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Novel Regulatory Properties of the IE1 and IE0 Transactivators Encoded by the Baculovirus *Autographa californica* Multicapsid Nuclear Polyhedrosis Virus

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The baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus expresses two immediate-early genes from the *Hin*dIII-G region (map units 90.4 to 96.8) of the genome. During the early phase of infection, nonspliced 1.9-kb and spliced 2.1-kb transcripts are expressed which encode the IE1 and IE0 (spliced IE1) gene products, respectively. These two gene products differ only in that IE0 contains an additional 54 amino acids at the amino terminus. RNA analysis of these two genes during infection revealed that they were differentially expressed. IE1 was expressed early and late, whereas IE0 was expressed only early in infection. The regulation of these two immediate-early genes was analyzed by transient expression assays. The IE1 gene product stimulated expression of IE1 promoter-directed expression but down-regulated expression from the IE0 promoter. The IE0 gene product also transactivated the IE1 promoter but did not affect expression from its own promoter. Unlike IE1, which transactivates the delayed early 39K gene in the presence and absence of the homologous region (*hr*) enhancers, IE0 transactivated the 39K promoter only in the presence of *cis*-linked *hr5* enhancer. The results of this study in conjunction with previous results suggest that the IE1 gene encodes a multifunctional gene product that may be involved in (i) repression of immediate-early gene expression, (ii) continued expression of its own gene product during infection, and (iii) transactivation of the delayed early and late classes of genes.

The baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus (Ac*M*NPV) is being used by many researchers as a model system for studies of viral gene expression and regulation in invertebrate cells, and as such it may serve as a prototype for comparing differential gene expression in higher eukaryotic systems. Baculovirus infection relies on the temporal regulation and sequential ordering of expression of approximately 100 viral genes (for reviews, see references 2 and 11). Infection is divided into an early (prereplication) phase and a late (postreplication) phase. Baculovirus genes are divided into four temporal classes (immediate early, delayed early, late, and very late) according to the time of expression and the requirements for both prior viral gene product expression and viral DNA synthesis. By definition, early genes are expressed prior to viral DNA replication. Most, if not all, immediate-early genes encode transcriptional regulatory proteins (3–5, 17, 19, 20, 36). They are distinguished from delayed-early genes by the fact that their transcription is independent of prior viral gene product expression in transient assays. Delayed-early gene expression depends on the prior expression of one or more immediate-early gene products (3, 12, 17, 23, 31). Some delayed-early genes are presumed to encode factors required for viral DNA synthesis (29, 33, 37). DNA replication activates the viral template in a manner not yet defined and enables the late and very late classes of genes to be expressed. Late and very late gene products are essential for virion assembly and viral occlusion formation.

Four Ac*M*NPV immediate-early genes have been cloned, and their expression, regulation, and activities have been partially characterized: IE1 (4, 14, 17–21), IE0 (7), IEN (3–5), and PE-38 (26). IE0 (7), IEN (3), and PE-38 (26) RNAs are expressed only during the early phase of infection. In contrast, IE1 RNA is expressed during both early and late phases of infection (7, 19). IE1 is a multifunctional transcriptional regulatory protein, transactivating a number of delayed-early genes (17, 19, 32) and at least one late gene (20). In addition to transactivating viral promoters, it stimulates expression of heterologous genes *cis* linked to homologous region (*hr* [8]) enhancer elements (18). IE1 is the only known viral factor that transactivates both promoters and enhancers. IE1 also down-regulates expression from the IEN promoter in transient assays (4). Electrophoretic mobility shift assays show that extracts prepared from insect cells expressing IE1 contain a specific *hr5* binding activity, suggesting that IE1 may be a DNA-binding protein (14). IE0 is expressed as a spliced mRNA with an open reading frame (ORF) that encodes a polypeptide identical to the IE1 gene product except for an additional 54 amino acids at the amino terminus (7). Prior to this report, no specific function had been attributed to the IE0 gene product. The IEN gene product transactivates immediate-early genes (IE1, IE0, and IEN), as determined by transient assay experiments, and contains several structural motifs present in transcriptional regulatory proteins, including a serine-threonine-rich region and a polyglutamine tract (5). The most recently isolated immediate-early gene, PE-38, contains a putative zinc finger and a leucine zipper structural motif, but its activities have not yet been determined (26). Based on the demonstrated transcriptional regulatory properties of two of the four known baculovirus immediate-early genes, it is reasonable to hypothesize that immediate-early gene products interact in combinatorial arrays, perhaps in ways analogous to the immediate-early gene products of herpes simplex virus, to

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regulate the expression of baculovirus genes during infection (9).

The first objective of this study was to examine the functional interactions of the IE1 and IE0 gene products with the IE1 and IE0 promoters. Analysis of RNAs expressed from 0.25 to 12 h postinfection (hpi) revealed that these two genes were expressed with different temporal patterns. Transient assay results showed that the IE1 gene product downregulates IE0 expression and stimulates its own expression. The IE0 gene product also stimulates IE1 expression but has no effect on its own expression. These data suggest that both IE1 and IE0 gene products may play a role in stimulating IE1 expression and that IE1 may be involved in the downregulation of IE0 expression during viral infection.

The second objective of this study was to investigate the function of the IE0 gene product in delayed-early gene regulation. The results of transient assays showed that the IE0 gene product does not transactivate the delayed-early 39K promoter (16) unless the *hr5* enhancer is cloned in *cis* to the 39K promoter. These experiments suggest that although a major portion of the IE0 gene product is structurally identical to that of IE1, it may have different transcriptional regulatory functions during infection.

MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* (Sf9) cells were passaged in spinner flasks at 27°C and infected as previously described by Summers and Smith (35). Cells were infected with the E2 strain of Ac*M*NPV at a multiplicity of 10 PFU per cell. Virus was added to cells and allowed to adsorb for 1 h; subsequently, virus was removed and cells were resuspended in fresh TNMFH medium. Time zero was defined as the time at which cells were inoculated with virus.

Plasmid constructions. A schematic representation of all plasmids used in this study is shown in Fig. 1B. All recombinant plasmids were constructed in vitro by standard cloning techniques (30) and sequenced by the method of Sanger et al. (34). pNheIE1 was constructed by digesting pIE1 (17) with *Nhe*I and *Sma*I, gel purifying the IE1-containing 3.0-kbp fragment, repairing the 5′ extensions with the large fragment of DNA polymerase I, and subsequently religating the ends. A nonsense mutant form of pNheIE1 (pIE1ΔORF) was constructed by site-directed mutagenesis as described by Kunkel (27). First, pNheIE1 was engineered to contain a unique *Bam*HI restriction site immediately downstream of the IE1 ATG translation initiation site, using a deoxyoligonucleotide 30-mer of the sequence 5′-CAAGTGACTATG GATCCAATTAATTTTAAC-3′ (ATG underlined; *Bam*HI site in bold type). The resulting plasmid was digested with *Bam*HI; 5′ extensions were repaired with the large fragment of DNA polymerase I and subsequently ligated to a synthetic linker of the sequence 5′-$(CTAG)_4$-3′. The resulting plasmid, pIE1ΔORF, contains a translation termination signal four codons downstream of the IE1 ATG. pGC123 was obtained from D. Henner (Genentech) and contains the IE0 (spliced IE1) cDNA cloned under the control of the IE0 promoter. pH3G was constructed by cloning the 8.2-kbp *Hin*dIII G restriction fragment of the Ac*M*NPV E2 strain into pUC9. A nonsense mutant of pH3G (pH3GΔORF) was derived by site-directed mutagenesis. Initially, the *Bam*HI site within the multiple cloning region of pH3G was destroyed by digestion with *Bam*HI, repair of 5′ extensions with the large fragment of DNA polymerase I, and religation. Subsequently, the 30-mer described above was used to engineer a unique *Bam*HI restriction site downstream of the IE1 ATG translation initiation signal. The resulting plasmid was digested with *Bam*HI, 5′ extensions were repaired with the large fragment of DNA polymerase I, and the synthetic linker 5′-$(CTAG)_4$-3′ was inserted. pH3GΔORF contains a translation termination signal four codons downstream of the IE1 ATG. pIE1-NFCAT was constructed by inserting an 8-mer *Bgl*II linker (New England BioLabs) at a unique *Hin*cII site within the untranslated leader in plasmid pIE1 and subsequently inserting a *Bam*HI 0.8-kbp fragment containing the chloramphenicol acetyltransferase (CAT) gene into the *Bgl*II site. pIE0-NFCAT was constructed by insert-

