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The *hr5* Transcriptional Enhancer Stimulates Early Expression from the *Autographa californica* Nuclear Polyhedrosis Virus Genome but Is Not Required for Virus Replication

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Autographa californica **nuclear polyhedrosis virus (Ac*M*NPV) contains five homologous regions (*hr*1 through *hr*5) interspersed throughout its genome. Analysis of plasmid transfections indicates that the *hr*s function as transcriptional enhancers and possible origins of viral DNA replication. The role of these repetitive elements in regulating expression from the Ac*M*NPV genome was examined by constructing a series of recombinant viruses that tested the effect of *hr*5 on expression of the adjacent p35 gene (*p35*). When embedded within the viral genome, *hr*5 stimulated transcription from the early *p35* promoter in a position- and orientation-independent manner. Moreover, *hr*5 and the upstream activating region of *p35* were functionally interchangeable. A 28-bp imperfect palindrome, repeated six times within *hr*5, was the minimal sequence required for *p35* promoter activation. *hr*5 also stimulated another early Ac*M*NPV promoter but not a late promoter or a host-derived promoter, suggesting that enhancement is promoter specific during infection. To investigate its role during Ac*M*NPV replication, *hr*5 was deleted from its normal position within the viral genome. The resulting *hr*5 mutants exhibited no apparent defects in replication, as judged by production of budded virus and levels of very late gene expression, even though steady-state levels of *p35* RNA were reduced. These results indicated for the first time that *hr*5 functions as a transcriptional enhancer within the viral genome. However, the element is not required for Ac*M*NPV replication in cultured cells. Thus, loss of one of five possible origins of DNA replication is not deleterious to viral growth. Since *p26* was removed from the *hr*5 deletion mutants, this gene is also nonessential for viral replication.**

The genome of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV), the prototype of the subgroup A baculoviruses, consists of a covalently closed circle of double-stranded DNA with a size of 131 kbp. Early and late viral genes are dispersed throughout the genome, and their proper expression results in the production of two morphologically and temporally distinct forms of Ac*M*NPV progeny, budded virus (BV) and occluded virus (for recent reviews, see references 1 and 31). Five homologous regions (designated *hr*1 through *hr*5), varying in size from 400 to 800 bp, are also interspersed throughout the genome (6). The *hr*s contain from two to eight imperfect palindromic repeats bisected by an *Eco*RI site (13, 16, 27). Because of their genomic distribution and repetitive nature, the *hr*s have been postulated to serve as origins of viral DNA replication (6). Consistent with this role is the observation that a single *hr* confers Ac*M*NPV-dependent replication to plasmids in transfected cells (23, 32). The transient amplification of plasmid DNA is proportional to the number of *hr* palindromic repeats (32). Analyses of defective Ac*M*NPV genomes indicated that the *hr*s are among those sequences retained during serial, undiluted passage of virus (23, 25). The amplification of regions that included *hr*3 and *hr*5 further suggests that these elements contain *cis*-acting sequences involved in DNA replication (23). Nonetheless, direct evidence that the *hr*s are required for Ac*M*NPV replication has yet to be obtained.

The *hr* elements of Ac*M*NPV also function as transcriptional enhancers when transfected into cultured cells on plasmid DNA. The *hr*s stimulate the rate of transcriptional initiation in a position- and orientation-independent manner (13, 16). Several Ac*M*NPV promoters, including those of the early 39K, IE-N, and p35 genes, are stimulated by *cis* linkage to the *hr*s (2, 16, 30). Although the *p35* and 39K promoters are responsive to *hr*5 alone, the activity of the enhancer is further augmented by the Ac*M*NPV transcriptional regulatory protein IE1 in transient assays (15, 30, 37). The *hr* elements may therefore have dual functions in a manner that is analogous to the enhancers of other DNA viruses (including polyomavirus, simian virus 40, adenovirus, and Epstein-Barr virus) which both stimulate transcription and serve as origins of DNA replication (reviewed in reference 7). The exact role of the *hr*s with respect to control of viral gene expression within the Ac*M*NPV genome remains to be determined.

The *hr*5 element is located immediately downstream from the Ac*M*NPV gene *p35* (Fig. 1). Proper expression of *p35* is required to maintain virus replication and to suppress premature cell death caused by virus-induced apoptosis that is cell line specific (5, 19). Both the proximity of *p35* and its responsiveness to *hr*5 in transient assays suggested that *hr*5 influences expression of this early viral gene (30). We have therefore investigated the function of *hr*5 with respect to *p35* regulation while embedded within the viral genome. Transcription of *p35*, directed by host RNA polymerase II, is detected within the first hour after Ac*M*NPV infection (10, 21, 30). The *p35* promoter is composed of a basal promoter element and an upstream activating region (UAR) extending from −110 to −30 (Fig. 1C). Although the TATA box of the basal promoter is the most influential element in determining the level of early transcription, multiple sequence motifs

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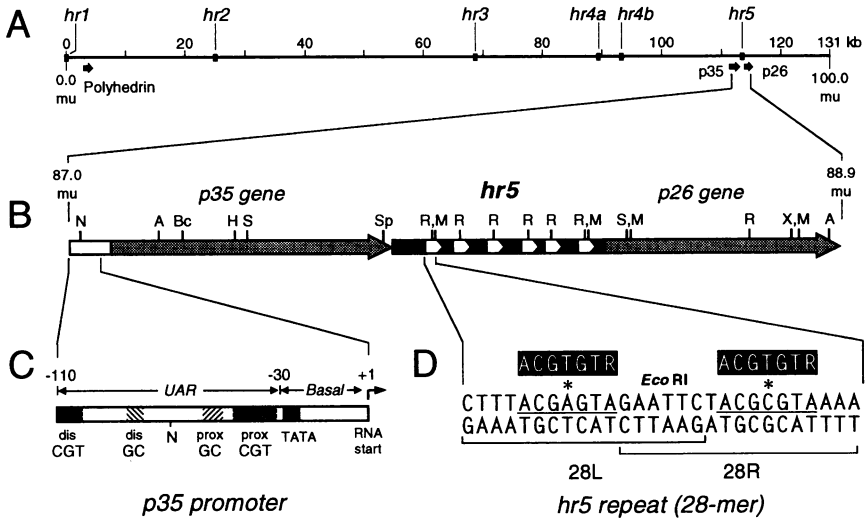

FIG. 1. Location of *hr5* within the Ac*M*NPV genome. (A) Linear map. The positions of the five homologous regions (*hr*1 through *hr5*) are shown with respect to the polyhedrin, p35, and p26 genes. The CAT reporter gene was inserted into the virus genome immediately upstream of the polyhedrin gene (3.96 map units [mu]). (B) Organization of the *hr5* locus. *p35* and *p26* flank either side of *hr5* (10, 16, 27); shaded arrows depict the coding region and direction of transcription of each gene. The six repetitive motifs within *hr5*, each containing an *Eco*RI site, are indicated by open arrows. (C) Organization of the *p35* promoter. The basal promoter includes the TATA box and RNA start site (positions −30 and +1, respectively), while the UAR extends from positions −110 to −30. Regulatory motifs are as described previously (8). (D) Nucleotide sequence of the 28-bp *hr5* repeat at the leftmost *Eco*RI site. The 28-bp repeat (28-mer) contains an imperfect palindrome centered at the *Eco*RI site. Underlined sequences correspond to the consensus sequence for the CGT motif (highlighted in black); asterisks indicate the nonconsensus nucleotides; brackets (28L and 28R) indicate the 17 nucleotides of the 28-mer found in plasmids pBAS35K-CAT/PA/28L and pBAS35K-CAT/PA/28R, respectively. Restriction site abbreviations: A, *Ava*II; Bc, *Bcl*I; H, *Hin*dIII; Hc, *Hin*cII; M, *Mlu*I; N, *Nru*I; P, *Pst*I; S, *Sal*I; Sp, *Spe*I; V, *Eco*RV; X, *Xho*I.

within the UAR are also involved (8). In particular, the CGT motif (consensus $A^{T}_{A}CGTGTR$) is required for full activity from the *p35* promoter and affects the activity of at least one other early Ac*M*NPV promoter (8, 14, 30). The CGT motif also resembles sequences within the palindromic repeats of *hr5* itself (Fig. 1D). This resemblance suggested that *hr5* and the UAR of early viral promoters have similar regulatory functions and may interact with common transcriptional factors.

