Specificity of the Endonuclease Activity of the Baculovirus Alkaline Nuclease for Single-stranded DNA*

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The Autographa californica multiple nucleocapsid nucleopolyhedrovirus (AcMNPV) alkaline nuclease (AN) likely participates in the maturation of virus genomes and in DNA recombination. AcMNPV AN was expressed in a recombinant baculovirus as a His$_6$-tagged fusion and obtained in pure form (AN) or as a complex with the baculoviral single-stranded DNA-binding protein LEF-3 (AN/L3). Both AN preparations possessed potent 5’ → 3’-exonuclease and weak endonuclease activities. Mutant *AN(S146A)/L3 with a change from serine to alanine at position 146 in a conservative motif was impaired in both activities. This proved that the endonuclease is an intrinsic activity of baculovirus AN. The AN endonuclease showed specificity for single-stranded DNA and converted supercoiled plasmid DNA (replication intermediates) into the open circular form (RFII) by a single strand break. Plasmid DNA relaxed with topoisomerase I was resistant to *AN/L3 indicating that the partially single-stranded regions in negatively supercoiled molecules served as targets for the endonuclease. Unwinding the supercoiled DNA with ethidium bromide also made DNA resistant to AN/L3. In reactions with nicked circular DNA (RFII), AN and AN/L3 hydrolyzed exonucleolytically the broken strand or cut endonucleolytically the intact strand at the position opposite the nick (gap). When LEF-3 was added to the assay, the balance between the exonucleolytic and endonucleolytic modes of hydrolysis shifted in favor of the exonuclease. The data suggest that the AN endonuclease may digest the intermediates in replication and recombination at positions of structural irregularities in DNA duplexes, whereas LEF-3 may further regulate processing of the intermediates by AN via the endonuclease and exonuclease pathways.

Baculoviruses contain double-stranded (ds), circular, supercoiled DNA genomes of 100–180 kbp and belong to the family Baculviridae, which includes two genera, the granuloviruses and the nucleopolyhedroviruses (NPVs) (1, 2). One NPV, Autographa californica multiple nucleocapsid NPV (AcMNPV), is widely used as a model for analysis of baculovirus replication in infected cells and for the generation of recombinant viruses for the expression of foreign genes. Replication of baculovirus genomes proceeds in discrete replication factories in the nuclei of infected cells (3, 4) presumably via a rolling-circle intermediate (5, 6), although the mechanisms of initiation, elongation, processing, and maturation have not been determined. Six viral factors including a transactivator of early gene transcription (IE-1), DNA polymerase, DNA helicase, DNA primase (LEF-1), an accessory factor (LEF-2), and ssDNA-binding protein (LEF-3) are necessary, and several other factors (P35, IE-2, PE38, and LEF-7) are stimulatory for replication of plasmids in the transient replication assays (7–12). The plasmid DNA synthesized in the presence of the essential and stimulatory replication factors in the transient assays is present as concatemeric molecules (5, 13) indicating that other viral products may be required for processing of nascent genomes. Two viral proteins, very late expression factor 1 (VLF-1) and alkaline nuclease (AN), have been predicted to participate in maturation of baculovirus genomes (14, 15). VLF-1 is required for expression of very late genes (16, 17) but also plays an unknown role in replication (14). VLF-1 is a member of the inte- grave/resolvase family of proteins (16), and it interacts preferentially with DNA crosses (18). This suggests that VLF-1 may function in the processing of branched intermediates in replication and recombination. The AN involvement in maturation of baculovirus genomes was predicted on the basis of structural homology of this enzyme and alkaline nuclease of viruses from the family Herpesviridae. Baculovirus AN contains five domains homologous to conserved motifs found in AN of alphaherpesviruses, although three other conserved motifs of the herpesvirus enzyme are not detected in the AcMNPV AN which is 1.5-fold smaller than the herpes simplex virus type 1 (HSV-1) homolog (15). Although HSV-1 AN is not essential for viral DNA synthesis (19), deletion of the gene encoding AN results in the accumulation of complex branched concatemeric genomes indicating that AN either cleaves or prevents the generation of these structures (20, 21). Because complex concatemeric intermediates are likely produced in DNA replication of baculoviruses (5, 6, 13), baculovirus AN may be also involved in the resolution of replication intermediates and genome maturation (15).

Computer analyses reveal that AN of baculoviruses and herpesviruses belongs to a protein family typified by bacteriophage λ exonuclease (22, 23), a toroidal trimeric enzyme (24) that produces single-stranded DNA overhangs that serve as intermediates in the repair and recombination of phage chromosomes. The λ exonuclease (Redα) interacts with the DNA-binding protein (Redβ), and both proteins mediate Red-dependent
homologous recombination (for review see Refs. 25–27). The HSV AN interacts with the major viral DNA-binding protein (mDBP) (28, 29), which facilitates annealing of complementary DNA strands (30–32) and the invasion of ssDNA into supercoiled DNA duplexes (33). In vitro both HSV-1 proteins, AN and mDBP, promote strand transfer from a linear duplex to ssDNA circle suggesting that a complex of AN and mDBP may function as a recombinaise (34). In a previous report we demonstrated that AcMNPV AN possesses a potent 5'→3' exonuclease activity and associates with the viral ssDNA-binding (SSB) protein LEF-3 (35). The polarity of the exonuclease and its association with the SSB protein LEF-3 suggest that baculovirus AN and LEF-3 may participate in homologous recombination of the baculovirus genome in a manner similar to that described for the Red-mediated recombination system of bacteriophage λ. Because efficient homologous recombination accompanies replication of herpesviruses (36–40) and baculoviruses (41–45), it may represent a general pathway for processing replication intermediates of large dsDNA viruses. In contrast to the prototypic phage λ exonuclease, herpesvirus AN possesses a weak endonuclease activity besides its potent exonuclease (46–50). The endonuclease associated with HSV-2 AN shows specificity for ssDNA (47), although a role of the endonuclease in infected cells remains unknown. Preliminary data from this laboratory suggest that baculovirus AN also possesses endonuclease activity (15). Because the endonuclease activity of baculovirus AN may contribute to the processing of replication and recombination intermediates, we have examined its specificity using plasmid and minicircle DNA as model substrates.