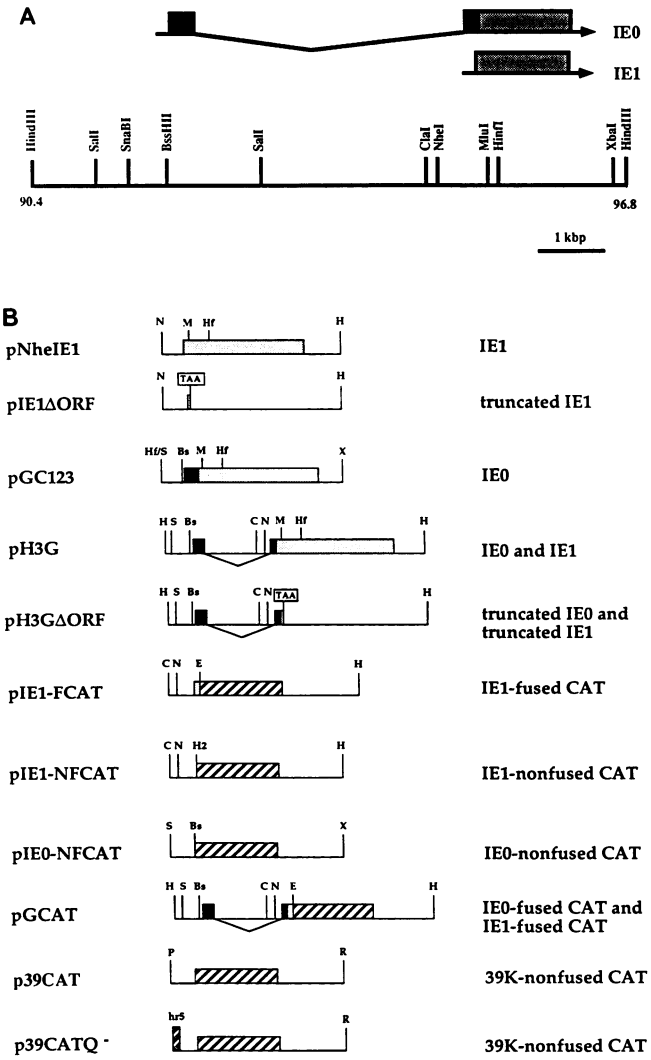

FIG. 1. (A) Transcription map of IE1 and IE0. Illustrated are the IE1 (nonspliced) and the IE0 (spliced) transcripts expressed from the *Hin*dIII-G region of Ac*M*NPV. Black boxes indicate exon 0 sequences, and gray boxes indicate exon 1 sequences. Above the map are shown pertinent restriction sites. Map units are shown below the map. (B) Schematic representation of effector and reporter plasmids used in this study. Plasmid nomenclature is shown to the left, and predicted expression products are shown to the right. Coding regions: ■, exon 0; ▦, exon 1; ▨, CAT. ▮, the enhancer element *hr5*. Abbreviations for restriction sites: Bs, *Bss*HII; C, *Cla*I; E, *Eco*47III; Hf, *Hin*fI; H2, *Hin*cII; H, *Hin*dIII; M, *Mlu*I; N, *Nhe*I; P, *Pst*I; R, *Eco*RI; S, *Sna*BI; X, *Xba*I. The TAA termination codon in the nonsense mutants is shown boxed. Diagrams are not drawn to scale.

ing a 10-mer *Bgl*II linker (New England BioLabs) into a unique *Bss*HII site located 40 bp upstream of the IE0 ATG in the untranslated leader region in pGC123. Subsequently, the 0.8-kbp CAT-containing fragment was inserted into the *Bgl*II site. pGCAT was constructed in several steps: (i) a 10-mer *Bgl*II linker was inserted into a repaired *Eco*47III site in pIE1, (ii) a 0.8-kbp *Bam*HI fragment containing the CAT ORF was inserted into the *Bgl*II site to construct pIE1-FCAT, and (iii) a 3.35-kbp *Nhe*I-*Xba*I fragment derived from pIE1-FCAT was inserted into *Nhe*I-*Xba*I-digested pH3G. The resulting plasmid, pGCAT, contains the CAT ORF inserted 36 bp downstream of the IE1 ATG. The construction of p39CAT and p39CATQ- has been previously described (18).

Transient CAT assay. Transfections were conducted essentially as described by Guarino and Summers (17). Briefly, 10^6 cells were seeded into six-well plates and allowed to attach for 1 h. Subsequently, cells were transfected with the indicated plasmids and pUC DNA as carrier for a total DNA concentration of 10 μg/10^6 cells. Lysates were prepared 24 h posttransfection, and CAT activity was assayed (13).

S1 nuclease assays. Total RNA was purified from cells by the method of Chirgwin et al. (6) and subsequently analyzed by the methods established by Berk and Sharp (1) as modified by Favolaro et al. (10).

Radioimmunoprecipitation assays. Cells were seeded at a density of 10^6 per well in six-well plates and transfected with the indicated plasmids. Approximately 24 h posttransfection, cells were incubated for 1 h in methionine-free Graces medium. Subsequently, cells were metabolically radiolabeled with 50 μCi of TRANS^{35}S-LABEL (a hydrolysate consisting of 15% [^{35}S]Cys and 75% [^{35}S]Met; ICN) per ml for 2 h at 27°C. Cells were harvested, and extracts were immunoprecipitated with polyclonal anti-CAT antiserum at a 1:250 dilution (5′-3′) as previously described (22, 24). Radiolabeled proteins were then separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (28), the gels were dried, and the proteins were visualized by autoradiography. Proteins were quantitated by using a model 300A computing densitometer (Molecular Dynamics).

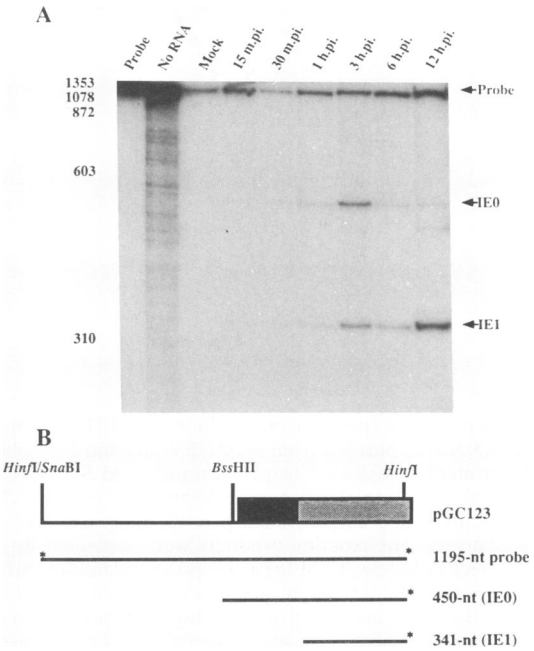

FIG. 2. Time course of IE1 and IE0 RNA expression in infected insect cells. (A) Total RNA was extracted from mock-infected and Ac*M*NPV-infected Sf9 cells at 0.25, 0.5, 1, 3, 6, and 12 hpi. An 1,195-nt *Hin*fI probe was hybridized to 20 μg of total RNA at 42°C for 15 h and then subjected to S1 nuclease digestion. The protected fragments were separated in a 7 M urea–5% polyacrylamide gel. Sizes (in nucleotides) of molecular weight standards (*Hae*III-digested ϕX174) are shown at the left; locations of the probe and protected fragments are shown at the right. An autoradiograph of the gel is illustrated. The probe lane contained undigested probe; the no-RNA lane contained digested probe. m.pi., minutes postinfection. (B) Partial restriction map of pGC123. Above the map are pertinent restriction sites. Also shown are the predicted sizes of protected fragments and of the probe. The black box represents exon 0; the shaded box represents a portion of exon 1; asterisks mark positions of the radiolabel.

