Identification of seven putative origins of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus DNA replication


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Seven putative origins of DNA replication (oris) were identified and located on the genome of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV), when an improved infection-dependent replication assay was used. A threefold higher yield of amplified plasmid was achieved when an m.o.i. of 1 was used (instead of 25), and another twofold increase was obtained when the interval between transfection and infection was extended from 5 to 24 h. Six of the putative oris were located in hr regions with homologous sequences. This suggests that all hrs in AcMNPV are bifunctional, i.e. have both ori and enhancer activity for transcription. In addition to the six hrs, the HindIII-K fragment of AcMNPV was also identified to carry a putative ori, although this fragment does not contain an hr region. However, the individual role of these seven oris during viral DNA replication, and whether they are all active simultaneously in vivo, is still unclear. The replication of an ori-containing plasmid starts at the same time (6 h post-infection) and proceeds at the same rate as viral DNA replication. A circular topology of ori-containing plasmids was a prerequisite for replication. Linear DNA, with an ori, did not replicate. Therefore, we suggest a theta structure or a rolling-circle as a model for baculovirus DNA replication.

Introduction

The genome of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) is a double-stranded, circular, covalently closed, supercoiled DNA molecule of approximately 130 kbp. The viral DNA consists primarily of unique sequences with the potential to encode more than 75 average-sized proteins (for reviews, see O'Reilly et al., 1992; Kool & Vlak, 1993). In addition, six homologous regions, designated hr1 to hr5 (hr4 contains two distinct hrs, designated hr4a and hr4b), of approximately 500 to 800 bp in length are interspersed at specific sites along the length of the genome (Cochran & Faulkner, 1983; Guarino et al., 1986). These hr regions contain sequence repeats centred around EcoRI sites, separated by 72 to 215 bp. Initial studies demonstrated that the hr regions function as enhancers for transcription, when placed in cis to the promoter of the AcMNPV delayed early gene 39K and trans-activated by the immediate early (IE)-1 gene (Guarino et al., 1986; Guarino & Summers, 1986). These results suggested that the hr enhancer function is dependent on IE-1 trans-activation but subsequent studies with the 35K gene (Nissen & Friesen, 1989) and IE-N gene (Carson et al., 1991) have shown that the hr region can also increase the expression of early genes in the absence of IE-1.

Homologous regions have been identified in four other baculoviruses to date, *Choristoneura fumiferana* MNPV (Arif & Doerfler, 1984; Kuzio & Faulkner, 1984), *Lymnantria dispar* MNPV (McClintock & Dougherty, 1988), *Bombyx mori* MNPV (Maeda & Majima, 1990) and *Orgyia pseudotsugata* MNPV (OpMNPV; Theilmann & Stewart, 1992). However, only for OpMNPV is it known that the identified hr regions can function as enhancer elements (Theilmann & Stewart, 1992).

Repeated sequences have also been described for a number of other eukaryotic DNA viruses. Unlike the baculovirus hrs, repeated sequences of vertebrate viral genomes usually consist of perfect or nearly perfect repeats of similar size. A variety of functions have been ascribed to such repeated sequences: the direct repeats of simian virus 40 and polyoma virus have a dual function, since both serve as enhancers of gene expression and as origins (oris) of DNA replication (Bergsmma et al., 1982; de Villiers et al., 1984); the replication of adenovirus DNA is initiated within the inverted terminal repeats (Tamanoi & Stillman, 1983), whereas in herpesvirus genomes terminal repeats are required for circularization during replication (Kitner & Sugden, 1979).
Recently, defective interfering virus particles (DIPs) have been described which are generated in continuous production of AcMNPV in bioreactors or upon serial, undiluted passage of virus in cell culture (Kool et al., 1991; Wickham et al., 1991; Lee & Krell, 1992). The defective viral genomes lack considerable portions of the standard viral genome, but apparently have retained the cis-acting sequences essential for DNA replication and encapsidation. At least two segments of the AcMNPV genome, containing the hr3 and hr5 regions, were maintained in defective genomes and could possibly serve as an origin of DNA replication (Kool et al., 1993). Hr1, which was also present in the DIPs, was found to be negative in the replication assay used. Pearson et al. (1992) showed that hr2 and hr5 could serve as origins of DNA replication and demonstrated in addition that a part of hr5, containing a single, complete palindrome, was sufficient to promote replication.