In this report we characterized the endonuclease activity of the purified complex of AcMNPV AN and LEF-3 that we designate AN/L3. To our knowledge, this is the first report describing endonuclease activity of AN associated with an SSB protein in a complex which presumably represents a major functional form of AN in infected cells. The endonuclease of AN/L3 showed specificity for ss regions in DNA and was inactive on relaxed DNA duplexes lacking free ends. A balance between the endonuclease and exonuclease modes of hydrolysis of DNA substrates was dependent on the structure of DNA substrates and the amount of SSB protein added to the reactions. These data suggest that the endonuclease of AN may participate in the processing of baculovirus replication and recombination intermediates by cutting the DNA products at positions of structural distortions, whereas LEF-3 may further regulate processing of the intermediates.

**EXPERIMENTAL PROCEDURES**

**Cells and Recombinant Baculoviruses—**Spodoptera frugiperda 9 (S9) cells were cultured in SF900II serum-free media (Invitrogen), penicillin G (50 units/ml), streptomycin (50 μg/ml), BioWhittaker), and fungizone (amphotericin B, 375 ng/ml, Flow Laboratories) as described previously (51). The recombinant baculoviruses AcHISAN and AcHISAN/S146A for overexpression of an AcMNPV His6-tagged alkaline endonuclease (AN) (open reading frame 133) and His6-tagged AN mutant with single amino acid altered at position 146 (S146A) in the conservative motif II of AcMNPV AN were described previously (15). For overexpression of AcMNPV LEF-3, the AN open reading frame was amplified with PCR primers, Ac67kpn5B (AGGTACCATGGCGACCAAAAGATCTTTGTC) and Ac76not3 (CCACCCTCCCCAATATTCACCATCAACCCTTCACCTCACTTCACTTCACTCCATATCCACTC) and inserted into a pFastBac1 vector (Invitrogen) under the polyh-promoter. Recombinant baculovirus AcLEF3 was produced using the BacToBac baculovirus expression system (Invitrogen) following the manufacturer's instructions.

**DNA Substrates—**Plasmid pS26 DNA (5.2 kb) produced by a HindIII-XbaI insertion of 2.0-kb h-fragment of AcMNPV genome into pHBluS (1) DNA (Stratagene, La Jolla) was used as a substrate for analysis of endonuclease activity. To obtain relaxed DNA, 10 μg of plasmid pS26 DNA was incubated with 20 units of calf thymus topoisomerase I (MBI Fermentas) in a 100-μl reaction mixture containing 35 mM Tris-HCl, pH 8.0, 72 mM KCl, 7 mM MgCl2, 200 μM BSA, and 2 mM dithiothreitol for 1 h at 37 °C. The reaction was terminated by the addition of 2 μl of 0.5 mM EDTA, pH 8.0. The DNA was extracted with phenol/chloroform (1:1), precipitated with ethanol, and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The control DNA sample for the experiments with the relaxed DNA was treated in the same manner but in the absence of topoisomerase I. The sonicated heat-denatured salmon sperm DNA was obtained by standard methods (52).

The ss minicircle DNA was produced as described previously (53, 54) with minor modifications. A 70-mer oligonucleotide (CACCATACCTTCAACCTTCTACCTCCACTCCCTATACACTCCTTCTCACTCCTCACTTCACTCCTGAGTGAATATTGGGGAGGGTGGAGGGTTTATGGGGTGTGTTATGCAGGATGTGAAAAGG) was labeled with [γ-32P]ATP (PerkinElmer Life Sciences). To obtain relaxed 70-mer DNA, the labeled supercoiled DNA was treated with S1 nuclease and a DNA polymerase and removed by phenol/chloroform extraction. The ss minicircle DNA was produced by dilute annealing of the 32P-labeled 70-mer with a 1.2-fold molar excess of 70-mer bridging oligonucleotide (GGTATGGTGAGGGTGTGATA) in dilute conditions followed by ligation using T4 DNA ligase at 16 °C. The ds minicircle DNA was produced by the dilute annealing of the 32P-labeled 70-mer with a 1.2-fold molar excess of 70-mer bridging oligonucleotide (TGATGGTGGAATATTGGGGGAGGGTGGAGGGTTTATGGGGTGTGTTATGCAGGATGTGAAAAGG) phosphorylated at the 5' end. The ends of both 70-mers in the generated ds circle were then covalently joined by T4 DNA ligase under incubation at 22 °C. The 32P-labeled minicircles were purified by electroelution on an 8% denaturing polyacrylamide gel. The yield of purified 32P-labeled ss and ds 70-mer minicircles was about 80 and 20%, respectively, of the linear ds DNA added. The 32P-labeled 70-mer is in a covalent circular structure of the ss and ds minicircles was confirmed by the anomalous behavior of these DNA substrates in the denaturing polyacrylamide. The ss 70-mer minicircle co-migrated with 80-nt linear DNA in the 8% polyacrylamide gel and with 150-nt linear DNA in the 14% polyacrylamide gel. The ds 70-mer minicircle migrated in these gels at the positions of about 100- and 500-nt linear DNA.