RESULTS

IE1 and IE0 RNAs are differentially expressed throughout infection. An S1 nuclease protection assay was used to quantitatively analyze the expression of IE1 and IE0 RNAs from 0 to 12 hpi (Fig. 2A). A *Hin*fI 1,195-nucleotide (nt) probe that should hybridize with both IE1 and IE0 mRNAs was derived from pGC123 (Fig. 2B). RNA was purified from Sf9 cells mock infected and infected with Ac*M*NPV at the indicated times (0 hpi was defined as the time at which cells were inoculated). Subsequently, 20 μg of total cellular RNA was incubated with the IE1/IE0-specific probe, and S1 nuclease assays were conducted. The S1 analysis shows that equivalent amounts of both IE1 and IE0 RNAs were expressed as early as 0.25 hpi. The steady-state levels of both transcripts rose equivalently from 0.25 to 3 hpi. After 3 hpi, IE1 and IE0 RNAs were differentially expressed. Steady-state levels of IE0 RNA peaked at 3 hpi and declined from 6 to 12 hpi, whereas steady-state levels of IE1 RNA increased from 3 to 12 hpi. A reproducible transient decrease in the steady-state level of IE1 RNA expressed at 6 hpi was also observed.

Expression of the cloned *Hin*dIII G fragment is regulated by the *trans*-acting IE1 gene product. Since the results in Fig. 2A showed that IE1 and IE0 were differentially expressed, transient expression assays were used to study the regulation of these two genes. To analyze transient expression of both IE1 and IE0 RNAs simultaneously, a plasmid (pH3G; Fig. 1B) that contains the entire IE1/IE0 transcription unit of Ac*M*NPV was constructed. RNA was purified from Ac*M*NPV-infected cells at 3 hpi or from cells mock-transfected or transfected with pH3G. These RNAs were analyzed by S1 nuclease protection assays with the *Hin*fI 1,195-nt probe that detects both IE1 and IE0 RNAs (Fig. 2B). We predicted that pH3G, containing the complete IE0 and IE1 genes, should express both transcripts. However, the results show that pH3G-transfected cells expressed only IE1 RNA; IE0 transcripts were not detected (Fig. 3).

The IE1 gene product had been previously shown to down-regulate expression from the IEN promoter in transient assays (4). Since pH3G-transfected cells should express the IE1 gene product, we hypothesized that IE0 expression may have been down-regulated by *trans*-acting IE1 protein. To determine whether IE0 RNA could be expressed in the absence of IE1 protein, a nonsense mutant form of pH3G (pH3GΔORF; Fig. 1B) was constructed. pH3GΔORF-transfected cells should not express IE1 or IE0 protein. S1 nuclease protection assays were conducted on RNA isolated from Sf9 cells transfected with pH3GΔORF

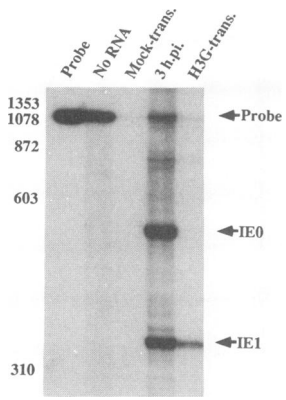

FIG. 3. Transient expression of the cloned *Hin*dIII G fragment in Sf9 cells. RNA was purified from Ac*M*NPV-infected Sf9 cells at 3 hpi or from mock-transfected or pH3G-transfected Sf9 cells. Subsequently, 20 μg of total RNA was hybridized to the 1,195-nt IE1/IE0-specific probe derived from pGC123 and subjected to S1 nuclease analysis. The reaction products were separated by denaturing gel electrophoresis (7 M urea–5% polyacrylamide). Sizes (in nucleotides) of molecular weight markers are indicated at the left; locations of the probe and the protected fragments are shown at the right.

(Fig. 4). A *Sna*BI-*Mlu*I 798-nt probe derived from pGC123, which should also hybridize to both IE1 and IE0 transcripts, was used in this experiment. The results show that a greater amount of IE0 RNA was expressed in the absence than in the presence of the IE1 and IE0 gene products (compare lanes 5 and 6). To determine whether expression of IE0 was down-regulated by *trans*-acting IE1 protein, RNA was analyzed from cells that were cotransfected with pH3GΔORF and pNheIE1. The results show that cells expressing the IE1 gene product, either from pH3G (*cis*) or from pNheIE1 (*trans*), expressed equivalently reduced levels of IE0 RNA (compare lanes 5 and 7). Cotransfection with a plasmid (pIE1ΔORF; Fig. 1B) that contains the IE1 gene but does not express IE1 protein had no effect on IE0 RNA expression (compare lanes 6 and 8). Finally, unlike IE0 RNA levels, IE1 RNA levels were significantly lower in the absence of the IE1 gene product (compare lanes 5 and 6). Conversely, IE1 RNA levels were significantly increased in the presence of the IE1 gene product (compare lanes 6, 7, and 8). These results show that the IE1 gene product has both down-regulating and autostimulatory activities.

