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The Baculovirus Transactivator IE1 Binds to Viral Enhancer Elements in the Absence of Insect Cell Factors

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The transregulatory IE1 protein of *Autographa californica* nuclear polyhedrosis virus binds to the viral enhancer element *hr*5. To test whether IE1 binds independently of host cell factors, IE1 was translated in rabbit reticulocyte extracts and tested for DNA binding activity by an electrophoretic mobility shift assay. Complexes with the *hr*5 probe were detected with translation reaction mixtures primed with *ie1* RNA but not with control translation reaction mixtures. However, the DNA-protein complexes formed with IE1 translated in vitro migrated more slowly than complexes formed with IE1 that was transiently expressed in insect cells. Phosphatase treatment of the translation reactions resulted in an increase in the mobility of the DNA-protein complexes, suggesting that hyperphosphorylation was responsible for the altered migration. To further verify that IE1 was capable of binding DNA in the absence of host cell factors, an N-terminal truncation of IE1 was synthesized in vitro, and shown to interact with *hr*5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of IE1 translated in vitro revealed that the mobility of the protein was heterogeneous. This pattern was altered by translation in the presence of an oligonucleotide corresponding to the IE1 specific binding site but was not affected by translation in the presence of a nonspecific DNA. These results suggest that binding of IE1 to DNA causes a conformational change in the protein that alters the accessibility of IE1 to protein kinases.

The identification of sequence-specific transcription factors that control viral transcription is essential for elucidation of the pathways of gene expression in virus-infected cells. The *ie1* gene of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) was originally identified as a gene whose protein product transactivated expression from other early viral promoters (8, 10). During virus infection, the *ie1* gene is transcribed as an unspliced 1.9-kb RNA with an open reading frame that encodes a 67-kDa protein (10). The *ie1* open reading frame is also expressed as a part of the spliced gene *ie0*. IE0 is identical to IE1 except for an additional 54 amino acids fused to the amino terminus of IE0 (3). IE0 is translated from a spliced mRNA that initiates from an upstream promoter. *ie0* is transiently expressed immediately after infection and during the late phase, while *ie1* is expressed throughout infection (13).

IE1 plays a central role in the regulation of viral gene expression. It regulates expression of all known early promoters as well as its own promoter. The essential function of IE1 during the early phase of infection was confirmed by the recent description of a temperature-sensitive mutant of Ac*M*NPV which has two amino acid substitutions in IE1 (18). The temperature-sensitive virus is delayed in DNA replication and budded virus formation at nonpermissive temperatures. IE1 also down-regulates expression of two other transregulatory factors, IE2 and IE0 (2, 14). IE1 is directly or indirectly required for expression of late genes (15). A functional dissection of IE1 has defined potential domains common to transactivators (12). The N-terminal portion of the protein is rich in acidic amino acid residues and is required for the *trans*-activation function of IE1. A region of about 70 amino acids at the C terminus is required for DNA binding and *trans* inhibition of expression from the *ie0* and *ie2* promoters. IE0 and IE2 increase expression of IE1, thus indirectly activating expression of all genes that are dependent on IE1 for high-level expression (14, 22). The *ie1* gene of *Orgyia pseudotsugata* nuclear polyhedrosis virus was isolated by its homology with *ie1* of Ac*M*NPV. The two IE1s have approximately 63% amino acid sequence identity (20), and the acidic residues at the C terminus are conserved.

Ac*M*NPV contains eight regions of homologous DNA of 76 to 800 bp that are interspersed throughout the genome (1). The five larger homologous regions (*hr*1 to *hr*5) were shown to have the ability to *cis*-activate expression of the early *39k* promoter (7). The nucleotide sequence of the 484-bp *hr*5 enhancer revealed that there are six copies of a 24-bp inverted repeat (IR24) within a 60-bp direct repeat (DR60) and three copies of a 30-bp direct repeat (DR30) (9). The *hr*s exhibit common characteristics of enhancer elements, including the ability to stimulate transcription in a position- and orientation-independent fashion (7, 9). Studies with recombinant viruses have also confirmed the function of *hr*5 as a transcriptional enhancer within the viral genome (19). In addition to their role in transcription, *hr*s stimulate plasmid DNA replication in a transient assay, suggesting that they may be origins of viral DNA replication (11, 16).

