A Baculovirus (Bombyx mori Nuclear Polyhedrosis Virus) Repeat Element Functions as a Powerful Constitutive Enhancer in Transfected Insect Cells*

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It has been previously reported that baculovirus homologous regions, the regions of baculovirus genomes that contain the origins of DNA replication, can augment the expression of a small number of baculovirus genes in vitro. We are now reporting that a region of the genome of Bombyx mori nuclear polyhedrosis virus (BmNPV) containing the homologous region 3 (HR3) acts as an enhancer for the promoter of a nonviral gene, the cytoplasmic actin gene of the silkworm B. mori. Incorporation of the HR3 sequences of BmNPV into an actin promoter-based expression cassette results in an augmentation of transgene expression in transfected cells by two orders of magnitude relative to the control recombinant expression cassette. This increase is due to a corresponding increase in the rate of transcription from the actin promoter and not to replication of the expression cassette and occurs only when the HR3 element is linked to the expression cassette in cis. A comparable degree of enhancement in the activity of the silkworm actin promoter occurs also in heterologous lepidopteran cells. Concomitant supplementation of transfected cells with the BmIE1 trans-activator, which was previously shown to be capable of functioning in vitro as a transcriptional co-activator of the cytoplasmic actin gene promoter, results in more than a 1,000-fold increase in the level of expression of recombinant proteins placed under the control of the actin gene promoter. These findings provide the foundation for the development of a nonlytic insect cell expression system for continuous high-level expression of recombinant proteins. Such a system should provide levels of expression of recombinant proteins comparable to those obtained from baculovirus expression systems and should also have the additional advantage of continuous production in a cellular environment that, in contrast to that generated by a baculovirus infection, supports continuously proper posttranslational modifications of recombinant proteins and the capability of expression of proteins from genomic as well as cDNA sequences.

Baculovirus homologous regions (HRs) are repeated sequences that are interspersed in the genomes of baculoviruses. They have been identified in all baculoviruses studied thus far, including Bombyx mori NPV (BmNPV), Autographa californica NPV (AcNPV), Choristoneura fumiferana NPV (CfNPV), Lymantria dispar NPV (LdNPV), and Orgyia pseudotsugata NPV (OpNPV) (1–8). The AcNPV and OpNPV HRs (HR1–HR5) are among the best characterized viral regulatory elements. They were shown to represent origins of viral DNA replication (9–13) and to be capable of functioning as strong enhancers for some, but not all, early viral genes such as 39k (3, 4), 35k (14, 15) and ec2 (16). In addition, the AcNPV HR5 element was also shown to be capable of enhancing another viral promoter of nonbaculovirus origin, the Rous sarcoma virus long terminal repeat promoter (14).

In a previous communication (17), we reported on the development of an in vitro expression system that employed the promoter of the silkworm A3 (cytoplasmic) actin gene (18, 19) as a driver of foreign gene expression in lepidopteran cells. Furthermore, we also reported previously that a baculovirus transcriptional activator, BmIE1, that is encoded by an immediate early gene of BmNPV, functions as co-activator of the cytoplasmic actin gene promoter in transfected cells and increases the level of transcription from this promoter by two orders of magnitude (20).

In an attempt to increase the general utility of the actin gene promoter as a component of expression vectors for production of foreign proteins in insect cells, we have looked for additional genetic elements that can stimulate further the expression of the actin promoter. Because baculoviruses have evolved both to cooperate and compete with cellular genes and to take advantage of cellular structures by enhancing general cell maintenance functions, and in view of the previous reports regarding the in vitro effects of baculovirus HRs for a few viral genes, we have asked whether the enhancing properties of HR elements can also be seen when these elements are linked to gene promoters of the host cell and introduced into lepidopteran insect cells.

We are now reporting that, when linked to gene constructs expressing reporter proteins under the control of the cytoplasmic actin gene promoter of B. mori, a DNA fragment containing the HR3 of BmNPV enhances significantly the in vitro expres-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) U77353.

