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An Additional Copy of the Homologous Region (hr1) Sequence in the *Autographa californica* Multinucleocapsid Polyhedrosis Virus Genome Promotes Hyperexpression of Foreign Genes†

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ABSTRACT: The *Autographa californica* multinucleocapsid nuclear polyhedrosis virus genome contains nine homologous region (hr1, hr1a, hr2, hr2a, hr3, hr4a, hr4b, hr4c, and hr5) sequences that are thought to be involved in viral replication and activation of transcription. Our results show that the 750 bp hr1 sequence is capable of functioning as an enhancer of transcription of foreign genes from the homologous late polyhderin gene promoter and the heterologous *Drosophila* heat shock protein (*hsp70*) promoter in insect cells. Introduction of an additional copy of the complete hr1 element downstream to the polyhedrin locus in the viral genome, while not affecting the stability of the recombinant virus for at least 30 serial passages, led to hyperexpression of reporter genes. The enhancement in the expression levels of foreign genes varied from 40 to 90-fold depending on the promoter used.

The baculovirus expression vector system (BEVS) utilizing *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (*Ac*MNPV)[1] continues to be widely used for the production of recombinant proteins in insect cells (*1*−*6*). This system employs the viral very late and powerful polyhedrin (*polh*) and p10 gene promoters that allow abundant expression of foreign genes in a temporally regulated manner. The circular supercoiled double-stranded DNA genome of 134 Kb encodes around 150 polypeptides (*7*, *8*). *Ac*MNPV expression undergoes tight regulation at the transcriptional level and occurs in an ordered fashion through early and very late phases (*9*, *10*). The viral genome consists of nine homologous region (hr1, hr1a, hr2, hr2a, hr3, hr4a, hr4b, hr4c, and hr5) sequences of 120−800 bp in length (*11*). Because of their high A + T content, symmetric location throughout the viral genome, presence of imperfect palindromes, and the ability to serve as origin of replication (*ori*) in transient replication assays, the hrs are thought to be involved in viral replication (*12*). Whether any of these are indeed essential for and function as viral replication origins in vivo is still not known. The hrs have also been shown to act as transcriptional enhancers of reporter genes driven by the baculovirus early and delayed-early promoters (*13*−*19*). We previously demonstrated that hr1, located ∼3.7 kb upstream of the *polh* gene in the wild-type *Ac*MNPV genome, enhances transcription from this very late viral promoter in a position and orientation independent manner in plasmid based transient expression assays (*20*, *21*). Enhancement of expression was demonstrated to be a direct result of enhanced transcription from the *polh* promoter and was independent of the *ori* function of hr1. We also showed that a 38 kDa host protein, hr1-binding protein (hr1-BP), interacts with high specificity and affinity with functionality relevant motifs at multiple sites within hr1 (*21*). We have now analyzed the effect of hr1 on the expression of reporter genes driven by the *Drosophila* heat shock protein (*hsp70*) promoter and *polh* promoter using recombinant viruses carrying the hr1 element cloned downstream in the polyhedrin locus in the baculovirus genome. A dramatic increase in foreign gene expression, driven by homologous and heterologous promoters is evident as a consequence of the presence of the extra copy of the hr1 element without affecting virus stability. This approach can be used for producing recombinant protein products of therapeutic interest (*22*) at much higher levels in the baculovirus insect cell system.

EXPERIMENTAL PROCEDURES

Virus and Cell Culture. *Spodoptera frugiperda* cells (*Sf*9 and *Sf*21) were maintained at 27 °C in TNMFH medium (Life Technologies Inc.) supplemented with 10% fetal calf serum (*23*). Cells were infected with virus at 0.1 plaque-forming units per cell (PFU/cell) for serial passage. For other

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[1] Abbreviations: *Ac*MNPV, *Autographa californica* multinucleocapsid polyhedrosis virus; hr1, homologous region 1; *polh*, polyhedrin; *hsp70*, *Drosophila* heat shock protein 70; hpi, hours post infection.

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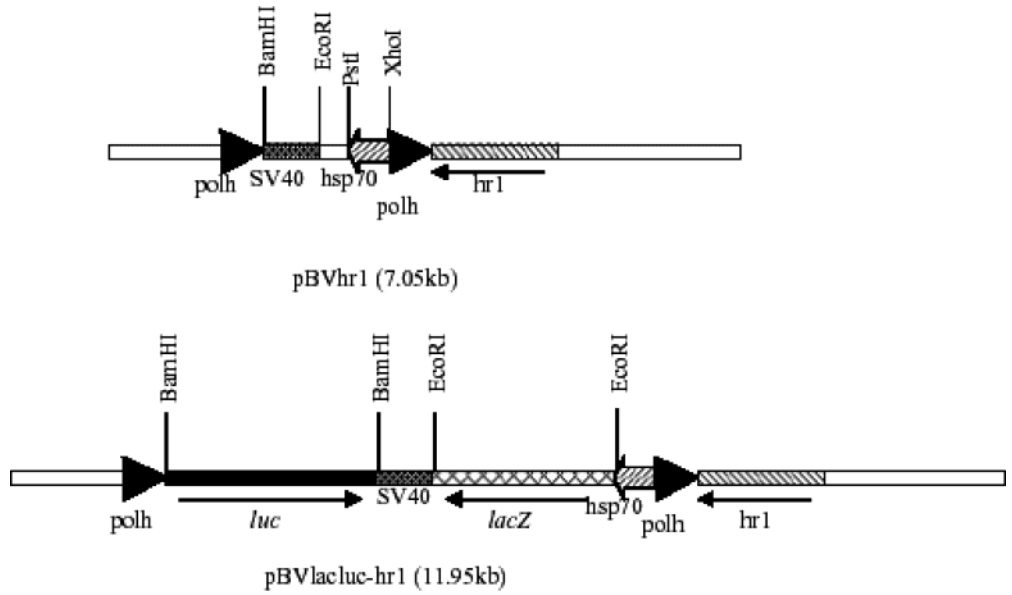

FIGURE 1: Recombinant transfer vector carrying the hr1 element. This plasmid carries *luc* and *lacZ* under the transcriptional control of *Ac*MNPV *polh* and the *Drosophila hsp70* promoters, respectively. The downstream *polh* does not carry any reporter gene. A SV40 terminator between the upstream *polh* and *hsp70* ensures termination of transcript from both the promoters. In this construct, the orientation of hr1 with respect to the *hsp70* promoters is the same as the normal orientation of hr1 with respect to the *polh* promoter in the wild-type viral genome.

experiments, cells were infected with virus at a multiplicity of infection of 10 PFU/cell. The viral inoculum was removed after 1 h infection followed by repeated washing with complete medium to remove any traces of residual virus. Mock-infected cells received identical treatment except that no virus was added to the cells.

Construction of Recombinant Plasmid. Plasmids propagated in *Escherichia coli* DH5α cells were purified in Qiagen columns as instructed by the manufacturer (Qiagen Inc.) Construction of recombinant plasmids carrying heterologous promoters and the baculovirus hr1 sequence was performed as described (*24*). The hr1 sequence, excised as 0.752 Kb SalI fragment from pSHhr1 (*20*), was cloned into the unique BglII site of pUlacluc (*24*) to construct the recombinant transfer vector pBVlacluc-hr1 (Figure 1).