In this report, we demonstrate that *hr5* stimulates gene expression in an orientation- and position-independent manner while embedded in the Ac*M*NPV genome. Thus, *hr5* can serve as a transcriptional enhancer during viral infection, confirming this function as demonstrated previously by transient assays (16). The minimum sequence required for stimulation of the *p35* promoter was a single 28-bp palindrome (28-mer) that contains sequences resembling the CGT motif. Thus, the repetitive 28-mer (Fig. 1) represents at least one of the enhancer modules of *hr5*. To directly address the role of *hr5* with respect to viral replication, we constructed Ac*M*NPV mutants that lacked this element by using a gene replacement strategy that took advantage of *p35* as a selectable genetic marker (26). Deletion of *hr5* and the adjacent gene, *p26*, had no effect on production of BV or on levels of very late gene expression. These findings demonstrated that *hr5* and *p26* are not required for Ac*M*NPV replication in cultured cells and suggested that deletion of one of the five possible origins of DNA replication is not detrimental to viral growth.

MATERIALS AND METHODS

Cells, virus, and transfections. *Spodoptera frugiperda* IPLB-SF21 (SF21) (38) and *Trichoplusia ni* TN368 (20) cell lines were propagated in TC100 growth medium (GIBCO Laboratories) supplemented with 2.6 mg of tryptose broth per ml and 10% heat-inactivated fetal bovine serum (FBS). For virus infection, SF21 monolayers were inoculated with the indicated Ac*M*NPV (L-1 strain) recombinants, using a multiplicity of infection (MOI) of 20 PFU per cell. After 1 h, the residual inoculum was removed, and the cells were washed, covered with growth medium, and incubated at 27°C. Time zero was defined as the point when virus was added. For transfections, calcium phosphate precipitates of plasmid DNA (10 μg) were added dropwise to SF21 monolayers (2×10^6 cells per plate) previously covered with Grace's insect medium and 10% FBS as described previously (30). When indicated, plasmid pIE-1 (1 μg) was included (30). After 4 h, the cells were washed and covered with TC100 containing 10% FBS. Cells were harvested 48 h after the addition of DNA.

Plasmid construction. *hr5* was cloned as a 484-bp *Mlu*I fragment from plasmid pHQ that was generated by inserting the Ac*M*NPV *Hin*dIII-Q genome fragment into pUC8. After end repair with the Klenow fragment and ligation to a *Bam*HI or *Xho*I linker, *hr5* was inserted at the *Bam*HI or *Xho*I site of the pBluescript (KS) vector (Stratagene), thereby generating plasmid p*hr5*^{B} or p*hr5*^{X}, respectively. The *hr5*-containing fragment from p*hr5*^{B} was then inserted at the unique *Bgl*II site located immediately upstream from the *p35* promoters of plasmids p(Δ3′-162/Δ5′-154)35K-CAT and p(Δ3′-162/Δ5′-30)35K-CAT (8), thereby generating plasmids pFL35K-CAT/*hr5*-up^{+} or -up^{-} and pBAS35K-CAT/*hr5*-up^{+} or -up^{-}, respectively, where superscript + or − designates the genome sense or opposite orientation of *hr5*. An *Xba*I-*Xho*I fragment containing the chloramphenicol acetyltransferase (CAT) gene from these plasmids was subsequently inserted into the transfer plasmid pEVocc^{+}/PA (8). To

construct plasmids in which *hr5* was located downstream of 35K-CAT sequences, the *Spe*I-*Xho*I fragment of p(Δ3′-162/Δ5′-154)35K-CAT and p(Δ3′-162/Δ5′-30)35K-CAT was inserted into pEVocc$^+$/HQPA, thereby generating plasmids pFL35K-CAT/*hr5*-dwn$^+$ and pBAS35K-CAT/*hr5*-dwn$^+$, respectively. pEVocc$^+$/HQPA was constructed by inserting a pHQ-derived 760-bp *Spe*I-*Hin*cII fragment into pEVocc$^+$/PA so that the existing polyadenylation (PA) signal was replaced with the *p35* PA signal and *hr5*.

Plasmids pIE1-CAT/PA/*hr5*-up$^+$ and -up$^-$ were constructed by inserting the 561-bp *hr5*-containing *Xho*I fragment from p*hr5*^{X} in either orientation into the *Xho*I site of pIE1-CAT/PA. Plasmid pIE1-CAT/PA contains IE1 promoter sequences from −161 to +11 relative to the mRNA start site (+1) linked to the CAT gene in pEVocc$^+$/PA.

Plasmid pLTR-CAT was made by inserting a 247-bp *Bgl*II fragment, containing sequences extending from the *Eco*RV to *Pst*I sites of the long terminal repeat (LTR) of the retrotransposon TED (11), immediately upstream of the CAT gene that was previously cloned into pBluescript. In plasmid pLTR^{mt}-CAT, the sequence TATAAG was replaced with the sequence CGCGAG by site-directed mutagenesis (24) using the oligonucleotide 5′-GCTAATTTATTACT CGCGAGAATTACAATAATTAA-3′. A 1,120-bp *Spe*I-*Xho*I fragment, containing either LTR-CAT or LTR^{mt}-CAT sequences, was cloned into pEVocc$^+$/PA and pEVocc$^+$/HQPA to generate plasmids pLTR-CAT/PA, pLTR^{mt}-CAT/PA, pLTR-CAT/*hr5*-dwn$^+$, and pLTR^{mt}-CAT/*hr5*-dwn$^+$, respectively. The *hr5*-containing *Xho*I fragment from p*hr5*^{X} was inserted into pLTR-CAT/PA and pLTR^{mt}-CAT/PA to generate plasmids pLTR-CAT/PA/*hr5*-up$^+$ and -up$^-$ and pLTR^{mt}-CAT/PA/*hr5*-up$^+$ and -up$^-$.

