A Mechanism for Negative Gene Regulation in *Autographa californica* Multinucleocapsid Nuclear Polyhedrosis Virus†

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The *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) *ie-1* gene product (IE-1) is thought to play a central role in stimulating early viral transcription. IE-1 has been demonstrated to activate several early viral gene promoters and to negatively regulate the promoters of two other AcMNPV regulatory genes, *ie-0* and *ie-2*. Our results indicate that IE-1 negatively regulates the expression of certain genes by binding directly, or as part of a complex, to promoter regions containing a specific IE-1-binding motif (5'-ACBYGTAA-3') near their mRNA start sites. The IE-1 binding motif was also found within the palindromic sequences of AcMNPV homologous repeat (*hr*) regions that have been shown to bind IE-1. The role of this IE-1 binding motif in the regulation of the *ie-2* and *pe-38* promoters was examined by introducing mutations in these promoters in which the central 6 bp were replaced with *Bgl*II sites. GUS reporter constructs containing *ie-2* and *pe-38* promoter fragments with and without these specific mutations were cotransfected into Sf9 cells with various amounts of an *ie-1*-containing plasmid (pIE-1). Comparisons of GUS expression produced by the mutant and wild-type constructs demonstrated that the IE-1 binding motif mediated a significant decrease in expression from the *ie-2* and *pe-38* promoters in response to increasing pIE-1 concentrations. Electrophoretic mobility shift assays with pIE-1-transfected cell extracts and supershift assays with IE-1-specific antiserum demonstrated that IE-1 binds to promoter fragments containing the IE-1 binding motif but does not bind to promoter fragments lacking this motif.

The *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) has a circular, supercoiled double-stranded DNA genome of about 134 kb (2) and replicates in the nucleus of infected insect cells. In AcMNPV-infected cells the expression of viral genes occurs in a temporally regulated fashion resulting in a cascade of early, late, and very late gene transcription (reviewed in reference 4). Transcription of early viral genes is mediated by the host RNA polymerase II transcription machinery. Many baculovirus early genes have a TATA box located 25 to 30 bp upstream of a conserved mRNA transcription initiation site that consists of the consensus sequence CAGT (3, 4). Baculovirus late transcription, beginning at or shortly after the onset of viral DNA replication, is associated with a virally induced, α -amanitin-resistant RNA polymerase activity (10, 15, 37). This virus-specific RNA polymerase activity is also responsible for transcription during the very late phase, resulting in high-level expression of the polyhedrin and p10 genes.

Our understanding of how the transcriptional cascade is regulated during baculovirus infection is limited. It is thought that the *ie-1* gene product (IE-1) plays a central role in stimulating early transcription. The *ie-1* gene has been demonstrated to activate several early promoters including 39K, *ie-2*, and p35 (6, 12, 13, 27). The stimulatory effect of *ie-1* is greatest when the target promoter is *cis* linked to palindromic sequences from the AcMNPV homologous repeat (*hr*) regions.

hr regions, which are present at eight dispersed locations on the AcMNPV genome, are composed of repeated imperfect palindromes within directly repeated sequences and have been shown to act as enhancers (5, 9, 13, 25–27). Electrophoretic mobility shift assays (EMSAs) have shown that in vitro-translated IE-1 binds to *hrs* (9). Whole-cell extracts prepared from Sf9 cells transfected with the *ie-1* gene also contain proteins that bind to *hrs* (11, 25, 30, 31), suggesting that IE-1 may activate transcription by binding to DNA. In addition to its stimulatory function, the *ie-1* gene has been shown to negatively regulate transcription from the promoters of two other AcMNPV regulatory genes, *ie-0* (20) and *ie-2* (formerly *ie-N*) (7).