In this paper we investigated whether all known hr regions can function as ori in an infection-dependent replication assay, and whether the negative result for hr1 is due to the conditions used in this assay. Therefore, the AcMNPV replication assay was optimized and all six known hr regions and adjacent sequences were tested for replication activity. The results indicated that all six hr regions as well as one additional non-hr sequence can, in principle, serve as origins of DNA replication of AcMNPV. However, it is not known whether the DNA replication of the AcMNPV genome involves the use of all these origins.

Methods

**Cells and virus.** *Spodoptera frugiperda* SF-AE-21 cells (SF; Vaughn et al., 1977) were cultured in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The E2 strain of AcMNPV (Smith & Summers, 1978) was used as wild-type (wt) virus. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers & Smith, 1987).

**Plasmid constructions.** AcMNPV fragments containing hr regions and surrounding sequences were cloned into pBR322, pUC19 or pDH119 (Hoheisel, 1989), in *Escherichia coli* JM101 using standard techniques (Sambrook et al., 1989). DNA isolation, purification in CsCl gradients, digestion with restriction enzymes and agarose gel electrophoresis were carried out using standard procedures (Sambrook et al., 1989).

**Replication assays.** The assay used to test for ori function was based on the observation that, in the presence of helper AcMNPV which provides trans-acting viral functions, plasmids carrying an AcMNPV ori will be amplified in insect cells (Kool et al., 1993; Stow & McMonagle, 1983). Test plasmids were amplified in *E. coli* JM101, which is Damin. The adenine residue within the GATC recognition sequence for DpnI is thus methylated and, hence, sensitive to DpnI digestion because DpnI cleaves only methylated GATC sequences. DNA that is replicated in insect cells is not methylated at GATC sequences and therefore is resistant to DpnI digestion. The plasmids were purified in CsCl gradients and 1 μg of plasmid DNA was employed to transfect 2×10⁶ SF cells using lipofectin (Bethesda Research Laboratories) (Groebe et al., 1990).

The replication assay was performed basically as described previously (Kool et al., 1993), but a few modifications were made. Before transfection the TNM-FH medium was removed and the cells were washed with TNM-FH medium without BSA and FCS. For transfection 1 μg plasmid DNA was mixed with 35 μl H2O and 15 μl of lipofectin in 1 ml of this medium and added to the cells. After incubation for 6 h at 27°C, the lipofectin-containing supernatant was removed by washing the cells twice with complete TNM-FH medium. The cells were further incubated in 2 ml of complete TNM-FH medium at 27°C. The next day, or 24 h after transfection, the cells were superinfected with AcMNPV at an m.o.i. of 1 TCID₉₀ per cell. After 1 h of incubation 2 ml of fresh medium was added and the cells were further incubated at 27°C.

**DNA analysis.** The cells were harvested 48 h post-infection (p.i.) with AcMNPV and total DNA was isolated from infected cells as described by Summers & Smith (1987). The DNA was digested with the restriction enzymes HindIII. To distinguish input plasmid DNA from plasmid DNA that has replicated in insect cells, DNA was digested with DpnI. After gel electrophoresis, the DNA was transferred to membrane filters (Hybond-N) by the method of Southern (1975). DNA probes for hybridization were radioactively labelled by nick translation (Rigby et al., 1977).

