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# Interspersed Homologous DNA of *Autographa californica* Nuclear Polyhedrosis Virus Enhances Delayed-Early Gene Expression

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**The five regions of homologous DNA which are interspersed in the genome of the baculovirus *Autographa californica* nuclear polyhedrosis virus increased the expression of a delayed-early gene of this virus. Although this activity was first observed as a 10-fold *trans* effect, the homologous region 5 (*hr*5) enhanced the expression of linked genes 1,000-fold. The *hr*5 enhancer also exhibited the other characteristics associated with viral enhancer elements, including orientation independence and the abilities to function at a distance from the linked promoter, to regulate heterologous promoters, and to increase the number of RNA polymerase molecules transcribing the linked genes. The expression of the immediate-early regulatory gene was not enhanced by *cis*-linked *hr*5, although the enhancer function may require the immediate-early regulatory gene product. The *hr*5 enhancer was relatively insensitive to competition by an excess of enhancer molecules. The nucleotide sequence of *hr*5 revealed two different conserved repeats separated by nonhomologous DNA. Deletion analysis of the *hr*5 enhancer indicated that a 30-base-pair inverted repeat was essential for enhancer function.**

The occluded baculoviruses are complex double-stranded DNA viruses which have two distinct infectious particles (for a review, see reference 13). Synthesis of the two forms is temporally regulated in infected cells. Extracellular virus is released from the cell by budding 12 to 18 h postinfection, and the viral infection spreads from cell to cell. Later in the infection (18 to 72 h postinfection), the virions are occluded in the nucleus. The occluded virions which are resistant to adverse environmental conditions are responsible for horizontal transmission of the virus.

Viral polypeptides are expressed in infected cells in a regulated cascade fashion and can be grouped into four distinct classes, referred to as immediate-early (α), delayed-early (β), late (γ), and very late (δ) proteins (19, 30, 37). The very late proteins, polyhedrin and the 10,000-molecular-weight protein (10K protein), are maximally expressed during the occlusion phase of viral replication. Several reports indicate that much of the temporal regulation of viral gene expression occurs at the level of transcription (1, 12, 46). Many regions of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome contain overlapping transcripts; some share a common 5′ end, while others have a common 3′ end (14, 34, 38). It has been hypothesized that these transcriptional units regulate the temporal expression of baculovirus genes (14). An AcNPV immediate-early regulatory gene (IE-1), which activates expression of a delayed-early gene, has been described (26), and the transition from delayed-early to late expression may be mediated by a virus-specific RNA polymerase (16).

The genome of the baculovirus AcNPV is a circular molecule of 130 kilobase pairs (kbp). Five regions interspersed along the length of the genome share homologous sequences (8). These homologous regions (*hr*s) are variable in length (500 to 800 base pairs [bp]) and contain repeats of *Eco*RI sites, separated by 72 to 215 bp. A similar pattern of interspersed homologous DNA is also found in another baculovirus, *Choristoneura fumeriferana* nuclear polyhedrosis virus (32), suggesting that the *hr*s play an important role in the life cycles of baculoviruses.

Repeated sequences have been described for a number of viruses. Unlike the baculovirus *hr*s, the repeated sequences of vertebrate viral genomes usually consist of perfect or nearly perfect repeats of similar size. A variety of functions have been ascribed to these repeated sequences: the direct repeats of simian virus 40 (SV40) and polyomaviruses serve as enhancers of gene expression and origins of DNA replication (3, 11); the replication of adenovirus DNA is initiated within the inverted terminal repeats (42); terminal repeats are required for the circularization of herpesvirus genomes (17, 31); and in Epstein-Barr virus, some of the repeated sequences are encoded into proteins (28). Here we demonstrate that at least one of the *hr*s of AcNPV is a very strong enhancer of gene expression.

## MATERIALS AND METHODS

**Transient expression assay.** The conditions for cell culture, transfections, and chloramphenicol acetyltransferase (CAT) assays have been described previously (26, 47).

**Construction of recombinant plasmids.** The plasmids were constructed by standard procedures. The plasmid p39CAT is similar to 39CAT-4 (26), except that the termination and polyadenylation signals are 39K sequences which were derived from a 1.3-kbp *Pvu*II fragment of *Pst*-K cloned into the *Sma*I site of pUC8. The promoter fragment of 39CAT-4 was cloned into the *Pst*I and *Bam*HI sites of the same plasmid, and the *Bam*HI CAT fragment was cloned into the *Bam*HI sites of the resulting plasmid. The *Hin*dIII site in the 39K coding region was removed by partial *Hin*dIII digestion, repair with the Klenow fragment of DNA polymerase 1, and religation. The *Hin*dIII site upstream of 39CAT is located in the multiple cloning site of pUC8. In 39CAT-Q$^+$, the orientation of AcNPV *Hin*dIII-Q is the same as that of the standard AcNPV genetic map (45). This is referred to as the genome sense (+) orientation, and the opposite orientation is referred to as the antisense (−) orientation. The plasmid pIE-1, which has been previously described (26), contains a

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3.0-kb *Cla*I-*Hin*dIII subclone of *Eco*RI-B of AcNPV DNA. In pIE-1down$^+$, *Hin*dIII-Q was cloned into the *Hin*dIII site downstream of the IE-1 coding region. In pIE-1down$^-$, the orientation of *Hin*dIII-Q was reversed. To construct pIE-1up$^+$, a 2.8-kb *Cla*I-*Xba*I subclone of *Eco*RI-B was cloned into the *Acc*I and *Bam*HI sites of pUC8 after *Xba*I and *Bam*HI sites were repaired with the Klenow fragment. This reversed the orientation of the insert relative to the vector so that the *Hin*dIII site was upstream of the IE-1 gene. *Hin*dIII-Q was cloned in the genome sense orientation (pIE-1up$^+$) or antisense orientation (pIE-1up$^-$).

