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Initiation of Baculovirus DNA Replication: Early Promoter Regions Can Function as Infection-Dependent Replicating Sequences in a Plasmid-Based Replication Assay

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From the results of transient plasmid-based replication assays, it has been postulated that homologous regions (*hr*s) of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) function as origins of viral DNA replication. However, these assays vary in specificity according to the methodology used and may not be dependent solely on the presence of *hr* sequences. To determine the role that *hr*s and other sequences might play in the replication process, a series of plasmids containing specific deletions of various *hr*s was generated and tested in a standardized replication assay. Deletion of the Ac*M*NPV *hr*2 and *hr*5 sequences abolished the ability of plasmids to replicate in the standard infection-dependent replication assay, while deletion of *hr*1, *hr*3, and *hr*4a sequences decreased but did not eliminate plasmid replication in this assay. Plasmids carrying the complete *ie-2* and *pe38* genes, the *ie-1* gene upstream region, or a variety of baculovirus genes including 11 early promoter regions were also able to replicate in virus-infected cells, suggesting that early viral promoter sequences could also function as putative origins of replication. These data suggest that the standard infection-dependent replication assay may identify a broad range of infection-dependent replicating sequences, only one or a few of which may represent genuine viral origins used by the virus in vivo. We propose a model suggesting that the selection of replication initiation sites may be imposed directly by chromatin structure and indirectly by primary sequence and that the process of viral DNA replication may be linked with viral transcription.

Autographa californica nuclear polyhedrosis virus (Ac*M* NPV), the type species baculovirus, has a closed circular, double-stranded DNA genome (33, 35) of 134 kb, potentially encoding over 150 polypeptides (1). Expression of Ac*M*NPV appears to be controlled mainly at the transcriptional level and occurs in an ordered cascade fashion through early and late phases (3). The genome contains eight A+T-rich homologous regions (*hr*s) interspersed around the genome, each containing two to eight 30-bp imperfect palindromes with a core *Eco*RI site (except *hr*4c) (15). Because of their symmetric location, high A+T content, and palindromic structure, *hr*s were originally postulated to function as viral origins of replication (5). Recent transient replication assays supported this hypothesis (15). A single palindrome containing an intact core *Eco*RI site was sufficient to support plasmid replication in virus-infected cells (20, 27). The only other region shown to support plasmid replication in this assay included several A+T-rich regions and imperfect palindromes within the *Hin*dIII-K (*ori*-K) fragment (16, 19). However, it is still not known whether any of these regions are essential for and function as replication origins in vivo. For instance, deletion of *hr*5 from Ac*M*NPV had no apparent effect on virus replication (30). In addition, it has been demonstrated that plasmids without baculovirus inserts can replicate when plasmid and viral DNAs are cotransfected into insect cells (10, 18). As well, when plasmids expressing a number of viral genes necessary for reporter plasmid replication were cotransfected, all plasmids in addition to the reporter plasmid replicated (14). Because only the reporter plasmid carried an *hr*, these data suggested to us that sequences other than *hr*s may act as origins of replication in this assay and clouded the issue of what constitutes a baculovirus origin of replication. We have now investigated other regions of the Ac*M*NPV genome for the ability to support plasmid replication. In this report, we demonstrate that plasmids carrying regions other than Ac*M*NPV *hr*s can replicate efficiently in an infection-dependent transient replication assay. Our results reveal a strong correlation between the presence of regions regulating viral transcription and an ability to initiate DNA replication. On the basis of these data, we propose a model to describe the specificity of this reaction and suggest a possible correlation between viral early transcription and initiation of viral DNA replication.

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MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* 21 (Sf21) cells were maintained by passage in TC-100 medium (Gibco BRL) supplemented with 10% fetal calf serum. Ac*M*NPV strain HR3 was prepared and titrated as previously described (6).

