Characterization of a Baculovirus Lacking the Alkaline Nuclease Gene

Kazuhiro Okano, Adam L. Vanarsdall, and George F. Rohrmann*

Department of Microbiology, Oregon State University, Corvallis, Oregon

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The Autographa californica multiple nucleocapsid nucleopolyhedrovirus (AcMNPV) alkaline nuclease (AN) associates with the baculovirus single-stranded DNA binding protein LEF-3 and possesses both a 5′-to-3′ exonuclease and an endonuclease activity. These activities are thought to be involved in DNA recombination and replication. To investigate the role of AN in AcMNPV replication, the λ Red system was used to replace the an open reading frame with a chloramphenicol acetyltransferase gene (cat) and a bacmid containing the AcMNPV genome in Escherichia coli. The AcMNPV an knockout bacmid (vAcAN-KO/GUS) was unable to propagate in SF9 cells, although an an-rescued bacmid (vAcAN-KO/GUS-Res) propagated normally. In addition, the mutant did not appear to produce budded virions. These data indicated that an is an essential baculovirus gene. Slot blot and DpnI assays of DNA replication in SF9 cells transfected with vAcAN-KO/GUS, vAcAN-KO/GUS-Res, and a wild-type bacmid showed that the vAcAN-KO/GUS bacmid was able to replicate to levels similar to those seen with the vAcAN-KO/GUS-Res and wild-type bacmids at early stages posttransfection. However, at later time points DNA did not accumulate to the levels seen with the repaired or wild-type bacmids. Northern analysis of SF9 cells transfected with bacmids vAcAN-KO/GUS showed that transcription of late and very late genes was lower at later times posttransfection relative to the results seen with wild-type and vAcAN-KO/GUS-Res bacmids. These data suggest that the an gene might be involved in the maturation of viral DNA or packaging of the DNA into virions.

Members of the Baculoviridae family have double-stranded circular DNA genomes of 100 to 180 kb, depending on the strain of virus (6). The Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the type NPV species and is widely used for the generation of recombinant viruses for expression of foreign genes. Production of such expression vectors is based on efficient homologous recombination of a gene of interest into the virus genome (21). Although homologous recombination of the AcMNPV genome has been widely employed, its mechanism is unknown and the viral gene products involved in this process have not been identified. In previous reports, it was shown that alkaline nuclease (AN) encoded by AcMNPV (open reading frame [ORF] 133 [ORF133]) associates with the baculovirus single-stranded DNA (ssDNA)-binding (SSB) protein, LEF-3, and that this complex possesses a 5′-to-3′ nuclease activity (11, 18).

Homologs of baculovirus ANs are widely distributed in eubacteria and archaea and are also found in eukaryotes (1, 2). These enzymes participate in the repair of double-strand breaks and in homologous recombination and therefore play a vital role in the maintenance of genome integrity. The best-studied recombination system is from bacteriophage λ and is called the Red (for “recombination defective”) system (for reviews, see references 9, 25, and 29). It includes λ exonuclease (Redξ), which degrades double-stranded DNA (dsDNA) from the 5′ ends, producing 3′ overhangs which serve as intermediates in recombination (13). The λ exonuclease forms a toroid-shaped trimer in solution with a channel in the center that can accommodate dsDNA at one end but only ssDNA at the other (8). During recombination, λ exonuclease interacts with a SSB protein (Redδ), which promotes renaturation of complementary strands, thereby mediating strand annealing and strand exchange reactions (3, 12). In studies of herpes simplex virus type 1 (HSV-1) when the gene encoding AN was deleted, viral DNA synthesis and late viral protein expression appeared to be normal, but the number of infectious progeny virus was reduced (31).

