No single homologous repeat region is essential for DNA replication of the baculovirus *Autographa californica multiple nucleopolyhedrovirus*

Eric B. Carstens and Yuntao Wu

INTRODUCTION

*Autographa californica multiple nucleopolyhedrovirus* is the type species of the genus *Nucleopolyhedrovirus* of the family *Baculoviridae*, a group of rod-shaped, enveloped viruses with large, circular, double-stranded DNA genomes. Baculovirus species members replicate only in invertebrates and, in general, exhibit a very specific host range, infecting only one or a few insect genera. A unique characteristic of almost all baculovirus genomes studied to date is the presence of homologous repeat (hr) regions, first described in the HR3 strain of *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) (Cochran & Faulkner, 1983). AcMNPV hrs comprise eight A/T-rich regions that contain between two and eight highly conserved repeated sequences of about 72 bp. Within this repeat sequence is a 30 bp imperfect palindrome carrying an EcoRI restriction site (except in hr4c). Because of the symmetric location on the genome (Fig. 1a), their repeated sequence, the high A/T content and the palindromic structure, hrs were originally postulated to represent baculovirus origins of replication (Cochran & Faulkner, 1983). Evidence to support the role of hrs in baculovirus DNA replication has come from the ability of hr-carrying bacterial plasmids to replicate in baculovirus-infected cells (Pearson et al., 1992). The presence of a single hr palindrome with an essential EcoRI core site is sufficient to support plasmid replication, although increasing the copy number of palindromes seems to enhance the relative replication efficiency (Leisy et al., 1995; Pearson et al., 1992). Additional evidence to support the role that hrs play in DNA replication has come from the isolation of defective interfering viruses. Serial passage of AcMNPV results in the presence of supermolar fragments in EcoRI digests of defective viral DNA genomes, suggesting the presence of hrs in these defective but replicating genomes (Kool et al., 1991, 1993). However, additional baculovirus regions have been retained in defective genomes that also support plasmid replication in transient assays (Habib & Hasnain, 2000; Kool et al., 1994; Lee & Krell, 1992, 1994; Wu & Carstens, 1996). Early promoter regions can also function as origins of replication, based on experiments where plasmids without hrs but carrying viral DNA sequences including the ie-1 gene promoter support replication in transient assays (Wu & Carstens, 1996).

The hrs also function as enhancers of AcMNPV early transcription when linked in cis to viral promoters. This has been demonstrated for early genes such as 39K (Guarino & Summers, 1986; Leisy et al., 1995), p143 (Lu & Carstens, 1993) and p35 (Nissen & Friesen, 1989; Rodems & Friesen, 1995). Promoter activity can be enhanced more than 1000-fold by the presence of an hr region and the expression of the
major transcription regulator, IE-1 (Guarino et al., 1986; Leisy et al., 1995; Nissen & Friesen, 1989). IE-1 binding has been correlated with its function during viral DNA replication (Leisy & Rohrmann, 2000; Rodems et al., 1997) and different domains have been identified as being involved separately in transcription and replication functions (Kovacs et al., 1992; Leisy & Rohrmann, 2000; Rodems et al., 1997; Slack & Blissard, 1997). Therefore, hrs are important components of baculovirus genomes, both because of their transcription enhancing function and as potential origins of replication.

A major question remains about baculovirus origin(s) of replication: do hrs act as origins of replication in vivo? It has been shown previously that hr5 can be deleted without any major affect on virus replication (Rodems & Friesen, 1993). However, no information is available for the hrs located in other regions of AcMNPV. Here, we investigated whether any of the other hrs is essential for in vivo virus replication.

METHODS

Cells and virus. Spodoptera frugiperda (Sf21) cells and AcMNPV (strain vAcLSXIV) (vAc) (Ooi et al., 1989) were propagated and maintained in TC100 growth medium (Gardiner & Stockdale, 1975) supplemented with 10% fetal bovine serum, as described previously (Lu & Carstens, 1991). In all experiments, the beginning of the adsorption period was taken as 0 h post-infection (p.i.).