Plasmid construction. All plasmids were propagated in *Escherichia coli* DH5α cells and purified on Qiagen Tip 500 columns as instructed by the manufacturer (Qiagen Inc.). The *Eco*RI site was deleted from pUC18 by digestion with *Eco*RI followed by incubation with DNA polymerase (Klenow fragment) and religation to produce pUC18ΔE. DNA fragments of Ac*M*NPV carrying the *hr*2 (*Pst*I-J), *hr*3 (a 6.2-kb *Sst*I-*Hin*dIII fragment of *Sst*I-D), *hr*4a (*Kpn*I-D), and *hr*5 (*Hin*dIII-Q) regions were cloned into pUC18ΔE. The *Kpn*I-D clone was further digested with *Hin*dIII, then partially digested with *Eco*RI to retain the right-end 3.5-kb viral fragment containing *Eco*RI-Q and the *hr*4a region, blunt ended with Klenow DNA polymerase, and religated to produce pAchr4a. The resulting plasmids were digested with *Eco*RI to delete the *hr* sequences, treated with S1 nuclease to destroy the residual *Eco*RI site within the core palindrome, and religated to generate pAcΔhr2, pAcΔhr3, pAcΔhr4a, and pAcΔhr5. The Ac*M*NPV *Hin*dIII-F fragment was cloned into pUC18, and then this plasmid was digested with *Cla*I and religated to generate pAcΔhr1. Subclones of the left (pAcHE4.3) end flanking *hr*1 and a 1.7-kb *Eco*RI-*Sca*I fragment of pAcHE4.3 [pAcHE4.3(ES)] have been previously described (23). The Ac*M*NPV *Pst*I-N fragment was cloned into pUC18 (pAcPstN) (23). *Pst*I digestion of pAcHE4.3 released a *Pst*I fragment containing the *pe38* gene, which was recovered and ligated into *Pst*I-digested pUC18 (pAcPE38). A *Hin*dIII-*Sca*I fragment of pAcHE4.3 was cloned into pUC18 to produce pAcIE-2. The 1.5-kb *Hin*dIII-*Sal*I fragment from pAcPstJ was cloned into the *Hin*dIII-*Sal*I site of pBSK$^-$ to generate pAchr2. The Ac*M*NPV

*Eco*RI-Q and *Eco*RI-S fragments were cloned into pBR322 to generate pAcHE65 and pAcp35, respectively. The *Hin*dIII-R fragment was cloned into pUC19 to generate pAc39K. A *Sca*I-*Xho*I fragment from pSTCHX-3 (34) was cloned into the *Sma*I-*Sal*I sites of pUC18 to generate pAclef4. A 3-kb *Hin*dIII-*Xba*I fragment from *Hin*dIII-E was cloned into the *Hin*dIII-*Xba*I site of pUC18 to generate pAcp47. A 4.7-kb *Eco*RI-*Ssp*I fragment from *Eco*RI-D was cloned into the *Eco*RI-*Sma*I site of pUC19 to generate pAcp143. A 1.4-kb *Eco*RI-*Nru*I fragment from *Eco*RI-O was cloned into the *Eco*RI-*Sma*I site of pUC19 to generate pAclef1. Plasmids carrying the *dnapol* (pAcDNApol) and *lef-3* (pAclef3) genes were previously described (22, 36). A plasmid containing the *ie-1* gene promoter linked to the *E. coli lacZ* gene (pIE1-lacZ) was a gift from Paul Friesen. The *ie-1* promoter was deleted from pIE1-lacZ by digestion with *Sma*I and *Eco*RV and religation of the large *Sma*I-*Eco*RV fragment to produce placZ(ORF). pIE1-P(CH) was subcloned from pIE1-lacZ by inserting the 558-bp *Cla*I-*Hin*cII fragment of the *ie-1* promoter region into the *Acc*I-*Hin*dIII site of pBS (Stratagene) by blunt-end ligation.

Various regions of the *ie-1* promoter were subcloned from pIE1-P(CH) (3.3 kb). The 1.1-kb *Ssp*I fragment of pIE1-P(CH) was ligated to a 1.8-kb *Ssp*I-*Pvu*II fragment of pIE1-P(CH) to generated pIE1-P(CS) (3.0 kb). Digestion of pIE1-P(CH) with *Afl*III and religation of the 2.7-kb fragment generated pIE1-P(CA) (2.7 kb). A 0.4-kb *Pvu*II fragment from pIE1-P(CS) was ligated with a 2.4-kb *Pvu*II fragment of pIE1-P(CH) to generate pIE1-P(CP) (2.8 kb). Digestion of pIE1-P(CH) with *Nhe*I and *Afl*III, filling in with DNA polymerase I (Klenow fragment), and ligation generated pIE1-P(CN) (2.5 kb). Digestion of pIE1-P(CH) with *Nhe*I and *Eco*RI, filling in with DNA polymerase I (Klenow fragment), and ligation generated pIE1-P(NH) (3.1 kb). Ligation of the 2.4- and 0.5-kb *Pvu*II fragments from pIE1-P(CH) generated pIE1-P(PH) (2.8 kb). Digestion of pIE1-P(CH) with *Mlu*I and *Eco*RI followed by filling in with DNA polymerase I (Klenow fragment) and ligation yielded pIE1-P(AH) (2.9 kb). Digestion of pIE1-P(CH) with *Ssp*I and religation generated pIE1-P(SH) (2.2 kb). The 0.3-kb *Pvu*II fragment of pIE1-P(NH) was ligated with a 2.4-kb *Pvu*II fragment of pIE1-P(CH) to generate pIE1-P(NP) (2.6 kb). Digestion of pIE1-P(PH) with *Afl*III and religation generated pIE1-P(PA) (2.3 kb). Digestion of pIE1-P(CS) with *Eco*RI and *Mlu*I, filling in with DNA polymerase I (Klenow fragment), and religation generated pIE1-P(AS) (2.6 kb).

