Published in final edited form as:

Virus Res. 2009 October ; 145(1): 106–111. doi:10.1016/j.virusres.2009.06.015.

CHARACTERIZATION OF SHORT-LIVED INTERMEDIATES PRODUCED DURING REPLICATION OF BACULOVIRUS DNA

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SUMMARY

In this report the short-lived DNA replication intermediates produced in both uninfected and *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) infected *Spodoptera frugiperda* cells were characterized. The methods used included pulse-labeling of DNA in permeabilized cells, treatment of nascent DNA with Mung bean nuclease, and electrophoresis in neutral and alkaline agarose gels. In contrast to uninfected cells that produced a population of small DNA fragments of about 200 bp, a population of heterogeneous fragments of up to 5 kb with an average size of 1 to 2 kb derived randomly from the virus genome was identified as the short-lived intermediates produced during AcMNPV replication. The intermediates likely include Okazaki fragments derived from the lagging strands in viral replication forks as well as fragments produced during the recombination-dependent replication.

Keywords

baculovirus; DNA replication; Okazaki fragments; Mung bean nuclease

INTRODUCTION

Baculoviruses belong to a family of large DNA-containing viruses that infect insect species of the orders *Hymenoptera*, *Diptera*, and *Lepidoptera*. They contain circular, double-stranded DNA genomes of 80 to 180 kb and replicate in the host nucleus by a mechanism that is poorly understood. The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most widely studied baculovirus and has a 134-kb genome that encodes approximately 150 genes (Ayres et al., 1994). Six viral factors are essential for replication in transient assays, a transcriptional transactivator called IE-1, DNA polymerase, DNA helicase, DNA primase (LEF-1), a primase accessory factor (LEF-2), and a single-stranded DNA-binding protein (LEF-3). A few other factors including LEF-11, DNA-binding protein (DBP) and alkaline nuclease (AN) are necessary for production of full-sized viral genomes in infected cells and presumably play an accessory role in replication (for review see (Rohrmann, 2008)). Viral replication starts in a limited number of foci dispersed in nuclei that may be related to the

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nuclear domain 10 (ND10) or PML bodies (Mainz et al., 2002; Okano et al., 1999). Initiation of DNA replication appears to take place at multiple origins that include homologous regions (*hrs*) that are comprised of 70-bp repeats that contain 30-bp imperfect palindromes in their center. In AcMNPV, *hrs* are distributed at about 8 different locations on the genome (Kool et al., 1994; Pearson et al., 1992). Other origins, including non-*hr* regions and early promoters, may also be utilized (Lee and Krell, 1992; Wu et al., 1999) with different efficiency during infection (Habib and Hasnain, 2000). Rolling circle and recombination-dependent mechanisms have been proposed for the elongation phase of baculovirus replication (Okano et al., 2007; Oppenheimer and Volkman, 1997). It is thought that baculoviruses may have a pattern of DNA replication similar to herpesviruses. They both have genomes of similar size, initiate DNA replication as circular intermediates in specific foci related to the ND10/PML domains in nuclei and produce concatamers in the course of replication. Both virus families encode similar sets of replicative proteins, although evidence of direct phylogenetic relatedness is lacking (for review see (Rohrmann, 2008)). Three different mechanisms, namely *theta* replication, rolling circle replication, and recombination-dependent replication were proposed for different stages in herpesvirus replication (for review see (Lehman and Boehmer, 1999)). HSV-1 DNA replication initiates by the *theta* mechanism. At some point, *theta* replication switches to the rolling circle mode. A similar switch in replication mode was also observed during bacteriophage λ replication (Stahl, 1998). At the late stage of HSV-1 replication, complex branched networks of concatamers of greater size than unit length are produced that arise presumably from recombination-dependent replication initiated by invasion of replication forks (Wilkinson and Weller, 2003). The linkage between replication and recombination has been well documented for bacteriophages T4 (Mosig, 1998) and λ (Stahl, 1998; Stahl et al., 2001). In the case of baculoviruses, numerous data also suggest a close association of replication with recombination (Croizier and Ribeiro, 1992; Hajos et al., 2000; Kamita et al., 2003; Summers et al., 1980; Wu et al., 1999). Nascent viral DNA appears to have a complex structure larger than unit length that is consistent with rolling circle or recombination-dependent replication or a combination of both these processes (Okano et al., 2007; Oppenheimer and Volkman, 1997; Wu et al., 1999). A mutant virus lacking the alkaline nuclease *an* gene, that is likely involved in recombination, is incapable of producing full-size progeny genomes (Okano et al., 2007). However, a role of recombination in baculovirus replication is still far from understood.

