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Evidence for rolling circle replication of *Autographa californica* M nucleopolyhedrovirus genomic DNA

Brief Report

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Summary. *Autographa californica* M nucleopolyhedrovirus (AcMNPV) is a large ds DNA virus restricted to larval lepidopteran insect hosts. Using field inversion gel electrophoresis and digestion with a restriction enzyme which cuts the AcMNPV genome once, we detected multiple unit-length genome fragments from replicating viral DNA. Our data suggest that AcMNPV replicates in a head-to-tail manner via rolling circle replication.

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In DNA replication, the formation of the replication complex is typically the rate-limiting step [8]. Thus, it is advantageous for large dsDNA viruses to use a method of replication which only requires the formation of the replication complex once for the generation of multiple copies of their genomes. One method that satisfies this requirement is rolling circle replication in which DNA synthesis proceeds unidirectionally around a circular template and, without stopping, continues beyond the origin of replication. This method of synthesis, used by lambda and herpes simplex virus type 1 [8], results in a linear DNA segment consisting of multiple copies of the genome. Evidence from origin-containing plasmid assays and defective interfering particles of AcMNPV suggests that this virus, with its large 134 kb dsDNA genome, might also use rolling circle replication [6, 7, 9, 10, 11]. We undertook, therefore, a study of genomic AcMNPV DNA to determine the mode(s) of replication used in an infection cycle.

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To study replicative intermediates of AcMNPV, we used a variant of pulsed field gel electrophoresis known as field inversion gel electrophoresis (FIGE) in order to resolve DNA forms up to one megabase in size. Sf-21 cells (1×10^7/10 cm plate) were infected at an MOI of 10 with wild-type AcMNPV (strain E2; [15]). At different hours post infection (hpi), where 0 hpi was defined as time of removal of inoculum after a 1 h adsorption period, cells were rinsed twice with phosphate-buffered saline containing 20 mM EDTA (PBSE) and encapsulated in agarose beads according to [18] with the following modifications: equilibration of low-melt agarose and mineral oil was performed at 37 °C, and the agarose beads were digested with 100 µg/ml proteinase K. Budded virus (BV) from 10 milliliters of tissue culture medium ($1–2 \times 10^8$ PFU/ml) was pelleted through a 25% sucrose cushion and encapsulated in a similar manner. DNA was resolved on FIGE gels with an MJ Research PPI-200 programmable pulse inverter, transferred to Nytran (Schleicher and Schuell), UV-crosslinked, and probed with [^{32}P]-dATP-labeled total viral DNA.

AcMNPV DNA exists as a covalently closed, circular (ccc) genome in virions [16, 17]. Within infected cells, cccDNA is found both in virions which have not yet budded and in occluded virus within the nucleus. To study the production of covalently closed circular (ccc) genomes in infected cells, we first conducted a time course of viral DNA synthesis and processing. In a FIGE gel, the cccDNA from budded virus resolved above 1500 kb (Fig. 1, lane 1). It has been shown previously that large supercoiled circular DNA resolves at a much greater apparent molecular weight than linear DNA [5]. Further, relaxed circular DNA does not enter the gel [2, 12]. Previous extractions of BV DNA have yielded between 20–50% cccDNA with the remainder mostly relaxed circular DNA [16, 17]. Thus, only a fraction of BV DNA can enter FIGE gels (Fig. 1, lane 1).

Parental viral DNA was unresolvable upon infection probably because of active transcription of early genes from the parental genome (Fig. 1, lane 2). CccDNA was detected by 12 hpi (Fig. 1, lane 6), coincident with the onset of BV production [3]. A smear of viral DNA between 200–750 kb was barely detectable in infected cells at 8 hpi but was easily detectable by 12 hpi (Fig. 1, lane 6). A large proportion of DNA remained in the wells of the gel; this DNA is probably a mixture of relaxed circular DNA and DNA that is too large and/or branched to be resolved by FIGE. Such retention of large amounts of DNA is typically observed in experiments characterizing herpesvirus genome replication where much of the retained DNA has been shown to be replicative intermediate DNA [13, 14, 18].

