A 38-kDa Host Factor Interacts with Functionally Important Motifs within the Autographa californica Multinucleocapsid Nuclear Polyhedrosis Virus Homologous Region (hr1) DNA Sequence*

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We recently demonstrated that the Autographa californica multinucleocapsid nuclear polyhedrosis virus homologous region (hr1) enhances transcription from the viral polyhedrin promoter and also functions as a putative origin of replication (ori). Hr1, carrying five 28-base pair core palindrome units, has also been mapped with respect to its enhancer and ori functions (Habib, S., Pandey, S., Chatterji, U., Burma, S., Ahmad, R., Jain, A., and Hasnain, S. E. (1996) DNA Cell Biol. 15, 737–747). A 38-kDa host factor termed hr1-binding protein (hr1-BP) binds with high specificity and affinity (K_{d} = 6.5 \times 10^{-11} M) to functionally important motifs within hr1. The core palindrome as well as sequences immediately flanking it are required for this interaction. Divalent cations are not essential, and ionic interactions play only a minor role in complex formation. hr1-BP binds through the minor groove of the double helix to multiple sites within hr1, and binding occurs as a function of the number of modules within hr1. Phosphorylation of hr1-BP is important for host factor-hr1 interaction. Hr1-BP differs in several respects from the other host factor, polyhedrin promoter-binding protein, described previously (Burma, S., Mukherjee, B., Jain, A., Habib, S., and Hasnain, S. E. (1994) J. Biol. Chem. 269, 2750–2757). When hr1-BP was sequestered out, in vivo, by a plasmid carrying hr1 alone, the hr1-mediated enhancement of reporter expression was abolished, demonstrating that the binding of hr1-BP may be crucial for the enhancer activity of the dual function hr1 element.

The genome of the Autographa californica multinucleocapsid nuclear polyhedrosis virus (MNPV)\(^{1}\) is a double-stranded, covalently closed, circular DNA molecule consisting largely of unique sequences. Eight homologous region sequences or hrs (hr1, hr1a, hr2, hr3, hr4a, hr4b, hr4c, and hr5) are distributed throughout the genome. These hrs vary in length from 150 to 800 bp and contain two to eight imperfect, 30-bp palindromes with an EcoRI site at the center of each palindromic core. The hrs have been demonstrated to be putative origins (oris) of viral DNA replication as well as enhancers of delayed-early gene transcription (3, 4).

hr1, located \(~3.7\) kilobase pairs upstream of the A. californica MNPV very late polyhedrin gene promoter, lies within an 880-bp ClaI fragment and contains five imperfect 28-bp palindromes (Fig. 1). hr1 has been demonstrated to be a putative origin of viral replication (5–8), and preliminary data have suggested its role as an enhancer of the immediate-early ie-n gene and the delayed-early 39K gene (9–11). We recently demonstrated that it also functions as a position- and orientation-independent enhancer of A. californica MNPV very late polyhedrin gene transcription in transient expression assays and that this enhancement effect is independent of the ori function of hr1 (1). An intact 28-bp core palindrome together with the palindrome-flanking sequences is essential for enhancement; a palindrome intermediate sequence flanked by two half-palindromes or a palindrome alone is incapable of enhancing polyhedrin promoter-driven transcription. The palindrome alone could, however, function as an infection-dependent ori in transient replication assays (1).

DNA-protein interactions have been previously investigated with hr1a and hr5. The virus-encoded immediate early protein IE-1 binds to sequences within both of these hrs (12–14), and potential IE-1 binding sites within these enhancer elements have been delineated (14, 15). In hr5, approximately half of the direct repeat (DR60) is sufficient for binding, and both the right and the left halves of DR60 bind the protein. Specific binding of IE-1 to the hr1a sequence has been detected for probes containing EcoRI core mutations, indicating that the protein binds to regions flanking the EcoRI core sequence (14). It has been recently demonstrated that IE-1 binding to hr5 is optimal when both palindrome half-sites are present and that a dimeric form of IE-1 binds to the hr5 28-mer palindromic core (16). The ie-1 gene product when translated in rabbit reticulocyte extracts can also bind hr5, thereby demonstrating that this DNA-protein interaction can occur in the absence of insect cell host factors (13). However, there has been no report so far of the involvement of a host-specific cellular factor interacting with any of the hr sequences.

