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The Homologous Region Sequence (hr1) of *Autographa californica* Multinucleocapsid Polyhedrosis Virus Can Enhance Transcription from Non-baculoviral Promoters in Mammalian Cells*

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From the ‡Laboratory of Molecular and Cellular Biology, Centre for DNA Fingerprinting and Diagnostics, Hyderabad 500076, India, the ¶National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India, and the ‖Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore 560064, India

The *Autographa californica* multinucleocapsid polyhedrosis virus homologous region sequence hr1 enhances transcription from the viral polyhedrin promoter in *Spodoptera frugiperda* insect cells and independently functions as an origin of replication (*ori*) sequence. The binding of the host nuclear protein, hr1-binding protein (hr1-BP), is crucial for the enhancer activity (Habib, S., Pandey, S., Chatterji, U., Burma, S., Ahmad, R., Jain, A., and Hasnain, S. E. (1996) *DNA Cell Biol.* 15, 737–747 and Habib, S., and Hasnain, S. E. (1996) *J. Biol. Chem.* 271, 28250–28258). We demonstrate that hr1 can also enhance transcription from non-baculoviral promoters like cytomegalovirus and *hsp70* in mammalian cells but does not support *ori* activity in these cells. Unlike insect cells, hr1 can also function in mammalian cells as an enhancer when present in *trans*. hr1 DNA sequence binds with high affinity and specificity to nuclear factors in the mammalian cells. The insect hr1-BP- and the hr1-BP-like proteins from mammalian cells (mhr1-BP) have different properties with respect to ion requirements, DNA groove binding, and molecular size. When mammalian cells are infected with a recombinant baculovirus containing two promoters, the baculovirus polyhedrin and *Drosophila hsp70* gene promoter, the *hsp70* gene promoter alone is active in these cells, and this activity is further enhanced by the presence of an additional hr1 in the recombinant virus. hr1 may thus also have a role in baculovirus-mediated gene delivery in mammalian cells.

Enhancers are genetic elements that up-regulate gene expression by enhancing transcription of genes, even when placed several thousand nucleotides away in a position or orientation-independent fashion. The SV40 enhancer, the first enhancer to be discovered (1, 2) and the most extensively studied, represents the best example of a prototypical enhancer that functions in a wide variety of cell types from different species. It is even active in non-mammalian systems like *Xenopus laevis* kidney cells or even in the green alga *Acetabularia*, and, in some cases, it can confer cell specificity or host-range response to the promoter (3, 4).

The baculovirus *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV)[1] genome consists largely of unique sequences. Interspersed, however, are nine homologous region (hr) sequences called hr1, hr1a, hr2, hr2a, hr3, hr4a, hr4b, hr4c, and hr5 (5, 6). The hrs vary in length from 150 to 800 bp and have a modular structure containing two to eight imperfect, 30-bp palindromes with an EcoRI site at the center of each palindrome. Homologous region 1 (hr1) has been shown to be an enhancer for the immediate early gene *ie-N* and delayed early gene *39k* (7). We earlier reported that hr1 enhances transcription from the polyhedrin promoter in a classic enhancer-like manner (8–10). hr1-mediated enhancement is position- and orientation-independent and can activate transcription to about 10-fold in a plasmid-based transient expression system and to more than 90-fold when inserted as an additional copy within the viral genome (10). Enhancement mediated by hr1 follows the temporal profile of very late gene expression (20–60 h.p.i.) and was a direct function of the number of modules (8). hr1 is bifunctional, *i.e.* it is both a transcriptional enhancer and an *ori* of replication (8), and these functions of hr1 are independent of each other. Although the palindrome alone is sufficient for the *ori* function, the palindrome plus the flanking sequence is essential for the enhancer function. A host cell protein, named hr1-BP (9), which binds very specifically to the palindrome plus the flanking sequence, is required for the enhancer function. We also showed that hr1 can stimulate expression from other homologous promoters like the *Ac*MNPV late promoter *cor* (8) and very late promoter *p10* and from heterologous promoters such as Drosophila *hsp70* in insect cells.[2]

In this study, we describe the ability of the baculovirus hr1 to function as an enhancer, both when placed in *cis* as well as *trans*, but not as an *ori* sequence in heterologous mammalian cells. These cells also contain protein factors that bind to hr1, but these binding proteins are distinct from hr1-BP present in insect cells. This enhancement of gene expression can be exploited for the development of improved vectors for baculovirus-mediated gene delivery in mammalian cells.

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[1] The abbreviations used are: AcMNPV, *A. californica* multinucleocapsid polyhedrosis virus; hr1, homologous region 1; hr1-BP, hr1-binding protein; mhr1-BP, hr1-binding protein from mammalian cells; *polh*, polyhedrin; EMSA, electrophoretic mobility shift assay; h.p.i., hours post infection; CMV, cytomegalovirus.

[2] P. Viswanathan and S. E. Hasnain, unpublished observation.

EXPERIMENTAL PROCEDURES

Cells and Virus—*Spodoptera frugiperda* cells (*Sf*9) were grown in TNMFH medium (Invitrogen) containing 10% fetal bovine serum as described previously (11). All the mammalian cell lines used namely, Vero (African monkey kidney epithelial cell line), COS-1 (African green monkey kidney fibroblast-like, SV40-transformed cell line), HepG2 (human hepatocyte cell line), HeLa (human adenocarcinoma cell line), and PS-1 (porcine kidney cell line) cell lines were cultured in Dulbecco's modified Eagle's medium containing high glucose. The C6 strain of wild type *Ac*MNPV was used in infection experiments. Recombinant viruses vAclacluc and vAclacluc-hr1 were constructed (10, 11) using pBacPAK8 (Clontech, Palo Alto, CA) baculovirus transfer vectors as per standard procedures. The recombinant viruses were purified and titrated, and viral infection was carried out at a multiplicity of infection of 10 for each virus. To ascertain that equal amounts of viral DNA from the different recombinants had entered the insect cells, equal amounts of the reaction mixtures were dot-blotted onto a nylon membrane after the luciferase assay, followed by probing with the *luc* cDNA and densitometric scanning (8).