In addition to *ie-1*, several other baculovirus genes are known to encode regulatory functions. IE-0 is identical to IE-1, except that a transcriptional splicing event results in the addition of 54 amino acids to the N terminus (8). Unlike IE-1, which has been shown to activate the 39K promoter both in the presence and in the absence of a *cis*-linked *hr*, activation by IE-0 has been shown to require an *hr* enhancer (20). A further difference between IE-0 and IE-1 is that IE-0 does not appear to downregulate the *ie-0* promoter (20). The *ie-2* gene was initially characterized as augmenting *ie-1* transactivation (5) and was subsequently shown to be capable of stimulating early gene expression independently of *ie-1* (6, 7, 38). The *pe-38* gene has been shown to activate expression from the p143 gene promoter (26). Other AcMNPV genes, such as *me-53* and *cg-30*, are also suspected of encoding *trans*-regulatory functions based on sequence comparisons with known transcriptional regulators from other systems (17, 36). Host proteins that interact with promoter and enhancer regions may also play important roles in regulating baculovirus gene expression. A 38-kDa host protein has been demonstrated to interact with high specificity and affinity to *hr1* sequence elements and has been implicated in the enhancer function of *hr1* (14).

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In this report, we demonstrate that a conserved IE-1 binding motif exists near the sites of transcriptional initiation in all promoters that are known to be negatively regulated by IE-1. Data from mutational analyses are presented indicating that binding of IE-1 to these sequences results in negative regulation of the *ie-2* and *pe-38* promoters.

MATERIALS AND METHODS

Virus and cell line. The E2 strain of AcMNPV (34) was used in this study. *Spodoptera frugiperda* (Sf9) cells (American Type Culture Collection [accession no. CRL1711]) were used for propagation of virus and in the experiments described here.

Construction and propagation of plasmids. Restriction and DNA modifying enzymes were purchased from New England Biolabs. Plasmids used for transfections were propagated in *Escherichia coli* DH5 α and purified on Qiagen columns (Qiagen, Inc.). Plasmids pIe-1, p39KGUSpl18, and pHdQ39KGUS have been described previously (25).

GUS expression clones. Plasmid clones containing the AcMNPV *ie-2* and *pe-38* genes were previously described (18). A *Bam*HI-*Eco*RV insert containing the *ie-2* gene and flanking sequences and a *Hind*III-*Eco*RI fragment containing the *pe-38* gene and flanking sequences were cloned into pBluescriptKS[−] (pBKS[−]) (Stratagene, Inc.), creating pKSle-2 and pKSPe-38, respectively. *Nco*I restriction sites were introduced at the positions of the first ATG codons of the *ie-2* and *pe-38* open reading frames (ORFs) via site-directed mutagenesis (23) to form pKS2le-2Nco and pKSPe-38Nco. The pKSle-2Nco and pKSPe-38Nco plasmids were digested with *Nco*I, blunt ended with T4 DNA polymerase in the presence of deoxynucleoside triphosphates (32), and redigested with *Pst*I, and then the 336-bp *ie-2* promoter-containing fragment and the 110-bp *pe-38* promoter-containing fragment were gel purified. pHdQ39KGUS (25) was digested with *Bam*HI, blunt ended, and redigested with *Pst*I, and then the larger fragment containing the AcMNPV *Hind*III-Q region, the β -glucuronidase (GUS) gene, and the vector sequences was gel purified. The gel-purified *ie-2* and *pe-38* fragments were ligated to the GUS vector fragment to construct the reporter plasmids pIe-2-QGUS and pPe-38-QGUS. pIe-2 $hs^−$ -QGUS and pPe-38 $hs^−$ -QGUS were constructed similarly with pKSle-2 $hs^−$ and pKSPe-38 $hs^−$ (see below) as the sources of the promoter fragments. pIe-2-GUS, pIe-2 $hs^−$ -GUS, pPe-38-GUS, and pPe-38 $hs^−$ -GUS were generated from pIe-2-QGUS, pIe-2 $hs^−$ -QGUS, pPe-38-QGUS, and pPe-38 $hs^−$ -QGUS, respectively, by digesting each plasmid with *Hind*III, which results in the loss of the AcMNPV *Hind*III-Q fragment upon subsequent religation.

Generation of promoter mutants. Plasmids containing 6-bp substitution mutations in the *ie-2* and *pe-38* promoter regions (see Fig. 1b) were generated via site-directed mutagenesis (23) of pKSle-2Nco with an oligonucleotide of sequence 5'-GCTGCCGTTCTAAGATCTAGCACAGTTCAG-3' and of pKSPe-38Nco with an oligonucleotide of sequence 5'-AGGCACTACCAAA GATCTAGCACAGTTCGTT-3'.

