The Early Baculovirus he65 Promoter: On the Mechanism of Transcriptional Activation by IE1

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We have initiated studies on the mechanism of early transcriptional activation of the early he65 promoter during infection with Autographa california multicapsid nuclear polyhedrosis virus. This analysis is based on a comparison of the sequences required for he65 promoter activation with those sequences that support specific protein binding. The he65 promoter is located immediately downstream of the homologous region (hr) 4a. The sequences of hr4a are characterized by two imperfect palindromes of 24 bp. The results of transient expression assays indicate promoter activation in the presence of both the proximal palindrome and the known viral trans-regulator IE1. The results of mobility shift assays and DNasel footprinting analyses reveal differences in specific protein binding at and close to the proximal palindrome depending on whether the nuclear protein extracts are prepared from uninfected or infected cells. The analysis of the protein binding complex at the proximal inverted repeat with extracts from infected cells suggests the involvement of both IE1 and IE0 as oligomers. The minimal protein binding sequences include the left half-site of the 24 bp repeat with 9 additional bp of the flanking sequences. The right half-site of the repeat also directs binding although with lower affinity as confirmed by phenanthroline-copper footprinting assays. Both half-sites of the repeat are thus essential for he65 promoter activation, suggesting that IE1 acts via cooperative binding. We conclude that the proximal inverted repeat is able to interact with both IE1 and IE0 although IE1 is sufficient for activation at least in transient expression assays.

INTRODUCTION

Gene expression of the baculovirus Autographa california multicapsid nuclear polyhedrosis virus (AcMNPV) can be divided into early, late and very late phases. Transcription of late and very late genes is mediated by a virus-induced RNA polymerase, while early promoters are recognized by the host RNA polymerase II (for reviews see O’Reilly et al., 1994; Blissard, 1996). As demonstrated by transient expression assays, the transcriptional activity of some early promoters depends exclusively on host factors while others need additional viral activators. To date, only two host proteins have been described that may be involved in the regulation of early viral transcription (Krappa et al., 1992; Kogan and Blissard, 1994). The best-studied viral transcription factor is IE1, which is highly conserved among baculoviruses and which is thought to act as a multifunctional protein during the infection cycle. As initially shown for the 39K and p35 promoters, IE1 can function as transcriptional activator of early promoters (Guarino and Summers, 1986b; Nissen and Friesen, 1989). Infection studies with cycloheximide as inhibitor of protein synthesis further support the view that IE1 modulates early gene transcription during the infection cycle (Ross and Guarino, 1997). In addition to the stimulatory effect of IE1, cotransfection studies demonstrate the potential of IE1 to regulate negatively the early promoters ie2 and pe38 (Kovacs et al., 1991; Leisy et al., 1997). Moreover, IE1 may be involved directly or indirectly in viral replication and late transcription (Passarelli and Miller, 1993; Lu and Miller, 1995). Infection with a temperature-sensitive mutant that carries a mutated ie1 gene leads to a delay of viral replication (Ribeiro et al., 1994). This finding suggests a functional role of IE1 during infection.

There is evidence that IE1 acts both by sequence-independent (Blissard and Rohrmann, 1991; Blissard et al., 1992) and by sequence-dependent mechanisms (Guarino and Summers, 1986a). The sequence-dependent activation relies on the presence of sequences from homologous regions (hrs), which are interspersed throughout the AcMNPV genome at eight different locations (Cochran and Faulkner, 1983; Ayres et al., 1994). These eight hrs (hr1, hr1a, hr2, hr3, hr4a, hr4b, hr4c, and hr5) are characterized by two to eight copies of imperfect palindromes each containing an EcoRI site at its center (Guarino et al., 1986). As mainly shown for hr5, the cis-linkage of hrs to early promoters leads to enhanced expression in the presence of IE1 (Guarino and Summers, 1986a; Guarino et al., 1986; Nissen and Friesen, 1989). The hrs may also act as origins of DNA replication (for review see Kool et al., 1995).

The only known spliced gene of AcMNPV is ie0, whose ORF is identical to the ie1 ORF with 54 additional
amino acids at the N terminus (Chisholm and Henner, 1988). Little is known about the role of IE0 as a trans-regulator (Kovacs et al., 1991).

Our interest is focused on how early viral promoters are regulated by host factors in concert with viral gene products during the infection cycle. We have analyzed the early he65 promoter since the close proximity to hr4a (formerly hr4aer), and the transcriptional delay in comparison with the major early genes ie1, ie2, me53, and pe38 has suggested a potential role of IE1 in the activation process in vivo (Becker and Knebel-Mörsdorf, 1993). Our recent results demonstrate that indeed IE1 can activate the he65 promoter in insect TN-368 and mammalian BHK21 cells (Murges et al., 1997). Here, we present the analysis of the he65 promoter sequences that are essential both for the activation by IE1 and for the binding of specific protein complexes that contain IE1 or IE1 and IE0. Thus our results provide the first evidence for the putative role of IE1 and IE0 in the regulation of the he65 promoter.

RESULTS

Experimental design

The regulation of the early he65 promoter was analyzed in the permissive cell lines S. frugiperda and TN-368. By transient expression and DNA–protein interaction studies, we have investigated the role of IE1 in the activation process. Various deletions in the he65 promoter were tested for activity to determine the sequences sufficient for transcriptional activation upon co-transfection with IE1-expressing plasmids. The DNA±protein complex formed at the activating sequences and the involvement of IE1 were studied by mobility shift assays and footprinting analyses.