In this study we describe a 38-kDa host factor, the hr1-binding protein (hr1-BP), that interacts with the hr1 sequence elements with high specificity and affinity. Analyses of DNA binding characteristics of this factor, its affinity for hr1, and the requirement of phosphorylation for binding are presented. The abolition of hr1-mediated enhancement of reporter gene expression in an \(\text{in vivo}\) transient expression assay by the presence of a competing hr1 sequence in \(\text{trans}\) points to the importance of this factor in hr1 function.

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\(^{1}\) The abbreviations used are: MNPV, multinucleocapsid nuclear polyhedrosis virus; bp, base pair(s); hr1-BP, hr1-binding protein; PPBP, polyhedrin promoter-binding protein.
MATERIALS AND METHODS

Cells and Virus—Spodoptera frugiperda cells (Sb9) were grown in TNEFH medium containing 10% fetal bovine serum as described by Summers and Smith (17). The cells were infected with A. californica MNPV E-5 strain at a multiplicity of infection of 10 plaque-forming units/cell for transient expression studies and for the preparation of nuclear protein extracts.

Transient Expression Assay—The expression of luciferase in Sb9 cells transfected with reporter plasmids was carried out using Lipofectin (1). Briefly, 2 × 10^6 cells were seeded in a 35-mm tissue culture plate, washed twice with serum-free TNEFH, and incubated for 2 h at 27°C. Reporter plasmid DNA (15 μg of DNA/2 × 10^6 cells) dissolved in 35 μl of water and 15 μl of (1 mg/ml) Lipofectin (Life Technologies, Inc.) were separately diluted to 500 μl in serum-free TNEFH, mixed, and added to the culture wells. After 8 h at 27°C, cells were washed twice with complete medium, infected with A. californica MNPV at a multiplicity of infection of 10 plaque-forming units/cell for 1 h and incubated in complete medium at 27°C. At 60 h postinfection, cells were dislodged in 400 μl of medium, diluted to 600 μl with 0.1 × Tris acetate (pH 7.75); 2 mM EDTA buffer, and assayed for luciferase in a buffer containing 40 μM luciferin, 14 mM MgCl₂, and 14 mM glycine (pH 7.6). Light emission was monitored using a luminometer (model 1250, Bio-Opti Oy, Turku, Finland) over an integration period of 10 s.

Preparation of Nuclear Extract—Nuclear protein extracts were prepared from Sb9 cells using a modification of the method of Dignam et al. (18) as described earlier (2, 19). All operations were carried out at 4°C. Briefly, the crude nuclear fraction was pelleted from cells lysed in a buffer containing 1% Nonidet P-40, 30 mM Tris-HCl, pH 7.5, and 10 mM magnesium acetate. The nuclei were purified by centrifugation at 28,000 rpm for 60 min in a Beckman SW41 rotor through a sucrose cushion (10 mM Hepes-NaOH, pH 7.5, 15 mM KCl, 2 mM sucrose, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). The purified nuclei were suspended in protein extraction buffer (10 mM Hepes-NaOH, pH 7.5, 420 mM NaCl, 1.5 mM MgCl₂, 25% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). After 30 min, the nuclei were pelleted at 11,000 rpm in a microcentrifuge; the supernatant was dialyzed against a buffer containing 20 mM Hepes, pH 7.9, 50% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol, aliquoted, and stored at −70°C.

Electrophoretic Mobility Shift Assay—Binding reactions with nuclear extracts from uninfected and infected cells were carried out by incubating 5 μg of nuclear extract with end-labeled fragments of hrl (Fig. 1). The binding reaction was carried out in a buffer containing 250 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM dithiothreitol, 20% glycerol, and 1 μg (−1000-fold excess) of poly(dI-dC) at 4°C for 15 min. The reaction was loaded on a 4.5% polyacrylamide gel and run at 150 V in TAE buffer. In competition experiments, a 20-fold molar excess of the unlabeled competitor was added to the reaction prior to the addition of the nuclear extract. In the experiment involving competition with oligonucleotides covering overlapping regions of hrl, up to a 200-fold molar excess of competitor was used.

