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(54) Title: RECOMBINANT DNA ELEMENTS FOR THE EXPRESSION OF RECOMBINANT PROTEINS IN A HOST CELL

(57) Abstract: Reagents and methods are provided that allow for an improved expression of a recombinant protein. More specifically, the introduction of recombinant DNA elements into a host cell allows for the increased expression of a recombinant protein, an improvement of the correct folding of said protein and an increase in cell viability and proliferation of the host cell. These recombinant DNA elements can be introduced into host cells, for example, via a recombinant baculovirus, which has incorporated said elements. The recombinant DNA elements include nucleic acids encoding transcriptional regulators, such as IE-0 and IE-1, transcriptional enhancer elements, such as the homologous region (*hr*) and promoters.

## RECOMBINANT DNA ELEMENTS FOR THE EXPRESSION OF RECOMBINANT PROTEINS IN A HOST CELL

### FIELD OF THE INVENTION

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The present invention may be included in the field of biotechnology and it covers nucleic acid sequences comprising such as promoters, homologous regions (*hr*) as enhancers, and sequences encoding transcriptional regulators, for example, the baculovirus *Ac-ie-01* cDNA, or any combination thereof, which are able to increase the quality and efficiency of recombinant protein production. Moreover, the present invention is also directed to the vectors themselves comprising the above mentioned nucleic acid sequences of the invention, cells infected, transformed or transfected with those sequences or vectors, and methods for producing proteins by using the aforesaid sequences, vectors or cells.

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### STATE OF THE ART

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The baculovirus expression vector system (BEVS) is a well-established method for the production of recombinant proteins to be used as vaccines, therapeutic molecules or diagnostic reagents. With its potential for over-expression and rapid speed of development, BEVS is one of the most attractive choices for producing recombinant proteins for any purpose. The most employed baculovirus used in industry for recombinant protein expression is based on *Autographa californica* multinuclear polyhedrosis virus (*AcMNPV*) with *Spodoptera frugiperda* 9 (*Sf9*) or 21 (*Sf21*) insect cells as suitable expression hosts (1), as well as *Trichoplusia ni* (*T. ni*) insect larvae as living biofactories (2). Since the BEVS was developed in the 80's (3), hundreds of recombinant proteins, ranging from cytosolic enzymes to membrane-bound proteins, have been successfully produced in baculovirus-infected insect cells.

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Efforts have been made to increase BEVS productivity (4). A variety of transfer vectors are available for the construction of recombinant baculoviruses, encoding resident fusion proteins, which have been reported to improve protein expression, including maltose binding protein, glutathione S transferase, SUMO and KDEL retention signal. Other attempts related to improve the stability of

expressed proteins have been investigated focusing on two genes in the baculovirus genome, which are not essential for growth of the virus in cell culture, namely *chiA* (chitinase) and *cath* (cathepsin). *ChiA* deletion appears to improve the production of secreted proteins by accumulating the protein in the 5 endoplasmic reticulum and processing the proteins through the secretory pathway of the cells. Additionally, the prevention of the formation of cathepsin protease may also contribute to improved product stability from *chiA*-viruses. Novel insect cell lines, such as High-Five™ (Hi-5) or BTI-TnAo38 cell lines from *T. ni*, have recently been developed to increase the baculovirus productivity with 10 significant improvements in the final amount of heterologous protein recovery (5, 6).

Accelerating recombinant protein expression, so that protein expression takes place before the machinery of insect cells is severely impaired by the baculovirus 15 infection, would be an important improvement of the BEVS. Late expression, driven by the conventional strong virus promoters of *polyhedrin* (*polh*) or *p10* genes, has serious disadvantages in the foreign protein post-translational modifications. Baculovirus promoters that allow for earlier expression than the conventionally used *polh* or *p10* promoters have been characterized and been 20 used for heterologous protein production, but showed a reduced productivity (7).

Another possibility for improving the BEVS would be to increase preservation of cell integrity at late times post-infection by reducing the virus-induced cell death. 25 Reduction in the severe impairment of the insect cell machinery at late times post-infection caused by BEVS should not only increase the time frame for producing and accumulating recombinant proteins (secreted or not), but also allow more time for the folding of complex proteins or any post-translational modification of the produced proteins.

