JOURNAL OF VIROLOGY, June 2000, p. 5182–5189 Vol. 74, No. 11
0022-538X/00/$04.00+0

Differential Activity of Two Non-*hr* Origins during Replication of the Baculovirus *Autographa californica* Nuclear Polyhedrosis Virus Genome†

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Received 28 September 1999/Accepted 13 March 2000

The identification of potential baculovirus origins of replication (*ori*) has involved the generation and characterization of defective interfering particles that contain major genomic deletions yet retain their capability to replicate by testing the replication ability of transiently transfected plasmids carrying viral sequences in infected cells. So far, there has not been any evidence to demonstrate the actual utilization of these putative origins in *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (*Ac*MNPV) replication. By using the method of origin mapping by competitive PCR, we have obtained quantitative data for the *ori* activity of the *Hin*dIII-K region and the *ie*-1 promoter sequence in *Ac*MNPV. We also provide evidence for differential activity of the two *ori* in the context of the viral genome through the replication phase of viral infection. Comparison of the number of molecules representing the *Hin*dIII-K and *ie-1* origins vis-à-vis the non-*ori polH* region in a size-selected nascent DNA preparation revealed that the *Hin*dIII-K *ori* is utilized ~14 times more efficiently than the *ie-1* region during the late phase of infection. *Hin*dIII-K also remains the more active *ori* through the early and middle replication phases. Our results provide in vivo evidence in support of the view that *Ac*MNPV replication involves multiple *ori* that are activated with vastly different efficiencies during the viral infection cycle.

The prototype baculovirus, *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (*Ac*MNPV), has a double-stranded, closed-circular genome of ~134 kb with a coding capacity of over 150 polypeptides (2). *Ac*MNPV gene expression is temporally regulated in an ordered cascade through early, late, and very late phases. Viral DNA replication precedes the late phase and initiates late/very late gene expression that ultimately results in the production of progeny virus (23).

Interspersed in the *Ac*MNPV genome are nine homologous regions (*hr*) that are adenine-plus-thymine-rich sequences containing two to eight 30-bp imperfect palindromes with an *Eco*RI site as the palindrome core (except *hr*4C) (7, 15). *hrs* were initially postulated to function as viral origins of replication (*ori*) because of their symmetric location in the genome, palindromic structure, and high A+T content (4). Subsequent analysis of these sequences by transient replication assays supported this hypothesis (1), and a single palindrome with an intact core was shown to be sufficient for *hr* plasmid replication in *Ac*MNPV-infected cells (9, 20). Non-*hr ori* have also been reported in *Ac*MNPV. These include sequences within the *Hin*dIII-K region (84.9 to 87.3 m.u.) that are tandemly repeated in defective viral genomes (18). Sequences within the *Hin*dIII-K fragment also support plasmid replication in transient replication assays (13). Additionally, early promoter regions of the virus, including the *ie-1* gene upstream region and 11 other early promoter regions, have been demonstrated to function as plasmid *ori* in these assays, suggesting that early viral promoter sequences can also function as putative *Ac*MNPV *ori* (35). A number of virally encoded genes involved in DNA replication have also been identified. These include five essential (*p143*, *ie-1*, *lef-1*, *lef-2*, and *lef-3*) and five stimulatory (*dnapol*, *p35*, *ie-2*, *lef-7*, and *pe-38*) genes from *Ac*MNPV (14, 21, 22).

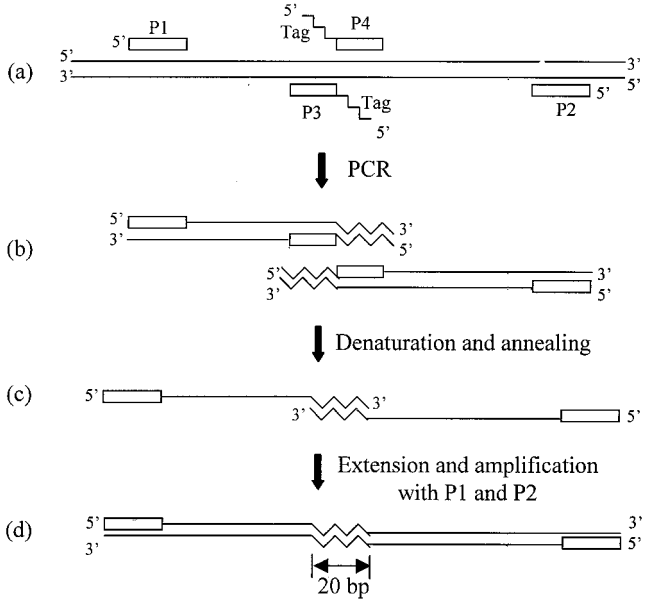

FIG. 1. Steps involved in competitor construction (Diviacco et al. [5]). P1-P3 and P2-P4 primer pairs were used to amplify DNA sequences adjacent to the target locus (a and b). The two products were then denatured and cooled, resulting in the annealing of the complementary 20-nt tail (c). The annealed product was subsequently extended and amplified by PCR by using primers P1 and P2 (d). The resultant competitor was 20 bp longer than the target locus.

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† This is CDRI communication no. 5989.

