A putative non-\textit{hr} origin of DNA replication in the \textit{HindIII-K} fragment of \textit{Autographa californica} multiple nucleocapsid nuclear polyhedrosis virus

M. Kool, R. W. Goldbach and J. M. Vlak*

Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

In addition to the seven known homologous regions (\textit{hrs}) of \textit{Autographa californica} multiple nucleocapsid polyhedrosis virus (AcMNPV) the \textit{HindIII-K} fragment was also found to carry a putative \textit{ori}, although this fragment does not contain an \textit{hr}. Deletion analysis showed that this \textit{ori} contains several segments essential for its activity and other 'auxiliary' sequences that enhance the \textit{ori} activity. Sequence analysis identified several structures often found in other viral replication \textit{oris}, such as palindromes and other repeated motifs.

Although most of the auxiliary sequences of this \textit{ori} were found to be deleted in the \textit{Bombyx mori} nucleocapsid polyhedrosis virus genome, the essential part of this \textit{ori}, containing the palindromes and the A/T-rich region, was retained. This and the fact that after prolonged serial passage of AcMNPV large replicating DNA molecules are found in which repeated sequences derived from the \textit{HindIII-K} fragment accumulate are consistent with this region being a putative origin of AcMNPV DNA replication.

Introduction

The \textit{Autographa californica} multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) is the type member of the insect virus family Baculoviridae (Francki et al., 1991). It has a circular, double-stranded DNA genome of approximately 131 kbp and replicates in the nuclei of infected insect cells. Knowledge of the molecular genetics of AcMNPV has greatly increased (for reviews, see Blissard & Rohrmann, 1990; Kool & Vlak, 1993) owing to the potential of this virus as a biological insecticide (Payne, 1988) and as an expression vector of foreign genes (Luckow & Summers, 1988; Miller, 1988).

Despite their widespread use, little is known about the mechanism by which baculoviruses replicate their DNA. Recently, eight cis-acting elements that may play a role in DNA replication have been identified (Pearson et al., 1992; Leisy & Rohrmann, 1993; Kool et al., 1993a, b). The nature and location of some of these cis-acting elements has firstly been inferred from the structure of defective viral genomes, which are generated in the 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). These defective viral genomes lack considerable portions of the standard viral genome, but apparently retain cis-acting elements that are essential for DNA replication and encapsidation. Plasmids containing these cis-acting elements replicate transiently in insect cells infected with AcMNPV and, hence, may contain putative origins of DNA replication (\textit{oris}).

Seven of the eight putative \textit{oris} are found in the homologous regions (\textit{hrs}) of AcMNPV (Kool et al., 1993b; Leisy & Rohrmann, 1993), which are interspersed along the genome (Cockran & Faulkner, 1983) (Fig. 1). Sequence analysis showed that \textit{hrs} contain two to eight 30 bp imperfect palindromes, interspaced by other repeated sequences, and that each palindrome contains a naturally occurring \textit{EcoRI} site at its core (Guarino et al., 1986).

In addition to the seven \textit{hrs}, a putative \textit{ori} was also found in the \textit{HindIII-K} fragment of AcMNPV (Kool et al., 1993b). Sequence analysis of this fragment showed no obvious homology with any of the \textit{hr} regions (Friesen & Miller, 1987; Guarino et al., 1986), indicating that either different sequences can function as \textit{ori} or that a common secondary structure and not the sequence itself, is recognized by the replication machinery. Lee & Krell (1992) reported that defective AcMNPV genomes, generated after extensive serial passage of this virus in insect cell culture, were comprised largely of reiterations of this \textit{HindIII-K} region. This also suggested that this region contained important cis-acting sequences suffi-
cient to initiate viral DNA replication. In this paper we have characterized this non-hr ori in more detail.

**Methods**

Cells and virus. *Spodoptera frugiperda* (Sf-AE-21) cells (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 were cloned into pUC19 and transformed into *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 a putative AcMNPV origin of replication will be amplified in insect cells (Kool et al., 1993a). Test plasmids were amplified in *E. coli* JM101, which is Dam−. The adenine residue within the GATC recognition sequence for DpnI is thus methylated and, hence, sensitive to DpnI digestion as 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 transfected into *S. frugiperda* cells using lipofectin (Bethesda Research Laboratories) (Groebbe et al., 1990).

The replication assay was performed in principle as described previously (Kool et al., 1993a, b) with the following modifications. Approximately 2 h prior to transfection the medium was removed and the cells (2 × 10⁶ cells per 35 mm diameter Petri dish) were washed with Hink’s medium without BSA and FCS. Plasmid DNA (1 μg) was mixed with 35 μl H₂O and 15 μl of lipofectin in 1 ml of Hink’s medium without BSA and FCS and added onto the cells. After incubation for 6 h at 27 °C, the lipofectin-containing supernatant was removed and the cells were treated as described before.

