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Expression of an Enhancer-Binding Protein in Insect Cells Transfected with the *Autographa californica* Nuclear Polyhedrosis Virus IE1 Gene

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The baculovirus *Autographa californica* nuclear polyhedrosis virus contains an element known as homologous region 5 (*hr5*) which is an enhancer of delayed-early viral gene expression. To begin to identify proteins that interact with *hr5*, DNA-protein interactions were analyzed by using extracts from *Spodoptera frugiperda* cells and a fragment of DNA containing the left half of the *hr5* enhancer. This 252-bp DNA fragment contains two copies of a 30-bp direct repeat (DR30) and two copies of a 24-bp imperfect palindrome contained within a 60-bp direct repeat (DR60). Extracts prepared from normal *S. frugiperda* cells and cells transfected with pUC8 lacked enhancer-binding proteins. However, when gel shift assays were performed with extracts from cells transfected with a plasmid containing the viral *trans*-activator IE1 gene, two DNA-protein complexes were formed. Both DNA-protein complexes were specifically inhibited by competition with a 60-bp oligonucleotide corresponding to DR60 but not by competition with a different oligonucleotide corresponding to DR30. Formation of the two complexes did not appear to involve cooperative interactions between binding proteins. When DR60 was used as a probe, a single complex was formed. To measure the enhancer activity of DR60, a reporter plasmid was constructed that contained DR60 cloned upstream of the reporter chloramphenicol acetyltransferase gene under the control of the delayed-early 39K promoter. Transient expression analysis indicated that the oligonucleotide increased expression of this gene 300-fold over the level obtained in the absence of any enhancer sequences.

The genome of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) is a double-stranded, circular, covalently closed, supercoiled DNA molecule of approximately 128 kbp (for a review, see reference 1). The viral DNA consists primarily of unique sequences with the potential capacity to encode more than 100 proteins. In addition, five homologous regions (*hr*1 to *hr*5) of approximately 500 to 800 bp in length are interspersed along the length of the genome (2, 6).

In infected cells, viral genes are expressed in a temporally regulated, sequential fashion (1, 12). The immediate-early genes are expressed in the absence of de novo viral protein synthesis. Functional immediate-early gene products are required for the expression of delayed-early genes. The synthesis of late genes is coincident with the onset of viral DNA replication. The very late genes are maximally expressed during the occlusion phase, after the peak of late gene expression. Central to the understanding of temporal gene regulation in Ac*M*NPV is the identification of *trans*-acting factors and *cis*-acting elements which control the expression of each class. For this purpose, a transient assay system was devised for the functional mapping of regulatory genes (7). A recombinant plasmid was constructed that contains the reporter gene encoding chloramphenicol acetyltransferase (CAT) under the control of the delayed-early 39K promoter (designated the 39CAT gene) and used to search for immediate-early genes regulating delayed-early gene expression (7). The immediate-early gene, IE1, was identified by this assay. In addition to 39CAT, IE-1 *trans* activates several other delayed early genes (9, 13).

Although IE1 is necessary for the *trans* activation of delayed-early genes in transient expression assays, subsequent experiments have shown that it is not sufficient for maximal induction. When DNA containing *hr5* cloned upstream of 39CAT was cotransfected with pIE1, CAT expression increased 1,000-fold. Besides its *cis*-activating capability, the *hr5* enhancer exhibited other characteristics of viral enhancer elements, including orientation independence, position independence, the ability to regulate heterologous promoters, and the ability to increase the number of RNA polymerase molecules transcribing the linked gene (8). The nucleotide sequence of the 484-bp *hr5* enhancer was determined, and two repeated sequences were found. There are six copies of a 24-bp inverted repeat, four of them contained within a 60-bp direct repeat (DR60), and three copies of a 30-bp repeat (DR30).