30 Some baculovirus DNA elements have been determined to be involved in the activation of late expression factor genes, which are necessary for virus propagation. One of them is the immediate early (ie) protein IE-1 and its splice

variant IE-0 from *AcMNPV* (*Autographa californica* multinuclear polyhedrosis virus). Translation of the *AcMNPV* mRNAs encoded by *Ac-ie-01* gene results in both IE-0 and IE-1 due to internal translation initiation. Both are thought to be critical mediators of baculovirus gene expression due to their potency as transcriptional regulators (8). Synthesized very early during infection, *AcMNPV* IE-1 is a 67-kDa dimeric DNA-binding protein that stimulates transcription in plasmid transfection assays through the activity of its N-terminal acidic domain (9, 10). IE-1 accumulates within the nucleus, where it is maintained through late times (11). Transactivation by IE-1 is enhanced by its binding as a homodimer to the baculovirus homologous region (*hr*) sequences, which function as transcriptional enhancers and origins of viral DNA replication. *AcMNPV* IE-0 is a 72.6-kDa 636 amino acid protein composed of 38 amino acids encoded by *orf141* (*exon0*), 16 amino acids encoded by the upstream nontranslated leader of *ie1*, and the entire 582 amino acid IE-1 protein. The final product is therefore identical to IE-1 except for the additional 54 amino acids fused to the N-terminus. Presumably due to their common sequences, IE-0 and IE-1 share biochemical activities, including *hr* enhancer binding and transcriptional regulation.

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There is a need of novel alternative BEVSs that allow a) stronger expression than the commercial BEVS that use the *polh* or *p10* promoters and b) long-term expression in the baculovirus system by reducing virus-induced cell damage.

25 **SUMMARY OF THE INVENTION**

The present invention provides products and methods for the improved expression of recombinant proteins using the BEVS.

30 The following items are preferred embodiments for allowing this improved expression:

1. Recombinant baculovirus comprising a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators, wherein the nucleic acid is selected from the group consisting of:

5 (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 1-5;

10 (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;

15 (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and

20 (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.

2. Recombinant baculovirus according to item 1, further comprising at least one recombinant homologous region (*hr*) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

30 3. Recombinant baculovirus according to item 2, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 27 (*hr1*).

4. Recombinant baculovirus according to item 2 or 3, wherein the promoter operably linked to the homologous region (*hr*) is selected from the group of nucleic acids comprising:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

5 (b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

10 5. Recombinant baculovirus according to any of the items 1-4, comprising a nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

15 6. Recombinant baculovirus according to any of the items 1-5, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of items 1-5.

20 7. Transfer vector suitable for producing a recombinant baculovirus according to any of the items 1-6, comprising said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof functioning as transcriptional regulators, further comprising a nucleic acid sequence suitable for integration or transposition in a baculovirus.

25 8. Transfer vector according to item 7, further comprising at least one recombinant homologous region (*hr*) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

30 9. Transfer vector according to item 8, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 27 (*hr1*).

10. Transfer vector according to item 8 or 9, wherein the promoter operably linked to the homologous region (*hr*) is selected from the group of nucleic acids comprising:

5 (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

15 11. Transfer vector according to any of the items 7-10, comprising a nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

20 12. Transfer vector according to any of the items 7-11, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of items 7-11.

25 13. Transfer vector according to any of the items 7-11, lacking a nucleic acid sequence encoding a recombinant protein.

14. Transfer vector according to any of the items 7-13, characterized in that the transfer vector is a bacmid.

30 15. Transfer vector according to any of the items 7-14, characterized in that the transfer vector is derived from any of the baculovirus expression systems "Bac-to-Bac®" (Invitrogen™), "BacPAK™" (Clontech™), "FlashBAC™" (Oxford Expression Technologies™), "BacuVance™"

(GenScript<sup>TM</sup>), "Bac-N-Blue DNA<sup>TM</sup>" (Invitrogen<sup>TM</sup>), "BaculoDirect<sup>TM</sup>" (Invitrogen<sup>TM</sup>), "BacVector<sup>®</sup>" 1000, 2000, 3000 (Novagen<sup>®</sup>), "DiamondBac<sup>TM</sup>" (Sigma-Aldrich<sup>®</sup>) or "BaculoGold<sup>TM</sup>" (BD Biosciences<sup>TM</sup>).

5 16. Cloning vector suitable for producing a recombinant baculovirus or transfer vector according to any of the items 1-15, comprising said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof functioning as transcriptional regulators, which is further suitable for bacterial replication.

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17. Cloning vector according to item 16, further comprising at least one recombinant homologous region (*hr*) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

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18. Cloning vector according to item 17, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 27 (*hr1*).

