroform extracted, and resuspended in 10 μ l of elution buffer (10 mM Tris, pH 8.0).

Initially, 1 ml of a fresh *E. coli* BW25113 culture harboring the AcMNPV bacmid (bMON14272) and temperature-sensitive plasmid

pKD46, which harbors the λ Red recombinase genes *gam*, *beta*, and *exo* (7), was transferred into 100 ml of fresh Luria-Bertani (LB) broth containing ampicillin and kanamycin and cultured at 30°C. At an optical density at 600 nm (OD_{600}) of 0.1, 1 ml of 10% L-arabinose was added to the culture to induce the expression of λ Red recombinase, and at an OD_{600} of 0.6, cells were made electrocompetent by concentrating the cells 100-fold and washing them three times in ice-cold 10% glycerol. The electroporation of the linear PCR product was done in a 2-mm-gap cuvette by using a Bio-Rad Gene Pulser II instrument at 2.3 kV, 25 μ F, and 200 Ω with 40 μ l of the cell suspension and 50 to 500 ng of the linear CAT PCR product. Following electroporation, the cells were allowed to recover in 800 μ l of LB broth devoid of antibiotics and then incubated at 37°C with gentle agitation (100 rpm) for 5 h. Two hundred microliters of the cell culture was plated onto LB plates containing kanamycin (30 μ g/ml) and chloramphenicol (34 μ g/ml) and incubated for 24 h at 37°C. Helper plasmid pKD46 was cured by incubation overnight at 43°C, followed by the selection of colonies on kanamycin and chloramphenicol plates.

Clones isolated from chloramphenicol and kanamycin plates following homologous recombination were further screened by PCR to ascertain the deletion of the *dnapol* gene from its locus in the AcMNPV bacmid and to verify the correct insertion and orientation of the CAT cassette into the *dnapol* locus by using primers listed in Table S1 in the supplemental material and Fig. 2D. Primers specific to the *dnapol* gene were used to verify the presence and absence of the *dnapol* gene in the AcMNPV bacmid and Ac^{KO} genomes.

Repair bacmid construction. A *dnapol* repair bacmid, expressing GFP to monitor replication, was constructed by using the knockout *dnapol* bacmid Ac^{KO} and plasmid pFACT-GFP (11). The 3.2-kb *dnapol* fragment containing the entire DNAPol ORF and its native promoter and poly(A) signal was PCR amplified from parental AcMNPV and the APC^r and ABC^r viruses and cloned into pGEM-T Easy vectors. Plasmids pGEM-APC, pGEM-ABC, and pGEM-WT were then sequenced to confirm the G-to-C transversion at AcMNPV DNA nt 53452 for both the APC^r and ABC^r viruses and the T-to-C transition at nt 53657 for the ABC^r virus, with the A residue of the first ATG codon of the AcMNPV *polyhedrin* gene defined as nucleotide position 1.

Plasmids pFACT-GFP-APC, pFACT-GFP-ABC, and pFACT-GFP-WT were constructed by isolating the 3.2-kb fragments from pGEM-APC, pGEM-ABC, and pGEM-WT, respectively, with SacI and XhoI and cloning them into pFACT-GFP. The entire *dnapol* inserts in pFACT-GFP were also sequenced to confirm the expanded sequence. The Tn7 cassettes from pFACT-GFP-APC, pFACT-GFP-ABC, and pFACT-GFP-WT were transferred to the Ac^{KO} bacmid, as described in the Bac-to-Bac expression manual (Invitrogen), creating the APC^{rep}, ABC^{rep}, and WT^{rep} viruses, respectively.

The altered nucleotides corresponding to the C543R and S611T mutations (see Fig. 2A) in each plasmid and virus were confirmed by the sequencing of a PCR product based on oligonucleotide primers DNAPolF53220 and DNAPolR53930.

Transfection. Bacmid DNA was purified from 500-ml LB cultures by using a Qiagen Midi plasmid kit, and concentrations were determined spectrophotometrically by using a NanoDrop ND-1000 instrument. Sf21 cells were plated at a density of 1×10^6 cells per well into six-well plates. Cells were transfected with 2 μ g of bacmid DNA by using Cellfectin (Invitrogen) according to the manufacturer's protocol. Cells were incubated in the presence of the transfection mixture for 5 h, at which time the transfection mixture was removed and replaced with fresh Grace's medium (11).

Drug susceptibility assays. The drug susceptibilities of parental and repaired viruses were determined by a GFP fluorescence infected-cell reduction assay in Sf21 cells. Briefly, Sf21 cells were inoculated at an MOI of 0.01, and after 1 h of viral adsorption, the inoculum was replaced with fresh medium containing aphidicolin (0.049, 0.098, 0.195, 0.39, and 0.78 μ M) or abacavir (6.25, 12.5, 25, 50, and 100 μ M). The results were recorded after 4 days of incubation at 28°C. At least three independent

experiments were performed to determine the concentration of compound required to reduce the number of GFP-fluorescent cells by 50% relative to that in non-drug-treated wells (EC_{50}).

Construction of defined *dnapol* mutations. The mutant *dnapol* gene with a single T53657C mutation was generated by using overlap-extension PCRs, as described previously (21). The mutagenic primers used are listed in Table S1 in the supplemental material; KOD DNA polymerase was used for all PCR amplifications. For the generation of the T53657C mutation (corresponding to the C543R mutation), pGEM-WT was used as the template for the following two PCRs: one with primers 55539XhoI and DNAPolT53657C^{up} and the other with primers DNAPolT53657C^{down} and 52314SacI (see Table S1 in the supplemental material). The PCR products were then annealed to each other, and external primers 55539XhoI and 52314SacI were used to generate the *dnapol* gene with the T53657C mutation. The T53657C fragment generated was cloned into the pGEM-T Easy vector and verified by sequencing. The T53657C fragment was then inserted into pFACT-GFP to generate pFACT-GFP-T53657C. The Tn7 cassette from pFACT-GFP-T53657C was transferred to the Ac^{KO} bacmid to create Ac^{KO}T53657C^{rep}.

Structure predictions. Structure predictions of the AcMNPV DNAPol were made based on the crystal structure of HSV-1 polymerase (Pol) and the ternary complex of yeast Pol δ as templates (28, 41) (Protein Data Bank accession numbers 2GV9 and 3IAY, respectively). The structure reported under Swiss-Prot accession number P18131 was used for modeling. We used Windows 7 Professional (64-bit operating system) with the DeepView v4.0 package of programs (Swiss Institute of Bioinformatics) and PyMOL 1.1 (DeLano Scientific); Magic Fit in DeepView was used for the superposition of chains to place the structure reported under Protein Data Bank accession number 2GV9 into the reference structure reported under accession number 3IAY. The modeling project was submitted to the SWISS-MODEL software program (<http://swissmodel.expasy.org/SWISS-MODEL.html>) (3, 39). PyMOL and DeepView were used for the production of Fig. 7.