Results

**Optimization of the replication assay**

As reported previously, two segments of the AcMNPV genome, containing hr3 and hr5, have been identified as harbouring putative oris of DNA replication (Kool et al., 1993). Since all hr regions have homologous sequences, it was logical to assume that they all could serve as oris and in view of their transcription enhancing activity (Guarino et al., 1986) would be bifunctional. However, when the HindIII-F fragment of AcMNPV, containing hr1, was tested for replication activity, no replication of the plasmid was found (Kool et al., 1993). In addition, under the conditions of the replication assay described by Pearson et al. (1992) we were unable to detect replication of our test plasmids. This prompted us to re-evaluate and optimize the assay, before testing all hr regions.

Starting with the assay conditions established and discussed in a previous paper (Kool et al., 1993), different amounts of plasmid were used for transfection, the m.o.i. of helper AcMNPV used for superinfection was varied, and different time intervals between infection and plasmid transfection were tested (Fig. 1). The plasmid used for these experiments was pAc7A (Kool et al., 1993), which contains hr5. Increase of the amount of transfected plasmid to 2 or 4 μg gave no higher yield of replicated plasmid, but resulted in a decrease of replication efficiency (Fig. 1a). Amounts of less than 1 μg were not further tested as the hybridization signal of the input plasmid then became too weak. Hence, for all the following experiments 1 μg of plasmid was used as the
standard amount for transfection. The Southern hybridizations with pUC19 as probe also gave a non-specific signal of about 2.4 kbp with viral DNA, but this signal could easily be distinguished from the signals caused by hybridization to the plasmid with a size of 3.7 kbp (Fig. 1c). When transfections were preceded by AcMNPV infection with an m.o.i. of 25, no plasmid replication could be detected (Kool et al., 1993). This result was also obtained when an m.o.i. of 10 was used (Fig. 1b). Surprisingly, at an m.o.i. of 1, replication did occur, although at a low level. Much higher levels of replication were obtained when the infection was carried out after the transfection, with the highest level at an m.o.i. of 1 (Fig. 1b). No further improvement of replication level was obtained when infections were carried out 5 h after transfection with an m.o.i. of 1, 0.5 or 0.1 (Fig. 1c). On the other hand, when infection was performed 24 h after transfection, this resulted in higher levels of replication, especially when an m.o.i. of 1 was used (Fig. 1c). Therefore, for all further experiments cells were transfected with 1 μg of plasmid and, 24 h later, infected with AcMNPV at an m.o.i. of 1.

**Time course of replication**

To gain more insight into the replicative behaviour of a plasmid containing an ori, the amplification of plasmid pAc7A was followed and compared with the replication of the wt viral genome. Ten 35 mm dishes with Sf cells were transfected with 1 μg of pAc7A, and infected with AcMNPV (m.o.i. of 1) 24 h later. The cells were subsequently incubated and harvested after 1, 6, 7, 8, 9, 10, 11, 12, 24 and 48 h.p.i., respectively. Newly replicated plasmid and viral DNA was analysed by digestion with HindIII with and without DpnI, and monitored by hybridization using the SstI-G fragment cloned in pUC19 as a probe (Fig. 2). The choice of SstI-G was based on its length, which closely corresponds with that of pUC19, and therefore will give similar hybridization signals for both the viral DNA and plasmid DNA. Progeny viral DNA and newly amplified plasmid DNA, i.e. DpnI-insensitive DNA, both became detectable 6 h p.i. Other experiments confirmed that DpnI-insensitive plasmid DNA was not detectably produced prior to 6 h p.i. (data not shown). This demonstrates that the replication of an ori-containing plasmid starts at the same time (6 h p.i.) as viral DNA replication begins (Tjia et al., 1979), and also that the rate of DNA synthesis in both processes is roughly comparable (Fig. 2).