cis activation of 39K by the *hr5* enhancer requires IE1, although the enhancers have little effect on the expression of IE1. These results raise interesting questions concerning the nature of the interaction between IE1 and the enhancer. Three possible mechanisms of action can be considered. One is that a host factor binds to the enhancer in the absence of IE1, but because transcription cannot occur in the absence of IE1, the effect of the enhancer is not observed. Another is that IE1 interacts with (complexes with, induces, or modifies) a host factor, thereby enabling it to bind to enhancer sequences. Finally, it is possible that IE1 activates directly through binding to the enhancer. To begin to investigate these possibilities, we tested extracts of *Spodoptera frugiperda* cells for the presence of factors which bind to the *hr5* enhancer. Cells transfected with IE1 express a protein which binds the *hr5* enhancer, while normal *S. frugiperda* cells do not. Competition with specific oligonucleotides

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FIG. 1. (A) Nucleotide sequence of the 252-bp enhancer fragment used for gel retardation experiments. The conserved 24-bp palindrome (IR24) is indicated by a double-headed arrow; the 30-bp direct repeat (DR30) is indicated by a single arrow. Sequences in boldface indicate relevant restriction sites; sequences in lowercase are derived from the vector. (B) Sequences of the oligonucleotides used for competition assays.

indicates that the factor binds within the 60-bp conserved sequences (DR60).

MATERIALS AND METHODS

Cell culture and CAT assays. The conditions for cell culture and CAT assays have been described previously (5, 7, 17). Plasmid DNAs were purified according to the boiling procedure (10), precipitated with polyethylene glycol (11), and purified on cesium chloride-ethidium bromide gradients. Transfections with plasmid DNAs were performed according to method I of Summers and Smith (17).

Preparation of cell extracts. Whole cell extracts were prepared by a modification of the procedure of Zimarino and Wu (18). *S. frugiperda* cells were grown to 10^6/ml in Spinner culture. For the initial experiments, 10^6 cells were plated in six-well plates (Corning) and transfected with 1 μg of pIE1 or pUC8 DNA exactly as described previously (7). After 24 h, cells were removed by scraping, washed twice in 1 ml of cold phosphate-buffered saline (PBS), and resuspended in 100-μl volumes of extraction buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.2], 0.4 M NaCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The cells were incubated on ice for 10 min and then microfuged at 2,000 rpm for 2 min. The supernatant was removed and used immediately in gel retardation assays.

For large-scale preparation of cell extracts, 500 ml of Spinner cells at 10^6 cells per ml were collected by centrifugation and resuspended in 125 ml of Grace's insect medium. An equal volume of transfection buffer (7) containing 500 μg of pIE1 was added. After 2 h at 27°C, the cells were collected by centrifugation and resuspended in 500 ml of complete medium. The cells were incubated at 27°C for 24 h and then washed three times by centrifugation in 100 ml of PBS. The packed cell volume was measured, and then the cells were resuspended in 4 volumes of extraction buffer. After 10 min on ice, the cells were pelleted by centrifugation. The supernatant was adjusted to 20% glycerol and frozen at −70°C. Extracts prepared in this way were active for over 6 months with no detectable loss in activity.

Gel retardation assays. A 484-bp *Mlu*I fragment of *hr5* was cloned into the *Mlu*I site of pIBI-24 (International Biotechnologies, Inc.). Probes were end labeled with T4 polynucleotide kinase at the *Hin*dIII site in the multiple cloning region (15). After digestion with *Eco*RV, the 252-bp fragment corresponding to the left half of *hr5* was purified by electrophoresis on 5% polyacrylamide gels. The probe (10,000 cpm; approximately 1 pmol) was incubated with 5 μl of nuclear extract in a total volume of 20 μl of 10 mM Tris (pH 7.5)–100 mM NaCl–1 mM dithiothreitol–20% glycerol–1 μg of poly (dI-dC) double-stranded heteropolymer (Boeringer Mannheim Biochemicals) for 20 min at 4°C. DNA-protein complexes (3, 4) were resolved on 5% polyacrylamide gels in 10 mM Tris (pH 7.5)–1 mM EDTA. The gels were run for 4 h at 200 V at 4°C, dried, and exposed for 16 h to XAR film.