In this report, we describe the deletion of the AcMNPV an gene by use of the λ Red homologous recombination system (4). After the gene deletion was performed, we then investigated DNA replication and gene expression by this construct. The an knockout bacmid produced no measurable levels of infectious virus progeny after transfection into Spodoptera frugiperda (Sf9) cells. However, up to 48 h posttransfection (p.t.), the DNA replication rate of the an knockout bacmid was similar to that of the wild-type bacmid or a repaired bacmid. In addition, Northern blot analysis indicated that expression levels of early, late, and very late genes of the wild-type, an knockout, and rescued bacmids were also similar until 48 h p.t. These data suggest that an is essential for virus progeny production but is not essential for DNA replication and transcription. With our previous biochemical studies, these data strongly suggested that an might be involved in maturation of replicated DNA or packaging of viral genome into virions.

MATERIALS AND METHODS

Cells, virus, plasmids, bacmids, and bacterial strains. Sf9 cells were cultured in SF900II serum-free medium (Invitrogen) with penicillin G (50 units/ml), streptomycin (Whittaker Bioproducts) (50 μg/ml), and Fungizone (amphotericin B [Invitrogen]) (375 ng/ml) as previously described (11). The Escherichia coli strains BW25113 (pKD46) (encoding genes for the λ Red system), BW25141 (pKD) (encoding a chloramphenicol acetyltransferase gene [cat]), and DH10B were kindly provided by G. W. Blissard (Boyece Thompson Institute, Cornell University, Ithaca, N.Y.) (4, 15). The E. coli strain DH10Bac (Invitrogen) was

* Corresponding author. Mailing address: Department of Microbiology, Nash Hall Room 220, Oregon State University, Corvallis, OR 97331-3804. Phone: (541) 737-1793. Fax: (541) 737-0496. E-mail: rohrmang@orst.edu.
used for isolation of the bacmid bMON14272 containing the AcMNPV genome and a helper plasmid (pMON7124) encoding a transposase (14). The concentrations of the antibiotics used for the bacmid and plasmid isolation were 100 μg/ml for kanamycin, 20 μg/ml for chloramphenicol, 10 μg/ml for tetracycline, and 7 μg/ml for gentamicin.

Use of the λ Red system for deletion of the alkaline nuclease gene. The production of an AcMNPV bacmid lacking an alkaline nuclease gene (an) was carried out in a manner similar to protocols described previously (15, 23). DH10B electrocompetent cells were made according to the method of Datsenko and Wanner (4). The bMON14272 bacmid DNA (50 ng in 2 μl of sterile water) was electroporated into the electrocompetent DH10B cells by use of a Bio-Rad DH10B electrocompetent cells were made according to the method of Datsenko and Wanner (4). The bMON14272 bacmid DNA (50 ng in 2 μl of sterile water) was electroporated into the electrocompetent DH10B cells by use of a Bio-Rad Gene Pulser II (2.3 kV, 200 μF) and 2-mm-diameter cuvettes. The cells were selected on a Luria-Bertani agar plate containing kanamycin at 37°C. The pKD46 plasmid (which encodes the λ Red system) was chemically transformed into DH10B cells harboring the bacmid, and the cells were selected with both kanamycin and ampicillin at 30°C.

To construct an alkaline nuclease deletion, a region of the AcMNPV genome (nucleotides [nt] 112630 to 113749) that encodes 364 amino acids from amino acids 34 to 397 of the an ORF was deleted from the bacmid DNA and replaced with a cat cassette by use of the λ Red system. The cat cassette was amplified from plasmid pKD3 (4) with primers KOP7-An133 and KOP8-An133, which have 5′ nt homologous to the an ORF and 24 nt of pKD3 sequences that flank the cat sequence (Table 1). PCR was carried out using Platinum Pfx DNA polymerase (Invitrogen) and 30 cycles of the following program: 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min. The PCR product was gel purified with a QIAquick gel extraction kit (QIAGEN), digested with DpnI to eliminate contamination by the template plasmid, ethanol precipitated, and dissolved in 5 μl of sterile H2O.

