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Expression of PE38 and IE2, Viral Members of the C_3HC_4 Finger Family, during Baculovirus Infection: PE38 and IE2 Localize to Distinct Nuclear Regions

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The pe38 gene of *Autographa californica* nuclear polyhedrosis virus represents one of the major early transcripts after viral infection. The function of the pe38 protein, which contains a C_3HC_4 zinc finger motif, is still not understood. We have raised polyclonal antiserum against the pe38 protein, PE38, produced in bacteria to investigate pe38 expression in the course of infection. A ~38-kDa polypeptide is first detectable at 2 h postinfection and decreases rapidly after 24 h. During the late phases of infection, a smaller protein of ~20 kDa which cross-reacts with the PE38-specific antiserum is visible at a constant level until 120 h postinfection. Since the pe38 gene shares a divergent promoter unit with the ie2 gene (formerly IEN), we have compared the expressions of the two genes. Polyclonal antibodies were raised against the bacterially expressed ie2 protein. The temporal expression pattern of the ~49-kDa ie2 protein is comparable to that of the ~38-kDa pe38 protein. Furthermore, both proteins are present in the nuclear fraction of *A. californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells, but the ~38-kDa pe38 protein is also detectable in the cytoplasm while the smaller protein of ~20 kDa is exclusively present in the cytoplasmic fraction. Immunofluorescence analysis reveals that PE38 and IE2 localize to distinct regions within the nucleus mainly detected after transfection of pe38- and ie2-expressing constructs.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) replicates in the nucleus of productively infected insect cell lines. Viral replication results in two infectious forms that have distinct roles during infection of the host organism.

The sequence of the circular DNA genome recently has been shown to comprise 133,894 bp with 337 potential open reading frames (ORFs) (1). Transcription of about 60 ORFs has been described (20).

The temporal gene expression of AcNPV is sequentially ordered in a cascade, and, to date, the molecular mechanism by which regulation is accomplished is poorly understood (for reviews, see references 3 and 29). One prominent feature of viral gene expression is the induction of an α-amanitin-resistant RNA polymerase activity which mediates transcription of the majority of late and very late genes (11, 44), while early viral promoters are recognized by the host RNA polymerase II.

Viral genes which are transcribed very early after infection are thought to have a regulatory function. Some of these genes, mainly the ie1 and ie2 (formerly IEN) genes, are able to act as transcriptional regulators in transient expression assays. The gene product IE1 has been shown to transactivate several early, late, and very late promoters (13, 14, 26, 31), while IE2 augments expression from these promoters (5, 6, 31, 46). Furthermore, IE1 is able to inhibit expression of the ie2 and ie0 promoters (7, 21). The putative regulatory functions of other early viral genes like pe38 (23), me53 (19), and cg30 (39) are based on sequence analysis. The amino acid sequence of the protein encoded by the pe38 gene contains putative DNA binding and dimerization domains (23). The carboxy terminus of the protein contains a leucine zipper, while the amino terminus includes a zinc finger motif, specifically, a C_3HC_4 or RING finger (10, 25). This zinc-binding domain has been found in a number of proteins of diverse function, many of which are thought to interact with DNA (25).

We have investigated the time course of pe38 expression and the intracellular localization of PE38 to provide first insights into the functional role of PE38 during AcNPV infection. Previous studies have shown that the transcriptional activities of the pe38 and ie2 genes, which are divergently transcribed, are similar during the course of infection. Transcription of both genes is first detectable at 1 h postinfection (p.i.), the genes maintaining a high level of transcription for about 12 h and decreasing in the late phases of infection (23). Therefore, we have investigated whether the corresponding transcriptional patterns predict similar time courses of translation for PE38 and IE2. Here we report that PE38 and IE2 are present during the early phase of infection and disappear during the late phase but that a smaller protein cross-reacting with PE38-specific antiserum is observed during the very late phase. Furthermore, the ~49-kDa IE2 and the ~38-kDa PE38 are targeted mainly to the nucleus, where the two gene products are associated with discrete nuclear structures.

MATERIALS AND METHODS

Cells, virus, and inhibitor treatment. The conditions for cell culture of *Spodoptera frugiperda* IPLB21 (41) and *Trichoplusia ni* TN-368 cells (16) and their infection with the AcNPV plaque isolate E (40) were as described earlier (23). *S. frugiperda* cells were treated with aphidicolin (5 μg/ml; Sigma) 1 h after AcNPV inoculation (33). AcNPV-infected cells were treated with cycloheximide (Sigma) at various times postinfection by adding fresh medium containing 1 mg of cycloheximide per ml. The inhibition of protein synthesis by cycloheximide was checked by Coomassie blue staining of proteins isolated at various times after AcNPV infection. In cycloheximide-treated cells (6 and 15 h p.i.), polyhedrin and p10 could not be detected.

Plasmid constructions. The constructions of pAcHind-F and its subclone pPE have been described previously (23). The plasmids pFHC and pAcPst-N were built by isolating the fragments *Hin*dIII-*Cla*I and *Pst*I-N, respectively, of the *Hin*dIII-F fragment of AcNPV DNA and inserting them into the vector pBlue-

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script (KS+), digested with *Hin*dIII, *Cla*I, or *Pst*I. The plasmid pPE-HR1 was generated by insertion of a 711-bp PCR-amplified homologous region 1 (HR1) fragment into the *Cla*I and *Xho*I sites of pAcPE. The PCR (35) fragment was amplified from the plasmid pAcHind-F by using the primers 5′ TTTTTGACAC TATCGATGATTGACCCC 3′ and 5′ TTTTTCTCGAGGCGGGAAACATTT TACACGC 3′. To construct pFHC-HR1, the PCR-generated HR1 fragment was inserted into the *Cla*I and *Xho*I sites of the pFHC plasmid.