RESULTS

Gel retardation analysis. To determine whether normal *S. frugiperda* cells contain factors that interact with the Ac*M*-NPV *hr5* enhancer or whether the IE1 gene product is required for binding, nuclear extracts were prepared from normal, mock-transfected, and pIE1-transfected cells. Gel retardation experiments were performed by using a 252-bp probe that contained the left half of *hr5* (Fig. 1). This fragment contains two copies of the 24-bp palindrome contained within a 60-bp direct repeat (DR60) that is conserved in all five enhancer regions and two copies of the direct repeat (DR30) that is found in *hr5* only (7). The *hr5* fragment was ^{32}P end labeled and incubated with the indicated amounts of nuclear extracts in the presence of poly(dI-dC). The resulting complexes were separated by electrophoresis on a nondenaturing polyacrylamide gel. Incubation of *hr5* with the cell extract transfected with pUC8 did not result in the formation of specific complexes (Fig. 2). Identical results were obtained with extracts prepared from normal cells (data not shown). During the course of these experiments, we

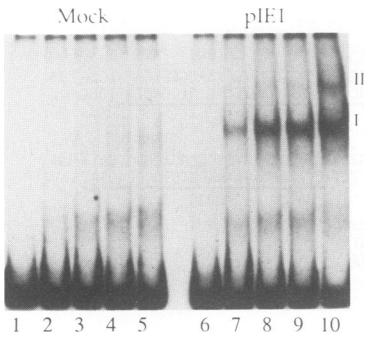

FIG. 2. Gel retardation analysis of *hr5*. An end-labeled 252-bp *Hin*dIII-*Eco*RV fragment was incubated with no cell extract (lanes 1 and 6) or with 1 μl (lanes 2 and 7), 2 μl (lanes 3 and 8), 3 μl (lanes 4 and 9), or 5 μl (lanes 5 and 10) of crude cell extract from cells transfected with pUC8 (lanes 1 to 5) or pIE1 (lanes 6 to 10).

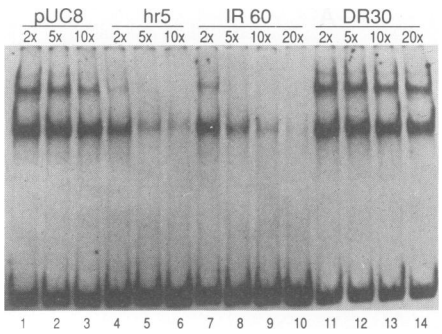

FIG. 4. Competition analysis of DNA-protein complexes. Binding reaction mixtures were prepared by using 5 μl of extract from cells transfected with pIE1. Prior to addition of the probe, competitors were added to the reaction mixtures in excess molar amounts as indicated: lanes 1 to 3, *Hae*-III-digested pUC8; lanes 4 to 6, unlabeled *Hin*dII-*Eco*RV fragment of *hr5*; lanes 6 to 10, DR60; lanes 11 to 14, DR30.

found that it was very important to maintain the probe in the presence of 0.1 M NaCl and to avoid heating the DNA. In the absence of these precautions, a nonspecific complex was sometimes formed with the extracts prepared from normal cells. The formation of this complex was variable, and we believe it to be due to partial denaturation of the probe, which has a low G+C content (40%). This phenomenon has previously been reported in other systems (16).

When *hr5* was incubated with extracts made from cells transfected with pIE1, two DNA-protein complexes were formed (complexes I and II). The fastest-migrating complex, complex I, was formed with the least amount of added extract, and complex II, the slower-migrating complex, was observed only with more extract. The amount of probe in the two complexes was determined by Cerenkov counting of the excised gel slices. The results are presented in Fig. 3 as a plot of radioactivity in each band against the amount of cell extract added. Both complexes increased linearly with increasing extract added, indicating that complex II formation is not cooperative.