**Analysis of gene expression.** Total cell RNA was purified and S1 nuclease analysis was performed as previously described (26, 47). The nuclei of transfected cells from three 6-cm plates were isolated (23), and nuclear transcription was performed as previously described (43). RNA was purified on guanidine isothiocyanate-CsCl gradients (7) and fragmented by partial alkali hydrolysis. For the analysis of in vitro-synthesized RNA, 1 μg each of p39CAT, pIE-1, and pUC8 was denatured and blotted onto nitrocellulose membranes, then hybridized to equal numbers of counts (41), washed, and exposed to Kodak X-AR5 film.

**DNA sequence analysis.** The *hr*5 enhancer was subcloned into M13 (36) and sequenced by the dideoxy chain termination procedure (39). The sequences were compiled and analyzed by the programs of Devereaux et al. (9).

## RESULTS

***hr* DNAs stimulating 39K expression.** Recently we reported that an immediate-early gene (IE-1) of AcNPV *trans*-activates expression of CAT under the control of the promoter for the delayed-early 39K gene (26). The genomic location of IE-1 was functionally mapped by a series of deletions of a plasmid containing *Eco*RI-B of AcNPV DNA (Fig. 1B). The plasmid p*Eco*RI-B activated expression of 39CAT (Fig. 2, lane 1). Therefore, we were surprised to observe in a subsequent experiment that *Eco*RI-restricted viral DNA did not activate 39CAT expression (Fig. 2, lane 3). This lack of activation could not be attributed to simple linearization of viral DNA, because digestion with several other restriction enzymes did not inhibit activation of 39CAT (26). This apparent contradiction suggests that although IE-1 efficiently induced 39K expression in a transient assay, an additional factor(s) was required for 39K expression in a viral infection.

First we determined that the *hr*s which are rich in *Eco*RI sites were involved in 39CAT expression. Five *Hin*dIII clones of AcNPV DNA (Fig. 1B) spanning the *hr* DNA were cotransfected with 39CAT in the presence or absence of *Eco*RI-digested viral DNA (Fig. 2). An equimolar mixture of all five *hr* plasmids did not stimulate CAT activity in the absence of viral DNA (Fig. 2A, lane 2). CAT activity also was not detected in cotransfections of 39CAT and individual *hr* plasmids (data not shown). However, when an equimolar mixture of the *hr* plasmids was cotransfected with *Eco*RI-digested DNA, CAT activity was induced (Fig. 2, lane 4). Cotransfection of the five plasmids individually with *Eco*RI-digested DNA stimulated CAT, indicating that all five *hr*s were not required for activation (Fig. 2, lanes 5 to 9).

The difference between cotransfection of p*Eco*RI-B, which stimulated 39CAT, and *Eco*RI-digested DNA, which did not stimulate 39CAT, was attributed to a combination of two factors, the concentration and the conformation of the plasmid DNA. Routinely, transfections were performed with 2 μg of 39CAT plasmid and 2 μg of viral DNA or *trans*-activating plasmid. Because p*Eco*RI-B is approximately 10 map units of the viral genome, the molar amount of *Eco*RI-B sequences cotransfected when the plasmid was tested corresponds to 10 times the amount cotransfected when viral DNA was tested. Cotransfection of an amount of p*Eco*RI-B equivalent to the molar amount (23 fmol) of that fragment in 2 μg of viral DNA stimulated the expression of 39CAT fourfold over background levels (Fig. 3A). Increasing the amount of p*Eco*RI-B two- or fivefold proportionally increased the level of CAT activity induced; 10 times as much p*Eco*RI-B was not significantly greater than 5 times (Fig. 2B, lanes 3 to 5). However, if p*Eco*RI-B was linearized by digestion with *Eco*RI before transfection, no activity was detected (data not shown), suggesting that transformation of the supercoiled plasmid DNA is more efficient. It has been reported for another system that supercoiled DNA is more efficiently expressed in transient assays than in linear or nicked DNA (21).

Alternatively, when the amount of p*Eco*RI-B was held constant at 23 fmol, CAT activity was stimulated up to 10-fold by the addition of a mixture of *hr* plasmids (Fig. 3B). Even the addition of *hr* sequences at 0.1 times the molar amount of *Eco*RI-B (2.3 fmol) stimulated CAT activity 3.5-fold. The results of this experiment indicate that the IE-1 gene was sufficient for the induction of 39CAT in the transient assay only when the gene was added in supercoiled form in extramolar amounts. When the amount of IE-1 was equivalent to the molar amount in 2 μg of viral DNA, then *hr* sequences were required for the activation of 39K.

The *hr*s are short repeated sequences (8), as are several enhancer-containing sequences which activate the expression of early regulatory genes in a number of viruses (18). However, the *hr*s activated 39CAT expression when cotransfected on separate plasmids, while enhancers acted only in *cis*. To determine whether the stimulation of 39CAT was indirectly due to in vivo recombination and the resultant *cis* activation of IE-1, we constructed plasmids containing AcNPV *Hin*dIII-Q (*hr*5) up- and downstream of IE-1 in both orientations. Cotransfection of p*Hin*dIII-Q with equimolar amounts of p39CAT and pIE-1 resulted in a 2.3-fold stimulation of activity compared with the results obtained with p39CAT and pIE-1 alone (Table 1). The activity obtained upon cotransfection of p39CAT and the four IE-1-*Hin*dIII-Q plasmids was not significantly greater, suggesting that the *hr* plasmids were not affecting the expression of IE-1. However, when *Hin*dIII-Q was cloned upstream of the 39K promoter, a dramatic increase in CAT activity was observed. The increase when *Hin*dIII-Q was cloned in the antisense orientation was significantly greater than in the genome sense orientation. Neither of the 39CAT plasmids containing *Hin*dIII-Q in *cis* was active in the absence of pIE-1.