Electron microscopy. Sf21 cells (1×10^6) were infected with BV (WT^{rep}, ABC^{rep}, or APC^{rep}) at an MOI of 10. Mock-infected cells were treated similarly but without the addition of BV. By 60 hpi, cells were dislodged and pelleted at $500 \times g$ for 5 min. At 60 hpi, WT^{rep}-, ABC^{rep}-, or APC^{rep}-infected Sf21 cells were fixed in 2.5% (vol/vol) buffered glutaraldehyde (cacodylate buffer) overnight at 4°C. Samples were washed in 0.1 M cacodylate buffer 3 times, postfixed in 1% buffered osmium tetroxide for 1 h at room temperature, washed thoroughly 3 times with double-distilled water (ddH₂O), dehydrated by using a graded ethanol series, infiltrated with 50:50 ethanol-LR White resin and 2 changes of 100% LR White resin, placed into gelatin capsules, and polymerized at 60°C. Ultrathin sections were taken by using a Porter-Blum MT-1 microtome (Sorvall). Sections were picked up on copper grids and stained with aqueous uranyl acetate and lead citrate. Samples were observed with a Philips CM10 transmission electron microscope at an accelerating voltage of 80 kV.

Determination of sizes of polyhedra. As the polyhedra resulting from infection with the different mutants appeared to be larger than expected, we measured their sizes. Polyhedra were isolated from virus-infected cells. Cells were broken by ultrasound, polyhedra were collected at $8,000 \times g$ for 10 min and then suspended with 500 μ l of 0.5% SDS, and the polyhedra were centrifuged at $8,000 \times g$. The pellet was washed twice and then resuspended in ddH₂O. The polyhedra were then examined by light microscopy, and their diameters were determined by using an internal ruler. Polyhedra were measured in random groups of 20, and this was repeated three times for each virus.

RESULTS

Selection and characterization of drug escape mutants. Two selected primary drug escape mutants, APC^r (aphidicolin resistant) and ABC^r (abacavir resistant), were generated by the sequential serial passage of the parental AcMNPV under conditions of in-

creasing concentrations of aphidicolin and abacavir, respectively. ABC^r and APC^r virus supernatants were harvested after 9 passages by increasing the concentrations of abacavir to 200 μ M and aphidicolin to 12.5 μ M and then plaque purified for three cycles in the presence of inhibitors at the same concentrations. In the presence of the inhibitors, both the APC^r and ABC^r viruses produced occlusion bodies (OBs) and exhibited typical CPE at various concentrations of aphidicolin and abacavir, unlike WT AcMNPV, which was inhibited by the drugs (data not shown). Also, some of the OBs of the APC^r and ABC^r viruses exhibited several large cuboidal morphologies in addition to the regular polyhedron-shaped morphologies noted for WT AcMNPV. Moreover, cells infected with either the APC^r or ABC^r virus contained typically fewer OBs/cell (9.33 ± 1.52 OBs/cell for the ABC^r virus and 18.33 ± 3.21 OBs/cell for the APC^r virus) than parental AcMNPV-infected cells (118.67 ± 21 OBs/cell) at 96 hpi. Both resistant phenotypes were maintained through four serial passages in the absence of the drugs, suggesting that the observed resistance phenotype was stable in infected cells.

Replication of APC^r and ABC^r viruses. Single-step growth curves and the kinetics of viral DNA accumulation for the parental virus and both mutants in the absence of the inhibitors were compared to determine the effects on virus replication and DNA synthesis in cell culture (Fig. 1A and B). Both drug-resistant mutants exhibited similar replication patterns and yields of infectious budded virions in untreated cells, comparable to those of the parental virus (Fig. 1A). Similarly, the kinetics of viral DNA accumulation were comparable for both the APC^r and ABC^r viruses and the parental virus in the absence of drugs (Fig. 1B). In contrast, viral DNA synthesis for both the APC^r and ABC^r viruses was resistant to both aphidicolin and abacavir, while that for WT AcMNPV was inhibited by both drugs at the concentrations used (Fig. 1C and D). While some minor DNA accumulation was detected in AcMNPV-infected cells in the presence of either aphidicolin or abacavir, it was much less than that for the two mutants. These results suggested that, in the absence of drugs, the drug escape mutants exhibited *in vitro* growth properties equivalent to those of the parental AcMNPV.

Sequence analysis of primary, drug-resistant ABC^r and APC^r viruses. Since resistance to the inhibitors was likely due to mutations in *dnapol*, and as DNAPol is sensitive to at least aphidicolin, we first sequenced the *dnapol* genes of the APC^r and ABC^r viruses to identify any *dnapol* mutations in the primary drug-resistant viruses. Sequence analyses of PCR products of the APC^r and ABC^r *dnapol* genes revealed a single point mutation (G-C) at position 53452 in both mutants and a second point mutation at nt position 53657 (T-C) of the ABC^r *dnapol* gene (Fig. 2A).

The G53452C transversion conferred a serine-to-threonine substitution at residue 611 (S611T) in conserved region III of the DNAPols of both mutant viruses, while the second genomic change, the T53657C transition in conserved region II, observed for the ABC^r virus conferred a cysteine-to-arginine change at amino acid position 543 (C543R) of the DNAPol (Fig. 2B).

A sequence alignment of DNAPols of all baculovirus genomes sequenced to date showed that the serine residue at position 611 is invariant and is conserved in all known baculoviruses (data not shown). This finding suggests that the residue (S611T) was selected for in the presence of aphidicolin and perhaps abacavir, and this change may have an important role in contributing to both aphidicolin and abacavir resistances in cell culture. On the other

FIG 1 Viral growth and DNA accumulation kinetics of the AcMNPV APC^r and ABC^r primary drug escape mutants. (A) Sf21 cells were independently infected with the APC^r and ABC^r mutants and parental AcMNPV (MOI = 0.1) in the absence of drugs. The production of budded virus was assayed by using TCID₅₀ endpoint dilution assays at different times postinfection (0 to 72 hpi). (B) Sf21 cells were infected with WT AcMNPV or the APC^r or ABC^r mutant at an MOI of 10. (C) The APC^r mutant and AcMNPV were grown in the presence of either abacavir (100 μM) or aphidicolin (6.25 μM) throughout, as indicated. (D) The ABC^r mutant and AcMNPV were grown in the presence of either abacavir (100 μM) or aphidicolin (6.25 μM) throughout, as indicated. For panels B to D, total intracellular DNA was extracted at different times postinfection from infected cells treated with aphidicolin and abacavir, and AcMNPV DNA copy numbers were determined by qPCR. Bars in the panels represent standard deviations determined from three independent replicates.

hand, the cysteine residue at position 543 is conserved only in group I alphabaculoviruses. The DNaPol of the deltabaculovirus *Culex nigripalpis* nucleopolyhedrovirus (24) contained an R substitution at position 543, equivalent to that observed for the ABC^r virus. Thus, this region might accommodate some variations without necessarily conferring resistance on its own.