Transcriptional mapping. S1 nuclease mapping of the 5′ ends of pe38 transcripts in AcNPV-infected *S. frugiperda* cells was performed as previously described (23) except that 20 μg of total cytoplasmic RNA was used instead of poly(A)$^+$ RNA. For primer extension analysis, cytoplasmic RNA (10 μg) was annealed to a pe38-specific 29-base primer (5′ TATGCTGTCCAAATTGTCT TGCAACTGTC 3′) or a p10-specific 30-base primer as described earlier (2).

Transfection experiments. Uninfected *S. frugiperda* cells were transfected with 10 μg of plasmid DNA by calcium phosphate precipitation as described previously (23). Cells were harvested 40 h after transfection, and total cellular protein extracts were prepared by lysis in sodium dodecyl sulfate (SDS) sample buffer.

Bacterial expression and purification of PE38 and IE2. A *Sty*I-*Xho*I fragment of cDNA clone pe38 (23), including the entire pe38 ORF apart from the initiating ATG codon, and the *Eco*RI-*Xho*I fragment of cDNA clone ie2 (formerly IEN) (23) were blunt ended with Klenow polymerase, ligated to 8-mer *Bam*HI adaptors (Boehringer), and inserted into the *Bam*HI site of the pQE-9 vector (Qiagen). The recombinant pe38 protein (rPE38), expressed from pQE-PE38, carried an N-terminal fusion of 11 amino acids with a tag of 6 histidine residues. Plasmid pQE-IE2 produced a recombinant ie2 protein (rIE2) with an N-terminal fusion of 21 amino acids including the histidine tag. The histidine-tagged proteins were expressed in *Escherichia coli* M15/pREP4 by induction with isopropyl-β-D-thiogalactopyranoside and purified under denaturing conditions.

Production of antibodies. Polyclonal antisera directed against denatured PE38 and IE2 were produced by immunizing rabbits with the respective purified proteins by standard procedures (15). The PE38 antiserum (anti-PE38) was precleared with an acetone powder of uninfected *S. frugiperda* cells according to published methods (15). The monoclonal antibody 58C9 against the *Drosophila* TATA box binding protein was kindly provided by Robert Weinzirl (University of California, Berkeley).

Cell extracts and subcellular fractionation. AcNPV-infected *S. frugiperda* cells were collected by low-speed centrifugation and washed twice with ice-cold Tris-saline. For total cellular protein extract, the pelleted cells were lysed in SDS sample buffer and boiled for 5 min. Detergent-based subcellular fractionation of *S. frugiperda* cells has been described previously (17). Briefly, the cell pellet was resuspended in TBN buffer (10 mM Tris-HCl [pH 6.5], 140 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 1 μg of aprotinin per ml) and incubated for 10 min on ice. After centrifugation for 1 min at 9,000 × *g*, the supernatant was taken as the cytoplasmic fraction and adjusted to 0.1 N NaOH. The pelleted nuclei were resuspended in an equal volume of radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 3 mM $MgCl_2$, 1% Nonidet P-40, 1 μg of aprotinin per ml). This nuclear fraction was also adjusted to 0.1 N NaOH. Both subcellular fractions were mixed with an equal volume of 2× SDS sample buffer and boiled for 5 min prior to analysis.

Detergent-free nuclear extracts were prepared according to the method of Parker and Topol (30) with minor modifications, as described previously (22). The supernatant of the centrifugation-clarified Dounce-homogenized cells was taken as the detergent-free cytoplasmic fraction. It was adjusted to 1 mM phenylmethylsulfonyl fluoride–230 ng of aprotinin per ml–10% glycerol and dialyzed for 4 h against 40 mM KCl–10 mM Tris-HCl of (pH 8.0)–0.1 mM EDTA–1 mM dithiothreitol–10% glycerol.

Immunoblotting. Proteins were resolved by SDS–12.5% polyacrylamide gel electrophoresis (24) and transferred to nitrocellulose membranes (Hybond-ECL; Amersham) by using a semidry blotting apparatus (Biometra). The membranes were blocked overnight at 5°C or for 1 h at room temperature in PBS-T (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 0.1% Tween-20) containing 5% nonfat milk powder. The primary antibodies were diluted in blocking buffer (polyclonal sera at a dilution of 1:20,000 and monoclonal anti-TBP at 1:1,000), incubated with the membranes for 1 h at room temperature, and washed once for 10 min and twice for 5 min in PBS-T. A 1-h incubation with the secondary, horseradish peroxidase-conjugated antibodies (donkey anti-rabbit at a dilution of 1:4,000 and sheep anti-mouse at 1:1,000; Amersham) was followed by extensive washing in PBS-T (once for 10 min, four times for 5 min). The antigen-antibody complexes were revealed by enhanced chemiluminescence (ECL system; Amersham).