**Construction and testing of plasmids containing different AcMNPV hr regions**

After optimization of the replication assay using a plasmid with hr5 as ori, all other hr regions of the AcMNPV genome were tested for their ability to initiate

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Fig. 1. Optimization of the DNA replication assay of AcMNPV ori-containing plasmids in insect cells. (a) Sf cells were transfected with 1, 2 or 4 μg plasmid DNA, and infected with AcMNPV (m.o.i. of 10) 5 h post-transfection. (b) Sf21 cells were transfected with 1 μg plasmid DNA and infected with AcMNPV 3 h prior to transfection or 5 h after transfection. (c) Sf21 cells were transfected with 1 μg plasmid DNA and infected with AcMNPV 5 h after transfection or 24 h after transfection. For all transfections cellular DNA was isolated 48 h p.i. and digested with HindIII with (+) or without (-) DpnI. Southern blot hybridization was carried out using 32P-labelled pUC19 as a probe.
DNA replication. The AcMNPV fragments used for cloning the \( hr \) regions and surrounding sequences are schematically drawn in Fig. 3. The \( hr1 \) region was cloned as \textit{HindIII-F} fragment in pBR322 (pAcHF). A \textit{PstI–SstII} subclone of this plasmid (pAc3A) was used to make three other constructs containing the left-hand (pAc3AL) and the right-hand (pAc3AR) border sequence of \( hr1 \), and the sequence of \( hr1 \) alone (pAcZ1) (Fig. 3a). For testing the \( hr2 \) region the \textit{PstI-J} fragment (pAcPJ) and \textit{HindIII-L} fragment (pAcHL) of AcMNPV were used to construct three subclones containing, respectively, the left-hand border of \( hr2 \) (pAcHE), and the sequence of \( hr2 \) alone in a longer (pAcSH) and shorter (pAcNH) version (Fig. 3b). For testing the \( hr3 \) region constructs pAc6AL and pAc6A were used (Kool \textit{et al.}, 1993) to generate four different subclones, pAcSL, pAcSE, pAcMH and pAcMS (Fig. 3c). For analysis of the \( hr4 \) region, which includes both \( hr4a \) and \( hr4b \), constructs pAcKD, pAcKS, pAcEQ, pAcEL, pAcNX, pAcKB and pAcEES were made (Fig. 3d). For testing region \( hr5 \), seven different constructs, pAc7A, pAcHK, pAcES, pAcHQ, pAcEP, pAcEX and pAcHQD, were used (Fig. 3e).

All 30 constructs obtained, containing either one of the six different \( hr \) regions and/or their surrounding sequences, were tested for their replication ability using the optimized assay conditions. As negative controls, the plasmids pUC19 and pJDH119 were used. Only for the regions \( hr1 \), \( hr2 \) and \( hr4 \) are the experimental data shown (Fig. 4a to c); the replication ability of all constructs is presented in Fig. 3. All plasmids that contained a \( hr \) region, including those with \( hr1 \) (Fig. 4a), were amplified in the presence of helper AcMNPV; all plasmids but one that did not contain an \( hr \) region were not amplified (Fig. 4).

One exception, however, was plasmid pAcHK, which contains the \textit{HindIII-K} fragment of AcMNPV and which does not contain \( hr \) sequences (Fig. 3). This plasmid was also amplified, although this was not found...
Seven origins of AcMNPV DNA replication

Fig. 4. Replication activity of AcMNPV regions hr1 (a), hr2 (b) and hr4 (c). Transfections were carried out with plasmids containing various AcMNPV fragments as indicated in Fig. 3(a, b and d), except for plasmid EES (Fig. 3d), which is not shown in (c). In addition, the plasmids pUC19 (C1) and pJDH119 (C2) were tested as negative controls (a). Sf cells were transfected with 1 μg plasmid DNA and infected with AcMNPV (m.o.i. of 1) 24 h after transfection. Cellular DNA was isolated 48 h p.i. and digested with HindIII with (+) or without (−) DpnI. Southern hybridization was carried out using 32P-labelled pUC19 as a probe.

in previous experiments (Kool et al., 1993). Inspection of the sequence of the HindIII-K fragment (Friesen & Miller, 1987) revealed no sequence homology with any of the hr regions. This means that either a non-hr sequence can function as ori or that a secondary structure, not the sequence itself, is important and is recognized by the replication machinery. No further attempts were made at this point to locate this origin in the HindIII-K fragment more precisely.