***hr*5 is a transcriptional regulator.** Quantitative S1 nuclease analysis was performed to determine whether the increase in CAT activity was due to a proportional increase in the 39CAT message (Fig. 4). As previously reported (26), 39K transcripts isolated 6 h postinfection are heterogeneous in length at the 5′ end. A similar heterogeneous pattern was seen for the 39CAT transcripts in cells cotransfected with pIE-1 and 39CAT plasmids (Fig. 4A, lanes 1 to 7). Twice as much of the 39CAT probe was protected in cells cotransfected with 39CAT, IE-1, and the *hr*5 plasmid (p*Hin*dIII-Q) as in those cells transfected with 39CAT and IE-1 alone. When cloned in *cis*, *hr*5 enhanced transcription of 39CAT by at least 2 orders of magnitude. The activity of the enhancer

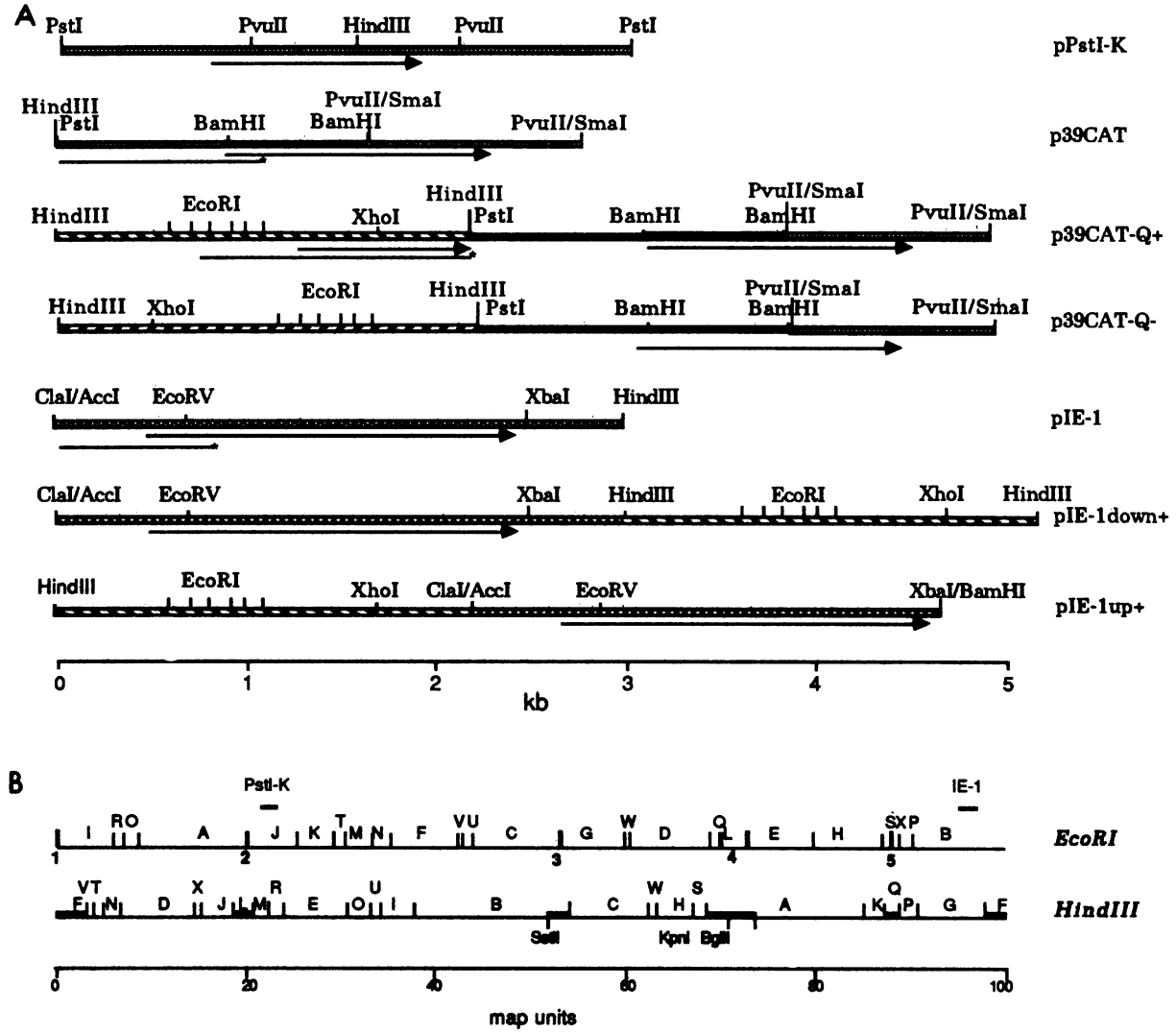


FIG. 1. Maps of the plasmids used in transfection studies and restriction maps of AcNPV DNA. (A) Plasmids constructed by standard techniques as described in Materials and Methods.$^+$, Genome sense orientation;$^-$, genome antisense orientation. (B) *Eco*RI restriction map of AcNPV DNA with the *hr*s denoted by heavy vertical lines numbered from 1 to 5. The *Hin*dIII clones which span the *Eco*RI sites are shown by shaded lines. To limit the size of the plasmids, a *Hin*dIII-*Sst*II subclone of *Hin*dIII-B was used for the *hr*3 plasmid, and a *Hin*dIII-*Bgl*II plasmid was used for *hr*4. The genomic locations of 39K (21.2 to 23.5 map units) and IE-1 (95.0 to 97.5 map units) are indicated.

cloned in the genome antisense orientation was fivefold greater than in the sense orientation.

To confirm the results of the CAT assay, which suggested that *cis* cloning of *Hin*dIII-Q in the pIE-1 plasmid did not affect the levels of IE-1 expression, we performed quantitative S1 nuclease analysis for cells transfected with pIE-1 and the four *cis* plasmids. The amount of the IE-1 probe protected by transfected cell RNA was similar for all plasmids (Fig. 4A).