**Purification of wt AN/L3, Mutant AN/L3, and Pure AN—**S9 cells at a density of 1.5 × 10⁶/ml in shaker flasks were infected with the recombinant viruses AcHISAN or AcHISAN/S146A at a multiplicity of infection of 4–5, and incubated with shaking for 48–72 h at 28 °C. The wt AN/L3 and the mutant complex AN/S146A/L3 were purified from 50- to 100-ml cultures sequentially on nickel-nitritotriacetic acid (Ni-NTA)-agarose (Qiagen), DEAE-Toyopearl 650 (Tosohaas), and heparin-Sepharose CL-6B (Amersham Biosciences) columns as described previously (35). The samples in buffer E (0.1 M KCl, 10 mM Tris-HCl, pH 7.5, 50% glycerol, 1 mM dithiothreitol, 0.2 mM EDTA) were stored at −20 °C for periods of 1–2 months or at −80 °C for long term storage. The yield of pure wt and mutant AN/L3 complexes was about 5–7 μg per ml of the infected cells cultures.

To obtain pure His6-tagged *AN, we dissociated the *AN/L3 complex by using the hydrophobic reagent ethylene glycol. The S9 cells were infected with the recombinant virus vAcHISAN, and the extract was processed with 1-ml portions of the buffer containing NaCl in final concentrations of 0.25, 0.3, 0.35, 0.4, 0.6, 0.8, and 1 M. Proteins from each fraction were analyzed by SDS–10% PAGE, and fractions containing pure *AN-L3 were combined and dialyzed against buffer containing 20 mM Tris-HCl, pH 8.5, 0.1 M KCl, 1% glycerol, and 5 mM 2-mercaptoethanol. The column was then washed with 2 ml of buffer C (20 mM Tris-HCl, pH 8.5, 75 mM KCl, 1% glycerol, 5 mM 2-mercaptoethanol) containing 20 mM imidazole, and protein was eluted with 4 ml of the buffer containing 200 mM imidazole. The sample was dialyzed overnight against buffer D (10 mM Tris-HCl, pH 7.5, 20% glycerol, 1 mM diithiothreitol, 1 mM EDTA) containing 75 mM KCl and loaded onto a 0.7-ml column of single-stranded DNA-agarose (Amersham Biosciences). The column was washed with several volumes of buffer D containing 0.2 mM NaCl and processed with 1-mI portions of the buffer containing NaCl in final concentrations of 0.25, 0.3, 0.35, 0.4, 0.6, 0.8, and 1 M. Proteins from each fraction were analyzed by SDS–10% PAGE, and fractions containing pure AN were combined and dialyzed against buffer containing 20 mM Tris-HCl, pH 8.5, 0.1 M KCl, 20% glycerol, and 5 mM 2-mercaptoethanol. The *AN sample was mixed with 1.4 volumes of buffer containing 87% ethylene glycol, 80 mM Tris-HCl, pH 8.5, 0.1 M KCl, and 5 mM 2-mercaptoethanol and incubated for 1 h at 4 °C while gently shaking the sample. To remove residual LEF-3, the *AN sample was subjected to Ni-NTA-agarose chromatography by Doug Laue and the fractions containing pure AN were dialyzed against buffer containing 87% ethylene glycol. After loading the sample onto a new column of Ni-NTA-agarose (0.6 ml) equilibrated with buffer containing 50% ethylene glycol, 50 mM Tris-HCl, pH 8.5, 0.1 M KCl, 10% glycerol, and 5 mM 2-mercaptoethanol, the column was washed with 6 ml of the same
buffer, and then with 3 ml of buffer containing 20 mM Tris-HCl, pH 8.5, 0.1 mM KCl, 20% glycerol, and 5 mM 2-mercaptoethanol. Protein was eluted with 3 ml of buffer C containing 200 mM imidazole, and fractions were analyzed by SDS-10% PAGE. The fractions were combined or dialyzed separately against buffer and stored at −20 °C for periods of 1–2 months or at −80 °C for long term storage.

Purification of LEP-3—AcMNPV LEP-3 was purified from Sf9 cells infected with wt AcMNPV or with the recombinant baculovirus vAcLEF3. The cells at a density of 1.5 × 10^7/ml in shaker flasks were infected with AcMNPV or vAcLEF3 at a multiplicity of infection of 4–5 and incubated with shaking at 28 °C for 22 and 72 h, respectively. LEP-3 was purified routinely from 50- or 100-ml cultures of infected cells by liquid chromatography sequentially on single-stranded DNA-cellulose (Sigma) and DEAE-Toyopearl 650 (TosoHaas) columns. The infected cells were pelleted by centrifugation for 5 min at 500 × g and resuspended in 7–10 ml of lysis buffer containing 50 mM Tris-HCl, pH 8.5, 200 mM KCl, 1% Nonidet P-40, 5 mM 2-mercaptoethanol, and fractions collected were analyzed by SDS-10% PAGE followed by staining with Coomassie Brilliant Blue, and those containing pure LEF-3 were combined or dialyzed separately against buffer E and stored at −20 °C for periods of 1–2 months or at −80 °C for long term storage.

Purification of Proteins—AN of AcMNPV was overexpressed in the recombinant baculovirus (15) as a His-6-tagged fusion and was purified from Sf9 cells as described previously (35). The highly purified samples contained two polypeptides, His-tagged AN (35 kDa) and viral ssDNA-binding (SSB) protein LEP-3 (44 kDa) (Fig. 1, lane 1). These are present predominately as a stable heterodimer designated as *AN/L3 (35). To compare a wild type (wt) AN with an AN mutant impaired in nuclease activity, we expressed and purified a His-tagged AN mutant with a single amino acid altered at position 146 (S146A) in the conservative motif II of AcMNPV AN (15). As shown previously, the mutant S146A has less than 10% the activity of wt AN in assay using linear DNA (15). SDS-PAGE analysis revealed that the mutant form of AN copurified with LEP-3, and the purified complex showed no change in the molar ratio of alkaline nuclease to LEP-3 (lane 2). This result suggests that the amino acid at position 146 in AcMNPV AN is not essential for interaction with LEP-3, and the association with LEP-3 does not depend on the level of nuclease activity of AN.