TABLE 1. Primer sequences and PCR product lengths of *ori* and control regions[a]

Primer	Primer coordinates (nt)	Sequence	Product length (bp)
HK-P1	114832–114854	5′-ACCCGATCATGCATTCTGTGTTG-3′	
HK-P2	115036–115056 (comp.)	5′-CTTTTACGGGCGCCATAGTGC-3′	Genomic, 225
HK-P3	114932–114951 (comp.)	5′-**ACCTGCAGGGATCCGTCGAC**TTATGTACTATTTGCTGTAC-3′	Competitor, 245
HK-P4	114952–114971	5′-**GTCGACGGATCCCTGCAGGT**GGCCCGCGTTAAACGCGACC-3′	
IE-P1	126808–126826	5′-TGCGCGTTACCACAAATCC-3′	
IE-P2	127004–127027 (comp.)	5′-ATAGAACATCCGCCGACATACAAT-3′	Genomic, 220
IE-P3	126888–126907 (comp.)	5′-**ACCTGCAGGGATCCGTCGAC**GGAGCGTACGTGATCAGCTG-3′	Competitor, 240
IE-P4	126908–126927	5′-**GTCGACGGATCCCTGCAGGT**TCGTGTTCCGTTCAAGGACG-3′	
PH-P1	5047–5068	5′-CGGCTGCCCAATAATGAACCTT-3′	
PH-P2	5234–5258 (comp.)	5′-TTAATACGCCGGACCAGTGAACAG-3′	Genomic, 211
PH-P3	5137–5156 (comp.)	5′-**ACCTGCAGGGATCCGTCGAC**CGGTACCGATGTAAACGATG-3′	Competitor, 231
PH-P4	5157–5176	5′-**GTCGACGGATCCCTGCAGGT**ACTCTGCTGAAGAGGAGGAA-3′	

[a] Primer sequences and PCR product lengths of *Ac*MNPV *ori* and control regions. Oligonucleotides used for the *Hin*dIII-K, *ie-1*, and *polH* regions are prefixed with HK, IE, and PH, respectively. P1 and P2 oligonucleotides represent external primers (left and right, respectively) used in competitor construction as well as the final competitive PCRs. P3 and P4 oligonucleotides, carrying a 20-nt tail at the 5′ end (tail nts shown in bold) represent internal primers used for competitor construction. The coordinates of the primers are from the *Ac*MNPV genome sequence of Ayres et al. (2) (GenBank accession no. L22858). P2 and P3 primer sequences are from the complementary (comp.) strand. The lengths of the genomic template and competitor DNA amplified by P1 and P2 are shown.

The identification of baculovirus replication *ori* has primarily been carried out by using two strategies. Putative *cis*-acting elements that may be involved in the initiation of DNA replication have been identified by the characterization of defective viral genomes generated by serial passage of the virus in tissue culture (11, 17) and by the analysis of the replication status of plasmids carrying these elements in transiently transfected cells in the presence of viral infection (9, 15, 19, 26, 27). However, it is still not known whether any of the putative *ori* thus identified are essential for or actually function as *ori* in vivo. Moreover, the individual roles of these multiple putative *ori* in DNA replication and whether they are active simultaneously and the relative efficiencies of utilization of these *ori* in a normal infection cycle have also not been worked out.

By using the method of origin mapping by competitive PCR, used previously for mapping mammalian DNA *ori* (6, 16, 28,

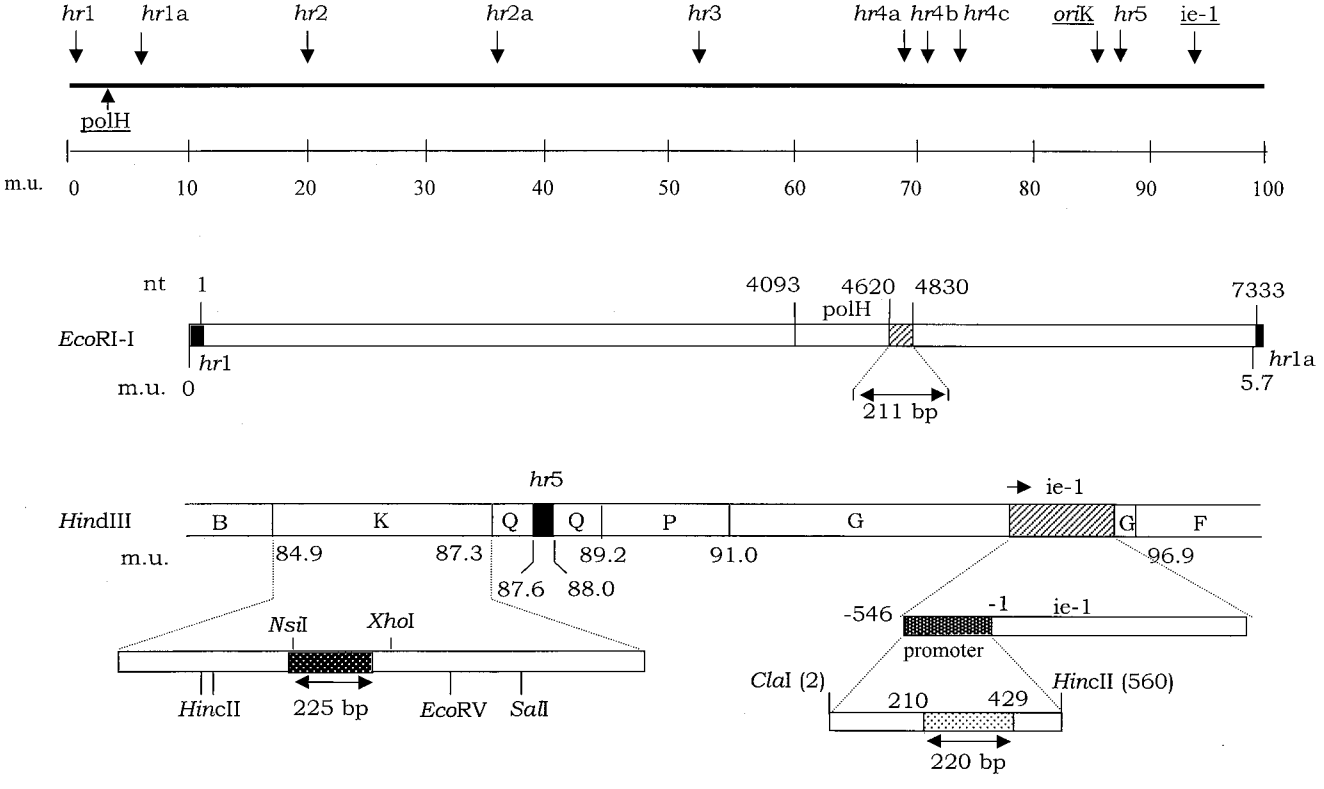

FIG. 2. Schematic representation of the positions of *hr* and the *Hin*dIII-K, *ie-1*, and *polH* regions in a linear map of the *Ac*MNPV genome. The *Eco*RI-I fragment containing the *polH* gene is shown together with the flanking *hr*1 and *hr*1a elements. The 211-bp 3′ end of the *polH* gene amplified by primers PH-P1 and PH-P2 is represented as a shaded box. Part of the *Ac*MNPV genome containing the *Hin*dIII-K, -Q, -P, and -G restriction fragments shows the location of the 225-bp *Hin*dIII-K region amplified by primers HK-P1 and HK-P2 as well as the 220-bp *ie-1* promoter region amplified by primers IE-P1 and IE-P2.