In contrast to the polar effect seen with *Hin*dIII-Q, orientation independence was observed when a 484-bp *Mlu*I fragment containing only *hr* sequences (see Fig. 7) was cloned upstream of 39CAT in both orientations. RNA purified from cells cotransfected with these plasmids protected an amount of the 39CAT probe similar to that protected by cells transfected with 39CAT-Q$^-$ (Fig. 4A, lanes 5 to 7). This result suggests that the polar effect observed with *Hin*dIII-Q is not due to the enhancer region itself but to the flanking sequences.

A possible explanation for the polar effect of *Hin*dIII-Q is that another promoter interferes with the enhancement of 39CAT. Four transcripts have been mapped to *Hin*dIII-Q. Two of these are δ genes and would not be expected to be transcribed in a transient assay. One of the other two, a 1.5-kb transcript, initiates transcription to the right of *hr*5 on the AcNPV genetic map and terminates outside of *Hin*dIII-Q (38). Therefore, in the plasmid p39CAT-Q$^+$, transcription from the 1.5-kb promoter may extend into the 39K gene, possibly decreasing initiation at the 39K promoter. However, in the 39CAT-Q$^-$ plasmid, the directions of transcription of the 1.5-kb transcript and 39CAT are opposite and are separated by vector DNA, so interference would not be expected. To determine whether the promoter for the 1.5-kb transcript was active in cells transfected with 39CAT-Q$^+$ and pIE-1, RNA purified from transfected cells was hybridized with a probe specific for the 1.5-kb transcript. S1 nuclease analysis (Fig. 4B) revealed a protected band of 1,050 nucleotides, as expected for the 1.5-kb transcript, indicating that

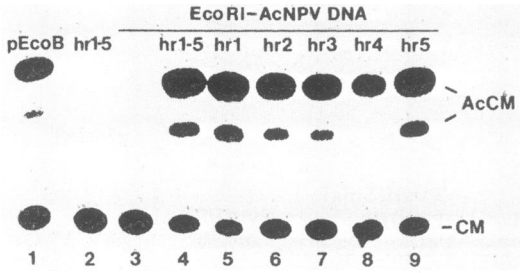


FIG. 2. Transient expression assays of 39CAT and homologous DNA regions. *S. frugiperda* cells were transfected and CAT assays were performed 24 h posttransfection as previously described (26), except that only 2 μl of extract was used for each assay. CM, Input unacetylated chloramphenicol; Ac-CM, acetylated chloramphenicol. Cells were transfected with 2 μg of p*Eco*RI-B (lane 1) or 2 μg of *Eco*RI-digested AcNPV DNA (lanes 3 to 9) plus an equimolar mixture of all five *hr*-containing plasmids (lanes 2 and 4) or an equimolar amount of *Hin*dIII-F (lane 5), *Hin*dIII-L (lane 6), *Hin*dIII-B (*Hin*dIII-*Sst*I subclone; lane 7), *Hin*dIII-A (*Hin*dIII-*Bgl*II subclone; lane 8), or *Hin*dIII-Q (lane 9).

the promoter for the 1.5-kb transcript was active in transfected cells.

The S1 nuclease analysis presented above indicates that the *hr5* enhancer increased the level of stable RNA but does not indicate whether *hr5* increased the rate of transcription. To determine whether the *hr5* enhancer increased the rate of transcription of 39CAT, we isolated nuclei from transfected cells and incubated them with [$^{32}$P]UTP under conditions in which elongation of previously initiated chains occurs. The relative amounts of RNA specific for 39CAT, IE-1, or vector DNA was determined by hybridization of in vitro-synthesized RNA to an excess of DNA immobilized on nitrocellulose. Because reinitiation was inhibited by the presence of 0.5% Sarkosyl, the amount of radioactive RNA that hybridized to a given fragment reflected the number of previously initiated RNA polymerase molecules transcribing a particular gene. The results of a nuclear transcription assay from *Spodoptera frugiperda* cells cotransfected with pIE-1 and p39CAT (lane 1) or pIE-1 and p39CAT-*hr5*$^+$ (lane 2) are shown in Fig. 5. The amounts of IE-1-specific RNA are equal in both lanes. However, the level of the 39CAT-specific transcript is 20-fold greater in nuclei isolated from cells transfected with the plasmid containing the *cis*-linked enhancer.

TABLE 1. *cis* and *trans* activation of 39CAT[a]

| Transfected plasmid(s) | CAT activity (pmol/min per $10^6$ cells) | Fold stimulation |
|---|---|---|
| p39CAT, pIE-1 | 1.2 | 1.0 |
| p39CAT, pIE-1, p*Hin*dIII-Q | 2.7 | 2.3 |
| p39CAT, pIE-1-Qup$^+$ | 3.1 | 2.6 |
| p39CAT, pIE-1-Qup$^-$ | 4.8 | 4.0 |
| p39CAT, pIE-1-Qdown$^+$ | 4.8 | 4.0 |
| p39CAT, pIE-1-Qdown$^-$ | 5.6 | 4.7 |
| p39CAT-Q$^+$, pIE-1 | 216.0 | 180.0 |
| p39CAT-Q$^-$, pIE-1 | 1,269.0 | 1,057.0 |
| p39CAT-Q$^+$ | 0.0 | 0 |
| p39CAT-Q$^-$ | 0.0 | 0 |

[a] *S. frugiperda* cells were transfected with 25 fmol of the indicated plasmids, and CAT assays were performed 24 h posttransfection. For accurate quantitation of CAT levels, cell extracts were diluted so that 30% or less of the input chloramphenicol was acetylated.

**Competition of 39CAT-Q with excess enhancer plasmid.** The results presented above indicate that *hr5* is a *cis*-active enhancer. A possible explanation for the observed *trans* stimulation is that some 39CAT and *hr* plasmid molecules recombine in vivo. This theory suggests that the activity of 39CAT-Q is fairly insensitive to competition by an excess of *Hin*dIII-Q plasmids, as most molecules probably do not recombine. To test this possibility, we cotransfected a constant amount of 39CAT-Q$^-$ with increasing amounts of p*Hin*dIII-Q. The addition of a 1.5-fold molar excess of p*Hin*dIII-Q had no effect on the level of CAT activity (Fig. 6). Cotransfection with p*Hin*dIII-Q in the range of a 3- to 150-fold molar excess over 39CAT-Q$^-$ actually increased CAT activity. Cotransfection greater than 150-fold molar excess decreased the enhancer activity. At the highest level of p*Hin*dIII-Q tested (1,500-fold excess), the CAT activity was decreased 86%, but even this value reflects a 140-fold enhancement of CAT activity compared with that obtained with p39CAT lacking the *hr5* enhancer.