The λ Red system-induced DH10B cells containing the bacmid and pKD46 were made electrocompetent using a method described previously (23). The PCR product of the cat cassette was electroporated into the λ Red system-induced cells. The cells were spread on Luria-Bertani agar plates containing kanamycin and chloramphenicol and incubated at 37°C for 2 to 3 days.

Purified bacmids were screened to confirm the replacement of the an ORF with the cat cassette by PCR with three different primer sets, Ac112160 + catR2, Ac114065R + catF, and Ac112160 + Ac114065R (Table 1). The isolated an knock- out bacmid was named vAcAN-KO. The vAcAN-KO bacmid DNA was transformed into DH10B cells with electroporation, selected with kanamycin and chloramphenicol, and used for marker gene insertion and an-rescued bacmid constructions.

Construction of a rescued an-knockout bacmid DNA. A bacmid with the an gene inserted in a nonnative locus was constructed to confirm that the behavior of the an knockout bacmid was due to the deletion of the an gene. To generate an an-rescued bacmid, two transfer plasmids were constructed on the basis of the use of pFastBac DUAL (Invitrogen) after deletion of the polyhedrin (polh) and p10 promoters. pFBEIGUS, a transfer plasmid, was constructed by inserting a fragment containing the ie-1 promoter (AcMNPV nt coordinates 126599 to 127198)−β-glucuronidase (gus) ORF upstream of HSV tk poly(A) signal. Another transfer plasmid, pFBIEGUSAN, was generated by inserting a PCR-amplified promoter and an ORF (nt 112160 to 138319) with primers Ac112160 and Ac133sec3 (Table 1) upstream of the simian virus 40 poly(A) signal of pFBIEGUS. pMONT7124, a helper plasmid encoding a transposase, and pFBIEGUS or pFBIEGUS-AN were transformed chemically into DH10B cells harboring VAcAN-KO to transpose the gus fragment or the gus fragment plus the an gene fragment into vAcAN-KO by Tn7 transposon. The cells were selected on medium containing kanamycin, gentamicin, tetracycline, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) following Bac to Bac kit protocol (Invitrogen). Screening of transposition-positive constructs into the polh locus was done by PCR employing a M13 forward- and reverse-primer set. The bacmid containing the gus ORF under the control of the ie-1 promoter was named vAcAN-KO/GUS. The rescued bacmid containing the an ORF under the control of its own promoter along with the gus gene was named vAcAN-KO/GUS-Res.

For a wide-type virus containing the gus construct, pFBIEGUS was transformed into DH10Bac and the promoter-gus fragment was transposed into the polh locus. The isolated bacmid was named vAcEIGUS. The bacmid DNAs were purified from 1- to 2-liter cultures with QIAGEN columns (QIAGEN) or CsCl gradients.

Miscellaneous procedures. Transfection of bacmid DNAs into S9 cells, GUS staining of transfected or infected cells, virus growth curves, slot blot and DpnI assays of viral DNA replication, and Northern blot analyses were done as previously described (30a).

RESULTS

Construction of alkaline nuclease-knockout and rescued Ac- MNPV bacmids. To investigate the function of an during the viral infection cycle, we constructed a knockout of the an ORF in bMON14272, the AcMNPV bacmid, by use of the λ Red homologous recombination system. To determine whether the an gene was deleted, PCR screening with primer sets Ac112160 + catR2 and Ac114065R + catF (Table 1 and Fig. 1A) was used. In all (10) of the bacmids that we tested, the an gene was replaced with the cat cassette. The result of PCR analysis of a representative bacmid clone called vAcAN-KO is shown (Fig. 1B). Primer sets Ac112160 + catR2 and Ac114065R + catF (Fig. 1A) were expected to produce 908- and 727-bp products, respectively, to confirm replacement of the an ORF by the cat cassette. PCR analysis of vAcAN-KO resulted in the predicted products, which were not amplified from the original bacmid bMON14272 (Fig. 1B, panels A + B and C + D). Furthermore, PCR products derived from the primer set Ac112160 + Ac114065R for vAcAN-KO and bMON14272 were predicted to result in products of 1.77 and 1.9 kb, respectively, from amplification of the cat cassette or the an ORF. To further confirm these results, both products were digested with BamHI. A BamHI site is located in the an ORF but not in the cat cassette. As would be predicted for the correct insert, the PCR product derived from vAcAN-KO was resistant whereas that from bMON14272 was digested with BamHI (Fig. 1B, panel A + D). Thus, these data indicated that the an ORF was replaced with the cat cassette by homologous recombination in vAcAN-KO.