Plasmids containing the 28-mer repeat of *hr5* (Fig. 1D) were constructed by annealing the oligonucleotides 5′-GAT CTCTTTACGAGTAGAATTCTACGCGTAAAAG-3′ and 5′-GATCCTTTTACGCGTAGAATTCTACTCGTAAAGA-3′ and then inserting this 34-bp *Bam*HI-*Bgl*II fragment into the corresponding sites of pBluescript (containing a *Bgl*II linker inserted at the *Sma*I site) to generate p28*mer*. The 34-bp fragment was also inserted into the *Bgl*II site of p(Δ3′-162/Δ5′-30)35K-CAT (8) and pEVocc$^+$/PA/BglII (a variant of pEVocc$^+$/PA containing a *Bgl*II site downstream of the PA signal) to generate four plasmids: pBAS35K-CAT/28*mer*-up$^+$, pBAS35K-CAT/28*mer*-up$^-$, pEVocc$^+$/PA/28*mer*-dwn$^+$, and pEVocc$^+$/PA/28*mer*-dwn$^-$. Proper insertion of the 28-mer was determined by nucleotide sequence analysis using the dideoxy-chain termination method (36). pBAS35K-CAT/28*mer*-dwn$^+$ and -dwn$^-$ were constructed by inserting the CAT-containing *Xba*I-*Xho*I fragment from p(Δ3′-162/Δ5′-30)35K-CAT into pEVocc$^+$/PA/28*mer*-dwn$^+$ and -dwn$^-$, respectively. A tandem 68-bp repeat (dimer) of the 34-bp *Bam*HI-*Bgl*II fragment was also cloned into pBluescript to generate plasmid p28Dimer, which was subsequently used to generate plasmids containing the 68-bp repeat as described above.

An 858-bp *Bgl*II-*Bam*HI fragment from p(Δ3′-162/Δ5′-30)35K-CAT containing the *p35* basal promoter linked to the CAT gene was inserted into the *Bam*HI site of plasmid p28R to generate pBAS35K-CAT/28R. p28R contains the rightmost 17 bp of the 28-mer (*Eco*RI-*Bam*HI) in pBluescript. A 921-bp *Xba*I-*Xho*I fragment from pBAS35K-CAT/28R was inserted into pEVocc$^+$/PA to give pBAS35K-CAT/PA/28R. A 315-bp *Eco*RI fragment from pBAS35K-CAT/PA/28*mer*-up$^-$ was inserted into the *Eco*RI sites of pBAS35K-CAT/28R to generate pBAS35K-CAT/28L, which contained the leftmost 17 bp of the 28-mer from *Bgl*II-*Eco*RI. A 921-bp *Xba*I-*Xho*I fragment from this plasmid was inserted into pEVocc$^+$/PA to give pBAS35K-CAT/PA/28L.

The transfer plasmid pPoly-lacZ/35K/Δhr5 was constructed by first inserting a 381-bp *Bgl*II-*Xho*I fragment from pHQ into the *Hin*dIII site downstream from the polyhedrin promoter-controlled *lacZ* gene of plasmid pPOLY-lacZ (19); each site was end repaired with the Klenow fragment. Next, the Ac*M*NPV *Eco*RI-S genome fragment, previously cloned into pBluescript, was inserted as a 1,514-bp *Xba*I-*Xho*I fragment into the corresponding sites located immediately upstream of the polyhedrin promoter-*lacZ* sequences. To construct transfer plasmid pPoly-lacZ/35K/hr5$^+$, the *hr5*-containing *Xho*I fragment of p*hr5*^{X} was inserted into the unique *Xho*I site of pPoly-lacZ/35K/Δhr5.

Construction of Ac*M*NPV recombinants. CAT-containing viruses were constructed by allelic replacement by using appropriate pEVocc$^+$/PA transfer plasmids as described previously (8). Plaque-purified viruses were verified for the proper insertion of desired sequences by restriction mapping of BV DNA. Because of its small size, genomic insertions of the 28-mer were verified by polymerase chain reaction amplification of BV DNA (29) and subsequent restriction mapping. Oligonucleotide primers complementary to sequences immediately upstream of the polyhedrin and open reading frame (ORF) 603 genes, respectively, were used to amplify such inserts.

To construct Ac*M*NPV *hr5* mutants, either pPoly-lacZ/35K/Δhr5 or pPoly-lacZ/35K/hr5$^+$ (10 μg) was mixed with *p35* deletion mutant vΔ35K (20,000 PFU) in Lipofectin (Bethesda Research Laboratories) and added to SF21 cells as described previously (19). BV was harvested 3 days later and plaque purified by using SF21 cells and 100 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. For virus amplification, 2.5×10^6 SF21 cells were inoculated with individual plaques. After 4 days, 0.25 ml of the BV-containing growth medium was applied to a Hybond-N membrane by using a dot blot apparatus and treated successively with 1.5 M NaCl–0.5 M NaOH and 1.5 M NaCl–0.5 M Tris (pH 7.5)–1 mM EDTA. The BV DNA was hybridized to DNA probes that were radiolabeled with [α-^{32}P]dATP (>5,000 Ci/mmol) by using the random prime method (Amersham). Individual DNA probes were derived from *p35*, *lacZ*, and *p26* to verify the presence or absence of the appropriate sequences. Putative recombinant viruses were further plaque purified and verified for the proper insertion of DNA sequences by restriction mapping and Southern blot analysis using *hr5*-specific DNA probes.

Assays for CAT and β-galactosidase. SF21 cells, either infected or transfected, were harvested and lysed by three freeze-thaw cycles. Clarified cell lysates (16,000 × *g*) were assayed for CAT activity as described previously (8) and quantitated by scintillation counting or a Betascope 603 blot analyzer. Cell lysates were assayed for β-galactosidase by using the substrate *p*-nitrophenyl-β-D-galactopyranoside essentially as described elsewhere (19). The extracts were diluted with 0.25 M Tris (pH 7.8) to ensure linearity of the assay.

RNA primer extension. Total RNA was isolated from SF21 cells at the indicated times after infection by using the guanidine isothiocyanate-cesium chloride method (3). Primer extension assays were performed as described previously (8, 30) by using 5′-^{32}P-end-labeled oligonucleotide primers. CAT RNA was annealed to a 39-base primer (positions +15 to +53 relative to the ATG codon), *p35* RNA was annealed to a 38-base primer (positions +53 to +90 relative to the early RNA start site), and IE1/IE0 RNA was

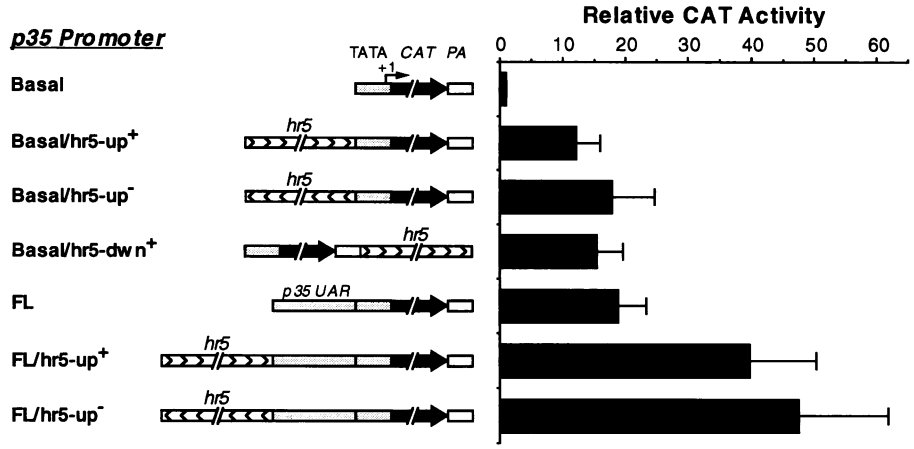

FIG. 2. Effect of *hr5* on expression from the *p35* promoter during infection. SF21 cells (2×10^6 per plate) were harvested 5 h after infection with the indicated viruses and assayed for CAT activity. The reported values were normalized to that obtained from the *p35* basal promoter and represent the averages of 8 to 16 independent assays. The positions of *hr5* and the *p35* promoter with respect to the CAT gene and its PA site are depicted for each recombinant virus. The *p35* basal promoter contains sequences from −30 to +12 which include the TATA box and RNA start site. The *p35* full-length (FL) promoter contains sequences from −226 to +12 which include the UAR (−110 to −30) and the basal promoter. Virus designations indicate the position (up or down) and orientation (+ or −) of *hr5* relative to the RNA start.

annealed to a 32-base primer (positions +99 to +130 relative to the early IE1 RNA start site).