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19. Cloning vector according to item 17 or 18, wherein the promoter operably linked to the homologous region (*hr*) is selected from the group of nucleic acids comprising:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

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20. Cloning vector according to any of the items 16-19, comprising a nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

21. Cloning vector according to any of the items 16-20, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of items 16-20.

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22. Cloning vector according to any of the items 16-20, lacking a nucleic acid sequence encoding a recombinant protein.

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23. Nucleic acid sequence suitable for producing a recombinant baculovirus, transfer vector or cloning vector according to any of the items 1-22, comprising said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof functioning as transcriptional regulators.

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24. Nucleic acid sequence according to item 23, further comprising at least one recombinant homologous region (*hr*) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

20

25. Nucleic acid sequence according to item 24, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 27 (*hr1*).

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26. Nucleic acid sequence according to item 24 or 25, wherein the promoter operably linked to the homologous region (*hr*) is selected from the group of nucleic acids comprising:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

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(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most

preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

27. Nucleic acid sequence according to any of the items 23-26, comprising a

5 nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

28. Nucleic acid sequence according to any of the items 23-27, further

10 comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of items 23-27.

15 29. Nucleic acid sequence according to any of the items 23-27, lacking a nucleic acid sequence encoding a recombinant protein.

30. A host cell infected, transfected, transduced or transformed with the

20 recombinant baculovirus, transfer vector, cloning vector or nucleic acid sequence of any of the items 1-29.

31. Infected, transfected, transduced or transformed host cell according to

item 30, characterized in that it is an insect cell line.

25 32. Infected, transfected, transduced or transformed host cell according to item 30 or 31, characterized in that it is derived from an insect belonging to the Lepidoptera or Diptera genus.

33. Infected, transfected, transduced or transformed host cell according to

30 any of the items 30-32, characterized in that it is derived from *Trichoplusia ni*, *Spodoptera frugiperda*, *Ascalapha odorata*, *Bombyx mori*, *Drosophila melanogaster* or *Aedes aegypti*.

34. Infected, transfected, transduced or transformed host cell according to

35 any of the items 30-33, characterized in that it is a cell line selected from

the group consisting of Hi-5™, *Sf9*, *Sf21*, BTI-Tn5B-1, Tn368, Express<sup>®</sup>, BTI-TnAo38, ATC-10 and Schneider's *Drosophila* Line 2.

35. Culture medium comprising the recombinant baculovirus, transfer vector,  
5 cloning vector or nucleic acid sequence of any of the items 1-29.

36. Method for producing a recombinant protein that comprises the infected,  
transfected, transduced or transformed host cell according to items 30-34  
and the extraction and purification of the recombinant protein by  
10 conventional means.

37. Method for producing a recombinant protein according to item 36,  
wherein the recombinant protein is selected from the group comprising  
subunit monomeric vaccine, subunit multimeric vaccine, virus like  
15 particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting  
factor, anticoagulant, receptor, hormone and diagnostic protein reagent.

38. Use of the transfer vector according to any of the items 7-15 for producing  
a recombinant baculovirus according to any of the items 1-6.

20 39. Use of the cloning vector according to any of the items 16-22 for  
producing a recombinant baculovirus or transfer vector according to any  
of the items 1-15.

25 40. Use of the nucleic acid sequence according to any of the items 23-29 for  
producing a recombinant baculovirus, transfer vector or cloning vector  
according to any of the items 1-22.

41. Transgenic cell line comprising a transgene, which is a nucleic acid  
30 selected from the group consisting of:

(a) a nucleic acid containing the nucleotide sequence indicated in any  
of SEQ ID NO: 1-5;

(b) a nucleic acid sequence having a sequence identity of at least 70%,  
preferably at least 75%, more preferably at least 80%, more  
35 preferably at least 85%, more preferably at least 90% and most

preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;

5 (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and

(d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.

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42. Transgenic cell line according to item 41, which is of insect, avian or mammalian origin.

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43. Method for producing a recombinant protein that comprises growth of a transgenic cell line according to item 41 or 42 and the extraction and purification of the recombinant protein by conventional means.

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44. Nucleic acid sequence comprising a nucleic acid sequence functioning as a promoter in a recombinant baculovirus, wherein the nucleic acid sequence is selected from the group consisting of:

(a) a nucleic acid containing the nucleotide sequence indicated in SEQ ID NO: 12, 14 or 15; and

(b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in SEQ ID NO: 12, 14 or 15.