Replication of HindIII-K and HindIII-Q without vector sequences

Because hr regions of AcMNPV have been also reported to possess enhancer activity (Guarino et al., 1986), the possibility that these regions would activate a bacterial ori or other cryptic, ori-like motifs in the bacterial vector sequences cannot be ruled out a priori. To exclude this possibility, fragment HindIII-Q, containing hr5, was tested in the absence of any vector sequences. Also, fragment HindIII-K with no hr region was tested without vector sequences to see whether it could independently replicate. To this end, plasmid pAc7A, which contains both HindIII-K and -Q (Fig. 3e), was digested with HindIII and the fragments HindIII-K and -Q were isolated from agarose gels and circularized using T4 DNA ligase. Transfections were performed for both fragments with 1 μg DNA of (i) intact plasmids pAcHK, pAcHQ and pUC19 (Fig. 5, lane 1) (ii) circularized fragments HindIII-K and -Q (Fig. 5, lanes 2), and (iii) linear fragments HindIII-K and -Q (Fig. 5, lanes 3). Plasmids pAcHK and pAcHQ served as positive controls, whereas plasmid pUC19 served as negative control. Transfections with linear fragments were carried out to
see whether a circular form of the DNA was essential for replication. Cellular DNA was isolated 48 h after infection of the cells and replication of transfected DNA was investigated by incubating EcoRI-digested DNA (HindIII-K and pUC19) or SstI-digested DNA (HindIII-Q) with DpnI. Digestion with HindIII was not appropriate in this case, since it would have been impossible to distinguish between newly amplified HindIII-K or -Q fragment and the corresponding fragments of replicated viral DNA. DNA blots were probed with 32P-labelled pAcES (Fig. 3e), which contained the EcoRI-S fragment and thus is able to detect both the HindIII-K and -Q fragments as well as pUC19 sequences.

The results obtained unequivocally demonstrated that both fragments HindIII-K and -Q were amplified in the absence of any vector sequences, but only when transfected in circularized form (Fig. 5, HK and HQ, lanes 1 and 2), whereas vector sequences alone, i.e. pUC19, did not replicate (Fig. 5, pUC19, lanes 1). The linear HindIII fragments also gave a weak signal at the same position as found for the circularized fragments (Fig. 5, HK and HQ, lanes 3, arrow). This did not mean that the linear fragments were amplified, because digestion of the linear fragments HindIII-K or -Q with EcoRI or SstI, respectively, results in two smaller fragments, only one of which will hybridize with EcoRI-S (Fig. 3e). These smaller fragments, respectively 844 and 1510 bp in size, can be seen in lanes 3 without DpnI digestion (asterisk), and are DpnI-sensitive, because they are not present in lanes 3 with DpnI digestion. Therefore the weak signals in lanes 3, which are DpnI-insensitive, are probably the result of amplification of spontaneously recircularized linear fragments. Digestion of the circularized forms of HindIII-K or -Q with either EcoRI or SstI indeed resulted in only a single fragment with the size of HindIII-K or -Q itself (2971 bp and 2189 bp, respectively) (lanes 2). From the results obtained it is concluded that both HindIII-Q, containing the hr5 region, and HindIII-K, without an hr region, can function as oris in the absence of any vector sequences but only when they are present in circularized form.