To compare the complex *AN/L3 with *AN samples lacking LEP-3, we attempted to dissociate the complex *AN/L3 using the hydrophobic agent ethylene glycol (see “Experimental Procedures”). Two subsequent runs on Ni-NTA columns in buffers containing 50% ethylene glycol were enough to remove most LEP-3 from the *AN samples (Fig. 1, lane 3). The final preparation of pure *AN contained less than 5% the LEP-3 present in samples obtained by the standard method.

Two different preparations of viral DNA-binding protein LEP-3 were used. Both LEP-3 samples were purified by conventional chromatography on columns of ssDNA-agarose and DEAE-Toyopearl, but the first sample was obtained from cells infected with wt AcMNPV, whereas the second sample was obtained from cells infected with the recombinant baculovirus vAcLEF3 that was engineered for overexpression of LEP-3 under the control of the polyhedrin promoter (see “Experimental Procedures”). The yield of LEP-3 from the recombinant virus was severalfold higher than that from the wt virus, but both LEP-3 samples showed the same mobility under SDS-PAGE (Fig. 1, lanes 4 and 5) and the same properties in the enzymatic assays, and they were used in experiments interchangeably.
Endonuclease activity has also been reported to associate with herpesvirus AN (46)

Endonuclease activity has also been reported to associate with herpesvirus AN (46). AN and HSV-2 AN perform both endonucleolytic and exonucleolytic digestion of DNA substrates, but the enzymes show only endonuclease activity in the presence of Mn$^{2+}$ ions in concentrations higher than 2 mM (47, 56). Therefore, in parallel to the standard reactions containing Mg$^{2+}$, we performed analysis of AcMNPV *AN/L3 in reactions containing Mn$^{2+}$. It was expected that Mn$^{2+}$ may selectively suppress the exonuclease activity of *AN/L3, as was shown earlier for herpesvirus AN (47, 56). Substitution of 2 mM MgCl$_2$ with 5 mM MnCl$_2$ resulted in approximately a 4–5-fold decrease in the rate of conversion of RFI into RFIII and in approximately 1 order of magnitude lower rate of exonuclease digestion of RFIII (compare Fig. 2, A and B). Although the nature of divalent cations appeared to affect the ratio of endonuclease and exonuclease activities of *AN/L3, the exonuclease of *AN/L3 remained active in the presence of 5 mM MnCl$_2$ (Fig. 2B). The same result was obtained with 2 mM MnCl$_2$ (data not shown). In this respect baculovirus AN differs from HSV-1 and HSV-2 AN which completely loses exonuclease activity at concentrations of MnCl$_2$ equal or above 2 mM (47, 56).

The complete transition from RFI to RFIII requires endonucleolytic scission of both strands of plasmid DNA, and this takes only a few minutes of incubation with *AN/L3 at standard assay conditions (Fig. 2). However, it was unclear whether these endonucleolytic acts proceed in a processive manner such that the enzyme complex incises the first DNA strand causing conversion RFI into RFIII, and then the same complex, without dissociation from the DNA, cuts the opposite strand accomplishing conversion into RFIII. The alternative distributive mechanism assumes that scission of the second DNA strand requires another single or multiple binding event as a result of random interaction of the enzyme complexes with previously nicked DNA molecules. In order to distinguish between these two processes, we compared the kinetics of conversion of RFI to RFIII at the standard assay conditions and in the presence of competitor DNA (Fig. 3). If the conversion of RFI to RFIII requires a single binding event, competitor DNA may affect the initial binding of *AN/L3 to RFI, but it should not change the rate of conversion RFI to RFIII, because scission of the second strand is carried out by the enzyme complex initially bound to the plasmid. In contrast, under a distributive mode, the competitor DNA should inhibit the conversion of RFI to RFIII because this conversion requires new binding events. As a
competitor, we used sonicated heat-denatured salmon sperm DNA that was added into the reaction mixture in 280-fold excess with respect to plasmid DNA. The reactions were carried out in the presence of 2 mM MgCl$_2$ (lanes 1–9) or 2 mM MnCl$_2$ (lanes 10–18). As expected, the competitor DNA decreased the rate of RFI digestion (compare lanes 2–5 with lanes 6–9 and lanes 11–14 with lanes 15–18) presumably due to less frequent interaction of $^*$AN/L3 with plasmid DNA in the presence of the competitor. More important, the competitor DNA efficiently inhibited conversion of RFII to RFIII. Although a large portion of plasmid DNA was presented in form RFII after a 5-min incubation in the presence of Mg$_2^+$ and the competitor (lane 6), further conversion to RFIII was blocked for about 10 min (lane 8) and was observed only after practically complete hydrolysis of the competitor DNA (lane 9). In contrast, incubation for 1.5 min was enough for conversion of most RFI into RFIII in the absence of the competitor DNA (lane 2). Efficient hydrolysis of competitor DNA in the course of incubation (lanes 6–9) confirmed that the competitor did not inactivate the nuclease activity of $^*$AN/L3 thus indirectly affecting the RFII to RFIII conversion. The inhibition of transition RFII $\rightarrow$ RFIII with the competitor DNA indicates that the initial $^*$AN/L3 binding to supercoiled DNA was not sufficient for its conversion into the linear form. The scission of the remaining intact strand in the RFII intermediate depended on new binding events. Similar conclusions could be drawn from the assay with Mn$_2^+$ (lanes 11–18). A major portion of RFI was converted to RFII after a 5-min incubation in the presence of competitor DNA (lane 15). However, a very small portion of RFII intermediates underwent further processing into RFIII under subsequent incubation for 30 min (lane 18). In contrast, incubation for 5 min was sufficient for conversion of most RFI molecules into RFIII in the absence of the competitor (lane 11). The data are in agreement with the distributive mode of transition RFI $\rightarrow$ RFII $\rightarrow$ RFIII when scission of each strand in plasmid DNA requires different $^*$AN/L3 binding events.