Plasmid Constructs—Plasmid pBVluc was constructed by cloning a 3.695-kb fragment from pCEP-X2-luc (containing the CMV promoter, luciferase reporter, and SV40 polyadenylation signal) into the SalI site within the multiple cloning site of pUC18 to generate a 6.38-kb plasmid. pBVluc was linearized by partially digesting with SalI and was ligated to a 750-bp hr1 fragment, released as SalI fragment from pSHhr1 (9), to construct various plasmids with different positions, orientation and number of hr1 (Fig. 1*A*). pBVluc-hr1-U_1 (7.13 kb) and pBVluc-hr1-U_2 (7.13 kb) have hr1 cloned upstream to the CMV promoter in the same or opposite orientation, respectively, as present in the viral genome relative to the polyhedrin promoter. pBVluc-hr1-U_1-U_1 (7.88 kb) and pBVluc-hr1-D_1-D_1 have two copies of hr1 in the correct orientation upstream or downstream to the promoter, respectively. pBVluc-hr1-D_1 and pBVluc-hr1-D_2 have a single hr1 placed downstream to the *CMV-luc* construct in the right or wrong orientation, respectively.

Transient Transfection Assays—Transfection of reporter plasmids into *Sf*9 insect cells was carried out using Lipofectin as described previously (8). Mammalian cell transfections were performed using the reagent LipofectAMINE Plus (Invitrogen). Equal numbers of cells were plated onto 6-well plates. Before transfection, the cells were repeatedly washed with serum-free media to remove all traces of sera. 3 μg of plasmid was diluted in 100 μl of serum-free medium, 6 μl of Plus reagent was added to this, and the DNA-Plus mix was incubated for 15 min. This was then added to another tube containing 4 μl of LipofectAMINE diluted in 100 μl of serum-free medium and incubated together for another 15 min, and the DNA LipofectAMINE complex was added to cells and the volume was made up to 1 ml. After 6 h, 1 ml of 20% fetal bovine serum-containing media was added to the cells followed by infection with either wild type or recombinant viruses or transfected with transfer vector plasmid DNAs. Aliquots of infected and/or transfected cells were harvested at different time points, and luciferase or β-galactosidase enzyme expression levels were monitored as described (8, 11). All the experiments, both transfections and viral infections, were repeated in duplicate, at least three times. To completely rule out artifacts caused by unequal amounts of transfected DNA entering the cells, it was ascertained that equal amounts of plasmid DNA from the different constructs had entered the insect cells. For this, equal amounts of the reaction mixture, after the respective reporter enzyme assays, were dot-blotted on a nylon membrane and probed with the *luc* DNA or *lacZ* DNA as described, followed by densitometric scanning of the dot blots.

Electrophoretic Mobility Shift Assay—Nuclear protein extracts were prepared from *Sf*9 cells or the mammalian cells using a modified method (9, 12). Binding reactions with nuclear extracts from both insect and mammalian cells were carried out by incubating 2 μg of nuclear extract with end-labeled fragments of hr1. The 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 fractionated at 150 V in TAE buffer (40 mM Tris-acetate and 1 mM EDTA). In competition experiments, a 20- or 40-fold molar excess of the unlabeled competitor was added to the reaction prior to the addition of the nuclear extract. For studying the effects of various ions on the DNA-protein binding, the salt containing the ion was added to the reaction buffer at the required concentration. To study the inhibition with minor and major groove-binding drugs, the hr1 probe was incubated for 30 min at room temperature with varying concentrations of distamycin A or methyl green and then analyzed for binding by incubation with the nuclear extract.

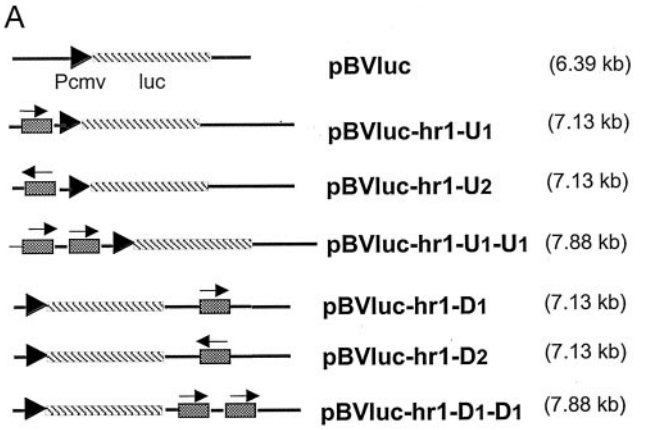
DpnI Sensitivity Assay—Plasmid DNA (2 μg) was transfected into insect and mammalian cells, followed by infection with wild type AcMNPV virus after 24 h or no infection at all as mentioned in the respective figure legends. The total cell DNA was isolated at different time points post transfection or infection as described (8) and resuspended in 25 μl of water. To assay for replication, 5 μl of DNA was digested with HindIII to linearize the plasmid and with both HindIII and DpnI to score for DpnI sensitivity (9). The digested DNA was Southern-transferred and hybridized to ^{32}P-labeled pUC18 probe.

RESULTS

hr1 Enhances Transcription from the CMV Promoter in a Position- and Orientation-independent Manner in Mammalian Cells—hr1 is a classic enhancer of transcription from the AcMNPV promoters in *Sf*9 insect cells. To check if hr1 can enhance CMV promoter-driven expression in mammalian cells, transient transfections were performed. The luciferase expression from Vero cells, transiently transfected with reporter plasmids (Fig. 1*A*) carrying hr1 sequence, was compared with cells transfected with pBVluc lacking the hr1 sequence. The presence of hr1 upstream and in the same orientation relative to the CMV promoter as in the viral genome (pBVluc-hr1-U_1), or upstream but in opposite orientation or downstream in either orientation results in ~3-fold increase in luciferase expression over the control plasmid pBVluc (Fig. 1*B*). Interestingly, constructs carrying two copies of the hr1 sequence in an upstream or downstream position did not generate any additional effect. Transient transfection experiments with the same constructs were performed in other mammalian cells. About similar enhancement of hr-1-mediated luciferase expression was observed in HepG2 cells and PS-1 cells irrespective of the position and orientation of hr1 relative to the CMV promoter (results not shown). When the same CMV promoter-containing plasmids were transfected into *Sf*9 cells, a 2.6-fold enhancement is observed (data not shown). These results suggest that hr1 can function as an enhancer of CMV promoter in both mammalian cells and *Sf*9 cells in a classic enhancer-like fashion.