Electrophoretic mobility shift assays showed that extracts prepared from pIE1-transfected *Spodoptera frugiperda* (Sf9) cells contain a protein that specifically binds to the enhancer elements (5). Furthermore, studies with a series of plasmids that encoded truncations of the IE1 protein revealed that deletion of amino acids from the N terminus of IE1 resulted in a corresponding increase in the mobility of protein-DNA complexes (12). These data prove that IE1 is a component of the DNA-protein complex. However, they do not indicate whether IE1 binds to enhancer elements alone or whether it binds in association with cellular factors.

We were interested in investigating whether IE1 could bind to enhancer elements in the absence of host transcription fac-

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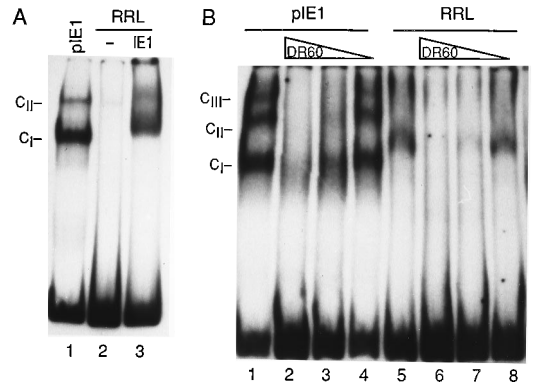

FIG. 1. DNA binding activity of IE1 translated in vitro. (A) Clone pBSIE1 (10) was linearized by digestion with *Xho*I, which cuts downstream of IE1 in the vector. After linearization, the plasmids were used for in vitro transcription with T7 polymerase, according to the procedure recommended by the manufacturer (Promega Biotec). The RNA transcript was purified from the DNA template by gel electrophoresis in low-melting-temperature agarose. After elution, the uncapped RNA transcript was translated in vitro with a rabbit reticulocyte lysate (RRL) according to the directions of the manufacturer (Promega Biotec). Mobility shift DNA binding assays were performed as previously described (5). A 252-bp *Eco*RV-*Hin*dIII fragment of *hr*5, corresponding to the left half of *hr*5, was end labeled with T4 polynucleotide kinase (New England Biolabs). The probes were incubated with 5 μl of whole-cell extracts prepared from pNheIE1-transfected cells (pIE1) after 24 h of transient expression (lane 1) or with 5 μl of reticulocyte extracts primed with pNheIE1 RNA (IE1) (lane 3) or the control translation reaction mixture (−) (lane 2). The binding reaction mixtures were incubated on ice for 20 min in a total volume of 20 μl of binding buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 20% glycerol, 1 μg of poly(dI-dC) (Boehringer Mannheim Biochemicals), 2 μg of sonicated calf thymus DNA]. The reaction mixtures were loaded onto a 5% nondenaturing gel and electrophoresed for 3 h in 10 mM Tris (pH 7.5)–1 mM EDTA at 4°C. (B) Competition of DNA binding activity with DR60. Binding reactions were performed with extracts prepared from cells transfected with pNheIE1 (lanes 1 to 4) (14) or IE1 translated in rabbit reticulocyte extracts (lanes 5 to 8). An oligonucleotide corresponding to DR60 was added to the binding reaction mixtures. Lanes 1 and 5, no DR60 added; lanes 2 and 6, 100-fold molar excess of DR60; lanes 3 and 7, 10-fold molar excess of DR60; lanes 4 and 8, equimolar amount of DR60 added to binding reaction mixtures. The positions of complexes I to III (C_I to C_{III}) are indicated at the left of the lanes.

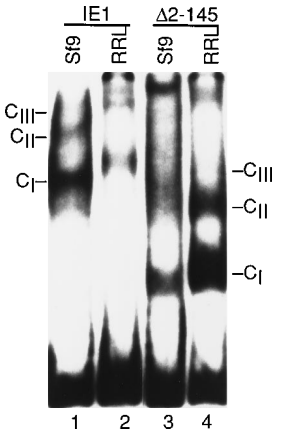

FIG. 2. Binding of IE1Δ2-145 to *hr*5-252. Plasmid pIE1Δ2-145 encodes an IE1 protein with residues 2 to 145 deleted, and expression of the protein is under the control of its own promoter (12). Plasmid pBSΔ2-145 was constructed from pIE1Δ2-145 by cloning a *Bam*HI-*Sau*3AI fragment into the *Bam*HI site of pBluescript KS (Stratagene). IE1Δ2-145 (lane 3) was translated in rabbit reticulocyte extracts (RRL) or in pIE1Δ2-145-transfected cells (lane 2), and 5 μl of the translation product was added to be used as a probe under standard conditions. DNA-protein complexes formed with IE1 expressed in insect cells are shown in lane 1. The DNA-protein complexes were resolved on a 5% polyacrylamide gel and visualized by autoradiography. The positions of complex I (C_I), complex II (C_{II}), and complex III (C_{III}) are indicated on the left for wild-type IE1 and on the right for IE1Δ2-145.