To confirm that the binding was specific for *hr5* DNA, competition experiments were performed with an excess of unlabeled probe or nonspecific DNA (Fig. 4). Excess unlabeled probe inhibited all three complexes (lanes 4 to 6), while addition of excess *Hae*III-digested pUC8 had no effect (lanes 1 to 3). To further define the sequences within the probe that

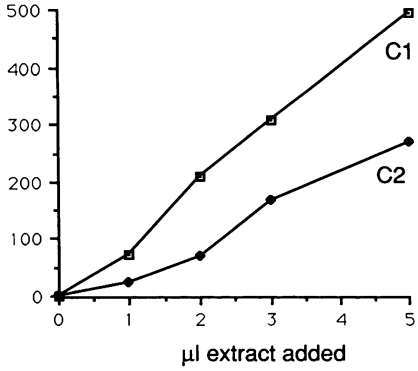

FIG. 3. Complex formation as a function of extract concentration. The regions of the gel corresponding to complex I and II were excised and quantitated by Cerenkov counting.

interact with proteins in the extract, synthetic oligonucleotides were synthesized and used in competition analyses. Competition with the 60-bp repeat containing the conserved 24-bp palindrome inhibited formation of all three DNA-protein complexes (lanes 7 to 10), indicating that both complexes were due to DNA-protein interactions at sequences within the 60-bp repeat. The addition of excess DNA corresponding to the smaller direct repeat (DR30) did not inhibit DNA protein interactions (lanes 13 to 16).

Functional analysis of 60-bp repeat. To confirm that sequences within the 60-bp oligonucleotide were sufficient for DNA-protein complex formation, the oligonucleotide was 5′ end labeled and used in gel retardation experiments (Fig. 5). A single, specific complex was formed with the 60-bp oligonucleotide. As a control, the 30-bp oligonucleotide was also labeled and tested with the same extract. Gel retardation assay indicated that DNA-protein complexes were not formed with this probe (data not shown).

To determine whether the 60-bp sequence was sufficient for enhancer function, the oligonucleotide was cloned upstream of the 39CAT promoter (39cat-DR60). *S. frugiperda* cells were transfected with pIE1 and p39cat-DR60. As controls, cells were transfected with pIE1 and either p39cat-*hr5*, which contains the entire *hr5* sequence, or p39cat, which has no enhancer sequences. As previously reported, the presence of the entire *hr5* enhancer upstream of 39CAT increases CAT activity more than 1,000-fold over the activity seen in the absence of enhancer sequences. The presence of the 60-bp oligonucleotide increased CAT expression approximately 300-fold (Table 1).

DISCUSSION

The IE1 gene product is essential for *trans* activation of both enhancers and delayed-early promoters (7–9). These observations suggest that IE1 may be bifunctional in nature; it *trans* activates both a promoter and an enhancer. Alternatively, it is possible that the mechanism of action of *trans* activation of both targets is the same. There are no obvious sequence homologies between the 39K promoter and the enhancer which could serve as IE1-responsive elements. However, both regions are A+T rich, and binding sites may be present but not obvious. The exact mechanism by which IE1 *trans* activates the 39K promoter is unknown, but it might involve direct binding to viral sequences or interac-

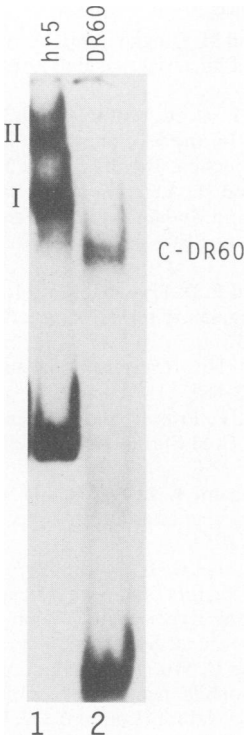

FIG. 5. Gel retardation assays with oligonucleotide probes. The top strand of the oligonucleotide sequence shown in Fig. 1B was 5′ end labeled with polynucleotide kinase. After hybridization with an equimolar amount of the complementary strand, binding reactions were performed. Lane 1, 252-bp *hr5* probe; lane 2, DR60.

tions with host transcription factors (14). The fact that IE1 activates many viral genes as well as heterologous genes (8, 9, 13) suggests that an indirect role may be more likely. The exact interaction between IE1 and the enhancer is also unknown. To begin to investigate these interactions, gel retardation experiments using extracts from cells transfected with pIE1 and enhancer DNA were performed.