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¶The abbreviations used are: HRs, homologous regions; NPV, nuclear polyhedrosis virus; kb, kilobase(s); CAT, chloramphenicol acetyl transferase; ORF, open reading frame; JHE, juvenile hormone esterase; bp, base pair(s); PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR.
sion of the reporter proteins. The observed stimulation is due to increases in the rate of transcription of the actin gene promoter. In accordance with the definition for enhancer elements, the BmNPV genomic fragment is capable of mediating its effect only in cis and in a position and orientation-independent manner. Finally, we show that linkage of the HR3 enhancer to the actin gene promoter combined with the presence of the BmIE1 protein in the transfected cells results in an augmentation in the level of actin promoter strength by three orders of magnitude relative to that of the basic actin gene promoter.

These results set the stage for the development of a stable insect cell transformation system for overexpression of recombinant proteins. Such a system should provide levels of expression similar to those achieved by recombinant baculoviruses but should be devoid of the limitations inherent to baculovirus expression systems. Well-known limitations of baculovirus expression systems include first, a reduction in proper posttranslational modification of recombinant proteins during the high production phase (late stages of infection) due to damaging of the insect cell's protein modification machinery; second, the general inability of baculovirus-infected cells to process during the late stages of infection, complex, intron-containing transcripts and produce recombinant proteins from genomic sequences; and third, difficulties associated with the purification of recombinant proteins as a result of cell lysis at the end of the infection process, and release of proteases that cause degradation of the overexpressed products. None of these problems should occur in an insect cell expression system in which the cells remain intact for the duration of the production phase.

MATERIALS AND METHODS

Cloning of the HR3 Element and Construction of Expression Plasmids—A 6.6-kb ClaI fragment of BmNPV genomic DNA (map units 49.94–54.94) containing genes p39, cg30, and p15 and a region with multiple EcoRI sites was previously cloned (Fig. 1A) (21, 22). A 1.2-kb SspI subfragment of the cloned fragment (map unit 51.8–52.7) containing multiple EcoRI sites (HR3 element of BmNPV) was subcloned into the Smal site of vector pBluescript-SK− (pBS; Stratagene). Two plasmids, p153 and p133, containing the 1.2-kb insert in opposite orientations were obtained and used subsequently for generating various expression cassettes. Plasmid pBmA.cat, which contains the Escherichia coli chloramphenicol acetyl transferase (CAT) open reading frame under the control of the cytoplasmic actin A3 gene promoter of chloramphenicol acetyl transferase (CAT) open reading frame expression cassettes. Plasmid pBmA.cat, which contains the ORF for JHE (kk) from plasmid pAcUW21-KK, was digested with SstI and a 3.0-kb HR3-mediated enhancement of gene expression in transfected Bombyx and Spodoptera Tissue Culture Cells—Derivatives of the expression vector pBmA.cat, which contains the CAT ORF under the control of the silkmoth cytoplasmic actin gene promoter, were constructed by inserting the 1.2-kb SspI fragment in four different combinations of position and orientation relative to the actin gene promoter (Fig. 2A). The ability of the HR3 fragment to enhance the actin promoter was assessed by transfecting Bm5 cells with pBmA.cat or each of the transfected plasmids. The double-enhanced expression cassettes pIE1/133A and pIE1/153A were generated by inserting a 2.2-kb SacI fragment containing the basic actin expression cassette from plasmid pBmA (17) into the unique SacI sites of plasmids p133 and p153, respectively, removing unwanted restriction sites in the polylinker of these plasmids by double digestion with SacI and BglII, blunts-terminating T4 DNA polymerase and self-ligating the resulting plasmids. The double-enhanced expression cassettes pIE1/133A and pIE1/153A were generated by inserting a 2.2-kb SacI fragment containing the basic actin expression cassette from plasmid pBmA (17) into the unique SacI sites of plasmids p133 and p153, respectively. The recombinant expression vectors pIE1/133A.cat and pIE1/153A.cat were generated by inserting a 0.9-kb BglII fragment, obtained from plasmid pBmA.cat and containing the ORF for CAT, into the unique BglII sites of the expression cassettes pIE1/133A and pIE1/153A, respectively. The construction of plasmid pBmA.jhe(kk) containing the ORF of a modified version of the juvenile hormone esterase (JHE) cDNA of Heliothis virescens (23) under the control of the actin gene promoter was previously reported (20). Finally, the double-enhanced expression vector pIE1/153A.jhe(kk) was generated by first isolating a 1.8-kb EcoRI fragment containing the ORF for JHE (kk) from plasmid pAcUW21-KK (23), ligating NotI linker to its ends, digesting with NotI, and inserting it into the unique NotI site of the double-enhanced expression cassette pIE1/153A.