hr1 Can Enhance Expression from Other Promoters and Reporters in Mammalian Cells—To check if hr1-mediated transcriptional enhancement works with other promoters and reporters, the plasmid pBVlacluc-hr1 was used. This plasmid contains the *hsp70* promoter-driven β-galactosidase, and the baculovirus polyhedrin promoter-driven luciferase along with a copy of the hr1 sequence element. The expression of the *lacZ* reporter from cells transfected with this plasmid was compared with a plasmid pUlacluc that did not carry the hr1 sequence. In Vero cells, about 3-fold increase in β-galactosidase expression in the hr1-containing plasmid over the control plasmid is observed (Fig. 2). The enhancement effected by hr1 is more or less similar irrespective of the constructs used (data not shown). COS-1 cells and HepG2 cells also show hr1-mediated increase in transcription, although to a slightly reduced level compared with Vero cells (data not shown). hr1 can, therefore, enhance expression of β-galactosidase from *hsp70* promoter in different heterologous host cells.

hr1 Can Also Function When Present in trans in Mammalian Cells—Enhancers are basically *cis*-acting DNA elements. In the recent years, there have been various reports of enhancers capable of working also in *trans* (13, 14). Experiments were accordingly designed to check if hr1, unlike in insect cells, can also function in *trans* in mammalian cells. pBVluc (a vector lacking hr1 sequence but with the CMV promoter driving luciferase reporter gene transcription) was co-transfected into Vero cells with different amounts of pSHhr1 (a plasmid containing only the hr1 element cloned in pUC18). pUC18 in appropriate concentration was added to the transfection mix to



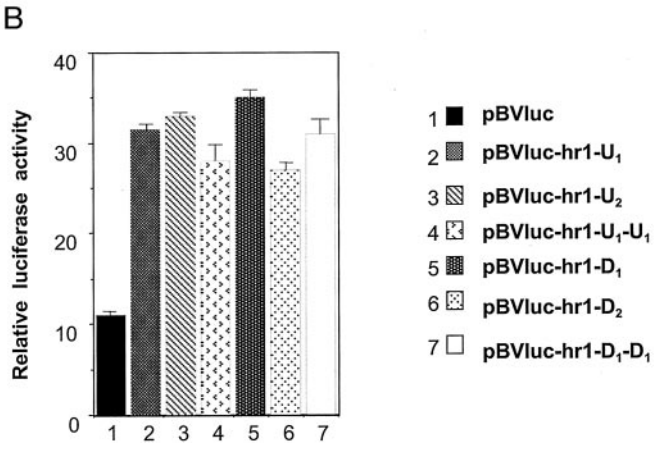


FIG. 1. *A*, a schematic representation of the plasmids used for transfections. pBVluc carries the CMV promoter driving the luciferase reporter. pBVluc-hr1-U_1 has hr1 cloned upstream of the CMV promoter in the same orientation as that in the virus. In pBVluc-hr1-U_2, hr1 is upstream but in the opposite orientation. pBVluc-hr1-U_1-U_1 has two hr sequences upstream, pBVluc-hr1-D_1 has hr1 downstream in the correct orientation, and pBVluc-hr1-D_2 has hr1 downstream but in the opposite orientation. pBVluc-hr1-D_1-D_1 has two hr sequences downstream to the promoter-reporter cassette. The *numbers* in the *parentheses* denote the sizes (in kb) of the respective plasmids. *B*, expression from the CMV promoter in mammalian cells is enhanced by hr1 in transient expression assays. Vero cells were transfected with control plasmid pBVluc (without hr1) and plasmids carrying hr1 placed in different positions and orientation as shown in Fig. 1*A*. The *first bar* represents the luciferase activity of the cells transfected with pBVluc, assayed 36 h post transfection. The *second*, *third*, *fourth*, *fifth*, *sixth*, and *seventh bars* show the luciferase activity after transfection with plasmid pBVluc-hr1-U_1, pBVluc-hr1-U_2, pBVluc-hr1-U_1-U_1, pBVluc-hr1-D_1, pBVluc-hr1-D_2, and pBVluc-hr1-D_1-D_1, respectively.

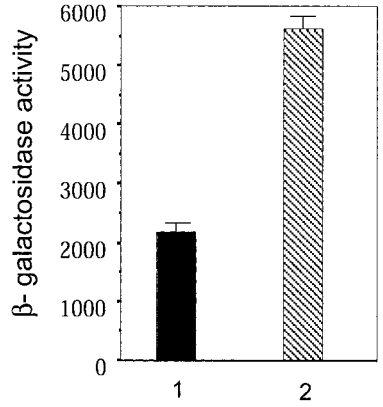
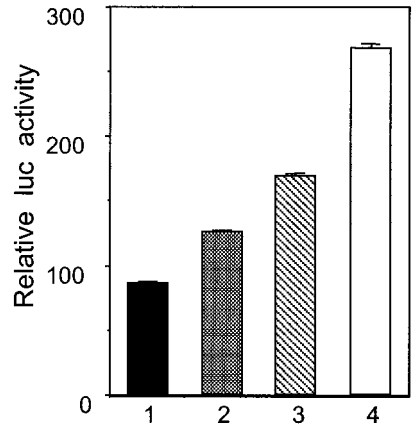


FIG. 2. **hr1 can enhance transcription from *hsp70* promoter in mammalian cells.** Vero cells were transfected with a control plasmid pUClacluc, containing *hsp70* promoter and β-galactosidase reporter (*filled bar*) and pBVlacluc-hr1, which has hr1 cloned downstream of the promoter-reporter (*shaded bar*). The levels of β-galactosidase activity in Vero cells assayed 36 h post transfection are shown.



pBVluc	2	2	2	2
pSHhr1	-	4	8	16

FIG. 3. **hr1 can as well function in *trans* in mammalian cells.** The luciferase activity in Vero cells were assayed 36 h after transfection with 2 μg of pBVluc alone (*1*) or along with 4 μg (*2*) or 8 μg (*3*) or 16 μg of pSHhr1 (*4*).

ensure identical amount of total transfected DNA. When Vero cells were transfected with the same amount of pBVluc and increasing amounts (4, 8, and 16 μg) of pSHhr1, an increase in luciferase expression is observed as a direct function of pSHhr1 concentration (Fig. 3). These results demonstrate that hr1 sequence can act as an enhancer when present in *trans*. Furthermore, this enhancement is a direct function of the concentration of the enhancer-carrying plasmid, pSHhr1, in transiently transfected cells.