Cloning and subcloning of viral DNA fragments. Plasmids pAChr1, pAChr1a, pAChr2, pAChr3, pAChr4a and pAChr4b, each carrying one specific AcMNPV hr region, have been described previously (Wu & Carstens, 1996). The series of hr deletion plasmids pAChr1, pAChr1a, pAChr2, pAChr3, pAChr4a and pAChr4b has also been described (Wu & Carstens, 1996). For the current study, these plasmids were digested with EcoRI to remove the hr sequences and ligated with a 4.0 kb EcoRI fragment carrying the ie-1 promoter driving the Escherichia coli ß-galactosidase (lacZ) gene (isolated by partial digestion of pE1-lacZ with EcoRI) to generate pAChr1-lacZ, pAChr1a-lacZ, pAChr2-lacZ, pAChr3-lacZ, pAChr4a-lacZ and pAChr4b-lacZ. A 4.5 kb HindIII–BamHI fragment from pE1-lacZ was cloned into the BglII–HindIII site of the EcoRI-P region of pAcEcoRI-P (vector pUC8) to generate pAcp10-lacZ. The plasmid pE1-lacZ was a gift from Dr Paul Friesen (University of Wisconsin–Madison, WI, USA). All constructs were confirmed by restriction digestion and sequence analysis.

Construction of hr knockout viruses. To knock out specific hrs, individual plasmids carrying viral regions in which hr1, hr1a, hr2, hr3, hr4a or hr4b were replaced by the lacZ gene were co-transfected with vAc DNA into Sf21 cells as described previously (Wu & Carstens, 1998). Cells were harvested 3 days after co-transfection and budded virus (BV) preparations were screened and purified by four to seven rounds of plaque assays. Recombinant viruses were identified by the production of blue plaques. To generate viruses where two hrs were deleted, DNA derived from vAChr3lacZ was co-transfected with plasmid pAChr3 DNA to remove the lacZ gene from the virus. Recombinants (white plaques) were plaque-purified (vAChr3). DNA from vAChr3 was co-transfected with pAChr2lacZ, and BV from the transfection supernatant was screened by plaque assays for the presence of blue plaques. Recombinant viruses where the hr3 region was deleted and the hr2 region was replaced by the lacZ gene (vAChr3hr2lacZ) were selected and plaque-purified six times to homogeneity. BV preparations were titrated by plaque assay and by TCID50 assay (Liu & Carstens, 1993). Titrations of BV by TCID50 were carried out with 10-fold dilutions to get approximate titres and then in triplicate with 2-fold dilutions to obtain accurate final titres.

Quantitative real-time PCR. Real-time PCR was performed with a Rotor-Gene 3000 cycler (Montreal Biotech) using a ready-to-use ‘hot start’ reaction mix (iQ SYBR Green Supermix; Bio-Rad). The mix contained Taq DNA polymerase and SYBR Green I for real-time detection of double-stranded DNA during the PCR. Reactions of 16 µl including 0.5 mM of each primer were performed for 20–30 cycles. Standard DNA samples, prepared from Sf21 cell DNA using an Easy-DNA kit (Invitrogen), serially diluted to 100, 10 and 1.0 ng and 100 and 10 pg, and from purified AcMNPV BV DNA, serially diluted to 1000, 100, 10, 1.0 and 0.1 pg were used. Sf21 cells were infected in triplicate (m.o.i. of 5) and, at various times after infection, total DNA was prepared using an Easy-DNA kit. The extracted DNA samples were diluted and 3 µl was used for real-time PCR. Primers designed to amplify a unique segment of genomic DNA (264 bp) were derived from the Sf21 hsp90 gene (Landais et al., 2001) (hsp90F2 and hsp90B2; Supplementary Table S1, available in JGV Online). Primers designed to amplify a segment of AcMNPV (369 bp) were derived from sequences upstream of the hr3 region (p95F1 and p95B1; Supplementary Table S1). Following PCR, the x-axis crossing point of each standard sample was plotted against the logarithm of concentration to produce a standard curve for both genomic and viral DNA standards. Genomic equivalents of DNA samples were determined by extrapolation from standard curves. One copy of AcMNPV genomic DNA is 1.36 × 10⁻⁴ pg. The size of the S. frugiperda genome is estimated to be 400 Mb (31 chromosomes, 0.412 pg per cell) (d’Alenc¸on et al., 2004). A melting-curve analysis of each amplified sample was carried out to check the specificity of each reaction. The results of replicates of real-time PCR runs were analysed using a freeware package from the R project for statistical computing (http://www.r-project.org/).