In this report we describe short-lived DNA replicative intermediates in both uninfected and AcMNPV infected *Spodoptera frugiperda* cells. Due to a 5' to 3' polymerization polarity of DNA polymerases, the lagging-strand in the replication fork is synthesized discontinuously with generation of Okazaki fragments. *Theta* DNA replication in the nucleus of eukaryotic cells is accompanied by the synthesis of Okazaki fragments of about 200 nt that roughly correspond to a nucleosome repeat. Short-lived DNA replication intermediates of this size were found in uninfected *S. frugiperda* cell. Larger Okazaki fragments, 1000–2000 nt, are synthesized during *theta* replication of bacterial chromosomes or during the rolling circle replication of bacteriophages (Kornberg and Baker, 1992). In order to understand DNA replication in baculovirus infected cells, we analyzed the short-lived replicative intermediates in permeabilized infected cells. Heterogeneous fragments up to 5000 nt with average size of 1000 to 2000 nt were identified as intermediates in virus replication. These likely represent Okazaki fragments. However, they could also be comprised of fragments synthesized during recombination-dependent replication.

MATERIALS AND METHODS

Cells

Spodoptera frugiperda 9 (Sf9) cells were cultured in Sf900II serum-free media (Invitrogen), penicillin G (50 units/ml), streptomycin (50 µg/ml, Whittaker Bioproducts), and fungizone (amphotericin B, 375 ng/ml, Flow Laboratories) as previously described (Harwood et al., 1998). Sf9 cells (20 ml, 1.5×10^6 cells/ml) were infected with AcMNPV at a MOI of about 5 and collected 16 hpi.

Labeling of nascent viral DNA in permeabilized cells

The method for labeling baculovirus replicative intermediates was based on a procedure described for Okazaki fragments in mammalian cells (Burhans et al., 1990). The cells were collected by centrifugation, washed once with fresh medium at room temperature (RT), suspended in ice-cold medium, and portions were transferred into 1.5 ml microcentrifuge tubes. After centrifugation at $800 \times g$ for 3 min, the supernatants were removed by aspiration, leaving ~20 µl of packed cells per tube. Each sample was resuspended in 25 µl of ice-cold 2 × reaction mixture containing: 60 mM HEPES (adjusted to pH 7.8 with KOH); 0.2 mM each of dGTP, dCTP, dTTP; 0.4 mM each of GTP, CTP, UTP; 7 mM ATP; 18 mM MgCl₂; 0.2 mg/ml BSA; 28% glycerol; 2 mM DTT, and then 2.5 µl of [α -³²P]dATP (10 mCi/ml, 3,000 Ci/mmol) was added to each tube. 10% Nonidet P-40 (NP-40) was then added to the tubes to a final concentration of 0.5%. Suspensions were mixed and incubated on ice for 2 min. Reactions were initiated by transferring the tubes to a 28°C water bath. After incubation for the indicated times, the reactions were stopped by adding 200 µl of termination buffer (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.4 M NaCl, 0.6% SDS, and 0.4 mg/ml proteinase K). For chase experiments, dATP was added after the labeling period to a final concentration of 0.4 to 1 mM, and the incubation continued for 9 to 15 min, and then stopped as described above. The lysates were incubated for 1.5 h at 37°C. Saturated NaCl (83 µl) was added to each tube, and they were gently mixed for 20 min at RT. DNA was precipitated by 2 volumes of absolute ethanol at RT and collected by centrifugation at $28,000 \times g$ for 15 min at 4°C. The DNA pellet was rinsed four times with 1-ml portions of cold 70% ethanol, air dried, and solubilized in 100 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA samples (8 µl) were digested with 4 units of Mung bean nuclease (New England BioLabs) at 37°C in reaction buffer supplied by the manufacturer in a final volume of 40 µl. Portions of 7.5 µl were taken from the reaction at 7, 15, and 30 min, mixed with 2.5 µl of 4 × loading buffer supplemented with 270 mM Tris-HCl (pH 8.0) and 33 mM EDTA, and analyzed by electrophoresis in a neutral 0.6% agarose gel.