Recognition of Nicks (Gaps) in Circular DNA by AN/L3—The data shown in Fig. 3 indicated that RFII DNA serves as substrate for $^*$AN/L3 binding followed by the endonucleolytic attack and conversion of nicked circular into linear molecules. There are two possible mechanisms for generation of linear molecules from nicked circular DNA. Linearization may be caused by a single breakage of the intact DNA strand at the position opposite or near the initial nick (gap) in RFII intermediates. Another possibility is accumulation of multiple breaks in both DNA strands, and the linearization of circular molecules resulted from random juxtaposition of breaks in two strands. To elucidate the mechanism of conversion RFII $\rightarrow$ RFIII, we determined the size of the DNA strands in nascent RFIII molecules. Heterogeneous, small sized DNA strands in RFIII molecules would indicate that $^*$AN/L3 produced multiple breaks in both strands of RFII intermediates. In contrast, genome size DNA strands in linear molecules would indicate that scission of the intact strand occurred at the position opposite the initial nick (gap) in the RFII intermediates. The results are shown in Fig. 4. When plasmid DNA was denatured by alkali and electrophoresed in a neutral agarose gel, a fraction of RFI molecules completely renatured and migrated at the original position of intact supercoiled molecules, whereas another fraction remained irreversibly denatured and migrated faster than RFI (lanes 1’ and 3’). The band of the topologically linked circular DNA strands is marked as RFII* in Fig. 4. Denaturation of RFII molecules present in the preparation of plasmid DNA resulted in the appearance of small quantities of linear full size strands marked as ssDNA in Fig. 4 (lanes 1’ and 3’). After incubation with $^*$AN/L3 in the presence of Mg$_2^+$ for 100 s or in the presence of Mn$_2^+$ for 10 min, most plasmid DNA was converted into form RFIII (respectively, lanes 2 and 4). Alkaline denaturation of these DNA samples liberated predominantly full size strands with the mobility of “ssDNA” (lanes 2’ and 4’) thus indicating that $^*$AN/L3 cut each strand of the plasmid DNA only once. In order to linearize the nicked circular molecule by hydrolysis of a single phosphodiester bond in the intact strand, $^*$AN/L3 should introduce a break at the
The mixtures were transferred to 37 °C in the presence of 0.15 M NaOH and rapidly chilled on ice. The temperature. Half of each sample was denatured by heating at 90 °C for 1 min in the presence of ethidium bromide (0.5 mg/ml). Reactions were terminated by 1.6 ml of stop solution (6% SDS, 150 mM EDTA) and treated with proteinase K (150 µg/ml) for 20 min at room temperature. Half of each sample was denatured by heating at 90 °C for 1 min in the presence of 0.15 M NaOH and rapidly chilled on ice. The denatured portion (marked by lane ssM) and the untreated portion were loaded onto parallel lanes and analyzed by electrophoresis in a 1% agarose gel in the presence of ethidium bromide (0.5 µg/ml). Lane dsM represents 1-kbp DNA ladder; lane ssM represents the ladder denatured by boiling for 5 min before loading onto the gel. RFI+ indicates a fraction of RFI irreversibly denatured after the treatment with alkalai; ssDNA indicates the full size ss DNA molecules resulted from denatured RFII and RFIII.

position opposite or very close to the nick (gap) in the RFII intermediates. This result indicates that *AN/L3 recognized nicks or gaps in circular molecules and specifically attacked endonucleolytically intact DNA strands at these positions.

Inability of AN/L3 to Digest Relaxed Plasmid DNA—The capability of *AN/L3 to recognize irregularities in DNA duplexes such as nicks and gaps suggests that ssDNA and ss regions in DNA duplexes may present true substrates for the endonuclease of baculovirus AN, whereas regular DNA duplexes lacking free ends are poor substrates or resistant to the enzyme. If this prediction is correct, *AN/L3 should be inactive on relaxed intact circular DNA molecules which, in contrast to supercoiled molecules, do not contain ss regions (57). To test this prediction, we obtained relaxed DNA by treatment of plasmid DNA with topoisomerase I and then compared hydrolysis with *AN/L3 of the relaxed DNA and control DNA that was not treated with topoisomerase (Fig. 5). The relaxed DNA was represented by a set of topoisomers with low numbers of superhelical turns, and it migrated in agarose gels in the absence of ethidium bromide near the position of nicked circular molecules (RFII) (Fig. 5A, lane 7). The control DNA was represented predominantly by negatively supercoiled molecules (RFI) and by a minor fraction of RFII molecules (Fig. 5A, lane 1). Incubation of the control DNA in the reaction mixture with *AN/L3 resulted in a dose-dependent conversion of DNA into form RFIII (Fig. 5A, lanes 2–6). Under the same conditions, the relaxed DNA appeared to be resistant to the *AN/L3 endonuclease (Fig. 5A, lanes 8–12). A minor fraction of RFII molecules seen after the treatment of the relaxed DNA presumably originated from RFII molecules initially present in the DNA sample. The resistance of the relaxed DNA to the endonuclease activity of *AN/L3 was confirmed by electrophoresis of the reaction products in an agarose gel in the presence of ethidium bromide (Fig. 5B). Fluorescence of the intact circular DNA in lanes of the gel was quantitatively estimated by optical densitometry, and the data obtained were used for drawing the graphs shown in Fig. 5C. Due to intercalation of ethidium bromide, the intact circular DNA molecules in both the relaxed and the control samples acquired positive superhelical turns and migrated in the gel at the same position marked as RFI in the figure. The supercoiled DNA in the control sample was sensitive to the *AN/L3 endonuclease, and it was digested with *AN/L3 in a dose-dependent manner (Fig. 5, B, lanes 1–6, and C, RFII). Under the same conditions, the relaxed intact circular DNA was resistant to *AN/L3 (Fig. 5, B, lanes 7–12, and C, rRFI). In a mixture of the relaxed DNA and control DNA samples, some of the intact circular molecules were sensitive to the *AN/L3 endonuclease, whereas other molecules were resistant (Fig. 5, B, lanes 13–18, and C, rRFI + RFII). The latter result as well as the sensitivity of RFII molecules in the sample of the relaxed DNA to *AN/L3 (Fig. 5B, lanes 7–12) eliminates the possibility that this DNA sample contained inhibitors that prevent digestion of the relaxed molecules.