All clones were confirmed by restriction enzyme mapping. All clones carrying *hr* sequence deletions and *ie-1* promoter deletions were confirmed by DNA sequence analysis.

DNA replication assays. Infection-dependent transient replication assays were performed according to a standard procedure (17) modified by transfecting 10^6 Sf21 cells with 1 to 2 μg of plasmid DNA followed 6 h later by infection with Ac*M*NPV (multiplicity of infection [MOI] of 1). Total intracellular DNA was harvested 48 h after infection. Cotransfections were carried out by mixing 1 to 2 μg of plasmid DNA with 0.5 to 1 μg of purified Ac*M*NPV DNA and 10 μl of Lipofectin in a total volume of 75 μl and then adding the mixture to washed cell monolayers. After 6 h of incubation at 28°C, the DNA-Lipofectin mixtures were removed, and the cells were washed twice, overlaid with fresh medium, and incubated at 28°C for 72 h. The ability of plasmid DNAs to replicate in Sf21 cells was monitored by their differential susceptibilities to the restriction enzyme *Dpn*I as previously reported (17, 27). The relative amount of DNA was determined by densitometry of X-ray films using the public domain computer program NIH Image (version 1.54).

The amounts of *Dpn*I-resistant plasmid DNA varied considerably among the panel of plasmids that we used, but so did the sizes of these plasmids. Because larger plasmids might be expected to replicate fewer copies than smaller plasmids within the same time period, it became important to determine the initiation efficiency of replication regardless of plasmid size. We assumed that once DNA replication had initiated, replication of the entire plasmid would continue at a constant rate. Thus, the efficiency of initiation was the critical parameter that needed to be examined. The replication process was therefore separated into two steps, initiation and elongation. The initiation efficiency ($K1$) is the ratio of initiated DNA (R_0) to uninitiated DNA (U), $K1 = [R_0]/[U]$. The rate of elongation ($K2$) is the ratio of fully replicated DNA (R) to initiated DNA (R_0), $K2 = [R]/[R_0]$. The rate of replication, once begun, is independent of initiation and is simply a function of the length (kilobases) of the DNA to be replicated and the supply of essential protein factors necessary for replication (f); therefore, $K2 = f$/kb. The total intracellular concentration of each plasmid DNA after replication [T] equals [U] + [R]. Therefore, $K1 = (R \cdot \text{kb})/[f \cdot (T - R)]$. The values for [$R$] (linearized *Dpn*I-resistant, replicated plasmid) and [T] (linearized total intracellular plasmid DNA including replicated and unreplicated plasmids) were determined by densitometer analysis of a variety of different exposures of three separate replication assay films, including those shown in Fig. 3. $K1$ of each plasmid was calculated for each experiment and averaged, assuming f to be 1.0 for an MOI of 1. The replication efficiency ($K1$) of the reporter plasmid pAchr2 was standardized as 100%, and all other plasmid $K1$ values were compared with this value.

RESULTS AND DISCUSSION

Plasmids with *hr* deletions replicate in virus-infected cells. To determine whether any of the Ac*M*NPV *hr* regions are specifically essential to support DNA replication, we deleted the *hr*s from several plasmids. pAcΔhr1 had a complete deletion of the *hr*1 region, plasmids pAcΔhr2, pAcΔhr3, and pAcΔhr4a contained a single palindrome with a disrupted central *Eco*RI sequence, and pAcΔhr5 contained only one half of a single palindrome. No replication of *hr*-deletion plasmids pAcΔhr2 and pAcΔhr5 was detected in standard infection-dependent transient replication assays, while the control *hr*2-containing plasmid pAcPstJ showed a strong replication signal (Fig. 1a). This result demonstrated that a single palindrome with a disrupted *Eco*RI core site (pAcΔhr2) or half a palindrome (pAcΔhr5) disabled the ability of *hr*s to function as DNA replication origins, consistent with published data (20). However, deletions of the *hr* from pAcΔhr1, pAcΔhr3, and pAcΔhr4a decreased but did not completely eliminate the ability of these plasmids to replicate (Fig. 1a, lanes 3, 5, and 6), indicating that other non-*hr* sequences in these plasmids could function as DNA replication origins. To explore the nature of non-*hr*-stimulated replication, regions of the plasmid pAcΔhr1 were subcloned and individual clones were tested in the standard replication assay. Both the left and right regions flanking *hr*1 and contained within *Hin*dIII-F fragment supported infection-dependent DNA replication (data not shown). A detailed dissection of plasmid pAcHE4.3 (the *hr*1 left flanking region) identified two regions that correlated with non-*hr* replication.