Transient Expression Assays. Transfection of reporter plasmids into *Sf*9 cells was carried out using Lipofectin (Gibco BRL). Equal amount of reference and recombinant plasmid DNAs were used for transfecting 2×10^6 *Sf*9 cells seeded in 35 mm tissue culture dishes. A total of 500 μL of transfection mixture containing plasmid DNA (20 μg) and lipofectin (15 μg) prepared in serum-free TNMFH was added to the culture wells. After incubating at 27 °C for 6−8 h, the cells were washed twice with complete medium and infected with wild-type *Ac*MNPV at an MOI of 10. The expression of the reporter genes cloned under polyhedrin and hsp70 promoters was monitored by scoring their enzyme activities (*24*) at the required time points postinfection.

Cells infected with either wild-type or recombinant viruses or transfected with transfer vector plasmid DNAs were harvested at different time points after infection. β-Galactosidase enzyme expression levels were monitored by a colorimetric assay by using the substrate *o*-nitrophenyl-β-galactopyranoside (ONPG) (*24*). The assay for luciferase activity was carried out as described (*20*).

Construction of Recombinant Viruses. Construction of recombinant baculoviruses was carried out as described earlier (*24*). A recombinant virus (v*Ac*lacluc-hr1) carrying an additional hr1 (a-hr1) along with two reporter genes was constructed by cotransfecting *Sf*9 insect cells with pBVlacluc-hr1 plasmid DNA and Bsu361 digested pBacPAK6 viral DNA (Clontech) using lipofectin (GibcoBRL). Methods of cotransfection, plaque assay, and purification of recombinant viruses have been described elsewhere (*24*). Individual plaques purified viruses were used as a source for the expression of reporter genes in insect cells to study the hr1 modulated transcription from both homologous and heterologous promoters. The recombinant virus carrying v*Ac*lacluc-hr1 or lacking the additional hr1 (a-hr1) (v*Ac*lac-luc) but carrying only the resident hr1 (r-hr1) was used to infect *Sf*9 cells. Assays for expression of reporter genes in virus-infected cells were performed similar to that for transient expression described earlier.

Northern Blot Hybridization. Total RNA was isolated from virus infected 2×10^6 *Sf*9 cells using guanidium thiocyante, as described (*25*). Cells were infected with appropriate viruses at an MOI of 10 and harvested at different hours postinfection. Cells were washed with ice-cold 1X phosphate buffered saline (PBS) prior to isolation of RNA. A total of 20 μg of RNA was subjected to electrophoresis (2 h) on a formaldehyde gel (1.4% agarose) in 1×3[*N*-morpholino] propanesulfonic acid (MOPS) buffer (0.02M MOPS (pH 7.0), 2 mM sodium acetate, 1 mM EDTA (pH 8.0)) at room temperature. After partial alkaline denaturation followed by overnight transfer carried out in 20xSSC (*25*), the membrane (Hybond N^+) was probed with radiolabeled 1.8 Kb *luc* and 3.07 Kb *lacZ* fragments separately and autoradiographed.

*Primer Extension Analysis of RNA***.** Total RNA (50 μg) extracted from virus infected cells at 60 hpi was annealed to 1.5 pmol of a ^{32}P end-labeled, 20 nt *luc*-specific primer as described earlier (*20*). Hybridization was carried out at 48 °C for 12 h in hybridization buffer as described previously. The primer 5′-AAGAATGTAACACAAAGGAA-3′, located 46 nt upstream to the luc ATG, was specific to the

5′ end of the *luc* gene. The annealed mix was precipitated with ethanol, washed in 70% ethanol, and desiccated. The pellet was resuspended in 50 μL of reverse transcriptase buffer containing 40 U of RNasin, 2.5 μg of actinomycin D, 1 mM dNTPs, and 250 U of AMV reverse transcriptase. The reverse transcription reaction was carried out at 37 °C for 1 h and stopped with the addition of 25 mM EDTA, and the products were precipitated and washed with 70% ethanol. The RNA was hydrolyzed by suspending the pellet in 4 μL of 0.1 N NaOH/1 mM EDTA at 30 °C for 25 min. The sample was denatured at 75 °C for 5 min after the addition of 4 μL of stop solution from the Sequenase Version 2.0 sequencing kit and fractionated on a sequencing gel. Sequencing of the pSHluc-*hr*U1 (*20*) was performed in the presence of [^{35}S] dATP employing the same primer. The sequencing reaction was run alongside the primer extension reaction product.

Polymerase Chain Reaction. To perform PCR reactions, cells were infected with the two viruses at the same MOI. and the presence of equal levels of both the viruses was confirmed by dot blot analysis of DNA from infected cells using luciferase as probe. Total cell DNA was isolated from *Sf*9 cells infected with virus of different passage numbers as described (*25*). For PCR, primers were designed such that an amplified fragment was generated from the residential hr1 element (r-hr1), and another fragment of a different size was generated from the additional hr1 element (a-hr1) of the recombinant viral genome. The primers used for generating a 0.766 Kb fragment from r-hr1 were 5′-TGTTTTAC-TATCTGTTCT-3′ (P1) and 5′- GTTGTCGATAAAACAT-TC-3′ (P2) (Figure 3A). For generating the 1.247 Kb fragment from a-hr1, the primers used were P1 and 5′-CGATGTTAAATATGTCCAAGC-3′ (P3) (Figure 3A). Virus-infected total cell DNA (20 ng) was taken in a reaction volume of 25 μL containing 10 pmol of each primer; 250 mM each dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim, Germany); 1xTaq of polymerase buffer; 0.5 units of Taq polymerase (Genei, India); and 2.5 mM $MgCl_2$. DNA amplification was carried out for 25 cycles comprising of denaturation (2 min, 90 °C), annealing (30 s, 50 °C), and elongation (1 min, 70 °C) in a Perkin-Elmer Cetus Thermal Cycler (PCR, Model 4800). The PCR products were analyzed by electrophoresis on a 1% agarose gel in 1X TAE Buffer.

FACS-gal Analysis. FACS-gal is a system that enables the fluorescence activated cell sorter (FACS) to sensitively assay expression of the *lacZ* (β-gal) reporter gene and sort viable cells based on the levels of expression of this enzyme. FACS-gal analysis was carried out as described earlier (*26*). Cells transfected with the plasmid or infected with the virus at the appropriate time point were harvested and centrifuged at 2000 rpm for 5 min, washed twice with cold PBS, and resuspended in 50 μL of complete medium. Meanwhile, a 2 mM FDG (fluorescein di-β-D-galactopyranoside) stock was dissolved by being maintained at 37 °C in a water bath. A total of 25 μL of cells were placed in a tube and equilibrated in a 37 °C water bath for few minutes. To this, 25 μL of prewarmed 2 mM FDG was added and mixed briefly by tapping the tube. After 1−3 min, the loading was stopped, and 250 μL of cold media was added and incubated on ice for 30 min (in case of infection experiments) and 2 h (in case of transfection experiments). The mix was then centrifuged, and the pellet was resuspended in 200 μL of medium.