Cell Cultures and Transfections—Bm5 and SF21 cells were maintained in IPL-41 medium with 10% fetal calf serum as described previously (24). They were transfected with various plasmid DNAs in 6-well microtiter plates (10^6 cells/well in 2 ml of medium) as described previously (20), except that cells in each well were transfected with 0.5 ml of transfection solution (20) containing 1 mg of pBmA.cat or equimolar amounts of each of the other expression plasmids described above and [3H]DNA to a combined final DNA concentration of 5 mg/ml.

CAT Assays and Dot Blot Hybridization—Transfected cells were pelleted 48 h posttransfection and washed three times with 1 ml of phosphate-buffered saline (10 mM KH_2PO_4, 2 mM NaH_2PO_4, 140 mM NaCl, 40 mM KCl). 90% of the cells were used to extract soluble protein, and 10-ng aliquots of the extracted protein were used for CAT assays as described (20). The remaining 10% of the transfected cells were used for Dot Blot DNA hybridization analysis. The cells were counted with a hemocytometer and Dot-blotted directly onto Hybond-N+ membrane (Amersham). The membranes were treated sequentially with 0.5 mM NaOH and 0.5 mM Tris-HCl, pH 7.5. Labeling of a 900-bp BamHI fragment of pBmA.cat containing the CAT ORF (17) with [*-32P]dCTP and hybridization of the immobilized DNA samples to the radioactive probe were as described (20).

JHE Assays—For JHE assays, cells were harvested as above, and their media were assayed as described previously (20). For quantitative measurements, samples were diluted to obtain juvenile hormone to juvenile hormone acid conversion rates between 10 and 50%, and activities were calculated on the basis of the dilution factors.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (PCR) Amplification—Total nucleic acids from transfected cells was extracted as described (20), treated with RNase-free DNase, and reverse-transcribed using as primer oligo(dT)12–18. Aliquots of cDNA reactions were subjected to semiquantitative PCR amplification using cat gene and chorion gene-specific primers as described previously (20).

Nuclear Run-on Transcription and Hybridization Assays—Nuclei were isolated from pBmA.cat and p13315-transfected cells, and nuclear run-on reactions were carried out as described (20). Nuclear RNA labeled with [*-32P]dCTP and [*-32P]dGTP was used as probe against a dilution series of a 900-bp BamHI cat gene fragment isolated from plasmid pBmA.cat (17) and immobilized on Hybond-N+ membrane as described previously (20).

RESULTS

Cloning of the HR3 Region—The nucleotide sequence of the portion of the 1.2-kb SspI fragment (GenBank™ accession no. U77353) containing the HR3 element of BmNPV is shown in Fig. 1B. The HR3 element consists of a 72-bp sequence motif that is repeated completely four times and incompletely once. The fourth repeat unit is missing 45 bp of the repeat, whereas the first one contains two insertions, 14 and 56 bp (Fig. 1B, X and Y, respectively). Each repeat unit contains an EcoRI site located at the center of a 30-bp imperfect palindrome. The nucleotide sequence of HR3 presented here is somewhat different from that reported previously (GenBank™ accession no. L33180), especially in the rightward half of the repeat unit (data not shown). This difference is probably due to the fact that different BmNPV isolates were used.