***trans* activation of heterologous promoters.** To investigate the specificity of the *trans* activation by the *hr5* enhancer, we cloned *Hin*dIII-Q up- and downstream of the CAT gene under the control of the Rous sarcoma virus (RSV) long terminal repeat (20). The RSV long terminal repeat promoter is active in infected *S. frugiperda* cells (6). In cells transfected with RSVcat, there was no CAT activity detected in the absence of pIE-1. (Table 2). Cotransfection of

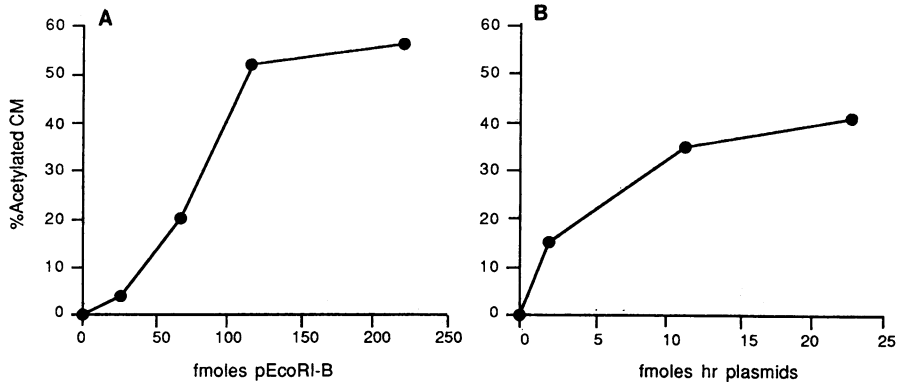


FIG. 3. 39CAT activity as a function of p*Eco*RI-B and p*Hin*dIII-Q concentration. *S. frugiperda* cells were transfected and assayed as described in the legend to Fig. 2. (A) Cells cotransfected with 2 μg of 39CAT and 23, 56, 115, or 230 fmol of p*Eco*RI-B (B). Cells cotransfected with 2 μg of 39CAT, 23 fmol of p*Eco*RI-B, and 2.3, 11.5, or 23 fmol of p*Hin*dIII-Q.