RESULTS

Generation of hr knockout

To address the status of hrs as origins of replication in vivo, we developed an approach to determine whether any of the AcMNPV hrs was essential for virus production by replacing each hr with the lacZ gene (Fig. 1b). A series of plasmids was generated where the bacterial lacZ gene, under the control of the AcMNPV ie-1 promoter, replaced one of the viral hrs cloned into a modified pUC18 vector (the single EcoRI site in the vector was removed) (Wu & Carstens, 1996). None of the lacZ inserts disrupted a specific AcMNPV open reading frame. We confirmed that the lacZ gene was inserted into the appropriate hr region in each plasmid by sequence analysis and restriction nuclease analysis. A series of specific primer pairs flanking each hr region was also designed (Supplementary Table S1, available in JGV Online) and used in PCRs to confirm the various knockout.

Primers located within viral sequences flanking each hr region were used in conjunction with primers specific for the lacZ gene (lacZF1 or lacZB1; Supplementary Table S1) to amplify the junction regions between viral sequences and the lacZ insert. The results of these reactions were all as expected, confirming the location of the lacZ gene within each hr region and mapping the orientation of the lacZ gene.
within the various hr regions (Fig. 2a). PCRs using the opposite lacZ primer were all negative, demonstrating that the plasmids were genetically clean (data not shown). These results validated the primer pairs for investigation of the hr knockout by lacZ insertion in recombinant viruses.

Fig. 2. PCR amplification of hr knockouts. (a) DNA from plasmids pAcΔhr1lacZ (hr1) pAcΔhr1alacZ (hr1a), pAcΔhr2lacZ (hr2), pAcΔhr3lacZ (hr3), pAcΔhr4lacZ (hr4a), pAcΔhr4blacZ (hr4b) or pAcΔp10lacZ (p10) (lanes 1–7, respectively) was used as template. Primers specific to sequences upstream (F1 primers; *F2 primer for Δhr4b) of each hr region and to sequences within the lacZ gene (B1 or F1 primer) were used in each reaction. (b) Primer pairs identical to those used in Fig. 2(b) were used in reactions with purified viral DNA from each knockout virus as template (vAcΔhr1lacZ, vAcΔhr1alacZ, vAcΔhr2lacZ, vAcΔhr3lacZ, vAcΔhr4alacZ, vAcΔhr4blacZ or vAcΔp10lacZ; lanes 1–7, respectively). The products were analysed by agarose-gel electrophoresis. The size of the products confirmed the presence and location of the lacZ gene in the appropriate position of each hr knockout. M, size markers (kbp).
Construction of hr knockout viruses

Each plasmid carrying a specific hr knockout was co-transfected with vAc DNA into Sf21 cells. The infected cell supernatants were then screened for the presence of recombinant viruses producing blue plaques. All transfection supernatants produced a low percentage of blue plaques. Representative plaques from each transfection supernatant were plaque-purified to homogeneity (vAcΔhr1lacZ, vAcΔhr1alacZ, vAcΔhr2lacZ, vAcΔhr3lacZ, vAcΔhr4lacZ, vAcΔhr5lacZ and vAcΔp10lacZ). The fact that viable BV was produced from each of the hr knockout infections suggested that none of the individual hrs was absolutely essential for virus replication or virus production.