Inhibition with Major/Minor Groove-binding Drugs—The 330-bp hrl probe was incubated for 30 min at room temperature with varying concentrations of distamycin A (10 μM to 1 mM), actinomycin D (10 μM to 1.4 mM), or methyl green (50 μM to 1.25 mM), drugs that specifically interact with the minor or major groove of the DNA double helix, respectively (20, 21). The DNA-drug mix was then analyzed for binding in a gel retardation assay by incubation with nuclear extract.

Southwestern Blot Analyses—All operations were carried out at 4°C unless stated otherwise. Nuclear protein extract (100 μg) was fractionated on a 12.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane in a buffer containing 25 mM Tris and 190 mM glycine at 30 mA for 16 h. The membrane was incubated with blocking buffer (2% nonfat dry milk, 1% bovine serum albumin, 10 mM Hepes-NaOH, pH 7.5, 0.1 mM EDTA, 200 mM NaCl, 50 mM MgCl₂, and 16 μg/ml sonicated salmon sperm DNA) for 2 h and then incubated with binding buffer (blocking buffer with 0.2% nonfat dry milk) containing 32P-labeled 170-bp hrl probe for 16 h, washed briefly, and subjected to autoradiography as described earlier (2).

Phosphorylation—Nuclear protein extract (50 μg) was treated with 200 units of calf intestine alkaline phosphatase (Boehringer Mannheim) for 4 h at 37°C (2, 22) in a dephosphorylation buffer (1 mM ZnCl₂, 1 mM MgCl₂, 10 mM Tris-HCl, pH 8.3). 200 units of phosphatase boiled for 30 min was used as a negative control. Phosphatase-treated nuclear extracts were assayed by Southwestern analysis as described above except that dry milk was omitted from both the

RESULTS

Host Factor(s) Interacts Specifically with hrl as a Function of Core Palindrome and Flanking Sequence Units—In order to determine the factors involved in hrl interactions, we carried out gel retardation assays using 32P-labeled hrl DNA sequences and nuclear extracts prepared from uninfected cells and from A. californica MNPV infected cells 3, 6, 12, 36, and 50 h postinfection. With the 330-bp fragment of hrl as probe (Fig. 1), a stronger shift of higher mobility (C1) and a weaker shift of lower mobility (CII) were detected at all time points. The intensities of the complexes were similar at all time points tested (Fig. 2). There was no visible difference in the complexes obtained with extracts from uninfected (lane 2) and infected (lanes 3, 4, 5, 6, and 7) cells, suggesting the involvement of host factor(s) in hrl interactions.

When the 420-, 330-, 170-, 158-, and 90-bp fragments representing different components of hrl (Fig. 1) were used as probes in gel retardation assays, three complexes with the 420-bp fragment (Fig. 3A), two complexes with the 330-bp (B) and 170-bp (C) fragments and a single complex each with the 158-bp (D) and 90-bp (E) fragments were visible. However, when a 28-bp synthetic oligonucleotide constituting just the complete palindrome without any flanking sequence was used as probe (Fig. 3F), the DNA-protein complex could not be detected. This suggested that a single palindromic unit alone is not sufficient for binding the host factor and that flanking sequences may play a major role in this DNA-protein interaction. The number of complexes observed corresponded to the number of palindrome-flanking sequences present in each probe. To confirm that the hrl-host factor complex represents a specific DNA-protein interaction, competition experiments were carried out. The addition of an excess of the unlabeled homologous competitor (probe fragment) to the binding reaction competed for complex formation (Fig. 3, A, lane 4, B–E, lane 3), while the complex remained unaffected in the presence of the nonspecific competitor pUC18 (Fig. 3, A, lane 5, B–E, lane 4). These results demonstrated that host factor(s) binds specifically to the hrl core palindrome along with flanking sequences and not to the palindrome alone. Furthermore, complex formation occurs as a function of this modular binding unit.