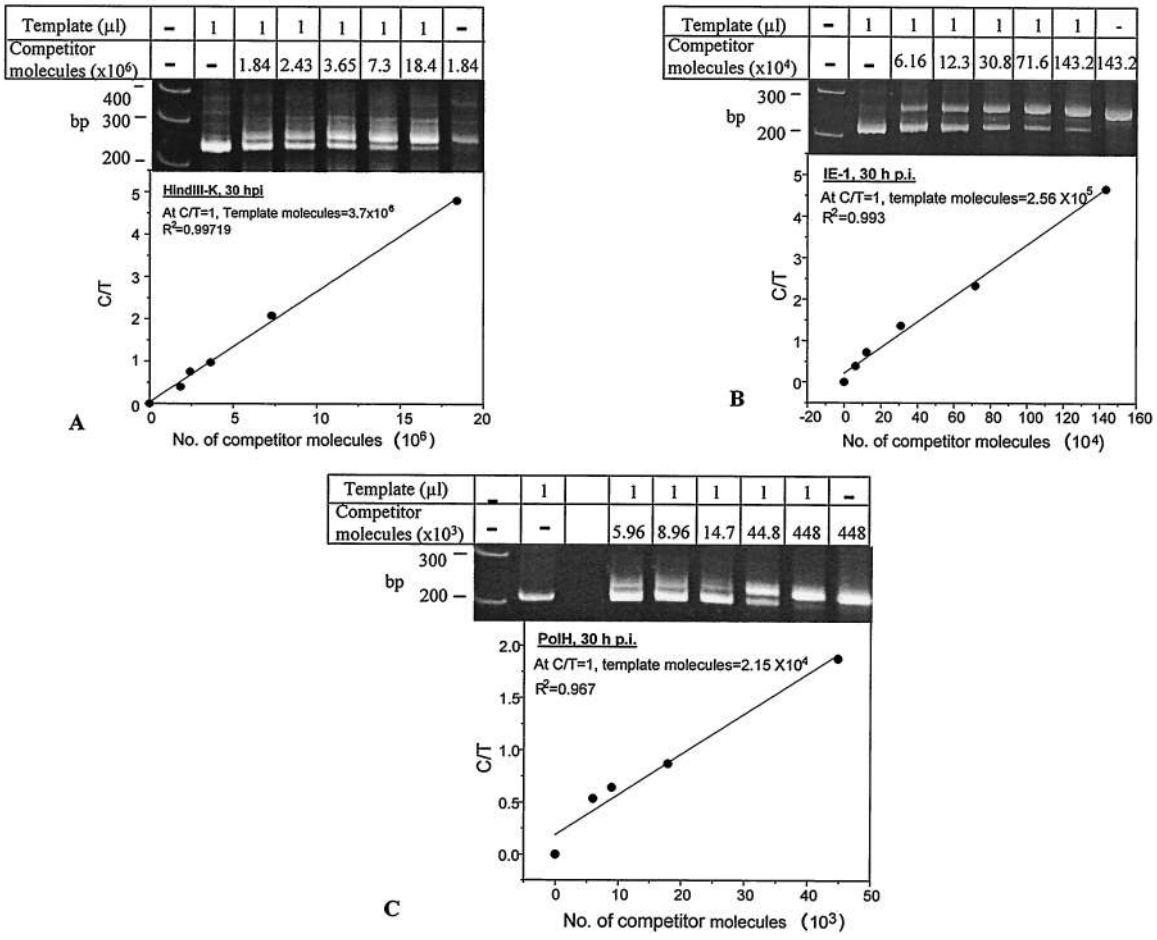

FIG. 3. Competitive PCR reveals greater abundance of *Hin*dIII-K and *ie-1 ori* regions compared to the non-*ori* control *polH* region in a 30-h p.i. nascent DNA preparation. Determinations of the numbers of molecules representing the *Hin*dIII-K, *ie-1*, and *polH* regions are shown in panels A, B, and C, respectively. A fixed amount (1 μl) of 30-h p.i. template DNA was added to competitive PCRs for the three regions. PCR products were resolved on an 8% polyacrylamide gel and were stained with ethidium bromide. The intensity of the bands corresponding to the template target (T) and competitor (C) DNAs was determined by densitometric analysis. The ratio between the two PCR products for each reaction (C/T) was plotted against the number of competitor molecules added to the reaction. A linear correlation between the C/T ratio and the quantity of competitor added to the reaction was observed. Correlation coefficients (R^2) are reported for each plot. The number of target template molecules, that equal the number of competitor molecules when C/T = 1, was calculated from the equation of the line fitting the experimental points.

32), we have been able to measure the efficiency of utilization of two putative non-*hr* origins (*Hin*dIII-K region and *ie-1* promoter region) vis-à-vis the control non-*ori* sequence within the polyhedrin (*polH*) gene of *Ac*MNPV. In this report, we provide in vivo evidence for utilization of multiple *ori* by the virus. Our results also support the view that different *Ac*MNPV *ori* may be activated with vastly different efficiencies during the viral infection cycle.

MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* cells (Sf9) were grown in TNMFH medium (31) containing 10% fetal bovine serum as described by Summers and Smith (31). The cells were infected with *Ac*MNPV (strain E-2) at a multiplicity of infection (MOI) of 50 PFU/cell for different time periods before isolation of total cell DNA.

Extraction and purification of nascent DNA. Total cell DNA was isolated from *Ac*MNPV-infected cells as described by Leisy and Rohrmann (19). Briefly, cells from an infected T75 flask were dislodged, centrifuged at 7,000 rpm for 3 min (SS-34 rotor, Sorval RC5C centrifuge), and washed twice with phosphate-buffered saline. The cell pellet was resuspended in 3 ml of DNA extraction buffer (10 mM Tris [pH 7.8], 0.6% sodium dodecyl sulfate, 10 mM EDTA) and then 15 μl of a solution containing 1 μg of RNase A/μl was added. After a 1-h incubation at 37°C, 375 μl of a 20-mg/ml solution of proteinase K was added, and the mix was further incubated for 12 to 16 h at 37°C. The samples were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform. DNA was precipitated with ethanol, was rinsed with 70% ethanol, was dried, and was resuspended in 300 μl of TE (0.01 M Tris [pH 8.0], 0.001 M EDTA).

Isolation of nascent DNA was carried out by sucrose gradient fractionation followed by further size selection of the fractionated nascent DNA by agarose gel electrophoresis (16, 32). Sucrose gradient fractionation was carried out according to the method of Kumar et al. (16). Briefly, total DNA from infected cells was denatured by a 10-min incubation in boiling water and was size separated on 17 ml of 5 to 30% continuous neutral sucrose gradient (150 μg of DNA per gradient) for 18 to 20 h at 26,000 rpm in a Beckman SW28 rotor at 15°C. Sucrose gradients were prepared in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.3 M NaCl. The bottom of the tube was punctured, and 500-μl fractions were collected from each tube. Fractions containing 0.3- to 1.5-kb segments of nascent DNA were identified by 1% agarose gel electrophoresis by using a 1-kb DNA ladder marker. These fractions were pooled and dialyzed against Tris-EDTA (0.5 M Tris [pH 8.0], 0.01 M EDTA) for at least 8 h. DNA was precipitated with sodium acetate and ethanol and was rinsed with 70% ethanol, dried, and suspended in TE. Further size selection of dialyzed nascent DNA was performed by fractionating the nascent DNA on a 1% preparative agarose gel and eluting 0.3- to 1.5-kb segments of DNA from the gel. After purification, the concentration of this DNA was determined and the preparation was used as template in competitive PCRs.

PCR amplification and competitor construction. Primers used for competitor construction and competitive PCRs for the *Hin*dIII-K, *ie-1*, and *polH* regions are shown in Table 1. Competitor construction for each of these regions was carried out as described by Diviacco et al. (5). Four specific oligonucleotides (two external primers, P1 and P2, and two internal primers, P3 and P4) were synthesized for each DNA region to be amplified (Fig. 1). The external primers were designed to amplify DNA regions in the range of 150 to 300 bp. The sequence of the upper (P1) and lower (P2) external primers is identical to the genomic region