Effect of Ethidium Bromide on Hydrolysis of Plasmid DNA—The resistance of the relaxed intact circular DNA to *AN/L3 was observed at different concentrations of MgCl2 (2–10 mM) and at different pH values of the Tris-HCl buffer (pH 7.5–8.8) (data not shown) and presumably reflects a true property of *AN/L3, an inability to endonucleolytically attack non-distorted DNA duplexes that do not contain ss regions. Because the digestion of negatively supercoiled DNA molecules with *AN/L3 apparently depended on the presence of partially denatured regions in these molecules (57), we attempted to eliminate negative superhelical turns and as a consequence the ss regions from plasmid DNA by using titration with an intercalating agent. It was expected that the intercalator in concentrations sufficient to remove negative super turns from circular DNA should provide resistance to the *AN/L3 endonuclease. In this case, the digestion of supercoiled DNA with *AN/L3 should be highly sensitive to intercalators such as ethidium bromide. To test this prediction, we analyzed digestion of the control supercoiled DNA and the relaxed DNA with *AN/L3 in the presence of ethidium bromide in different concentrations (Fig. 6). The graphs in Fig. 6B show changes in fluorescence of the intact circular DNA estimated by optical densitometry for each lane of the gel shown in Fig. 6A. Hydrolysis of the control DNA was actually very sensitive to the intercalator (Fig. 6, A, lanes 1–9, and B, RFI). The 50% inhibition of the hydrolysis was achieved at the ethidium bromide concentrations in a range 0.25–0.5 µg/ml, and the hydrolysis was blocked completely at dye concentrations above 1 µg/ml. In contrast, the relaxed DNA was resistant to *AN/L3 in the absence of intercalator as well as in the presence of ethidium bromide in concentrations up to 4 µg/ml (Fig. 6, A, lanes 10–18, and B, rRFI). The increase in ethidium bromide concentrations above 4 µg/ml resulted in distortion of the RFI bands in the gel (data not shown), and we have not analyzed in detail interaction of *AN/L3 with DNA in this range of dye concentrations. At the concentrations used in the experiment shown in Fig. 6, ethidium bromide did not inhibit digestion of RFII molecules with *AN/L3 in either sample, the control or the relaxed DNA. In a control experiment, the 50% inhibition of hydrolysis of RFII molecules was observed at ethidium bromide concentrations in a range of 15–20 µg/ml (data not shown). This result confirmed that the inhibition of the digestion of supercoiled DNA with ethidium bromide
in concentrations of 0.25–1 μg/ml was caused by interaction of the dye with DNA substrate, but not by its influence on the enzyme. The resistance of the supercoiled DNA to the *AN/L3 endonuclease acquired in the presence of ethidium bromide suggests that at these conditions the negative super turns and ss regions were removed from the DNA. Further intercalation of ethidium bromide should induce positive super turns in plasmid DNA. Positive super turns should also be induced upon intercalation of ethidium bromide into the relaxed circular DNA. Because the positively supercoiled DNA appeared to be resistant to *AN/L3 (Fig. 6A, lanes 11–18), the positive superhelical turns themselves do not make plasmid DNA sensitive to the endonuclease. This result is in agreement with the idea that ss regions but not superhelical turns are essential for productive interaction of the *AN/L3 endonuclease with the negatively supercoiled DNA. The data confirmed that the endonuclease of *AN/L3 has specificity for ssDNA.

Hydrolysis of ss Minicircle DNA—To confirm directly the capability of baculovirus AN to endonucleolytically digest ssDNA, we performed an experiment with 32P-labeled ss 70-mer minicircle DNA. This substrate does not possess free ends required for exonucleolytic hydrolysis; therefore, the digestion of minicircle DNA should be initiated by an endonucleolytic attack. For control DNA substrates, we used a linear ss 70-mer DNA that should be sensitive to the nuclease activity of AN/L3 (35) and a ds 70-mer minicircle that was expected to be resistant to AN/L3. The linear ss 70-mers labeled with 32P at the 5'-terminus were rapidly hydrolyzed by *AN/L3 producing radioactive nucleotides (Fig. 7, lanes 1–4). This digestion pattern reflected removal of 5'-terminal nucleotides from linear oligonucleotides by the exonuclease activity of AN/L3 (35). *AN/L3 also efficiently hydrolyzed the labeled ss minicircle DNA producing radioactive nucleotides and oligonucleotides shorter than the 10-nt marker (lanes 5–8). The digestion of minicircle DNA proved that AN/L3 has the ability to endonucleolytically cut ssDNA. Labeled linear 70-mers served presumably as intermediates in the hydrolysis of ss minicircles, and they were clearly seen after overexposure of the gel (data not shown). Previous experiments with 5'- and 3'-labeled oligonucleotides showed...
that the digestion rate by *AN/L3 drops dramatically when the size of oligonucleotides is decreased to about 10 nt (35). If the minicircle is linearized at a position distal to the 32P-labeled nucleotide and then undergone the exonuclease hydrolysis, radioactive nucleotides should eventually be liberated. In contrast, if DNA is broken at a position 3′-proximal to the 32P-labeled nucleotide, the label should remain in short oligonucleotides after the reaction. The appearance of both radioactive products, mononucleotides and short oligonucleotides after the reaction. The fluorescence of the intact circular dsDNA after treatment of supercoiled DNA (RFI) and relaxed DNA (rRFI) with *AN/L3 in the presence of ethidium bromide. The gel shown in A was used for quantification. The fluorescence of the RFI band in each lane is expressed as a ratio to the total DNA fluorescence of the sample (cumulative fluorescence of the RFI, RFII, and RFIII bands).