hr1-BP-like Factors in the Mammalian Nuclear Extract Interact Specifically with hr1—The complete hr1 is a 750-bp fragment containing five imperfect palindromes (shown as *double-outward arrows* in Fig. 4*A*) with EcoRI site at the center of each palindrome. A complete enhancer module carries an EcoRI palindrome and flanking sequences. Using suitable restriction enzyme sites hr1 was digested into smaller fragments to generate constructs with varying number of palindromes and flanking sequences (Fig. 4*A*). The 170-bp single-enhancer module was used in an electrophoretic mobility shift assay (EMSA) to detect for the presence of hr1-binding proteins in mammalian cells. A distinct retardation in the migration of the labeled 170-bp fragment can be seen when the fragment is incubated with mammalian Vero cell nuclear extract (Fig. 4*B*). The binding of one or more of the Vero cell nuclear factors to hr1 is specific, and this is evident from the observation that the complex can be competed with cold 170-bp hr1 but not with heterologous competitor like pUC18, even when present in high molar excesses. 20- and 40-fold molar excess of cold 170-bp hr1 competitor abolishes the binding (*lanes 3* and *4*), whereas similar molar excesses of pUC18 (a heterologous competitor) has no effect (*lane 5* and *6*), demonstrating the specificity of the binding. 90-, 330-, and 420-bp fragments could also bind with high specificity to the mammalian hr1-binding protein (mhr1-BP) present within the mammalian cell nuclear extract (data not shown). These results demonstrate that factors present in mammalian cells interact with high specificity with the hr1 element.

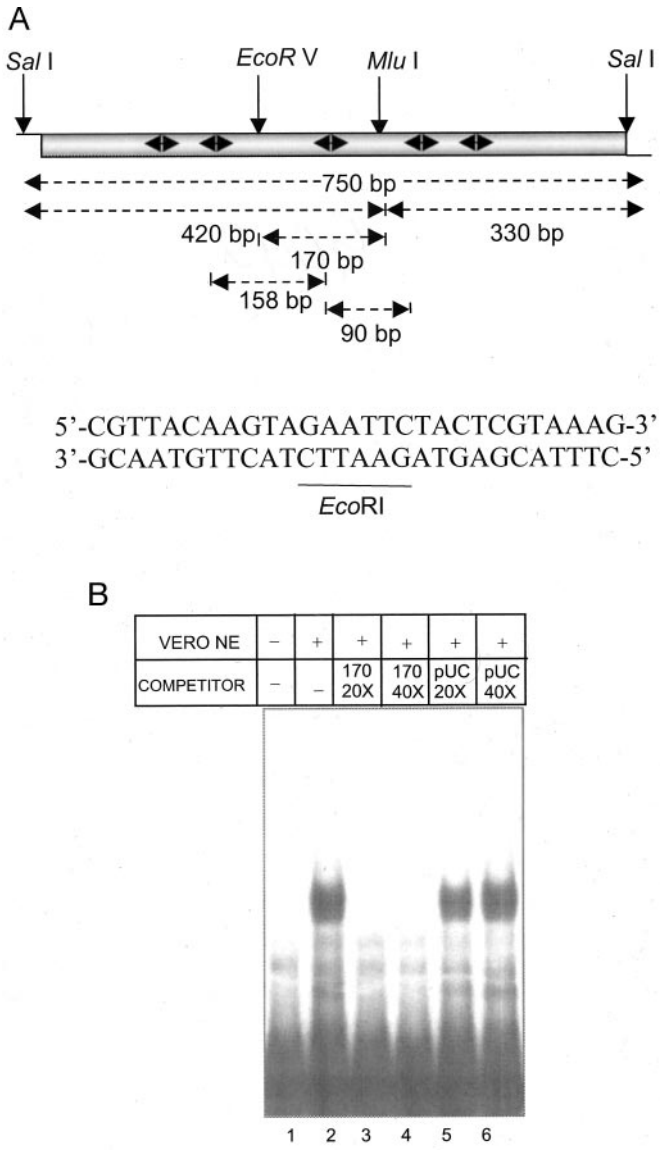


FIG. 4. *A*, a schematic map of the 750-bp hr1. The five 28-bp core palindromes are shown as *filled double arrows*. There is an EcoRI site at the center of each palindrome the sequence of which is shown. Using different restriction enzymes, hr1 can be fragmented to generate different combinations of palindrome and inter-palindrome sequences. *B*, hr1 binds with high affinity to a nuclear factor in mammalian cells. Nuclear extract (2 μg) from Vero cells was incubated with 1 ng of the 170-bp radiolabeled hr1 probe in binding reactions for electrophoretic mobility shift assays. The mobility of free probe is shown in *lane 1*. A distinct retardation in the mobility of the probe is seen when it is incubated with Vero nuclear extract (*lane 2*). 20- and 40-fold molar excess of cold 170-bp hr1 competitor abolishes the binding (*lane 3* and *4*). Similar molar excesses of pUC18 (a heterologous competitor) did not affect the retardation, showing the specificity of the binding (*lane 5* and *6*).

Effect of DNA Binding Drugs on the Enhancer-Nuclear Factor Interaction—Actinomycin D is a drug that binds to the minor groove of the DNA, whereas methyl green binds to the major groove. The 170-bp hr1 probe was incubated alone (Fig. 5, *lane 1*), or with 0.5 mM actinomycin D (*lane 2*), or 0.5 mM methyl green (*lane 8*), or with the addition of Vero cell nuclear extract along with different concentrations of actinomycin D (*lanes 4–7*) or methyl green (*lanes 10–13*). Complex formation was analyzed by gel retardation assay. It could be seen that increasing concentrations of actinomycin D or methyl green causes a reduction of the DNA-protein complex (Fig. 5, shown by an *arrow*), although this effect was more evident at higher concentrations of the drugs. As both the major and minor groove-binding drugs at higher concentrations inhibit the hr1-host factor interaction, it appears likely that the interaction of mhr1-BP with the hr1 DNA overlaps both the grooves.

Divalent Cations Destabilize the Binding of hr1 to hr1-BP, Whereas Monovalent Cations Stabilize the Binding—In an EMSA where the Vero cell nuclear extract and the 170-bp hr1 probe is incubated with increasing concentration (5–125 mM) of $MgCl_2$, a gradual decrease in the binding is apparent (Fig. 6 shown by an *arrow*). At concentrations higher than 50 mM a total loss of binding is seen (Fig. 6). Incubation with monovalent cation KCl generates an opposite pattern. At lower than 125 mM concentration of KCl, binding is very weak, however, at a concentration of 1000 mM, a very tight binding is observed (data not shown). These results demonstrate that, although divalent cations destabilize the hr1-mhr1-BP interactions, monovalent cations stabilize the same.