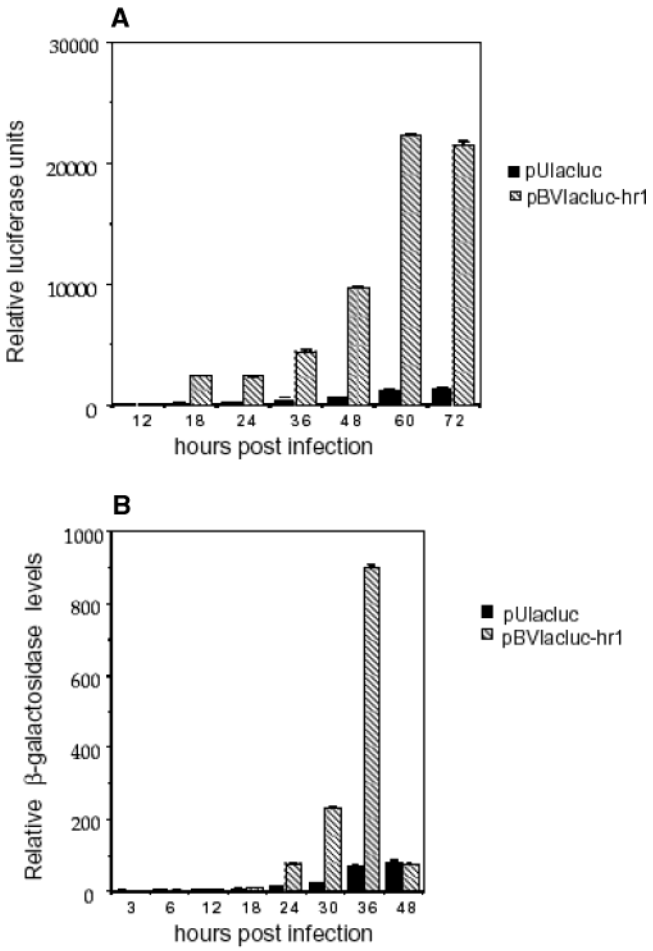

FIGURE 2: Hr1 enhances expression of reporter genes driven by the *polh* or *hsp70* promoters. Equal amounts of the two plasmids pUlacluc and pBVlacluc-hr1 were transfected into Sf9 cells followed by infection with wild-type virus. **(**A**)** Expression of the *luc* gene cloned under the *polh* promoter was measured at different time points post infection. **(**B**)** Influence of hr1 on the expression of *lacZ* cloned under the *hsp70* promoter.

Just prior to analysis, 200 μL of 10 μL/mL propidium iodide (PI) was added.

RESULTS

hr1 Enhances Expression of Reporter Genes in Transient Expression Assays. Transient expression assays were carried out to study the effect of the hr1 element on reporter gene expression from the *hsp70* promoter, and the same was compared with the homologous *polh* promoter. The recombinant transfer vector pBVlacluc-hr1 carrying the firefly luciferase (*luc*) and *E. coli* β-galactosidase (lacZ) genes cloned under *polh* and *hsp70* promoters, respectively, was a derivative of the expression vector plasmid pBVhr1, carrying the hr1 element, two *polh* promoters, and a single *hsp70* promoter (Figure 1). The downstream *polh* promoter did not have any reporter gene under its control. pUlacluc (*24*), which did not carry hr1 but only had the *polh-luc* and *hsp70-lacZ* cassettes, was used as a control plasmid for comparison in expression studies. Enhancement of expression from the *polh* promoter was monitored by measuring luciferase activity in a luminometer (*20*) at various points post infection (hpi), while an assay for β-galactosidase (*24*) was used to study enhancement from the *hsp70* promoter. A promoter-

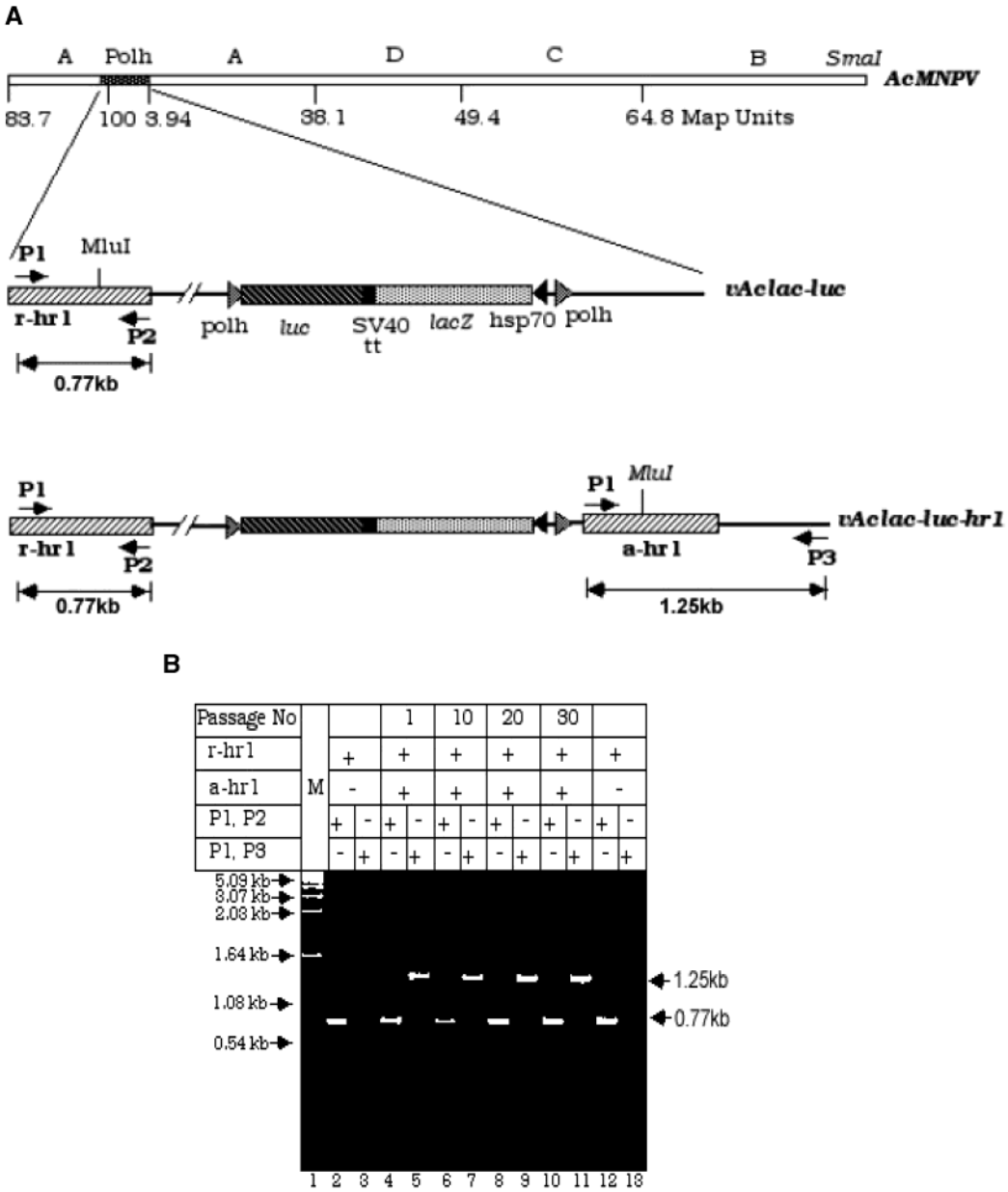

FIGURE 3: Presence of an additional copy of hr1 does not affect the stability of the recombinant virus. (A) Genomic map of recombinant baculovirus carrying the additional hr1 sequence. The top panel depicts linearized *Sma*I map of *Ac*MNPV. The polyhedrin locus, located within the A fragment, is enlarged to show the hr1 introduced into the viral genome. The position of PCR primers used to amplify the r-hr1 (P1, P2) or the a-hr1 (P1, P3) and the size of the corresponding amplified regions are indicated. (B) PCR analysis of the DNA isolated from v*Ac*ulacluc-hr1 and v*Ac*lac-luc at different passages (1, 10, 20, and 30). The primer pairs P1 and P2 or P1 and P3 were designed to generate a 0.766 kb (lanes 2, 4, 6, 8, 10, and 12) or 1.247 kb (lanes 3, 5, 7, 9, 11, and 13) fragment after specific amplification of the r-hr1 locus or a-hr1 locus, respectively. A 1 Kb DNA ladder was run alongside as molecular size marker (M, lane 1). Amplification of 1.247 Kb fragment is not observed from the DNA isolated from v*Ac*lacluc as it does not carry a-hr1 locus (lanes 2, 3 and 12, 13).

dependent increase in reporter gene expression from both the promoters, modulated by hr1, was observed as a function of time postinfection in transient expression assays of *Sf*9 cells. The level of enhancement of luciferase under the control of *polh* promoter increased as infection progressed with maximum enhancement (15−18-fold) attained at 60 hpi (Figure 2A). The *polh* promoter is hyperactive at very late hpi in *Ac*MNPV-infected *Sf*9 cells, and this temporal activation profile of the promoter was maintained in the presence of hr1. Detectable levels of the lacZ gene product synthesized under *hsp70* promoter control were observed early in infection showing maximum enhancement of 8−12-fold at 36 hpi (Figure 2B). At 48 hpi, there is a fall in the level of luciferase in transfection with both constructs. hr1 therefore, not only enhances expression of reporter genes under the transcriptional control of the viral *polh* promoter but also the heterologous *hsp70* promoter in transient expression assays.