To confirm that individual hrs had been removed, purified DNA from each knockout virus was investigated by PCR analysis using the same primer pairs used above to orient the lacZ gene within the knockout plasmids (Fig. 2b). The results demonstrated that each knockout virus carried the lacZ gene and its orientation was the same as in the original plasmid transfer vectors used for the co-transfections (compare Fig. 2a and b). Therefore, each individual hr region in the viral DNA had been replaced by the lacZ gene. However, we have previously shown that plasmid DNA can become incorporated into BVs and can be continuously present in passaged virus stocks (Wu et al., 1999). We confirmed that the signals obtained from these PCRs were amplified from an integrated copy of the lacZ gene inserted into the corresponding region of the viral genome and not from a copy of the original plasmid co-packaged into virions and present as a contaminant in the infected cells with another series of primers designed from viral sequences lying outside the region present in the original plasmid transfer vectors (Supplementary Table S1, available in JGV Online). When used in conjunction with a specific lacZ primer, the PCR would amplify a product only if the lacZ gene was integrated into the viral genome, as the viral primer site was not included in the sequences cloned into the original plasmids. All knockout viruses carried integrated copies of lacZ, which were amplified by primers specific to the expected hr region (Fig. 3, compare with Fig. 2b). These results confirmed the integration of lacZ into the viral genomes and the replacement of the hr region with this reporter gene.

**Effect of hr knockouts on BV production**

It was possible that deletion of one of the hrs had a subtle effect on the virus replication cycle that was not detected during the screening process. For example, deletion of an hr might result in a delay in the onset of BV production or in a decrease in the total yield of BV. These possibilities were investigated using one-step growth curves. As a control to normalize possible effects of the expressed lacZ gene on virus growth, a knockout virus with intact hrs but with the lacZ reporter gene inserted into the viral p10 region (vAcΔp10lacZ) was included. P10 is a very late gene product, so a knockout of this gene would not be expected to affect early events or BV production (Weyer et al., 1990). Sf21 cells were infected and harvested at 1.5, 8, 10, 12, 18, 24, 36 and 48 h p.i. and the titre of BV at each time point was determined using TCID50 assays. The infections were carried out in duplicate and each time point was titrated in triplicate. Deletion of any single hr from the viral genome had no significant effect on the production of BV, either on the time of onset of BV production or on the final titre obtained (Fig. 4). We concluded that none of the hrs is essential for virus production in vivo.

![Fig. 3. PCR amplification to confirm the location of the lacZ gene in hr knockout viruses. DNA from recombinant AcMNPV knockout viruses (vAcΔhr1lacZ, vAcΔhr1alacZ, vAcΔhr2lacZ, vAcΔhr3lacZ, vAcΔhr4lacZ, vAcΔhr5lacZ or vAcΔp10lacZ; lanes 1–7, respectively) was used as template in PCRs with one primer specific for viral sequences upstream of the hr region and outside the region of viral DNA included in the plasmid transfer vector and one primer specific for the lacZ gene. The amplification products (lanes: 1, 4.7 kb; 2, 1.5 kb; 3, 1.0 kb; 4, 0.75 kb; 5, 1.7 kb; 6, 4.3 kb; 7, 2.1 kb) confirmed the location and orientation of the lacZ gene integrated into the viral genomes. M, size markers (kbp).](http://vir.sgmjournals.org)

![Fig. 4. Knockout virus growth curves. Sf21 cells were infected with each knockout virus (m.o.i. of 5). At the indicated time points, supernatants were collected and BV was titrated using TCID50 assays using a 2-fold dilution series. Infections were done in duplicate and each time point was titrated in triplicate. Mean virus titres at each time point are shown.](http://vir.sgmjournals.org)
Characterization of a double hr knockout virus