Activation of the he65 promoter in AcMNPV-infected S. frugiperda and TN-368 cells

We cloned he65 promoter fragments in front of the reporter gene CAT (Fig. 1b) and tested the activity of these constructs in uninfected or AcMNPV-infected S. frugiperda and TN-368 cells, respectively. All promoter constructs include the transcriptional and translational start sites of the he65 gene but vary in the lengths of their upstream sequences (Fig. 1b). Plasmid pHE65-CAT474 contains the complete hr4a, whereas pHE65-CAT474 carries the proximal of the two inverted repeats present in hr4a (Fig. 1b). Plasmid pHE65-CAT184 lacks one-half of the proximal 24-bp inverted repeat (Fig. 1b). After transfection of these he65 promoter constructs, no activity was observed in uninfected S. frugiperda or in uninfected TN-368 cells (Fig. 2), indicating that viral factors were needed for the activation of the he65 promoter. When S. frugiperda cells were infected with AcMNPV prior to transfection, the constructs pHE65-CAT474 and pHE65-CAT220 were activated while no significant CAT expression over basal levels was observed from the shorter construct pHE65-CAT184 (Fig. 2). In three independent experiments, pHE65-CAT220 was about three-fold less active than pHE65-CAT474. In infected TN-368 cells, only pHE65-CAT474 was efficiently expressed (Fig. 2), although some experiments also indicated a slight activation of pHE65-CAT220 (data not shown). These results imply that the complete hr4a supports transcriptional activation by viral factors, whereas promoter constructs without hr4a (pHE65-CAT184) are not activated. The presence of the complete proximal inverted repeat (pHE65-CAT220) led to increased CAT expression in infected S. frugiperda cells but was barely detectable in infected TN-368 cells.

Transfection experiments in TN-368 cells demonstrate that in contrast to pHE65-CAT184, pHE65-CAT220 is activated by coexpression of IE1 (Murges et al., 1997). In the present study, we have demonstrated the activation of pHE65-CAT220 and pHE65-CAT474 in S. frugiperda and TN-368 cells after cotransfection with IE1-expressing plasmids (Fig. 2, lanes +). The higher expression level in TN-368 cells was probably due to a higher transfection efficiency in comparison with S. frugiperda cells. The observation that both constructs (pHE65-CAT220 and pHE65-CAT474) were active in cotransfected TN-368 cells was in contrast to the reduced activity of pHE65-CAT220 in infected TN-368 cells. Differences in the amount of IE1 in transfected versus infected cells might account for this apparent discrepancy. However, the proximal inverted repeat might be sufficient for activation when only IE1 is present. Additional factors present in infected cells might interfere with the action of IE1. This putative interference would be compensated by the presence of the complete hr4a. The involvement of additional factors during AcMNPV infection is supported by the finding that the time course of he65 transcription differs in TN-368 and S. frugiperda cells (data not shown) although the level of IE1 expression is comparable (Roncarati and Knebel-Mörsdorf, 1997; see Fig. 10b).

Protein binding to the he65 promoter in nuclear extracts from uninfected and infected cells

Since activation of the he65 promoter was dependent on viral factors, we investigated whether a difference in factor binding was detectable when nuclear extracts from uninfected or infected cells were used. In vitro DNasel footprinting of the 474-bp Xhol/Styl fragment, representing the coding strand (Fig. 3c), was performed with crude nuclear extracts from uninfected or infected (4 h p.i.) S. frugiperda cells. With poly(dI-dC) as nonspecific competitor, four major protected regions were visible in the presence of nuclear extracts from either uninfected or infected cells (Fig. 3a, gray boxes). The protected regions are flanking each of the two inverted repeats in
the hr4a sequences (Fig. 3c). Further protected sequences were detectable in extracts both from uninfected and infected cells with poly(dG-dC) as nonspecific competitor (Fig. 3b, gray box). These sequences are located at the proximal inverted repeat between nucleotides 2140 to 2137 relative to the transcriptional start site (Fig. 3c). With poly(dA-dT), reproducible data were not obtained (data not shown).

Although no striking differences in factor binding were observed (Figs. 3a and 3b, gray boxes), minor changes were visible in the presence of poly(dI-dC) (Fig. 3a, filled and open boxes). Hypersensitive sites were detectable downstream of the protected region II and upstream of the proximal inverted repeat in extracts from infected cells but not in extracts from uninfected cells (Fig. 3a, filled box). These sites are located between nucleotides 2170 and 2150 relative to the transcriptional start site (Figs. 3a and 3c). Furthermore, weak protection was
observed in extracts from infected cells at nucleotides −140 to −130 (Fig. 3a, open box). These protected sequences overlapped with the 4 nt protected in extracts both from uninfected and infected cells when poly(dG-dC) was used as nonspecific competitor (Fig. 3b, for summary, see 3c). This finding suggests that the left half-site of the proximal repeat can interact with cellular factors that might compete for binding when viral factors are present. We conclude from the results of the DNaseI footprinting assays that differences in factor binding are restricted to sequences of the proximal inverted repeat and the upstream flanking region.

Specific protein binding to sequences at and close to the proximal inverted repeat in nuclear extracts from infected cells

We performed mobility shift experiments to investigate the potential difference in specific DNA–protein binding at the proximal inverted repeat and its flanking sequences in extracts from uninfected and infected cells. The oligonucleotide IRprox, which was used as probe, corresponds to the upstream promoter sequences in pHE65-CAT220 (Fig. 5). This DNA probe contains both the sequences for specific binding in protein extracts prepared from infected S. frugiperda cells and the sequences that were sufficient to support promoter activation.

After incubation of the probe IRprox with nuclear extracts from S. frugiperda cells prepared at 4 h p.i., a tripartite DNA–protein complex was visible with poly(dA-dT) and poly(dl-dC) as nonspecific competitors (Fig. 4, arrowhead). This complex was not detectable with extracts from uninfected cells (Fig. 4). The specificity of the DNA–protein complex was demonstrated by competition with an excess of unlabeled IRprox, whereas the unrelated oligonucleotide FX did not interfere with protein binding (Fig. 4). The observation that IRprox forms complexes with factors from either uninfected or infected cells is in line with our results of the DNaseI footprinting assays.