The One or More Nuclear Factors That Bind to hr1 in Sf9 and Mammalian Cells Are Not the Same—The AcMNPV hr1 binds to the *Sf*9 insect cell host protein, hr1-BP, and this binding is crucial for the enhancer function. Having shown that mammalian cells, even though they are not natural hosts to *Ac*MNPV infection, do contain one or more factors that binds to hr1 we investigated whether these factor(s) is(are) similar to those present in *Sf*9 insect cells. In an EMSA, the 170-bp probe was incubated with 2 μg of *Sf*9 nuclear extract and 2 μg of Vero nuclear extract. The DNA-protein complex in the two cases has different mobilities (Fig. 7, compare *lane 1* with *lane 2*). When 2 μg of the Vero and *Sf*9 extracts are incubated together, independent complexes corresponding to complexes generated by the individual extracts are seen (Fig. 7, *lane 3*). Moreover, when binding reactions were carried out under limiting concentrations of probe and 2 μg each of *Sf*9 and Vero extracts, the results were similar. This implies that the hr1-binding proteins in the two nuclear extracts are of different molecular sizes and perhaps involve different protein-protein interactions, whereas complexing with hr1 and/or bind to different regions within the hr1, hence they do not compete out each other. These results suggest that, although the mammalian cells indeed contain one or more factors that bind to hr1, these factors are different from the *Sf*9 hr1-BP in terms of their molecular sizes and possible cognate sequence motifs and/or protein partners involved in such an interaction.

hr1 Cannot Function as a Replication Origin in Mammalian Cells—In *Sf*9 cells, hr1 also functions as an origin of replication. The *ori* and enhancer activities are independent of each other and are detected using different transfection regimes (8). The *ori* activity is scored by a standard method, which is based on the sensitivity of the isolated plasmid to DpnI restriction digestion, which recognizes the target site to be methylated with Dam methylase (15). *Escherichia coli*, which is Dam^+ will methylate adenine residue within the GATC recognition site for DpnI, and consequently all its DNA will be digested by the enzyme. However, plasmid DNA that is replicated in mammalian cells (Dam^-) will not be methylated at GATC sequence and will therefore be resistant to DpnI digestion. Thus, DpnI can differentiate input plasmid DNA from that which has indeed replicated in the eukaryotic cell. DNA was isolated from mammalian cells transfected with pUC18 and pSHhr1, followed by AcMNPV viral infection after a 24-h gap to ensure the presence of viral factors required for *ori* function. A control where cells were transfected with pSHhr1 but not infected with virus was also included to check if the hr1 *ori* activity can occur even in the absence of viral infection. Total DNA was isolated after different time points (6, 12, 24, and 36 h) post-infection or post-transfection (in case of uninfected cells) and assayed for DpnI digestibility. In the control *Sf*9 cells, a high molecular

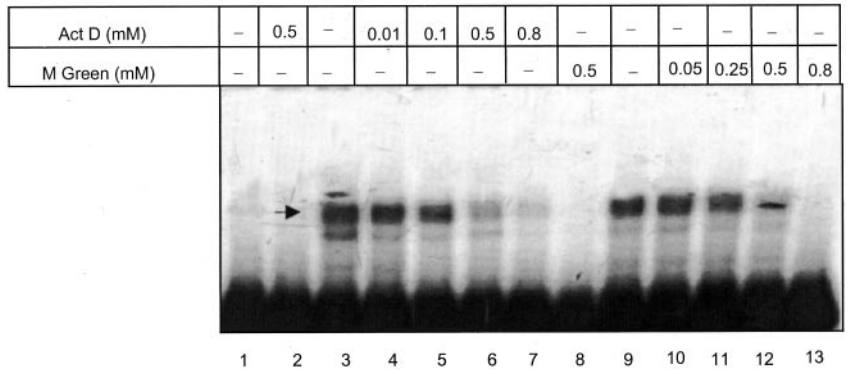


FIG. 5. **Both the major and minor groove binding drugs have an effect on the interaction of hr1 to mhr1-BP.** The 170-bp hr1 probe was incubated alone (*lane 1*), or with 0.5 mM actinomycin D (*lane 2*), or with 0.5 mM methyl green (*lane 8*) or with just the nuclear extract (*lanes 3* and *9*). The probe was incubated with different concentrations of actinomycin D as shown (*lanes 4–7*) or methyl green (*lanes 10–13*), and Vero cell nuclear extract was added subsequently. Complex formation (shown by an *arrow*) was analyzed by gel retardation assay.

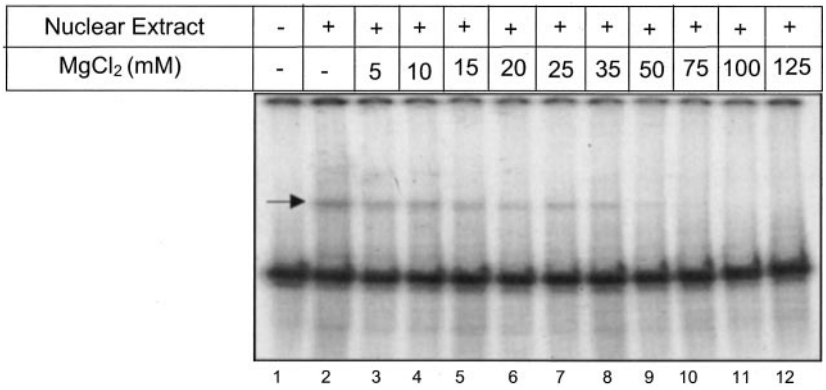


FIG. 6. **Divalent cations destabilize the binding of hr1 to mhr1-BP.** Binding reactions were carried out with Vero nuclear extract and the 170-bp probe at $MgCl_2$ concentrations ranging from 5 to 125 mM (*lanes 3–12*). *Lane 1*, free probe. *Lane 2*, probe with nuclear extract with no additional $MgCl_2$. With increasing amounts of $MgCl_2$ there is a decrease in binding (shown by an *arrow*).