RESULTS

***hr5* stimulates expression from the *p35* promoter while embedded within the genome of AcMNPV recombinants.** The position of *hr5* immediately downstream of *p35* (Fig. 1) and its capacity to stimulate *p35* promoter activity in transient expression assays (30) suggested that *hr5* plays a role in regulating *p35* expression during viral infection. To test this hypothesis, we first constructed a series of recombinant viruses in which *hr5* was inserted adjacent to the CAT gene linked to the *p35* basal promoter (−30 through the RNA start site, +1) or the *p35* full-length promoter (from −226 through the RNA start). Reporter sequences were inserted into the AcMNPV genome at a nonessential site upstream from the intact polyhedrin gene through allelic replacement. The effect of *hr5* on promoter activity was determined by monitoring CAT expression early (5 h) after infection, the period of maximum *p35* transcription.

When *hr5* was placed upstream from the *p35* basal promoter, early CAT expression increased approximately 15-fold compared with that directed by the basal promoter alone (Fig. 2). This increase was independent of the orientation of *hr5* when located upstream from the RNA start. Comparable stimulation was observed when *hr5* was placed downstream from the reporter gene. Both *hr5* and the *p35* UAR exhibited similar capacities to stimulate early CAT expression from the basal promoter (Fig. 2). When *hr5* and the UAR were placed in tandem, the stimulatory effect was additive; CAT expression was 40- to 50-fold higher than that provided by the basal promoter alone. In other experiments, *hr5* provided a comparable fold stimulation from the *p35* promoter when assayed as early as 1 and 2 h after infection (data not shown). Thus, *hr5*'s effect was evident well before the initiation of viral DNA replication (6 to 8 h after infection).

Primer extension assays verified that *hr5*-mediated stimulation was due to a proportional increase in steady-state levels of p35-CAT RNA when examined 5 h after infection (Fig. 3). For each virus, transcription initiated from the early *p35* RNA start site (position +1). Higher levels of steady-state RNA were detected when *hr5* was placed in either orientation upstream from the basal or full-length promoter. Thus, the *hr5*-mediated increase in CAT levels was due to transcription from the *p35* promoter rather than the initiation of new RNAs from *hr5*. Taken together, these results indicated that *hr5* has many of the properties characteristic of a transcriptional enhancer when embedded within the viral genome. Moreover, *hr5*-mediated stimulation was not dependent on the presence of UAR sequences.

A 28-bp palindrome within *hr5* stimulates early viral gene

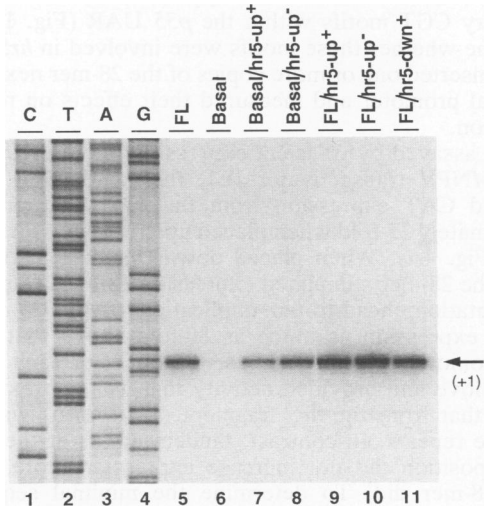

FIG. 3. Primer extension quantitation of p35-CAT RNAs. Total RNA was isolated from SF21 cells 5 h after infection with the indicated viruses and annealed to a 5'-end-labeled oligonucleotide primer complementary to the CAT gene. The extension products generated from equivalent amounts (7 μg) of RNA were subjected to polyacrylamide gel electrophoresis and autoradiography. The arrow denotes the *p35* RNA start site, +1. A dideoxy-chain sequencing ladder (lanes 1 to 4) of the *p35* promoter region was generated by using the same primer and plasmid p(Δ3'-162/Δ5'-154)35K-CAT.

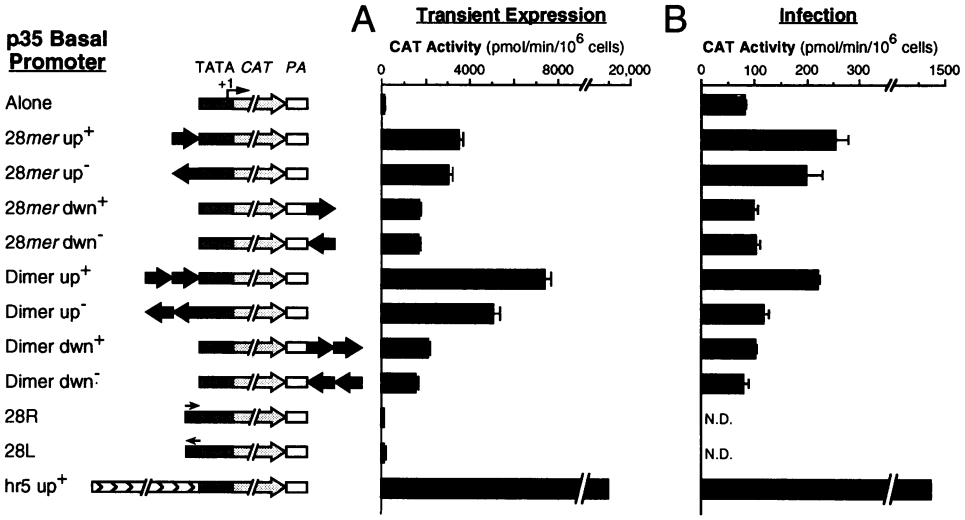

FIG. 4. Effect of the *hr5* 28-mer on expression from the *p35* basal promoter. (A) Transient expression. SF21 cells (2×10^6 per plate) were transfected with plasmid pIE-1 and plasmids containing the *p35* promoter-CAT sequences diagrammed at the left. Cell lysates were prepared 48 h later and assayed for CAT activity. The values reported represent the averages of triplicate transfections. CAT activity obtained after transfection with the *hr5* control (Basal/hr5-up$^+$) was 18,960 ± 3,330 pmol/min/10^6 cells. (B) Expression from recombinant viruses. SF21 cells were inoculated with viruses containing the *p35* promoter-CAT sequences shown on the left. Lysates were prepared 5 h after infection and assayed for CAT activity. The reported values represent the averages of duplicate assays conducted on two independently isolated viruses except for viruses Dimer-dwn$^+$ and Dimer-dwn$^-$, in which cases single isolates were used. CAT activity obtained after infection with the *hr5* control virus (Basal/hr5-up$^+$) was 1,470 ± 170 pmol/min/10^6 cells. The organization of the *p35* basal promoter and the 28-mer motifs are shown on the left; the dark arrow depicts the orientation of the 28-mer, either the same as (+) or opposite (−) that shown in Fig. 1D. Dimers were constructed by head-to-tail joining of two 28-mers. 28R and 28L contain only the rightmost and leftmost 17 nucleotides, respectively, of the 28-mer (see the legend to Fig. 1). N.D., not determined.

expression. Our finding that *hr5* compensated for loss of the *p35* UAR and that early transcriptional motifs within the UAR resemble repetitive sequences within *hr5* (8, 30) suggested that both contain common regulatory elements. Indeed, the 28-bp repeat (28-mer) derived from the leftmost palindrome of *hr5* contains two sequences that resemble the regulatory CGT motifs within the *p35* UAR (Fig. 1D). To determine whether these motifs were involved in *hr5* activity, we inserted one or more copies of the 28-mer next to the *p35* basal promoter and measured their effects on reporter expression.