For the next step, we constructed an alkaline nuclease repair bacmid to ensure that the traits of the an knockout were due to the removal of the an gene (Fig. 1C). The gus reporter, which is under the control of the AcMNPV ie-1 promoter linked to the an ORF with 400 bp of 5′ flanking sequence predicted to contain the an promoter sequence, was used to rescue the an knockout bacmid by Tn7-mediated transposition at the polh locus of vAcAN-KO; the resulting construct was named vAcAN-KO/GUS-Res. A similar construct with gus expressed from the ie-1 promoter was also produced in vAcAN-KO and called vAcAN-KO/GUS (Fig. 1C). Transpositions were confirmed by PCR with M13 forward and reverse primers (Fig. 1C) that were predicted to amplify a fragment including a polh

<table>
<thead>
<tr>
<th>Primer</th>
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<tr>
<td>KOP7-An133</td>
<td>ATTTAACAACTTTGAAAGCAGTCATGGGTGTTGAGTCAACAGGCATGTTGGACACAGCTG</td>
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<tr>
<td>KOP8-An133</td>
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<td>Act33esco3</td>
<td>TAGAGCGATCAGTTGCGCAGCTATTTTTCATGACGTTTGGTGGG</td>
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TABLE 1. Primers used in this study
locus with the Tn7 transposition site (Fig. 1C, panel R/H11001). The gus expression cassette was also inserted into the parent AcMNPV bacmid bMON14727. The resulting bacmid was named vAcIEGUS and was used for a wild-type bacmid (data not shown).

**Analysis of viral propagation in Sf9 cells by the an knockout bacmid.** To examine the effect of the deletion of the alkaline nuclease gene on baculovirus replication, the bacmids with the an gene removed (vAcAN-KO/GUS), repaired by insertion of an at the polh locus (vAcAN-KO/GUS-Res), or the wild-type control (vAcIEGUS) was transposed into Sf9 cells. At 5 days p.t., supernatants were removed, added to newly plated Sf9 cells, and incubated for 3 days. The cells that were transfected or incubated with supernatants from transfected cells were observed under light microscopy and stained with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) after fixation to visualize GUS expression (Fig. 2A). There were a few GUS-stained cells in vAcAN-KO/GUS-transfected cells after 5 days p.t., and these cells did not show cytopathic effects (CPE) (Fig. 2A, panel A). When the supernatant from these cells was incubated with Sf9 cells for 3 days, no GUS staining or CPE was evident (Fig. 2A, panel B). We interpret this to indicate that the limited number of GUS-stained cells evident after the initial transfection was caused by GUS expression from the ie-1 promoter and a wild-type an ORF under the control of its own putative promoter region were transposed into the polyhedrin (polh) locus. The bars flanking the cat cassette indicate primer regions homologous to the 5′- and 3′-flanking regions of the an ORF. Transposition into the polh locus was confirmed by PCR with the M13 forward and reverse primers (arrows F and R). The result of the PCR confirmation assay is shown in panel R+F. The expected sizes of PCR products (vAcAN-KO/GUS, 4.9 kb; vAcAN-KO/GUS-Res, 6.7 kb) are indicated by arrows on left side of the panel. Lanes M, 1, and 2 indicate a 1-kb DNA ladder marker, vAcAN-KO/GUS, and vAcAN-KO/GUS-Res, respectively.