Labeling of nascent viral DNA in isolated nuclei

The cells (20 ml) were collected by centrifugation, washed once with phosphate buffered saline at RT, and then suspended in 5.0 ml of ice-cold lysis buffer (LB): 0.25 M sucrose, 30 mM HEPES (pH 7.8), 50 mM KCl, 2 mM MgCl₂, 0.5 mM DTT, and 0.5% NP-40. The cells were disrupted in a glass Dounce homogenizer by 5 strokes of a tight-fitting pestle, and nuclei were pelleted at $1,000 \times g$ for 10 min. The pellet was suspended in 1.8 ml of LB containing 0.1% NP-40, and nuclei were pelleted at $1,500 \times g$ for 5 min. Finally, the nuclei were suspended in 160 µl of LB containing 0.1% NP-40 and stored on ice. Reaction was initiated by adding 25 µl of ice-cold nuclear suspension to 25 µl of 2 × reaction mixture (200 µCi/ml [α -³²P]dATP (3,000 Ci/mmol); 30 mM HEPES (pH 7.8); 0.2 mM each of dGTP, dCTP, dTTP; 0.4 mM each of GTP, CTP, UTP; 6 mM ATP; 8 mM MgCl₂; 50 mM KCl; 0.2 mg/ml BSA; 20% glycerol, and 2 mM DTT) that was pre-incubated in a 28°C water bath. After incubation for 1 min at 28°C, the reactions were stopped by adding 200 µl of the termination buffer. DNA was purified as described above.

Agarose gel electrophoresis

Electrophoresis in agarose gels was performed as described (Sambrook et al., 1989) with TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and the alkaline buffer (50 mM NaOH, 1 mM EDTA) used respectively for neutral and alkaline gels. The DNA samples for alkaline electrophoresis were denatured at 100°C for 3 min and were loaded onto the gel after adding 6 × loading buffer (300 mM NaOH, 6 mM EDTA, 18% Ficoll 400, and tracking dyes). Electrophoresis was carried out at 2 V/cm at RT for 6 h. The gel containing ³²P-labeled DNA was incubated for 30 min in 7% TCA, dried on Whatman 3MM paper, exposed to X-ray film, and analyzed by a PhosphorImager (Molecular Dynamics). A DNA molecular mass standard was visualized by staining with ethidium bromide after neutralization of the gel.

Southern blotting and hybridization of nascent viral DNA

AcMNPV DNA was purified from budded viruses as described (O'Reilly et al., 1992) and digested with restriction enzymes *Hind*III and *Pst*I. The DNA fragments were separated by electrophoresis in a 0.6% agarose gel and blotted onto a Hybond-N+ membrane (Amersham, Biosciences). The pulse-labeled viral DNA was purified from permeabilized AcMNPV-infected cells that were incubated for 1 min in a reaction mixture of 100 µl containing 50 µCi [α -³²P]dATP as described above. The labeled DNA was fractionated by electrophoresis in an alkaline 1.5% agarose gel, and three sections containing fragments of 100 to 300 nt, 300 to 700 nt, and 700 to 1,000 nt were cut from the gel. The DNA fragments were electroeluted and concentrated as described (Sambrook et al., 1989). RNA was digested by incubation in 0.2 N NaOH for 18 h at 37°C. The sample was neutralized, extracted with phenol/chloroform (1:1), precipitated with ethanol, and resuspended in TE. Hybridization was performed at high-stringency conditions.