Analyses of IE0 and IE1 promoter-directed CAT expression. To specifically analyze the regulatory effects of the IE1 and IE0 gene products on the IE0 promoter, a plasmid (pIE0-NFCAT; Fig. 1B) that contains the CAT-coding region under the transcriptional control of the IE0 promoter was constructed. This plasmid should express a nonfused CAT (NFCAT) protein. Sf9 cells were cotransfected with the reporter pIE0-NFCAT together with pNheIE1 or pGC123 (IE0 expressing), and CAT activity was quantitated (Fig. 5A). The results show that the IE0 promoter directed the expression of measurable levels of CAT activity in the absence of any viral factors. Cotransfection with IE1-expressing plasmid resulted in about a threefold decrease in the amount of CAT expressed from the IE0 promoter, supporting the S1 nuclease assay results shown in Fig. 4. Cotransfection of pIE0-NFCAT with IE0-expressing plasmid had no significant effect on CAT expression, suggesting that the IE0 gene product has no detectable autoregulatory activity.

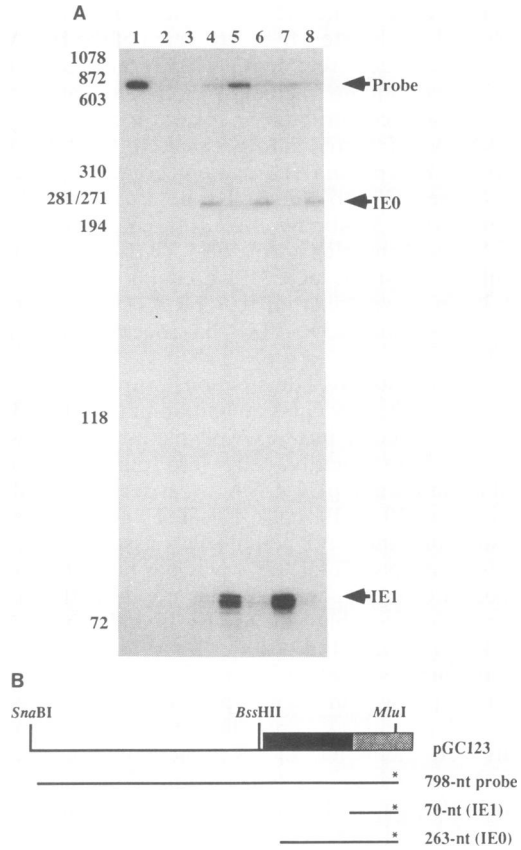

FIG. 4. Down-regulation of IE0 and stimulation of IE1 expression by the IE1 gene product. (A) S1 nuclease analysis of RNAs probed with a 798-nt probe derived from pGC123, specific for IE1 and IE0 RNAs. Total RNA (20 μg) was hybridized to the probe at 42°C for 15 h and subjected to S1 nuclease analysis. The reaction products were separated in a 7 M urea–5% polyacrylamide gel. An autoradiogram of the dried gel is shown. Lanes: 1, unreacted probe; 2, digested probe; 3, mock transfected; 4, 3 hpi; 5, pH3G transfected; 6, pH3GΔORF transfected; 7, pH3GΔORF plus pNheIE1 cotransfected; 8, pH3GΔORF plus pIE1ΔORF cotransfected. The sizes (in nucleotides) of *Hae*III-digested ϕX174 molecular weight standards are shown at the left; locations of the probe and the protected fragments are shown at the right. (B) Partial map of pGC123. The sizes of the probe and the predicted protected fragments are shown at the right. Pertinent restriction sites are shown above the map. The black box represents exon 0; the shaded box represents part of exon 1; asterisks mark positions of the radiolabel.

To further study the effects of the IE1 and IE0 gene products on the IE1 promoter, the reporter pIE1-NFCAT was constructed and cotransfected with either pNheIE1 or pGC123. At 24 h posttransfection, cells were harvested and lysates were analyzed for CAT activity (Fig. 5B). The results show that both IE1 and IE0 gene products stimulated expression of the reporter pIE1-NFCAT approximately two- to fourfold. Increasing the amount of effector plasmid increased the amount of pIE1-NFCAT expression.

During Ac*M*NPV infection, IE1 is expressed as a 1.9-kb unspliced RNA and IE0 is expressed as a 2.1-kb spliced RNA from the *Hin*dIII-G region of Ac*M*NPV (7). A reporter plasmid, pGCAT (Fig. 1B), was derived from pH3G by inserting the CAT-coding region 36 bp downstream of the IE1 ATG translation initiation signal. Upon transfection into Sf9 cells, pGCAT should express an unspliced 2.7-kb RNA