The Same Host Factor Interacts with Different Components of hrl—A series of gel retardation assays were carried out using the 330-, 170-, and 90-bp fragments corresponding to different components of hrl as probes and challenging the binding with
unlabeled fragments of hr1 in a cross-competition assay. The DNA-protein complexes obtained with the 170-bp fragment (Fig. 4A, lane 2) were efficiently competed out with an excess of unlabeled 330-bp (lane 3) and 158-bp (lane 4) fragments but not with a similar excess of unlabeled 28-bp core palindrome alone (lane 5) or a nonspecific competitor pUC18 (lane 6). Similarly, the single complex obtained with the 158-bp fragment (Fig. 4B, lane 2) carrying an intermediate sequence flanked by two half-palindromes as probe disappeared in the presence of a molar excess of the 330-bp (lane 3), 170-bp (lane 4), and 90-bp (lane 5) fragments but was not affected when the 28-bp EcoRI core palindrome (lane 6) or nonspecific pUC18 (lane 7) was used as competitor. These cross-competition analyses clearly demonstrated that the same host factor is involved in interacting with different fragments of hr1.

The Palindrome and Sequences Immediately Flanking It Are Essential for hr1-Host Factor Interaction—Nine overlapping double-stranded oligodeoxyribonucleotides encompassing different regions of an hr1-palindrome and its 5’- and 3’-flanking sequences (Table I) as well as the CANNTG motif recognized by helix-loop-helix proteins (1) were used as competitors in a binding reaction with the 170-bp fragment of hr1 (Fig. 5A). None of the oligonucleotides, including those that carried a half or full palindrome with only the 5’- or the 3’-flanking sequence, could compete significantly for binding even when a 200-fold molar excess of the unlabeled competitors was used (Fig. 5A, lanes 3–10). The CANNTG motif was found to be present within the hr1 element (1); therefore, oligonucleotides were designed to explore the significance of this motif in hr1-BP interaction. The slight decrease in the intensity of the complex seen with respect to competition with the oligonucleotide M+ (lane 9) that carries the CANNTG motif (Table I) was not consistently observed. Competition was, however, clearly seen with the 46-bp oligonucleotide 5P3 that carries the full palindrome along with 9 bp each of the 5’- and 3’-flanking sequences (lane 11). We then used this oligonucleotide as probe and observed two specific shifts in the normal binding reaction (Fig. 5B, lane 2). The complex could not be competed out with the oligonucleotides P5F (full palindrome with 5’-flanking sequence) (lane 4) and P (full palindrome alone) (lane 5) but was competed out when the oligonucleotide mFP (full palindrome with shuffled 9 bp each of the 5’- and 3’-flanking sequences) (lane 3) and the 170-bp hr1 fragment were used as competitors. Use of the nonspecific competitor pUC18 did not affect complex formation. These results demonstrate that short segments not containing both the 5’- and 3’-flanking sequences are required for the hr1-BP interaction. A half-palindrome, a palindrome alone, or flanking sequences without the palindrome are not sufficient for the interaction. Moreover, the fact that an oligonucleotide carrying an intact palindrome with shuffled flanking sequences could act as a competitor indicates promiscuity in the sequence requirement of the 9-bp 5’ and 3’ palindrome-flanking segments. The nucleotide sequence of the flanking segments required for binding of hr1-BP, therefore, does not appear to be precise. The
two shifts observed with 5P3 as probe suggest binding of an hr1-BP dimer to this sequence.

Binding Does Not Involve an RNA Component—We checked whether the factor binding to hr1 was a protein or whether it also involved an RNA component. Binding of the 32P-labeled 330-bp hr1 fragment was carried out after pretreating the nuclear extract (2 μg) with RNase A (1 μg) or proteinase K (20 μg) at 37 °C for 1 h. Complex formation, observed in a gel retardation assay (Fig. 6, lane 1), was abolished when nuclear extracts were treated with proteinase K (lane 3) but remained unaffected after treatment with RNase (lane 2). These results clearly rule out the involvement of any RNA component in the host factor-hr1 interaction.