Indirect immunofluorescence. *S. frugiperda* cells were seeded on glass coverslips in six-well plates (Falcon) at 10^6 cells per well, allowed to attach for 4 h at 27°C, and transfected with 5 μg of plasmid pAcHind-F, pPE-HR1, or pAcPst-N by calcium phosphate precipitation, as previously described (23). After 45 to 48 h, the cells on the coverslips were rinsed with PBS, fixed by submersion in 100% acetone (precooled to −70°C) for 15 min at room temperature, and finally rehydrated. TN-368 cells were also seeded on glass coverslips but allowed to attach overnight. The cells were infected with AcNPV at a multiplicity of 10 to 20 PFU per cell and stained with anti-IE2 at 5 h p.i. Fixation of the cells was performed with 2% paraformaldehyde for 20 min at room temperature followed by a quenching step in 50 mM NH_4Cl in PBS for 10 min at room temperature. The cells were permeabilized by incubation in 0.4% Triton X-100 for 4 min. The cells, fixed either by acetone or by paraformaldehyde, were blocked for 30 min at room temperature with PBS containing 1% bovine serum albumin and then incubated for 45 min with a 1:1,000 dilution of polyclonal PE38 or IE2 antisera. The cells were washed three times for 10 min in PBS and incubated for 45 min with a 1:100 dilution of fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin G (Nordic Immunologicals). After three washes in PBS for 10 min, the cells were mounted on microscope slides in 9:1 glycerol-PBS–2.3% 1,4-diazobicyclo[2.2.2]octan. Cells were stained for DNA with 100 ng of bisbenzimide (Hoechst 33258) per ml added during the final wash and then mounted in 9:1 glycerol-PBS–5% *n*-propyl gallate. Photographs were taken by using the Olympus BH2-RFC microscope-camera system with Kodak Ektachrome 400HC film.

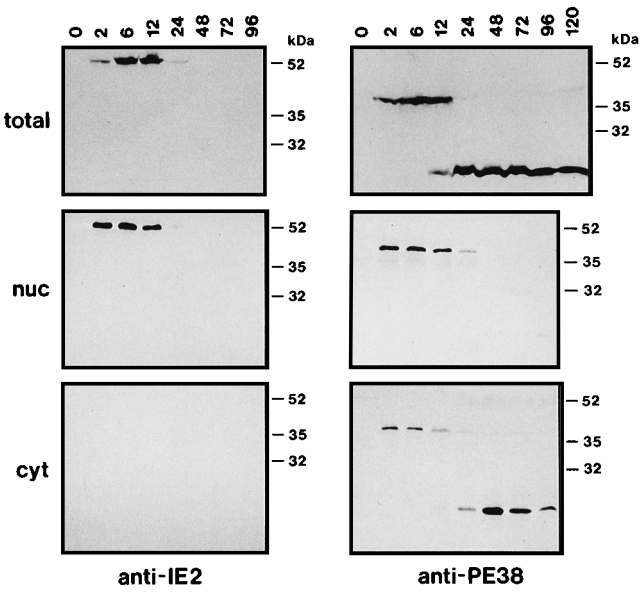

FIG. 1. Expression of PE38 and IE2 during the course of infection and their subcellular localization. Cell extracts were prepared from uninfected *S. frugiperda* cells (lane 0) or from cells at 2, 6, 12, 24, 48, 72, 96, and 120 h p.i. (lanes 2, 6, 12, 24, 48, 72, 96, and 120) and electrophoresed on SDS–12.5% polyacrylamide gels. The proteins of total cellular extracts (total) and of the cytoplasmic (cyt) and nuclear (nuc) fractions were analyzed by immunoblotting with polyclonal antisera raised against PE38 (anti-PE38) or IE2 (anti-IE2). Protein size markers are indicated on the right.

RESULTS

Expression of PE38 and IE2 during the course of infection. Previous analysis indicated that expression of pe38 transcripts occurs not only during the early phase of infection but also late in infection (23). In this study, we have examined whether the pe38 gene product is detectable throughout the different temporal phases of infection. Western blot (immunoblot) analysis of total cell protein extracts of AcNPV-infected *S. frugiperda* cells isolated at various times after infection revealed a single specific ~38-kDa polypeptide, which matches the predicted size of the putative pe38 ORF. This protein was visible from 2 h p.i. and was maintained until 12 h p.i. The level of the ~38-kDa protein at 24 h p.i. varied with the efficiency of infection, but no expression was detected later than 24 h p.i. (Fig. 1). Surprisingly, concomitant with the rapid decrease in the level of the ~38-kDa PE38, a smaller polypeptide of ~20 kDa which cross-reacted with the PE38 antiserum was observed (Fig. 1). This smaller polypeptide was expressed at a constant level until at least 120 h p.i., the last time point assayed.

Recent studies have shown that the transcriptional pattern of the ie2 gene, which is transcribed in the opposite direction relative to the pe38 gene, is comparable to pe38 transcriptional

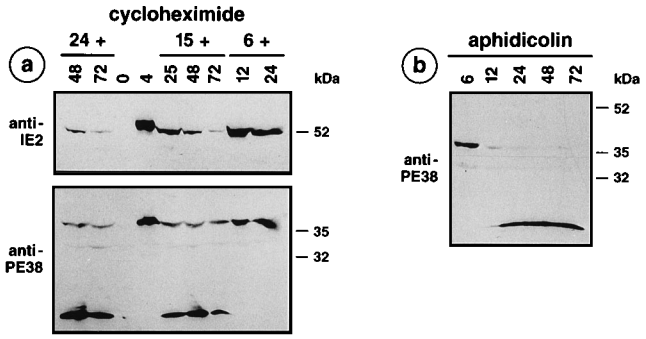

FIG. 2. Expression of PE38 and IE2 in cycloheximide- or aphidicolin-treated AcNPV-infected cells. (a) Total cellular extracts were prepared from uninfected *S. frugiperda* cells (lane 0), from cells at 4 h p.i. (lane 4), or from cells after treatment with cycloheximide at 6, 15, or 24 h p.i. (lanes 6+, 15+, and 24+) and harvested at 12, 24, 25, 48, and 72 h p.i., respectively (lanes 12, 24, 25, 48, and 72). (b) Total cellular extracts were prepared from aphidicolin-treated cells at 6, 12, 24, 48, and 72 h p.i. (lanes 6, 12, 24, 48, and 72). Proteins were resolved on SDS–12.5% polyacrylamide gels and stained with anti-PE38 or anti-IE2 antisera. Protein size markers are indicated on the right.