Although we identified mutations in the *dnaPol* genes of the two mutants, mutations in other parts of the genome might have been responsible for this resistance. To eliminate the possibility of a mutation in some other known *trans*-acting factors involved in AcMNPV DNA replication, we generated and sequenced PCR products for LEF-1 (DNA primase), LEF-2 (primase accessory factor), LEF-3 (single-stranded DNA binding protein), P143 (DNA helicase), IE-1 (immediate-early protein 1), and PCNA (proliferating cell nuclear antigen), but no mutations were found in any of these factors. Furthermore, there were no mutations identified following sequence analysis of the *fp25k* gene (Ac61), a viral gene that encodes the few polyhedra (FP) phenotype, although both the APC^r and ABC^r drug escape mutants exhibited an FP phenotype commonly attributed to mutations in *fp25k* in other baculoviruses (20).

Generation and characterization of the GFP reporter *dnaPol* knockout, mutant and wild-type *dnaPol* repair, and WT AcMNPV bacmids. In order to confirm that the mutations from the *dnaPol* genes of the selected mutants and not mutations elsewhere in the genome were exclusively responsible for resistance, we duplicated those same *dnaPol* mutations in a common bacmid

background. Thus, the only difference would be the mutations that we introduced into *dnaPol*. We first generated a *dnaPol*-null mutant bacmid, Ac^{KO} (Fig. 2C). This Ac^{KO} bacmid (lacking *dnaPol*) was then used as the backbone to reintroduce (repair), under the control of the native *dnaPol* promoter and poly(A) site, the WT *dnaPol* (WT^{rep}), the APC^r *dnaPol* (APC^{rep}), and the ABC^r *dnaPol* (ABC^{rep}) genes into the polyhedrin locus of the bacmid. The *polyhedrin* and *gfp* genes were also introduced by transposition into the same locus (Fig. 2E). Sequence analysis confirmed the accuracy of this bacmid cloning of WT *dnaPol* and the two *dnaPol* repair viruses (single and double point mutations). The positions of the PCR primer pairs used to confirm the knockout sites of these viruses are shown in Fig. 2D. All constructs were confirmed by PCR analysis. AcGFP was used as a positive control for WT *dnaPol* in its native locus.

Ac^{KO}GFP, APC^{rep}, ABC^{rep}, WT^{rep}, and AcGFP bacmids were transfected into Sf21 cells, and virus replication and spread were monitored by GFP fluorescence. The numbers of GFP-positive cells at 24 hpt were similar for all five bacmid-transfected monolayers, indicating equivalent transfection efficiencies (Fig. 3A). Only a limited spread of fluorescence was observed, with GFP-positive cells being found either individually or in small clusters of two to three cells each for Ac^{KO}GFP at all times posttransfection. However, there was an increased spread of fluorescence to neighboring cells for the APC^{rep}, ABC^{rep}, WT^{rep}, and AcGFP bacmids by 48 hpt. By 72 hpt, fluorescence was observed in almost all cells transfected by the APC^{rep}, WT^{rep}, and AcGFP bacmids. By 96 hpt,

FIG 2 DNA polymerase mutations, construction of knockout AcMNPV bacmids, and schematic diagram of the WT and three mutant *dnapol* repair viruses. (A) The entire *dnapol* gene was PCR amplified by using *Pfu* polymerase and sequenced. Sequence analysis of the PCR products of the *dnapol* genes of the AcMNPV escape mutants showed that the APC^r and ABC^r escape mutants had a single-nucleotide change of G53452C in the genome, and an additional change of T53657C was found for the ABC^r mutant. (B) DNAPol conserved domains I to VII and amino acid residues associated with drug resistance. Filled boxes depict the relative positions of conserved regions I to VII of the AcMNPV DNAPol ORF. The numbers above the boxes refer to the range of amino acid residues of each of the conserved regions. The amino acid sequence alignment of conserved regions II and III of both DNAPols and those of the WT DNA polymerase are indicated by the dotted lines for the ABC^r and APC^r mutants. The corresponding amino acids for the HSV DNAPol are in the bottom row. Both the APC^r and ABC^r DNAPols have amino acid substitutions (boxed) within conserved regions III (S611T) and II (C543R), respectively. (C) Strategy for construction of a DNAPol knockout bacmid containing a deletion of the AcMNPV *dnapol* gene by recombination in *E. coli*. An internal 1,749-bp portion of a *dnapol* ORF was deleted and replaced with the chloramphenicol acetyltransferase (CAT) gene. (D) PCR strategy to show the presence or absence of sequence modifications in the Ac^{KO} bacmid. Positions of primer pairs and probes used to confirm the disruption of the DNAPol ORF and the correct insertion of the CAT cassette (see Table S1 in the supplemental material) are shown above and below the map. (E) Schematic of WT^{rep}, ABC^{rep}, APC^{rep}, and Ac^{KO}GFP viruses showing the *polyhedrin* (*polh*) and *gfp* genes inserted into the *polyhedrin* locus by Tn7-mediated transposition.

GFP fluorescence spread completely from the initially transfected cells to adjacent cells for the APC^{rep}, WT^{rep}, AcGFP, and ABC^{rep} bacmids. Even by 96 hpt, there was no increase in the number of fluorescent cells for the Ac^{KO}GFP bacmid, indicating that there was no spread of the virus from the initially transfected cells for the *dnapol* KO bacmid. Occlusion bodies were detected microscopi-

cally in APC^{rep}-, ABC^{rep}-, WT^{rep}-, and AcGFP-transfected cells by 96 hpt, while no occlusion bodies were found in the Ac^{KO}GFP-transfected cells (Fig. 3B). This indicated that *dnapol* was essential for native polyhedron production and permissive infection of the cells. This also indicated that the APC^r and ABC^r versions of *dnapol* were equally able to repair the *dnapol* knockout.

FIG 3 GFP fluorescence and cytopathology of repair virus bacmids and the Ac^{KO}GFP bacmid in Sf21 cells. (A) Fluorescence images of monolayers of ABC^{rep}-, APC^{rep}-, WT^{rep}-, Ac^{KO}GFP-, and AcGFP-transfected cells at 24, 48, 72, and 96 hpt. (B) Light microscopy images of monolayers of ABC^{rep}-, APC^{rep}-, WT^{rep}-, Ac^{KO}GFP-, and AcGFP-transfected cells at 96 hpt.

***dnapol* point mutations affected virus replication.** Virus replication was monitored in Sf21 cells transfected with 5 μ g of bacmid DNA of either the Ac^{KO}GFP, APC^{rep}, ABC^{rep}, or WT^{rep} virus for up to 120 hpt. Sf21 cells transfected with the APC^{rep}, ABC^{rep},

and WT^{rep} viruses all revealed a steady increase in the level of virus production up to 120 hpt (Fig. 4A). No significant difference was found at 24 hpt among the APC^{rep}, ABC^{rep}, and WT^{rep} viruses. However, the level of virus production was somewhat lower for

FIG 4 Analysis of virus production and viral DNA replication of WT^{rep}, APC^{rep}, and ABC^{rep} repair viruses. (A) Monolayers were transfected with 5 μ g of APC^{rep}, ABC^{rep}, WT^{rep}, or Ac^{KO}GFP bacmid in the absence of the drugs. (B) Sf21 cells were infected by APC^{rep}, ABC^{rep}, and WT^{rep} viruses at an MOI of 0.01 in the absence of the drugs. For panels A and B, supernatant samples were collected at the indicated time points and assayed for the production of infectious virus by a TCID₅₀ assay. (C) Sf21 cells were infected by APC^{rep}, ABC^{rep}, and WT^{rep} viruses at an MOI of 10 in the absence of the drugs. Total intracellular DNA was extracted at different times postinfection. The quantification of AcMNPV DNA copy numbers was performed by qPCR. Each datum point represents the average value from three independent transfections, and error bars represent standard errors.