Presence of an Extra Copy of the hr1 Element in a Recombinant Baculovirus Does Not Affect Virus Stability but Leads to Overexpression of Reporter Genes. The effect of hr1 was studied under conditions where this enhancer element was present in the viral genome in addition to the native resident hr1 (r-hr1). A recombinant baculovirus, v*Ac*lacluc-hr1 (Figure 3A), carrying the additional hr1 (a-hr1) along with two reporter genes was constructed from

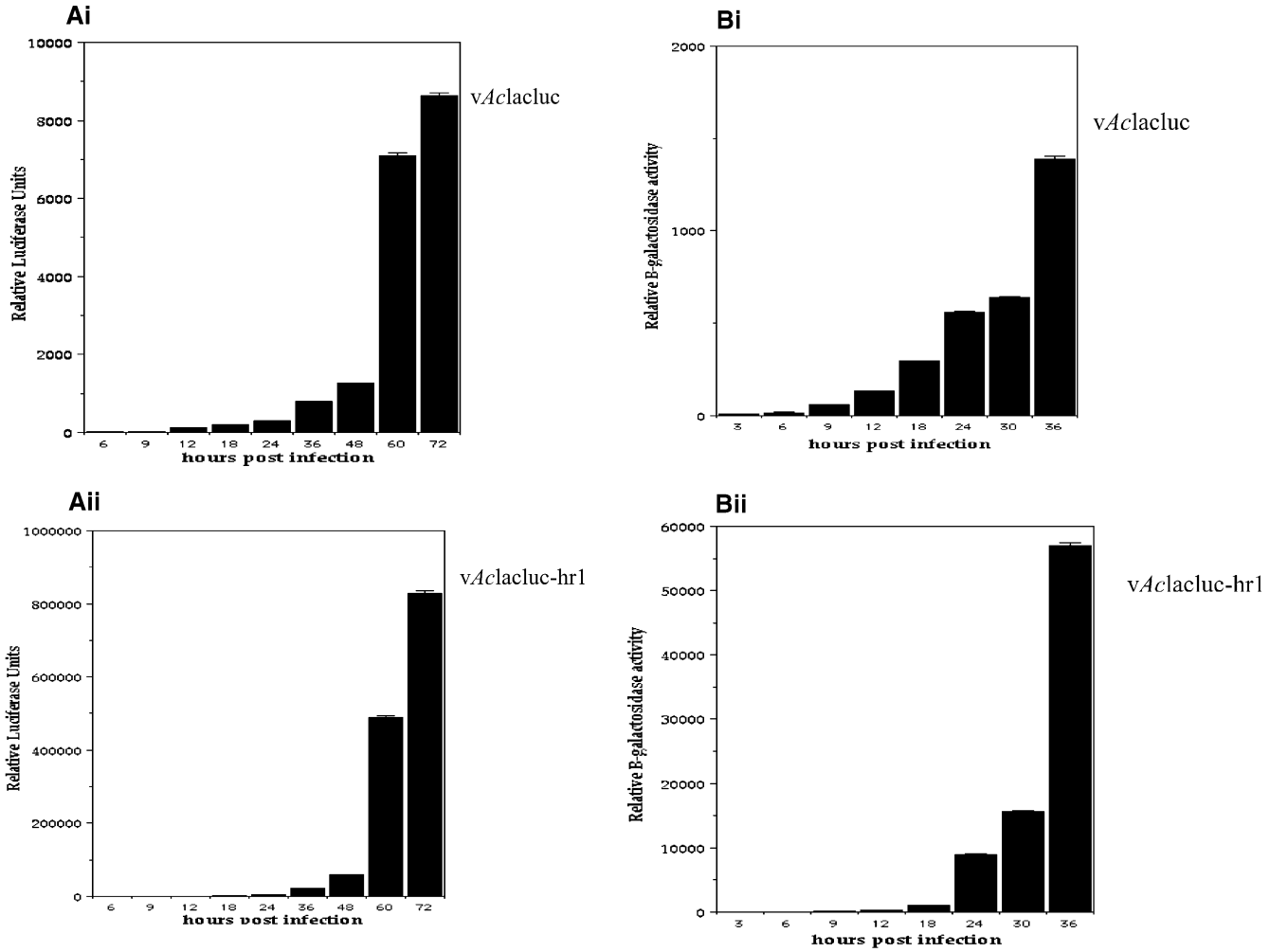

FIGURE 4: Presence of an additional hr1 element in the viral genome enhances heterologous gene expression. (A) hr1 causes ~95-fold enhancement of polyhedrin driven *luc* expression. Panel A(i**)** shows the *luc* expression profile for cells infected with v*Ac*lacluc, and panel A(ii) represents the luciferase levels for cells infected with v*Ac*lacluc-hr1. (B) Expression of *β*-galactosidase from the *hsp70* promoter is enhanced by ~40-fold by hr1. Panels B(i) and B(ii) show the *lacZ* expression of cells infected with v*Ac*lacluc and v*Ac*laluc-hr1 viruses, respectively.

the plasmid pBVlacluc-hr1. In v*Ac*lacluc-hr1, the r-hr1 is located about 4 Kb upstream, whereas the a-hr1 is placed downstream of the *polh-luc* reporter cassette. Presence of the a-hr1 sequence could possibly create impediments to the stability of the recombinant virus as homologous recombination mediated deletion, the insertion of large DNA segments are known to occur during serial passages (*23*, *27*), and this could therefore be a matter of possible concern. To exclude this possibility, the stability of the recombinant virus v*Ac*lacluc-hr1 was directly monitored for up to 30 serial passages by PCR amplification. Total DNA isolated from *Sf*9 cells infected with v*Ac*lacluc or v*Ac*lac-luc-hr1 at different passages was used as template. PCR amplification, using primers specific for sequence flanking the hr1 elements (Figure 3A), generated bands of two different sizes (Figure 3B). A 0.776 Kb fragment corresponding to r-hr1 was amplified from v*Ac*lacluc-hr1 infected cells with primers P1 and P2, while in v*Ac*lacluc-hr1 infected cells, primers P1 and P3 generated an additional 1.247 Kb fragment corresponding to the a-hr1 locus. Results of PCR amplification of the first, 10th, 20th, and 30th passages clearly demonstrate that the stability of the virus is not affected by the presence of an additional copy of the hr1 element.