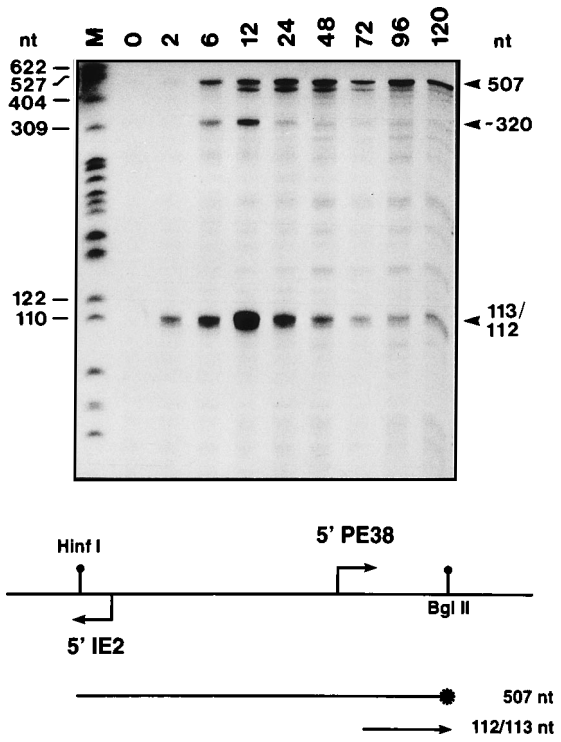

FIG. 3. S1 analysis of pe38 transcription during the course of infection. Cytoplasmic RNAs (20 μg) isolated from uninfected *S. frugiperda* cells (lane 0) or from cells at 2, 6, 12, 24, 48, 72, 96, and 120 h p.i. (lanes 2, 6, 12, 24, 48, 72, 96, and 120) were hybridized to a 5′-end-labeled DNA probe of 507 nt. The hybrids were analyzed on a 6% polyacrylamide gel. The major protected hybrids of 113 and 112 nt and the protected DNA probe of 507 nt are indicated by arrows. Positions of DNA size markers (lane M) are shown on the left.

appearance (23). Here we provide evidence that the proteins IE2 and PE38 are expressed in a similar fashion. As early as 2 h p.i., the IE2 antiserum specifically recognized a single ~49-kDa protein, which had decreased markedly by 24 h p.i. (Fig. 1). The size of 49 kDa correlates with the prediction from the ie2 sequence analysis (6).

The decrease in abundance of both proteins, IE2 and the 38-kDa PE38, at 24 h p.i. and the conversion from the 38-kDa form to the 20-kDa polypeptide were further investigated by blocking protein synthesis at various times p.i. If the infected cells were treated with cycloheximide at 6 h p.i., the 38-kDa PE38 and the 49-kDa IE2 remained stable until 24 h p.i. Furthermore, the small polypeptide was not observed (Fig. 2). Inhibition by cycloheximide at later times after infection demonstrated the stability of the proteins. If cycloheximide was added at 15 or 24 h p.i., IE2 was detectable until 72 h p.i. indicating active degradation of IE2 during the late phase of infection (Fig. 2). In addition, the treatment with cycloheximide at 15 h p.i. revealed that the 38-kDa PE38 and the smaller polypeptide were already present before 15 h p.i., but the 38-kDa PE38 did not disappear at 48 and 72 h p.i., as was observed in vivo.

Correlation of temporal pe38 transcription and translation during infection. The appearance of a small polypeptide cross-reacting with anti-PE38 late during infection raised the question of whether it was translated from a late pe38 transcript. Northern (RNA) blot analysis was previously performed with poly(A)$^+$ RNA prepared from AcNPV-infected cells at various times p.i. This indicated no smaller pe38-specific transcript in addition to the major pe38 transcript of 1,300 nucleotides (nt), which is present during early and late phases of infection (23). In this study, we have investigated by S1 analysis the early transcriptional start site of the PE38 gene during the course of infection. Cytoplasmic RNA isolated at various times p.i. was hybridized to a 5′-end-labeled *Bgl*II-*Hin*fI DNA probe of 507 nt in length. Protected fragments of 113 and 112 nt indicated transcriptional initiation, which occurred by 2 h p.i. and increased until 12 to 24 h p.i. The level of this transcript decreased by 48 h p.i.; however, it remained weakly detectable until 120 h p.i. (Fig. 3). We also observed protection of a ~320-nt fragment that accumulated at 6 to 12 h p.i., which was not investigated in further detail.

The transcriptional mapping of the pe38 gene suggests that the early transcript, which initiates from the divergent promoter unit of the pe38 and ie2 genes, is also present during the late phases of infection. The presence of this transcript during late phases of infection could be due to late transcriptional initiation or simply RNA stability. To address the question of whether the 20-kDa polypeptide is made from a late pe38 transcript, we have analyzed pe38 expression in AcNPV-infected cells treated with aphidicolin. The inhibitory effect of aphidicolin on late viral transcription has been demonstrated for the late p10 gene by primer extension studies (Fig. 4b). In contrast, aphidicolin had no effect on early pe38 transcription at 6, 24, and 48 h p.i. (Fig. 4a). Furthermore, the conversion of the 38-kDa PE38 to the smaller polypeptide was not influenced by aphidicolin, as shown by Western blot analysis of total cell protein extracts (Fig. 2b). These results support the notion that the smaller pe38 protein detectable during the late phases of infection is not expressed from a late transcript. After aphidicolin treatment, IE2 was still present (data not shown).