We have concentrated on studies of the protein complex formed in infected cells. Competition experiments were performed with oligonucleotides containing different deletions in the upstream promoter sequences to identify the stretch of sequences that was responsible for the specific protein binding (Fig. 5). In extracts from infected cells, binding to the IRprox probe was competed for by oligonucleotide IR containing a deletion of 16 bp (Fig. 5 and Fig. 6a, lane 3). However, almost no competition was observed for IRS, which is 8 bp shorter than IR (Fig. 5 and Fig. 6a, lane 6). When IR was used as the probe, binding was comparable with IRprox, whereas only a slightly shifted complex was detectable with the IRS probe (data not shown). These results indicate that a stretch of 9 bp located upstream of the proximal 24-bp inverted repeat is involved in specific complex formation.

The oligonucleotide UF was used as the probe to investigate whether the upstream flanking sequences of the inverted repeat were sufficient for protein binding (Fig. 5). Since no binding was observed (Fig. 6c, lane 6), we investigated binding to oligonucleotides that contained the left half-site of the inverted repeat in addition to the upstream flanking sequences of different lengths (Fig. 5). Oligonucleotides IRL-UF, IRL-UF16, and IRL-UF8 (Fig. 5) supported binding of a protein complex (Fig. 6c, lanes 5, 2, and 3) that could be competed for by the

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**FIG. 2.** CAT activity of he65 promoter constructs in S. frugiperda and TN-368 cells. The constructs pHE65-CAT47, pHE65-CAT220, and pHE65-CAT184 were transfected into uninfected (lanes −) or infected cells (lanes +) or cotransfected with the IE1-expressing plasmid pIE1 (lanes +). The percentage of acetylated CAM is given underneath the autoradiogram.
FIG. 3. In vitro DNaseI footprinting of the he65 promoter with nuclear extracts from uninfected or infected (4 h p.i.) S. frugiperda cells. (a) The coding strand was labeled by 3'-end-filling of the StyI site. The labeled DNA was incubated with crude nuclear extracts from uninfected cells (lanes un) or from cells prepared at 4 h p.i. (lanes 4). The control reaction containing naked DNA (lanes no) was digested with 10 ng (lane 10), 5 ng (lanes 5), or 2.5 ng (lane 2.5) of DNaseI. For the DNA incubated with extracts, 100 ng (lanes 100), 40 ng (lanes 40), or 20 ng (lane 20) of DNaseI were used. Each reaction mixture contained 5 μg of poly(dI-dC) as nonspecific competitor. DNA size markers (lane M), and a sequencing ladder (lanes A, T, C, and
oligonucleotides IRprox and IR (data not shown) and vice versa (Fig. 6a, lanes 4 and 7). Oligonucleotide IRL containing only the left half-site of the inverted repeat (Fig. 5) showed neither protein binding (Fig. 6c, lane 4) nor competition for the complex formed at IRprox (Fig. 6a, lane 8). Since IRL might be too short for stable complex formation, we also used the oligonucleotide IRLs with 8 supplemented nt that were unrelated to hr4a (Fig. 5). The observation that IRLs served as a weak competitor and the oligonucleotide of the same length, IRL-UF8, competed efficiently (Fig. 6a, lanes 11 and 7) led us to the conclusion that both the sequences of the inverted repeat and the 9 bp of the flanking region had to be involved in binding.

In summary, the mobility shift assays demonstrate that a specific tripartite complex is formed in extracts from infected cells at the left half-site of the proximal inverted repeat and at least 9 nt of the upstream flanking sequences. The right half-site with the downstream flanking sequences competes for the tripartite complex with low affinity.

Since the presence of the proximal inverted repeat was sufficient for activation of the he65 promoter by IE1, we have analyzed whether the same sequences that interact with factors from infected cells also interact with factors from cells transfected with IE1-expressing plasmids. Competition experiments were performed in nuclear extracts from TN-368 cells transfected with IE1-expressing plasmids and with IRprox as the probe. Oligonucleotides that competed for binding in extracts from infected cells also competed for the complex formed in extracts from transfected cells (Fig. 6b). Only IRLs that competed weakly for binding in extracts from infected cells showed stronger competition in extracts from transfected cells (Figs. 6a, lane 11, and 6b, lane 11). Thus we conclude that the same sequences are involved in binding when extracts from infected and transfected cells have been used.

Activation of the minimal he65 promoter by coexpression of IE1

We have tested he65 promoter constructs for activity after coexpression of IE1 to address the question of whether sequences that support the specific binding complex observed either in extracts from infected and transfected cells correlate with those sequences that are essential for the activation by IE1. The 5′ ends of the he65 promoter constructs corresponded to the sequences of the oligonucleotides that were used for the binding studies. Transient expression assays were per-

FIG. 4. Specific protein binding at the proximal inverted repeat of hr4a. Mobility shift assays with nuclear extracts from uninfected (lanes un) or infected S. frugiperda cells prepared at 4 h p.i. (lanes 4) were performed with poly(dA-dT), poly(dG-dC), or poly(dI-dC) as nonspecific competitors. The 3′-end labeled oligonucleotide IRprox was competed for by 5, 50, and 125 ng of unlabeled IRprox competitor and by 125 ng of the unrelated competitor FX. Lanes —, no further competitor added. The position of the tripartite complex and of the probe are marked by an arrowhead and by an asterisk, respectively.
formed in TN-368 cells which showed a higher expression level of the transfected plasmids as compared to S. frugiperda cells.