Transfection experiments. Sf9 cells were propagated at 27°C in TNM-FH medium (35) containing 10% fetal bovine serum, penicillin G (50 U/ml), streptomycin (50 μ g/ml; Whittaker Bioproducts), and fungizone (375-ng/ml amphotericin B; Flow Laboratories). Log-phase cells were seeded into six-well plates (10⁶ cells/well) and allowed to attach for 12 to 16 h. The medium was removed from the attached cells and replaced with 0.5 ml of Grace's insect cell culture medium (Life Technologies, Inc.) with 10% fetal bovine serum. An equal volume of transfection buffer (25 mM HEPES [pH 7.1], 140 mM NaCl, 125 mM CaCl₂·2H₂O) containing plasmid DNA was applied dropwise to the well and mixed thoroughly by gentle rotation of the titer plate. After 4 h, the transfection mixture was removed and replaced with 1 ml of fresh TNM-FH medium, and the cells were incubated for 24 h at 28°C. The cells were harvested by scraping with a rubber policeman and processed for GUS assays as described below.

Transfection and infection experiments. The transfection and infection experiments were done similarly, except that the cells were seeded at 1.25 $\times$ 10⁶ cells/well and the transfection mixture was added after the cells had attached for 4 h. The cells were incubated with the transfection mixture for 4 h, and then the transfection mixture was removed and replaced with TNM-FH. Twelve hours after replacement of the transfection mixture with TNM-FH, the medium was removed and replaced with virus-containing TNM-FH medium (multiplicity of infection of 10). At various times postinfection (p.i.), the cells were harvested by scraping with a rubber policeman and processed for GUS assays as described below.

GUS assay. The detached cells were transferred to an Eppendorf tube and pelleted by centrifugation at 7,000 rpm for 3 min in a Microfuge. The cell pellets were resuspended in 200 μ l of phosphate-buffered saline (120 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ [pH 7.4]) and lysed by three freeze-thaw cycles. Insoluble debris was removed by centrifugation at 7,000 rpm for 3 min, and the supernatants were assayed for GUS activity by a fluorogenic assay (16).

Preparation of whole-cell extracts. Whole-cell extracts were prepared as described previously (25).

Production of IE-1 antiserum. An *Nco*I site was introduced at the position of the first ATG codon of the *ie-1* gene in pIe-1 (25) by site-directed mutagenesis

of pIe-1 to create pIe-1Nco. A seven-histidine N-terminal fusion construct of *ie-1* (pHT-IE1) was made by cloning the *ie-1* gene containing the *Nco*I-*Kpn*I fragment from pIe-1Nco into pTrc-7Hpro (a gift from Bill Dougherty), a modified form of pTrc99A (Pharmacia Biotech) that contains an *Nco*I site downstream of seven histidine codons. The histidine-tagged-IE1 fusion protein was expressed in pHT-IE1-transformed *E. coli* BL21 and was purified by chromatography on Ni-nitrilotriacetic acid resin (Qiagen) according to the manufacturer's instructions. Purified protein (500 μ g) emulsified in complete Freund's adjuvant was intradermally injected into a New Zealand White rabbit. Subsequent injections of 100 to 200 μ g of fusion protein emulsified in incomplete Freund's adjuvant were given biweekly starting during the third week after the initial injection. Immune serum was collected 7 days after each booster injection. For these experiments, we used antisera collected 11 days after the third booster injection.

EMSAs. pIe-2-GUS and pIe-2 $hs^−$ -GUS were digested with *Nco*I and *Sca*I, and pPe-38-GUS and pPe-38 $hs^−$ -GUS were digested with *Hind*III and *Bam*HI. The resulting fragments were dephosphorylated with calf intestinal phosphatase, and the promoter-containing fragments from each clone were isolated and end labeled with T4 polynucleotide kinase in the presence of [γ -³²P]ATP (32). After purification on Midi-Select G-50 spin columns (Intermountain Scientific Corporation), the radiolabeled probes were used for EMSAs as described previously (25). Supershift analyses were done by including IE-1-specific rabbit polyclonal antiserum in the reactions. A preimmune rabbit serum was used in the control reactions.