FIG 5 GFP fluorescence assay of APC^{rep}, ABC^{rep}, and WT^{rep} virus spread in Sf21 cells at different concentrations of aphidicolin and abacavir. Shown are fluorescence images of monolayers of cells infected at an MOI of 0.01 with APC^{rep}, ABC^{rep}, and WT^{rep} viruses sampled at 24 and 96 hpi and grown in the presence of abacavir (6.25, 12.5, 25, 50, and 100 μ M) (A) and aphidicolin (0.098, 0.195, 0.39, 0.78, and 1.57 μ M) (B), as indicated next to each row. Rows 0 for both panels are control cells infected by viruses without drugs.

both the ABC^{rep} and APC^{rep} viruses than for the WT^{rep} virus at later times posttransfection. By 120 hpt, the BV titer of the APC^{rep} virus was about half that of the WT^{rep} virus, while that of the ABC^{rep} virus was 11-fold lower (Fig. 4A). In contrast, BV was not detectable at any time point up to 120 hpt for Ac^{KO}GFP-transfected cells (Fig. 4A). These data showed that the *dnapol* repair viruses produced infectious virus but with a slightly lower yield than that of the wild type.

The effect of the point mutations on virus replication was determined by viral growth curves using viruses as well. Cells were infected by virus derived from the APC^{rep}, ABC^{rep}, and WT^{rep} viruses at a multiplicity of infection (MOI) of 0.01. These growth curves also showed a steady increase in the level of virus production during infection. By 120 hpi, the titers of the APC^{rep} and ABC^{rep} viruses were about 4.9 logs and 4.1 logs higher than those at time zero, respectively, compared to a 5.5-log increase in titers for the WT^{rep} virus (Fig. 4B). Both the transfection and infection data revealed that the *dnapol* point mutant viruses were somewhat compromised in virus replication and yield.

A quantitative PCR assay was utilized to monitor the accumulation of intracellular total viral DNA in the absence of aphidicolin and abacavir and restricted to a single replication cycle over the first 24 hpi. Sf21 cells were infected with the APC^{rep}, ABC^{rep}, or WT^{rep} virus at an MOI of 10. There was no increase in viral DNA content at 6 hpi for any virus. DNA started to accumulate at 12 hpi. By 24 hpi, the levels of intracellular DNA for the APC^{rep} and ABC^{rep} viruses increased about 3.0 logs and 1.4 logs over those at time zero, respectively, while DNA accumulation for the WT^{rep} virus increased about 3.3 logs over the same time course (Fig. 4C). These results revealed that the viral DNA replication of point mutant viruses was somewhat compromised.

***dnapol* mutant analysis.** The APC^r virus has a single mutation at position G53452C, and the ABC^r virus has a double mutation of G53452C and T53657C. To investigate whether the T53657C point mutation alone could contribute to drug resistance, we also constructed a recombinant virus containing only the T53657C

single point mutation within conserved region II of DNApol. Drug sensitivity assays were performed to evaluate the sensitivity of Ac^{KO}T53657C^{rep} to aphidicolin and abacavir. The T53657C recombinant was sensitive to both aphidicolin and abacavir. The EC₅₀s of aphidicolin and abacavir for Ac^{KO}T53657C^{rep} were 0.14 μ M and 9.19 μ M, respectively, which were not different from that of the WT^{rep} virus. This finding suggests that although this mutation was detected in the ABC^r virus, it conferred, at least on its own, only a low level of resistance to both abacavir and aphidicolin.

***dnapol* point mutations conferred drug resistance.** To measure the level of resistance of viruses with the point mutations in *dnapol* to the two inhibitors, Sf21 cells were infected with the three recombinant repair viruses, APC^{rep}, ABC^{rep}, and WT^{rep}, and the replication-dependent spread of GFP fluorescence was measured by using various concentrations of aphidicolin and abacavir. Sensitivity to these inhibitors would be reflected in a reduced level of spread of GFP fluorescence at 96 hpi. The GFP fluorescence of the APC^{rep} or ABC^{rep} virus spread to other cells in the absence or presence of aphidicolin (from 0.098 to 0.39 μ M) or abacavir (from 6.25 to 50 μ M) by 96 hpi, respectively, showing that both the APC^{rep} and ABC^{rep} viruses were resistant to both aphidicolin and abacavir, while the WT^{rep} virus was sensitive to both inhibitors at all concentrations tested (Fig. 5). Values for the 50% effective concentration (EC₅₀), the concentration of drug needed to reduce the number of GFP-fluorescent cells at 96 hpi by 50%, were determined directly from GFP fluorescence reduction curves. The EC₅₀s of aphidicolin and abacavir for the APC^{rep} virus were 0.56 μ M and 29 μ M, respectively, while for the ABC^{rep} virus, the EC₅₀s were 0.15 μ M and 78 μ M, respectively.

Virus growth curve analysis was also performed to monitor the replication of the APC^{rep} and ABC^{rep} viruses in the presence of aphidicolin and abacavir, respectively. Sf21 cells were infected with recombinant viruses at an MOI of 0.01, and at selected time points, the virus titers were determined by TCID₅₀ endpoint dilution assays. The level of virus production increased steadily in cells

FIG 6 Analysis of virus resistance to aphidicolin and abacavir. (A) APC^{rep} and WT^{rep} viruses were grown in Sf21 cells with or without aphidicolin. (B) ABC^{rep} and WT^{rep} viruses were grown in Sf21 cells with or without abacavir. For panels A and B, supernatant samples were collected at the indicated time points and assayed for the production of infectious virus by a TCID₅₀ assay. (C) Analysis of viral DNA accumulation of APC^{rep} and WT^{rep} viruses grown in the presence or absence of aphidicolin. (D) Analysis of viral DNA accumulation of ABC^{rep} and WT^{rep} viruses grown in the presence or absence of abacavir. For panels C and D, the quantification of AcMNPV DNA copy numbers was performed by qPCR by using an external standard calibration curve. Each datum point represents the average value from three independent infections, and error bars represent standard errors.

infected with the APC^{rep} or ABC^{rep} virus in the presence of 0.39 μ M aphidicolin (Fig. 6A) or 50 μ M abacavir (Fig. 6B). The growth curves of the ABC^{rep} and APC^{rep} viruses in the presence of aphidicolin or abacavir were indistinguishable from each other. However, in cells infected with the WT^{rep} virus in the presence of either abacavir or aphidicolin, very little virus was detected. Only single GFP-positive cells were observed by 120 hpi, probably reflecting only the initially WT^{rep}-infected cells. The yields of the APC^{rep} and ABC^{rep} viruses were both reduced somewhat in the presence of aphidicolin and abacavir, respectively (Fig. 6A and B) compared to the yields in the absence of the inhibitors.