After cotransfection of the IE1-expressing plasmid pIE1, the pHE65CAT-IR construct (Fig. 1b) was as efficiently activated as pHE65-CAT220 (Fig. 7). CAT expression of pHE65CAT-IRS containing only the inverted repeat without upstream flanking sequences (Fig. 1b) was significantly reduced (Fig. 7) as observed in four independent experiments. Activation by IE1 was observed neither for pHE65CAT-IRL/UF8 nor for pHE65CAT-IRL/UF8s (Fig. 7), which both contain only the left half-site of the inverted repeat (Fig. 1b). The construct pHE65CAT-IRL/UF8 carries a deletion of the right half-site which was substituted by unrelated sequences in pHE65CAT-IRL/UF8s (Fig. 1b). Thus the he65 promoter requires a complete copy of the proximal inverted repeat with at least 9 bp of the upstream flanking sequences for the activation by IE1. By replacing the right half-site of the repeat by unrelated sequences, we could demonstrate that sequence specificity rather than spacing of promoter sequences were essential for activation. The deletion of 8 bp of the upstream flanking sequences resulted in reduced promoter activation. These sequences were also required for the formation of the specific protein complex in extracts from infected and transfected cells (Fig. 5). Thus weak expression of pHE65CAT-IRS correlates with the reduced protein binding at IRS (Fig. 5). We argue that in the case of IRS, weak binding occurs at the left half-site of the repeat and is stabilized by the interaction with downstream sequences. Weak promoter activity ensues. When the 8 bp of the upstream flanking sequences are present, binding and activation are more efficiently accomplished.

As control, we performed transfection experiments which demonstrate the compatibility of various promoter constructs. Activation by IE1 was shown for pHE65CAT-IRprox and pHE65-CAT220P (Fig. 7), which both contain the same he65 promoter sequences as pHE65-CAT220 but differ in the cloning sites of the promoter fragment (see Fig. 1b). Accordingly, pHE65-CAT184 corresponds to pHE65CAT-IRR/DF, which carries the promoter fragment in the same sites of pBLCAT3 as pHE65CAT-IRL/UF8s, -IRL/UF8, -IRS, -IR, and -IRprox (see Fig. 1b).

The role of IE1 in the binding complex at the inverted repeat

We performed mobility shift experiments using IRprox as probe with nuclear extracts from infected S. frugiperda cells, infected TN-368 cells, or from TN-368 cells transfected with IE1-expressing plasmids to compare the complexes formed in each extract. The tripartite complex formed in extracts from either infected TN-368 or S. frugiperda cells migrated at the same position (Fig. 8, lanes 1 and 13, shown by a filled arrowhead) and could be competed for by the same specific oligonucleotides (data not shown). The complex formed in extracts from transfected cells (Fig. 8, lanes 5 and 9) migrated differently than that from infected cells (lanes 1 and 13). The tripartite complex (Fig. 8, filled arrowhead) was replaced by one major complex (Fig. 8, open arrowhead) that migrated at the same position as the fastest migrating band of the tripartite complex.
Since IE1 activated the he65 promoter, we analyzed whether IE1 was involved in the binding complex. The complexes formed in extracts from either infected or transfected cells were incubated with polyclonal IE1 antisem which led to a supershift of the retarded bands (Fig. 8, lanes 2, 6, 10, and 14). These results confirm that IE1 was at least part of both the tripartite binding complex (Fig. 8, lanes 2 and 14) and the complex formed in extracts from transfected cells (Fig. 8, lanes 6 and 10). As control, preimmune and anti-IE2 sera were used which did not affect complex migration (Fig.7, lanes 3, 4, 7, 8, 11, 12, 15, and 16).

We performed protein clipping bandshift assays (Schreiber et al., 1988; Schoeler et al., 1989) to investigate whether the tripartite complex formed in extracts from infected cells results from binding of different proteins. This method is based on the limited proteolytic cleavage of the preformed DNA-protein complex by adding specific proteases. After electrophoresis, the resulting pattern of retarded bands represents the binding peptide(s). Hence this method allows determination of whether one or more peptides have been bound to DNA. The diluted protease chymotrypsin was added to the DNA-protein complex that had formed in extracts from infected cells. The resulting complexes were analyzed by electrophoresis, which demonstrated that the tripartite complex was replaced by one faster migrating band (Fig. 9).

Similar results were obtained after adding increasing amounts of the protease dispase (Fig. 9). These results suggest that one peptide binds to DNA and raise the question of whether the tripartite complex reflects binding of a protein that forms oligomers. We investigated whether the formation of the DNA-protein complex was based on protein-protein interactions by adding the dissociating agent deoxycholate (DOC) to the preformed DNA-protein complex. When DOC was added in low concentrations, binding was inhibited without generating faster migrating complexes (Fig. 9). The inhibition of binding could be restored by adding an excess of the nonionic detergent NP-40 (Fig. 9). Thus the DOC experiments suggest that only dimers or oligomers support DNA binding.