For monitoring the effect of hr1 on foreign gene expression, v*Ac*lacluc, which lacked the a-hr1 but only carried the r-hr1, was used as a control virus in expression studies. *Sf*9 cells were separately infected at the same MOI with recombinant viruses v*Ac*lacluc and v*Ac*lacluc-hr1. In addition, the presence of equal levels of v*Ac*lacluc and v*Ac*lacluc-hr1 DNA was further ensured by dot blot analysis of DNA from infected cells using *luc* gene as a probe (data not shown). Reporter expression analysis showed that the temporal activation profile for the luciferase gene under a polyhedrin promoter followed that expected for a gene under a very late *Ac*MNPV promoter. In the presence of the a-hr1, enhanced levels of luciferase was observed at all times postinfection (with maximum enhancement of 95-fold) over the basal virus v*Ac*lacluc attained at 72 hpi. Figure 4A(i) shows the expression profile of cells infected with v*Ac*lacluc, and Figure 4A(ii) shows the luciferase expression of cells infected with v*Ac*lacluc-hr1. The additional enhancer sequence also enhanced the expression of the *β*-galactosidase gene under the *hsp70* promoter up to 40-fold. Maximum enhancement of *lacZ* expression was observed between 30 and 36 hpi. Figure 4B(i) and 4B(ii) represents the *β*-galactosidase levels at various hpi of cells infected with viruses

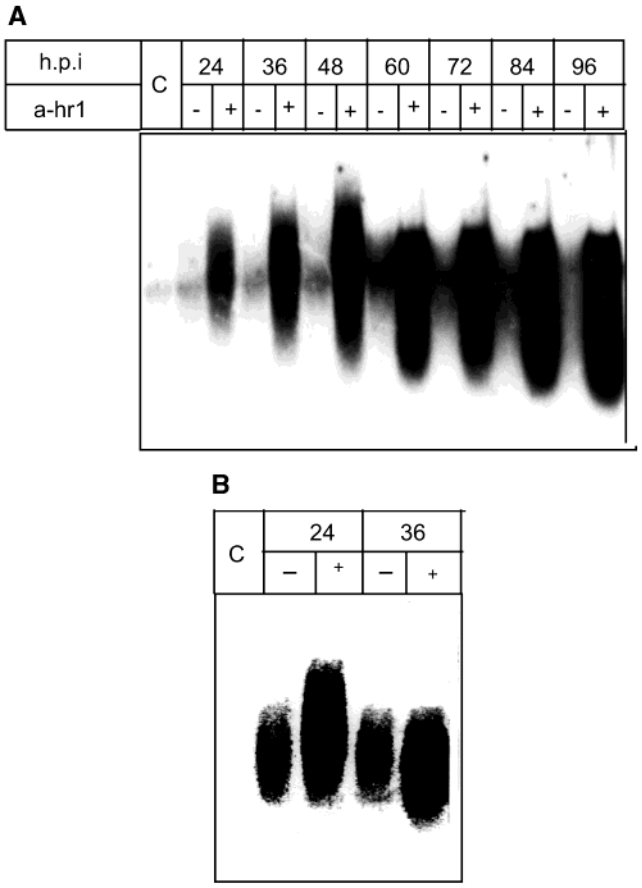

FIGURE 5: Hr1-mediated enhancement is at the level of transcription. Total cellular RNA isolated by standard procedures from in vitro cultured *Sf*9 cells (lane C) or those infected with v*Ac*lacluc (lane−) or v*Ac*lacluc-hr 1 (lane+) was transferred into nylon membrane (Hybond N+) and probed with radiolabeled *luc* (A) or *lacZ* (B) gene.

v*Ac*lacluc and v*Ac*lalуc-hr1, respectively. At time points after 36 hpi, there was a reduction in the β-galactosidase activity as observed in the transient transfection assay. These results demonstrate that an extra copy of the hr1 element is thus capable of substantially enhancing foreign gene expression over and above the resident hr1 element. Furthermore, hr1 can also function as an enhancer for a heterologous promoter (*hsp70*) in recombinant baculovirus infected cells.

Enhanced Expression of the Reporter Genes Is a Direct Result of Enhanced Transcription from the Promoters in the Presence of hr1. Northern blot analysis was carried out to compare the relative amounts of RNA produced from cells infected with v*Ac*lacluc and v*Ac*lacluc-hr1 at various time points postinfection. Total cellular RNA was isolated from virus infected *Sf*9 cells, and equal amounts of total RNA were loaded on a gel, then transferred to nylon membrane (Hybond N+) and probed with a radiolabeled 1.8 Kb fragment corresponding to the complete *luc* gene (Figure 5A). An identical blot was probed with the 3.07kb *lacZ* fragment (Figure 5B). A drastic increase in steady-state levels of RNA was evident in the presence of the a-hr1 element. Also, as in the protein expression profile, maximum increase in luciferase transcript levels was observed at late hpi.

Enhancement of Transcription from the Polyhedrin Gene Promoter in the Presence of the a-hr1 Sequence Was Also Evident from Primer Extension Analysis. Experiments were performed to determine whether the initiation of transcription of the *luc* is from the authentic polyhedrin transcription start site. Total RNA from cells infected with v*Ac*lacluc and v*Ac*lacluc-hr1 were analyzed by primer extension. Transcription of luciferase reporter gene in both the viruses was found to initiate within the TAAGTATT sequence with the major band at the 5′A residue of the TAAGTATT motif of the polyhedrin promoter. This position corresponds to the authentic start point of polyhedrin transcription in wild-type *Ac*MNPV (*23*, *28*). Moreover, a high level of transcriptional enhancement in the presence of additional hr1 sequence was also evident (Figure 6, compare hr1 lane with L lane). These data, while complementing earlier results on increased expression in the presence of hr1 element, further document that hr1-mediated enhanced transcription initiates from authentic start sites within the viral *polh* promoter.

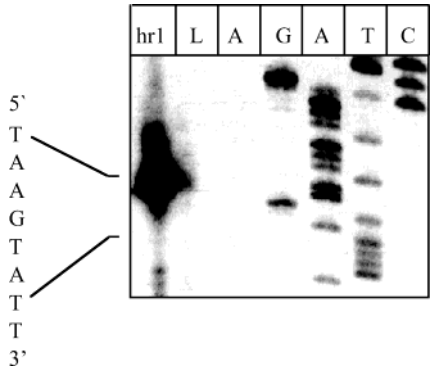

FIGURE 6: Enhanced transcription effected by hr1 is initiated from authentic transcription start sites. Primer extension analysis of *luc* transcripts in v*Ac*lacluc-hr1 or v*Ac*lacluc infected cells was carried out at 60 hpi (*15*) to determine transcription start site. The lanes represented by hr1 and L denote the primer extension of RNA from cells infected with v*Ac*lacluc-hr1 and v*Ac*lacluc, respectively. *Ac* represents primer extension of RNA from wild-type *Ac*MNPV virus infected cells. G, A, T, and C are guanine, adenine, thymine, and cytosine, respectively.