As deletion of a single hr did not affect virus production, we tested the effects of deleting more than one hr region. As the largest region of the AcMNPV genome that does not carry any hr sequence is located between hr2 and hr3 (see Fig. 1a), we postulated that if there were any subtle effects on the virus replication cycle related to the position of hrs, it might be detected by removing these two specific hrs. First, pAcΔhr3 plasmid and vAcΔhr3lacZ viral DNA were co-transfected into Sf21 cells and the resulting supernatants were screened for the presence of recombinant viruses producing white plaques (loss of lacZ expression). Several white plaques were obtained and these viruses, designated vAcΔhr3, were plaque-purified. Deletion of the lacZ gene from the hr3 region of this virus was confirmed by PCR using the flanking primer pair hr3F and hr3B4 (expected 297 bp product) and hr3F and hr3R (expected 616 bp product) (Fig. 5a, lanes 1 and 2). Sf21 cells were then co-transfected with vAcΔhr3 viral and pAcΔhr2lacZ plasmid DNA and the supernatants were screened for recombinant viruses expressing the lacZ gene (blue plaques). Many blue plaques were seen in the initial plaque screens but when these plaques were picked and replaqued, many of the progeny plaques were still lacZ-negative (white). It required four to five plaque purifications before virus stocks were obtained that expressed only pure blue plaques. Working virus stocks were prepared from isolates, designated vAcΔhr3/hr2lacZ. DNA from vAcΔhr3/hr2lacZ was isolated and examined by PCR. Amplification with primer pair hr3F and hr3B4 (297 bp fragment) and hr3F and hr3R (616 bp fragment) confirmed that vAcΔhr3/hr2lacZ carried the hr3 knockout (Fig. 5a, lanes 5 and 6). Integration of lacZ in place of hr2 was also confirmed by PCR using primer pair hr2F1 and lacZB1 (expected 599 bp fragment) (Fig. 5a, lanes 3 and 7) and lacZF1 and hr2B2 (expected 1037 bp fragment) (Fig. 5a, lanes 4 and 8). The latter reaction supported the conclusion that the double hr3/hr2 knockout virus carried an integrated copy of lacZ in the hr2 region.

We confirmed these results by PCR amplification of pAcΔhr2lacZ, vAcΔhr3lacZ and two different plaque isolates of the double-knockout recombinant virus vAcΔhr3/hr2lacZ, picked plaque 1 (PP1) and 2 (PP2) (Fig. 5b). The results clearly showed that when paired with the hr2F1 primer, the hr2B1 primer but not the hr2B2 primer produced a product with pAcΔhr2lacZ DNA, whilst both primer pairs produced fragments of the expected size with vAcΔhr3, demonstrating that the hr2 region was intact, as expected, in the parental viral DNA prior to recombination with pACΔhr2lacZ. Analysis of the double-knockout viruses showed that both of these viruses carried a large insert in the hr2 region, which was amplifiable using hr2F1 and either the hr2B1 or hr2B2 primers (Fig. 5b, lanes 5–8). Taken together with the results shown in Figs 3 and 5a, this indicated that both hr2 and hr3 were deleted in this virus. The isolation of these double knockouts demonstrated that at least two hrs could be deleted without totally disrupting viral DNA replication or BV production. The double knockout was also included in the virus growth experiments. No detectable effects on the kinetics of virus production were seen (Fig. 4).

Protein synthesis in recombinant viruses

Although hrs are suspected to act as enhancers of mRNA expression, based on experiments using reporter plasmids in which hrs are placed in close proximity to viral promoters, as far as we know, it has not been demonstrated that this enhancement results in higher protein synthesis levels in virus-infected cells. To determine whether the deletion of specific hrs detectably affected protein expression and

![Fig. 5. PCR confirmation of the double-knockout virus vAcΔhr3/hr2lacZ.](image)
accumulation, SF21 cells infected with knockout virus (m.o.i. of 5) were investigated by immunoblotting. Extracts of equal quantities of cells, harvested at 12 h p.i., were analysed to determine the relative amounts of immediate-early (IE-1), delayed-early (P143, LEF-3, P47), early and late (GP64), and late (VP39) genes expressed by each knockout virus. Representative blots are shown in Fig. 6. The experiments were repeated three times and at least two separate blots were analysed for each experiment. Although this approach is not quantitative, in general, the results did not reveal any consistent differences in the amount of these proteins detected in any of the single hr knockout virus infections (Fig. 6, lanes 2–5 and 7–9). However, cells infected with the double knockout consistently revealed a reduction in vp39 expression at 12 h p.i. (Fig. 6, lane 6). However, this difference had disappeared by 18 h p.i. (not shown). VP39 is the major capsid protein produced in large amounts during normal virus infection.