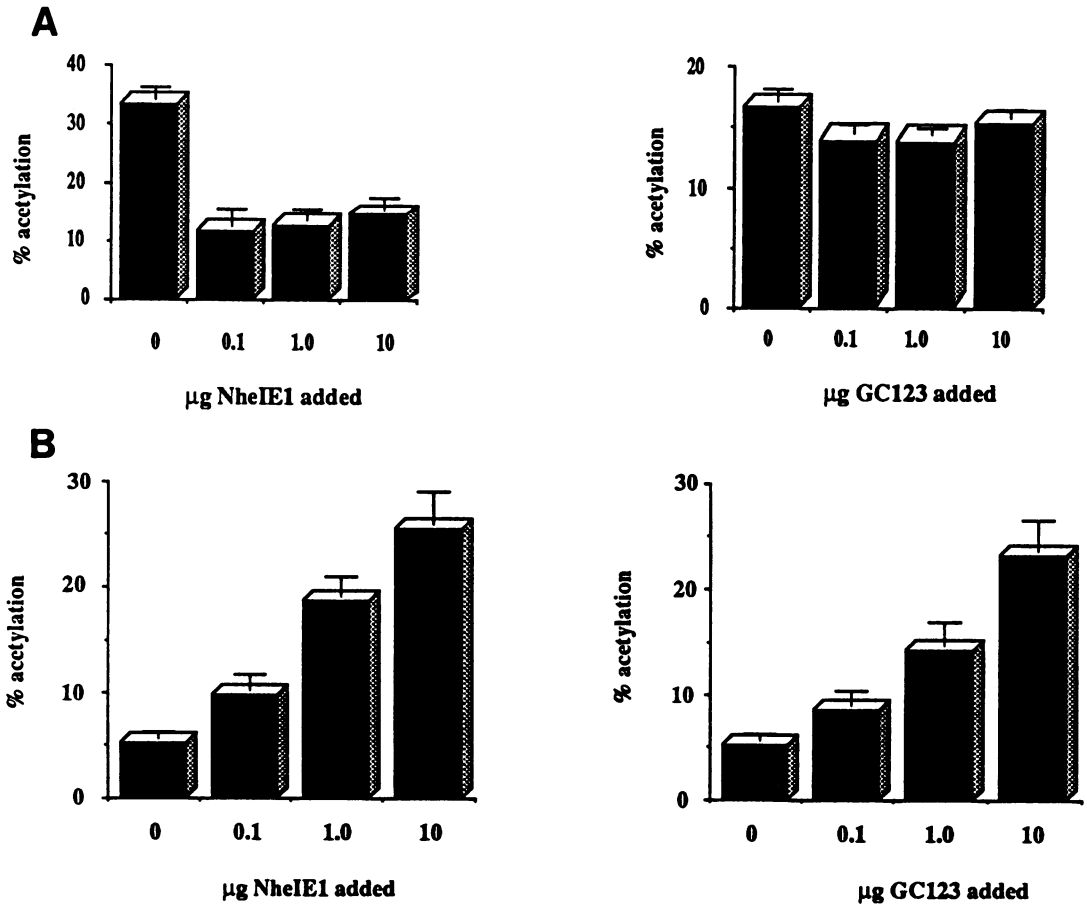

FIG. 5. Regulation of IE1 and IE0 promoter-directed expression of CAT. Sf9 cells (10^6) were transfected with 1 μg of the reporter pIE0-NFCAT (A) or pIE1-NFCAT (B) and the indicated amounts of the effector pNheIE1 or pGC123. At 24 h posttransfection, cell lysates were analyzed for CAT activity. The averages of three independent experiments are plotted as percent acetylated chloramphenicol per reaction. Error bars are indicated above the columns.

encoding a 24.6-kDa IE1 fused CAT (FCAT) protein and a spliced 2.9-kb RNA encoding a 28.9-kDa IE0 FCAT protein. This plasmid was used to simultaneously monitor expression from both the IE1 and IE0 promoters in the presence and absence of the IE1 or IE0 gene product. Sf9 cells were transfected with 1 μg of pGCAT alone or in combination with 1 or 5 μg of pNheIE1, pIE1ΔORF (nonsense mutant), or pGC123 (IE0 expressing). At 24 h posttransfection, cells were metabolically radiolabeled and lysates were immunoprecipitated with polyclonal anti-CAT antiserum. Figure 6 shows the effects of IE1 and IE0 gene products on pGCAT expression. Two CAT-related proteins corresponding to the predicted sizes of IE0 FCAT and IE1 FCAT were expressed in pGCAT-transfected cells. Coexpression of IE1 resulted in a 5-fold decrease in the amount of IE0 CAT and a 5- to 10-fold increase in the amount of IE1 CAT. Cotransfection with the IE1 nonsense mutant (pIE1ΔORF) had no significant effect on the expression of either CAT protein. Coexpression of IE0 increased the amount of IE1 CAT fivefold but did not have a significant effect on IE0 CAT expression.

The IE0 gene product transactivates the delayed-early 39K gene only in an enhancer-dependent manner. The IE1 gene product transactivates numerous delayed-early gene promoters (enhancer-independent activity [17, 19]). In addition, IE1 transactivates delayed-early and heterologous promoters *cis* linked to Ac*M*NPV *hr* enhancers (enhancer-dependent activity [15, 18, 32]). The sequence of the IE0 ORF predicts a protein of 636 amino acids (7). The amino-terminal 54 amino acids are unique, but the remainder of the polypeptide is identical in primary sequence to the predicted IE1 gene product. Because these two gene products are structurally similar, it was reasonable to expect that they would have functional similarities. The Ac*M*NPV delayed-early gene reporter plasmids 39CAT (17) and 39CATQ- (18) were used in transient cotransfection assays to analyze IE0 transcriptional regulatory activities. Figure 7 shows the effects of IE1 and IE0 gene products on the expression of these two reporter plasmids. As previously shown by Guarino and Summers (18), IE1 transactivated both p39CAT and p39CATQ- (*hr5* linked); however, IE0 transactivated the 39K promoter only in the presence of *cis*-linked *hr5*.

DISCUSSION

Although much is known about the transcriptional regulatory properties of IE1, prior to this study no function had been attributed to the IE0 gene product. To learn more about these two genes, we compared their temporal expression and tested the effects that each gene product has on IE1, IE0, and 39K promoter-directed expression.