FACS-gal Analysis Confirms hr1 Mediated Enhancement of Reporter Gene. FACS-gal analysis provides an ideal method for analyzing β-galactosidase expression at the cellular level. Experiments were designed to physically monitor the expression, at the cellular level using FACS, of one of the reporter gene (*lacZ*) in *Sf*9 cells transfected with plasmids or viruses with or without hr1. Equal numbers of cells were transfected with the plasmids pUlacluc and pBVlacluc-hr1 and subjected to FACS-gal analysis. Figure 7A(i) shows the FACS analysis of 10 000 cells transfected with pUlacluc where *lacZ* is under the control of *hsp70* promoter but without hr1. FL1 shows the level of FDG (Fluorescein di-β-D-galactopyranoside) fluorescence, which is also a reflection of the β-galactosidase level and FL2 represents fluorescence of propidium iodide (PI), a marker that distinguishes between live and dead cells. Live cells do not take up PI, whereas dead cells take up the stain. The region R1 represents live cells, and R2 shows live and expressing cells. FACS analysis of cells transfected with pBVlacluc-hr1, which has hr1 cloned downstream of the hsp70-lacZ cassette, shows (Figure 7A(ii)) that in the presence of hr1, the FDG fluorescence is significantly increased. This is very clearly seen as a shift in the fluorescent channel in the histogram plot of the FACS results that corresponds to a 6.5-fold increase in reporter expression

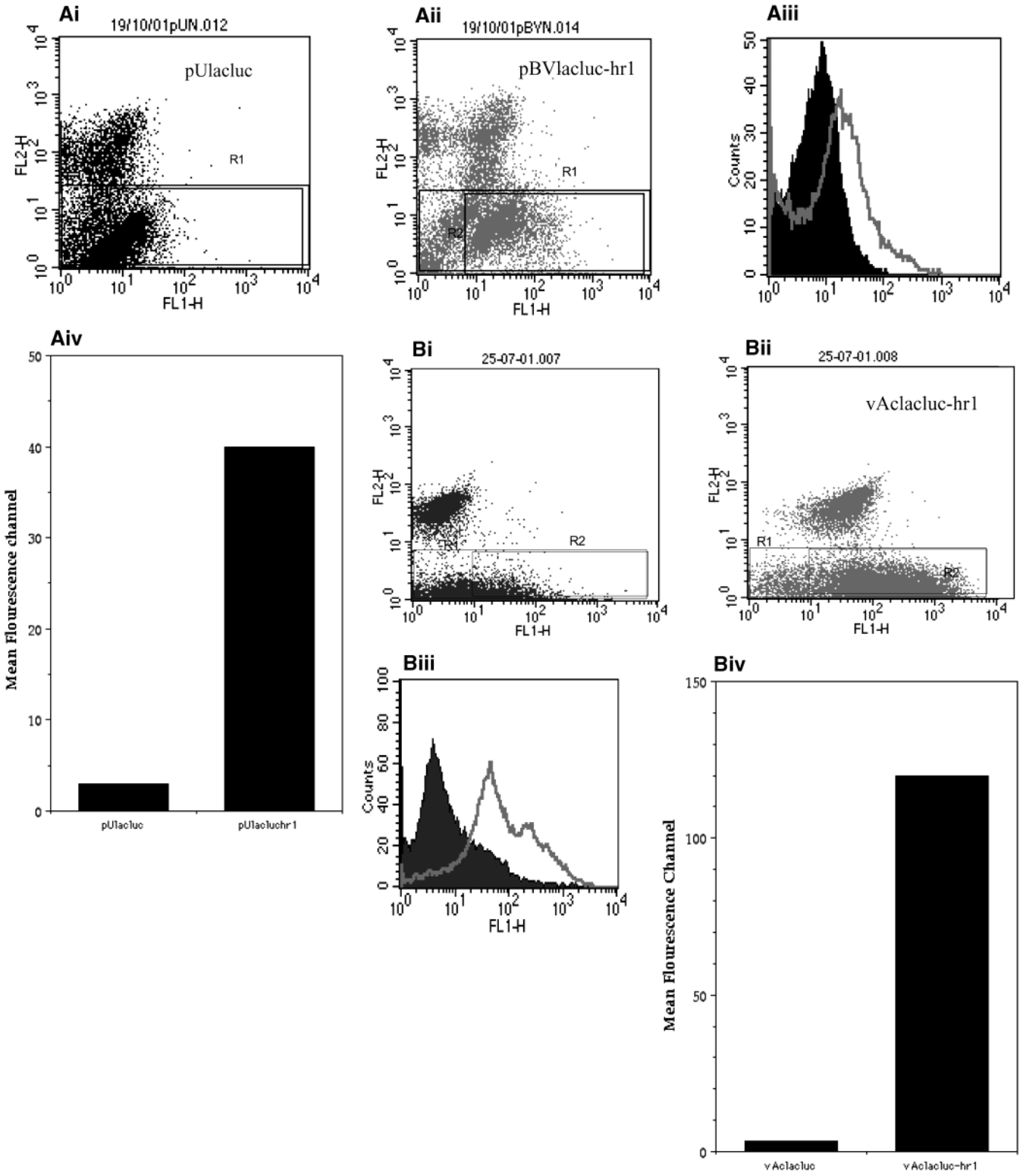

FIGURE 7: (A) FACS analysis of cells transfected with pUlacluc (i) and pBVlacluc-hr1 (ii) at 30 h posttransfection. FL1 shows the level of FDG fluorescence or the level of β-galactosidase expression, and FL2 indicates fluorescence due to propidium iodide. (iii) Cells transfected with plasmid containing hr1 show higher level of lacZ expression seen as a shift in fluorescence in the plot. (iv) The histogram plot shows a difference of around 6.5-fold in the presence of hr1. (B) FACS analysis of cells infected with virus v*Ac*lacluc (i) and v*Ac*lacluc-hr1 (ii) at 30 hpi. The plot (iii) shows that the shift in FDG fluorescence is expectedly much greater in case of the additional hr1 carrying virus, and (iv) the fold difference seen in the histogram plot is almost 35-fold.

(Figure 7A(iii) and (iv)). As opposed to plasmid transfection experiments (Figure 7A), in infection studies where cells were infected with the either v*Ac*lacluc (Figure 7B(i)) or v*Ac*lacluc-hr1 (Figure 7B(ii)) and subjected to FACS-gal analysis, the shift in FDG fluorescence is remarkably higher. As noticed in earlier experiments (Figure 4B(i) and (ii)), with the presence of an additional hr1 in the virus v*Ac*lacluc-hr1, the increase in β-galactosidase expression monitored by FACS analysis is almost 35-fold. These results correlate well with the fold increase in the reporter assay experiments and

thus convincingly demonstrate that hr1 enhances the expression of heterologous promoter driven lacZ reporter (Figure 7B(iii) and (iv)).

DISCUSSION

Our results demonstrate that the AcMPNV hr1 sequence can be used as an enhancer for increased expression of foreign genes in the baculovirus mediated insect expression system. hr1 functions by directly enhancing transcription, which was evident at the level of RNA as well as by FACS analysis. Earlier, we showed that hr1-mediated enhancement of transient expression is not due to the putative ori function (*20*) of this sequence element. Enhancers are known to have sequence-specific transcription factor binding site(s) that function from either an upstream or downstream position and stimulate weak promoters in a tissue specific manner (*29*−*31*). The 750 bp *Ac*MPNV hr1 element is composed of enhancer modules that contribute to the overall activity and serve as binding sites for a 38 kDa host protein (*20*, *21*) that is distinct from the other host nuclear proteins, namely, the polyhedrin promoter binding protein (PPBP) that interacts with *Ac*MPNV very late gene promoters (*32*−*37*) or the Sp-1 like host factor present in these cells (*38*). The palindromes and palindrome-flanking sequences of hr1 represent the repeating structural modules within this enhancer that bring about their enhancer action by recruiting cellular protein hr1-BP and the enhancement is a function of the number of such modules (*21*). The presence of an additional hr1 sequence, together with the resident hr1 element, would increase the number of these enhancer modules thereby enabling more recruitment of cellular factors to the enhancer. Cellular factors have been previously known to control the activity of viral enhancers (*31*) by appropriately interfacing independent signals generated at the core promoter and at the enhancer element (*39*). Another possible mode of action is related to the chromatin structure. Enhancer stimulated promoter activity is also observed under conditions where the DNA template is assembled into a chromatin structure that represses promoter activity (*40*−*42*). Enhancer elements cause perturbation in the nucleosome structure leading to alleviation of the chromatin mediated repression of promoters (*43*−*47*). The hr1-mediated activation of homologous or heterologous promoters could be a reflection of the synergistic effect of recruitment of the basal transcription factors and RNA polymerase to the adjacent promoters in the simple recruitment model for gene activation (*30*, *48*−*54*) and the relieving of the chromatin mediated repression of transcription.