RESULTS

For identification of short-lived intermediates in baculovirus replication, we initially used agarose gel electrophoresis under denaturing condition to examine viral DNA that had been pulse-labeled with [α -³²P]dATP in AcMNPV-infected Sf9 cells. The infected cells were collected at 16 hpi, i.e. at a stage prior the budded virus release when synthesis of host nuclear DNA is blocked and replication of the virus genome proceeds efficiently (Rosinski et al., 2002). To allow access of the radioactive precursor to the viral replication machinery, the AcMNPV-infected cells were permeabilized by treatment with the non-ionic detergent Nonidet P-40. This method has been used previously for detection of Okazaki fragments in various eukaryotic systems (Burhans et al., 1990). When it was applied to the control mock-infected Sf9 cells, a picture typical for replication of the nuclear DNA was revealed (Fig. 1A). Nascent DNA labeled by [α -³²P]dATP during a 1-min *pulse* was present in alkaline agarose by two distinct fractions (Fig. 1A, lane 1). A large portion of labeled DNA was short fragments of 80 up to approximately 200 nucleotides (nt), whereas the rest were long fragments in a range of several thousand nt. The size of the short labeled fragments corresponds to Okazaki fragments synthesized in the lagging strands of nuclear DNA (Kornberg and Baker, 1992). According to a generally accepted model of the replication fork during eukaryotic cell replication, fifty percent of the DNA precursors should be incorporated into the lagging strands and transiently present in Okazaki fragments during replication. After a *chase*, when the reaction mixture, after 1-min incubation with [α -³²P]dATP, was diluted with an excess of unlabeled dATP and incubated for additional 15 min, all labeled DNA was converted into long fragments (Fig. 1A, lane 2). This *pulse-chase* experiment demonstrated efficient maturation of the Okazaki fragments into a high molecular weight nuclear DNA in the permeabilized Sf9 cells. Conversion of the pulse-labeled short fragments into high molecular weight DNA, although less efficient, was also observed in a pulse-chase experiment with nuclei isolated from Sf9 cells (Fig. 1B, lanes 1–2).

Both experimental assays described above, with permeabilized Sf9 cells and with isolated nuclei, were used for analysis of short-lived replicative intermediates of AcMNPV. The incorporation of label from [α - 32 P]dATP into DNA of the virus-infected cells was several times higher than that of mock-infected cells. The viral DNA labeled during a 1-min pulse in permeabilized AcMNPV-infected cells migrated in alkaline agarose as heterogeneous fragments predominantly larger than 200 nt in length (Fig. 1C, lane 1). A similar pattern of nascent viral DNA in alkaline agarose was obtained in experiments when the permeabilized infected cells were labeled with [α - 32 P]dATP by pulses less than 1 min (data not shown). No distinct fraction of labeled Okazaki fragments similar in size to those observed in uninfected cells was present. The subsequent incubation for 10 min in the presence of an excess of unlabeled dATP (*chase*) shifted the distribution of the labeled DNA to higher molecular masses (Fig. 1C, lane 2). This shift presumably reflects elongation of the shorter viral replicative intermediates. However, a large portion of radioactivity remained in fragments in a range from 1 to 3 kb after the chase. Pulse-chase experiment with nuclei isolated from AcMNPV-infected cells (Fig. 1D) showed that elongation of viral DNA in isolated nuclei proceeds less efficiently than in permeabilized cells. Moreover, noticeable fragmentation of viral DNA was observed during prolonged incubation of the nuclei isolated from infected cells (data not shown), and this system was not used in further experiments. Overall, the experiments described above showed that infection with AcMNPV changes the mechanism of DNA synthesis in nucleus and results in viral short-lived replicative intermediates significantly larger than the Okazaki fragments of about 100 to 200 nt observed in uninfected cells.