Under the conditions used for these assays, extracts prepared from normal or mock-transfected *S. frugiperda* cells do not interact with the baculovirus *hr5* enhancer in vitro. However, we cannot eliminate the possibility that factors are present in normal cells but were not detected. Cells transfected with pIE1 formed two specific complexes with an *hr5* fragment. Competition analyses indicated that formation of complexes I and II was due to interactions of proteins and DNA sequences within the conserved 60-bp direct repeats. The fragment of *hr5* used in this analysis contains two of the 60-bp repeats. When DR60 was used as a probe, a single complex was formed. These data are consistent with the hypothesis that formation of complex I, the fastest-migrating complex, is due to the binding of protein to either one of the repeats, and formation of complex II is due to binding of protein to both repeats. Proof of this hypothesis will required purification of the factors binding to *hr5*.

TABLE 1. Enhancer activity of a synthetic 60-bp oligonucleotide[a]

Reporter plasmid	CAT activity[b]	Fold stimulation
39cat	0.04	1
39cat-*hr5*	45.0	1,125
39cat-DR60	13.3	310

[a] Cells were transfected with 1 μg of the indicated CAT plasmid and 0.1 μg of pIE1. After 24 h, the CAT activity was determined.

[b] Expressed as nanomoles of chloramphenicol acetylated per minute per 10^6 cells.

It is not known exactly where protein binds within the 60-bp repeat. We have synthesized several smaller oligonucleotides containing conserved sequences and attempted to use them as competitors or probes. Thus far, we have been unable to identify smaller sequences containing IR24 that efficiently interact with DNA-binding proteins in extracts from pIE1-transfected cells (data not shown). Our attempts at DNase I footprinting and methylation protection have been unsuccessful. We have also been unable to purify the binding activity by using either conventional chromatography or DNA affinity chromatography. Although the binding activity in crude cell extracts is stable, we have observed a rapid loss of activity in our purification techniques (data not shown).

Quantitation of the amount of probe in each retarded band revealed that complex formation increased linearly with increasing amount of extract added to the binding reactions. A linear increase indicates that the binding is not cooperative. If complexes I and II were formed in a cooperative manner, we would expect to see a sigmoidal increase in the formation of complex II and a decrease in the formation of complex I as the amount of added extract was increased. The observed results are inconsistent with such a model, as there was a linear relationship between amount of extract and binding activity. However, this does not rule out the possibility that once bound, the proteins enhance transcription in a cooperative manner.

To analyze the enhancer function of IR60, a reporter plasmid containing IR60 upstream of 39CAT was constructed. Transient assay analysis revealed that a single copy of the 60-bp repeat enhances 39CAT expression 300-fold over that seen in the absence of enhancer sequences. In the same experiment, the 484-bp *Mlu*I fragment of *hr5* region which contains four 60-bp repeats enhances expression 1,100-fold. It is not known whether the higher activity of the larger fragment is due to an additive effect of the repeats or is due to the sequences not represented in the 60-bp oligonucleotide.

The results presented here indicate that *S. frugiperda* cells transfected with IE1 contain factors capable of interacting with the *hr5* enhancer. Under the same conditions, binding was not detected with extracts prepared from normal cells. Currently, we cannot distinguish between the possibilities that IE1 binds directly and that it mediates its activity through a host cell factor. We are now involved in identifying other proteins that may be involved in DNA-protein interactions.

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