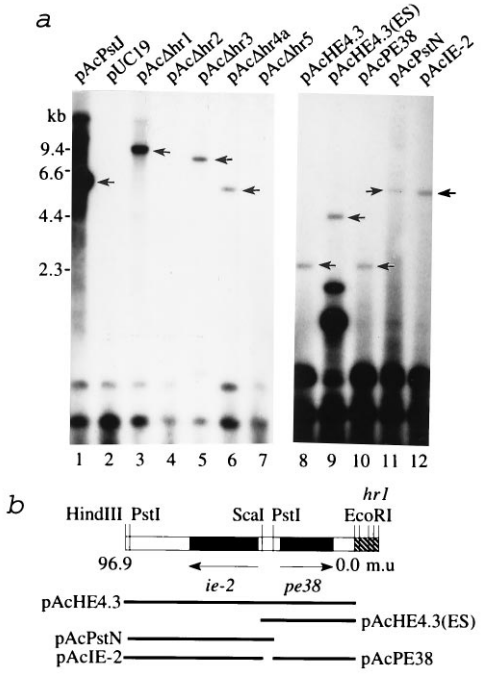

FIG. 1. Infection-dependent transient replication of plasmids containing *hr* sequence deletions. (a) Sf21 cells (10^6) were transfected with 1 μg of *hr*-deletion plasmid pAcΔhr1 (lane 3), pAcΔhr2 (lane 4), pAcΔhr3 (lane 5), pAcΔhr4a (lane 6), or pAcΔhr5 (lane 7) or plasmids containing regions flanking *hr*1 (lanes 8 to 12). The same amounts of pAcPstJ (lane 1) and pUC19 (lane 2) were used in control transfections. Plasmid-transfected cells were infected with Ac*M*NPV (MOI of 1) at 6 h posttransfection. Total cellular DNA was purified at 48 h postinfection and doubly digested with *Dpn*I plus *Sma*I (lanes 1 to 5 and 7), *Kpn*I (lane 6), *Hin*dIII (lanes 8 and 11), or *Pst*I (lanes 9, 10, and 12) to linearize the replicated plasmid DNA (lanes 1 to 7, 9, 11, and 12) or to release viral inserts from vectors (lanes 8 and 10). After electrophoresis, DNA samples were transferred to Qiagen nylon membranes and hybridized with ^{32}P-labeled pUC18 DNA. The *Dpn*I-resistant fragments are indicated by arrows. (b) Physical locations of viral DNA inserts derived from the left flanking region of *hr*1 and present in pAcHE4.3 (lane 8), pAcHE4.3(ES) (lane 9), pAcPE38 (lane 10), pAcPstN (lane 11), and pAcIE-2 (lane 12). m.u., map units.