Electrophoresis of the pulse-labeled DNA in alkaline agarose gels (Fig. 1) indicated that viral replicative intermediates might exceed the size of the eukaryotic nuclear Okazaki fragments but this analysis did not provide quantitative estimates. Therefore, a new method was developed to isolate short-lived fragments of viral DNA and to improve the resolution of the fragments in the range of 0.5 to 10 kb. This involved digestion of the single-stranded (ss) regions that are present at both ends of Okazaki fragments by Mung bean nuclease (MBN) treatment of nascent viral DNA and then fractionation by electrophoresis in a neutral 0.6% agarose gel. In contrast to the alkaline electrophoresis shown in Fig. 1 which examines ssDNA produced by boiling and alkali treatment, the MBN protocol does not employ these harsh conditions and resolves double-stranded DNA fragments. In addition, RNA primers would not be removed by this protocol. The 0.6% agarose gel resolves well dsDNA fragments in a range of 0.5 to 10 kb. Fragments larger than 50 kb, if they enter the gel, form an unresolved band close to the position of phage λ DNA (48.5 kb). Small fragments form a band of a high mobility. Before treatment with MBN, labeled viral DNA had a large molecular mass and probably a branched structure and did not enter a 0.6% agarose (Fig. 2A). Hydrolysis of pulse-labeled viral DNA by MBN released heterogeneous fragments in a range predominantly from 0.2 to 5 kb with an average size of 1 to 2 kb (Fig. 2B, lanes 1–3; Fig. 2C, line 2). A portion of high molecular weight labeled fragments migrated near the phage λ DNA marker. Although MBN partially hydrolyzed nascent viral DNA, a general pattern of radioactive fragments in the agarose gels remained the same during 30-min of treatment with the enzyme. Incubation of the pulse-labeled permeabilized cells for additional 9 min after dilution of [α - 32 P]dATP with nonradioactive dATP (*chase*) caused conversion of most radioactive fragments into DNA of a larger molecular mass that was relatively resistant to MBN and migrated near the phage λ DNA marker (Fig. 2B, lanes 4–6). This result indicated that efficient release of heterogeneous labeled fragments from the pulse-labeled DNA seen in lanes 1–3 (Fig. 2B) was due to the presence of ssDNA regions exposed transiently during synthesis of viral DNA. Control *pulse-chase* experiments with permeabilized mock-infected cells confirmed that MBN is actually capable of recognizing and hydrolyzing ssDNA regions surrounding normal Okazaki fragments (Fig. 2B, lanes 7–12). About one half of the label incorporated into the host nuclear DNA during a 90-sec pulse was released by MBN treatment as fragments of approximately 200 nt (Fig. 2B, lanes 7–9, Fig. 2C, line 8). After a 9-min chase, these radioactive fragments were converted into large fragments

that migrated near the phage λ marker (Fig. 2B, lanes 10–12). This conversion presumably reflected the maturation of the Okazaki fragments by their ligation to larger fragments in the lagging strands.

In order to characterize the heterogeneous fragments released by MBN from nascent viral DNA, it was important to ensure that the treatment with MBN specifically and efficiently liberates the Okazaki fragments from replicative forks in nuclear DNA. To confirm the specificity of the MBN action, we used two-dimensional agarose gel electrophoresis (Fig. 3). DNA pulse-labeled in permeabilized uninfected cells was treated by MBN and then fractionated in a neutral 1% agarose gel in the first direction. Two distinct fractions comprising the small and large labeled fragments were obtained. This pattern was typical for nascent host DNA and was similar to those shown in Fig. 2B, lanes 7–9. After electrophoresis in the first direction at pH 8.0, DNA was denatured by incubation of the gel in alkali, and electrophoresis was continued in the second direction at alkaline conditions. The resulting distribution of radioactive fragments indicated that practically all the Okazaki-type labeled fragments were released from the native nuclear DNA by the MBN treatment. Therefore MBN treatment provides a method useful for the analysis of newly synthesized DNA.

At least two different initiation events may accompany replication of viral DNA in infected cells. The short-lived intermediates such as Okazaki fragments should be initiated *de novo* during lagging strand synthesis in a replication fork. In addition, replication of daughter strands should be initiated *de novo* at a single or a few unique sites (*origins of replication*) in the viral genome. Because both the mechanism of baculovirus replication in infected cells and the nature of short labeled fragments liberated by MBN from pulse-labeled viral DNA remain unknown, we analyzed the distribution of short fragments of the nascent viral DNA on the virus genome by Southern blotting. AcMNPV DNA isolated from the virus-infected cells was treated by the *HindIII* or *PstI* endonucleases, fractionated in a neutral agarose gel, and blotted onto a membrane. To obtain the radioactive intermediates for hybridization, nascent viral DNA was extracted from the pulse-labeled permeabilized infected cells and fractionated by electrophoresis in an alkaline agarose as shown in Fig. 1C, lane 1. Three fractions of fragments in the size ranges from 100 to 300, 300 to 700, and 700 to 1000 nt, were cut from the gel and separately hybridized under conditions of high-stringency to the restriction fragments of AcMNPV DNA. For all three size ranges, the distribution of the labeled fragments in the virus genome appeared to be random showing hybridization to fragments representative of the complete genome. The hybridization pattern for the nascent fragments 300 to 700 nt is shown in Fig. 4. The apparently random distribution of the short-lived replicative intermediates in the virus genome is consistent with priming of DNA synthesis at locations throughout the genome.