Expression of the ie1 and ie0 genes after transfection and during the course of infection

The expression of the IE1 gene is observed throughout the course of infection, whereas IE0 is only expressed during the early phase (Ohresser et al., 1994; Choi and Guarino, 1995a). Recent studies provide evidence that distinct domains of IE1 are responsible for DNA binding, transactivation, or negative regulation (Kovacs et al., 1992; Rodems et al., 1997; Slack and Blissard, 1997). Since IE1 and IE0 are identical in their C terminus, both contain the same DNA binding domain. Thus the nuclear extracts which we prepared from cells at 4 h p.i. might provide IE1 and IE0, which potentially can bind to the same sequences. In contrast, in cells transfected with pIE1, only IE1 is expressed. We performed transient expression assays with the plasmids pIE1 and pHindG to investigate whether the presence or absence of IE0 may account for the differences in the shifted complex formed in extracts from infected and transfected cells. Construct pHindG contains the fragment HindG of the AcMNPV genome potentially encoding both IE1 and IE0 (Kovacs et al., 1991; Ohresser et al., 1994). Interestingly, no difference in binding was observed when extracts were used from cells transfected with either pIE1 or pHindG (Fig. 8). We compared IE1 and IE0 expression by Western blot.
analyses in nuclear extracts prepared from infected cells with extracts from cells transfected with either of the plasmids pIE1 or pHindG to confirm whether indeed both proteins were expressed. In extracts prepared at 4 h p.i., two polypeptides were detectable with the IE1 antiserum directed against a common N-terminal domain of IE1 and IE0 (Fig. 10c). The predicted molecular weights of IE0 and IE1 are 73 and 67 kDa, respectively (Chisholm and Henner, 1988). Thus the polypeptides of approximately 82 and 76 kDa may reflect IE0 and IE1 expression, respectively (Figs. 10b and 10c). The IE1-specific polypeptide was also observed in extracts from cells transfected with pIE1, although no expression of IE0 was detectable in extracts from cells transfected with pHindG (Fig. 10c). This finding may explain why DNA binding did not differ in extracts from cells transfected with either pIE1 or pHindG (Fig. 8). The presence of IE1 and IE0 in extracts prepared from infected cells with extracts from cells transfected with either of the plasmids pIE1 or pHindG to confirm whether indeed both proteins were expressed. In extracts prepared at 4 h p.i., two polypeptides were detectable with the IE1 antiserum directed against a common N-terminal domain of IE1 and

FIG. 7. CAT activity of he65 promoter constructs in TN-368 cells after cotransfection with IE1-expressing plasmids. Each of the promoter constructs was transfected into uninfected cells (lanes –) or cells transfected with IE1-expressing plasmids (lanes +). The relative CAT activity is shown below the autoradiogram.

FIG. 8. IE1 is part of the specific complex formed in nuclear extracts from infected and transfected cells. Mobility shift assays were performed in nuclear extracts from infected (4 h p.i.) S. frugiperda cells (lanes 4 (Sf)), from infected TN-368 cells (lanes 4 (TN-368)), and from TN-368 cells transfected with either pIE1 (lanes IE1) or pHindG (lanes G) with oligonucleotide IRprox as probe and poly(dA-dT) as nonspecific competitor. The polyclonal anti-IE1 and anti-IE2 sera and the preimmune serum were used for supershift experiments. Lanes –, no sera were added. The position of the tripartite complex and of the complex formed in transfected cells are marked by a filled and open arrowhead, respectively. The lanes are numbered.

FIG. 9. The tripartite complex is based on protein–protein interactions with one binding peptide. Mobility shift assays were performed in nuclear extracts from infected (4 h p.i.) S. frugiperda cells with oligonucleotide IRprox as probe and poly(dA-dT) as nonspecific competitor (lanes 4 (AT)). Protein–DNA complexes were treated with 0.0001, 0.0005, and 0.001 U of chymotrypsin or with 0.5, 1, 5, 10, and 20 μg of dispase. The detergent DOC was added to a final concentration of 0.05, 0.1, 0.2, and 0.3%. In addition to 0.2% DOC, NP-40 was added to a final concentration of 1% as control.
nuclear extracts from infected cells may correlate with the formation of the tripartite complex. If IE1 and IE0 interact with sequences of the inverted repeat only as homo- and heterodimers, binding may result in three differently shifted complexes.

We next prepared nuclear extracts at different times p.i., which were tested for the presence of IE1 and IE0 (Fig. 10b). The same extracts were used for mobility shift assays to analyze whether the varying ratios of IE1 and IE0 in the course of infection result in a different composition of the tripartite complex. Our results indeed indicate a change in the intensity of each of the three parts of the complex (Fig. 10a). The two more slowly migrating bands were the strongest with extracts prepared at 4 or 6 h p.i., whereas the fastest migrating band became more intense with extracts prepared at 12, 24, or 48 h p.i. and correlated with the disappearance of the slowest migrating band (Fig. 10a). Alterations of the tripartite complex may result from the decreasing IE0 expression during infection.

Sequences involved in complex formation with extracts from cells expressing IE1 and IE0

Our results from mobility shift assays indicate that sequences of the proximal inverted repeat are able to interact with factors, presumably IE1 and IE0 in infected and in transfected cells. Furthermore, binding appears to occur with different affinity at the two half-sites of the repeat. We performed phenanthroline-copper footprinting of the complex preformed either in extracts from infected cells or from cells expressing only IE1 to identify the sequences that were involved in protein binding. Initially, mobility shift assays were performed with a promoter fragment of 298 bp as the probe that corresponds to the promoter sequences in construct pHE65-CAT220P (see Fig. 1b). The complexes formed with extracts either from TN-368 cells prepared at 4 h p.i. or from TN-368 cells transfected with pHindG were resolved by electrophoresis on 5% polyacrylamide gels followed by the chemical cleavage of the unprotected sequences. The resulting sequences were analyzed on 6% sequencing gels (Fig. 11). Our results indicate that the complete inverted repeat in addition to the upstream and downstream flanking sequences are protected by factors present both in infected and in transfected cells (Fig. 11). It is worth mentioning that the protected sequences are only observed with the noncoding strand, whereas no significant protection is visible with the coding strand. Protection of nucleotides −153 to −123 relative to the transcriptional start site was stronger than the protection of the nucleotides −123 to −109 (Fig. 11). This finding indicates differences in the binding affinity of either half-site of the inverted repeat.