Analysis of DNA replication following infection with hr knockout viruses

By isolating recombinant viruses with specific hr knockouts, we have demonstrated for the first time that no specific hr is essential for virus multiplication in cell culture. However, it is possible that specific hrs might have an influence on the efficiency or rate of viral DNA replication. Cells infected with knockout virus were harvested at 4, 8, 12, 18 and 25 h p.i., total intracellular DNA was harvested and the amount of virus-specific DNA was quantified by real-time PCR. Each reaction was normalized to the SF21 genomic DNA present in each total intracellular DNA preparation. We assumed that the cellular genomic DNA would serve as an internal standard that could be applied to determine the relative amount of viral DNA at each time point. We also assumed that the efficiency of purification of cellular DNA and viral DNA from the infected cells was equivalent so that this ratio would reflect the relative copy number of viral genomes per cell. Samples were prepared from infection triplicates. An excellent linear correlation was obtained for the standard curves using SF21 genomic DNA with the hsp90 primers and AcMNPV genomic DNA with the p95 primers (Fig. 7a). Melting-curve analysis revealed that the PCR products were specific and unique (not shown). However, although we expected the standard curves of SF21 and AcMNPV genomic DNA to overlap, the SF21 genomic curve was displaced, suggesting that the estimate of 0.412 pg DNA per cell was too low. The data suggested that the genome complement of SF21 cells is 1.6 pg per cell (four genome copies per cell). This value was used in determining the cell numbers and then the ratio of viral to cell copy numbers for each knockout virus at each time point. A best-fit line was generated for each virus, based on the model that the square root of the ratio of viral to cellular DNA was linear with time, with different slopes for each knockout virus but with all viruses having the same value at 4 h p.i.

At 4 h p.i., a mean of 0.6 copies of viral DNA per cell was detected, close to the expected value of 1 (m.o.i. of 5). There was an increase in viral DNA for all knockout virus DNA samples between 4 and 8 h p.i., indicating that viral DNA replication was initiated during this time (Fig. 7b). These results are consistent with our previously published data (Tija et al., 1979). There was no obvious difference in any of the knockouts with respect to the time of initiation of replication. In addition, the amount of viral DNA per cell with each knockout virus increased up to 25 h p.i. to approximately $1.7 \times 10^3$–$2.7 \times 10^3$ genomic copies per cell, clearly demonstrating that viral DNA replication was occurring with genomes carrying specific hr deletions. The sensitivity of real-time PCR highlighted differences between the accumulation of viral DNA in some infected cells when compared with the control virus, vAcΔp10 (estimated to be $2.0 \times 10^3$ copies per cell), vAcΔhr4a and vAcΔhr3 produced significantly more viral DNA (estimated to be $2.7 \times 10^3$ copies per cell) over the entire time course ($P<0.0001$). vAcΔhr4b produced more (estimated to be $2.2 \times 10^3$ copies per cell) ($P=0.0039$) and vAchr1a produced less (estimated to be $1.7 \times 10^3$ copies per cell) ($P=0.0033$). The double knockout, vAcΔhr3Δhr2, also produced less DNA (estimated to be $1.8 \times 10^3$ copies per cell) than vAcΔp10 ($P=0.0243$), whilst vAchr1 and vAchr2 were indistinguishable from vAcΔp10 ($P=0.4563–0.4892$). As the graphs show, there was a broadening of the data points with increasing time after infection, suggesting an increase in variation of analysis with time. This was probably a result of the non-synchronized state of a normal virus infection.