Several lines of evidence suggest that the 200–750 kb smear could represent an intermediate form of replicative viral DNA. First, full length genomes could be resolved from this smear (data not shown) following digestion with *Avr* II, an enzyme that cuts the AcMNPV genome only once (see below). Second, this smear was detectable by 8 hpi, before cccDNA could be detected. Third, the smear was present throughout the time course of DNA synthesis. Since circular forms of the genome would not be resolved in this region of the gel, the smear

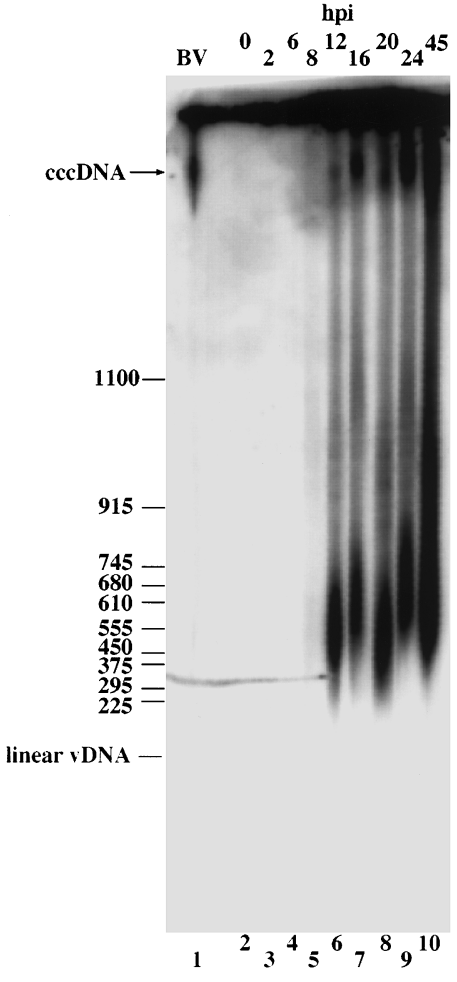

Fig. 1. Time course of AcMNPV DNA synthesis. Cells were embedded in agarose microbeads at different hpi. Undigested DNA was resolved by FIGE (program 9 on PPI-200). cccDNA: covalently closed, circular DNA. Positions of yeast chromosome markers (New England Biolabs) and the *Avr* II-digested purified viral DNA marker are shown in kilobases. The image was captured on a Molecular Dynamics PhosphorImager using ImageQuant 3.3, transferred to a Macintosh 6100/60 computer and lettered using Canvas 3.5.3

must have consisted of linear DNA greater than one genome in length. This smear probably represents a legitimate form of replicative intermediate which is processed into monomeric genomes and packaged. It is possible, however, that such a smear might arise through shearing of rolling circle concatemers from the parental genome during processing of infected cells (and thus would be an artifact). Regardless, this type of smear has not been observed in studies of herpesvirus or lambda virus genome replication. The intense smear at 45 hpi (Fig. 1, lane 10) may be the result of random degradation of viral DNA; DNAase activity has been detected in infected cells late in infection [4].

One way of studying the structure of replicative intermediate DNA is to conduct partial digestions of this DNA. Partial digestions are most effective for visualizing concatemeric DNA fragments, indicative of rolling circle replication, when there is a single, unique restriction site within the genome being studied. Our first step toward determining whether AcMNPV uses rolling circle replication, therefore, was to search the AcMNPV genome for single, unique restriction sites. Computer analysis of the recently sequenced 134 kb genome of

AcMNPV strain C6 [1] revealed a unique *Avr* II restriction site located at 8.8 map units and within the *egt* gene. We confirmed that a unique *Avr* II site existed in the E2 strain at the same location (data not shown).