DISCUSSION

By studying the regulation of the he65 promoter in transient expression assays, we have demonstrated that IE1 is sufficient for promoter activation in the presence of hr4a. Furthermore, our results indicate the putative involvement of IE0 in the regulation of the he65 gene during the infection cycle. Since IE1 activation depends on the presence of at least the proximal inverted repeat of hr4a, we have performed studies on DNA-protein interactions. Our results of DNasel assays have shown

FIG. 10. Time course of IE1 and IE0 expression and the formation of the tripartite complex. (a) Shift assays were performed with oligonucleotide IRprox as probe in extracts from uninfected S. frugiperda cells with poly(dG-dC) as nonspecific competitor (lanes GC). Poly(dA-dT) (lanes AT) was used for shift assays in extracts from S. frugiperda cells prepared at 4, 6, 12, 24, and 48 h p.i. (lanes 4, 6, 12, 24, and 48). (b) The same extracts used for the shift experiments were analyzed by immunoblotting with the polyclonal serum anti-IE1. Protein size markers are indicated on the left. (c) Crude nuclear extracts were prepared from uninfected TN-368 cells (lane un), from cells at 4 h p.i. (lane 4), or from cells transfected with either pIE1 (lane IE1) or pHindG (lane G). Protein size markers are indicated on the right. The position of IE1 and IE0, respectively, are indicated by arrowheads.
FIG. 11. Phenanthroline-copper footprinting of the complex formed in extracts from infected or transfected TN-368 cells. The 3’-end labeled HindIII/StyI fragment of pHE65-CAT220P was incubated with extracts from TN-368 cells prepared at 4 h p.i. or with extracts from cells transfected with pHindG. The formed complex (lanes bound) and the free probe (lanes free) were digested and resolved on a 6% sequencing gel. A sequencing ladder (lanes G, C, T, and A) is shown; DNA fragment sizes are depicted on the left. Strong protection is indicated by a filled bar and weak protection is indicated by a hatched bar. The protected sequences close to and at the proximal inverted repeat are shown underneath the autoradiogram. The numbers on the right and on top of the sequences refer to the nucleotides relative to the he65 transcriptional start.
that host factors present in S. frugiperda cells can interact with the flanking sequences of the two inverted repeats of hr4a. Specific interactions with factors present only in nuclear extracts from infected cells were detectable at the left half-site of the proximal inverted repeat. The potential of these sequences to form complexes in infected cells was further investigated by mobility shift assays, indicating that IE1 is part of the DNA binding complex in extracts from infected S. frugiperda and TN-368 cells. The specific binding complex migrates as a tripartite complex that may reflect binding of oligomers with a single binding peptide as indicated by protein clipping bandshifts and treatment with DOC, respectively. The possible candidates are IE1 and IE0 because both contain the same DNA binding domain (Kovacs et al., 1991, 1992; Rodems et al., 1997). Our evidence for the involvement of IE0 in the binding complex is based on the observation that the changing ratios of IE1 and IE0 during infection correlate with changes of the tripartite complex. When almost no IE0 was present in extracts from infected cells, the faster migrating band of the tripartite complex comigrated with the complex observed with extracts from cells transfected with IE1-expressing plasmids.

To identify the minimal sequences that are required for complex formation, we tested half-sites of the proximal inverted repeat with varying lengths of the flanking sequences for binding. Our results indicate that the left half-site of the repeat in addition to at least 9 bp of the flanking sequences are sufficient to form the tripartite binding complex. The right half-site of the repeat shows weak binding affinity which is in line with the finding that sequences immediately downstream of the inverted repeat differ from the upstream flanking sequences.

Previous studies also suggest the interaction of IE1 with sequences of the hrs. Mobility shift assays initially indicate that proteins of whole-cell extracts prepared from S. frugiperda cells transfected with IE1-expressing plasmids interact with oligonucleotides containing one half-site of a palindrome within hr5 (Guarino and Dong, 1991, 1994). Evidence for the presence of IE1 in the DNA-protein binding complex emerges from supershift experiments with antibodies against IE1 (Choi and Guarino, 1995a), and deletion studies of the IE1 gene indicating that the C terminus is required for the binding activity (Kovacs et al., 1992). The direct interaction has been shown for in vitro synthesized IE1 (Choi and Guarino, 1995b; Rodems and Friesen, 1995), which is able to interact as a dimeric complex with both half-sites of the palindrome (Rodems and Friesen, 1995). Recently, the existence of a specific IE1 binding motif has been postulated that is located within the palindromic sequences of hrs (Leisy et al., 1997). All previous studies have concentrated on the inverted repeat although the oligonucleotides used as probes in mobility shift assays have included the flanking sequences (Fig. 12). Our results are consistent with the observations on the inverted repeat as a potential binding site, but in addition, our studies reveal the important role of the flanking sequences in the complex formation. The different binding affinities of both half-sites of the proximal repeat in hr4a further support our conclusion that both the inverted repeat and the flanking sequences are involved in DNA-protein interactions. As depicted in Fig. 12, there are extensive homologies of sequences in the repeat and the flanking regions with sequences including the pe38 transcriptional start site. Leisy et al. (1997) provide evidence that the 8-bp element immediately upstream of the transcriptional start site is involved in the negative regulation of the pe38 promoter by IE1. We have used an oligonucleotide with all conserved nucleotides as efficient competitor in mobility shift assays (data not shown), confirming the involvement of IE1 in the complex that is formed either at the pe38 promoter and the inverted repeat in hr4a. Furthermore, our results of phenanthroline-copper footprinting and mobility shift assays predict that the conserved sequences upstream of the left half-site of the repeat are essential for the binding complex (Fig. 12).