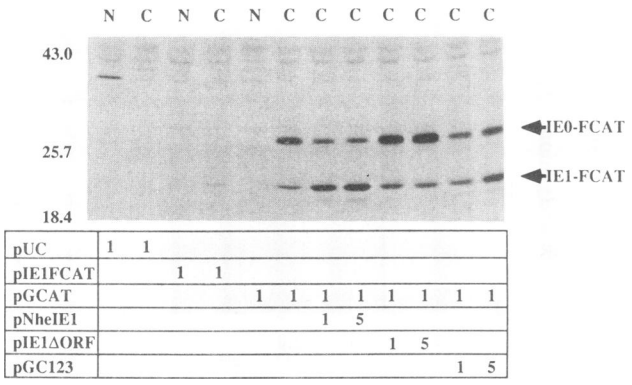

FIG. 6. Regulation of pGCAT expression in the presence of the IE1 or IE0 gene product. Sf9 cells (10^6) were transfected with the indicated amounts (in micrograms) of plasmids. At 24 h posttransfection, cells were radiolabeled for 2 h with TRANS^{35}S-LABEL. Subsequently, lysates were prepared and immunoprecipitated with polyclonal anti-CAT antiserum. Immunoprecipitates were collected and electrophoresed in a 12% SDS-polyacrylamide gel. Gels were dried and subjected to autoradiography. The sizes (in kilodaltons) of pertinent molecular mass markers are shown at the left; locations of the predicted IE0 FCAT and IE1 FCAT polypeptides are shown at the right. N, normal preimmune rabbit serum; C, polyclonal anti-CAT antiserum.

Previous studies on IE1 and IE0 RNA expression revealed that both genes were expressed very early in the infection. The maximum steady-state level of IE1 RNA was reached 1 h after addition of virus and was maintained relatively constant throughout 24 hpi (7, 19). The level of IE0 RNA increased to a maximum 3 h after addition of virus and thereafter decreased to undetectable levels (7). To further study IE1 and IE0 gene expression and regulation, a temporal analysis of IE1 and IE0 RNA expressed earlier in infection was conducted. Our data extend and differ from those presented in two previous studies (7, 19). Our results show that both RNAs were expressed at equivalent levels as early as 0.25 hpi (time zero referring to the time at which virus was added to cells). Steady-state levels of both IE1 and IE0 RNAs increased equivalently during the early phase of infection to peak at about 3 hpi. After that time, steady-state levels of IE0 RNA progressively decreased through 12 hpi. Interestingly, a reproducible drop in the steady-state levels of IE1 RNA was observed at 6 hpi. Thereafter, the expression of IE1 RNA increased to a maximum level observed at 24 hpi. The kinetics of expression of IE1 and IE0 are identical during the early phase of infection but differ late in infection, which suggests that the two genes may be regulated during infection. A previous study using Northern (RNA) and primer extension analyses to study IE0 RNA found that IE0 RNA expression was below detectable levels after 3 hpi (7). The fact that IE0 RNA was detected after 3 hpi in our study using S1 nuclease protection assays may be a result of the intrinsic properties of the different assays used. To our surprise, an S1 protection fragment, approximately 54 nt shorter than the fragment corresponding to IE0, was observed at 12 hpi (Fig. 2). Upon further investigation, it was found that this band corresponds to another spliced form of IE0 expressed during the late phase of infection (25). Thus, IE0 gene expression may be regulated at multiple levels during infection. Steady-state levels of IE1 RNA were not constant throughout the course of this study (Fig. 2) as they were in previous studies (7, 19). The probes used in our study were derived from a plasmid (pGC123) that contains the IE0 cDNA under the control of the IE0 promoter (Fig. 1B). These probes hybridize to both IE1 and IE0 RNAs and allow a quantitative assessment of both RNAs simultaneously. Guarino and Summers (19) used an S1 probe derived from pIE1. This IE1-specific probe would hybridize to both IE1 and IE0 RNAs but would not distinguish between the two RNAs. Thus, the S1 probe derived from pIE1 cannot be used to quantitatively measure IE1 RNA expression. Overall, the patterns of IE1 and IE0 RNA expression showed that these genes are differentially expressed throughout the course of infection, suggesting that the two genes are regulated differently.

Transient assay experiments were used to further study the regulation of IE1 and IE0. The *Hin*dIII G fragment (90.4 to 96.8 map units) of Ac*M*NPV was cloned (pH3G) and transfected into Sf9 cells to determine whether these two genes could be expressed transiently. This fragment contains all of the IE1 transcriptional regulatory sequences and most, if not all, of the IE0 regulatory sequences. Therefore, it was presumed that both genes would be transcribed in pH3G-transfected cells. However, an analysis of RNAs isolated from pH3G-transfected cells showed that only IE1 RNA was expressed; the IE0 gene was transcriptionally silent. This result suggested either that the IE0 gene is not of the immediate-early class (that is, it requires a viral factor for its expression) or that *trans*-acting IE1 and/or IE0 gene products, expressed from pH3G, down-regulate IE0 expression. Previously, Carson et al. (4) showed that the IE1 gene product down-regulated expression of the IEN gene. To determine whether the IE1 gene product in pH3G-transfected cells down-regulated IE0 expression, a nonsense mutant form of pH3G (pH3GΔORF) was constructed. When this plasmid was transfected into Sf9 cells, higher levels of IE0 RNA were expressed, suggesting that the IE1 or IE0 gene product, expressed in pH3G-transfected cells, re-