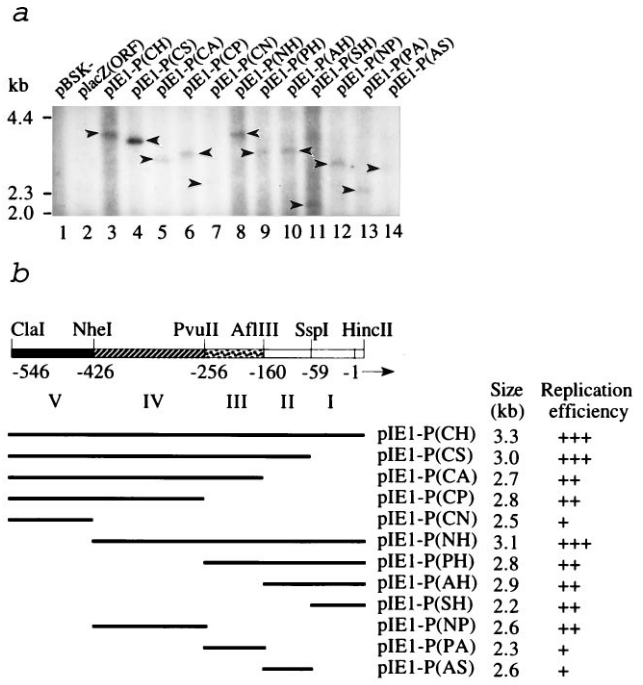

FIG. 2. The *ie-1* promoter region functions as DNA replication origins. (a) Infection-dependent transient replication of plasmids carrying the Ac*M*NPV *ie-1* gene promoter region and its subdomains. Sf21 cells were transfected with pBSK$^-$ (lane 1), placZ(ORF) (lane 2), pIE1-(CH) (lane 3), pIE1-P(CS) (lane 4), pIE1-P(CA) (lane 5), pIE1-P(CP) (lane 6), pIE1-P(CN) (lane 7), pIE1-P(NH) (lane 8), pIE1-P(PH) (lane 9), pIE1-P(AH) (lane 10), pIE1-P(SH) (lane 11), pIE1-P(NP) (lane 12), pIE1-P(PA) (lane 13), or pIE1-P(AS) (lane 14) and infected with Ac*M*NPV as described for Fig. 1. Total cellular DNA was purified and doubly digested with *Dpn*I plus *Sma*I (lanes 1 and 2) or *Xmn*I (lanes 3 to 14). Blotting and hybridization conditions are outlined in the legend to Fig. 1. The *Dpn*I-resistant fragments are indicated by arrowheads. (b) Restriction map of the 558-bp *Cla*I-*Hin*cII fragment containing the *ie-1* promoter region. Locations of the five domains (I to V) tested in the replication assay are shown.

One contained the complete open reading frame of the early *pe38* gene plus 94 bp of upstream sequence (Fig. 1a, lane 10), while the other contained the complete open reading frame for the early *ie-2* gene plus 91 bp of upstream sequence (Fig. 1a, lane 12). During normal virus infection, both of these genes are expressed immediately after infection, but they are also regulated by another viral early gene, called *ie-1* (9, 28, 29). These experiments demonstrated that regions other than *hr*s can stimulate plasmid replication in virus-infected cells and suggested that the presence of early genes may be responsible for this property.

Initiation of DNA replication by the *ie-1* promoter region. To investigate this possibility further, we analyzed the replication ability of the promoter region of the *ie-1* gene in the standard infection-dependent replication assay. Plasmid pIE1-P(CH) carrying the 558-bp *Cla*I-*Hin*cII region of the *ie-1* promoter clearly replicated (Fig. 2a, lane 3), while placZ(ORF), lacking the *ie-1* promoter, did not (Fig. 2a, lane 2), demonstrating that DNA sequences found within the *ie-1* promoter region could act as replication initiation sites. To identify functional motifs within this region, a series of plasmids containing deletions of the *ie-1* upstream sequence was constructed (Fig. 2b) and tested in the replication assay. Replication of subclones individually containing only one of the five regions demonstrated that any one of these regions could support DNA replication. The signals from region V (Fig. 2a, lane 7) and region II (Fig. 2a, lane 14) were very weak but were visible in longer exposures. In contrast, subclones pIE1-P(CS) and pIE1-P(NH) replicated almost as efficiently as the whole promoter plasmid pIE1-P(CH), suggesting that replication efficiency increased with increasing insert size. Deletion of region I had little effect on initiation of DNA replication (Fig. 2a, lane 4) and therefore was not essential for replication initiation in the assays.

The *ie-1* promoter region does not contain any sequence homology with previously described origins. It does contain one 24-bp imperfect palindrome within region IV, but this region could be deleted without abolishing replication (Fig. 2a, lanes 7 and 9 to 11). Three A+T-rich domains are located within regions I, II, and V (55 to 67% A+T over 70 to 119 nucleotides), but the replication of the lower-A+T-content region IV (46% A+T over 169 nucleotides), as well as the fact that one A+T-rich domain between regions I and II was disrupted, did not support a specific role for these A+T-rich domains in replication. Analysis of the replication negative plasmids pAcΔhr2 and pAcΔhr5 revealed that they also contain regions of about 140 nucleotides which are about 80% A+T. In addition, all of the control plasmids lacking viral inserts also contain regions with high A+T content (74% A+T over 100 nucleotides), but they were completely negative in our experiments. Therefore, high A+T content is not sufficient to impart replication ability. We conclude that retaining the viral promoter region intact is important in determining maximal replication ability. This ability did not appear to directly correlate with transcriptional activation but rather appeared to indicate the potential role that these regions play in binding transcription factors prior to early transcription. The *ie-1* promoter region I, which contains the TATA box and CAGT initiator sequences, known to be essential for accurate initiation of transcription (2, 8, 29), was not essential for replication. In addition, although the upstream regions of the *ie-1* promoter, including regions II to V in our study, may be nonessential for transcription in vivo, they can enhance *ie-1* expression when transfected into cells (28), suggesting that other transcription factor binding sites likely exist within these regions. Evidence suggesting that these regions carry regulatory elements responsive to host cell factors has also been published (28). Therefore, our data are in agreement with other studies in which the presence of binding sites for a range of cellular transcription factors near the simian virus 40 origin of replication stimulated viral DNA replication but did not correlate with the ability to stimulate transcription (13).