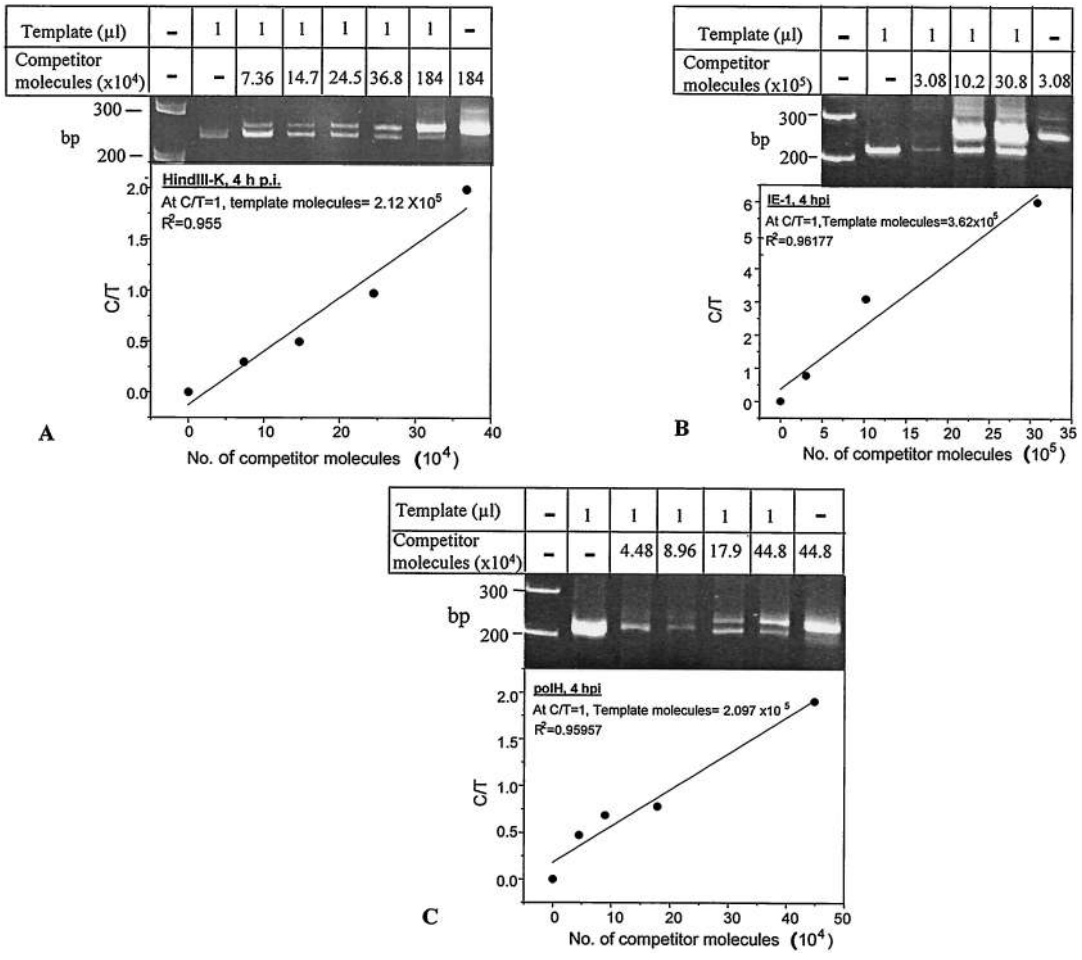

FIG. 4. *Ac*MNPV *ori* and control regions are represented equally in a prereplication nascent DNA preparation from infected Sf9 cells at 4 h p.i. Panels A, B, and C depict the determinations of the numbers of molecules representing the *Hin*dIII-K, *ie-1*, and *polH* regions by competitive PCR, respectively. Evaluation of the number of target template molecules representing each region was carried out as described in the legend to Fig. 3. Best curve fit for the experimental points was obtained by fitting the points to a linear equation. The number of target template molecules calculated for C/T = 1 from the equation of the line fitting the experimental points is reported inside the plot frame.

to be amplified. The upper (P4) and lower (P3) internal primers have 3′ ends identical to contiguous sequences on the upper and lower genomic strands, respectively, and 5′ ends that carry a 20-nucleotide (nt) tag. The 20-nt tags of the internal upper (P4) and lower (P3) primers are complementary to each other and are unrelated to the target sequence to be amplified. For each primer set, competitor DNA segments carrying the corresponding genomic sequence with the addition of 20 extra nts in the middle were constructed. These would allow gel electrophoretic resolution of the template and competitor amplification products. For competitor construction, the four primers were used to carry out two separate PCR amplifications. Amplification products of the P1-P3 and P2-P4 reactions, which contain a single overlapping region of 20 bp, were annealed together by first denaturing at 94°C for 1 min followed by lowering the temperature to 50°C (over a period of 10 min). After further incubation for 2 min at 50°C, the annealed products were extended by incubation at 72°C for 5 min and were amplified by using the following PCR conditions: cycles 1 to 5, 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and cycles 6 to 30, 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. One or more subsequent reamplification steps of the full-length competitor were needed to enrich for the competitor product and allow its quantification by radioactive labelling. All amplification reactions were carried out in an advanced version of the ThermostarII thermal cycler (34).

Quantification of the competitor template for each DNA region was obtained by measuring the amount of incorporated [α-^{32}P]dCTP in a competitor reamplification PCR cycle. A small amount of competitor, picked by touching a needle to the band on a polyacrylamide gel and immersing the tip in TE, was used as template. The PCR amplification mixture (50 µl) contained the standard amount of cold dCTP (10 nmol) and 0.2 µl (0.5 pmol) of [α-^{32}P]dCTP (Jonaki, Hyderabad, India) (4,000 Ci/mmol and 10 mCi/ml), corresponding to 1.94×10^6 cpm as measured by Cerenkov counting in a β-counter. The amplification products were resolved on a 8% polyacrylamide gel, and the radioactive competitor band was eluted in 150 µl of water. Five microliters of the eluted DNA was counted, and the concentration of the competitor (number of molecules per microliter) was determined from the final specific activity of [α-^{32}P]dCTP and the number of nucleotides incorporated. Dilutions of this competitor preparation were used as template in competitive PCRs.

Competitive PCR experiments. Competitive PCR was first carried out by using 10-fold serial dilutions of competitor with a fixed amount of nascent DNA template for each region in the presence of primers P1 and P2. The range within which the point of equivalence between competitor and template lay was thus determined. Similar reactions were then conducted, using further dilutions of the competitor within the range. Competitive PCR for each region was carried out in 30 cycles with the following conditions: denaturation, 94°C, 1 min; annealing, 55°C, 1 min; and extension, 72°C, 1 min.

RESULTS AND DISCUSSION

Evaluation of in vivo *ori* activity of two non-*hr* putative *Ac*MNPV *ori* by competitive PCR. Two putative non-*hr* origins, the *Hin*dIII-K region (*ori K*) and the promoter region of the *ie-1* gene, were selected for analysis of in vivo *ori* activity by competitive PCR. A region of the *polh* gene that does not support replication of transiently transfected plasmids in *Ac*MNPV-infected cells (data not shown) was used as a non-*ori* control region for measurement of background DNA levels. PCR primers selected for the *Hin*dIII-K region (84.9 to 87.3 m.u.) amplified a 225-bp sequence within region V and a small