The fact that hr1 acts not only on commonly used baculovirus promoters such as *polh* and core gene promoter but can also exert itself in an orientation and position independent manner on other heterologous promoters, such as CMV promoter in noninsect cell environment (*55*), is of significance. Such action of hr1 in enhancing transcription without supporting *ori* activity points to the differential requirement of host factor for these two activities of the hr elements (*20*, *21*, *56*), which has a bearing on the use of baculovirus as delivery system in gene therapy (*57*). This is particularly relevant for the new generation of multiple expression vectors carrying a combination of different promoters (*24*, *58*) for simultaneous expression of different genes. It has been shown that hr1 functions synergistically with the polyhedrin upstream sequences in upregulating the CMV minimal promoter (*59*). hr1-mediated enhancement of gene expression is directly a consequence of increased transcription at the level of RNA. While the increased RNA level also leads to increase in the reporter protein levels as evident from Western blot analysis and metabolic labeling experiments (unpublished observations), there is a lack of one-to-one correlation between RNA and protein levels. This is in tune with the emerging evidences on insufficient correlation between RNA and protein levels (*60*, *61*). Invariant steady state levels of certain proteins have been reportedly observed with mRNA transcript levels that varied as much as 30-fold (*61*). Interestingly, we also observed that the presence of an additional enhancer surprisingly resulted in enhanced activity and stability of the protein (manuscript in preparation). That the presence of an additional enhancer hr1, the only difference between the two recombinant viruses, has implications not only in the protein quantity but also quality throws open a number of questions about other possible modes of enhancer action (*44*, *62*) that are being explored.

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REFERENCES

1. Luckow, V. A., and Summers, M. D. (1988) Trends in the development of baculovirus expression vectors, *Virology 6*, 47−55.
2. Kidd, M., and Emery, V. C. (1993) The use of baculoviruses as expression vectors, *Appl. Biochem. Biotech. 42*, 137−159.
3. Sridhar, P., Awasthi, A. K., Azim, C. A., Burma, S., Habib, S., Jain, A., Mukherjee, B., Ranjan, A., and Hasnain, S. E. (1994) Baculovirus vector mediated expression of heterologous genes in insect cells, *J. Biosci. 19*, 603−614.
4. Possee, R. D. (1997) Baculoviruses as expression vectors, *Curr. Opin. Biotechnol. 5*, 569−572.
5. Kost, T. A., and Condreay, J. P. (1999) Recombinant baculoviruses as expression vectors for insect and mammalian cells, *Curr. Opin. Biotechnol. 10*, 428−433.
6. Ramachandran, A., Bashyam, M. D., Viswanathan, P., Ghosh, S., Kumar, M. S., and Hasnain, S. E. (2001) The bountiful and baffling baculovirus: The story of polyhedrin transcription, *Curr. Sci. 81*, 998−1010.
7. Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M., and Possee, R. D. (1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus, *Virology 202*, 586−605.
8. Summers, M. D., and Anderson, D. L. (1973) Characterization of nuclear polyhedrosis virus DNAs, *J. Virol. 12*, 1336−1346.
9. Blissard, G. W., and Rohrmann, G. F. (1990) Baculovirus diversity and molecular biology, *Annu. Rev. Entomol. 35*, 127−155.
10. Bashyam, M. D., Ramachandran, A., Ghosh, S., Viswanathan, P., and Hasnain, S. E. (2000) Multiple regulatory circuits for transcription from the polyhedrin gene promoter of the *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus, *Proc. Indian Natl. Sci. Acad. B66*, 83−96.
11. Kool, M., Ahrens, C. H., Vlak, J. M., and Rohrmann, G. F. (1995) Replication of baculovirus DNA, *J. Gen. Virol. 76*, 2103−2118.
12. Cochran, M. A., and Faulkner, P. (1983) Location of homologous DNA sequences interspersed at five regions in the baculovirus *Ac*MNPV genome, *J. Virol. 45*, 961−970.
13. Carson, D. D., Summers, M. D., and Guarino, L. A. (1991) Transient expression of the *Autographa californica* nuclear polyhedrosis virus immediate-early gene, IE-N, is regulated by three viral elements, *J. Virol. 65*, 945−951.
14. Guarino, L. A., and Summers, M. D. (1986) Interspersed homologous DNA of *Autographa californica* nuclear polyhedrosis virus enhances delayed early gene expression, *J. Virol. 60*, 215−223.
15. Guarino, L. A., and Dong, W. (1991) Expression of an enhancer binding protein in insect cells transfected with the *Autographa californica* polyhedrosis virus IE1 gene, *J. Virol. 65*, 3676−3680.
16. Guarino, L. A., and Dong, W. (1994) Functional dissection of the *Autographa californica* nuclear polyhedrosis virus enhancer element hr5, *Virology 200*, 328−335.