**Fig. 6.** Effect of ethidium bromide on endonucleolytic hydrolysis of supercoiled and relaxed circular DNAs. A, the assay was carried out in 10-μl reaction mixtures containing 200 ng of supercoiled plasmid DNA (RFI, lanes 1–9) or 200 ng of relaxed plasmid DNA (rRFI, lanes 10–18), 20 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 50 mM NaCl, 10 mM KCl, 2 mM DTT, 10% glycerol, and 100 μg/ml BSA. Ethidium bromide was added to the reaction mixtures with ds minicircles to obtain 1-kbp DNA ladder. B, fluorescence of the intact circular dsDNA after treatment of supercoiled DNA (RFI) and relaxed DNA (rRFI) with *AN/L3 in the presence of ethidium bromide. The gel shown in A was used for quantification. The fluorescence of the RFI band in each lane is expressed as a ratio to the total DNA fluorescence of the sample (cumulative fluorescence of the RFI, RFII, and RFIII bands).
LEF-3 from the *AN samples (Fig. 1, lane 3). The pure *AN was capable of digesting RFI DNA (Fig. 9, lane 6), although the specific endonuclease activity of the pure *AN sample was ~2–3-fold lower than its activity in the complex with LEF-3. LEF-3 in concentrations of 2–10 µg/ml (a molar ratio of LEF-3 to *AN of 3–15) increased hydrolysis of RFI (Fig. 9, A, lanes 7–9, and B, RFI). Fig. 9A (lanes 2–5) also shows that the LEF-3 sample was apparently free of contamination with endonucleases. The RFI hydrolysis with *AN in this range of LEF-3 concentrations was accompanied by accumulation of RFII DNA (Fig. 9B, RFII). Further increase in LEF-3 concentration from 10 to 20 µg/ml (a molar ratio of LEF-3 to *AN of 15–30) did not affect the hydrolysis of RFI but caused accumulation of RFII presumably due to inhibition of its conversion into RFIII. The marked increase in electrophoretic mobility of RFII molecules in the presence of LEF-3 in a concentration 20 µg/ml (compare lane 10 with lanes 6–8) indicated that LEF-3 stimulated exonucleolytic digestion of broken strands in nicked circular molecules, whereas it inhibited endonucleolytic attack of the intact strands in these molecules. Heating of the LEF-3 sample for 15 min at 65 °C before addition into the reaction completely inactivated its capability to affect the digestion of plasmid DNA with *AN (data not shown). In summary, the following conclusions can be drawn from the data shown in Fig. 9. (i) Pure *AN possesses endonuclease activity and is capable of digesting supercoiled DNA. (ii) LEF-3 affects the digestion of supercoiled DNA with *AN and in moderate concentrations stimulates the hydrolysis. (iii) In the reaction of *AN with nicked circular molecules, LEF-3 in high concentrations shifts a balance between the endonucleolytic hydrolysis of intact strands and the exonucleolytic hydrolysis of broken strands in favor of the exonucleolytic pathway. The particular effect of LEF-3 on *AN function depends on the nature of the DNA substrate used in the assay and LEF-3 concentration that may determine both the interaction of LEF-3 with *AN and the level of DNA saturation with protein.

**Effect of LEF-3 on Nuclease Activities of AN/L3.**—In infected cells AN presumably acts in a complex with LEF-3. Therefore, we repeated the experiment described above with the complex *AN/L3 (Fig. 10). The addition of LEF-3 in concentrations up to 20 µg/ml to the reaction mixtures with *AN/L3 stimulated digestion of RFI, whereas further increase in LEF-3 concentrations to 80 µg/ml reversed the stimulation (Fig. 10B, RFI). LEF-3 in concentrations higher than 20 µg/ml efficiently inhib-
presence of ethidium bromide (0.5 μg/ml) as described under "Experimental Procedures." After briefly mixing, the samples were preincubated for 10 min on ice and then incubated for 5 min at 37 °C. The reactions were terminated, and the samples were analyzed by electrophoresis in a 1% agarose gel in the presence of ethidium bromide (0.5 μg/ml) as described under "Experimental Procedures." The change from serine to alanine at position 146 in the conservative motif of AcMNPV AN impaired both the exonuclease and the endonuclease activities of *AN/L3 (Fig. 8A). This result proved that the endonuclease activity was not caused by contamination in the AN samples, but it represents an intrinsic property of baculovirus AN. Similar conclusions could be drawn from the experiment on the dissociation of AN/L3 complexes with ethylene glycol (Fig. 9). Because AN samples lacking LEF-3 also showed endonuclease activity, this activity was not acquired upon interaction with LEF-3, although manifestation of the endonuclease was enhanced by LEF-3 added to the assay. The specific endonuclease activity of *AN/L3 in the reaction with plasmid DNA was about 4 orders of magnitude lower than the nuclease activity of this complex under standard assay with ssDNA. Therefore, the endonuclease might be considered as a minor enzymatic activity of bacu-
lovius AN. However, the capability of AN to endonucleolytically attack DNA at the internal positions may determine specific pathways for processing of intermediates in replication and recombination, and the endonuclease should be taken into account when considering the role of AN in the baculovirus infection cycle. The endonuclease in particular allowed the initiation of hydrolysis of supercoiled DNA.