The hr1-binding Host Protein Is Distinct from the Other Host Factor, Which Binds to the Polyhedrin Promoter—In order to check whether the host factor binding to hr1 was the same as the cellular 30-kDa polyhedrin promoter-binding protein (PPBP) reported earlier (2, 24), we challenged the complexes obtained with the 330-bp probe with unlabeled domain B, which is a 32-bp synthetic oligonucleotide that carries the AATAAAATAAGTTT sequence essential for the PPBP-polyhedrin promoter interaction (2) (Fig. 7A). The two complexes (Fig. 7A, lane 2) remained unaffected in the presence of a 30-fold molar excess of the competitor (lane 3) as well as in the presence of a similar excess of pUC18 (lane 4). In a complementary experiment, in which the domain B-PPBP complex was challenged with an excess of the unlabeled 420-bp fragment, the complex was not competed with an excess of the 420-bp hr1 competitor (Fig. 7B, lane 4) but disappeared in the presence of the homologous B domain oligonucleotide (lane 3). This confirmed that the host factor hr1-BP interacting with hr1 is not the same as the other host factor PPBP, which binds to the AT-rich polyhedrin initiator promoter.

Binding Does Not Require Divalent Cations, and Ionic Interactions May Only Be Marginally Involved—Experiments were carried out to determine the interactions involved in hr1-host factor binding. When the binding reaction was carried out using the 170-bp hr1 fragment in the presence of increasing amounts of NaCl, complex formation gradually decreased as a function of salt concentration (Fig. 8A, lanes 2–7) and was abolished only at salt concentrations as high as 2.5 M (lane 8). This suggested that ionic interactions may be only marginally involved in complex formation. When binding was carried out in the presence of different concentrations of EDTA (Fig. 8B), the complex remained unaffected and was stable even at a concentration of 100 mM EDTA (lane 6), demonstrating that divalent cations are not required for the hr1-BP-hr1 interaction.

The Host Factor Displays High Affinity for hr1—The affinity of the host protein for hr1 was determined by calculating the dissociation constant (Kd) of the complex. The apparent Kd value for the specific DNA-protein interaction was estimated by a method based on the gel retardation assay (2, 25). A constant amount of protein (2 μg (Fig. 9A) and 3 μg (Fig. 9B) in two separate determinations) and increasing amounts of the 170-bp radiolabeled hr1 probe were used in separate binding reactions. The extent of complex formation was quantitated by scintillation counting of excised labeled bands obtained in the gel retardation assay. A plot of bound/free versus the bound probe (Fig. 9C) yielded a straight line with the inverse of the slope equal to the dissociation constant. From two separate determinations, the Kd value was estimated to be 6.5 (± 0.2) μM. The palindrome sequences are shown in boldface type, and the CANNTG motif is underlined. The sequence of the upper strand alone is shown.

**Table I**  
Oligodeoxyribonucleotides used to fine map the binding motif

| Oligonucleotide name | Double-stranded oligonucleotide sequence
|----------------------|---------------------------------------------|
| 3F                   | 5’-CGTTCTCGTATGACGAACTGACAT-3’             |
| 5F                   | 5’-CACTAGTAATGACGAACTGACATGACTGCACGCTGTCCTGTCCTTGG-3’ |
| 2P3F                 | 5’-TCTACTCTGGTAAACCGATCTCGTTATGACGCTGAGG-3’ |
| 2P5F                 | 5’-TGACATTCACTGCTATGCTCGTATGACGCTGACGCTGAGG-3’ |
| P5F                  | 5’-TCTACTCTGGTATGACGACGCTGACGCTGACGCTGAGG-3’ |
| P3F                  | 5’-CGGATCTCACTGCTATGCTCGTATGACGCTGACGCTGAGG-3’ |
| 5P3                  | 5’-CACTAGTAATGACGAACTGACATGACTGCACGCTGTCCTGTCCTTGG-3’ |
| mFP                  | 5’-CTACTCTGGTATGACGACGCTGACGCTGACGCTGAGG-3’ |
| M+                   | 5’-CTACTCTGGTATGACGACGCTGACGCTGACGCTGAGG-3’ |
| M−                   | 5’-CTACTCTGGTATGACGACGCTGACGCTGACGCTGAGG-3’ |

a The palindrome sequences are shown in boldface type, and the CANNTG motif is underlined. The sequence of the upper strand alone is shown.
Host Factor Binding to *A. californica* MNPV hr1 Enhancer Element