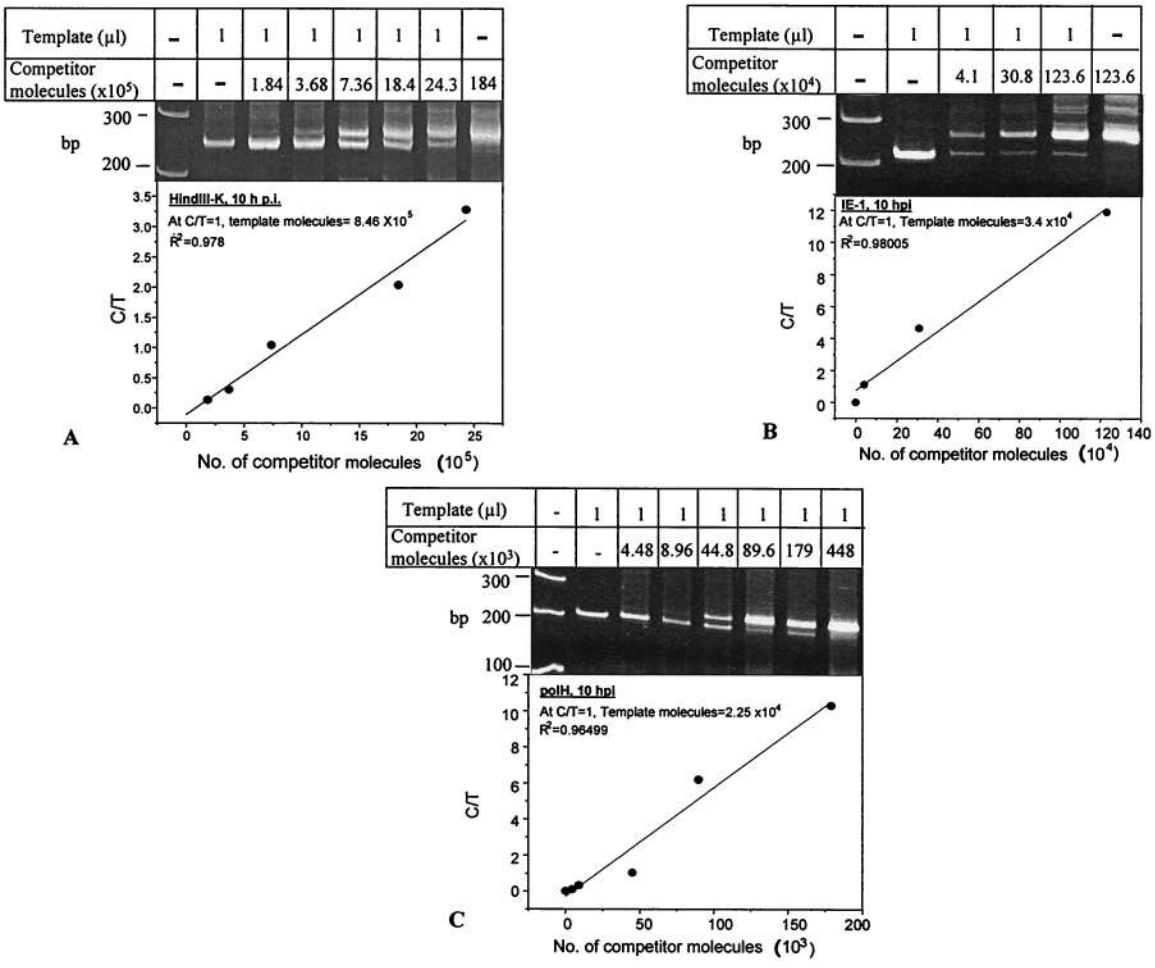

FIG. 5. Competitive PCR analysis reveals abundance of nascent DNA molecules representing the *Hin*dIII-K region in DNA prepared 10 h p.i. Determinations of numbers of molecules representing the *Hin*dIII-K, *ie-1*, and *polH* regions are shown in panels A, B, and C, respectively. Best curve fit for the experimental points was obtained by fitting the points to a linear equation, and the number of target template molecules when C/T = 1 was calculated.

portion of region IV (13) of the *Hin*dIII-K fragment (Fig. 2). Since the *hr*5 element (87.6 to 88 m.u.), which is known to be a putative viral *ori*, is located close to the *Hin*dIII-K region, we ensured that the primers for the *Hin*dIII-K region would not amplify *hr*5 *ori*-derived DNA in the nascent DNA preparation. This was done by specifically selecting nascent DNA in the size range of 0.3 to 1.5 kb from the sucrose gradient fractions as well as from the second gel-purification step (Materials and Methods). Since the distance between the 3′ end of the 225-bp *Hin*dIII-K region being amplified and the 5′ end of the *hr*5 element is greater than 2.2 kb (Fig. 2), the size of selected nascent DNA fragments would ensure that nascent DNA derived from the *hr*5 *ori* is not amplified by the primers specific for the *Hin*dIII-K region. Primers for the *ie-1* locus amplified a 220-bp sequence within the *Cla*I-*Hin*cII region of the *ie-1* promoter while a 211-bp fragment of the *polH* gene was amplified by the external primers designed for this locus (Fig. 2). Again, the size of the selected nascent DNA fragments (0.3 to 1.5 kb) ensured that nascent DNA derived from the *hr*1a putative *ori* sequence is not amplified by primers specific for the *polH* region. The distance between the 3′ end of the *polH* region amplified by the external primers and the *hr*1a element is ~2.5 kb (Fig. 2). Our attempts at amplification of a portion of the putative *hr*5 *ori* for competitive PCR analysis of *ori* activity were rendered unsuccessful by the generation of multiple bands due to extensive homologies with other *hr*.

Competitive PCR is used for the absolute quantification of low amounts of DNA and has been used for mapping *ori* in mammalian cells (6, 16, 28) as well as for determination of the abundance of sequences within origin regions in nascent DNA preparations (32). A fixed amount of DNA sample enriched in nascent DNA (i.e., low-molecular-weight DNA emanating from *ori*) is coamplified with increasing amounts of a quantified reference template (competitor), so that the two templates compete for the same primer set and subsequently amplify at the same rate. The ratio between the final amplification products of the two species is evaluated for each point. This ratio is a precise reflection of the ratio between the initial amounts of the two templates and is used to evaluate the amount of the unknown nascent DNA template. For sequences that are believed to be at or near *ori*, this method of quantification of nascent DNA templates has shown a high level of sensitivity and fidelity (32, 37). The isolation of nascent DNA in the size range of 0.3 to 1.5 kb ensures maximal elimination of broken genomic parental DNA and large nascent DNA fragments, including sheared DNA (typically ranging from 25 to 50 kb). As a result, sequences located at a significant distance from *ori* would not be detected. Since Okazaki fragments at mammalian