The following model may describe the initial stages in the hydrolysis of plasmid DNA with baculovirus AN. Pure AN or the AN/L3 complex endonucleolytically attacks one strand in supercoiled DNA molecule (RFII) at a partially denatured region and converts it into the open circular form (RFII). The free 5' end generated in the nicked strand of RFII molecule serves as a substrate for the exonuclease of AN or AN/L3 (these intermediates are indicated by white arrows in Fig. 2). At the same time, a nick (or gap generated by limited exonucleolytic digestion) exposes a ss region in the opposite, intact strand of the RFII molecule, which serves as a target for the endonuclease attack by AN or AN/L3 accompanied by the conversion of the circular molecule into the linear form (RFIII). The scission of the intact strand in the RFII molecule requires a new enzyme binding event. The proposed scheme assumes that the endonuclease of baculovirus AN has specificity for ssDNA and produces single strand breaks in DNA. The specificity for ssDNA was revealed by the capability of AN/L3 to hydrolyze negatively supercoiled DNA and to recognize nicks or small gaps in circular molecules. The resistance of plasmid DNA relaxed with topoisomerase I to the endonuclease (Fig. 5), and a high sensitivity of RFII hydrolysis to ethidium bromide (Fig. 6) confirmed that the ss regions in negatively supercoiled molecules serve as a primary target for the endonuclease attack by AN/L3. The experiment with minicircle DNA (Fig. 7) also confirmed the specificity of the endonuclease activity of baculovirus AN for ssDNA.

The two different modes for processing nicked circular molecules (RFII) with *AN and *AN/L3, endonucleolytic and exonucleolytic, suggest competition between both AN activities for DNA substrates. A balance between both activities was affected by divalent cations. Although the replacement of Mg2+ with Mn2+ ions inhibited the exonuclease activity of AN/L3 more than its endonuclease activity, we did not observe complete inhibition of the exonuclease in the presence of 2 or 5 mM MnCl2 (Fig. 2) that was previously shown for HSV-1 and HSV-2 (47, 56). Manifestation of each activity and a balance between them also depended on the structure of the DNA substrate and the amount of LEF-3 added to the reactions (Figs. 9 and 10). LEF-3 in moderate concentrations (up to 20–40 μg/ml) stimulated digestion of supercoiled RFII DNA with *AN or *AN/L3. The mechanism of the LEF-3 stimulation effect on AN endonuclease remains unknown. The ability of AN to bind LEF-3 may presumably facilitate AN interaction with ss regions in the supercoiled molecules due to the high affinity of LEF-3 for ssDNA. Hypothetically, LEF-3 may also stabilize the ss regions in RFII molecules due to the high affinity of LEF-3 for ssDNA. Hypothetically, LEF-3 may also stabilize the ss regions in the supercoiled molecules due to the high affinity of LEF-3 for ssDNA. Hypothetically, LEF-3 may also stabilize the ss regions in negatively supercoiled molecules thus increasing the concentration of target DNA in the reactions. Another apparent effect of LEF-3 is a shift in the balance between endonucleolytic and exonucleolytic digestion of RFII molecules in favor of the exonucleolytic pathway. LEF-3 at the maximum concentration used in the experiments (80 μg/ml) almost completely blocked the endonuclease hydrolysis of RFII molecules and their conversion into form RFIII, whereas exonucleolytic digestion of RFII molecules proceeded efficiently (Fig. 10, lane 6). The inhibition of the RFII conversion into RFIII at high concentrations of LEF-3 suggests that saturation of ssDNA with viral SSB protein protects this DNA from the endonuclease of viral AN. This prediction is in agreement with our previous observation that LEF-3 inhibits hydrolysis of short and long ssDNA molecules by *AN/L3 (35). Interestingly, the LEF-3 concentrations that completely blocked the endonuclease hydrolysis of RFII molecules did not prevent the hydrolysis of RFII molecules (Fig. 10). This suggests that the AN endonuclease may function at local distortions in DNA duplexes even in the presence of excessive amounts of viral SSB protein. The apparent stimulatory effect of LEF-3 on the exonucleolytic digestion of RFII molecules may also result from the melting activity of this SSB protein (58). Recent analysis of bacteriophage λ exonuclease revealed that melting of base pair precedes scission of the phosphodiester bond, and the melting step is a rate-limiting step in each exonuclease act (59). The 5’ to 3’ direction of the exonuclease hydrolysis of ssDNA with AN coincides with the preferential direction of LEF-3 movement along the DNA strand as it melts the DNA duplex (58). Therefore, the LEF-3 subunit in the AN/L3 complex may facilitate both the translocation of the enzyme complex in a 5’ to 3’ direction and the melting of base pairs prior to the complete prior the exonucleolytic attack by the AN subunit. The proposed mechanisms will be the subject of subsequent analysis.

The specificity of the AN endonuclease for ssDNA and its inability to hydrolyze non-distorted DNA duplexes lacking free ends conforms to the proposed function of AN in processing of the intermediates in replication and recombination of baculoviruses (15, 35). The AN endonuclease may initiate processing by cutting the concatemeric and branched intermediates that are likely produced in replication of the baculovirus genomes (5, 6, 13) at positions of nicks, gaps, or in different ss regions such as hairpins. Another viral protein, VLF-1, has the ability to recognize branched DNA structures, may play a critical role in this process (18). Further processing of the intermediates may proceed under the concerted action of AN/L3 and LEF-3 via the recombination pathway that has been well studied for the Red-mediated homologous recombination system of bacteriophage λ (for a review see Refs. 25–27). According to this model, the 5’ to 3’ exonuclease of AN/L3 may digest the 5’-terminal regions in the strands of circular and linear molecules thus producing 3’ overhangs that are involved in recombinational exchanges by the annealing of complementary regions or by invasion of the overhangs into duplex DNA under control of SS protein LEF-3. Similar roles in processing of the replication and recombination intermediates were suggested recently for AN and ssDNA-binding protein ICP8 of HSV-1 (33, 34). The capability of baculovirus AN and LEF-3 to perform the strand exchange reactions is under investigation.

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Specificity of the Endonuclease Activity of the Baculovirus Alkaline Nuclease for Single-stranded DNA

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