**Results**

When the regions flanking hr5 were tested for replication activity, it was found that the HindIII-K fragment [map unit (m.u.) 84.9 to 87.2] also contained a putative origin of replication (Kool et al., 1993b). To locate the putative non-hr ori in the HindIII-K fragment more precisely, subclones were made and tested in the transient replication assay. Subclones pAcHKA to pAcHKF were made using the internal SalI, EcoRI, EcoRV, XhoI, NsiI and HindIII restriction sites, respectively, of the HindIII-K fragment (Fig. 2a). The complete HindIII-K fragment (pAcHK) and the subclones were tested in a replication assay along with pAcES and pAcH9 [containing the fragments EcoRI-S and HindIII-Q (hr5), respectively] as negative and positive controls. The results in Fig. 2(b) show that the relative efficiency of replication was slightly lower for pAcH9 as compared to pAcHQ, which contains hr5. Subclones pAcHKA to pAcHKL replicated more or less at the same level as pAcH9. Subclone pAcHKE replicated significantly less than HindIII-K, whereas subclone pAcHKF did not replicate at all. This suggested that the ori activity of HindIII-K is located between the HindIII (m.u. 84.9) and XhoI sites (m.u. 85.9) of pAcH9K, and that essential ori sequences are located in the part of pAcHKE that is lacking in pAcH9K (Fig. 2a).

To investigate these essential sequences further, several subclones were made of pAcH9K (Fig. 3a). To construct pAcHJ, the HindIII–HinII fragment in pAcH9K was deleted, and sites were filled up with Klenow enzyme and religated. Plasmid pAcHKK was also derived from pAcH9K by deleting the HindIII–NsiI fragment. To make pAcHKL, pAcHJ was digested with NsiI and SmaI, using the SmaI site in the vector, and sites were filled up with Klenow enzyme and religated. Plasmid pAcHKO was made from pAcH9K by deleting the internal 61 bp of the HinII fragment in pAcH9K and religating the remaining part. Plasmid pAcHKP contains a 158 bp Dral fragment cloned into the HinII site of pUC19. All these subclones were subsequently tested in a replication assay (Fig. 3b). Subclones pAcHKF and
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Fig. 2. (a) Schematic diagram showing the position of fragments EcoRI-S (ES), HindIII-Q (HQ), HindIII-K (HK) and subclones HKA to HKF. A plus (+) or a minus (−) indicates the replication ability of the various fragments in the replication assay. E, EcoRI; m.u., map unit. (b) Replication activity of the plasmids containing HindIII-K (HK), subclones HKA to HKF, EcoRI-S (ES) and HindIII-Q (HQ). *S. frugiperda* cells were transfected with 1 μg plasmid DNA and infected with AcMNPV (m.o.i. = 1) 24 h after transfection. Cellular DNA was isolated 48 h post-infection and digested with HindIII with (+) or without (−) DpnI. Southern hybridization was carried out using 32P-labelled pUC19 as probe.

**Sequence analysis of the HKD fragment**

The sequence of the HindIII–XhoI fragment (pAcHKD) of HindIII-K is part of the C-terminal coding region of

pAcHKP (Fig. 3b) as well as pAcHKK and pAcHKL (not shown) did not replicate. Subclone pAcHKO, which lacked the internal 61 bp HincII fragment, replicated, but significantly less efficiently than the parental pAcHKT clone, indicating that these 61 bp are not essential for ori activity, but contain auxiliary sequences (Fig. 3b). Subclone pAcHKJ, which lacked in addition to the 61 bp HincII fragment the HindIII–HincII fragment also, was still able to replicate, although at a very low level (Fig. 3b). This suggests that pAcHKJ still contained all the essential ori sequences, but the lower replication efficiency of pAcHKT compared to pAcHKO showed that the HindIII–HincII fragment also contained auxiliary sequences.