RESULTS

Identification of putative IE-1-binding sites in AcMNPV negatively regulated promoters. Mutational analysis of *hr* palindromes from both *hr1a* (25) and *hr5* (11, 31) suggests that IE-1 binds to specific sequences immediately flanking the *Eco*RI site that is present at the center of each palindrome. Since the promoters of the *ie-0* and *ie-2* genes have been shown to be negatively regulated by *ie-1* (references 20 and 7, respectively), we examined the possibility that such negative regulation might be mediated through IE-1 binding sites within these promoter regions. A Gibbs sampling strategy for multiple sequence alignments (24) was used to compare nucleotide sequences that have the ability to specifically bind IE-1. Deletion and substitution mutations in *hr1a* (25) and *hr5* (11, 31) that still retained the ability to bind IE-1 were included in the analysis. We also included information about the number of complexes formed during EMSAs with each *hr*-containing fragment (11, 25, 31). This allowed us to identify similar sequence motifs within a particular DNA fragment. An 8-bp sequence, AC(C,G,T)(C,T)GTAA, was identified as a possible IE-1 binding motif (Fig. 1a). A search of the AcMNPV genome revealed 111 matches to this sequence, 72 of which are clustered in *hr* regions. Every *hr* palindrome was found to have two copies of this sequence oriented in opposite directions flanking a central *Eco*RI site. In both the *ie-0* and the *ie-2* promoters, which have been shown to be negatively regulated by IE-1, an IE-1 binding motif was found positioned 3 to 4 bp upstream of a CAGT motif which is near the sites of transcriptional initiation (Fig. 1a). We refer to the IE-1 binding motif in these promoters as a half-site (*hs*) sequence because of its resemblance to the IE-1 binding site consensus found in each half of the *hr* palindrome. Our analysis revealed that the *pe-38* promoter also contains an *hs* sequence near the transcription initiation site (Fig. 1a). Of the remaining IE-1 binding motifs, there is a set of oppositely oriented overlapping pairs in the promoter regions of ORF 121 (2); several are positioned downstream of ORFs, and the majority are found within ORFs. The functions for these sequences have not yet been established.

Construction of *hs*[−] mutants. A mutational strategy was used to examine the role that the *hs* sequence may play in the transcriptional regulation of the *ie-2* and *pe-38* promoters by IE-1. Site-directed mutagenesis was used to substitute a *Bgl*II site for the central 6 bp of the *ie-2* and *pe-38* *hs* sequences (Fig. 1b). Promoter fragments with and without these specific mu-

FIG. 1. Sequence comparison of negatively regulated promoters and mutational strategy for analysis of *hs* sequence function. (a) Sequences of the *ie-0*, *ie-2*, and *pe-38* promoters compared with the consensus sequence from the *hr* palindromes. Nucleotides are expressed with standard IUB nomenclature. The conserved 8-mer sequences are boxed. In each *hr* palindrome, there are two of these conserved sequences flanking an *Eco*RI site (underlined). In the promoter sequences, conserved TATA and CAGT motifs are underlined. Transcriptional start sites determined for *ie-0* (19), *ie-2* (7), and *pe-38* (16a, 21) are indicated by arrows. P, start sites determined by primer extension analysis; S, those determined by S1 analysis. The method used to determine the *ie-2* start site was not specified (6). (b) Site-specific mutagenesis was used to substitute the central 6 bp of the *hs* sequence in the *ie-2* and *pe-38* promoters with a *Bgl*II recognition site (see Materials and Methods).

tations were cloned into GUS reporter gene constructs, and their expression levels when cotransfected into Sf9 cells with various concentrations of pIe-1 were assayed. Mutant and wild-type promoter fragments were also assayed for their abilities to bind factors specific to pIe-1-transfected cells.

Expression of *hs⁻* mutant and wild-type promoters in transfected Sf9 cells. To determine whether the *hs* sequence was involved in negative regulation by *ie-1*, DNA fragments containing the *ie-2* and *pe-38* promoter regions, with and without *hs⁻* mutations, were subcloned into GUS expression vectors, and the expression levels were monitored after cotransfection

of Sf9 cells with various concentrations of the *ie-1* gene-containing plasmid pIe-1. In order to improve overall expression levels, the reporter constructs initially used contained a fragment (*AcMNPV Hind*III-Q) harboring the *hr5* enhancer.