Fig. 6. Western blot analysis of cells infected with knockout virus. SF21 cells were infected in triplicate with vAcΔhr1lacZ, vAcΔhr1alacZ, vAcΔhr2lacZ, vAcΔhr3lacZ, vAcΔhr3Δhr2lacZ, vAcΔhr4alacZ, vAcΔhr4blacZ or vAcΔp10lacZ (lanes 2–9, respectively) (m.o.i. of 5) or mock-infected (lane 1) and harvested at 12 h p.i. Whole-cell extracts were prepared and analysed by Western blotting using antibodies against IE1-1, P143, LEF-3, P47, VP39 or GP64. Only vAcΔhr3Δhr2lacZ-infected cells probed with anti-VP39 revealed a consistent reduction in protein accumulation in the infected cells (lane 6).
of the number of viral genomes per cell for each virus at each sample was analysed in triplicate. A best-fit line was used for each virus at each time point (infections were carried out in triplicate) using the data generated from the standard curves shown to cellular DNA was determined for each sample at each time point. (b) The ratio of copies of viral DNA measured. Ct values were plotted against the genomic copy number of the target template. The number of PCR cycles to reach the fluorescence threshold in each dilution. The number of PCR cycles to reach the fluorescence threshold in each sample was defined as the cycle threshold (Ct). Ct is inversely proportional to the copy number of the target template: the higher the template concentration, the lower the Ct measured. Ct values were plotted against the genomic copy numbers for each sample. (b) The ratio of copies of viral DNA to cellular DNA was determined for each sample at each time point using the data generated from the standard curves shown in (a). A total of nine determinations is shown for each knockout virus at each time point (infections were carried out in triplicate and each sample was analysed in triplicate). A best-fit line of the number of viral genomes per cell for each virus at each time point after infection is shown.

Fig. 7. Time course of viral DNA replication. Sf21 cells were infected in triplicate with each knockout virus (m.o.i. of 5). At 4, 8, 12, 18 and 25 h p.i., total intracellular DNA was purified and used as template in real-time PCR. Each sample was analysed in triplicate. (a) Standard curves of Sf21 genomic DNA and AcMNPV genomic DNA were generated from 10-fold dilutions of purified samples of DNA. Genome copy number was calculated from the known amounts of DNA in each dilution. The number of PCR cycles to reach the fluorescence threshold in each sample was defined as the cycle threshold (Ct). Ct is inversely proportional to the copy number of the target template: the higher the template concentration, the lower the Ct measured. Ct values were plotted against the genomic copy numbers for each sample. (b) The ratio of copies of viral DNA to cellular DNA was determined for each sample at each time point using the data generated from the standard curves shown in (a). A total of nine determinations is shown for each knockout virus at each time point (infections were carried out in triplicate and each sample was analysed in triplicate). A best-fit line of the number of viral genomes per cell for each virus at each time point after infection is shown.

DISCUSSION

We successfully generated recombinant viruses where hr1, hr1a, hr2, hr3, hr4a or hr4b were knocked out. A control virus in which the p10 gene was replaced by the ie-1–lacZ construct was also used. The fact that each of these constructs resulted in viable BV indicated that no particular hr region was absolutely essential for virus production in cell cultures.

The virus growth curves of the hr knockout viruses were indistinguishable from the control virus. In addition, these growth curves were comparable with those of normal AcMNPV infections in time of appearance of BV, the slope of increase in virus titre with time and the final titre of virus obtained. Therefore, none of the hrs tested was essential for production of normal levels of BV nor did any of the knockouts detectably affect the temporal formation of BV. These results are consistent with a previous report where deletion of hr5 had no apparent effect on the production of BV (Rodems & Friesen, 1993).