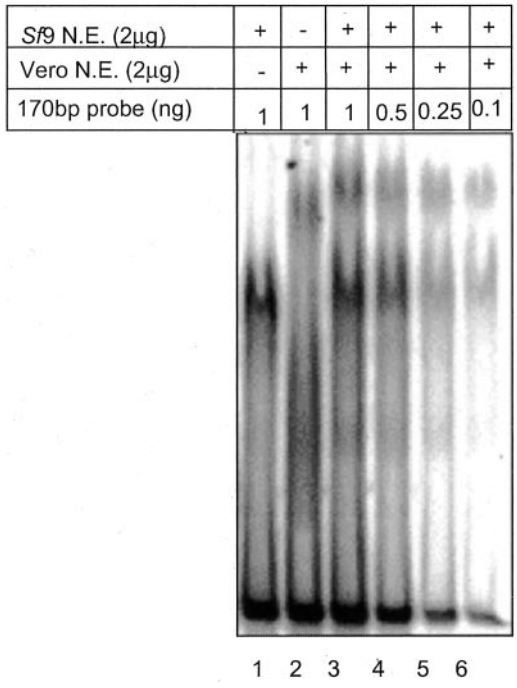


FIG. 7. **The *Sf*9 hr1-BP and mhr1-BP are distinct from each other.** 1 ng of 170-bp hr1 probe was incubated with 2 μg each of *Sf*9 (*lane 1*), Vero (*lane 2*), or both Vero and *Sf*9 extracts (*lane 3*). In competition experiments, 2 μg each of *Sf*9 and Vero extracts were incubated with either 0.5 ng (*lane 4*) or 0.25 ng (*lane 5*) or 0.1 ng (*lane 6*) of 170-bp hr1 probe.

weight DpnI-resistant form is seen in cells transfected with pSHhr1 followed by viral infection (Fig. 8*A*, *lanes 6*, *12*, *18*, and *24*), whereas cells transfected with pSHhr1 but not followed by viral infection do not show any resistant form but were easily digested with DpnI (Fig. 8*A*, *lanes 4*, *10*, *16*, and *22*) pointing to their unreplicated status. Cells transfected with pUC18 followed by viral infection are also sensitive to DpnI (Fig. 8*A*, *lanes 2*, *8*, *14*, and *20*) reflecting their unreplicated status. In contrast, a DpnI-resistant band is not detected at any point in mammalian HepG2 cells, even in cells transfected with pSHhr1 followed by viral infection (Fig. 8*B*, *lanes 6*, *12*, *18*, and *24*). Thus, although in the control *Sf*9 insect cells, the hr1-containing plasmid can support replication in the presence of viral infection hr1 fails to function as an origin of replication in mammalian cells.

A Recombinant Virus Containing Two Promoters Can Infect Mammalian Cells, However, Only the hsp70 Promoter Is Functional Not the Polyhedrin Promoter—Baculoviruses do not replicate in mammalian cells but can enter the cells through a passive endosomal pathway. A recombinant virus containing two promoters and two reporters, luciferase under the control of the baculoviral *polh* promoter and β-galactosidase under the *hsp*70 promoter, was used to infect different mammalian cells and control *Sf*9 cells. After different time points post infection luciferase and β-galactosidase assays were performed. In the control *Sf*9 cells (Fig. 9*A*), luciferase activity follows the typical time profile of *polh* promoter. Luciferase is detectable at 12 h.p.i., increases steadily from 24 to 36 h.p.i., and is very high at 48 h.p.i. In mammalian cells, there is no luciferase activity (data not shown), which is expected given the fact that *polh* promoter requires baculovirus encoded factors. The β-galactosidase activity follows a completely different profile. In *Sf*9 cells this activity, detectable as early as 6 h.p.i., increases only marginally from 12 h.p.i. up to 48 h.p.i. (Fig. 9*B*, *filled bars*). The β-galactosidase activity in mammalian cells is very much lower than in insect cells. In COS-1 cells (Fig. 9*B*, *white bars*) and HepG2 cells (Fig. 9*B*, *shaded bars*), the β-galactosidase activity is detectable at 6 h.p.i., increasing only marginally from 6 to 48 h.p.i. It is interesting to highlight that the observed overwhelming quantitative differences between *Sf*9 insect cells *vis-à-vis* mammalian cells is a combined reflection of the replication of baculovirus in the insect cells as well as increased efficiency of the *hsp*70 promoter. The Drosophila