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## BRIEF DESCRIPTION OF THE FIGURES

**Figure 1:** Schematic representation of the baculovirus recombinant DNA elements of the invention, comprising four principal elements: a sequence 5 encoding for transcriptional regulators (**A**; e.g. IE0 and IE1), which expression is driven by a promoter (**B**; e.g. *polh* or *pB2<sub>9</sub>*); an enhancer homologous region (*hr*) sequence (**C**; e.g. *hr1*), upstream of the promoters (**D**; e.g. *p10*, *polh*, *pB2<sub>9</sub>p10* or 10 *p6.9p10*) driving the expression of the foreign gene coding for the recombinant protein. The scheme shows the theoretical mechanism of interaction between the recombinant DNA elements of the present invention that results in the unprecedented overexpression of the recombinant protein.

**Figure 2:** Different strategies that result in the generation of recombinant baculoviruses by the “Bac-to-Bac®” cloning system (invitrogen™).

**Figure 3:** General scheme for the generation of cloning, donor and transfer vectors compatible with other commercial technologies used to generate recombinant baculoviruses.

**Figure 4: A)** Fluorimetric assays for the measurement of the increment in GFP protein accumulated in infected *Sf21* cells at different times post-infection when expressed in the baculovirus expression cassettes of the invention, containing *hr1p10*, *hr1pB2<sub>9</sub>p10* or *hr1p6.9p10* and the *Ac-ie-01* cDNA expressed under the control of *pB2<sub>9</sub>* or *polh* promoters. All GFP expression levels were compared to that obtained with the *polh* promoter in a conventional baculovirus vector. The graph represents the mean values of three independent expression experiments for each baculovirus with standard deviations lower than 5 % in each case. This figure also shows representative fluorescence micrographs showing *Sf21* cells at different times post-infection with a wild-type baculovirus (control), with a conventional baculovirus expressing the GFP under the control of *polh* promoter or with a baculovirus expressing the GFP by the expression cassette of the invention *polhAc-ie-01/hr1pB2<sub>9</sub>p10GFP*. Cells were infected in **A)** and **B)** at a multiplicity of infection (MOI) of 5 or in **C)** at a MOI of 0.1.

**Figure 5: A)** Comparison of the amounts of recombinant GFP protein in *Sf21* insect cells grown in monolayer at different times post-infection expressed by a conventional baculovirus vector under the control of a *polh* promoter (light grey) or by the baculovirus vector engineered with an expression cassette of the invention, composed of the elements *polhAc-ie-01/hr1pB29p10GFP* (dark grey) and measured by microfluidic protein analysis (Experion™; BioRad™, USA). Cells were infected at a MOI of 5 with both viruses; **B)** Comparison of recombinant GFP protein accumulated in *Sf9* insect cells grown in suspension at different times post-infection expressed by a conventional baculovirus vector under the control of a *polh* promoter (light grey) or by the baculovirus vector engineered with an expression cassette of the invention, composed of the elements *polhAc-ie-01/hr1p6.p10GFP* (dark grey) and measured by microfluidic protein analysis (Experion™; BioRad™, USA). Cells were infected at a MOI of 0.1 with both viruses; **15** Discontinued lines indicate the percentage of increment of recombinant GFP produced by the baculoviruses containing the expression cassette of the invention in comparison to that obtained with the conventional baculovirus expressing the GFP under the control of the *polh* promoter; **C)** Coomassie blue staining of SDS-PAGE gels resolving the infected cell extracts of the experiment described in panel A; **D)** Coomassie blue staining of SDS-PAGE gels resolving the infected cell extracts of the experiment described in panel B.

**Figure 6.** *Sf9* insect cells were cultured in suspension and infected by a baculovirus overexpressing the *Ac-ie-01* cDNA under the control of *polh* or by a **25** baculovirus expressing a reporter protein GFP in the context of the baculovirus expression cassette *polhAc-ie-01/hr1p6.9p10GFP* of the present invention to assess the cell density **(A)** and viability **(B)** of these cells. As a control, a conventional baculovirus expressing the GFP protein under the control of *polh* was used. Cells were infected in suspension at a MOI of 0.1. **(A)** The cells were **30** counted at different times post-infection (0, 24 and 48 hours) to calculate the cell density. A more detailed analysis of the precise moment in which cell proliferation is produced by the overexpression of the *Ac-ie-01* cDNA is shown in the insert for cells infected with *polhGFP* or *polhAc-ie-01/hr1p6.9p10GFP*. **(B)** Cell

viability was assessed by Trypan blue staining (dilution 1:1 of suspended cells and colorant at 0.4 % in PBS buffer). This staining allows the differentiation between live and death cells. Cell viability was calculated by the percentage of living cells with respect to the total number of cells at different times post-infection (from 0 to 120 hours). Micrographs of Hi-5™ insect cell monolayers infected at a MOI of 5 with a control conventional baculovirus overexpressing the reporter protein GFP under the *polh* promoter (C) or with a baculovirus overexpressing the *Ac-ie-01* cDNA under the control of the *polh* promoter (D). Micrographs were obtained at 96 hours post-infection at a 20X magnification in an inverted microscope Leica™ DMIL™.