Taking the above results together with the results of protein synthesis inhibition, we postulate that the smaller polypeptide is generated by proteolytic cleavage of the 38-kDa PE38, although an alternative mechanism cannot be excluded. Factors involved in this processing are probably expressed during the early phase, since inhibition of protein synthesis at 6 h p.i. abolished the conversion. The appearance of the 20-kDa polypeptide until 120 h p.i. is probably due to protein stability.

The degradation of IE2 was delayed by inhibition of protein synthesis and was not affected by blocking late transcription. These results suggest an activity present after viral replication which could be involved in the degradation process. Although

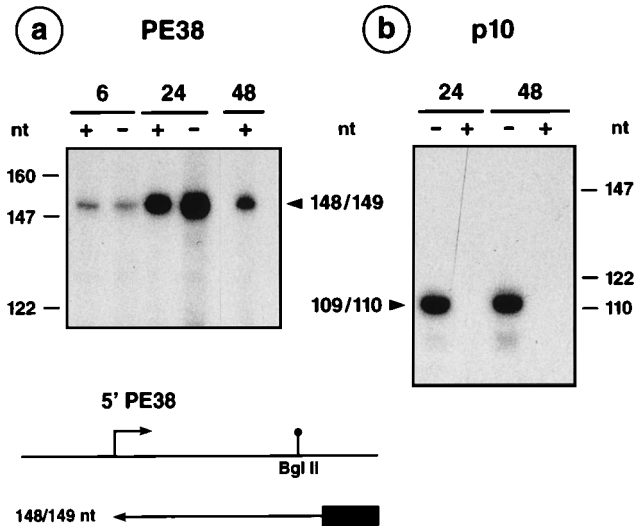

FIG. 4. Primer extension analysis of the effect of aphidicolin on pe38 and p10 transcription. (a) Cytoplasmic RNAs (10 μg) prepared at 6, 24, and 48 h p.i. (lanes 6, 24, and 48) from untreated (lanes −) or aphidicolin-treated (lanes +) cells were hybridized to the pe38-specific primer. (b) Cytoplasmic RNAs (5 μg) prepared at 24 or 48 h p.i. (lanes 24 and 48) from untreated (lanes −) or aphidicolin-treated (lanes +) cells were hybridized to the p10-specific primer. The extended products were analyzed on a 6% polyacrylamide gel. The times of exposure for the autoradiograms were overnight (a) and 1 h (b). Positions of DNA size markers are shown on the left (a) and right (b).

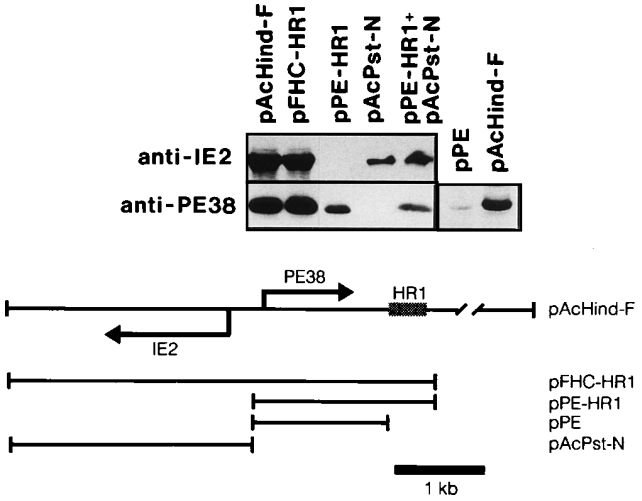

FIG. 5. Expression of PE38 and IE2 after transfection of *S. frugiperda* cells. Cells transfected with each of the plasmids pAcHind-F, pPE, pFHC-HR1, and pAcPst-N or cotransfected with pPE-HR1 and pAcPst-N were harvested 40 h after transfection, and cell extracts were tested for IE2 or PE38 expression by using either polyclonal anti-IE2 or anti-PE38 antisera. Length and location of each transfected plasmid are indicated below the gel. The arrows represent the transcriptional start sites of ie2 and pe38 genes.

PE38 and IE2 disappeared at the same time during infection, the involved mechanisms seem to be quite different.

Expression of PE38 from transfected DNA. To investigate which PE38 polypeptide is expressed from transfected plasmids and whether the levels of PE38 and IE2 expression are mutually influenced, we have performed transient expression studies. Uninfected *S. frugiperda* cells were transfected with the plasmid pPE, which contains only the pe38 ORF and 5′ sequences of about 80 bp upstream of the ATG start codon. These 5′ sequences, including putative TATA sequences, are required for basal transcriptional activity, as shown by previous pe38 promoter studies (22). In this study we have demonstrated that after transfection of the plasmid pPE, weak expression of a single polypeptide of ~38 kDa was detected with the PE38 antiserum (Fig. 5). The lack of the 20-kDa polypeptide confirms that viral factors are necessary for its occurrence.

Transfection of the pAcHind-F plasmid resulted in relatively strong expression of PE38 (Fig. 5). This plasmid includes the ie2 and pe38 ORFs as well as the HR1 (12), the lef-2 gene (31), and other potential ORFs. To distinguish whether IE2 or *cis*-acting sequences influence the stronger expression of PE38, various deletions were investigated. Deletion of sequences downstream of HR1 did not influence the level of pe38 expression (Fig. 5, pFHC-HR1). Indeed, the presence of the HR1 element downstream of the pe38 ORF (pPE-HR1) led to an increase of pe38 expression compared with the expression level for the HR1-deficient construct pPE, but it did not restore the relatively high level of pe38 expression observed with plasmids pAcHind-F and pFHC-HR1 (Fig. 5). Previous studies have shown that the HRs stimulate expression of some early genes, even in uninfected *S. frugiperda* cells (26, 28, 34).