We have previously shown that expression of the AcMNPV p143 promoter can be stimulated up to 20-fold in transient expression assays by including hr5 in cis on the reporter plasmid (Lu & Carstens, 1993). This suggests that hrs may play a role in gene enhancement during virus infection. However, it is not known whether any particular hr is required for this enhancement or if this level of enhancement occurs in vivo. The specific locations of hrs on the genome may reflect positional requirements for transcription enhancers in vivo. If this were the case, then deletion of an hr located closer to a particular gene might affect the expression of that proximal gene more strongly than a more distant one. However, our data provided no evidence to support this hypothesis (Fig. 1). In fact, our results support those of a previous report where deletion of hr5 had little or no effect on late gene expression but had a promoter-specific effect perhaps limited to viral promoters responsive to the host RNA polymerase II (Rodems & Friesen, 1993). However, these studies were based on analysis of the expression of p35, a gene immediately upstream of hr5. Expression of p35 mRNA in the absence of hr5 was reduced approximately 2-fold. The data presented here show that the accumulation of early gene products required for DNA replication such as IE-1, P143 and LEF-3 was unaffected by any of the knockouts. However, the genes investigated were located much further from any hr region than p35 and hr5. Deletion of hr3 or hr4a, which are located 12.8 kbp upstream and 9.2 kbp downstream of p143, did not reduce P143 accumulation at 12 h p.i. compared with its expression in cells infected with any of the other knockout viruses. Likewise, deletion of hr2 did not result in any observable alteration in P47 accumulation, even though hr2 is located only about 3.5 kbp away from p47. The accumulation of these early or delayed-early genes did not appear to be altered by any single knockout or by the double knockout (hr2 and hr3). However, there was a consistent reduction in or delayed accumulation of VP39, the major capsid protein, in cells infected with the double-knockout hr2 and hr3.
virus. This reduction did not affect virus production as the growth curves for this virus were almost identical to all of the other knockout viruses. This suggests that the major capsid protein is produced in excess during the replication cycle and a reduction in accumulation at 12 h p.i. does not affect the production of BV significantly.

When we examined the DNA replication patterns of the single- and double-knockout viruses using real-time PCR, the time and rate of increase of intracellular viral DNA, as well as the total accumulation of viral DNA, was not affected dramatically by the single hr knockouts, at least up to 25 h p.i. The estimate of the number of copies of viral DNA per cell presented here is based on our assumption of 1.6 pg genomic DNA (four copies) per Sf21 cell in culture. There was an increase in the amount of accumulated DNA in cells infected with vAc hr3 and vAc hr4a at 25 h p.i. compared with the control virus vAc AP10. The effect of inserting the lacZ gene in these knockouts would simply increase the distance between hr65 and orf106 in the hr4a region and between p95 and orf84 in the hr3 region. It is unclear how this might stimulate an increase in viral DNA at late times after infection. We have shown previously that plasmids in which hr3 or hr4a are deleted still replicate. However, the amount of replicated DNA was not quantified in that study (Wu & Carstens, 1996). All other single knockouts were comparable to the control vAc AP10 knockout, supporting the conclusion that no particular hr region is essential for viral DNA replication or gene expression. Even deletion of two hrs did not result in a significant reduction in viral DNA accumulation. Apparently, deleting hr2 in addition to hr3 negated any stimulation of viral DNA accumulation by the single deletion of hr3. The lack of reduction in the early accumulation of viral DNA in cells infected with the double hr knockout suggests that the presence of multiple copies of hrs does not increase the efficiency of viral DNA replication. Therefore, it is possible that only a single hr is essential for virus replication. With the exception of Trichoplusia ni single nucleopolyhedrovirus (Willis et al., 2005), all baculovirus genomes sequenced to date carry variable numbers of hrs (Okano et al., 2006), but the number and location of the various hrs are stable in individual baculoviruses, with no evidence of their involvement in intra-hr inversions during replication. All of our results are consistent with the hypothesis that, in AcMNPV, multiple hrs result in redundance of origin function and that no single hr is essential for replication of AcMNPV in cell culture. Therefore, the functional significance of multiple origins of DNA replication in baculovirus remains unclear.

ACKNOWLEDGEMENTS

We gratefully acknowledge the technical assistance including real-time PCR of Maike Bossert and Daniela Sahri. We thank Richard Mather and Markus Waldmueller for their assistance with virus titrations and Professor Malcolm Griffin for assistance with the statistical analysis. This research was supported by grants from the Canadian Institute of Health Research and the Ontario Genomics Institute.

REFERENCES