Replication of plasmids containing Ac*M*NPV early genes and promoters. From the results presented above, we postulated that viral early gene promoter regions could function as putative DNA replication origins. We therefore investigated a number of early Ac*M*NPV genes and their promoter regions for the ability to stimulate plasmid replication. Surprisingly, almost all plasmids carrying viral DNA inserts expected to be expressed early after infection were capable of replication (Fig. 3). These plasmids carried a variety of genes (open reading frames plus their upstream regions), including the *E. coli lacZ* gene (driven by the *ie-1* promoter), the apoptosis-repressing gene (*p35*), the early *39K* gene, the immediate-early *he65*, *dnapol*, *p143*, *lef-1*, *lef-3*, *lef-4*, *p47*, *p43*, and *gta* Ac*M*NPV genes. The vector plasmids used in our cloning experiments without viral sequences (pBSK$^-$, pBR322, and pUC19) were completely *Dpn*I sensitive under our assay conditions (Fig. 3, lanes 2 to 4). By calculating the replication efficiencies of these various plasmids, taking into account their various sizes, we determined that plasmids carrying sequences including *lef-1*, *pe38*, *39K*, and *ie-2* showed low but detectable levels of replication (less than 5% of the value for the reporter pAchr2).

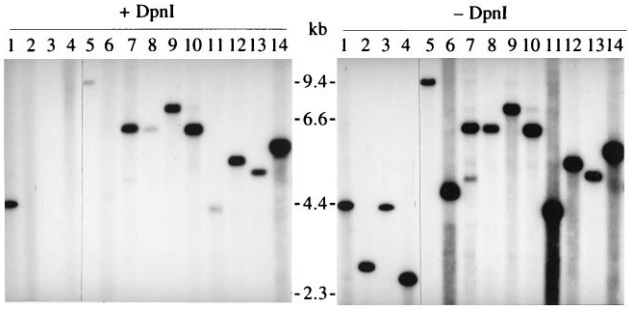

FIG. 3. Infection-dependent transient replication of plasmids carrying Ac*M* NPV genes and promoters. Sf21 cells were transfected with pAchr2 (lanes 1), pBSK$^-$ (lanes 2), pBR322 (lanes 3), pUC19 (lanes 4), pIE1-lacZ (lanes 5), pAc39K (lanes 6), pAcp35 (lanes 7), pAcHE65 (lanes 8), pAcDNApol (lanes 9), pAcp143 (lanes 10), pAclef1 (lanes 11), pAclef3 (lanes 12), pAclef4 (lanes 13), or pAcp47 (lanes 14) and infected with Ac*M*NPV as described for Fig. 1. Total intracellular DNA was purified and digested with *Sma*I (lanes 1, 2, 4, 5, 6, and 9), *Pst*I (lanes 3, 7, 8, 10, 11, and 13), or *Hin*dIII (lanes 12 and 14) to linearize the total intracellular plasmid DNA (except that in pAcp143, which has two *Pst*I sites, only the 6.3-kb site hybridized). (−*Dpn*I) or with the same restriction enzymes plus *Dpn*I (+DpnI) to identify replicated plasmid DNA. Blotting and hybridization conditions were the same as for Fig. 1.

Plasmids carrying the *he65* gene or the *ie-1* promoter replicated at about 15 to 20% of the reporter level. Plasmids carrying the *lef-3*, *lef-4*, *p35*, or *dnapol* gene replicated with efficiencies between 33 and 78% of that of the reporter, while plasmids carrying the *p143* gene or a region containing the *p47*, *p43*, and *gta* gene promoter regions replicated as efficiently as or better than the reporter plasmid (112 to 370%). Plasmids pAcΔhr2 and pAcΔhr5 did not carry any early genes or promoters and did not replicate (Fig. 1). Given that pAcΔhr5 carried the late *p10* promoter, these results clearly support our hypothesis that viral early genes and specifically their promoter regions can function in some cases as efficiently as *hr*s in supporting plasmid DNA replication initiation in virus-infected cells.