IE1 can bind as a dimer to the inverted repeat (Rodems and Friesen, 1995), alternatively one might envision a higher order complex that consists of a homodimer formed by IE1 and IE0 at the left half-site and the flanking region. Cooperativity would lead to the binding at the right half-site that might trigger the interaction with host factors. The recent finding of multiple IE1 domains involved in DNA binding supports this notion (Rodems et al., 1997).

Deletion studies of the he65 promoter indicate that both half-sites in addition to the upstream flanking region are essential for IE1 activation, which is in line with previous studies (Guarino and Dong, 1994; Rodems and Friesen, 1993). The minimal he65 promoter containing the proximal inverted repeat is less active than the promoter construct with the complete hr4a when viral factors in addition to IE1 are present. Thus the complete hr4a seems to be involved in he65 regulation during infection. Our results then suggest that both IE1 and IE0 play a role in the regulation of the he65 promoter although the exact mode of regulation remains to be defined.

MATERIALS AND METHODS

Cells and virus

Trichoplusia ni TN-368 (Hink, 1970) and Spodoptera frugiperda IPLB21 cells (Vaughn et al., 1977) were grown as previously described (Knebel-Mørsdorf et al., 1993). Infection with the AcMNPV plaque isolate E (Tjia et al., 1979) was performed at a multiplicity of infection of 10 PFU/cell. Time zero was defined as the time when the inoculum was added to the cells. Cells were grown in
suspension, and then prior to infection or transfection, they were plated into monolayer cultures.

Plasmid constructions and oligonucleotides

The plasmids pEcoQ (Becker and Knebel-Mörsdorf, 1993), pIE1, pHE65-CAT220, and pHE65-CAT184 were described earlier (Murges et al., 1997). The duplicated 29-bp oligonucleotide, 5\textsuperscript{9} AGCTTTGCAGGTCGACTCTAGGAGGATCCAGATC3 \textsuperscript{9} was inserted into the HindIII site of pHE65-CAT220 to construct pHE65-CAT220P. The inserted oligonucleotide in this plasmid is located upstream of the he65 promoter sequences and carries SalI, XbaI, and BamHI sites. The plasmid pAcHindA1-HK contains a 4-kb HindIII-KpnI fragment isolated from the cloned fragment HindIII A1 of AcMNPV DNA (Becker and Knebel-Mörsdorf, unpublished results). The plasmid pHE65-CAT47 4 was generated by isolating the fragment StyI (blunt-ended)-XhoII of plasmid pAcHindA1-HK and inserting it into the BamHI and blunt-ended XhoI sites of pBLCAT3 (Luckow and Schütz, 1987). The resulting construct pHE65-CAT220 contains the entire hr4a region (Guarino et al., 1986a) in addition to the he65 promoter sequences. Plasmids containing various lengths of hr4a sequences at the 5' end of the he65 promoter sequences were all constructed by inserting the corresponding oligonucleotides (Fig. 1b) into pHE65-CAT184.

All oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer. After heating at 95°C for 5 min, complementary pairs were annealed for 25 min each at 65 and 37°C and ligated into pHE65-CAT184. Each of the plasmids pHE65-CAT-IRprox, pHE65-CAT-IRS, and pHE65-CAT carries the oligonucleotides within the ClaI site located in the he65 promoter and the PstI site. The oligonucleotides IRL-UF8, IRR-DF, and IRL-UF8s were inserted into the ClaI and blunt-ended SalI sites generating plasmids pHE65CAT-IRL/UF8, pHE65CAT-IRR/DF, and pHE65CAT-IRL/UF8s (see Fig. 1b).

Transfection experiments

S. frugiperda (3×10^5) or TN-368 cells in 60-mm dishes were transfected by calcium phosphate precipitation with 10 μg of a promoter-CAT gene construct (see Fig. 1b) and with either 5 μg of the IE1-expressing plasmid or pBluescript (Stratagene) as negative control. Cells were infected with AcMNPV (10 PFU/cell) at 4 h prior to transfection. Alternatively, liposome-mediated transfection (Felgner et al., 1987) of TN-368 cells was performed. The liposome-solution was prepared by mixing dimethyl-dioctadecylammonium bromide (Sigma) and L-phosphatidylethanolamine dioleoyl (Sigma) in a 0.5:1 molar ratio dissolved in 100% ethanol (Campbell, 1995). After a 1:20 dilution in H\textsubscript{2}O, 30 μl of liposome solution was used in mobility shift assays by Rodems and Friesen (1995). The homologous nucleotides to pe38 located at the upstream flanking region are underlined. (c) Sequences of the pe38 promoter and the consensus sequence from the hr palindromes with the boxed 8-bp element as suggested by Leisy et al. (1997). The asterisks indicate the conserved nucleotides. The arrow shows the transcriptional start site of the pe38 promoter.

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**FIG. 12.** Potential nucleotides close to and at inverted repeats that interact with IE1 and IE0. (a) The nucleotide sequence of the proximal inverted repeat of hr4a. The 24-bp repeat is boxed. The numbers refer to the nucleotides relative to the transcriptional start site of the he65 promoter. The filled and hatched bar indicates the protected sequences observed by phenanthroline-copper footprinting. The shaded sequences indicate interaction with host factors as shown by DNAseI footprinting analyses. The asterisks above and underneath the sequences depicts the conserved nucleotides to the pe38 promoter sequences. The 8-bp element predicted as IE1 binding motif by Leisy et al. (1997) is shown by a broken line. (b) Nucleotide sequence of the leftmost palindrome of hr5 used as probe in mobility shift assays by Rodems and Friesen (1995). The homologous nucleotides to pe38 located at the upstream flanking region are underlined. (c) Sequences of the pe38 promoter and the consensus sequence from the hr palindromes with the boxed 8-bp element as suggested by Leisy et al. (1997). The asterisks indicate the conserved nucleotides. The arrow shows the transcriptional start site of the pe38 promoter.
added to 15 μg of plasmid DNA, which was used for transfection of 2 × 10⁵ cells in 35-mm dishes. For both transfection procedures, the ratio of the reporter and the IE1-expressing plasmids was 2:1. Cells were harvested 45 h after transfection, and cell extracts were tested for CAT activity as described (Krappa et al., 1992).