tors. Therefore, we translated IE1 in rabbit reticulocyte extracts and performed electrophoretic mobility shift assays under standard reaction conditions (Fig. 1). The 252-bp probe used for these reactions is derived from the left half of *hr*5, and it contains three potential binding sites for IE1. Extracts prepared from pIE1-transfected extracts form one, two, or three complexes depending on the relative amounts of IE1 expressed in the cells (6). In this particular experiment, two DNA-protein complexes were detected with extracts prepared from pIE1-transfected cells (Fig. 1A, lane 1). Two complexes were also observed in the reactions with IE1 translated in rabbit reticulocyte extracts (Fig. 1A, lane 3), and no DNA-protein complexes were detected in the control reticulocyte reaction mixture (Fig. 1A, lane 2). However, the mobilities of the complexes formed with IE1 produced in the cell-free system did not correspond with those formed with extracts prepared from pIE1-transfected cells. The complexes formed with in vitro-translated proteins migrated more slowly than the complexes from transfected cells. Also the DNA-protein complexes formed with the translation products migrated as more diffuse bands than the corresponding complexes formed with IE1 synthesized in insect cells.

A competition experiment was performed to demonstrate that the DNA-protein complexes were formed as a result of protein binding to specific sequences within the *hr*5 probe. Previously, we have shown that the formation of DNA-protein complexes with *hr*5 and IE1 produced in insect cells is inhibited by the addition of DR60 (5, 6). Similarly, complex formation with IE1 produced in the reticulocyte extracts was inhibited by the addition of increasing amounts of DR60 (Fig. 1B). The competition patterns with IE1 produced in the reticulocyte extracts and in *S. frugiperda* cells were essentially identical.

To further confirm that IE1 binds to *hr*5 in the absence of host cell factors, we used rabbit reticulocyte extracts to translate an N-terminal deletion mutant of IE1 and tested the activity of this protein by DNA-binding assays (Fig. 2). As previously shown (12), complexes containing IE1Δ2-145, a truncated form of IE1, migrate faster than the complexes containing wild-type IE1 (compare lanes 1 and 3). When the truncated protein was produced in reticulocyte extracts, it was capable of forming DNA-protein complexes with the *hr*5 probe. The migration of the Δ2-145 complexes formed with the in vitro-translated protein also migrated more slowly than those formed with the protein expressed in insect cells (compare lanes 3 and 4). However, the difference was not as obvious as with the full-length protein.

Together, these experiments suggest that IE1 binds to the baculovirus enhancer elements in the absence of host cell factors. However, we cannot eliminate the possibility that an insect cellular protein is normally a component of DNA-protein complexes and that this protein is replaced by a reticulocyte protein. However, the reticulocytes are enucleated cells and it seems unlikely that they contain transcription factors. Thus far, we have been unable to purify IE1 to homogeneity and demonstrate directly that it binds to *hr*5 in the absence of other proteins.

We have previously observed that IE1 is hyperphosphorylated during the late phase of virus infection (3a). The migration of the DNA-protein complexes formed with IE1 expressed during the late phase of virus infection is similar to that observed with IE1 produced in reticulocyte extracts (Fig. 3, compare lanes 2 and 8). To test whether the altered mobilities of the reticulocyte-translated complexes were due to hyperphos-

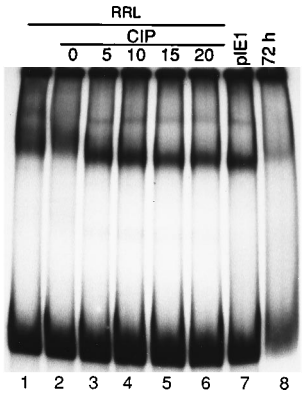

FIG. 3. CIP treatment of IE1 translated in cell extracts. IE1 was translated in rabbit reticulocyte extracts (RRL) (lanes 1 to 6). Aliquots (5 μl) of the translation reaction mixture were separately incubated with the indicated amounts of calf intestinal alkaline phosphatase (United States Biochemical) in a final reaction volume of 20 μl in phosphatase buffer (100 mM glycine–NaOH [pH 9.6], 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mM *p*-nitrophenyl phosphate) at 30°C for 30 min. Lane 1, aliquot kept on ice for 30 min; lane 2, no CIP but with incubation at 30°C for 30 min in CIP buffer; lane 3, 5 U of CIP; lane 4, 10 U of CIP; lane 5, 15 U of CIP; lane 6, 20 U of CIP. After the 30-min incubation, the samples were added to binding reaction mixtures containing the uniformly labeled *hr*5 probe generated by PCR in the presence of [^{32}P]dCTP. Whole-cell extracts were prepared in the presence of phosphatase inhibitors from cells transfected with pIE1 or from infected cells at 72 h postinfection.