HR3-mediated Enhancement of Gene Expression in Transfected Bombyx and Spodoptera Tissue Culture Cells—Derivatives of the expression vector pBmA.cat, which contains the CAT ORF under the control of the cytoplasmic actin gene promoter, were constructed by inserting the 1.2-kb SspI fragment in four different combinations of position and orientation relative to the actin gene promoter (Fig. 2A). The ability of the HR3 fragment to enhance the actin promoter was assessed by transfecting Bm5 cells with pBmA.cat or each of the four HR3-containing plasmids. As shown in Fig. 2B, although some variability was observed between CAT activities obtained from constructs containing the HR3 element in various spatial arrangements relative to the actin promoter, the same fundamental result was obtained from all four HR3 configurations tested; cells transfected with actin expression cassettes containing the HR3 element resulted in expression of higher CAT activities than cells transfected with the basal expression vector. Quantitative analysis of the results shown in Fig. 2B revealed a 35-, 88-, 15-, and 23-fold stimulation in actin promoter-driven expression of cat gene expression in cells transfected
with plasmids p13314, p13315, p15316, and p15317, respectively. For example, whereas Bm5 cells transfected with pBmA.cat contained 0.33 units CAT activity per mg of soluble cellular protein, cells transfected with vector p13315 contained 29.5 units per mg (not shown).

To ensure that the observed differences in CAT activity in transfected cells were not due to differences in transfection efficiencies between constructs or to plasmid DNA replication, we carried out hybridization-based determinations of plasmid efficiencies between constructs or to plasmid DNA replication, transfected cells were not due to differences in transfection efficiencies between constructs or to plasmid DNA replication.

To exclude the possibility that an unknown gene product encoded by sequences present in the 1.2 kb sequence was responsible for the observed increases in actin promoter-driven cat gene expression, we carried out co-transfections of Bm5 cells with pBmA.cat and p133 or p153, each of which contains the 1.2-kb HR3 fragment. The CAT assays shown in Fig. 2D showed that neither p133 nor p153 was able to increase CAT expression from pBmA.cat in trans. Therefore, because the HR3 element is capable of stimulating the level of expression of the actin gene promoter only in cis and in a position- and orientation-independent manner, it can be defined as an enhancer of the cytoplasmic actin gene promoter.

Finally, to test whether the HR3 element is also functional in other lepidopteran cells, we transfected Spodoptera frugiperda (Sf21) tissue culture cells with plasmids pBmA.cat and p13315. As shown in Fig. 2E, the CAT assays revealed that the BmNPV HR3 element functions in these cells in a manner analogous to that seen in Bm5 cells.

**HR3-mediated Activation Involves Increases in Transcription Rates**—To find out whether the observed increases in CAT activity obtained in the presence of the HR3 element were due to corresponding increases in the accumulation of CAT mRNA in the transfected cells, we carried out a semiquantitative RT-PCR analysis of CAT mRNA present in cells transfected with different plasmids. As shown in Fig. 3A, cells transfected with vector p13315, which contains the enhancer upstream of the actin promoter (see Fig. 2A for its orientation), contained much higher quantities of CAT mRNA than cells transfected with vector pBmA.cat.

Nuclear run-on transcription assays were also performed to determine whether the observed increases in CAT mRNA accumulation observed in the presence of the HR3 element were due to corresponding changes in rates of transcription. As shown in Fig. 3B, an average of a 16-fold higher hybridization signal was detected with the run-on RNA probe obtained from nuclei of p13315-transfected cells than with the probe obtained from pBmA.cat-transfected cells. These results indicate that the HR3 element stimulates the expression of the actin.cat fusion gene by increasing the rate at which this gene is transcribed.

**Super-activation of Transgene Expression by BmIE1 and HR3**—The immediate early gene ie1 of baculoviruses is transcribed in infected lepidopteran cells by the host transcriptional machinery in the absence of any accessory viral products. We have previously shown that BmIE1, the protein encoded by the ie1 gene of BmNPV, functions as co-activator of the actin gene promoter in vitro and potentiates the level of expression of the actin gene promoter also by approximately two orders of magnitude (20). To determine whether BmIE1 and HR3 can act synergistically on the actin gene promoter, we co-transfected Bm5 cells with plasmid pBmAIE1 and each of the four HR3/actin-cat constructs. As shown in Fig. 4, the CAT assays showed that CAT activity in the presence of both BmIE1 and HR3 was higher than that obtained in the absence of either BmIE1 or HR3, irrespective of whether the BmIE1 gene was present in the same plasmid as the HR3-enhanced actin promoter construct or supplied to the cells as a separate plasmid.