We also performed a quantitative PCR assay to monitor the accumulation of total intracellular viral DNA in the presence of aphidicolin or abacavir during a single replication cycle over the first 24 hpi. Sf21 cells were infected with the APC^{rep}, ABC^{rep}, or WT^{rep} virus at an MOI of 10. The three viruses were grown in the presence of 0.325 μ M aphidicolin (Fig. 6C) or 50 μ M abacavir (Fig. 6D). There was some increase in viral DNA levels evident at 6 hpi for some of the viruses. By 24 hpi, there was an approximately 2.8-log increase over the level at time zero for the APC^{rep} virus in the presence of aphidicolin, while there was an approximately 2.3-log increase in viral DNA accumulation for the ABC^{rep} virus in the presence of abacavir. There were no obvious differences in viral DNA accumulation with or without drug for both the APC^{rep} and ABC^{rep} viruses. However, there was little detectable increase in viral DNA accumulation for the WT^{rep} virus in the presence of either aphidicolin or abacavir. These results demon-

strated that the *dnapol* point mutations were responsible for the increased resistance to drugs.

AcMNPV DNapol mutant structure predictions. The crystal structures of both prokaryotic and eukaryotic DNA polymerases have been reported, allowing us to perform structure predictions for the AcMNPV DNapol mutants (34). As the closest homologues of the AcMNPV DNapol were the HSV DNapol and yeast Pol δ , we used those DNA polymerases for our structural comparison. The AcMNPV DNapol domain (aa 500 to 984) shows 26% identity (42% positive) to the HSV-1 DNapol and 23% identity (43% positive) to the yeast Pol δ . The AcMNPV DNapol three-dimensional (3D) structure was predicted by using comparative modeling based on the HSV-1 DNapol. The QMEAN6 scores (3), a measure of the quality of the protein structure, for WT^{rep}, APC^{rep}, and ABC^{rep} DNapols were 0.703, 0.601, and 0.637, respectively. AcMNPV DNapol amino acid S611, the one that is S611T in the two *dnapol* mutants, is equivalent to HSV-1 DNapol residue S816 and yeast DNA polymerase δ residue S706. These serine amino acids are all located in highly conserved DNapol region III, and they impinge on the dG-dCTG bases and stack against the dCTP sugar (41). Structurally, this residue is outside the finger domain, which is formed primarily by two long antiparallel α -helices and includes conserved regions III and IV (41). Region III (Kx3NSxYG [“S” is equivalent to S611]) is involved in dNTP binding (12, 13, 41). Because of the decreased sensitivity to aphidicolin and abacavir of the T611 variant compared to that of the S611 variant, we consider that AcMNPV DNapol residue 611 plays an important role in dNTP binding (Fig. 7C and D). The S-to-T change at position 611 might affect the interaction of residue 611 with nucleotide binding by slightly modifying the DNapol tertiary structure and may thereby result in altered drug sensitivity. For example, the space-filling models of the polymerase region show a more open cleft for S611T than for S611 (Fig. 7B and D) (2, 24). The other variant amino acid (C543R) belongs to conserved DNapol region II; this residue is outside the palm domain (Fig. 7E and F). The palm interacts with the replicative end of the DNA and catalyzes the nucleotidyl transfer reaction (41). DNapol residue 543 may play a vital role in catalysis, as our results showed that the single point mutation at this position (T53657C) had no effect on drug resistance but showed decreased virus replication. The ABC^{rep} virus, containing both the S611T and C543R mutations, also compromised viral production. The structure predictions for the ABC^{rep} DNapol variant domain protein (Fig. 7E and F) lend support to this hypothesis.

***dnapol* point mutations altered occluded virus virion morphogenesis.** By light microscopy, we initially noticed a difference in OB morphologies for the two *dnapol* mutants, particularly for the ABC^{rep} mutant, which produced fewer but larger and more cuboidal polyhedra (Fig. 3B). To determine if the *dnapol* point mutations in the APC^{rep} and ABC^{rep} mutants in comparison to the WT^{rep} virus affected virion and polyhedral morphogenesis, sections from infected cells were visualized by transmission electron microscopy. Cells infected with the WT^{rep} virus showed morphological characteristics typical of baculovirus infection by 60 hpt (Fig. 8). This included the virogenic stroma (VS), an electron-dense, net-shaped baculovirus-induced structure containing numerous rod-shaped nucleocapsids, virus-induced intranuclear microvesicles, bundles of enveloped nucleocapsids aligning within the ring zone, and the mature multiply enveloped nucleocapsids within the ring zone or embedded in polyhedra.

FIG 7 Structural modeling of finger and palm domains of protein variants of the AcMNPV DNAPol region (residues 511 to 770) of the wild type and the drug-resistant mutants. Shown is the prediction of structural changes caused by the residue changes C543R and S611T in DNAPol. (A) Ribbon diagram of the AcMNPV DNAPol C543R and S611T mutations based on the HSV-1 and yeast DNAPol δ crystal structures. (B) Space-filling diagram highlighting the unmutated region on the outside surface of the finger domains. (C and D) Ribbon diagram (C) and space-filling diagram (D) of predictions of structural changes resulting from the single-residue change of T611S in DNAPol on the outside surface of the finger domain. (E and F) Ribbon diagram (E) and space-filling diagram (F) of predictions of structural changes resulting in the double-residue changes of T611S and R543C in DNAPol. Positions of altered amino acids are in red.

The VS and rod-shaped nucleocapsids were also observed in the nucleus of APC^{rep}- and ABC^{rep}-infected cells (Fig. 8). Intranuclear microvesicles appeared within the ring zone of these cells (Fig. 8C and F). Within the ring zone, for the WT^{rep} virus, there were 3% $\pm$ 3% single-, 7% $\pm$ 3% double-, and 90% $\pm$ 7% multiple-nucleocapsid ODVs, and in the polyhedra, there were 5% $\pm$ 3% single-, 11% $\pm$ 2% double-, and 85% $\pm$ 4% multiple-nucleocapsid ODVs, reflecting the typical preponderance of multiple-nucleocapsid ODVs for AcMNPV. In contrast, for APC^{rep}-infected cells, we observed ODVs with 61% $\pm$ 6% single and only rarely two (25% $\pm$ 2%) or more (14% $\pm$ 5%) enveloped nucleocapsids in the ring zone and 55% $\pm$ 13% single and only rarely two (35% $\pm$ 12%) or more (10% $\pm$ 1%) enveloped nucleocapsids in polyhedra (Fig. 8B and E). In ABC^{rep}-infected cells, there were 89% $\pm$ 2% single-, 9% $\pm$ 2% double-, and 1% $\pm$ 1% multiple-nucleocapsid ODVs in the ring zone and 86% $\pm$ 2% single-, 12% $\pm$ 2% double-, and 2% $\pm$ 2% multiple-nucleocapsid ODVs in polyhedra (Fig. 8A and D). This finding indicated that the *dnapol* point mutation affected the number of nucleocapsids enveloped in ODV in the ring zone and the occlusion of ODV in polyhedra.