On the basis of these results five regions can be identified in the HindIII–XhoI fragment in pAcHKD that contribute to the replication ability of this fragment to different extents (Fig. 3a). None of the regions alone is sufficient to allow replication as shown by the negative results with clones pAcHKF, pAcHKK, pAcHKL and pAcHKP. The regions I, II and V contain auxiliary cis-acting sequences, because deletion of any of these regions reduces the replication efficiency. Only regions III and/or IV seem to be essential as deletion of one or both of these regions abolishes replication ability. However, the combination of regions III and IV (clone pAcHKL) or regions II and III (clone pAcHKT) is not sufficient for replication.
Fig. 3. (a) Schematic diagram showing the position of various subclones of HKD. A plus (+) or a minus (−) indicates the replication activity of the various fragments in the replication assay. m.u., map unit. (b) Replication activity of the plasmids containing the fragments as indicated in (a). *S. frugiperda* cells were transfected with 1 μg plasmid DNA and infected with AcMNPV (m.o.i. = 1) 24 h after transfection. Cellular DNA was isolated 48 h post-infection and digested with *EcoRI* and *DpnI*. Southern hybridization was carried out using ³²P-labelled pUC19 as probe.
the p94 gene (Friesen & Miller, 1987). The function of this early transcribed gene is unknown, but it is not essential for virus replication (Friesen & Nissen, 1990). Sequence analysis of the five regions in pAcHKD showed no obvious homology with any of the hr regions, but inspection of essential regions III and IV revealed two overlapping, imperfect palindromes in region III (Figs 4a and 5). Palindromes are also found in the hr regions of AcMNPV and seem to be essential for ori activity (Pearson et al., 1992). It is characteristic of the hr palindromes that they all contain an EcoRI site at their core. Although the palindromes in region III of pAcHKD do not contain an EcoRI site, one (HdK-B) contains an EcoRI-like sequence at its core, differing in only one position. However, there is almost no homology with the hr palindromes. Four repeats of a previously undescribed palindrome motif were identified in hr4b of Bombyx mori NPV (BmNPV) (Fig. 4), but there is no homology with the ones in HindIII-K. A recently identified putative ori in Orgyia pseudotsugata MNPV (OpMNPV) shows no obvious homology with the sequence of HindIII-K or the hr regions in AcMNPV or with other sequences in the OpMNPV genome (Pearson et al., 1993). Two palindromes are present in the essential part of the OpMNPV ori, a 16 bp perfect palindrome and a 61 bp imperfect one, with some homology to each other but with no homology with the palindromes in HindIII-K (Fig. 4).

Other structures, besides palindromes, often found to be associated with replication origins are direct repeats and A/T-rich regions in proximity to palindromes (DePamphilis, 1993). The palindromes in the hr regions are also interspersed by many direct repeats and A/T-rich regions. Inspection of the sequence in HindIII-K around the palindromic sequences reveals that the region upstream of palindrome A and part of palindrome B is indeed A/T-rich. A search for direct repeats in the sequence of pAcHKD resulted in numerous repeated motifs throughout the sequence. Two copies of a 23 bp repeated motif (repeat 1: GGACG/ATGTCGTAAA-AACATGTT) were found at the borders of regions I and II and regions III and IV (Figs 4b and 5). A second 10 bp repeated motif with the sequence GTTC/GGTC AAC (repeat 2) was found at the borders of region II (Figs 4 and 5). Another repeated motif with the consensus sequence GATGATGTCATI_TGTI_7 was found in many copies in the hr regions of AcMNPV. This motif is also conserved in the hr regions of BmNPV (Majima et al., 1993), and is present, though less conserved, in the hr region OpE 5 of OpMNPV (Theilmann & Stewart, 1992) (Fig. 4b). Several copies of this motif with only one or a few mismatches, mainly in the first six nucleotides, are also found in the sequence of pAcHKD and in the sequence of OpMNPV fragment HindIII-N, which contains a putative OpMNPV ori (Pearson et al., 1993) (Fig. 4b). The function of this motif is unknown, but it resembles the -CANNTG- motif recognized by helix-loop-helix group proteins and in particular (Fig. 4b) the binding site for major late transcription factor USF found in the adenovirus major late promoter and in the immunoglobulin M heavy-chain enhancer (μE3) (Gregor et al., 1990).

Discussion

Previous results identified seven putative oris in the hr regions and another putative ori located in the HindIII-K fragment of the AcMNPV genome (Pearson et al., 1992; Leisy & Rohrmann, 1993; Kool et al., 1993a, b). The results in this paper demonstrate that the structure and sequence of this putative ori in HindIII-K is distinctly different from those of the oris in the hr regions. Deletion analysis of the HindIII-K fragment showed that all the sequences involved in the replication ability of this fragment are located between the HindIII (m.u. 84'9) and the XhoI site (m.u. 85'9) (Fig. 3a). Further analysis of this subfragment showed that it can be divided into five regions of which only regions III and/or IV seem to be essential (Fig. 3a, b). The other regions are not essential, but contain auxiliary sequences that stimulate the replication efficiency of this ori. However, plasmids containing regions III and IV failed to replicate (Fig. 3a, pAcHKL), suggesting that at least one of the auxiliary regions is necessary for the ori to replicate. It is also possible that the ori contains multiple partially redundant regions, one of which may be absolutely essential as long as several other regions are present. Sequence analysis of these regions identified several structural features often found in other replication oris of eukaryotic viruses (DePamphilis, 1993). Two imperfect palindromes, one with a EcoRI-like site at its core, were identified within region III, preceded by an A/T-rich region. The sequences of these palindromes do not exhibit homology to previously identified palindromes in other baculovirus replication oris. Furthermore, several other repeated motifs were found in the ori-containing sequence of HindIII-K, some of which show homology with repeats found in other baculovirus replication oris, but their relevance in replication is unclear.