Figure 2a shows the expression of *ie-2* promoter constructs (pIe-2-QGUS and pIe-2*hs⁻*-QGUS) when they are cotransfected with pIe-1 at concentrations varying from 0 to 5.0 μ g of DNA per well. We observed that GUS expression with the unmodified *ie-2* promoter construct pIe-2-QGUS was slightly stimulated by the addition of a low level of pIe-1, reaching a

FIG. 2. Negative regulation of the *ie-2* and *pe-38* promoters and stimulation of *ie-2hs⁻* and *pe-38hs⁻* promoters by cotransfection with pIe-1. Various concentrations of pIe-1, as indicated, were cotransfected with 1.0 μ g of pIe-2-QGUS (black histograms) or 1.0 μ g pIe-2*hs⁻*-QGUS (striped histograms) (a) and 1.0 μ g of pPe-38-QGUS (black histograms) or 1.0 μ g of pPe-38*hs⁻*-QGUS (striped histograms) (b). Each column represents the average of three independent transfections, with the standard deviations indicated.

FIG. 3. Negative regulation of the *ie-2* and *pe-38* promoters and stimulation of *ie-2^{hs}* and *pe-38^{hs}* promoters in the absence of a *cis*-linked *hr5* enhancer. Various concentrations of pIe-1, as indicated, were cotransfected with 1.0 µg of pIe-2-GUS (black histograms) or 1.0 µg of pIe-2^{hs}-GUS (striped histograms) (a) and 1.0 µg of pPe-38-GUS (black histograms) or 1.0 µg of pPe-38^{hs}-GUS (striped histograms) (b). Each column represents the average of three independent transfections, with the standard deviations indicated.

maximum of 1.35-fold stimulation at 0.01 µg/well. As concentrations of pIe-1 were increased, GUS activity declined. At the highest pIe-1 concentration tested (5.0 µg/well), GUS activity was 38% of that observed without pIe-1 cotransfection. In contrast, expression from the *hs*⁻ mutant pIe-2^{hs}-QGUS was stimulated at all concentration levels of pIe-1 assayed, reaching a maximum of 10.4-fold stimulation at 5 µg/well.

The results observed with the *pe-38* promoter constructs (Fig. 2b) were similar to those observed with the *ie-2* promoter constructs. The *pe-38* promoter fragment used in these studies lacks upstream elements that are needed for full expression (unpublished data) but retains the sequences needed for IE-1 downregulation. When the reporter construct containing the unmodified *pe-38* promoter fragment (pPe-38-QGUS) was assayed, GUS expression was stimulated 1.2-fold by the addition of 0.01 µg of pIe-1 per well, and expression levels declined as the pIe-1 concentration was increased. At 5 µg of pIe-1 per well, pPe-38-QGUS-transfected cells produced only 20% of the GUS activity that was seen when Sf9 cells were not cotransfected with pIe-1. In contrast, cells transfected with the *hs*⁻ mutant pPe-38^{hs}-QGUS showed stimulation of GUS activity at all pIe-1 concentrations tested. Stimulation of GUS activity appeared to reach a maximum at fivefold the unstimulated level with pIe-1 concentrations of 0.1 µg/well or greater.

We also tested GUS expression constructs lacking the *hr5* enhancer element (Fig. 3). As expected, overall expression levels were lower with these constructs. GUS activities in extracts from the pIe-2-GUS- and pPe-38-GUS-transfected cells were not stimulated at low concentrations of pIe-1, as was observed when the enhancer-containing constructs were used. Significant inhibition of GUS expression was evident in cells cotransfected with pIe-1 at concentrations of 0.1 µg/well or greater. In contrast, GUS activity from the *hs*⁻ mutant constructs pIe-2^{hs}-GUS and pPe-38^{hs}-GUS was stimulated five- to sixfold by cotransfection with pIe-1. Collectively, these results suggest that the putative IE-1 binding motif plays a major role in expression of the *ie-2* and *pe-38* promoters.