The differences in the sizes of polyhedra were confirmed by light microscopy using purified polyhedra. The average diameters of the parental virus and the WT^{rep} virus were 1.31 $\pm$ 0.02 and 1.45 $\pm$ 0.02 μ m, respectively, which were not significantly different. All of the mutant viruses produced larger polyhedra than these. The diameters of polyhedra from the ABC^r (the originally

selected ABC mutant) and the ABC^{rep} viruses were 2.30 $\pm$ 0.11 and 2.30 $\pm$ 0.06 μ m, respectively, which were not significantly different. The diameters of polyhedra from the APC^r and APC^{rep} viruses were 1.96 $\pm$ 0.09 μ m and 1.71 $\pm$ 0.04 μ m, respectively, a bit smaller than those from the abacavir mutants but still significantly larger than the polyhedra from the parental or WT^{rep} virus. Thus, not only did the mutant viruses produce fewer polyhedra per nucleus, they were also larger and with mostly singly enveloped ODVs.

DISCUSSION

In the present study, we demonstrate that AcMNPV mutants resistant to the DNA replication inhibitors aphidicolin and abacavir can be selected for and that these mutants are due to point mutations in *dnapol*. We demonstrated that two specific point mutations (single or double mutations) in *dnapol* are responsible for the increased resistance to both drugs but somewhat compromised viral DNA replication and virus production. Surprisingly, these mutations also resulted in an increased proportion of single-nucleocapsid ODVs during their morphogenesis, fewer polyhedra per cell, and the production of larger polyhedra.

The use of bacmid technology allowed us to determine the effect of only the two identified *dnapol* point mutations on virus and viral DNA replication and occlusion-derived-virus morphogenesis. The *dnapol* knockout bacmid was unable to propagate virus after transfection into Sf21 cells and was unable to replicate viral DNA. This demonstrates that viral *dnapol* is essential to the virus replication cycle. This finding agrees with the conclusion drawn previously by Vanarsdall et al. (48), using a *dnapol*-null mutant. However, in that study, in addition to deleting part of the *dnapol* gene, those authors also eliminated part of the adjacent AcORF66 gene, which was shown by Ke et al. (25) to be essential. Thus, in the study by Vanarsdall et al., the lack of replication of the DNA-null mutant could have been due to the elimination of the essential Ac66 gene rather than the removal of the *dnapol* gene itself. In our studies, we took pains to eliminate only the DNAPol ORF, leaving the adjacent predicted regulatory and coding sequences intact. The lack of replication of this *dnapol* KO proved more convincingly that the viral *dnapol* gene is essential.

Nevertheless, the defect in the replication of viral DNA and virus production could be rescued by the reinsertion of the wild-type *dnapol* gene into the *polyhedrin* locus of the knockout bacmid. Thus, the *dnapol* KO bacmid allowed us to engineer the virus so that the only changes in the entire bacmid were the two point mutations in *dnapol* identified in the originally selected drug-resistant viruses, and any altered characteristics would be because of these *dnapol* point mutations only. Real-time PCR and viral growth curve analysis revealed that both the APC^{rep} and ABC^{rep} mutants showed minor decreases in levels of viral DNA replication and a somewhat reduced overall viral DNA yield. Compared with the WT^{rep} virus, the level of viral DNA synthesized by the APC^{rep} single point mutant was reduced about one-half, a result similar to that reported previously for vaccinia virus *dnapol* mutants (42). However, infection with the ABC^{rep} double point mutant resulted in at least an 11-fold reduction in virus titers. Both mutations are located within conserved regions III and II, indicating that the two conserved domains might play a key role in viral DNA replication but can accommodate minor mutations.

Each of the resistant and equivalent repair viruses had EC₅₀s higher than those for the wild type for both drugs. For example,

FIG 8 Electron microscopic analysis of Sf21 cells infected with ABC^{rep}, APC^{rep}, and WT^{rep} viruses. (A) Single-nucleocapsid ODVs in the ring zone of ABC^{rep} virus-infected cells. (B) Single- and multiple-nucleocapsid ODVs in the ring zone of APC^{rep} virus-infected cells. (C) Multiple-nucleocapsid ODVs in the ring zone of WT^{rep} virus-infected cells. (D) Image of a polyhedron within the nucleus of a cell infected with the ABC^{rep} virus showing only single-nucleocapsid ODVs embedded in the polyhedron. (E) Polyhedron within a nucleus of a cell infected with the APC^{rep} virus showing mostly single- and only a few multiple-nucleocapsid ODVs embedded in the polyhedron. (F) Polyhedron within the nucleus of a cell infected with the WT^{rep} virus showing single- and multiple-nucleocapsid ODVs embedded in the polyhedron. Bars are 500 nm for all panels. Arrows point to single-nucleocapsid ODVs, and double arrowheads point to multiple-nucleocapsid ODVs.

while the WT DNAPol virus was sensitive to 0.07 μ M aphidicolin and 4.75 μ M abacavir, the EC₅₀ of aphidicolin for the APC^{rep} mutant was 0.56 μ M, and that of abacavir was 29 μ M, equivalent to an 8-fold-higher concentration of aphidicolin and a 6-fold-higher concentration of abacavir. Similarly, the EC₅₀s of aphidicolin for the ABC^{rep} mutant was 0.15 μ M, and that of abacavir was 78 μ M, an approximately 2-fold-higher concentration of aphidicolin and over a 16-fold-higher concentration of abacavir. Thus, both drug-resistant viruses (and their repair virus equivalents) were resistant to higher concentrations than the parental virus (and the repair WT DNAPol virus equivalent). Moreover, each drug-resistant virus showed resistance to both aphidicolin and abacavir irrespective of the drug used for selection. In comparison to the AcMNPV DNAPol, an HSV-2 DNAPol Y-to-H amino acid change (equivalent to Y696H within conserved region II of HSV-1 DNAPol) conferred resistance to aphidicolin (at 14.8 μ M) (47). In contrast, an L774F mutation (region VI) in the HSV-1 DNA polymerase was hypersensitive to aphidicolin, with an EC₅₀ of 0.10 μ M, compared to 0.15 μ M for the parental virus (23). A vaccinia virus DNAPol with an A498T/V mutation in domain II was selected at 85 μ M (42). With respect to abacavir resistance, the mutations M184V and L74V/I of HIV reverse transcriptase conferred resistance to abacavir (29). Moreover, combinations of mutations (e.g., of M184V, L74V, K65R, and Y115F) gave rise to mutant viruses with 50% inhibitory concentrations (IC₅₀s) of abacavir of

up to 58 μ M (44) and conferred as much as an 11-fold-increased resistance to abacavir, comparable to the 6- to 16-fold increase observed for our abacavir-resistant *dnapol* mutants (36).

dnapol point mutations in both mutants conferred drug resistance to both aphidicolin and abacavir irrespective of the drug used for selection. The C543R and S611T mutations are located in conserved regions II and III, respectively, which belong to the palm and finger domains, both forming the two antiparallel α -helix N (dNTP binding site) and P (the primer site) sites (28, 50). The finger domain in the structure of the bacteriophage RB69 protein-DNA complex is probably in the open conformation (15). Upon nucleotide binding, the fingers would rotate to form the closed conformation of the Pol-DNA-dNTP ternary structure for the delivery of dNTP to the polymerase (15). In the absence of a detailed crystallographic analysis of the AcMNPV DNAPol, as reported previously for that of HSV-1 (28), the following inferences of how the two mutations in the AcMNPV DNAPol might reduce its interaction with aphidicolin and abacavir are only speculative. The analysis of the secondary structure of the α -like polymerase implies that S611 of the AcMNPV DNAPol is a consensus residue in the region III sequence. A replacement at this position could lead to a subtle local structural change in the finger domain, as shown in the structure prediction in Fig. 7. This may induce the finger to change to a different orientation, thereby affecting the efficiency of the polymerase interaction with dNTP.