**Figure 7:** A) *Sf9* insect cells were infected by a conventional baculovirus expressing the GFP protein under the control of the *polh* promoter (1) or by a baculovirus vector engineered with the expression cassette of the invention 15 containing the elements *polhAc-ie-01/hr1p6.9p10GFP*, overexpressing the transcriptional regulators encoded by *Ac-ie-01* cDNA (2). Cells were sampled at different times post-infection (0 to 120 hours) and cell extracts analysed by SDS-PAGE and Western blot with an antiserum against GFP or against the cellular actin protein. B) The functionality of the GFP protein expressed in *Sf9* insect cells 20 as analyzed in panel A was measured by fluorimetry. The fluorescence values obtained at different hours post-infection with the GFP protein produced by a conventional recombinant baculovirus (grey bars) was compared to that produced by the recombinant baculovirus with the baculovirus cassette of the present invention (black bars).

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**Figure 8:** Schematic representation of the preferred elements contained in the baculovirus expression cassettes of the invention, comprising encoding sequences for transcriptional regulators, homologous regions (*hr*) enhancing the transcription from promoter(s) of a foreign gene encoding a recombinant 30 protein.

**Figure 9:** Sequence analysis of the *pB2* promoter isolated from *T. ni* and determination of the transcriptional regulatory element *pB2<sub>9</sub>* sequence. A)

Nucleotide sequence of the *pB2* promoter region of BJHSP2 (Genbank accession no **U41640**) isolated from *T. ni*. The previously described transcription start site is indicated by a triangle, whereas the TATA box is underlined and the potential cis-acting elements are enclosed in boxes in the *pB2* sequence. Shaded nucleotide 5 residues indicate the sequence incorporated into the *pB2<sub>9</sub>* promoter. The predicted Br-C and EcR putative binding sites are also indicated in the figure by transparent boxes **B)** Schematic illustration of the *pB2* DNA fragment and its derivative promoter *pB2<sub>9</sub>*. Their promoter activity was analyzed by fluorimetric analysis using the GFP protein as the reporter gene. The GFP expression was 10 quantified 72 hours post- infection in all experiments and is indicated as the arithmetic media with standard deviations of three independent experiments.

**Figure 10:** GFP expression levels mediated by the use of different promoters or combination of promoters. **A)** Fluorimetric analysis at 24 hours post-infection of 15 *Sf21* cells infected with different recombinant baculoviruses expressing the GFP under the control of different individual or chimeric promoters. **B)** Time course study of the GFP expression in *Sf21* cells infected with the same recombinant baculoviruses as in panel A, measured by a fluorimetric assay. All the experiments were done at a MOI of 5 and the figure shows the arithmetic media 20 of three independent experiments with the corresponding standard deviations.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention improves the expression of recombinant proteins in the 25 BEVS by means of the introduction of recombinant DNA elements into baculoviruses.

The recombinant DNA elements of the present invention are sequences that cause the expression of baculovirus transcriptional regulators above endogenous 30 levels and optionally enhancer homologous regions (*hr*) and promoters operably linked to these aforementioned elements.

Furthermore, the recombinant DNA elements may form part of an expression cassette.

“Expression cassette” refers to a nucleic acid sequence that contains 5 recombinant DNA elements, which control (e.g. the promoter) and/or are required (e.g. the gene itself) for gene expression. The expression cassette can be introduced in a recombinant vector or baculovirus.

10 The recombinant DNA elements may be incorporated in a single nucleic acid sequence, cloning vector, transfer vector, recombinant baculovirus or cell. However, they can also be present in different nucleic acid sequences, cloning vectors, transfer vectors or recombinant baculoviruses and be introduced into the same cell.

15 The present invention surprisingly shows that introduction of sequences that cause the expression of baculovirus transcriptional regulators above endogenous levels and optionally the introduction of an enhancer homologous region (*hr*) sequence, a promoter or a combination of promoters is able to increase the production of a recombinant protein to unprecedented levels from early (6 to 8 20 hours post-infection) to late (48 to 96-120 hours post-infection) times post-infection.