To determine whether the DNA retained in wells was replicative intermediate DNA, we performed partial digests on this DNA after having first separated it from those species of DNA that freely entered the gel as shown in Fig. 1. Specifically, we harvested approximately 3×10^6 infected cells at 10 and 20 hpi, embedded them in agarose beads and subjected them to FIGE as in Fig. 1, but then recovered the agarose beads from the wells. These beads were subsequently melted in restriction enzyme buffer (total volume of 200 µl) at 68°C, and a sample was taken before enzyme (2 U of *Avr* II) was added (Fig. 2, lane 2). Aliquots (20 µl) were removed at various times during digestion, and the digestion was stopped by the addition of 5 µl 0.5 M ethylenediaminetetraacetate. The sample was immediately loaded onto a gel. After all samples

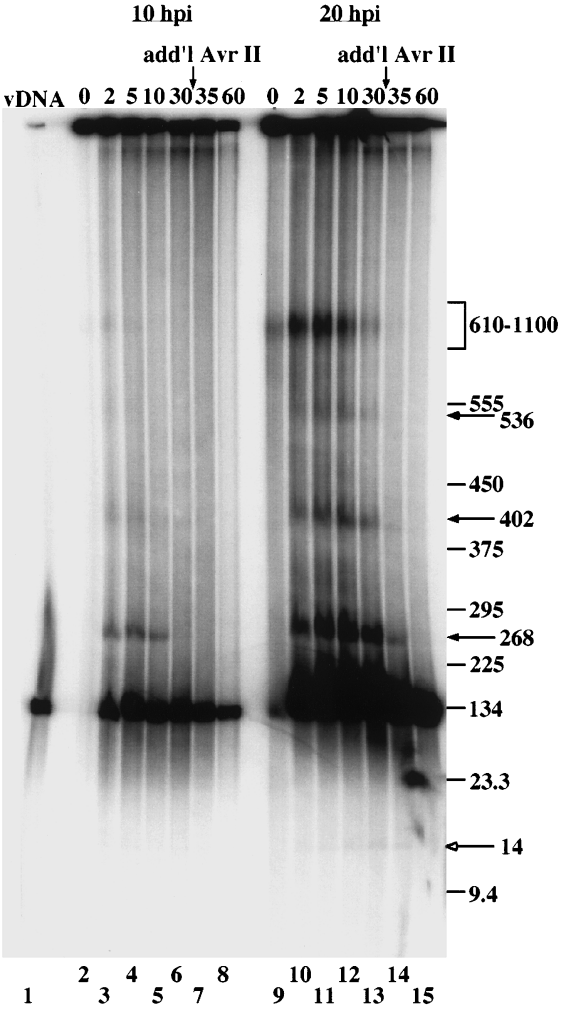

Fig. 2. Partial digestion of viral replicative intermediate DNA from AcMNPV-infected cells. *Avr* II digestion is shown in minutes. Total DNA was resolved by FIGE (program 5 on PPI-200). *2–8* 10 hpi replicative intermediate DNA. *9–15* 20 hpi replicative intermediate DNA. vDNA: purified viral DNA digested with *Avr* II. Closed arrows indicate multimers of the viral genome. The open arrow indicates a subgenomic fragment. Positions of yeast chromosome markers (New England Biolabs), lambda/*Hind* III markers and the *Avr* II-digested purified viral DNA are indicated in kilobases. The image was captured on a Molecular Dynamics PhosphorImager using ImageQuant 3.3, transferred to a Macintosh 6100/60 computer and lettered using Canvas 3.5.3

were collected, the gel was subjected to FIGE, blotted and probed with total viral DNA.

Very little cccDNA or linear DNA was detected in the undigested lane indicating that the initial separation procedure had been effective (Fig. 2, lane 2). Within 2 min of digestion with *Avr* II, however, three bands with molecular weights of 134 kb, 268 kb, and 402 kb were detected from the 10 hpi replicative intermediate DNA. The molecular weights of the bands were consistent with DNA fragments containing 1, 2 and 3 unit lengths of linearized genomic DNA (Fig. 2, lane 3). Consistent with this interpretation, the 402 and 268 kb fragments became less intense with continued digestion. After 30 min of digestion, only the genome-sized fragment and residual replicative intermediate DNA that failed to enter the gel were detected (Fig. 2, lane 6).