**FIG. 1.** Construction of bacmids. Results for the an-knockout bacmid vAcAN-KO/GUS and the an-rescued bacmid vAcAN-KO/GUS-Res are shown. (A) AcMNPV gene organization near the an locus and confirmation of the deletion of an. A cat gene PCR cassette amplified with KOP7-Ac133 and KOP8-Ac133 (Table 1), each of which has 50 nt homologous to the an ORF of 5′ or 3′ ends and 24 nt homologous to the 5′ or 3′ cat ORF, was used to replace the an ORF by homologous recombination. To confirm the replacement of the an ORF with the cat cassette, PCR was used with two primers located outside of the an sequence and two internal primers in the cat ORF. (Numbers above and below the lines connecting A to B, C to D, and A to D indicate the sizes of the predicted products. The primers listed in Table 1 are indicated in the figure as follows: primer A, AC112160; primer B, CatR2; primer C, CatF; primer D, Ac114065R). (B) Results of the PCR analysis of the an deletion bacmid (vAcAN-KO) and the wild-type bacmid (wt bacmid) (bMON14272). Three panels labeled A+B, C+D, and A+D (see panel A legend) indicate the primers used. The expected sizes of products are also indicated with arrows on left sides of the panels. PCR products with primers A+D were digested by BamHI. The an gene has a single BamHI site (Bam +) which allows it to be distinguished from the knockout containing the cat gene which lacks BamHI sites (Bam −); results for the constructs are shown in panel A+D. Lanes M, 1-kb DNA ladder marker (Invitrogen). (C) Construction of the alkaline nuclease knockout (vAcAN-KO/GUS) and -rescued (vAcAN-KO/GUS-Res) bacmids. The gus marker gene under the control of ie-1 promoter and a wild-type an ORF under the control of its own putative promoter region were transposed into the polyhedrin (polh) locus. The bars flanking the cat cassette indicate primer regions homologous to the 5′- and 3′-flanking regions of the an ORF. Transposition into the polh locus was confirmed by PCR with the M13 forward and reverse primers (arrows F and R). The result of the PCR confirmation assay is shown in panel R+F. The expected sizes of PCR products (vAcAN-KO/GUS, 4.9 kb; vAcAN-KO/GUS-Res, 6.7 kb) are indicated by arrows on left side of the panel. Lanes M, 1, and 2 indicate a 1-kb DNA ladder marker, vAcAN-KO/GUS, and vAcAN-KO/GUS-Res, respectively.
Each experiment was performed in triplicate. Supernatants of transfected and infected cells. The virus titer was determined by measurement of doses that were 50% tissue infective for Sf9 cells. Cells were stained at 3 days p.i. The white arrows in image A indicate two cells that tested positive for GUS expression. (B) Virus growth curves from were stained at 5 days p.t. Images B, D, and F: cells were infected with supernatants from the cells shown in images A, C, and E, respectively, and stained at 3 days p.i. The white arrows in image A indicate two cells that tested positive for GUS expression.

These results suggested that deletion of the an gene is essential for progeny virus production in Sf9 cells. For more-detailed characterization of the phenotype of the bacmids, virus growth curves were determined for supernatants from Sf9 cells transfected with vAcAN-KO/GUS, vAcAN-KO/GUS-Res, and vAcIEGUS or from infected cells with supernatants derived from cells transfected with vAcAN-KO/GUS-Res and vAcIEGUS (Fig. 2B). There was no virus titer detected in the supernatant from vAcAN-KO/GUS-transfected cells at any time point up to 120 h p.t. In contrast, virus was detected in the supernatant from vAcAN-KO/GUS-Res-transfected cells by 24 h p.t. and the titer increased at a rate similar to that seen with the control bacmid, vAcIEGUS. The viruses obtained from vAcAN-KO/GUS-Res or vAcIEGUS-transfected cells were used to infect Sf9 cells at a multiplicity of infection of 5, and the virus titers were determined. The an-rescued virus showed normal growth kinetics and increased in titer to a level similar to that seen with the wild-type virus. These data suggested that deletion of the an gene caused a defect in the production of progeny virus and that the defect was rescued by the reinsertion of the an ORF expressed from its own promoter. They also indicated that the defect in virus production was due solely to the an deletion.