The host factor interaction requires an intact palindrome and is promiscuous with respect to the requirement of 5′- and 3′-flanking sequences. A, the complexes formed with the 170-bp hr1 probe were competed with a 200-fold excess of the oligonucleotides described in Table I (lanes 3–11). Lane 1, free probe; lane 2, normal binding reaction in the absence of competitor. B, complex formation with the oligonucleotide SFP3 as probe. Lanes 3–7 represent competitions with a 100-fold excess of cold-competitors. Lane 1, free probe; lane 2, complex formation in the absence of a competitor.

10−11 m. The host protein hr1-BP thus interacts with hr1 with very high affinity.

**The Host Protein has a Molecular Mass of ~38 kDa**—Southwestern analysis of the total nuclear extract using the radiolabeled 170-bp fragment as a probe was carried out to determine the molecular mass of the hr1-BP. Nuclear extract from cells very late in infection (50 h postinfection) was fractionated on a 12.5% SDS-polyacrylamide gel, blotted onto nitrocellulose membrane, and probed with the labeled 170-bp fragment (Fig. 10C). A band obtained in the 38-kDa region demonstrated that the apparent molecular mass of the protein was around this value. Hybridization with radiolabeled pUC18, when used as a negative control, did not give any signal (Fig. 10A). The 28-bp palindrome that does not bind hr1-BP in gel retardation assays also did not give a band when used as probe in the Southwestern assay (Fig. 10B). These results point to the specificity of the hr1-binding protein detected in the Southwestern blots.

**Phosphorylation of the Host Factor Is Necessary for Binding to hr1**—Experiments were carried out to determine the effect of phosphorylation on DNA-binding activity of hr1-BP. Nuclear extracts prepared from infected cells very late in infection (50 h postinfection), when the polyhedrin promoter is hyperactive, were treated with calf intestine alkaline phosphatase and analyzed for binding by Southwestern blotting using the 170-bp hr1 fragment as a probe. Phosphatase treatment completely abolished DNA binding (Fig. 11, lane P), while binding was unaffected in the untreated extract (lane U) or in the extract treated with boiled phosphatase (lane BP). These results, therefore, demonstrated that phosphorylation of the host factor is essential for binding to the hr1 sequence.

**The Host Protein Interacts with hr1 through the Minor Groove of the DNA Molecule**—Experiments were carried out to determine whether the factor binding to hr1 interacts through the major or the minor groove of the DNA double helix. Binding reactions were carried out in the presence of actinomycin D or methyl green that interact specifically with the minor and the major groove, respectively (20, 21). Incubation of the radiolabeled 330-bp probe with actinomycin D (lane 2) or methyl green (lane 9) alone did not affect the mobility of the probe. The hr1-protein complex (Fig. 12A, lane 3) was inhibited by actinomycin D in a dose-dependent manner (lanes 4–8) in gel retardation assays. The complex (lane 10) was, however, not affected upon preincubation of the DNA probe with methyl green even when 1.25 mM of the drug was used in the binding reaction. Specific binding of hr1-BP to the minor groove was further confirmed by incubation of the 170-bp probe with distamycin A, another specific minor groove-binding drug (Fig. 12B). Formation of the hr1-host factor complex (lane 1) was also inhibited by distamycin A in a dose-dependent manner (lanes 4–9). Distamycin has sequence-dependent affinity for DNA in the low micromolar range. The requirement of higher concentrations of distamycin for complete inhibition of binding to hr1 suggests that minor groove interactions with the protein may occur at sites with low affinity for distamycin. These results demonstrate that while hr1-BP interacts with hr1 primarily through the minor groove of the DNA double-helix, it is not clear where potential minor groove interactions may be occurring.