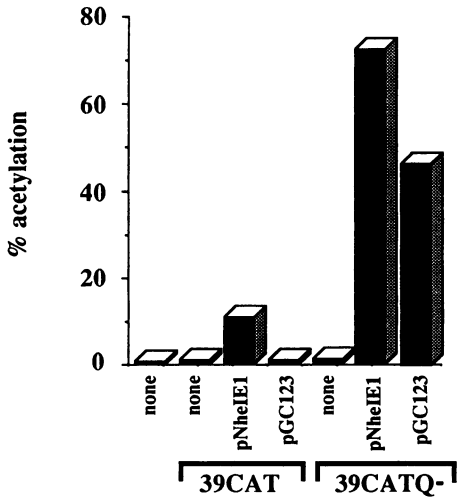

FIG. 7. Transactivation of the 39K gene by the IE0 gene product only in the presence of enhancer *hr*5. Sf9 cells (10^6) were cotransfected with 1 μg of each effector plasmid indicated and 1 μg of either p39CAT or p39CATQ-. Lysates were prepared 24 h posttransfection and assayed for CAT activity. A 50-fold-greater amount of p39CAT-expressing lysate was assayed to compensate for the greater amount of CAT expressed from p39CATQ-. Results are the averages of three independent experiments.

pressed expression of the IE0 gene. Previous to this study it was not known whether the IE0 promoter was active in the absence of any viral gene products. These results showed that IE0 RNA is expressed in the absence of viral gene products and hence by definition is an immediate-early gene. In contrast to IE0, levels of IE1 RNA decreased in the absence of functional IE1 and IE0 gene products. When the IE1 gene product was provided in *trans* on a different plasmid, steady-state levels of IE0 RNA decreased and IE1 RNA levels increased. The fact that a nonsense mutant of IE1 (pIE1ΔORF) had no effect on the expression of either IE1 or IE0 supports the observation that the IE1 gene product has a negative regulatory effect on IE0 and a positive autoregulatory effect on its own promoter. A recent report by Theilmann and Stewart (36) showed that the IE1 gene product of the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus also has autoregulatory activity.

To further analyze and quantitate the effects of the IE1 and IE0 gene products on their promoters, reporter plasmids (pIE1-NFCAT and pIE0-NFCAT) expressing CAT under the transcriptional control of the IE1 and IE0 promoters were constructed. These plasmids were subsequently cotransfected with either IE1- or IE0-expressing plasmids. The results of CAT assays support our previous RNA analysis. The IE1 gene product stimulated its own expression and repressed expression of IE0. The IE0 gene product also transactivated IE1, but it did not have an autoregulatory activity. To determine the effects of the IE1 and IE0 gene products on a plasmid that expresses both IE1 (unspliced) and IE0 (spliced) genes, the reporter pGCAT was constructed. This plasmid contains the CAT gene inserted into the IE1 ORF and is expected to express a spliced IE0 CAT RNA and a nonspliced IE1 CAT RNA from the IE0 and IE1 promoters, respectively. Cotransfection of pGCAT with pNheIE1 resulted in an approximate 5-fold decrease in IE0 CAT protein and a 5- to 10-fold increase in IE1 CAT protein expression. The nonsense mutant, pIE1ΔORF, had no effect on the expression of either CAT protein. Cotransfection of pGCAT with pGC123 had no effect on IE0 CAT expression but stimulated the expression of IE1 CAT. Together, these results further support the observation that the IE1 gene product has both positive and negative transcriptional regulatory properties. In addition, although IE1 and IE0 have structural similarities, the additional 54 amino acids at the amino terminus of IE0 confer different transcriptional regulatory properties on the gene product.

IE1 has previously been shown to have enhancer-independent and enhancer-dependent delayed-early gene transactivation activity (17, 18). To determine whether IE0 could transactivate delayed-early expression, transient cotransfection experiments using the reporter plasmids 39CAT (minus enhancer) and 39CATQ- (plus *hr5* enhancer) were conducted. These reporter plasmids express CAT under the control of the delayed-early 39K gene promoter. Although the IE1 and IE0 gene products have a large region that is identical in primary sequence, they regulate the expression of the 39K gene differently. Unlike IE1, IE0 did not transactivate the 39K promoter in the absence of the enhancer *hr5*. The effect of IE0 on the other four *hr* enhancers remains to be tested. However, this experiment suggests that IE1 and IE0 may have different transcriptional regulatory functions during infection.

Ac*M*NPV expresses at least three immediate-early genes (IE1, IE0, and IEN) with transcriptional regulatory properties. Transient assay experiments have been used to study how these gene products regulate viral gene expression. Early gene regulation may be regulated by a combinatorial interplay between these three gene products. All Ac*M*MPV immediate-early genes isolated to date except IE1 are transcriptionally silent during the late phase of infection. The fact that IE1 down-regulates the expression of both IE0 and IEN in transient assays suggests that it may function to shut off immediate-early gene expression during the late phase. IE1 also stimulated its own expression, suggesting that it may positively regulate its expression during infection. In sum, the IE1 gene product has been shown to (i) transactivate a number of different delayed-early genes (17, 32), (ii) down-regulate IEN (4) and IE0 expression, (iii) stimulate its own expression, and (iv) possibly have DNA-binding activity (14). We are currently pursuing a functional dissection of the IE1 gene product to determine the structural elements essential for its transcriptional regulatory activities.

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