DNA replication analysis of the an knockout bacmid. To investigate the effect of knocking out the an gene on DNA replication, the levels of DNA synthesis for cells transfected with vAcAN-KO/GUS, vAcAN-KO/GUS-Res, and vAcIEGUS at various time points were examined by slot blot analysis and DpnI assays (Fig. 3). The results of the slot blot (Fig. 3A) were quantified by measuring relative intensities from triplicate experiments (Fig. 3B). Increases in DNA levels became evident by 48 h p.t. for all three bacmids. Whereas the wild-type and repaired bacmids showed increases in DNA replication at up to 120 h p.t., vAcAN-KO/GUS DNA replication did not appear to increase significantly after 72 h p.t. and the final levels were about 20% of those for the wild-type and rescued virus (vAcIEGUS and vAcAN-KO/GUS-Res). Because the same number of cells was used at each time point, the increases in DNA level for wild-type and rescued bacmids suggested that secondary and tertiary infection of cells occurred. These data suggested that AN is not essential for viral DNA replication but does affect the amount of progeny viral DNA that is produced.

To determine the possibility that the an knockout bacmid was able to generate budded virions from the replicated DNA, slot blot analysis was performed with DNA isolated from supernatants of Sf9 cells transfected with vAcAN-KO/GUS or vAcIEGUS bacmid. A strong signal for viral DNA was detected with supernatant from cells transfected with the wild-type bacmid at 72 and 96 h p.t. (Fig. 3C). In contrast, no signal was detected above the background level at any time point from supernatants of cells transfected with the an knockout bacmid (Fig. 3C). Therefore, although not required for viral DNA synthesis, AN appeared to be required for the production of infectious budded virions in cell culture.

To investigate viral DNA replication at early stages after transfection, DpnI assays were performed to detect only replicated viral genome DNA which would be resistant to DpnI. The bacmid DNA which was produced in bacteria should have been methylated at the DpnI site and sensitive to this enzyme; however, the site on replicated DNA in eukaryotic cells is unmethylated and thus resistant to DpnI digestion (22). Total

![FIG. 2. Analysis of bacmid and virus propagation in Sf9 cells. (A) GUS-staining assays of Sf9 cells transfected or infected with vAcAN-KO/GUS, vAcAN-KO/GUS-Res, and vAcIEGUS. The transfected cells were incubated for 5 days. A total of 100 µl of supernatants from each transfected sample was added to freshly prepared Sf9 cells and incubated for 3 days. After fixation, cells were stained with X-Gluc and incubated overnight at 37°C. Images A, C, and E; cells were transfected with vAcAN-KO/GUS, vAcAN-KO/GUS-Res and vAcIEGUS, respectively, and they were also stained with X-Gluc and incubated overnight at 37°C. Images B, D, and F: cells were infected with supernatants from the cells shown in images A, C, and E, respectively, and stained at 3 days p.t. The white arrows in image A indicate two cells that tested positive for GUS expression. (B) Virus growth curves from supernatants of transfected and infected cells. The virus titer was determined by measurement of doses that were 50% tissue infective for Sf9 cells. Each experiment was performed in triplicate.](http://jvi.asm.org/)
DNA was extracted from cells transfected with vAcAN-KO/GUS or vAcIEGUS, digested with XhoI or XhoI-DpnI, and examined by Southern blot analysis with a labeled gus fragment as the probe (Fig. 3D). The genomic DNA samples applied to the gel from the 48- and 72-h-p.t. time points were a quarter of the volume used at the earlier time points. The signal from the gus fragment produced by XhoI digestion for vAcIEGUS purified from E. coli was predicted to be 4.2 kb, and since it was replicated in E. coli, it was sensitive to DpnI (Fig. 3D, lanes 11 and 12). Although signals were present in the non-DpnI-treated lanes at 0 and 12 h p.t. (Fig. 3D, lanes 1 and 3 and lanes 13 and 15), they were not DpnI resistant (Fig. 3D, lanes 2 and 4 and lanes 14 and 16), indicating that they represent input bacmid DNA. A DpnI-resistant band appeared in vAcAN-KO/GUS- and vAcIEGUS-transfected cells at 24 h p.t. (Fig. 3D, lanes 6 and 18). Therefore, by 24 h p.t. replication of the transfected bacmid DNA had occurred. The intensities of DpnI-resistant bands in vAcIEGUS increased significantly at 48 and 72 h p.t. likely because of secondary and tertiary propagation to uninfected cells (Fig. 3D, lanes 20 and 22). The patterns of the DpnI assays for the an-rescued bacmid in vAcAN-KO/GUS-Res-transfected cells were similar for vAcIEGUS at all stages (data not shown). In contrast, the DpnI-resistant band in vAcAN-KO/GUS was observed but at the later time point was of significantly lower intensity than for the other constructs (Fig. 3D, vAcAN-KO/GUS, 72 h). (Note that lanes 19 to 22 were exposed for a quarter of the time of exposure of the rest of the blot). These results indicated that AN was not required for the initial stages in DNA replication but was likely required for a later stage in virus production such as maturation of viral genome DNA or packaging of viral DNA into virions.