**hr1-BP Appears to Be Directly Involved in the Enhancer Function of hr1**—To provide evidence for the functional role of hr1-BP, we performed in *vivo* competition by hr1 in transient expression assays using the plasmids pSHluc and pSHuc-hr1 (1). The latter carries a copy of the hr1 sequence 730 bp upstream to the polyhedrin promoter-luciferase reporter cassette. We have previously demonstrated that transient expression of luciferase from pSHluc-hr1, in infected Sf9 cells is several-fold higher than that from pSHluc. The construct pSHhr1, which carries only the hr1 sequence cloned in pUC18 (1), was used as a competitor to sequester the hr1-BP present in the cells, thereby making it unavailable for binding to the hr1 sequence in the reporter plasmid. Sf9 cells co-transfected with the reporter construct as well as different amounts of the competitor plasmid were assayed for luciferase expression 60 h postinfection (Fig. 13). As expected, the presence of 10 μg of the competitor pSHhr1 did not have any effect on the expression of...
Host Factor Binding to A. californica MNPV hr1 Enhancer Element

DISCUSSION

The enhancer activity for all hrs (9, 10) and primarily that of hr5 has previously been demonstrated only for the immediate-early and delayed-early genes in transient expression assays (15, 26). We have shown that apart from stimulating early and delayed early genes in transient expression assays hr1 can also enhance transcription from the immediate early IE-N and delayed early 39K (15, 26). We have shown that apart from stimulating expression of the 330-bp hr1 promoter (free probe (lane 1); normal binding with nuclear extract (lane 2)) was challenged with a 30-fold molar excess of an unlabeled oligonucleotide encompassing the B domain (2) of the polyhedrin promoter (lane 3) and a similar excess of pUC18 (lane 4). B, the PPBP-domain B complex (lane 2) was challenged with an excess of unlabeled domain B (lane 3) or with a molar excess of the 420-bp hr1 fragment (lane 4). Lane 1 is the free B domain probe.

pSHluc, which did not carry the hr1 element. The expression from pSHluc-hr1-U1, which had been enhanced several fold by the enhancer effect of hr1, was reduced proportionately as the amount of competitor was increased to 1, 5, and 10 μg. When 10 and 20 μg of competitor was used, the reporter expression level was down brought to that of the basal plasmid pSHluc. This demonstrates that the nonavailability of hr1-BP due to the binding of this factor to an hr1 sequence present in trans abolishes the enhancement effect. The factor is therefore important for mediating the enhancer function of hr1 vis-a-vis the polyhedrin promoter of A. californica MNPV.

for binding to occur. Moreover, the position and intensity of the retarded complex does not change throughout infection, suggesting that either the factor is not modified during the infection cycle or that its DNA-binding activity is not drastically altered by such modification(s). The dephosphorylated protein is incapable of binding hr1, suggesting that phosphorylation/dephosphorylation of hr1-BP may play a regulatory role in mediating hr1 function.

Characterization of the host factor-hr1 complex revealed some striking features of the interaction. Although there was a slight concentration-dependent decrease in complex formation in the presence of salt, the fact that the complex was observed even at 2 M NaCl suggested that ionic interactions may play a marginal role in complex formation. The host factor-hr1 complex was also not disrupted in the presence of a high concentration of EDTA (up to 100 mM), indicating that divalent cations are not required for binding. hr1-BP also displays unusually high affinity for hr1 with an apparent $K_d$ value of 6.5 (± 0.2) × 10$^{-11}$ M. An extremely low $K_d$ value (~3.7 × 10$^{-12}$ M) has also been reported (2) for another host protein, the 30-kDa host factor PPBP, that interacts with transcriptionally important motifs within the polyhedrin promoter and another A. californica MNPV very late p10 gene promoter (27). The high affinity of these cellular factors for viral sequences may be crucial for the virus to recruit them from their normal sites of action in the host insect cell.