The additional C543R substitution might reduce the efficiency of dNTP binding, leading to a further reduction in DNA replication. In particular, the C543R mutation might restrict the binding of the two DNApol inhibitors aphidicolin and abacavir to the extent that they are no longer effective as inhibitors. It is not known if C543 participates in the formation of a disulfide bond in the AcMNPV DNApol, which might play an important role in maintaining the structure of the finger and palm subdomains at the active site. In the absence of a Cys at position 543, in C543R, the nearby C545 residue might replace C543 to maintain the structure. Further analysis of the crystal structure of the AcMNPV DNA polymerase is required to verify such a disulfide bond. Our results showed that the ABC^{rep} double point mutant decreased virus production 11-fold compared to that of the WT^{rep} virus, but the APC^{rep} single point mutant reduced replication only about one-half. Nevertheless, conserved regions II and III of the AcMNPV DNApol, together with other conserved regions, presumably play a major role in DNA polymerization and interactions with dNTP. Further biochemical analyses will be necessary to confirm this hypothesis.

Electron microscopy of the ABC^{rep} and APC^{rep} mutants demonstrated the formation of the electron-dense VS, where viral DNA replication takes place. During the late phase of viral infection, viral DNA is condensed and packaged into capsids to form nucleocapsids within the VS (52). Our results showed that the point mutations did not affect the formation of the VS, nucleocapsid assembly, the formation of intranuclear microvesicles, or polyhedra. However, to our surprise, and in contrast to WT ODVs, containing mostly multiple nucleocapsids with only a few single nucleocapsids, very few or no multiple-nucleocapsid ODVs were observed in occlusion bodies from the two *dnapol* mutants. Although the mechanisms of nucleocapsid envelopment in ODVs probably differ, the lack of multiple-nucleocapsid ODVs in the ABC^{rep} double point mutant was similar to that of the nonviable Ac92 and viable Ac23 knockout viruses (53, 54). Moreover, the drug-resistant viruses generated polyhedra which were larger than those generated by the WT^{rep} virus. This finding suggests that the DNA polymerase may play an important structural or accessory role in the virion formation of ODV but not of BV. It is not clear if DNApol plays a direct role in determining polyhedral size, and the larger size may simply reflect the longer time provided for polyhedron production due to the lower rate of viral DNA replication of the mutant viruses. This is supported in part by the observation that the ABC^{rep} mutant replicates more slowly than the APC^{rep} mutant and also produces larger polyhedra.

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REFERENCES

1. Ayres MD, Howard SC, Kuzio J, Lopez-Ferber M, Possee RD. 1994. The complete DNA sequence of Autographa californica nuclear polyhedrosis virus. *Virology* 202:586–605.
2. Bestman-Smith J, Boivin G. 2003. Drug resistance patterns of recombinant herpes simplex virus DNA polymerase mutants generated with a set of overlapping cosmid and plasmids. *J. Virol.* 77:7820–7829.
3. Bordoli L, et al. 2009. Protein structure homology modeling using SWISS-MODEL workspace. *Nat. Protoc.* 4:1–13.
4. Bouchonnet F, et al. 2005. Quantification of the effects on viral DNA synthesis of reverse transcriptase mutations conferring human immunodeficiency virus type 1 resistance to nucleoside analogues. *J. Virol.* 79:812–822.
5. Chaeychomsri S, Ikeda M, Kobayashi M. 1995. Nucleotide sequence and transcriptional analysis of the DNA polymerase gene of Bombyx mori nuclear polyhedrosis virus. *Virology* 206:435–447.
6. Crawford AM, Miller LK. 1988. Characterization of an early gene accelerating expression of late genes of the baculovirus Autographa californica nuclear polyhedrosis virus. *J. Virol.* 62:2773–2781.
7. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97:6640–6645.
8. De Clercq E. 2005. Antiviral drug discovery and development: where chemistry meets with biomedicine. *Antiviral Res.* 67:56–75.
9. DeFilippes FM. 1984. Effect of aphidicolin on vaccinia virus: isolation of an aphidicolin-resistant mutant. *J. Virol.* 52:474–482.
10. DeFilippes FM. 1989. Site of the base change in the vaccinia virus DNA polymerase gene which confers aphidicolin resistance. *J. Virol.* 63:4060–4063.
11. de Jong J, Arif BM, Theilmann DA, Krell PJ. 2009. Autographa californica multiple nucleopolyhedrovirus me53 (ac140) is a nonessential gene required for efficient budded-virus production. *J. Virol.* 83:7440–7448.
12. Dong Q, Copeland WC, Wang TS. 1993. Mutational studies of human DNA polymerase alpha. Identification of residues critical for deoxynucleotide binding and misinsertion fidelity of DNA synthesis. *J. Biol. Chem.* 268:24163–24174.
13. Dong Q, Wang TS. 1995. Mutational studies of human DNA polymerase alpha. Lysine 950 in the third most conserved region of alpha-like DNA polymerases is involved in binding the deoxynucleoside triphosphate. *J. Biol. Chem.* 270:21563–21570.
14. Federici BA. 1997. Baculovirus pathogenesis, p 33–56. *In* Miller LK (ed), *The baculoviruses*. Plenum Press, New York, NY.
15. Franklin MC, Wang J, Steitz TA. 2001. Structure of the replicating complex of a pol alpha family DNA polymerase. *Cell* 105:657–667.
16. Gao HQ, Boyer PL, Sarafianos SG, Arnold E, Hughes SH. 2000. The role of steric hindrance in 3TC resistance of human immunodeficiency virus type-1 reverse transcriptase. *J. Mol. Biol.* 300:403–418.
17. Hall JD, Woodward S. 1989. Aphidicolin resistance in herpes simplex virus type 1 appears to alter substrate specificity in the DNA polymerase. *J. Virol.* 63:2874–2876.
18. Hang X, Guarino LA. 1999. Purification of Autographa californica nucleopolyhedrovirus DNA polymerase from infected insect cells. *J. Gen. Virol.* 80(Pt 9):2519–2526.
19. Harrison RL, Lynn DE. 2007. Genomic sequence analysis of a nucleopolyhedrovirus isolated from the diamondback moth, *Plutella xylostella*. *Virus Genes* 35:857–873.
20. Harrison RL, Summers MD. 1995. Mutations in the Autographa californica multinucleocapsid nuclear polyhedrosis virus 25 kDa protein gene result in reduced virion occlusion, altered intranuclear envelopment and enhanced virus production. *J. Gen. Virol.* 76(Pt 6):1451–1459.
21. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51–59.
22. Hwang CB, Ruffner KL, Coen DM. 1992. A point mutation within a distinct conserved region of the herpes simplex virus DNA polymerase gene confers drug resistance. *J. Virol.* 66:1774–1776.
23. Hwang YT, Zuccola HJ, Lu Q, Hwang CB. 2004. A point mutation within conserved region VI of herpes simplex virus type 1 DNA polymerase confers altered drug sensitivity and enhances replication fidelity. *J. Virol.* 78:650–657.
24. Jehle JA, et al. 2006. On the classification and nomenclature of baculoviruses: a proposal for revision. *Arch. Virol.* 151:1257–1266.
25. Ke J, Wang J, Deng R, Wang X. 2008. Autographa californica multiple nucleopolyhedrovirus ac66 is required for the efficient egress of nucleocapsids from the nucleus, general synthesis of preoccluded virions and occlusion body formation. *Virology* 374:421–431.
26. Kelly DC. 1984. Baculovirus replication: effects of inhibitors of macromolecular synthesis. *J. Antimicrob. Chemother.* 14(Suppl A):43–55.
27. Kool M, Ahrens CH, Goldbach RW, Rohrmann GF, Vlak JM. 1994. Identification of genes involved in DNA replication of the Autographa californica baculovirus. *Proc. Natl. Acad. Sci. U. S. A.* 91:11212–11216.
28. Liu S, et al. 2006. Crystal structure of the herpes simplex virus 1 DNA polymerase. *J. Biol. Chem.* 281:18193–18200.