It is worth mentioning that IE2 is also expressed much more efficiently from the pAcHind-F construct than from plasmid pAcPst-N, which contains only the ie2 ORF (Fig. 5). To investigate whether the ie2 gene product *trans*-activated the pe38 gene or vice versa, *S. frugiperda* cells were transfected with plasmids which solely encode either pe38 (pPE-HR1) or ie2 (pAcPst-N) genes. The ~49-kDa ie2 protein was detected to comparable extents in cells transfected with pAcPst-N and cells transfected with both plasmids, pPE-HR1 and pAcPst-N (Fig. 5). This suggests that PE38 plays no significant role in the stimulation of ie2 expression and that IE2 is not involved in pe38 regulation. These results suggest that *cis*-acting elements are probably responsible for very efficient expression of PE38 and IE2.

Furthermore, we have tested whether the different levels of pe38 translation from the transfected plasmids correlate with the levels of transcriptional activation. Our experimental data indicate that the pe38 gene is most efficiently transcribed from the transfected plasmid pAcHind-F, pointing to regulatory events at the transcriptional level (data not shown). However, further experiments are needed to elucidate the role of potential *cis*-acting elements in the regulation of pe38 and ie2 expression.

Subcellular localization of PE38 and IE2. In order to determine the intracellular distribution of PE38 and IE2 during the course of infection, we have analyzed cytoplasmic and nuclear fractions of *S. frugiperda* cells at various times after infection with AcNPV by a detergent-based procedure. As depicted in Fig. 1, the ~38-kDa PE38 was detected mainly in the nuclear fraction but was also present in the cytoplasmic cell extract, while the ~20-kDa protein was exclusively present in the cytoplasmic fraction. In contrast, IE2 could be detected only in the nuclear cell extract. These results are in agreement with the recent observation that the ie2 gene product is localized in the nucleus after transient expression (45). The investigation of nuclear salt extracts (see Materials and Methods) confirmed the nuclear localization of PE38 and IE2 until 12 to 24 h p.i. (Fig. 6). However, the 20-kDa protein was not observed in the cytoplasmic or the nuclear fractions. Since this cytoplasmic fraction represents only the soluble protein extract, the failure to detect the small PE38 protein could point to its association with membranes or other insoluble structures that are present in the cytoplasmic cell extract prepared by the detergent-based procedure. As a control, the nuclear salt extract was also ex-

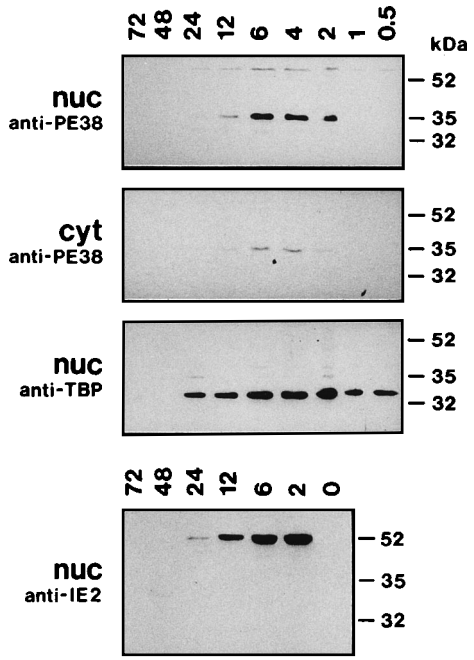

FIG. 6. Expression of the 38-kDa PE38 and the 49-kDa IE2 during the infection. Nuclear salt and cytoplasmic extracts (see Materials and Methods) were prepared from infected *S. frugiperda* cells at 0.5, 1, 2, 4, 6, 12, 24, 48, and 72 h p.i. (lanes 0.5, 1, 2, 4, 6, 12, 24, 48, and 72). The proteins were analyzed as described in the legend for Fig. 1. As a control, expression of TBP was investigated by using monoclonal antibodies raised against *Drosophila* TBP (anti-TBP). Protein size markers are indicated on the right. Abbreviations are as described in the legend for Fig. 1.

amined for the presence of a known nuclear protein, the TATA-binding protein (TBP). A polypeptide of about 33 kDa was visible only in the nuclear fraction of uninfected and infected *S. frugiperda* cells until 24 h p.i. when *Drosophila melanogaster* anti-TBP 58C9 was used (Fig. 6). It should be noted that these results conflict with a previous study in which it was demonstrated that although transcription of the *S. frugiperda* TBP declined after 24 h p.i., two polypeptides of 36 and 38 kDa could be detected with anti-TBP 58C9 as long as 72 h p.i. (32).

Localization of PE38 and IE2 to punctate structures in the nucleus. The localization of PE38 and IE2 within the nucleus was confirmed by indirect immunofluorescence. *S. frugiperda* cells were transfected with the plasmid pAcHind-F, which produces the ~38-kDa PE38 and the ~49-kDa IE2. The cells were examined 45 to 48 h after transfection by using polyclonal antisera raised against PE38 (Fig. 7c and g) and IE2 (d and h) and the corresponding preimmune sera (a and b). The nuclei were stained with bis-benzimide (Fig. 7 e and f). Interestingly, the staining pattern observed with the antibodies indicated that the viral gene products are both localized to similar structures within the nucleus. The number and size of the stained nuclear structures varied among individual transfected cells (Fig. 7 c and d). Moreover, this pattern was not observed after staining with the respective preimmune sera (Fig. 7 a and b) or in mock-transfected cells incubated with either anti-PE38 or anti-IE2 (data not shown). Both PE38 and IE2 appeared to be associated with these nuclear structures, since neither antibody cross-reacted with the product of the other gene.