DISCUSSION

This report describes for the first time the short-lived replicative intermediates produced during baculovirus DNA replication. Although, the size of the baculovirus genome is similar to that of an average cellular nuclear DNA domain containing one replicon (Gasser and Laemmli, 1986) and host histones appear to be involved in viral chromatin structure (Wilson and Miller, 1986), based on the size of the short-lived replication intermediates, the replication of baculovirus DNA proceeds by a mechanism markedly different from the replication of host nuclear DNA, or the DNA of small viruses such as the *Papovaviridae* (Anderson and DePamphilis, 1979). Okazaki fragments of a standard size typical for eukaryotes (~200 nt) were not seen as intermediates in DNA replication of baculoviruses (Fig. 1). In order to isolate short-lived replicative intermediates of AcMNPV, we developed a new method based on the assumption that before ligation to the lagging strand, the Okazaki fragments should be transiently surrounded at the 3'- and 5'-ends by single-stranded (ss) regions that should be susceptible to nucleases specific for ssDNA. Therefore, we used Mung bean nuclease (MBN)

that endonucleolytically hydrolyzes ssDNA and ssRNA (Kowalski et al., 1976) to characterize these fragments. Subsequent electrophoresis of the DNA digests in neutral 0.6% agarose gels allowed fractionation of DNA fragments of a broad size range under mild conditions. This method proved to be efficient for the isolation of Okazaki fragments of nuclear DNA from uninfected Sf9 cells (Fig. 3). In the case of AcMNPV-infected cells, this approach resulted in the identification of transient replicative intermediates that ranged in size up to approximately 5 kb with an average size of about 1 to 2 kb (Fig. 2C). Hybridization analysis indicated that these fragments were distributed randomly on the genome (Fig. 4). Most of these fragments were converted into fragments larger than 50 kb during chase incubation indicating that they actually represent intermediates in DNA synthesis. The size of the replicative intermediates of baculoviruses appeared to be similar to the Okazaki fragments of prokaryotes and bacteriophages (Kornberg and Baker, 1992) in which fragments substantially larger than 200 bp have been identified. Large Okazaki fragments were also synthesized in a model system of herpesvirus replication. The replisome of HSV-1 reconstituted *in vitro* carries out the rolling circle replication on circular DNA and produces leading strands that exceed 10 kb and Okazaki fragments of 3 kb (Falkenberg et al., 2000). Most of the short-lived intermediates of AcMNPV could be attributed to Okazaki fragments derived from the lagging strands of viral replication forks. Their size indicates that the priming of the lagging strands in viral replication forks occurs at a lower frequency than in host cell DNA and a larger ssDNA region is generated by the viral replication fork before initiation of a new Okazaki fragment. In addition, if baculoviruses also use a recombination-dependent process for replication, ss regions susceptible to nucleases might be exposed in both leading and lagging strands. In this case, some short-lived fragments could be released from intermediates in recombination and may reflect DNA synthesis initiated by the invasion of replication forks. Although, the size of replicative intermediates does not allow discriminating between the *theta*, rolling circle, and recombination-dependent mode of replication, it provides an essential feature of DNA synthesis in infected cells. Experiments with purified replicative proteins of baculoviruses might help to clarify a complex process of baculovirus replication.

Acknowledgments

This research was supported by grants from the Russian Foundation for Basic Research (09-04-00423) to V.S.M. and from the NIH (GM9982536) to G.F.R.