When assayed by transient expression in the presence of the Ac*M*NPV transactivator IE1, the *hr5*-derived 28-mer increased CAT expression from the *p35* basal promoter approximately 35-fold when placed upstream in either orientation (Fig. 4A). When placed downstream from the promoter, the 28-mer stimulated expression 18-fold. Depending on orientation, head-to-tail duplication of the 28-mer increased expression as much as 80-fold above that of the basal promoter (Fig. 4A). Thus, the effect of each 28-mer was additive and provided activity that was approximately 40% of that from the *hr5* fragment (*Mlu*I-*Mlu*I) with four complete repeats. In contrast, tandem motifs at the downstream position did not increase expression more than a single 28-mer did. To determine the minimal sequences required for enhancer activity, the CGT-like motifs were independently deleted from the 28-mer. In each case, the leftmost or rightmost 17 nucleotides of the 28-mer alone failed to stimulate expression from the basal promoter (Fig. 4A). Thus, both halves of the palindrome, each containing a CGT-like motif, are required for minimal enhancer activity.

To determine the effect of the 28-mer when inserted into the viral genome, we constructed recombinant viruses in which the same *p35* promoter arrangements were used to direct expression of the CAT reporter gene. In both orientations upstream from the basal promoter, the 28-mer produced a 2.5- to 3-fold stimulation of expression when assayed early (5 h) after infection (Fig. 4B). This stimulation was sixfold lower than that provided by *hr5* itself. In contrast to the transient assays, upstream duplication of the 28-mer provided no additional stimulatory effect compared with a single 28-mer; moreover, stimulation was observed only in the sense orientation. When positioned downstream, the 28-mer exhibited no effect on *p35* basal promoter activity, even when duplicated. These findings suggested that additional regulatory motifs within *hr5* are required for maximum enhancer activity during infection.

Effects of *hr5* on other early and late viral promoters within the Ac*M*NPV genome. To determine whether *hr5*-mediated enhancement was limited to the *p35* promoter, we constructed recombinant viruses in which other promoters were linked to the CAT gene. When *hr5* was placed in either orientation upstream from the early Ac*M*NPV IE1 promoter, consisting of sequences from −161 to +11 (relative to the RNA start site, +1), CAT expression increased from two- to threefold when assayed early after infection (Fig. 5A). The IE1 promoter is active within the first hour of infection (17, 34). Quantitative primer extension assays demonstrated that CAT expression was proportional to the steady-state levels of IE1-CAT RNA initiated from the proper RNA start site that is composed of a conserved CAGT motif (data not shown). Insertion of *hr5* immediately upstream of the IE1 basal promoter containing only the TATA box (position −30) and RNA start site produced a 4- to 5-fold increase in early CAT expression from the virus genome and a 600-fold stimulation in transient expression assays (34).

In contrast, *hr5* had no effect on CAT expression directed

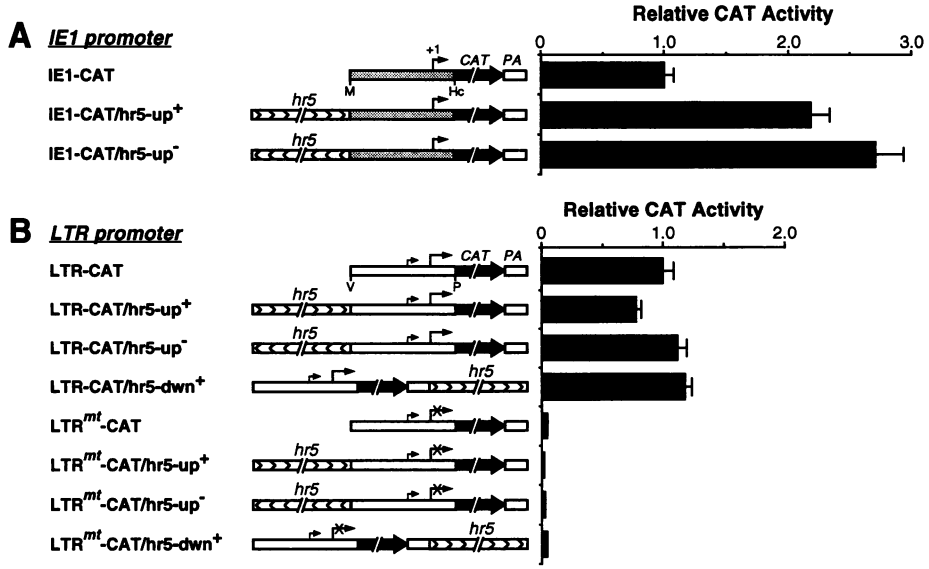

FIG. 5. Effect of *hr5* on expression from the IE1 and TED LTR promoters during infection. (A) IE1 promoter. Cell lysates prepared 5 h after infection with viruses containing the indicated IE1 promoter-CAT sequences were assayed for CAT activity, and results were normalized to that for virus IE1-CAT (without *hr5*). Reported values represent the averages of duplicate assays conducted on two independently isolated viruses; standard deviations (bars) are shown. The IE1 promoter fragment extends from −161 to +11 relative to the IE1 RNA start site, +1. (B) TED LTR. Lysates prepared 25 h after infection with viruses containing the indicated LTR-CAT sequences were assayed for CAT activity. Values were normalized to that for virus LTR-CAT and represent the averages of duplicate assays on single virus isolates. The RNA start site at the late baculovirus promoter (large arrow) and the U3-R junction (small arrow) are shown within the TED LTR. The late start (ATAAG) was replaced by the sequence GCGAG in LTR^{mt}-CAT viruses. Restriction site abbreviations are listed in the legend to Fig. 1.

by the LTR of the retrotransposon TED. The TED LTR contains a highly active late baculovirus promoter which is located 55 bp downstream from the normal transcriptional initiation site at the U3-R junction (11, 12). Levels of late (25 h) CAT expression were unaffected when *hr5* was placed upstream in either orientation or downstream from the LTR (Fig. 5B). Mutagenesis of the late baculovirus promoter motif (ATAAG) within the LTR dramatically reduced CAT levels, thereby demonstrating that the late promoter was the major contributor to late expression. *hr5* exhibited little, if any, effect on expression from the mutated LTRs (Fig. 5B).