Transfection of cells with pAcHind-F resulted in a high accumulation of the ~38-kDa PE38. To investigate whether the nuclear structures stained by anti-PE38 antiserum were a result of the high level of the ~38-kDa protein, we have ana-

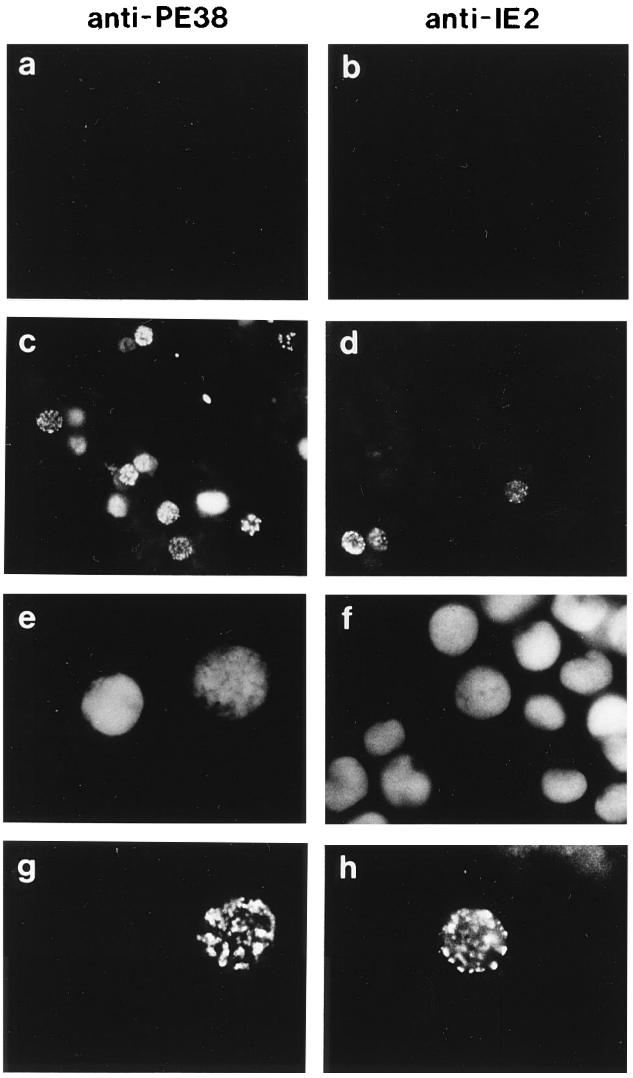

FIG. 7. Localization of PE38 and IE2 to punctate nuclear structures after transient expression in transfected *S. frugiperda* cells. Cells were transfected with the plasmid pAcHind-F, which encodes the pe38 and ie2 genes, and were stained with anti-PE38 preimmune serum (a) or anti-IE2 preimmune serum (b). The nuclear structures were detected with anti-PE38 (c and g) and anti-IE2 (d and h). The nuclear localization of the cellular DNA was visible after staining with bis-benzimide (e and f). The sections shown in panels e and f correspond to panels g and h, respectively.

lyzed cells transfected with the plasmid pPE-HR1. This plasmid expressed the full-size PE38 protein at a significantly lower level than pAcHind-F did (Fig. 5). As depicted in Fig. 8a, discrete nuclear structures were also detected by PE38 antibodies after transfection of plasmid pPE-HR1. Similar results were obtained for expression of the ie2 gene. When IE2 was expressed at a lower level from the plasmid pAcPst-N, the staining pattern was exactly as previously observed. Furthermore, these results indicate that association of the viral gene products PE38 and IE2 with nuclear structures was not dependent on simultaneous expression of both proteins.

The detection of PE38- and IE2-specific signals was more difficult after AcNPV infection than after transfection. One possible explanation could be the relatively low level of expression in individual cells after infection. Figure 9b presents the staining pattern with anti-IE2 obtained with TN-368 cells 5 h

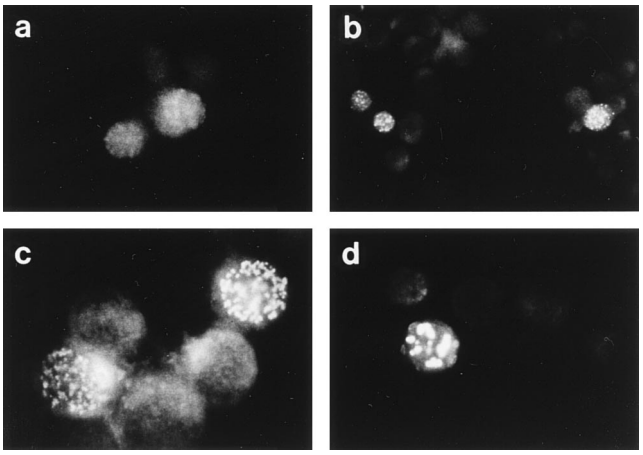

FIG. 8. Punctate distribution of PE38 and/or IE2 proteins within the nucleus. *S. frugiperda* cells were transfected with the plasmid pPE-HR1, which encodes only PE38, and were stained with anti-PE38 antiserum (a) or were transfected with plasmid pAcPst-N, which encodes the ie2 gene, and stained with IE2 antiserum (b). (c and d) Variability in number and size of nuclear structures stained by anti-PE38 after transfection with the plasmid pAcHind-F.

p.i. A similar staining pattern was observed with anti-PE38. The IE2- and PE38-specific signals were less abundant than they were after transfection, but they were not visible in uninfected cells or after staining of the infected cells with preimmune sera (Fig. 9a and c).