Although the complex structure of the ori in HindIII-K is different from the structure in the hr oris, the replication efficiency of HindIII-K is almost the same as that of HindIII-Q, which contains hr3. Pearson et al. (1992) suggested that the replication efficiency of the hr regions is correlated with the number of palindromes. However, our results with the HindIII-K ori suggest that other sequences are involved as well. Recently, an ori that also has a complex structure has been identified in
OpMNPV, but it has no homology with the hr regions in AcMNPV (Pearson et al., 1993). Complex origins composed of multiple elements have been found in several other viral DNA genomes. The simian cytomegalovirus (SCMV) and the related human cytomegalovirus (HCMV), for instance, contain complex origins spanning 1.3 to 2.6 kbp, respectively (Anders & Punturieri, 1991; Anders et al., 1992).

The question remains whether each of the seven hr regions and the HindIII-K region also function as an ori in vivo. Deletion or absence of hr5 from the AcMNPV or BmNPV genomes, respectively, has no apparent effect on the replication of these viruses (Rodems & Friesen, 1993; Majima et al., 1993). This suggests that the complete set of identifiedoris is not necessary or that maybe none of the identified hr oris are necessary for replication or function as an ori in vivo. Similar results have been found for herpes simplex virus 1 (HSV-1). HSV-1 contains three oris, one of which is essential for replication (Longnecker & Roizman, 1986; Polvino-Bodnar et al., 1987; Igaraschi et al., 1993). It is not known whether the ori in HindIII-K can be deleted from the AcMNPV genome. Friesen & Nissen (1990)

Fig. 4. Comparison of palindrome motifs (a) and other repeated motifs (b) identified in the AcMNPV (Ac) HindIII-K (HdK) region and other putative baculovirus origins of BmNPV (Bm) and OpMNPV (Op). The EcoRI site and the EcoRI-like site in the palindromes of Ac hr5 and Ac HdK B, respectively, are shown in bold. Repeats 3 were also aligned with the binding site for the major late transcription factor (MLTF) found in the adenovirus major late promoter (AdML) and in the immunoglobulin M heavy-chain enhancer (µE3).
reported that the \textit{p94} gene, containing the \textit{ori} in its sequence, is not essential. However, the \textit{p94} gene itself was not deleted; instead the open reading frame of \textit{p94} was interrupted. Attempts are being made to delete the complete \textit{p94} gene from the AcMNPV genome.

Generation of defective genomes by serial, undiluted passage of AcMNPV in \textit{S. frugiperda} cells showed that after 81 passages the majority of the defective genomes had a size of approximately 50 kb but appeared to retain less than 28 kbp of the original AcMNPV genome derived from the \textit{HindIII-K} fragment (Lee & Krell, 1992). Very recently the same authors (Lee & Krell, 1994) investigated these presumably repeated sequences further and cloned and analysed two abundant hypermolar \textit{XhoI} fragments. These two DNA segments collectively covered 2371 bp of the standard AcMNPV DNA with a 1174 bp overlap around the \textit{XhoI} site at m.u. 85.9. Comparison with the data in this report shows that the regions II, III, IV, V and part of region I are present in these 1174 bp, indicating the importance of these regions for carrying essential cis-acting sequences.

The importance of the putative \textit{ori} in the AcMNPV \textit{HindIII-K} fragment \textit{in vivo} is further supported by sequence data of the closely related BmNPV (Kamita \textit{et al.}, 1993). In this virus the region, spanning the \textit{p94} and \textit{p35} genes upstream of \textit{hr5}, contains an almost identical \textit{p35} gene, as compared to the AcMNPV \textit{p35}, and a region homologous to \textit{hr5} of AcMNPV. However, most sequences corresponding to the \textit{p94} gene were missing. Only 151 nucleotides of the \textit{p94} gene, with a high degree (96.1\%) of sequence similarity to the corresponding sequence of AcMNPV \textit{p94}, were found in BmNPV. These 151 nucleotides map exactly with regions II and III, identified in this paper, of the \textit{ori} in AcMNPV \textit{HindIII-K}. The repeats, A/T-rich region and palindromic motifs found in these regions are shown.

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References


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