Primer extension assays demonstrated that *hr5* had no effect on the steady-state levels of RNAs directed by either the late viral ATAAG promoter or the earlier U3-R promoter within the TED LTR (Fig. 6). Similar levels of LTR transcripts were detected 11 h after infection whether or not *hr5* was present, either upstream or downstream from the reporter gene. By 25 h after infection, the U3-R transcripts

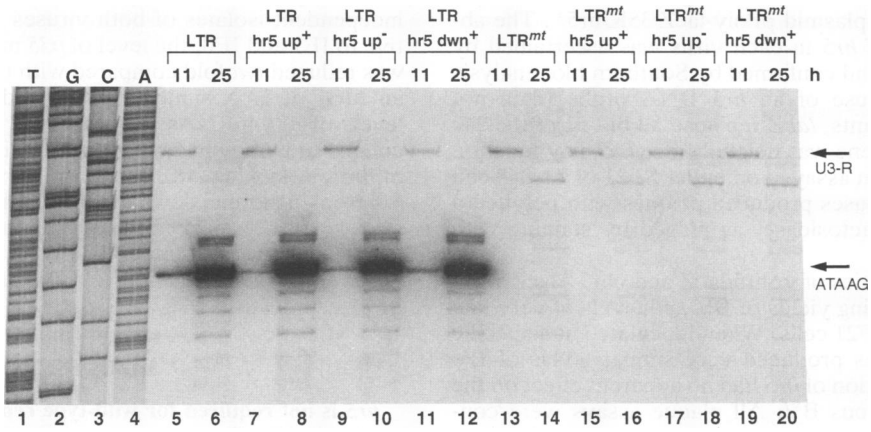

FIG. 6. Primer extension quantitation of RNAs initiated from the TED LTR with or without *hr5*. Total RNA isolated from SF21 cells 11 and 25 h after infection with the indicated LTR-CAT viruses was annealed to a 5′-end-labeled primer complementary to the CAT gene. The extension products generated from equivalent amounts (7 μg) of RNA were subjected to polyacrylamide gel electrophoresis and autoradiography. The positions of the RNA start at the ATAAG motif and the U3-R junction are indicated on the right. A sequencing ladder (lanes 1 to 4) of the LTR was generated by using the same primer and plasmid pLTR-CAT/PA/*hr5*-up$^+$.

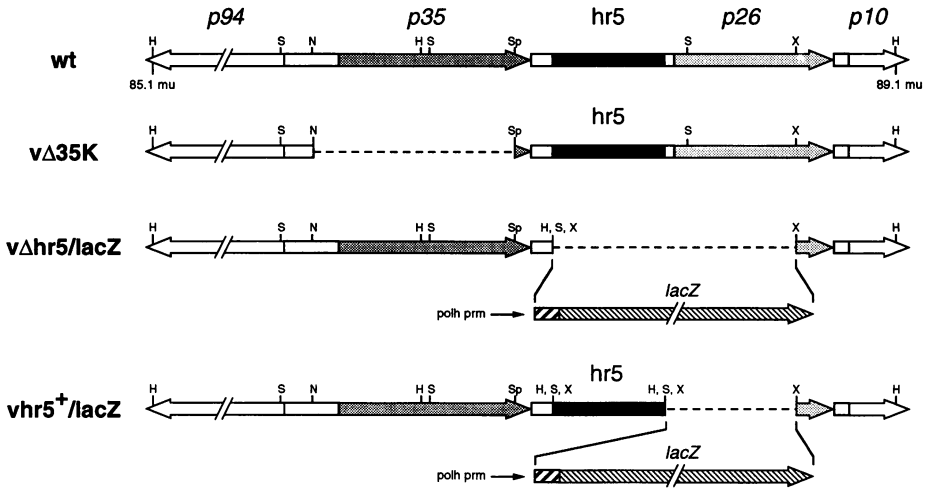

FIG. 7. Organization of the *hr5* locus of Ac*M*NPV mutants vΔhr5/lacZ and vhr5$^+$/lacZ. In the wild-type (wt) genome, *hr5* (black box) is located between *p35* and *p26*. Deletion mutant vΔ35K, which lacks *p35* and its promoter (dashed line), was used to generate *hr5* deletion mutant vΔhr5/lacZ and *hr5* insertion mutant vhr5$^+$/lacZ. In both cases, *p35* was linked to *lacZ* under control of the polyhedrin promoter (cross-hatched box) and inserted into the vΔ35K genome, thereby replacing *hr5* and most of *p26*. vhr5$^+$/lacZ contains a copy of *hr5* (*Mlu*I-*Mlu*I) inserted in the wild-type orientation. Restriction site abbreviations are listed in the legend to Fig. 1.

disappeared, whereas the level of the late ATAAG transcripts increased. Mutagenesis of the late ATAAG motif abolished transcription from this site and had no effect on transcription from the U3-R promoter (Fig. 6). Thus, the lack of detectable effects on transcription from the TED LTR suggested that *hr5* exhibits selective enhancement of promoter activity while embedded in the viral genome.

Deletion of *hr5* from the Ac*M*NPV genome. To directly assess *hr5*'s role in viral replication, we constructed Ac*M*-NPV mutants in which *hr5* was deleted from its native position (88 map units). Our mutagenesis strategy employed *p35* as a selectable marker during allelic replacement in which viral sequences were replaced with transfected plasmid sequences (26). Deletion mutant vΔhr5/lacZ (Fig. 7) was obtained by using transfer plasmid pPoly-lacZ/35K/Δhr5 and the parent virus vΔ35K, which lacked *p35*. The progeny virus obtained after transfection was screened for the acquisition of *p35* and *lacZ* as well as the loss of *p26*. The *hr5*-containing mutant vhr5$^+$/lacZ (Fig. 7) was isolated similarly, using transfer plasmid pPoly-lacZ/35K/hr5$^+$. The absence or presence of *hr5* in each virus was ascertained by restriction mapping and confirmed by Southern blot analysis of viral DNA with use of an *hr5* DNA probe (data not shown). In both mutants, *lacZ* replaced all but 51 of the 240 codons of *p26*; this gene was deleted since *hr5* may function as its promoter. When assayed on either SF21 or TN368 cell monolayers, both viruses produced plaques with polyhedra and expressed β-galactosidase, as judged by staining with X-Gal.

The growth properties of vΔhr5/lacZ and vhr5$^+$/lacZ were compared by measuring yields of BV and levels of very late gene expression in SF21 cells. When inoculated at a specific MOI, the two viruses produced very similar levels of BV (Fig. 8A). Thus, deletion of *hr5* had no apparent effect on the production of infectious BV. All plaque assays were conducted by using TN368 cells, in which viral growth is not dependent on *p35* expression (see below). The two viruses also exhibited comparable levels of very late gene expression, as demonstrated by the intracellular accumulation of β-galactosidase under control of the polyhedrin promoter (Fig. 8B). Although vhr5$^+$/lacZ produced a twofold-higher level of β-galactosidase at the lowest MOI, this difference diminished with increasing MOI. Taken together, these results indicated that *hr5* is not required for viral replication and that *hr5* does not confer a detectable growth advantage to Ac*M*NPV even at low multiplicities in cultured cells. Second, on the basis that both vΔhr5/lacZ and vhr5$^+$/lacZ produced levels of BV similar to that of wild-type virus, *p26* is not required for viral growth in SF21 cells.