Quantification of the observed enzyme activities (Table I) revealed that cells co-transfected with pBmAIE1 and each of HR3/actin-cat constructs contained more than 1,000-fold higher CAT activity than cells transfected with plasmid pBmA.cat. Primer extension experiments (not shown) revealed that the increases in actin-cat gene expression mediated by BmAIE1 and HR3 resulted from increases in transcription rates and that the transcription start sites were the same as those observed with the basal actin expression cassette.

Finally, to demonstrate that the observed increases in transgene expression observed in the presence of the HR3 enhancer and the BmAIE1 factor were not transgene specific, we tested the activity of two additional plasmids, pBmA.jhe(kk) and pIE1/153A.jhe(kk), in transfected Bm5 cells. These two plasmids contain the ORF for a modified version of the enzyme juvenile hormone esterase (JHE), a secreted glycoprotein of Heliothis virescens, under the control of the basal actin promoter or under the control of the double-enhanced (HR3 and BmAIE1) actin promoter, respectively. As shown in Table I, JHE activity in the medium of cells transfected with plasmid pIE1/
dot-blotted onto a Hybond-N+ cated plasmids and collected 2 days posttransfection, and 10 µg aliquots of soluble cellular protein were assayed for CAT activity. Cells were transfected with the indicated plasmids and collected 2 days posttransfection, and 10 µg aliquots of soluble cellular protein were assayed for CAT activity.

**DISCUSSION**

The HR3 enhancer is based on three criteria: first, this fragment is 1,000-fold higher than that obtained from cells transfected with the basic recombinant vector pBmA.cat (kk). Therefore, the HR3-enhancing effect on transgene expression under the control of the silkmoth cytoplasmic gene promoter is promoter rather than transgene specific. Furthermore, considering that BmIE1 alone increases the activity of the actin promoter by approximately 100-fold (Table I), these results also demonstrate that the BmIE1 protein and the HR3 enhancer are capable of increasing each other’s effect on the in vitro transcriptional stimulation of the cytoplasmic actin gene promoter by an additional order of magnitude.

It was previously shown that AcNPV HRs can enhance the expression of some early baculovirus genes, such as 35k and 39k, and ie2, as well as that of the Rous sarcoma virus long terminal repeat promoter (3, 4, 15, 16).

In the present study, we have demonstrated that a DNA fragment containing the HR3 sequences of BmNPV can function in vitro as an enhancer for the cytoplasmic actin promoter of B. mori. The definition of the HR3-containing fragment as an enhancer is based on three criteria: first, this fragment is

**FIG. 2.** Enhancement of CAT expression by the HR3 element in transfected cells. Panel A, plasmids used for transfection contain the actin promoter region (Pact), the CAT ORF (cat), and the actin termination and polyadenylation region (TA). The arrow indicates the orientation of the HR3 element sequence relative to that in the BmNPV genome (Fig. 1A). Panel B, CAT activity in transfected Bm5 cells. Cells were transfected with the indicated plasmids and collected 2 days posttransfection, and 10 µg aliquots of soluble cellular protein were assayed for CAT activity. C and Ac-C indicate the positions of migration of the nonacetylated and acetylated CAT, respectively. Panel C, Dot Blot hybridization. 10,000 (top row), 20,000 (middle row), and 50,000 (bottom row) transfected cells were Dot Blotted onto a Hybond-N+ membrane and hybridized with the cat gene probe. Panel D, the HR3 element does not increase CAT expression in trans. Bm5 cells were transfected with plasmids pBmA.cat (lane 1), pBmA.cat and p13313 (lane 2) or pBmA.cat and p13315 (lane 3), and CAT activity in the transfected cells was assayed as in panel B. Other designations are as in panel B. Panel E, HR3-mediated enhancement of CAT expression in Sf21 cells. Sf21 cells were transfected with pBS (lane 1), pBmA.cat (lane 2), or pBmA.cat and p13315 (lane 3) and assayed for CAT activity as described in panel B. Other designations are as in panel B.