Discussion

Using an optimized replication assay at least seven distinct regions of the AcMNPV genome displayed replicating activity. These regions include the six hr regions, originally described by Cochran & Faulkner (1983), and one non-hr region. The results confirm previous observations for hr2, hr3 and hr5 (Pearson et al., 1992; Kool et al., 1993) and indicate that all these regions contain sequences that may serve as oris of AcMNPV DNA replication. The six hr regions contain homologous repetitive sequences, which are also involved in enhancement of transcription (Guarino et al., 1986). This result implies that all hrs in AcMNPV are bifunctional, i.e. have both enhancer and ori activity, as has been reported also for certain sequences in simian virus 40 and polyoma virus (Bergsma et al., 1983; Tyndall et al., 1981). In addition to the six hrs, an additional genomic segment (HindIII-K) was found to contain a putative ori, although this segment did not contain a conventional hr region.

A possible reason why hr1 and the HindIII-K fragment could now also be identified as oris, although these elements were negative in previous experiments (Kool et al., 1993), could be that the assay conditions to promote replication were improved, resulting in a 10-fold higher rate of amplification of ori-containing plasmids. Using an m.o.i. of 1 for infection (instead of 25) another threefold higher yield of amplified plasmid was achieved (Fig. 1b), and another twofold increase was obtained by extending the interval between transfection and infection from 5 to 24 h (Fig. 1c).

Seven putative origins of DNA replication, six of which are located in the hr regions, have now been identified in the AcMNPV genome. The individual role of these oris during viral DNA replication, and whether they are all active simultaneously in vivo, is still unclear. From experiments with DIPs generated by serial passaging, it can be deduced that not all the oris are necessary for replication of the genome. After 40 serial, undiluted passages three small segments of the genome were predominantly found to be retained, harbouring only the hr1, hr3 and hr5 regions (Kool et al., 1993). This observation may suggest that hr2 and hr4 are less important or not essential. Analysis of viral DNA of multiply passaged virus showed that the defective genomes mainly contain a small 2.8 kbp fragment which maps to the HindIII-K fragment (Lee & Krell, 1992). This would mean that hr1, hr3 and hr5 are also not essential and that the ori in the HindIII-K fragment alone is sufficient to support DIP replication. The construction of viable recombinant virus, in which hr5 was deleted, also means that not all hr regions need to be present in AcMNPV for replication (P. Friesen, personal communication).

The occurrence of multiple oris is not unique for baculoviruses, but has also been reported for herpes simplex virus 1 (HSV-1) and Chilo iridescent virus (CIV). The genome of HSV-1 contains three oris, oriS and two copies of oriL (for review, see Fields & Knipe, 1990), which can support replication in an assay similar to that used in this paper (Stow & MacMonagle, 1983). For HSV-1 it has been reported that the presence of any single ori is sufficient for replication (Longnecker & Roizman, 1986; Polvino-Bodnar et al., 1987; Igarashi et al., 1993). In CIV at least six putative oris have been
identified (Handermann et al., 1992). It remains to be seen whether in the case of baculoviruses each of the seven putative oris is necessary for viral replication. When the oris are indeed functionally redundant, the presence of multiple origins in the viral genome may increase the frequency of initiation and thus increase the speed of DNA replication. Subsequent deletion of each of the possible oris may shed light on their relative importance.

The experiments in this paper also demonstrated that a circular topology is a prerequisite for replication of ori-containing plasmids. Linear DNA, even if it contained an ori, was not replicated (Fig. 5). These results are in line with the circular nature of baculovirus DNA and suggest a theta structure or a rolling-circle model for baculovirus replication. The latter model is supported by the finding of defective genomes with many reiterations (concatemers) of a 28 kb segment, mainly mapping in the HindIII-K fragment (Lee & Krell, 1992). However, in a rolling-circle model the dependence on the origins is minimal and occurs at most only once at the initiation of DNA synthesis (Kornberg & Baker, 1992). It is possible that in vivo the oris are not equivalent or that they are not all functional. The relative abundance of HindIII-K in DIPs thus requires further investigation.

The authors wish to thank Magda Usmany for technical assistance and Dr Douwe Zuidema for his continued interest and helpful discussions. This research was supported in part by the Dutch Programme Committee for Agricultural Biotechnology.

References


(Received 22 April 1993; Accepted 20 July 1993)