In vitro binding inhibition with the minor groove-binding
drugs demonstrates that the insect cellular factor approaches the hr1 element through contact points within the minor groove of the DNA double helix. Some eukaryotic enhancer-binding proteins that interact with the minor groove of DNA induce DNA bending. Examples of such proteins include the lymphoid enhancer-binding factor 1, which interacts with the mouse T cell receptor a gene enhancer (28) and the HMG1(Y) protein, which contains a highly charged basic repeat sequence required for its interaction with the AT-rich duplex DNA of the virus-inducible enhancer of the human interferon-β gene (29). A number of viral genes required for DNA replication have been identified (30, 31). The baculovirus immediate-early ie-1 gene product is the only hr-binding factor identified so far. IE-1 binds the DR60 sequence, and sequences flanking the EcoRI core may be important for this interaction (15). Dual palindrome half-sites of hr5 mediate binding of a dimeric form of IE-1. Studies on protein interactions with both hr5 and hr1a have failed to detect a specific host factor-hr interaction although the possibility of host factor(s) interacting with hr1a has not been completely negated (14). There are reports of

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**Fig. 9.** The host protein interacts with hr1 with very high affinity. Binding reactions were carried out with a constant amount of protein (2 μg in A and 3 μg in B), using increasing concentrations of the 170-bp probe. C, the ratio of bound to free DNA probe is plotted versus the concentration of bound (in pM). The filled circles and filled triangles represent data from experiments using 2 μg of protein (A) and 3 μg of protein (B), respectively.

**Fig. 10.** The molecular mass of the host factor is ~38 kDa. Southwestern blotting was carried out with the 50 h postinfection nuclear extract using the 170-bp hr1 fragment as probe (C). Panels A and B represent blots using labeled pUC18 and the 28-bp oligonucleotide encompassing a palindrome as probes, respectively. Protein molecular size markers in kDa (M) are indicated on the right.

**Fig. 11.** Dephosphorylation of the factor abolishes binding. Nuclear extract, prepared 50 h postinfection, was treated with 200 units of calf intestine alkaline phosphatase (lane P) or boiled phosphatase (lane BP) and analyzed for binding by Southwestern analysis using radiolabeled 170-bp hr1 DNA as probe. Lane U shows the 38-kDa band obtained with the untreated extract. Protein molecular size markers are indicated (lane M).
enhancer-binding cellular proteins in other viruses. At least four different host cellular factors have been found to bind to distinct cis-elements in the U3 region of the long terminal repeats of the Rous sarcoma virus (32, 33). The nuclear factor EF-C binds as a dimer to functionally important inverted repeat sequences in the polyoma virus and hepatitis B virus enhancer regions (34, 35). We have observed complexes with the uninfected nuclear extract, irrespective of the region of hr1 being used as probe, with the number of complexes being a function of the length of the hr1 probe and the nature of sequences therein. Complex formation does not occur with the 28-bp core palindrome alone, suggesting the requirement of core-flanking sequences for binding. This is confirmed by a series of competitions with overlapping hr1 oligonucleotides. Although short segments of both the 5' and 3' palindromeflanking sequences are essential for the host factor-hr1 interaction, there exists a degeneracy in the sequence requirement, as is evident from the fact that hr1-BP binds even to the oligonucleotide in which the flanking sequences have been shuffled. This may be expected in view of the differences that exist between the sequences flanking the five palindromes of hr1 with which hr1-BP interacts.

We previously showed that the replication and enhancer functions of hr1 can be distinguished in a transient expression assay (1). It is likely that the two functions (ori- and enhancer-like) are carried out by different sequence elements within hr1. Some of the regions may overlap as suggested by our earlier results that a palindrome alone is sufficient for ori function; for enhancer function, however, a palindrome alone is necessary but not sufficient (1). That this host factor, present throughout infection, does not bind to the 28-bp core palindrome alone makes it tempting to speculate that our hr1-binding protein may be important for the enhancer function and not for the ori function of hr1. Indeed, sequestering of this factor by competition in vivo abolishes hr1-mediated enhancement in a transient expression assay. The binding of the host factor to hr1 may contribute to low levels of basal transcription relatively early in the infection cycle. Subsequently, as infection proceeds, the host factor may help to create an environment that would make overall transcription more responsive to the presence of yet unidentified inducible host or viral proteins bound to nearby sites in hr1. The interaction of host factors with both the polyhedrin promoter and hr1 enhancer element represents an interesting molecular interaction during baculovirus pathogenesis.

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