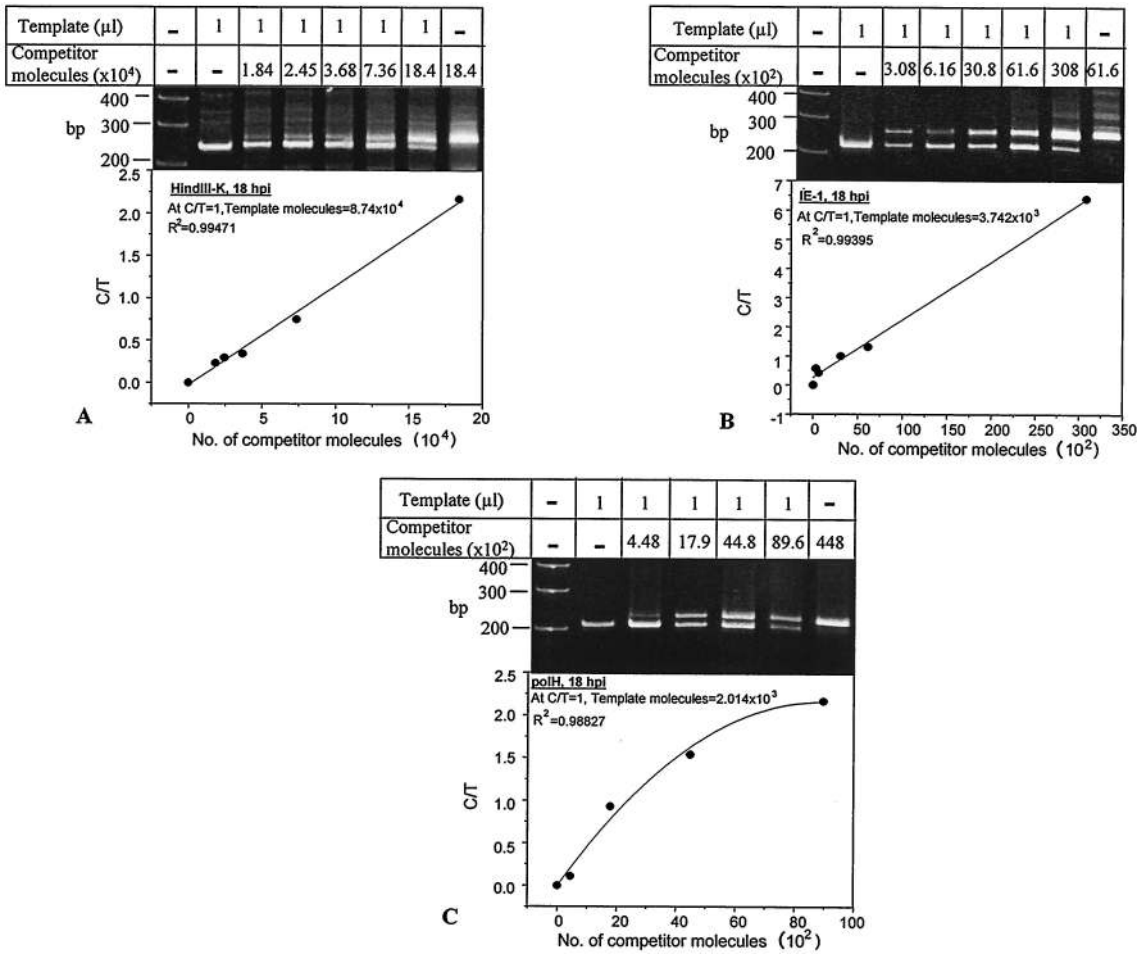

FIG. 6. *Hin*dIII-K continues as an active *ori* 18 h p.i. Competitive PCR analyses for determination of the number of molecules representing the *Hin*dIII-K, *ie-1*, and *polH* regions in nascent DNA prepared from *Ac*MNPV-infected Sf9 cells 18 h p.i. are shown in panels A, B, and C, respectively. The experimental points fitted to a linear (panels A and B) or quadratic (panel C) equation were used to calculate the number of target template molecules in the nascent DNA preparation.

replication forks range in size from 25 to 300 nt (3), it is presumed that Okazaki fragments from viral replication forks would also be eliminated by size selection of segments of nascent DNA greater than 0.3 kb.

Competitor DNA fragments constructed for the *Hin*dIII-K, *ie-1*, and *polH* regions were used as competing templates in competitive PCRs for each locus. A constant amount of nascent DNA template was used for all reactions carried out for the quantification of the number of template (nascent DNA) molecules representing each region at a particular time postinfection (p.i.). A fixed amount of nascent DNA prepared from *Ac*MNPV-infected cells harvested at 30 h p.i. was added to the PCR mix together with increasing amounts of the corresponding competitor DNA. The ratio of the competitor and template reaction products (C/T) was calculated by densitometric analysis of the ethidium-stained gels (ImageMaster 1D Elite software; Amersham Pharmacia Biotech) and was plotted against the number of competitor molecules added to each reaction (Fig. 3). The number of competitor molecules when C/T = 1 was calculated from the plot equation. This value corresponds to the precise number of molecules of the target template (nascent DNA) added to the PCRs. For nascent DNA isolated 30 h p.i., competitive PCR analysis carried out for each region revealed that the *Hin*dIII-K and *ie-1* regions were represented by ~170- and ~12-times-higher number of molecules compared to the control *polH* region, respectively (3.7×10^6 molecules of the *Hin*dIII-K region and 2.56×10^5 molecules of the *ie-1* region compared to 2.15×10^4 molecules of the *polH* region) (Fig. 3). Repeat experiments using another 30-h p.i. DNA sample gave similar relative values for the three loci

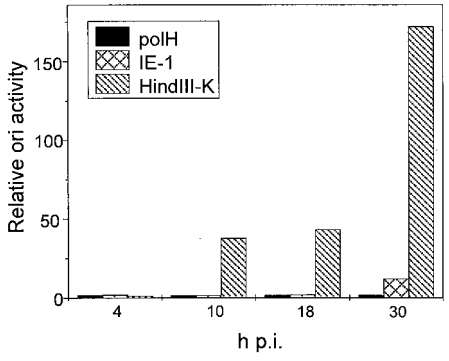

FIG. 7. Comparison of *ori* activities of the *Hin*dIII-K and *ie-1* regions at different times p.i. The ratio of number of molecules representing the *Hin*dIII-K and *ie-1* regions relative to the *polH* control region for 4, 10, 18, and 30 h p.i. is plotted as relative *ori* activity.