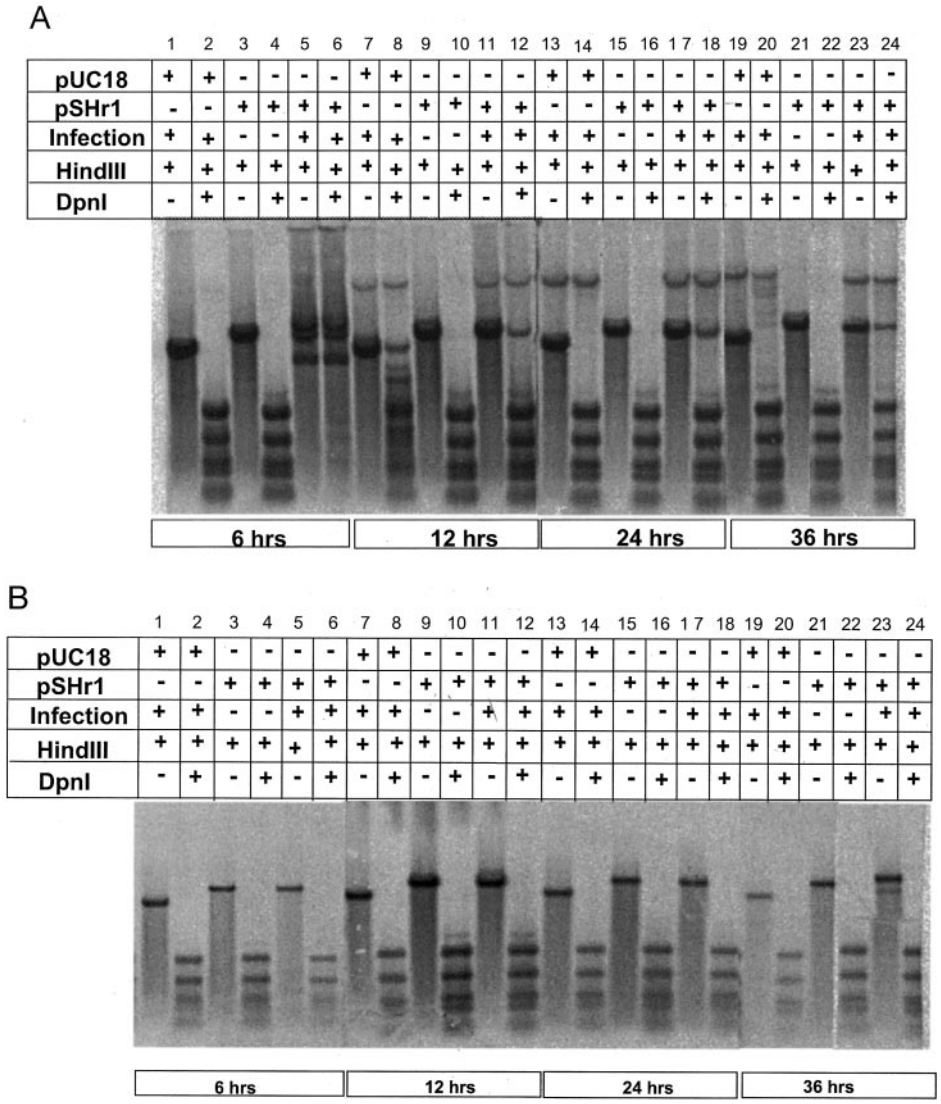


FIG. 8. *A*, hr1 does not function as an *ori* of replication in mammalian cells. The control *Sf*9 cells were transfected with pUC18 followed by infection with AcMNPV (*lanes 1*, *2*, *7*, *8*, *13*, *14*, *19*, and *20*), and total DNA was isolated at different time points (6, 12, 24, and 36 h) post infection. In another experiment, cells were transfected with an hr1-containing plasmid (*pSHhr1*) without any viral infection (*lanes 3*, *4*, *9*, *10*, *15*, *16*, *21*, and *22*) or followed by infection (*lanes 5*, *6*, *11*, *12*, *17*, *18*, *23*, and *24*). The total DNA isolated was further digested with HindIII alone to linearize the plasmid (all *odd lanes*) or digested with both HindIII and DpnI (all *even lanes*) and analyzed for DpnI sensitivity. Lanes where cells were transfected with pSHhr1 followed by infection with virus show the appearance of a DpnI-insensitive band indicating that hr1 works as an *ori* of replication in these cells. *B*, DpnI sensitivity assay to score for hr1 *ori* activity in HepG2 mammalian cells. HepG2 cells were transfected with pUC18 (*lanes 1*, *2*, *7*, *8*, *13*, *14*, *19*, and *20*) followed by infection with *Ac*MNPV. The total DNA was isolated at different time points (6, 12, 24, and 36 h) post infection. In parallel experiment, HepG2 cells were transfected with a hr1 containing plasmid (*pSHhr1*) without any viral infection (*lanes 3*, *4*, *9*, *10*, *15*, *16*, *21*, and *22*) or followed by infection (*lanes 5*, *6*, *11*, *12*, *17*, *18*, *23*, and *24*). The total DNA isolated was further digested with HindIII alone to linearize the plasmid (all *odd lanes*) or digested with both HindIII and DpnI (all *even lanes*) and analyzed for DpnI sensitivity. Absence of any DpnI-insensitive band implies that hr1 cannot function as an *ori* in mammalian cells.

*hsp*70 promoter is expectedly more active in a homologous insect cell environment than in a mammalian cell.

Introduction of an Additional hr1 in the Recombinant Virus Increases the Expression from the hsp70 Promoter in Mammalian Cells—Having shown that the hr1 sequence element can enhance transcription from *hsp*70 promoter in transient transfections in mammalian cells, we checked for hr1-mediated enhancement when it is present as an additional copy in the virus. A recombinant virus (vAclacluc-hr1) was constructed by introducing an additional copy of the hr1 enhancer stably integrated into the vAclacluc (10, 11). More than 2-fold enhancement of expression of β-galactosidase in mammalian cells from *hsp70* promoter was seen when vAclacluc-hr1 was used as compared with vAclacluc (Fig. 10). It is important to note that, unlike *Sf*9 insect cells, mammalian cells do not support baculovirus replication and even then hr1 could enhance expression of a reporter gene driven by a relatively inefficient heterologous insect promoter.

DISCUSSION

The baculovirus AcMNPV hr1, the enhancer under study, is bifunctional, in that it can function both as an enhancer of transcription and an origin of replication. *Spodoptera frugiperda* insect cells (*Sf*9) are the natural hosts for *Ac*MNPV, which follows a temporal profile of gene expression and has been used to express heterologous genes. hr1 can act to enhance the expression of genes during all the different phases of infection; the immediate early gene *ie-N* (or *ie-2*), the delayed early *39k* gene (7, 15, 16), the late gene *cor* and very late *polh* (8). A number of reasons such as the dual role of hr1, its ability to enhance transcription from a wide range of promoters within the host system by recruiting a host factor hr1-BP (9), the

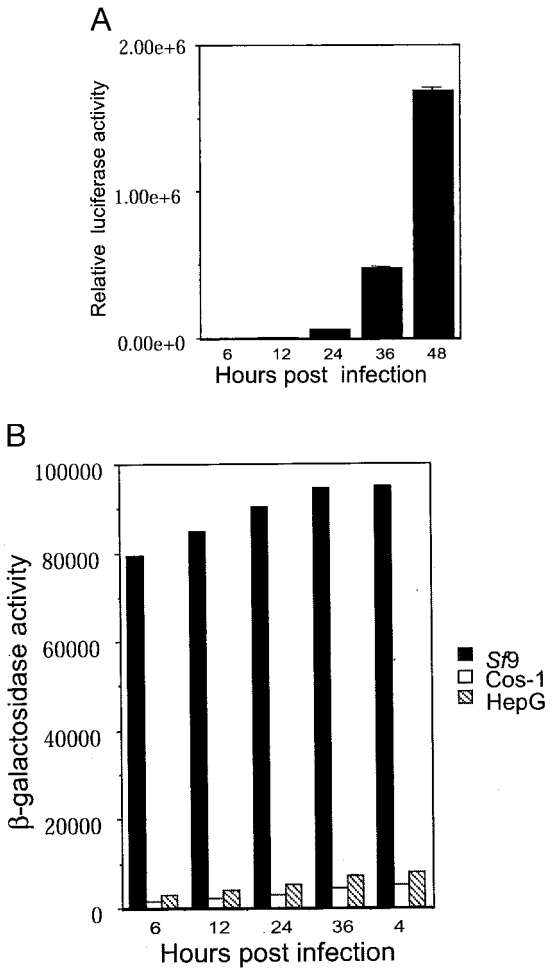


FIG. 9. **Differential reporter gene expression in *Sf*9 and mammalian cells.** Mammalian cells and control *Sf*9 cells were infected with the virus vAclacluc at 10 multiplicity of infection. *A*, luciferase activity of *Sf*9 cells was assayed at different time points post infection 6 h (*bar 1*), 12 h (*bar 2*), 24 h (*bar 3*), 36 h (*bar 4*), and 48 h (*bar 5*). *B*, the β-galactosidase of activity of mammalian cells (COS-1 cells, *white bars*; HepG2 cells, *shaded bars*) and control *Sf*9 cells (*filled bars*) at different h.p.i. is shown.