A correlation between transcription and DNA replication has been demonstrated in several other eukaryotic systems (for a review, see reference 12). The transcription activator NF1, which specifically prevents repression of simian virus 40 DNA replication by chromatin assembly, stimulates DNA replication 20-fold in vivo (4). The transcription factors VP16, GAL4, and p53 can bind to the large subunit of replication protein A, stimulating polyomavirus DNA replication through an influence on a very early stage of the initiation process such as initiation complex assembly (11, 21). The fact that a single *ie-1* promoter or its subdomains, as well as a number of other early promoter-containing regions of Ac*M*NPV, when present in plasmids, can lead to DNA replication suggests that the processes of baculovirus early gene transcription and replication may be intimately connected. This concept is also supported by our finding that the deletion of *hr*2 or *hr*5 inactivated the plasmid's ability to replicate; neither pAcΔhr2 nor pAcΔhr5 carried early promoters, although pAcΔhr5 maintained an intact late *p10* promoter. The binding of transcription factors prior to early transcription initiation may expose the DNA to the viral replication machinery, allowing for initiation of DNA replication. Alternatively, the interaction between transcription factors and the virus replication machinery may facilitate the assembly of replication factors necessary for DNA replication. Finally, factors which recognize early viral promoters may inhibit or dislodge nucleosomes, allowing access to DNA domains, which then function as replication origins. The conformation of a plasmid can affect its transcriptional activity during a transient expression assay, and supercoiling of plasmids is crucial for maximum transcription activity (26). Coincidentally, linearization of supercoiled plasmids containing *hr*s completely abolishes replication ability in the Ac*M*NPV infection-dependent replication assay (17). Because prolonged storage of plasmids or repeated extraction of plasmid DNA with phenol can greatly reduce the DNA's replication efficiency (37), DNA conformation may be an important criterion for both transcriptional and replication activation.

Specificity of plasmid replication. One major paradox in defining Ac*M*NPV DNA replication origins is the nonspecific replication of plasmid DNA even in the absence of any integrated viral sequences upon cotransfection with Ac*M*NPV DNA into Sf21 cells (10, 17, 37, 38). The replication efficiency for this vector DNA can be as high as that of an *hr*-containing plasmid and is not a result of recombination or acquisition of *hr*s following cotransfection (37). In addition, when the essential genes for baculovirus replication are cotransfected into insect cells, all plasmids including those not carrying *hr*s replicate (14, 18, 24), and this replication does not result from recombination between the *hr*-containing reporter plasmid and the plasmids expressing the replication genes (22a). These results suggest that the Ac*M*NPV DNA replication system does not require *hr*s for initiation; instead, virtually any sequences can be replicated when plasmids and viral replication-essential genes are introduced into the cells at the same time. However, if the plasmid DNA is introduced into cells prior to virus infection, the virus replication system is very sequence specific, replicating only plasmids containing certain viral sequences.

As a working hypothesis to resolve this paradox, we propose a model (Fig. 4c) suggesting that the specificity of baculovirus replication initiation in the infection-dependent replication assay is determined by features of chromatin structure. When a plasmid is transfected into a cell, it may assemble into a chromatin structure with cellular proteins. Following virus infection of the cell, viral transcription factors and replication proteins are expressed. If the plasmid contains binding sites (such as viral promoter regions or *hr*s) recognized by the early viral proteins, binding of these factors could disrupt the chromatin structure and expose the DNA to replication proteins. If no viral factor binding sequences are present, the chromatin structure could prevent replication proteins from interacting with the plasmid DNA, effectively repressing replication. Alternatively, the assembly of the replication proteins onto DNA may simply require binding factors to open the chromatin structure. In this case, the specificity of replication initiation would be based directly on the presence of recognizable binding sequences. On the other hand, following cotransfection of purified, naked plasmid DNA and viral DNA, the replication proteins may directly assemble onto the naked plasmid DNA (possibly at A+T-rich regions) prior to formation of the chromatin structure, initiating nonspecific DNA replication.

We tested this theory by infecting cells at several time points early after transfection of vector plasmid into cells. Nonspecific replication of pUC18 DNA was detected following infection with Ac*M*NPV at 2, 3, or 4 h after transfection. However, no replication of pUC18 DNA was detectable after 5 h posttransfection (Fig. 4b). Plasmid containing the *hr*2 region was recognized as a template and replicated at all times after transfection (Fig. 4a). Although there is no direct evidence yet, we suspect that after 5 h, the transfected pUC18 DNA is structurally altered and is no longer recognizable by viral replication proteins. This hypothesis will be testable once in vitro replication assays for baculovirus are available.