Expression of *ie-2* and *ie-2^{hs}* promoters in infected Sf9 cells. As a method of assessing whether the *hs* sequence plays a biologically significant role during the course of AcMNPV

infection, Sf9 cells that had previously been transfected with pIe-2-QGUS and pIe-2^{hs}-QGUS were infected with AcMNPV and monitored for GUS activity levels at various times p.i. Figure 4 shows that before 9 h p.i., there was little difference in the accumulation of GUS activity in cells transfected with these two constructs. However, after 9 h p.i., the cells transfected with the pIe-2^{hs}-QGUS construct accumulated GUS activity at a faster rate, so that at 24 h p.i. there was eightfold more GUS activity in pIe-2^{hs}-QGUS-transfected cells than in those transfected with pIe-2-QGUS. After 24 h p.i., GUS activity declined slightly in pIe-2-QGUS-transfected cells but continued to increase in cells transfected with pIe-2^{hs}-QGUS. At 36 h p.i., when the experiment was terminated, there was nearly 20 times as much GUS activity in pIe-2^{hs}-QGUS transfected cells than in pIe-2-QGUS-transfected cells.

FIG. 4. Expression of transfected *ie-2* and *ie-2^{hs}* promoter-GUS constructs in AcMNPV-infected cells. A 1.0-µg amount of pIe-2-QGUS (open squares) or 1.0 µg of pIe-2^{hs}-QGUS (filled triangles) was transfected into Sf9 cells and subsequently infected with AcMNPV (multiplicity of infection of 10) as described in Materials and Methods. GUS activities were determined at various times p.i. Inset shows expression at early time points.

FIG. 5. EMSA of *ie-2* and *pe-38* promoter fragments. Prior to electrophoresis, various amounts of extract from pKS⁻ or ple-1-transfected cells were incubated with a 93-bp ³²P-end-labeled fragment encompassing the transcriptional initiation site of *ie-2* (lanes 1 to 6) or a similar fragment containing the *hs⁻* mutation (lanes 7 to 12) (a) and a 132-bp ³²P-end-labeled fragment encompassing the transcriptional initiation site of *pe-38* (lanes 1 to 6) or a similar fragment containing an *hs⁻* mutation (lanes 7 to 12) (b). The volume of extract (in microliters) used in each reaction is indicated above each lane. Extract concentrations for pKS⁻ and ple-1 were 2.3 and 2.9 μg/ml, respectively. The faint band across several lanes just above the free probe is a gel artifact.

EMSAs of mutant and wild-type *ie-2* and *pe-38* promoter fragments. IE-1 has been shown to bind specifically to sequences in each half of an *hr* palindrome (11, 25, 31). Since we found that *hs* sequences appear to mediate negative regulation of the *ie-2* and *pe-38* promoters by *ie-1* in cotransfection experiments, we were interested in determining whether IE-1 interacts with these sequences. Figure 5a (lane 1) shows that incubation of a ³²P-labeled *hs* sequence-containing 93-bp probe derived from the *ie-2* promoter (*ie-2* probe) with extracts from pKS⁻ transfected Sf9 cells did not produce a detectable shift in the mobility of the probe (Fig. 5a, lane 2). In contrast, addition of extracts prepared from ple-1-transfected cells resulted in the formation of a DNA-protein complex.

Furthermore, addition of increasing amounts of ple-1-trans-

fected extracts resulted in a proportional increase in the amount of shifted probe (Fig. 5a, lanes 3 to 6). EMSA analysis with a similar probe containing the 6-bp *hs⁻* mutation (*ie-2hs⁻* probe) showed no detectable mobility shifts after incubation with ple-1-transfected cell extracts (Fig. 5a, lanes 9 to 12). A 132-bp probe derived from the *pe-38* promoter was also shifted in the presence of ple-1-transfected cell extract (Fig. 5b, lanes 3 to 6), but a similar probe with the *hs⁻* mutation (*pe-38hs⁻* probe) was not shifted (Fig. 5b, lanes 9 to 12).

Supershift analysis was performed to demonstrate that IE-1 is a component of the shifted complexes. Incubation of the *ie-2* and *pe-38* probes with ple-1-transfected cell extract in the presence of polyclonal antibody to IE-1 resulted in supershifting of the complexes (Fig. 6a and b, lanes 7 to 9), whereas incubation with preimmune serum did not affect the migration of the complexes (lanes 4 to 6). These results indicate that an IE-1-containing complex binds to the proposed IE-1 binding motif to affect regulation of the *pe-38* and *ie-2* genes.