29. McColl DJ, Chappey C, Parkin NT, Miller MD. 2008. Prevalence, genotypic associations and phenotypic characterization of K65R, L74V and other HIV-1 RT resistance mutations in a commercial database. *Antivir. Ther.* 13:189–197.
30. Miller LK. 1981. Construction of a genetic map of the baculovirus *Autographa californica* nuclear polyhedrosis virus by marker rescue of temperature-sensitive mutants. *J. Virol.* 39:973–976.
31. Okano K, Vanarsdall AL, Mikhailov VS, Rohrmann GF. 2006. Conserved molecular systems of the Baculoviridae. *Virology* 344:77–87.
32. O'Reilly DR, Miller LK, Luckow VA. 1992. *Baculovirus expression vectors: a laboratory manual*. WH Freeman and Co, New York, NY.
33. O'Reilly DR, Crawford AM, Miller LK. 1989. Viral proliferating cell nuclear antigen. *Nature* 337:606. doi:10.1038/337606a0.
34. Patel PH, Loeb LA. 2001. Getting a grip on how DNA polymerases function. *Nat. Struct. Biol.* 8:656–659.
35. Ray AS, Anderson KS. 2001. Mechanistic studies to understand the inhibition of wild type and mutant HIV-1 reverse transcriptase by carbovir-triphosphate. *Nucleosides Nucleotides Nucleic Acids* 20:1247–1250.
36. Ray AS, et al. 2002. Insights into the molecular mechanism of inhibition and drug resistance for HIV-1 RT with carbovir triphosphate. *Biochemistry* 41:5150–5162.
37. Rice WC, Miller LK. 1986. Baculovirus transcription in the presence of inhibitors and in nonpermissive *Drosophila* cells. *Virus Res.* 6:155–172.
38. Sarafianos SG, et al. 1999. Touching the heart of HIV-1 drug resistance: the fingers close down on the dNTP at the polymerase active site. *Chem. Biol.* 6:R137–R146. doi:10.1016/S1074-5521(99)80071-4.
39. Schwede T, Kopp J, Guex N, Peitsch MC. 2003. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* 31:3381–3385.
40. Sturmer M, Staszewski S, Doerr HW. 2007. Quadruple nucleoside therapy with zidovudine, lamivudine, abacavir and tenofovir in the treatment of HIV. *Antivir. Ther.* 12:695–703.
41. Swan MK, Johnson RE, Prakash L, Prakash S, Aggarwal AK. 2009. Structural basis of high-fidelity DNA synthesis by yeast DNA polymerase delta. *Nat. Struct. Mol. Biol.* 16:979–986.
42. Taddie JA, Traktman P. 1991. Genetic characterization of the vaccinia virus DNA polymerase: identification of point mutations conferring altered drug sensitivities and reduced fidelity. *J. Virol.* 65:869–879.
43. Thumbi DK, Arif BM, Krell PJ. 2007. Susceptibility of *Autographa californica* multiple nucleopolyhedrovirus to inhibitors of DNA replication. *J. Virol. Methods* 146:209–217.
44. Tisdale M, Alnadaf T, Cousens D. 1997. Combination of mutations in human immunodeficiency virus type 1 reverse transcriptase required for resistance to the carbocyclic nucleoside 1592U89. *Antimicrob. Agents Chemother.* 41:1094–1098.
45. Tisdale M, Kemp SD, Parry NR, Larder BA. 1993. Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc. Natl. Acad. Sci. U. S. A.* 90:5653–5656.
46. Tomalski MD, Wu JG, Miller LK. 1988. The location, sequence, transcription, and regulation of a baculovirus DNA polymerase gene. *Virology* 167:591–600.
47. Tsurumi T, Maeno K, Nishiyama Y. 1987. A single-base change within the DNA polymerase locus of herpes simplex virus type 2 can confer resistance to aphidicolin. *J. Virol.* 61:388–394.
48. Vanarsdall AL, Okano K, Rohrmann GF. 2005. Characterization of the replication of a baculovirus mutant lacking the DNA polymerase gene. *Virology* 331:175–180.
49. Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P. 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). *In Vitro* 13:213–217.
50. Wang J, et al. 1997. Crystal structure of a pol alpha family replication DNA polymerase from bacteriophage RB69. *Cell* 89:1087–1099.
51. Wang X, Lescott T, De Clercq E, Kelly DC. 1983. Baculovirus replication: inhibition of *Trichoplusia ni* multiple nuclear polyhedrosis virus by [E]-5-(2-bromovinyl)-2'-deoxyuridine. *J. Gen. Virol.* 64(Pt 6):1221–1227.
52. Williams GV, Faulkner P. 1997. Cytological changes and viral morphogenesis during baculovirus infection, p 61–107. *In* Miller LK (ed), *The baculoviruses*. Plenum Press, New York, NY.
53. Wu W, Passarelli AL. 2010. *Autographa californica* multiple nucleopolyhedrovirus Ac92 (ORF92, P33) is required for budded virus production and multiply enveloped occlusion-derived virus formation. *J. Virol.* 84:12351–12361.
54. Yu IL, Bray D, Lin YC, Lung O. 2009. *Autographa californica* multiple nucleopolyhedrovirus ORF 23 null mutant produces occlusion-derived virions with fewer nucleocapsids. *J. Gen. Virol.* 90:1499–1504.