Production of polyclonal antibodies

The production of polyclonal antisera directed against PE38 and IE2 was described previously (Krappa et al., 1995). The polyclonal anti-IE1 serum is directed against the N terminus of the ie1 gene, which was overexpressed as a fusion protein with mouse dihydrofolate reductase (DHFR). The fragment BamHI/Stul of the cDNA clone ie1 (Krappa and Knebel-Mörsdorf, 1991), which includes 31 bp of the 5’ untranslated region and 124 aa of the N terminus of the ie1 gene, was inserted into the BglII and Smal sites of the pQE-40 vector (Qiagen). The resulting plasmid pQE-IE1(0.5) carries the histidine-tagged IE1-DHFR fusion gene, which was overexpressed in Escherichia coli M15/pREP4 by induction with isopropyl-β-D-thiogalactopyranoside and purified under denaturing conditions. Polyclonal antiserum was produced by immunizing rabbits with denatured IE1-DHFR by standard procedures (Harlow and Lane, 1988).

Immunoblotting

Proteins were resolved by SDS±10% polyacrylamide gel electrophoresis (Laemmli, 1970) and processed as described earlier (Krappa et al., 1995). Briefly, the polyclonal anti-IE1 serum was used at a dilution of 1:10,000 or 1:20,000, incubated with the secondary, horseradish peroxidase-conjugated antibodies, and the antigen-antibody complexes were revealed by enhanced chemiluminescence (ECL system, Amersham).

Preparation of nuclear extracts

Nuclear extracts were prepared from uninfected cells and from cells at different times p.i. with AcMNPV as described earlier (Krappa et al., 1992). Accordingly, nuclear extracts from TN-368 cells were prepared at 48 h after transfection with 0.5±1 mg of IE1-expressing plasmid per 1 × 10⁶ cells.

DNasel footprinting

The conditions for the DNA-binding reactions, the DNasel cleavage, and the extraction and analysis of the cleavage products were as described earlier (Krappa et al., 1992).

Gel mobility shift assays and competition studies

The preparation of radiolabeled oligonucleotides and the DNA-protein binding conditions were previously described (Krappa et al. 1992). About 100 ng of the labeled oligonucleotide was incubated with 2 μg of nuclear extract at room temperature for 20 min in the presence of 1 μg of poly(dA-dT), poly(dI-dC), or poly(dG-dC). The DNA-protein complexes were resolved at room temperature on 4 or 5% polyacrylamide gels in 45 mM Tris/HCl, 45 mM sodium borate, 1 mM EDTA (0.5× TBE) by electrophoresis at 250 V for 2 h. For competition experiments, usually 100 ng of unlabeled, double-stranded oligonucleotide was added to the DNA-protein binding reaction mixtures prior to addition of the labeled nucleotide probe. The reaction mixtures were allowed to preincubate at room temperature for 5 min.

Supershift analysis, DOC and protein clipping bandshift assays

Polyclonal antisera were added to the preformed DNA-protein complexes, and incubation was continued at room temperature for 10 min. The shifted complexes were analyzed by electrophoresis on 5% PAA gels.

DOC was added to the reaction mixture at the concentrations indicated (see Fig. 9) 8 min after the labeled nucleotide probe, and the mixtures were incubated at room temperature for additional 10 min (Baeuerle and Baltimore, 1988). As a control, NP-40 was added to a final concentration of 1% to reverse the function of DOC as detergent.

Protein clipping bandshift assays (Schreiber et al., 1988) were performed by adding the proteases chymotrypsin or dispase to the DNA-protein complexes at the concentrations indicated (see Fig. 9). The DNA-protein complexes were preformed for 10 min followed by the addition of the proteases and incubation at room temperature for 5 min.

Phenanthroline-copper footprinting analysis

The HindIII/Styl fragment of pHE65-CAT220P, including the hr4a/he65 promoter sequences, was 3’-end labeled at the HindIII restriction site by fill-in reaction with Klone polymerase and after purification used as DNA probe. Published procedures (Papavassiliou and Silverstein, 1990) were adapted as follows. Binding reactions were scaled up 15-fold as compared to the mobility shift assays and fractionated by polyacrylamide gel electrophoresis as described above. In situ digestion of the DNA by the nuclease activity of 110-phenanthroline-cuprous complex was allowed to proceed for 15±20 min at 18°C. DNAs from free and bound fractions were visualized by autoradiography of the wet gel for 2 h at 4°C, then the radioactive bands were eluted from the corresponding areas of the gel in 500 μl elution buffer (0.5 M CH₃COONH₄, pH 7.5, 1 mM EDTA, 0.1% SDS) overnight at 37°C. The DNA was extracted with phenol/chloroform/isoamyl alcohol to which 5 μg of glycogen (Boehringer Mannheim) per sample was added. Equal Cerenkov counts of the bound and free DNA fractions, respectively,
were loaded immediately on a 6% polyacrylamide, 7 M urea sequencing gel and electrophoresed in 0.5 × TBE buffer at constant power (40 W) for 45 ± 60 min. Following electrophoresis, the gel was transferred to Whatman 3M paper, vacuum-dried, and exposed to Kodak XAR-5 or BIOMAX films with an intensifying screen for 1 ± 3 days at room temperature or at −70°C.

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