DISCUSSION

In this report, we have provided evidence that the previously described negative regulation by *ie-1* of the *ie-2* promoter (7) is mediated by the interaction of IE-1 with a specific sequence in this promoter. We have also provided evidence that the *pe-38* promoter is subject to negative regulation by a similar mechanism. We identified a common 8-bp sequence element (*hs* sequence) in the *ie-2*, *pe-38*, and *ie-0* promoters that is similar to sequences present in each half of every *hr* palindrome. In cotransfection assays, we showed that mutations abolishing the *hs* sequences in the *ie-2* and *pe-38* promoters convert them from promoters that are negatively regulated by *ie-1* to promoters that are induced by *ie-1*. The negative regulation of the wild-type promoters and activation of the mutant promoters occurred independently of a *cis*-linked *hr* enhancer, except at very low concentrations of ple-1. However, the expression of the wild-type promoters was stimulated slightly upon cotransfection with very low ple-1 concentrations (0.01 μg/10⁶ cells) when *cis* linked to *hr5* enhancer sequences (compare Fig. 2a and 3a, and Fig. 2b and 3b). This may be due to differences in the binding affinity of IE-1 for enhancer sequences and the promoter *hs* sequences due to cooperativity. Each *hr* contains 2 to 16 copies of the IE-1 binding motif, allowing the possibility of cooperative binding of IE-1 to multiple binding sites. On the other hand, the promoter *hs* sequences occur in isolation, and cooperative binding is unlikely to occur. As a result of cooperativity, *hrs* may be bound at concentrations of IE-1 lower than those required for binding to the *ie-2* and *pe-38* promoters.

hr sequences in a number of baculoviruses including the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus (OpMNPV) have been characterized. Both AcMNPV *hr* and OpMNPV *hr* sequences contain 30-bp imperfect palindromes (1). Although none of the OpMNPV palindromes contain sequences that match perfectly with the AcMNPV IE-1 binding motif, each half of the consensus OpMNPV palindrome contains a sequence that matches the AcMNPV *hr* motif except for one or two positions, suggesting that the IE-1 recognition sequences in OpMNPV and AcMNPV may be similar but not identical. The promoters for the OpMNPV *ie-0*, *ie-2*, and *ie-3* (homolog of the AcMNPV *pe-38*) genes each contain sequences located 2 to 3 bases upstream of CAGT transcriptional initiation motifs that match the OpMNPV half-palindrome consensus sequence in at least 11 of 13 positions. Since these sequences have the same relation to the CAGT motif as do the IE-1 binding motifs in the homologous

FIG. 6. Supershift analysis of *ie-2* and *pe-38* promoter fragments. Prior to electrophoresis, radiolabeled fragments from the *ie-2* (a) and *pe-38* (b) promoters were incubated with 2 μ l of whole-cell extracts in the presence of preimmune rabbit antiserum (lanes 4 to 6) or an IE-1-specific polyclonal rabbit antiserum (lanes 7 to 9). Antiserum dilutions are as indicated. Control reactions included no extract (lane 1), 2 μ l of pKS⁻-transfected cell extract without serum addition (lane 2), and 2 μ l of ple-1-transfected cell extract without serum addition (lane 3).

AcMNPV genes, this suggests that these may be binding motifs used by the OpMNPV IE-1 protein for downregulation of these genes.

EMSAs were used to characterize the binding of IE-1 to *hs* sequences. The electrophoretic mobility of *hs* sequence-containing fragments was shifted after incubation with extracts from ple-1-transfected cells. No shifts were evident when *hs*⁻ mutant fragments were used or when pKS⁻-transfected control extracts were incubated with wild-type fragments. These results indicate that factors specific to ple-1-transfected extracts bind DNA-containing *hs* sequences. Supershift assays with IE-1-specific antiserum demonstrated that IE-1 is present in the shifted complexes and, therefore, that IE-1 binds either directly or as part of a complex to the *hs* sequence. It should be noted that the probes used in these assays are smaller than the promoter fragments that were cloned and assayed for their abilities to drive GUS expression in transfection assays. It is possible that upstream regions not present on these probes contain IE-1 binding sequences that are important for the observed upregulation of the *hs*⁻ constructs. Alternatively, it is possible that IE-1 upregulates minimal promoters by direct protein-protein interactions with components of the transcriptional machinery in the absence of a specific IE-1 binding site.