When replicative intermediate DNA from cells harvested at 20 hpi was digested with *Avr* II, bands of 134, 268, 402 and 536 kb, were detected after a 2 min digestion period (Fig. 2, lane 10). As in the 10 hpi sample, the molecular weights of these bands were consistent with monomers, dimers, trimers and tetramers of viral genomes, respectively. A relatively intense band also was detected higher on the gel where resolution was much lower (Fig. 2, lanes 10–12). This band probably represented multiple high molecular weight viral DNA concatemers. As digestion progressed, the high molecular weight band, the 536 kb, 402 kb and 268 kb bands sequentially disappeared but the 134 kb band remained (Fig. 2, lanes 12–15), suggesting not only that these bands resolved into monomer-sized fragments but that concatemers of viral DNA were a component of AcMNPV replicative intermediate DNA. We also detected a faint 14 kb band in these partial digests (Fig. 2, lanes 10–15). One end of this fragment was identified as the *Avr* II site, while the other end was mapped to a region between the pe38 and ie2 genes. This region of DNA (between the pe38 and ie2 genes) had no origin-replicating ability in a plasmid-based assay [(D.I.O., unpubl. data) [9]].

Further treatment of this DNA with 2 U more of *Avr* II and an additional 30 min of digestion yielded no additional viral DNA which could be resolved by the gel. Further, a substantial amount of viral DNA remained in the wells. It is very likely that this DNA may have had some sort of branched or replicating theta structure as partially replicated circular genomes held together by unreplicated regions would not have migrated into the gel (Fig. 2, lanes 8 and 15). Lambda bacteriophage, for example, initially undergoes several rounds of theta replication in which a single copy is made from a template genome before switching to rolling circle replication [8, 14]. Theta structures would not be linearized by digestion at a single site and therefore would not be expected to enter the gel. Herpes simplex virus type 1, on the other hand, appears to form a branched network consisting of multiple replication forks during rolling circle replication. Analysis of HSV-1 replicative intermediates following complete digestion with an enzyme that cuts only once per genome has revealed that a considerable amount of replicative intermediate DNA is retained in the wells while DNA concatemers no larger than two genomes in length have been

resolved [13, 14]. Thus, there is a precedent for unresolvable replicative intermediate DNA structures of large dsDNA viruses being retained in wells following complete digestion and electrophoresis.

We detected similar viral DNA banding patterns on FIGE gels using hemocytes harvested from AcMNPV infected *Heliothis virescens* larvae as from infected Sf-21 cells, suggesting that the genomic replication patterns described are representative of viral DNA replication in vivo (data not shown). Both sets of observations are consistent with a rolling circle mechanism of replication. Further, all detected bands (except for the very faint band at 14 kb) were unit-length, which is suggestive of head-to-tail replication. Head-head or tail-tail replication forms would have different restriction patterns than the patterns shown here. For example, if the 14 kb band were indicative of head-head and/or tail-tail replication, then a corresponding 254 kb fragment, with an intensity of approximately twenty times that of the 14 kb band should be evident in Fig 2, lane 15, and it is not. It is possible that the formation of concatemers occurred by recombination between individual genomes rather than by rolling circle replication. Although we cannot rule out this possibility at present, there is evidence that recombination does not occur between replicating baculovirus origin-containing plasmids because they are not resolvable without restriction enzyme digestion [11], and one would expect to be able to resolve, without digestion, a ladder of circular DNAs consisting of monomers, dimers and larger forms if circular plasmids had recombined. In summary, our studies provide strong evidence that AcMNPV replicates in a head-to tail manner via rolling circle replication.

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