**FIG. 3.** Transcriptional activation of the actin gene promoter by the HR3 element. A, semiquantitative analysis of cat gene transcripts in transfected Bm5 cells by RT-PCR. Total nucleic acid was extracted from control Bm5 cells (lanes 1 and 2) and from cells transfected with pBS, pBmA.cat, or p13315 DNA and reverse-transcribed following addition of 0.1 µg of a chorion gene template (20). One-tenth of each reverse-transcription reaction was subjected to 26 or 20 rounds of PCR amplification in the presence of [α-32P]dCTP (20) using cat (top) or chorion gene-specific primers (bottom), respectively. The PCR products are indicated by arrows. B, nuclear run-on analysis of cat gene expression in transfected Bm5 cells. Aliquots (nanogram as indicated at the top of autoradiogram) of a denatured 900-bp BamHI fragment containing the CAT ORF were immobilized on hybridization membranes and hybridized with nuclear RNA probes obtained from pulse-labeled nuclei of cells transfected with pBmA.cat (top) or p13315 (bottom).

**FIG. 4.** Enhancement of actin-cat expression by BmIE1 and the HR3 element. Bm5 cells were co-transfected as indicated above the autoradiogram, and 10-µg aliquots of soluble protein from transfected cells were assayed for CAT activity 48 h posttransfection. The vectors used for the transfections were as follows: lane 1, pBmA.cat; lane 2, pBmA.cat and pBmIE1; lanes 3–6, p13314, p13315, p15316, and p15317, respectively; lanes 7–10, same as lanes 3–6 but in the presence of pBmE1 provided in the form of a separate plasmid; lanes 11 and 12, pIE1/133A.cat and pIE1/153A.cat, respectively; and lane 13, pBS.
and augments the level of the actin promoter in the absence of HR3 element (Fig. 4) (20), is acting exclusively on the actin promoter conserved during evolution.

fected cells, must be controlled by host factors that have been shown, however, that an AcNPV HR element-binding protein could be detected in insect cells following transfection with the AcNPV ie1 gene and that no obvious binding activity could be detected in control cells (37). Thus, it appears that in addition to binding to the actin promoter sequences, the BmIE1 protein may also bind to the HR3 enhancer and further augment its enhancing activity.

In conclusion, the work described in this paper has demonstrated that two baculovirus-specific genetic elements, the HR3 sequence-containing fragment and the ie1 gene product, are capable of enhancing cooperatively the activity of the silkmoth cytoplasmic actin gene promoter by three orders of magnitude in a virus-free environment. This finding provides an opportunity to develop a recombinant gene expression system in a baculovirus-free insect system. We anticipate that coupling of the enhanced actin promoter-based expression cassette with an appropriate antibiotic selection scheme for generating stably transformed cell lines containing chromosomally integrated copies of this recombinant expression cassette, should result in an insect cell expression system capable of yielding quantities of recombinant proteins comparable to those achieved through conventional infection with baculovirus expression vectors. Such a system should have the additional advantages of continuous production in a cellular environment that, in contrast to that generated by a baculovirus infection, supports continuously proper posttranslational modifications of recombinant proteins.

TABLE I
Baculovirus Enhancer in Transfected Insect Cells

| Transfection plasmid | Average units of activity | Average relative activity |
|----------------------|---------------------------|--------------------------|
| pBS                  | 0 (3)                     | 0                        |
| pBmA.cat             | 0.336 (3)                 | 1                        |
| HR3/pBmA.cat (p13315)| 29.47 (3)                 | 87.7                     |
| pBmA.cat + BmIE1     | 22.92 (2)                 | 68.2                     |
| HR3/pBmA.cat (p13315) + BmIE1 | 382.80 (1) | 1,139.3                  |
| BmIE1/HR3/pBmA.cat (pIE1/133A.cat and pIE1/153A.cat) | 549.09 (2) | 1,634.2                  |
| pBmA.jhe(kk)         | 1.18 × 10−3 (5)           | 1                        |
| pBmA.jhe(kk) + BmIE1 | 0.114 (5)                 | 96.9                     |
| BmIE1/HR3/pBmA.jhe(kk) | 1.37 (3)                 | 1,160                    |

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