(*Hin*dIII-K–*ie-1–polH* ratio of 120:7.3:1) (data not shown). Additionally, comparison of the number of template molecules from the *Hin*dIII-K *ori* and control *polH* regions in a 30-h p.i. preparation from cells infected with *Ac*MNPV at 10 MOI gave a ratio of 103:1, thus demonstrating that the *Hin*dIII-K region exhibited high *ori* activity even at a lower MOI (data not shown). These results demonstrate that both the *Hin*dIII-K and the *ie-1* regions function as *ori* in vivo, although at 30 h p.i. the *Hin*dIII-K *ori* region is utilized much more efficiently than the *ie-1* locus.

***Hin*dIII-K and *ie-1 ori* exhibit differential activities during the *Ac*MNPV infection cycle.** To determine whether there were any differences in the relative utilizations of the *Hin*dIII-K and *ie-1 ori* during the viral infection cycle, we isolated nascent DNA from infected cells at different times p.i. and used these as templates in competitive PCR. Nascent DNA isolated from cells 4, 10, and 18 h p.i. was evaluated for DNA molecules representing the two non-*hr ori* and the control *polH* region. In *Ac*MNPV-infected Sf9 cells, DNA replication is detected by about 6 h p.i. and continues until about 18 h p.i., after which the level of replication declines (23, 33). Thus, nascent DNA isolated at 4 h p.i. would give background relative values for each region prior to replication initiation while DNA isolated at 10 and 18 h p.i. would indicate relative *ori* activity of the *Hin*dIII-K and *ie-1* regions vis à vis the *polH* control region when viral DNA replication activity is high in infected Sf9 cells.

Competitive PCR experiments for the three regions, carried out by using nascent DNA isolated 4 h p.i. as template, gave a *Hin*dIII-K–*ie-1–polH* template molecule ratio of 1.01:1.7:1 (i.e., 2.12×10^5:3.62×10^5:2.097×10^5 molecules) (Fig. 4). Near-equal representation of the two *ori* regions (*Hin*dIII-K and *ie-1*) and the non-*ori* control region *polH* in this prereplication DNA preparation confirmed that differences in the number of template molecules obtained for other time points are an actual indication of their relative *ori* activities. Infection of Sf9 cells with *Ac*MNPV at an MOI of 50, prior to the isolation of nascent DNA at different times p.i., ensured that all cells were infected at the same time. Competitive PCR with nascent DNA isolated 10 h p.i. yielded a *Hin*dIII-K–*ie-1–polH* template molecule ratio of 37.6:1.5:1 (i.e., 8.46×10^5:3.4×10^4:2.25×10^4 molecules) (Fig. 5), demonstrating that the *Hin*dIII-K *ori* is active at 10 h p.i. while the *ie-1* region does not show *ori* activity at this time point. Quantification of nascent DNA isolated 18 h p.i. revealed slightly higher relative *ori* activity of the *Hin*dIII-K region, although the change in relative *ie-1 ori* activity was insignificant compared to the activity 10 h p.i. The *Hin*dIII-K–*ie-1–polH* template molecule ratio obtained at 18 h p.i. was 43.4:1.8:1 (i.e., 8.74×10^4:3.742×10^3:2.014×10^3 molecules) (Fig. 6). These results indicate that replication is initiated at the *Hin*dIII-K region throughout the viral replication phase with maximal utilization of the *Hin*dIII-K *ori* in the late replication phase (30 h p.i.). On the other hand, the *ie-1* promoter region is utilized as an *ori* primarily in the late phase of replication. *Hin*dIII-K, however, remains the more active *ori* even in the late replication phase (Fig. 7). The lower *ori* activity of the *ie-1* region suggests that although active as an *ori* in the late phase, *ie-1* is not a preferred origin of *Ac*MNPV replication.

The mechanism by which baculoviruses generate mature, circular, unit-length genomes after replication is still not clear. Kool et al. (12) demonstrated that a circular topology is a prerequisite for the replication of *ori*-containing plasmids in *Ac*MNPV-infected cells, thus suggesting that baculovirus DNA replication involves a theta or a rolling circle intermediate. Replicated *ori*-containing plasmids organized into high-molecular-weight concatemers containing multiple plasmid copies in virus-infected cells (19) and multimers of viral DNA were detected in infected cells (25), indicating that *Ac*MNPV replication may use a rolling circle mechanism. A role for recombination has also recently been suggested for baculovirus replication (35). Irrespective of the mechanism of replication, a population of viral genomes may utilize multiple *ori* with differing levels of initiation efficiency. Rapid initial amplification of circular templates (by the theta or the rolling circle mode) could take place by replication initiation primarily at the *hr* origins. The non-*hr Hin*dIII-K *ori* is utilized both in the early and late replication phases. As replication proceeds and factors required for the specific initiation of replication become limiting, additional *ori* such as the *ie-1* region may also be activated. Differential activity of the *Hin*dIII-K and *ie-1 ori* in the viral genome context confirms that multiple *ori* are utilized during *Ac*MNPV replication in a temporally regulated manner. Determination of the activation profile of other non-*hr* putative *ori* sequences (35) by using the competitive PCR method could help delineate the order and efficiency of activation of these origins. Activation of *ori* could be regulated by the interaction of a specific viral-origin-binding protein(s) such as the *ie-1* gene product that binds to *hr* (8, 24, 29, 30) and host factors such as the 38-kDa protein that interacts specifically with *hr*1 (10). The regulated activation of multiple *ori* represents an interesting molecular event in baculovirus pathogenesis.

ACKNOWLEDGMENTS

We thank Anshu Chaturvedi, R. K. Srivastava, and Divya Singh for technical assistance; Pramod Upadhyay for the gift of the thermalcycler; and Amit Misra for help in manuscript preparation.

This work was supported by a Young Scientist Grant of the Indian National Science Academy to S.H.

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