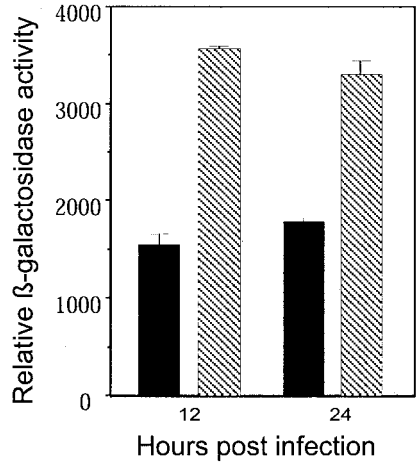


FIG. 10. **Presence of an additional copy of hr1 in the virus enhances the expression of hsp70 promoter-driven β-galactosidase expression in mammalian cells.** Mammalian cells were infected with two recombinant viruses, vAclacluc (*filled bars*) and vAclacluc-hr1 (*shaded bars*) and assayed for β-galactosidase activity at different time points post infection. The virus containing additional hr1 expresses to higher levels than the virus with only one copy of hr1.

utility of baculovirus-mediated gene transfer into mammalian cells, etc., tempted us to analyze the role of hr1 in mammalian cells and the identification of host factors that could interact with hr1. A search for sequence motifs that interact with known enhancer binding proteins and transcription factors in other viral and animal systems revealed that indeed quite a few of these motifs were present in hr1. The most interesting were the motifs with high homology to the consensus enhancer element motif recognized by the C/EBP family of transcription factors in the long terminal repeat enhancer of the avian leukemia virus (17). This motif was repeated (with 1- or 2-bp changes) 3′ to each palindrome in hr1. This motif is incidentally within the region in the flanking sequences, which exhibit high levels of homology.

We have demonstrated that hr1 behaves as a classic enhancer of transcription from the CMV promoter in mammalian cells such as kidney cells (Vero and COS-1), liver cells (HepG2), HeLa and porcine cells (PS-1). Enhancement was also seen when the Drosophila *hsp70* promoter is used. Furthermore, hr1-mediated action was independent of the reporter gene used. The maximum enhancement was observed in Vero cells, where a consistent 3-fold enhancement was observed in hr1-containing plasmids over control non-hr1 plasmids. In *Sf*9 cells, the binding of host factor hr1-BP to hr1 is critical for its enhancer function. Identification of specific nuclear factors present in mammalian cells (mhr1-BP) that bind with high affinity and specificity to different regions of hr1 was consistent with the ability of hr1 to function in non-insect host environment. mhr1-BP and hr1-BP are distinct from each other in terms of their molecular sizes, their ion requirements for binding, and the manner in which they approach the DNA.

Enhancers are usually *cis*-acting DNA sequences that upregulate gene expression, the exact mechanism of which is not clear though several models have been put forward. We have demonstrated for the first time that hr1 is capable of enhancing transcription even in *trans*, *i.e.* when it is present on another plasmid during co-transfections. There are reports that an enhancer can have effect on another DNA molecule when present in *trans* (13, 14). The long range action of enhancers include their ability to stimulate transcription across homologues. In *Drosophila*, these effects are called transvection. In certain combinations the cuticle pigmentation gene, called *yellow*, display intra-allelic complementation, as a result of the action of tissue-specific *yellow* enhancers present on one chromosome stimulating transcription from the *yellow* promoter located on the paired homologue (18). Although hr1 can exert its influence when present both in "*cis*" and "*trans*" in mammalian cells, it can act only in "*cis*" in insect cells. This could be due to the difference in the properties of the enhancer binding proteins in the two cases (*Sf*9 hr1-BP and mhr1-BP). mhr1-BP by virtue of its protein-protein interactions may be able to bring about enhancement of transcription from promoters present on another plasmid molecule.

hr1-BP has little or no role in the *ori* function of hr1 (8, 9), therefore the presence of hr1-BP like factors in mammalian cells should not be of any consequence as far as the *ori* function is concerned. This was indeed the case as was evident from the inability of the hr1 to support replication in mammalian cells. This also explains why baculoviruses can though enter and transiently express foreign genes in mammalian cells, they cannot replicate in these cells. One could speculate that replication is a more host-specific phenomenon than transcription involving hr and non-hr origins of replication (19). Several recent reports have demonstrated that primary human hepatocytes, cell lines of hepatic origin, and other tumor cell lines were able to efficiently take up and transiently express foreign genes under the control of constitutive mammalian promoters

using baculoviruses as delivery vehicles (20–24). High level reporter gene expression from heterologous promoters (8, 21) was observed in human and rabbit hepatocytes *in vitro*, whereas mouse hepatocytes and some other epithelial cell types are targeted at lower rates. The efficiency of baculovirus-mediated entry of foreign genes via an endosomal pathway considerably exceeds that by lipid transfection (20). Another report shows that baculovirus promoters in mammalian cells can be activated by adenovirus functions (25). Baculoviruses, therefore, offer an interesting alternative for a safe, self-limiting gene delivery system for gene therapy (24, 26). These viruses provide an added advantage by their ability to package inserts greater than 10 kb. The dual promoter reporter recombinant virus used here provides an ideal tool to study baculovirus-mediated gene delivery into mammalian cells. The baculovirus polyhedrin gene promoter requires insect cell host factors (27–33) for its activation and therefore cannot function in a non-insect environment. The transcription ability of the constitutive *hsp70* promoter, however, is relatively uniform in different cell lines, except that HepG2 cells show higher levels of reporter activity, consistent with earlier reports that these cells are able to take up baculoviruses more efficiently than other cell lines. The presence of an additional copy of hr1 increases the β-galactosidase expression thereby providing a system for improving the expression of genes delivered via baculovirus in mammalian cells. hr1 therefore can play a major role in enhancing expression of foreign gene in these cells.

Baculoviruses have long been used as biopesticides, owing to their small host range. It is possible now to manipulate the virus so as to increase its host range. Although the ability of the hr1 enhancer (10, 34) to activate transcription and support DNA replication in insect cells can be further exploited (35), the fact that hr1 can positively regulate non-baculoviral promoters both in transient transfections and viral infections without affecting replication makes it an ideal candidate for value addition in baculovirus-mediated gene delivery in mammalian cells (22, 36).

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