DISCUSSION

An understanding of the functional role of the pe38 gene product during AcNPV infection requires a detailed knowledge of its site of action. With polyclonal antibodies available, we have examined the accumulation of PE38 in infected *S. frugiperda* cells and compared it with expression of the protein encoded by the ie2 gene. During the early phase of infection, the ~38-kDa PE38 and the ~49-kDa IE2 were detected until 12 to 24 h p.i., while a smaller PE38 polypeptide of ~20 kDa was observed during the late phases of infection. The ~38-kDa PE38 is present prior to the ~20-kDa protein. It is interesting that the 34-kDa protein derived from the p34 gene of the related baculovirus *Orgyia pseudotsugata* nuclear polyhedrosis virus, which shows about 37% homology to the pe38 gene of AcNPV (38), was detectable from 2.5 to 120 h p.i., while a p34 product of 20 kDa appeared from 24 to 120 h p.i. (43). The accumulation of the smaller p34 protein correlated with the expression of a late p34-specific transcript, which led to the proposal that alternative transcriptional initiation might occur (43). However, a different mechanism accounts for the presence of the ~20-kDa pe38 protein, since inhibition of late transcription did not influence the occurrence of the smaller protein. Our data suggest that expression of viral factors before the onset of viral replication is necessary for the conversion of the 38-kDa PE38 to the 20-kDa protein. To date, the nature of these postulated factors is still unclear.

In contrast to the situation with the 38-kDa PE38, the disappearance of IE2 during the late phase of infection is probably due to a late viral function, as suggested by inhibitor experiments. Recent studies based on transient expression assays suggest the involvement of IE2 in very late gene expression (31). Since IE2, like the 38-kDa PE38, was not detectable during the very late phase, it remains to be shown whether IE2 is involved in the regulation of very late promoters in vivo.

The full-size gene products of PE38 and IE2 colocalize in the nucleus. Recent experiments have indicated that deletions in the C terminus of IE2 affected the localization of this protein, although a specific nuclear localization signal (NLS) has not been identified (45). If transport of PE38 to the nucleus is mediated by NLSs (36), the sequence YKKTQ within the pe38 protein might be a potential candidate. Using indirect immunofluorescence, we have observed that the two proteins, PE38 and IE2, are colocalized in discrete subnuclear structures mainly after transfection of *S. frugiperda* cells. Our data also suggest that the intracellular localization of neither the ~38-kDa PE38 nor IE2 depends on the presence of the other protein. The observed localization of PE38 and IE2 in punctate structures was more easily detected after transient expression than it was after infection. This could be due to the fact that there is less of the proteins in the infected cells. However, the nuclear structures with which PE38 and IE2 associate may be reorganized as a consequence of viral infection.

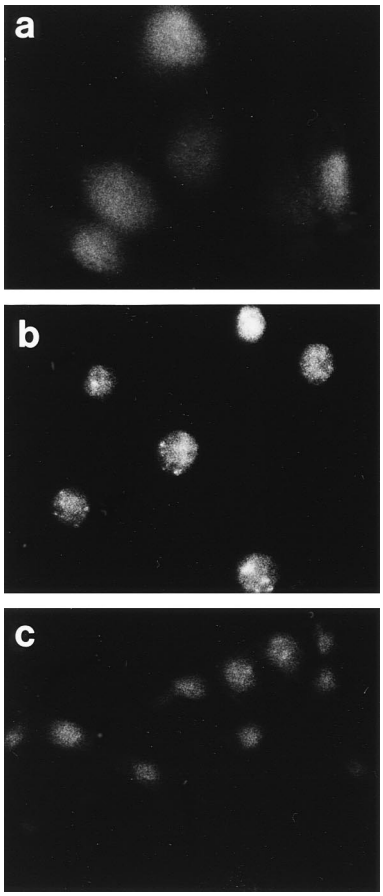

FIG. 9. Punctate nuclear distribution of IE2 after AcNPV infection of TN-368 cells. Uninfected cells (a) or cells 5 h p.i. (b) were stained with anti-IE2 antiserum. As a control, infected cells were stained with anti-IE2 preimmune serum at 5 h p.i. (c).

The existence of subnuclear bodies has been known for a long time. Some of these structures have been shown to colocalize with antibodies that stain components of the splicing machinery (4, 9, 37). Another class of nuclear bodies, referred to as PODs (promyelocyte [PML] oncogenic domains), which are thought to be a multiprotein complex but whose function is still unknown, have been identified by association with the vertebrate PML gene product (8, 42). PODs are probably not restricted to vetebrates, since overexpression of PML in

Schneider S2 *Drosophila* cells is reported also to result in a punctate nuclear localization pattern (8). Like the pe38 gene, the PML gene encodes a member of the C_3HC_4 zinc finger family. Mutations within the zinc finger motif of PML abolish the discrete nuclear pattern obtained in transfected cells (18). The nuclear location of PML in discrete structures is disrupted by infection with herpes simplex virus type 1. Interestingly, herpes simplex virus type 1 encodes another member of the C_3HC_4 zinc finger family, ICP0, that is suggested to affect the localization of PML (27). It will be of great interest to determine whether the distinct nuclear pattern observed with baculovirus gene products corresponds to structures recognized by the cellular gene product PML and the viral ICP0 proteins.

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