Transcriptional analysis of representative genes expressed by the bacmid lacking an. To investigate the effect of the an knockout on AcMNPV bacmid transcription, Sf9 cells were transfected with vAcAN-KO/GUS, vAcAN-KO/GUS-Res, and vAcIEGUS and harvested at various time points. Total RNA was extracted from the cells, and the RNA was examined by Northern blot analysis using probes to ie-1, vp39, and p10 genes, which are representative of AcMNPV early and late, late, and very late genes, respectively (Fig. 4). Transcripts of ie-1 were detected in all of the bacmids by 12 h p.t., and the signal increased by 48 h p.t. The wild-type and rescued bacmids showed high levels of expression by 72 h p.t., whereas the signal from the an deletion bacmid appeared to plateau at 48 h p.t. (Fig. 4, top panel). Transcripts of the late gene, vp39, and the
very late gene, p10, were observed from 48 h.p.t., and the levels of the transcript were similar for all three constructs at this time point (Fig. 4, upper and lower center panels). The vp39 transcript increased only slightly for the time point (Fig. 4, upper and lower center panels). The vp39 transcript increased only slightly for the time point (Fig. 4, upper and lower center panels). In addition, the transcripts of the late genes p24 and gp16 (ORF129 and ORF130) behaved similarly to those of vp39 (data not shown). In contrast, the expression of p10 increased significantly between 48 and 72 h.p.t. for all the bacmids, although the levels for the an knockout were lower than for the wild-type and rescued constructs. These data indicate that the deletion of an does not appear to affect the initial levels of transcription of the different gene categories but that it does affect their later expression levels. This was likely due to the lack of secondary or tertiary infections by this construct.

**DISCUSSION**

In this report, we describe the use of bacmid technology and the λ Red homologous recombination system to delete the gene encoding AN from the AcMNPV genome. This allowed us to investigate the role of the an gene in aspects of the AcMNPV life cycle. We found that AN is essential for the production of infectious progeny virus. In addition, we found that although it was not essential for DNA replication, the level of transcription was reduced. This was likely due to the lack of production of virus progeny and the consequent result that the infection could not spread to other cells. Moreover, we demonstrated that deletion of the gene did not affect the levels of transcription of representatives of major gene categories early after transfection but that the levels plateaued prematurely and at lower levels than virus possessing the an gene. Similar to the reduction in levels of replicated DNA, the reduction in transcription levels could be due to the lack of spread of the virus to other cells and the consequent reduction in the numbers of gene copies present.