17. Leisy, D. J., Rasmussen, C., Kim, H. T., and Rohrmann, G. F. (1995) The *Autographa californica* nuclear polyhedrosis virus homologous region 1a: identical sequences are essential for DNA replication activity and transcriptional enhancer function, *Virology 208*, 742−752.
18. Lu, A., and Carstens, E. B. (1993) Immediate-early baculovirus genes transactivate the p143 gene promoter of *Autographa californica* nuclear polyhedrosis virus, *Virology 195*, 710−718.
19. Nissen, M. S. and Friesen, P. D. (1989) Molecular analysis of the transcriptional regulatory region of an early baculovirus gene, *J. Virol. 63*, 493−503.
20. Habib, S., Pandey, S., Chatterji, U., Burma, S., Ahmad, R., Jain, A., and Hasnain, S. E. (1996) Bifuncationality of the *Ac*MNPV homologous region sequence (hr1): enhancer and ori functions have different sequence requirements, *DNA Cell Biol. 15*, 737−747.
21. Habib, S., and Hasnain, S. E. (1996) A 38 kDa host factor interacts with functionally important motifs within the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus homologous region (hr1) DNA sequence, *J. Biol. Chem. 271*, 28250−28258.
22. Shi, Y. P., Hasnain, S. E., Sacci, J. B., Holloway, B. P., Fujioka, H., Kumar, N., Wohlhueter, R., Hoffman, S. L., Collins, W. E., and Lal, A. A. (1999) Immunogenecity and in vitro protective efficacy of a recombinant multistage *Plasmodium falciparum* candidate vaccine, *Proc. Natl. Acad. Sci. U.S.A. 96*, 1615−1620.
23. O'Reilly D. R., Miller, L. K., and Luckow, V. A. (1992) *Baculovirus expression vectors: A laboratory manual*, WH Freeman and Co., New York.
24. Chatterji, U., Raies, A., Venkaiah, B., and Hasnain, S. E. (1996) A recombination-efficient baculovirus vector for simultaneous expression of multiple genes, *Gene 171*, 209−213.
25. Summers, M. D., and Smith, G. E. (1988) *A Manual of Methods for Baculovirus Vectors and Insect Cells Culture Procedures*, Texas Agricultural Experiment Station Bulletin No. 1555 Texas Agricultural Experimental Station: College Station, TX.
26. Fiering, S. N., Roederer, M., and Nolan, G. P. (1991) Imrproved FACS-Gal: flow cytometric analysis and sorting of viable eukaryotic cells expressing reporter constructs, *Cytometry 12*, 291−301.
27. Kumar, S., and Miller, L. K. (1987) Effects of serial passage of *Autographa californica* nuclear polyhedrosis virus in cell culture, *Virus Res. 7*, 335−349.
28. Rankin, C., Ooi, B. G. and Miller, L. K. (1988) Eight base pairs encompassing the transcriptional start point are the major determinant for baculovirus polyhedrin gene expression, *Gene 70*, 30−50.
29. Blackwood, E. M., and Kadonaga, J. T. (1998) Going the distance: a current view of enhancer action, *Science 281*, 61−63.
30. Farrell, S., Simkovich, N., Wu, Y., Barberis, A., and Ptashne, M. (1996) Gene activation by recruitment of the RNA polymerase II holoenzyme, *Genes Dev. 10*, 2359−2367.
31. Ueda, K., Wei, Y., and Ganem, D. J. (1996) Cellular factors controlling the activity of woodchuck hepatitis virus enhancer II, *J. Virol. 70*, 4714−4723.
32. Burma, S., Mukherjee, B., Jain, A., Habib, S., and Hasnain, S. E. (1994) An unusual 30 kDa protein binding to the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus, *J. Biol. Chem. 269*, 2750−2757.
33. Hasnain, S. E., Jain, A., Habib, S., Ghosh, S., Chatterji, U., Ramachandran, A., Das, P., Venkaiah, B., Pandey, S., Liang, B., Ranjan, A., Natarajan, K., and Azim, C. A. (1997) Involvement of host factors in transcription from the baculovirus very late promoters—a review, *Gene 190*, 113−118.
34. Jain, A., and Hasnain, S. E. (1996) A 30 kDa host-protein binds to two very late baculovirus promoters, *Eur. J. Biochem. 239*, 384−390.
35. Mukherjee, B., Burma, S. and Hasnain, S. E. (1995) The 30 kDa protein binding to the "initiator" of the baculovirus polyhedrin promoter also binds specifically to the coding strand, *J. Biol. Chem. 270*, 4405−4411.
36. Mukherjee, B., Burma, S., Talwar, G. P., and Hasnain, S. E. (1995) Transcriptional regulation of cell-line dependent, baculovirus-mediated expression of foreign genes, *DNA Cell Biol. 14*, 7−14.
37. Ghosh, S., Jain, A., Mukherjee, B., Habib, S., and Hasnain, S. E. (1998) The host factor polyhedrin promoter binding protein (PPBP) is involved in transcription from the baculovirus polyhedrin gene promoter, *J. Virol. 72*, 7484−7493.
38. Ramachandran, A., Jain, A., Arora, P., Bashyam, M. D., Chatterjee, U., Ghosh, S., Parnaik, V. K., and Hasnain, S. E. (2001) Novel Sp familylike transcription factors are present in adult insect cells and are involved in transcription from the polyhedrin gene initiator promoter, *J. Biol. Chem. 276*, 23440−23449.
39. Novina, C. D., and Roy, A. L. (1996) Core promoters and transcriptional control, *Trends Genet. 12*, 351−355.
40. Majumder, S., and DePamphilis, M. L. (1995) A unique role for enhancers is revealed during early mouse development, *BioEssays 17*, 879−889.
41. Paranjape, S. M., Kamakaka, R. T., and Kadonaga, J. T. (1994) Role of chromatin structure in the regulation of transcription by RNA polymerase II, *Annu. Rev. Biochem. 63*, 265−297.
42. Majumder, S., and DePamphilis, M. L. (1994) TATA-dependent enhancer stimulation of promoter activity in mice is developmentally acquired, *Mol. Cell Biol. 14*, 4258−4268.
43. Felsenfeld G. (1992) Chromatin as an essential part of the transcriptional mechanism, *Nature 355*, 219−24.
44. Struhl, K. (1996) Chromatin structure and RNA polymerase II connection: implication for transcription, *Cell 84*, 179−182.
45. Studitsky, V. M., Clark, D. J., and Felsenfeld, G. (1995) Overcoming a nucleosomal barrier to transcription, *Cell 83*, 19−27.
46. Wolffe, A. P., and Pruss, D. (1996) Targeting chromatin disruption: Transcription regulators that acetylate histones, *Cell 84*, 817−819.
47. Workman, J. L., and Buchman, A. R. (1993) Multiple functions of nucleosomes and regulatory factors in transcription, *Trends Biochem. Sci. 18*, 90−9548.
48. Barberis, A., Pearlberg, J., Simkovich, N., Farrell, S., Reinagel, P., Bamdad, C., Sigal, G., and Ptashne, M. (1995) Contact with a component of the polymerase II holoenzyme suffices for gene activation, *Cell 81*, 359−368.
49. Ptashne, M., and Gann, A. (1997) Transcriptional activation by recruitment, *Nature 386*, 569−77.
50. Gaudreau, L., Adam, M., and Ptashne, M. (1998) Activation of transcription in vitro by recruitment of the yeast RNA polymerase II holoenzyme, *Mol. Cell 1*, 913−916.
51. Zaman, Z., Ansari, A. Z., Gaudreau, L., Nevado, J., and Ptashne, M. (1998) Gene transcription by recruitment, *Cold Spring Harb. Symp. Quantum Biol. 63*, 167−171.
52. Kim, A., and Dean, A. (2003) Human globin enhancer causes both discrete and widespread alterations in chromatin structure, *Mol. Cell. Biol. 22*, 8099−8109.
53. Kuras, L., Borggrefe, T., and Kornberg, R. D. (2003) Association of the mediator complex with enhancers of active genes, *Proc. Natl. Acad. Sci. U.S.A. 100*, 13887−13891.
54. Marmorstein, R. (2003) Transcription initiation at its most basic level, *Cell 115*, 370−372.
55. Viswanathan, P., Venkaiah, B., Kumar, M. S., Rasheedi, S., Vrati, S., Bashyam, M. D., and Hasnain, S. E. (2003) The homologous region sequence (hr1) of *Autographa californica* multinucleocapsid polyhedrosis virus can enhance transcription from nonbaculoviral promoters in mammalian cells, *J. Biol. Chem. 278*, 52564−52571.
56. Rodems, S. M., and Friesen, P. D. (1993) The hr5 transcriptional enhancer stimulates early expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication, *J. Virol. 67*, 5776−5785.
57. Ghosh, S., Parvez, M. K., Banerjee, K., Sarin, S. K., and Hasnain, S. E. (2002) Baculovirus as mammalian cell expression vector for gene therapy: an emerging strategy, *Mol. Ther. 6*, 5−11.
58. Belyaev, A. S., Hails, R. S., and Roy, P. (1995) High-level expression of five foreign genes by a single recombinant baculovirus, *Gene 156*, 229−233.
59. Lo, H. R., Chou, C. C., Wu, T. Y., Yuen, J. P., and Chao, Y. C. (2002) Novel baculovirus DNA elements strongly stimulate activities of exogenous and endogenous promoters, *J. Biol. Chem. 277*, 5256−5264.
60. Zohar, M., Mesika, A., and Reich, Z. (2001) Analysis of genetic control elements in eukaryotes: transcriptional activity or nuclear hitchhiking? *BioEssays 23*, 1176−1179.
61. Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast, *Mol. Cell. Biol. 19*, 1720−1730.
62. Walters, M. C., Fiering, S., Eidemiller, J., Magis, W., Groudine, M., and Martin, D. I. (1995) Enhancers increase the probability but not the level of gene expression, *Proc. Natl. Acad. Sci. U.S.A. 92*, 7125−7129.

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