Direct effect of *hr5* on levels of *p35* mRNA. Our finding that *hr5* deletion mutant vΔhr5/lacZ exhibited wild-type growth properties suggested that loss of *hr5* caused only minimal alterations in the level of *p35* expression that is required to block premature cell death of SF21 cells (5, 19). To directly assess *hr5*'s effect on transcription at the *p35* locus, we used primer extension assays to measure the steady-state levels of early *p35* mRNA (Fig. 9). By simultaneously including a primer complementary to the RNA transcripts of the immediate-early genes IE1 and IE0 (4), early mRNA levels from independent isolates of both viruses were compared. Relative to IE1 and IE0, the level of *p35* mRNA from vΔhr5/lacZ was reduced twofold compared with that from vhr5$^+$/lacZ at an MOI of 1. A similar twofold reduction in steady-state levels of *p35* mRNAs was observed when vΔhr5/lacZ was compared with wild-type virus containing a full-length copy of *hr5* but lacking *lacZ* (data not shown). Thus, removal of *hr5* from its normal downstream position reduced the steady-state levels of *p35* expression to about 50% of the wild-type level. This twofold reduction therefore had no apparent effect on viral replication or normal suppression of early SF21 cell death.

DISCUSSION

***hr5* is not required for wild-type replication of Ac*M*NPV in cultured cells.** By using a series of recombinant viruses, including *hr5* deletion mutants, we have investigated the function of the *hr5* element while embedded in the viral genome. Analysis of the growth properties of the *hr5* deletion mutant vΔhr5/lacZ indicated that loss of *hr5* had little or

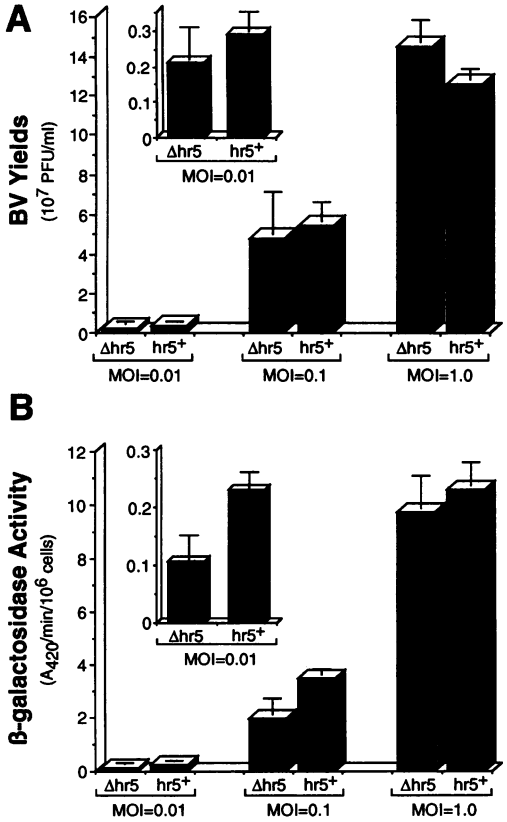

FIG. 8. Comparison of BV yields and late gene expression of AcMNPV *hr5* mutants. SF21 cells (2×10^6 per plate) were inoculated with the indicated MOI of either vΔhr5/lacZ (Δhr5) or vhr5$^+$/lacZ (hr5$^+$). After 1 h, the cells were washed and covered with growth medium. The growth medium and cells were harvested 48 h after infection and assayed for BV yield and β-galactosidase activity, respectively. (A) BV yields. BV in the extracellular growth medium was measured by plaque assay on TN368 cells, using X-Gal. Each value represents the average of triplicate assays performed by using four independently isolated viruses. (B) Polyhedrin promoter-directed *lacZ* expression. Intracellular β-galactosidase activity was determined by using lysates prepared from cells infected with four independently isolated viruses. The values shown represent the averages of duplicate infections for each of the four viruses. The insets show the values obtained with an MOI of 0.01 PFU per cell. In every case, the MOI was calculated from virus titers determined by using TN368 cells.

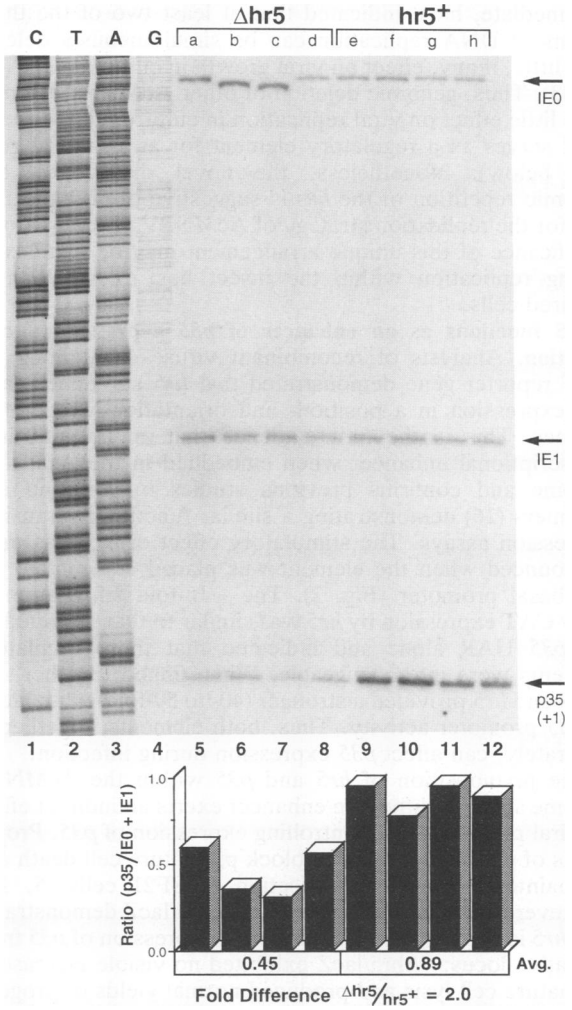

FIG. 9. Comparison of intracellular levels of *p35* mRNA in the presence and absence of *hr5*. SF21 cells were inoculated with a multiplicity of 1 PFU per cell, using four independent isolates of vΔhr5/lacZ (a to d; lanes 5 to 8) and vhr5$^+$/lacZ (e to h; lanes 9 to 12). Total RNA was isolated 5 h after infection and annealed simultaneously to 5′-end-labeled primers complementary to the *p35* and IE1/IE0 mRNAs. After extension with reverse transcriptase, the products were subjected to gel electrophoresis. In the autoradiogram shown, the sample volume loaded was adjusted so that IE1-specific extension products were similar. Individual extension products were measured, and the ratio of *p35* to the sum of IE1 and IE0 was calculated and is displayed as a histogram below each lane. The sequencing ladder (lanes 1 to 4) was generated by using a *p35*-specific primer and a plasmid containing *p35*. The slight alteration in mobility of IE0-specific extension products of Δ*hr5* viruses b and c (lanes 6 and 7) was not observed in other experiments (not shown).

no effect on the yield of BV or the levels of late gene expression over a range of virus multiplicities (Fig. 8). Deletion of *hr5* from its normal genomic position removed all but 17 bp of the leftmost *Eco*RI-containing palindrome and included the adjacent gene, *p26* (Fig. 7). Multiplication of vΔhr5/lacZ and vhr5$^+$/lacZ was indistinguishable from that of wild-type virus. In addition, both mutants produced plaques with polyhedra in TN368 and SF21 cells. Thus, *hr5* and *p26* are nonessential for viral replication in cultured cells; the function of *p26* is unknown. Together with findings of others (5, 10, 19, 39), our results suggest that the entire 5.3-kb region (Fig. 7) that includes *p94*, *p35*, *hr5*, *p26*, and *p10* (from 85 to 89 map units) is dispensable for AcMNPV replication in culture.