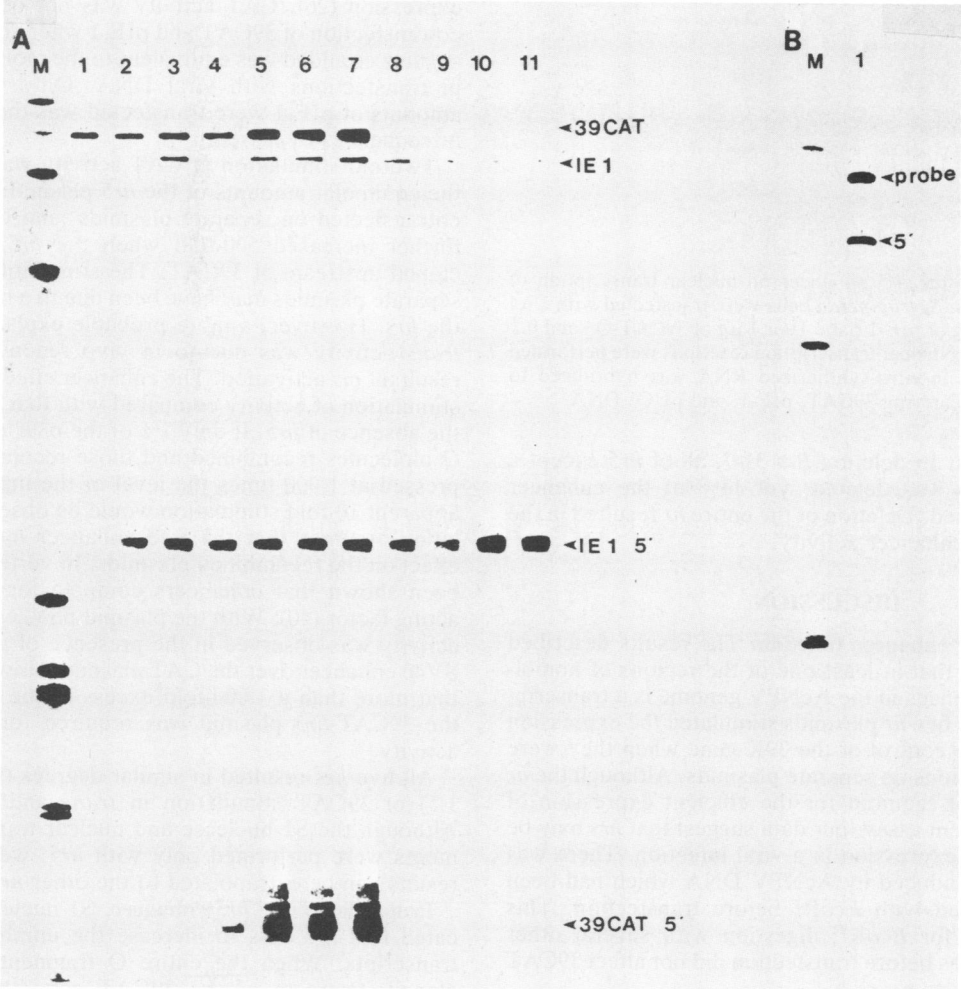


FIG. 4. The effect of *hr*5 on the quantity of stable 39K RNA in transfected *S. frugiperda* cells. Cytoplasmic RNA (20 μg) from transfected *S. frugiperda* cells was hybridized to an excess of an end-labeled DNA probe. The hybrids were digested with S1 nuclease, denatured, fractionated by polyacrylamide gel electrophoresis in the presence of 7 M urea, and autoradiographed as previously described (26). The positions of the probes and the 5′ ends of correctly initiated transcripts are indicated. The relative amounts of transcripts were estimated by densitometric scanning of autoradiograms from several experiments. An overexposure (not shown) was used to quantitate 39CAT probes in lanes 2 and 3. M, end-labeled markers. (A) S1 nuclease analysis of RNA from cells transfected with 0.1 μg of pIE-1 (lanes 2 to 11) and 1 μg of the indicated 39CAT plasmids and hybridized with a 940-nucleotide *Pst*I-*Pvu*II fragment end labeled at the *Pvu*II site (lanes 1 to 7) and an 850-nucleotide *Bam*HI-*Hin*fI fragment exclusively labeled at the *Hin*fI site (lanes 2 to 11). Lanes: 1, 39CAT in the absence of pIE-1; 2, 39CAT; 3, 39CAT and 0.1 μg of p*Hin*dIII-Q; 4, 39CAT-Q$^+$; 5, 39CAT-Q$^-$; 6, 39CAT-*hr*5$^+$; 7, 39CAT-*hr*5$^-$; 8, pIE-1-Qup$^+$; 9, pIE-1-Q-up$^-$; 10, pIE-1-Qdown$^+$; 11, pIE-1-Qdown$^-$. (B) S1 nuclease analysis of RNA from cells transfected with 0.1 μg of pIE-1 and 1 μg of 39CAT-Q$^+$ and hybridized with a 1,250-nucleotide *Eco*RV-*Hin*dIII fragment exclusively labeled at the *Hin*dIII site.

the AcNPV immediate-early regulatory gene IE-1 with RSVcat stimulated expression of CAT, and the addition of p*Hin*dIII-Q increased the level of expression eightfold. Cloning *hr*5 sequences up- or downstream of RSVcat stimulated CAT activity to a degree similar to that caused by the addition of IE-1 in *trans*. A dramatic effect on the expression of RSVcat was observed when RSVcat plasmids containing *hr*5 in *cis* were cotransfected with pIE-1.

**Nucleotide sequence of *hr*5.** The nucleotide sequence of *hr*5 is presented in Fig. 7. There are two conserved repeated sequences in *hr*5. A 30-bp sequence is repeated three times, twice perfectly and once with a 1-bp deletion. A 34-bp sequence is repeated six times, although with less fidelity (94 to 97%), and one repeat is incomplete. The *Eco*RI sites are part of a 30-bp imperfect palindrome contained in the 34-bp imperfect direct repeat. The conserved repeats are separated by nonhomologous DNA.

**Analysis of enhancer function by deletion mutagenesis.** To identify the functionally essential components of the enhancer region, we constructed deletion mutants of *hr*5. The plasmid 39CAT-Q$^-$ was digested with *Eco*RV, which cuts in the middle of *hr*5 and does not cleave elsewhere in the plasmid. After treatment with *Bal* 31 for various lengths of time, the plasmid was religated. Several deletions were analyzed for CAT activity in transfected *S. frugiperda* cells and were sequenced to determine the extent of deletion. The results indicate that most of the *Eco*RI region could be deleted with little effect (Fig. 8). A 50% loss of activity was seen with deletion *Bal* 31-1, which retained one of the conserved 30-bp repeats and one complete and one incom-

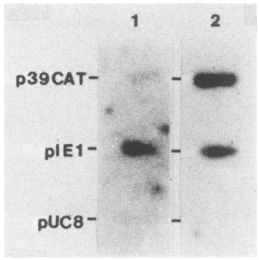


FIG. 5. Effect of the *hr5* enhancer on nuclear transcription of transfected plasmids. *S. frugiperda* cells were transfected with 1 μg of 39CAT and 0.1 μg of pIE-1 (lane 1) or 1 μg of 39CAT-Q⁻ and 0.1 μg of pIE-1 (lane 2). Nuclear transcription reactions were performed 24 h later, and the in-vitro-synthesized RNA was hybridized to nitrocellulose blots carrying 39CAT, pIE-1, and pUC8 DNA.

plete 34-bp repeat. In deletion *Bal* 31-7, all of *hr5* except a 30-bp palindrome was deleted, yet 13% of the enhancer activity was retained. Deletion of the entire *hr* resulted in the complete loss of enhancer activity.

## DISCUSSION

**Identification of enhancer function.** The results described here demonstrate that at least one of the regions of homologous DNA sequences in the AcNPV genome is a transcription enhancer. All five *hr* plasmids stimulated the expression of CAT under the control of the 39K gene when they were added to transfections on separate plasmids. Although the *hr* sequences are not required for the efficient expression of 39CAT in a transient assay, our data suggest that *hr*s may be essential for 39K expression in a viral infection. There was no CAT activity induced by AcNPV DNA which had been previously digested with *Eco*RI before transfection. This result is specific for *Eco*RI; digestion with several other restriction enzymes before transfection did not affect 39CAT expression (26). CAT activity was not observed upon the cotransfection of 39CAT and pIE-1 when the amount of IE-1 sequences added was equivalent to the molar amount of IE-1 in transfections with viral DNA. Only when extramolar amounts of pIE-1 were transfected was the requirement for *hr* sequences bypassed.