phorylation of IE1, the in vitro translation reaction mixtures were treated with calf intestinal phosphatase (CIP) prior to the addition of DNA (Fig. 3). This treatment resulted in an increase in the mobility of the DNA-protein complexes; the complexes formed with CIP-treated extracts migrated to a position similar to that of DNA protein complexes with IE1 transiently expressed in *S. frugiperda* cells (compare lanes 6 and 7). This result suggests that hyperphosphorylation of IE1 in the rabbit reticulocyte extracts is responsible for the altered mobility of DNA-protein complexes formed with IE1 translated in vitro.

To demonstrate that IE1 was phosphorylated in the reticulocyte extracts, we analyzed the protein by sodium dodecyl sulfate-gel electrophoresis after incubation of translation reaction mixtures in the presence or absence of CIP. When the translation products were analyzed directly, IE1 migrated as three electrophoretically distinct species and the major band was one of the more slowly migrating forms. After CIP treatment, the fastest-migrating species was the major band, consistent with the hypothesis that IE1 was phosphorylated in the reticulocyte extracts (Fig. 4, compare lanes 1 and 4). Some forms of IE1 with altered mobility were apparently resistant to CIP treatment. These species of IE1 may have a different type of modification, or they may contain phosphorylated residues

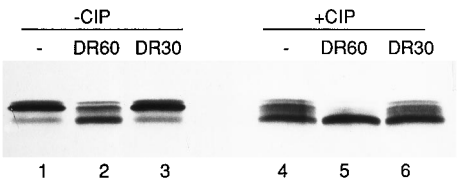

FIG. 4. Electrophoretic mobility of IE1 is altered by translation in the presence of DR60. IE1 was translated in the absence of oligonucleotide (−) (lanes 1 and 4) or in the presence of 1 μg of DR60 (lanes 2 and 5) or DR30 (lanes 3 and 6) double-stranded oligonucleotides. After translation, half of the samples were analyzed directly (−CIP) and half were treated with 26 U of CIP (+CIP) prior to gel electrophoresis.

that are resistant to CIP. It is commonly observed that CIP is unable to remove all phosphates from certain proteins. For example, both simian virus 40 and polyomavirus T antigens have specific phosphorylated residues that are completely resistant to treatment with CIP (4, 21).

When IE1 was translated in the presence of an oligonucleotide corresponding to DR60 that contains the binding site for IE1, the relative abundances of the three species of IE1 differed from those generated in the absence of DR60 (Fig. 4, compare lanes 1 and 2). In the presence of DNA, the major species was the fastest species; this pattern was similar to that observed after CIP treatment of protein translated in the absence of the DNA binding site for IE1 (compare lanes 2 and 4). The sample translated in the presence of DR60 also contained some slower forms of IE1. This heterogeneity was apparently due to phosphorylation, as the mobility of the bands was increased by treatment with CIP (compare lanes 2 and 5). When IE1 was translated in the presence of DR30, a repeated sequence in *hr*5 that does not interact with IE1, the migration pattern of the bands was the same as that seen in the absence of DNA. The fact that the DNA effect was specific for DR60 indicates that specific interactions between the protein and the oligonucleotide were responsible for the reduced levels of phosphorylation. This result suggests that the binding of IE1 to enhancer DNA alters the tertiary structure of the protein and thus affects the accessibility of certain phosphorylation sites to protein kinases.

Phosphorylation is a common means of posttranslational regulation of protein function. For example, the T antigens of simian virus 40 and polyomavirus are differentially phosphorylated during infection. Certain phosphorylation states of the T antigens preferentially bind to the viral origins of DNA replication or to sites that function as enhancers of early transcription (17, 21). The baculovirus IE1 protein has functional homologies with simian virus 40. Both are viral transcription factors that bind specific sequences within enhancer elements; both are required for DNA replication, although it is not known whether IE1 has a specific role in the initiation of DNA replication. These similarities suggest that the replication functions of baculovirus IE1 may be regulated by phosphorylation. The studies presented here strengthen this hypothesis by showing that the binding of IE1 to enhancer DNA affects the pattern of phosphorylation of IE1.

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