Mutational analyses of *hr* palindromes have demonstrated that two closely opposed IE-1 binding sites are required for enhancer activity, even though IE-1 can bind to a single half-site sequence (11, 25, 31). Furthermore, alterations in the spacing between *hr* half-sites abolish enhancer function (25, 31). In contrast, the IE-1 binding motifs involved in negative regulation occur as single copies downstream from a TATA box and 3 or 4 bases upstream of a conserved CAGT motif. A CAGT motif has been associated with the transcriptional initiation sites of many baculovirus early genes (3). The position of the IE-1 binding motif suggests a mechanism whereby the binding of IE-1 to the *hs* sequence may sterically interfere with initiation by RNA polymerase II. Studies of the interaction of the herpes simplex virus type 1 transregulator ICP4 with its binding site suggests, however, that the mechanism of inhibition could be more complex. Like IE-1, ICP4 activates transcription of some genes and represses transcription of others (28). ICP4 has been shown to interact with the RNA polymer-

ase II transcription factors TBP and TFIIB, resulting in the formation of a tripartite complex that binds cooperatively to the TATA box and the ICP4 binding site (33). Kuddas et al. (22) found that changing the ICP4 binding site orientation or increasing the spacing between this site and the TATA box leads to concomitant decreases in both repression and tripartite complex formation. They also found evidence that stereoaxial phasing influenced repression and tripartite complex formation, although this was not as important as absolute distance. They speculated that binding of the complex may interfere with either the further assembly of the transcription complex or the ability of upstream activating proteins to interact with TBP-associated factors.

Our data suggest that binding of IE-1 to the *hs* sequence is a mechanism that AcMNPV uses to repress transcription of selected genes during infection. In order to determine if this mechanism could account for the decrease in *ie-2* mRNA levels that has been observed during infection (7, 21), we compared GUS activity levels after AcMNPV infection of Sf9 cells that had been transfected with reporter constructs containing mutant and wild-type *ie-2* promoters. The reporter constructs used also contained *hr5* enhancer elements, and, therefore, the amounts of GUS activity accumulated at various times p.i. are expected to be influenced by both the transcriptional activation functions of IE-1 mediated by the enhancers and the repression functions mediated by the promoter *hs* sequences. By immunoblot analysis, Choi and Guarino (9) showed that IE-1 increases steadily during infection, reaching a level at 72 h p.i. approximately 20-fold higher than that observed at 4 h p.i. We found very little difference in the accumulation of GUS activity with our mutant and wild-type constructs as late as 9 h p.i. (Fig. 4, inset), suggesting that IE-1 had not reached a concentration high enough to bind effectively to the promoter *hs*⁻ sequence and cause repression. This is in agreement with a Northern blot analysis (21), which showed relatively constant steady-state levels of *ie-2* mRNA between 2 and 12 h p.i., but is in contrast to the results of an S1 nuclease analysis (7), which showed that peak levels of *ie-2* mRNA occurred between 0 and 3 h p.i. We found that although GUS activity continued to accumulate in cells transfected with either reporter from 9 until 24 h p.i., the rate of accumulation was dramatically higher

in cells transfected with the mutant *ie-2* promoter construct. This suggests either that IE-1 concentrations are insufficient to effect full repression or that the promoter is repressed but that mRNA produced prior to this time is relatively stable and continues to be translated, allowing further GUS accumulation. Beyond 24 h p.i., there was no further accumulation of GUS activity in the wild-type construct, and, in fact, activity levels decreased slightly, suggesting that full repression had been obtained.

Our results suggest that binding of IE-1 to *hs* sequences near the transcriptional initiation site is one mechanism used by the virus to repress gene expression; however, we found no evidence of *ie-1*-negative regulation of several other baculovirus promoters in additional cotransfection assays (data not shown). Among the promoters tested was the DNA polymerase gene promoter for which expression has been shown to be at maximal levels 6 h p.i., decreasing to very low levels by 10 h p.i. (29). In fact, we observed stimulation of expression from the DNA polymerase promoter at pIE-1 concentrations of as high as 5 µg/10⁶ cells, suggesting that an alternative mechanism is used to counteract the stimulatory effects of IE-1 on this promoter. Therefore, in addition to the mechanism that we have described for repression of *ie-2* and *pe-38* gene expression, baculoviruses may have other mechanisms that they use to repress the expression of specific genes.

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