It was previously demonstrated that AN has a preference for digesting ssDNA (11), digests this DNA in the 5’→3’ direction, and forms a stable complex with the ssDNA binding protein LEF-3 (18). We have also found that it has an endonuclease activity and that this activity appears to be specific for single-stranded regions of DNA most likely caused by physical distortions that occur as a result of the negative superhelicity of the DNA (19). Furthermore, this endonucleolytic activity was associated with a high degree of specificity for secondary cleavage on the strand opposite the initial nick such that, rather than random endonucleolytic cleavage, the result is specific cleavage of both DNA strands at or very near the same position. This could be an essential function for the reduction in size of large concatemeric DNA structures that are likely produced during baculovirus DNA replication (10, 20). Once the endonuclease activity produces free DNA ends, the highly active AN 5’→3’ nuclease activity could produce single-stranded 3’ ends that could be involved in recombination via association of homologous sequences or by strand invasion for the production of complete genomes segments or for the circularization of such segments into mature genomes. Whereas the an knockout resulted in significant levels of DNA replication and early, late, and very late genes were transcribed, no budded virus was produced. These results are consistent with the biochemical activities that we have previously reported and the likely function of AN in the processing of replicated DNA into mature infectious genomes or in the production of DNA-containing budded virions.

A homolog of the baculovirus an gene is present in members of the *Herpesviridae* family, and the HSV-1 AN forms a complex with HSV-1 SSB protein ICP8 (30). This is similar to the results seen with AcMNPV AN and SSB (LEF-3), which also form a complex (18). AN null mutants of HSV-1 have also been described. However, the results of investigations of these mutants differ from those of investigations we have carried out on AcMNPV, as the HSV-1 null mutants were propagated in a helper cell line that expressed HSV-1 AN. Therefore, the HSV-1 genomes lacking the an gene were packaged as virions. When these virions were used to infect non-helper-cell lines, normal levels of viral DNA and late protein synthesis were observed (31). However, these virions were deficient in the production of infectious virions, with their titers reduced to 0.1 to 1% of wild-type levels (24, 28). In addition, the DNA was present in complex branched structures, suggesting that HSV-1 AN either cleaved or prevented the accumulation of these aberrant structures (5, 16). Moreover, the amount of DNA packaged into virions which were found in cell supernatants was reduced by 15- to 20-fold (24). Furthermore, DNA isolated from virions produced by the HSV-1 an null mutant infection of nonhelper cells was not infectious when transfected into a helper (AN-expressing) cell line (24).

The AcMNPV an deletion mutant that we characterized appears to have a number of properties similar to those of the HSV-1 an null mutants. We observed significant levels of DNA replication and transcription of early, late, and very late genes. However, in contrast to the results of studies of HSV-1, we did not detect any infectious virus. This latter difference could reflect the different approaches used for production of the
viruses with an gene deletions. The HSV-1 constructs were amplified and packaged in helper cell lines, whereas we generated our constructs in bacteria and our investigations were done using transfected DNA.

Another gene called very late expression factor 1 (vlf-1) may also be involved in baculovirus genome processing. It is found in all baculovirus genomes that have been sequenced (6, 7), and it is essential for virus production (17, 32), but a homolog has not been identified in members of the Herpesviridae family. VLF-1 has a number of domains related to those of the integrase family of proteins and binds to complex DNA structures such as cruciforms (27), as would be expected of an enzyme involved in the resolution of complex branched structures. We recently deleted the vlf-1 gene from the AcMNPV bacmid. Similar to the results of an deletion, this knockout showed wild-type levels of DNA replication early after transfection, but then synthesis plateaued at levels lower than those seen with the wild-type and rescued controls, and no infectious virus was produced (Vanarsdall et al., submitted). Therefore, there may be at least two baculovirus gene products, AN and VLF-1, that are involved in baculovirus genome processing, and they may act in concert (possibly with additional unidentified proteins) to produce infectious virus genomes.

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REFERENCES