Several independent studies have suggested that the AcMNPV *hr*s serve as origins of DNA replication (6, 23, 32). Our data indicated that removal of one of these putative origins had little, if any, effect on viral DNA replication, since BV production and late gene expression were not significantly altered. Nonetheless, the direct effect of *hr5* deletion on the level or timing of viral DNA replication remains to be determined. Because of their genomic redundancy, loss of one *hr* may be compensated for by the remaining elements. Alternatively, other regions of the genome may provide the *cis*-acting signals for viral DNA replication (25). Analogous deletion studies with herpes simplex virus type 1, which also uses a circular DNA

intermediate, have indicated that at least two of the three origins of DNA replication can be simultaneously deleted with little, if any, effect on viral growth in cultured cells (22, 28, 33). Thus, genomic deletion of other Ac*M*NPV *hr*s may have little effect on viral replication in culture, unless the *hr* itself serves as a regulatory element for an essential gene (see below). Nonetheless, the novel distribution and genomic repetition of the *hr*s is suggestive of an important role for the replication strategy of Ac*M*NPV. The functional significance of this unique arrangement may be manifested during replication within the insect host rather than in cultured cells.

***hr5* functions as an enhancer of *p35* expression during infection.** Analysis of recombinant viruses containing the CAT reporter gene demonstrated that *hr5* stimulates early *p35* expression in a position- and orientation-independent manner. This is the first evidence that an *hr* acts as a transcriptional enhancer when embedded in the Ac*M*NPV genome and confirms previous studies by Guarino and Summers (16) demonstrating a similar function in transient expression assays. The stimulatory effect of *hr5* was most pronounced when the element was placed adjacent to the *p35* basal promoter (Fig. 2). The ~15-fold stimulation of early CAT expression by *hr5* was similar to that provided by the *p35* UAR alone and indicated that these regulatory elements were interchangeable. When linked together, the UAR and *hr5* provided a stronger (40- to 50-fold) stimulation of *p35* promoter activity. Thus, both elements, together or separately, can affect *p35* expression during infection.

The juxtaposition of *hr5* and *p35* within the Ac*M*NPV genome suggested that the enhancer exerts an indirect effect on viral replication by controlling expression of *p35*. Proper levels of p35 are required to block premature cell death and to maintain Ac*M*NPV replication in SF21 cells (5, 19). However, the growth properties of vΔhr5/lacZ demonstrated that *hr5* is not required for functional expression of *p35* from its native locus; vΔhr5/lacZ exhibited no visible increase in premature cell lysis and produced normal yields of progeny BV. Nonetheless, loss of *hr5* reduced early *p35* RNA levels by 50% (Fig. 9). Thus, the enhancer functions to supplement transcriptional activity provided by the *p35* UAR in SF21 cells. In the event of a deficiency of UAR-specific transcription factors in certain cell types, *hr5* may compensate by providing adequate promoter activity. Thus, the *hr5* enhancer may have a more influential role during replication in specific host tissues or different insect species. *hr5* may also function directly as the UAR for the adjacent p26 gene (Fig. 7).

***hr5* enhancement is promoter specific.** When embedded in the viral genome, *hr5* stimulated expression from the TATA-containing promoters of the immediate-early genes, *p35* and IE1 (Fig. 2 and 5). In contrast, *hr5* had no effect on the U3-R promoter or the late baculovirus-like (ATAAG) promoter of the TED LTR (Fig. 5 and 6). At high multiplicities, the accumulated levels of β-galactosidase under control of the polyhedrin promoter were identical for vΔhr5/lacZ and vhr5$^+$/lacZ (Fig. 7), suggesting that *hr5* has no direct effect on this very late viral promoter. *hr5* also failed to affect the polyhedrin promoter in transient expression assays (35). Collectively, these results suggest that *hr5* stimulation within the viral genome is promoter specific. *hr*-mediated enhancement may be limited to viral promoters that are responsive to RNA polymerase II at early times after infection. Our analyses also demonstrated more than 10-fold-lower levels of *hr5*-mediated promoter stimulation in viral infections compared with plasmid transfections. The greater effect of *hr5* in transient assays may be due to a combination of factors, including the lack of potential negative regulators present during infection and higher levels of the transactivator IE1. Although the lessened influence of *hr5* may also be affected by the redundancy of *hr*s within the Ac*M*NPV genome, this study underscores the importance of comparing transient assays with infection assays when evaluating the influence of transcriptional control elements.

Our data are consistent with a mechanism(s) in which *hr5* stimulates expression by increasing the rate of transcriptional initiation rather than by increasing the level of DNA template by affecting viral DNA replication. First, *hr5* stimulation of early *p35* and IE1 promoters was detected 1 h after infection, well before the expected start (6 to 8 h) of viral DNA synthesis. Second, the level of enhancement differed between early promoters (Fig. 2 and 5). Finally, *hr5* had no effect on transcription levels from the TED U3-R or ATAAG promoter even after the initiation of viral DNA replication (Fig. 6). These observations are incompatible with the possibility that increased levels of viral DNA caused an apparent enhancement of transcription. Thus, *hr5* is expected to affect rates of transcriptional initiation from the viral genome, supporting the same conclusion obtained by transient expression assays (16).

Structural organization of the *hr5* enhancer. In general, eukaryotic enhancers possess a modular organization in which the repetition of individual regulatory motifs provide expanded stimulatory activity (reviewed in references 9 and 18). As demonstrated by transient expression assays (Fig. 4A), the 28-mer alone was sufficient for enhancer activity, providing an 18- to 35-fold stimulation of *p35* promoter activity that was position and orientation independent. Consistent with a modular organization of *hr5*, duplication of the 28-mer resulted in increased stimulation that was only 2.5-fold lower than that conferred by *hr5* itself (Fig. 4A). Since both halves of the palindrome were required, the 28-mer represents the minimal sequence for activation. Progressive deletions of *hr5* also showed that partial stimulation was obtained from sequences that included a single palindromic repeat (16). Although the 28-mer alone was functional within the viral genome, it stimulated expression only when positioned immediately upstream from the *p35* promoter (Fig. 4B). Thus, additional *hr5* motifs may be required for long-range, enhancerlike activity during infection. Alternatively, an optimal spacing of repetitive 28-mers may be required.

Enhancer modules are often bipartite, consisting of individual motifs (enhansons) that represent the binding sites for transcriptional activators. A single enhanson may require a nearby identical or nonidentical motif for activity (9, 18). Our data are consistent with a hypothesis that the 28-mer is an *hr5* enhancer module, whereas the CGT-like motifs represent internal enhansons. Characteristic of enhansons, tandem CGT-like motifs may be required since both halves of the 28-mer were necessary for promoter stimulation. Our finding that the proximal CGT motif within the *p35* UAR is required for full *p35* promoter activity (8) but is not functional by itself (34) is suggestive of the cooperative nature of CGT motifs. Studies are under way to further compare the structural arrangement of activating motifs within *hr5* and different UARs as well as to examine the protein interactions involved.

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