We have now identified a number of regions of the genome

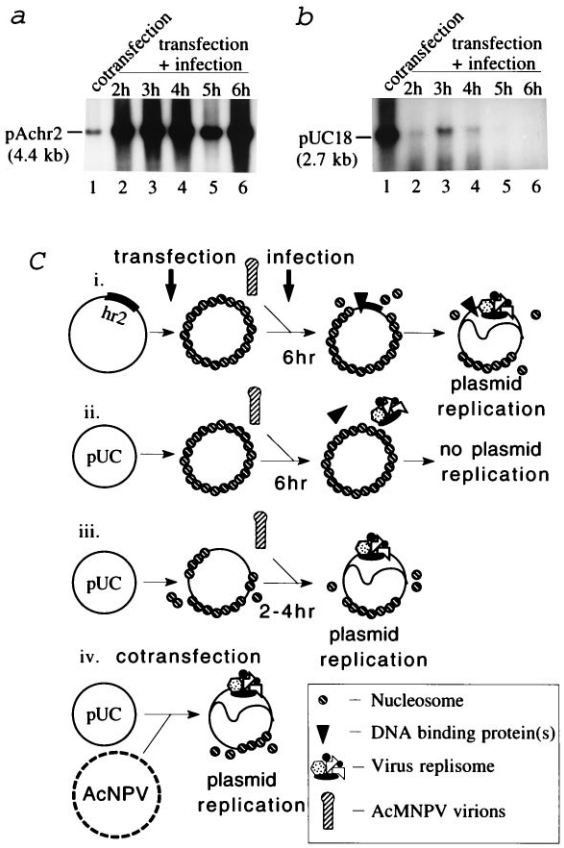

FIG. 4. Replication potential of plasmid DNA changes with time after transfection. Sf21 cells were cotransfected for 2 h with 1 μg of Ac*M*NPV DNA plus 2 μg of pAchr2 (a, lane 1) or 1 μg of Ac*M*NPV DNA plus 2 μg of pUC18 (b, lane 1). For infection-dependent transient replication assays, 2 μg of pAchr2 (a, lanes 2 to 6) or 2 μg of pUC18 (b, lanes 2 to 6) was incubated for 2 h with cells, and then monolayers were washed immediately and infected with virus (MOI of 1) at 2, 3, 4, 5, and 6 h (lanes 2 to 6, respectively) after transfection (the time of addition of DNA to cells was time zero). Total cellular DNAs were purified at 72 h after cotransfection or 48 h after virus infection and digested with *Dpn*I plus *Sma*I. Blotting and hybridization conditions were as for Fig. 1. (c) We propose that plasmid DNA, when transfected into cells, slowly forms a chromatin structure which affects the way the plasmid is recognized as a template for virus-induced replication. In this model, plasmid containing a viral DNA insert recognized by transcription factors would bind these factors, opening up the chromatin structure and serving as a platform for the assembly of the replisome and initiation of replication (i). Otherwise, a complete chromatin structure could preclude the assembly of the replisome (ii). If introduction of the plasmid DNA into cells is followed shortly by virus infection, there would not be enough time to form a complete chromatin structure, and so the virus replisome could assemble on any naked region of plasmids and initiate replication (iii). This conclusion is supported by data in panel b, lanes 2 to 4, showing that pUC18 (without any viral insert) replicated when virus infection occurred within 4 h after transfection. If plasmid and viral DNA were cotransfected into cells, the immediately synthesized viral replisome could assemble on any naked plasmid DNA and initiate replication (iv). These conclusions are supported by data in panels a and b, lanes 1.

in addition to *hr*s and ori-K which can function as putative DNA replication origins. However, we cannot exclude the possibility that more DNA elements are involved in this process. The situation is reminiscent of the widely used yeast autonomously replicating sequence (ARS) high-frequency transformation assay, which successfully identified the first eukaryotic chromosomal origin of replication, ARS1, and later identified many other yeast ARS elements (25, 31, 32). However, it was subsequently discovered, by using a variety of approaches, that while all yeast chromosomal origins are ARS elements, not all ARS elements function as origins in the yeast chromosome (7). Furthermore, not all yeast origins can initiate replication at the same time (7). We have now shown that in the infection-dependent replication assay, regions capable of binding host and viral transcription factors can function as origins of replication. We suggest that these putative origins be called infection-dependent replicating sequences. It remains to be determined which of these sequences are used during the virus replication cycle as genuine origins of replication.

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