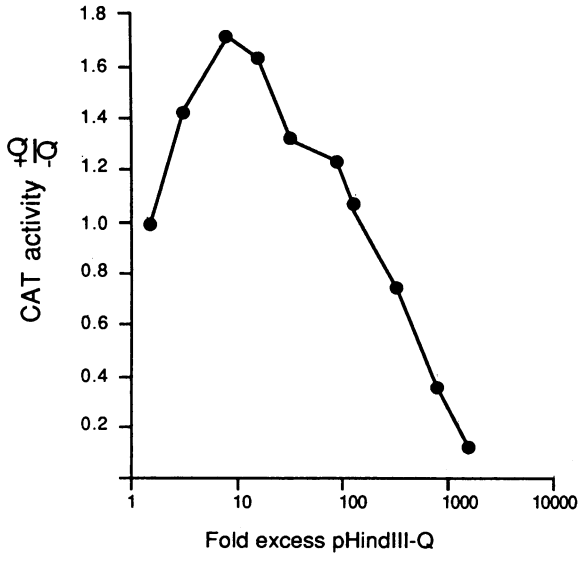


FIG. 6. Competition of 39CAT-Q⁻ and excess p*Hin*dIII-Q. Cells were cotransfected with 0.01 μg (2 fmol) of 39CAT-Q⁻ and increasing amounts of p*Hin*dIII-Q. Where necessary the total amount of DNA was adjusted to 10 μg with the addition of pUC8 DNA. The levels of CAT activity were plotted as the ratio of competing to noncompeting reactions against the logarithm of the amount of p*Hin*dIII-Q.

Twofold stimulation of CAT activity was observed when the equimolar amounts of the *hr5* plasmid and 39CAT were cotransfected on separate plasmids, and CAT activity was further increased 500-fold when the *hr5* sequences were cloned upstream of 39CAT. The stimulation observed with separate plasmids may have been due to a true *trans* effect of the *hr*s. However, a more probable explanation is that the *trans* activity was due to in vivo recombination and the resultant *cis* activation. The enhancer effect was a 1,000-fold stimulation of activity compared with that of transfection in the absence of *hr5*. If only 1% of the p39CAT and p*Hin*dIII-Q molecules recombined and those recombinants were expressed at 1,000 times the level of the input molecules, an apparent 10-fold stimulation would be observed. This calculation assumes that the free enhancer molecules have no effect on the recombined plasmids. In vertebrate cells, it has been shown that enhancers compete for a limiting *trans*-acting factor (40). With the plasmid pSV2cat, an 80% loss of activity was observed in the presence of a 2-fold excess of SV40 enhancer over the CAT plasmid. However, we showed that more than a 1,000-fold excess of the *hr5* plasmid over the 39CAT-*hr5* plasmid was required for an 80% loss of activity.

All five *hr*s resulted in similar degrees (within a factor of 1.5) of 39CAT stimulation in *trans* and also in *cis* (25). Although the S1 nuclease and nuclear transcription experiments were performed only with *hr5*, we believe that the results can be extrapolated to the other *hr*s.

**Properties of the *hr5* enhancer.** S1 nuclease analysis indicated that *hr5* acts to increase the number of stable 39K transcripts. When the entire Q fragment of *Hin*dIII was cloned upstream of the 39CAT gene, the *hr5* enhancer showed a polar effect. The enhancer was 10 times stronger in the antisense orientation than in the sense orientation. This is believed to be due to the proximity of another promoter which directs transcription into the 39K promoter. In the opposite orientation the promoter would direct transcription into the vector DNA, where it apparently does not interfere with 39CAT transcription. When a 484-bp fragment containing most of the enhancer region but lacking the adjacent promoter was cloned upstream of the 39CAT gene, this polar effect of orientation was not seen.

Nuclear run-on transcription indicated that *hr5*, like the SV40 enhancer (43, 48), acts to increase the number of RNA polymerase II molecules transcribing the linked gene. The

TABLE 2. *hr5*-enhanced expression of RSVcat[a]

| Transfected plasmid(s) | CAT activity (pmol/min per $10^6$ cells) |
|---|---|
| RSVcat | 0.0 |
| RSVcat, pIE-1 | 7.1 |
| RSVcat, pIE-1, p*Hin*dIII-Q | 58.4 |
| RSVcat-Qup | 5.9 |
| RSVcat-Qup, pIE-1 | 4,438.0 |
| RSVcat-Qdown | 20.5 |
| RSVcat-Qdown, pIE-1 | 7,700.0 |

[a] *S. frugiperda* cells were transfected with 0.25 pmol of the indicated plasmids, and CAT assays were performed 24 h posttransfection. For accurate quantitation of CAT levels, cell extracts were diluted so that 30% or less of the input chloramphenicol was acetylated.

```
        10                  30                  50                  70                  90
TTGAAAATTTATTGCCTAATATTATTTTTGTCAGTTCGTTGTCATTTATTAATTTGGATGATGTCCATTTGTTTTTAAAATTGAACTGGCTTTACGAGTA

       110                 130                 150                 170                 190
GAATTCTACGCGTAAAACACAATCAAGTATGAGTCATAAGCTGATGTCATGTTTTGCACACGGCTCATAACCGAACTGGCTTTACGAGTAGAATTCTACT
Eco RI  Mlu I                                                                          Eco RI

       210                 230                 250                 270                 290
TGTAACGCACGATCGAGTGGATGATGGTCATTTGTTTTTCAAATCGAGATGATGTCATGTTTTGCACACGGGCTCATAAACTGCTTTACGAGTAGAATTC
                                                                                       Eco RI

       310                 330                 350                 370                 390
TACGTGTAACGCACGATCGATTGATGAGTCATTTGTTTTGCAATATGATATCATACAATATGACTCATTTGTTTTTCAAAACCGAACTTGATTTACGGGT
                                               Eco RV

       410                 430                 450                 470                 490
AGAATTCTACTCGTAAAGCACAATCAAAAAGATGATGTCATTTGTTTTTCAAAACTGAACTCTCGGCTTTACGAGTAGAATTCTACGTGTAAAACACAAT
 Eco RI                                                                 Eco RI

       510                 530                 550                 570                 590
CAAGAAATGATGTCATTTGTTATAAAAATAAAAGCTGATGTCATGTTTTGCACATGGCTCATAACTAAACTCGCTTTACGGGTAGAATTCTACGCGTAAA
                                                                                       Eco RI  Mlu I

       610                 630                 650                 670                 690
ACATGATTGATAATTAAATAATTCATTTGCAAAGCTATACGTTAAATCAAACGGACGTTATGGAATTGTATAATATTAAATATGCAATTGATCCAACAAA

       710
TAAAATTATAATAGAGCAAGTCGAC
                  Sal I
```

FIG. 7. Nucleotide sequence of the AcNPV *hr*5 enhancer and flanking DNA. Two classes of imperfect repeats are overlined: ---, 34-bp repeat; •••, 30-bp repeat. Some restriction sites are indicated.

enhancer effect observed in the nuclear transcription experiment was significantly less than the enhancer effect observed in the CAT assay and S1 nuclease analysis. A similar discrepancy has been noted for the SV40 enhancer (43).