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Fig. 1.

Analysis of *Spodoptera frugiperda* (Sf9) cell and AcMNPV replicative intermediates by alkaline agarose gel electrophoresis. A and C, permeabilized Sf9 cells, mock-infected (A) or AcMNPV-infected (C), were incubated for 1 min in a reaction mixture with [α - 32 P]dATP. DNA was purified from the cells immediately after the pulse (P, lanes 1) or after additional incubation for 15 min in the presence of excess of unlabeled dATP (Pulse plus Chase - P+C, lanes 2). B and D, nuclei isolated from the mock-infected (B) or AcMNPV-infected (D) Sf9 cells were incubated for 1 min in a reaction mixture with [α - 32 P]dATP. DNA was purified immediately after the pulse (P, lanes 1) or after additional incubation for 10 min in the presence of an excess of unlabeled dATP (P+C, lanes 2). The purified DNA was denatured for 3 min at 100°C, and subjected to electrophoresis in an alkaline 1.5% agarose gel. The gels were dried and exposed to X-ray film. Migration of DNA standards (in kb) is shown on the left.

Fig. 2.

Analysis of Sf9 cell and AcMNPV replicative intermediates by treatment with Mung bean nuclease (MBN) followed by electrophoresis in neutral agarose gels. *A*, permeabilized AcMNPV-infected Sf9 cells were incubated for 40 sec in a reaction mixture with [α - 32 P]dATP. DNA was purified from the cells immediately after the pulse (*P*, lane 1) or after additional incubation for 9 min in the presence of excess of unlabeled dATP (*P+C*, lane 2) and was subjected to electrophoresis in a neutral 0.6% agarose gel. *B*, AcMNPV-infected or mock-infected permeabilized Sf9 cells were incubated in a reaction mixture with [α - 32 P]dATP for 50 or 90 sec, respectively. DNA was purified from the cells immediately (*Pulse*) or after additional incubation for 9 min in the presence of excess of unlabeled dATP (*Pulse+Chase*).

The purified DNA was digested with MBN for 7, 15, or 30 min, and subjected to electrophoresis in a neutral 0.6% agarose gel. DNA standards (in kb) and an unresolved band of λ DNA standard are shown on the sides of the gels. *C*, densitometry of *lane 2* (the pulse-labeled DNA in AcMNPV-infected cells, 15-min digestion with MBN) and *lane 8* (the pulse-labeled DNA in mock-infected cells, 15-min digestion with MBN).

Fig. 3.

Two-dimensional electrophoresis of DNA pulse-labeled in mock-infected Sf9 cells and treated with Mung bean nuclease. DNA was purified from permeabilized mock-infected Sf9 cells incubated for 90 sec in a reaction mixture with [α - 32 P]dATP. The labeled DNA was loaded onto two lanes and subjected to electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0) in the first direction. One lane was cut from the gel and exposed to X-ray film (shown above the panel). The remaining section of the gel was incubated for 1 h in several volumes of an alkaline buffer (50 mM NaOH, 1 mM EDTA) and then subjected to electrophoresis in this buffer in the second direction.

Fig. 4. Hybridization of DNA pulse-labeled in AcMNPV-infected cells to restriction fragments of AcMNPV DNA. Unlabeled viral DNA was purified from budded viruses, digested with endonucleases *HindIII* (A) or *PstI* (B), fractionated by electrophoresis in a neutral 0.6% agarose gel, and blotted onto Hybond-N+ membrane. Nascent ³²P-labeled DNA was purified from AcMNPV-infected cells incubated for 1 min in a reaction mixture with [α -³²P]dATP. The labeled DNA was fractionated by electrophoresis in an alkaline 1.5% agarose gel, and fragments of 300 to 700 nt were extracted from the gel. Lanes 2 and 3 show hybridization of the ³²P-labeled DNA probe to the restriction fragments of viral DNA. Lanes 3 contains twice the amount of viral DNA as lane 2. Lanes 1 shows the ethidium bromide staining pattern of

the restriction fragments loaded onto *lanes* 2. DNA standards (in kb) and an unresolved band of λ DNA standard are shown on the left.