Viral enhancers were originally identified as upstream promoter elements required for efficient early transcription of regulatory genes in SV40 and polyomaviruses (2, 10, 15, 24, 44). Since then, enhancers have been found upstream of the adenovirus EIA gene (27, 49), upstream of the promoter for IE mRNA 3 of herpes simplex virus type 1 (33), upstream of a major IE RNA of human cytomegalovirus (5), and downstream of a bovine papilloma virus major IE transcript (35). Because of this consistent association of enhancers and early regulatory genes, we expected that the *hr*s would increase the expression of 39CAT indirectly by their enhancement of the transcription of IE-1, which regulates 39CAT expression. However, *hr*5 was found to increase transcription of 39CAT directly and to have little effect on transcription of IE-1. It will be of interest to determine whether any of the other *hr*s enhance expression of IE-1 or whether IE-1 is regulated by a different type of enhancer or no enhancer.

Although the *hr*5 enhancer did not affect IE-1, enhancer activity may require the immediate-early gene. Neither CAT activity nor transcription from the 39K promoter was detected in the absence of IE-1, even in the presence of a linked enhancer. The RSV long terminal repeat promoter was activated by *cis-hr*5, but the addition of pIE-1 further stimulated expression 375- to 750-fold. The adenovirus and herpesvirus transcriptional regulatory proteins can substitute for enhancers (22), and a requirement for the adenovirus EIA protein can be circumvented by the E1A enhancer (29). A combination of these transcription regulatory factors, however, did not produce a synergistic effect as did the AcNPV enhancer and IE-1 gene. Further experiments are needed to determine whether IE-1 interacts with the enhancer or whether the requirement for IE-1 merely reflects the low level of transcription from these promoters in its

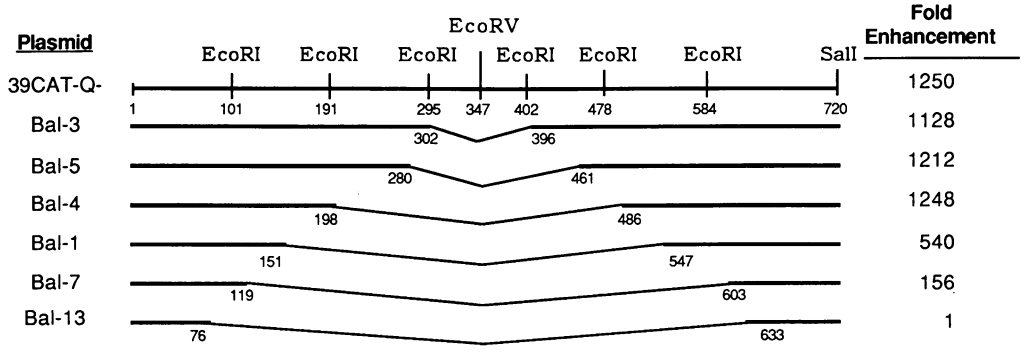


FIG. 8. Construction and activity of 39CAT-Q⁻ plasmids with deletions in the enhancer region. The plasmid p39CAT-Q⁻ was digested with *Eco*RV and incubated with *Bal* 31 for different lengths of time. Deletions of various sizes were tested for CAT activity in the transient assays. The extent of deletion was determined by DNA sequence analysis and indicated by sloping lines. The numbers refer to the base numbers in Fig. 7 which are adjacent in the deletion mutant.

absence. In this respect IE-1 differs from the adenovirus E1A protein, which negatively regulates enhancers (4). The AcNPV *hr* enhancers also differ from other viral enhancers in that the *hr*s are not physically associated with the immediate-early regulatory gene but are interspersed throughout the genome. *hr*5, which is closest to IE-1 on the genetic map, is located approximately 5 kb upstream of the IE-1 promoter.

**Nucleotide sequence of the *hr*5 enhancer.** The AcNPV *hr*5 contains repeated sequences, as do many viral enhancers. Unlike the perfect tandem repeats of SV40, *hr*5 contains imperfect repeats separated by nonhomologous DNA. In this respect, the *hr*s are more similar to the human cytomegalovirus enhancer than to other viral enhancers (5). The enhancer elements of both human cytomegalovirus and AcNPV are quite large, approximately 500 bp. At the nucleotide level, however, there is no significant homology between the *hr*5 enhancer and the human cytomegalovirus enhancer. There are no sequence motifs common to all enhancers; however, many enhancers contain the TGG$^{AAA}_{TTT}$G motif (50). Analysis of *hr*5 did not reveal any significant homology to this sequence.

Deletion analysis of the *hr*5 enhancer indicated that the multiple repeats are not essential for activity. Most of *hr*5 can be deleted without a loss of activity. The minimum requirement for activity that we have defined is one of the 30-bp inverted repeats. In other viruses which contain repeated sequences, it has also been shown that the repeats are not essential for activity but may potentiate activity (11, 48).

The AcNPV enhancer is similar to vertebrate viral enhancers with respect to orientation independence, ability to function at a distance, ability to regulate heterologous promoters, and ability to increase the number of RNA polymerase molecules transcribing the linked gene. *hr*5 differs from other enhancers in its lack of physical association with the immediate-early regulatory gene and its apparent inability to function in the absence of the immediate-early gene product.

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