25 Additionally, the introduction of these recombinant DNA elements also increases the proliferation of baculovirus-infected cells (particularly at early times post-infection), the viability at late times post-infection and the integrity of the molecular cell machinery and morphology of said baculovirus-infected cells. An improvement in the integrity of cell functions during baculovirus infections also contributes to the correct post-translational processing of the recombinant protein.

30

Introduction of these recombinant DNA elements also increases the recombinant protein production in host cells compared to the conventional *polh* or *p10* promoters.

Thus, one aspect of the invention relates to a recombinant baculovirus that contains a nucleic acid sequence that allows for the expression above endogenous levels of transcriptional regulators. In a preferred embodiment, the 5 transcriptional regulators are IE-1, IE-0 and/or fragments thereof.

“Baculovirus” refers to a family of infectious viruses for invertebrates, mainly infecting insects and arthropods. A “recombinant baculovirus” has further introduced recombinant DNA through, for example, homologous recombination 10 or transposition. The recombinant baculovirus preferably originates from *AcMNPV*.

“Recombinant DNA” refers to a form of artificial DNA that is engineered through the combination or insertion of one or more DNA strands, thereby combining 15 DNA that would normally not occur together.

“Recombinant DNA element” refers to a functional element within recombinant DNA, such as a promoter, enhancer or a gene. As mentioned above, the recombinant DNA elements of the present invention are sequences that cause 20 the expression of baculovirus transcriptional regulators above endogenous levels, enhancer homologous regions (*hr*) and promoters operably linked to these aforementioned elements.

“Transcriptional regulator” refers to a regulatory protein that has the ability to 25 modulate the transcription of specific genes by, for example, binding to enhancer or repressor regions and/or recruiting further proteins that are involved in transcription.

IE-1 and its splice variant IE-0 are transcriptional regulators that are 30 endogenously expressed during baculovirus infection. According to the present invention, IE-1, IE-0 and/or fragments thereof are recombinantly expressed to increase the total level of these proteins above endogenous levels. This can be achieved through, for example, introducing further copies of the endogenous

gene or manipulating the expression of the promoter of the endogenous gene. Further, copies of the endogenous genes can be introduced as transgenes under the control of a suitable promoter such as *polh* or *pB29*.

5 The expression level of the proteins IE-1, IE-0 and/or fragments thereof can be determined at both the mRNA and at the protein level with methods conventionally known to the person skilled in the art, such as quantitative PCR and Western Blot analysis.

10 According to the invention, IE-1, IE-0 and fragments thereof are encoded by the nucleic acids of SEQ ID NO: 1 (also referred to as *Ac-ie-01*) to SEQ ID NO: 5. SEQ ID NO: 1 is the *Ac-ie-01* cDNA that encodes both IE-1 and IE-0, SEQ ID NO: 2 is the coding sequence (CDS) of IE-1 and SEQ ID NO: 3 is the CDS of IE-0. SEQ ID NO: 4 and 5 are the CDSs of the N-terminal domains of IE-1 and IE-0 respectively that

15 retain the catalytic transcriptional regulator activity. The proteins that are encoded by SEQ ID NO: 2-5 are represented by SEQ ID NO: 6-9 respectively.

The present invention furthermore discloses variants of SEQ ID NO: 1-9 that are or encode amino acids that are able to function as a transcriptional regulator.

20 These variants are nucleic or amino acids whose nucleotide or amino acid sequence differs in one or more positions from these parental nucleic or amino acids, whereby differences might be additions, deletions and/or substitutions of nucleotides or amino acid residues.

25 Nucleic and amino acid sequences of the present invention shall be distinguished from other nucleic and amino acid sequences by their degree of sequence identity or similarity respectively as determined using EMBOSS Needle with the default parameters ([http://www.ebi.ac.uk/Tools/psa/emboss\\_needle/](http://www.ebi.ac.uk/Tools/psa/emboss_needle/)). Methods for the generation of such variants include random or site directed

30 mutagenesis, site-saturation mutagenesis, PCR-based fragment assembly, DNA shuffling, homologous recombination *in vitro* or *in vivo*, and methods of gene-synthesis.

The sequence of the variants of SEQ ID NO: 1-5 is at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% identical to the sequences of SEQ ID NO: 1-5.

5

The sequence of the variants of SEQ ID NO: 6-9 is at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% similar to the sequences of SEQ ID NO: 6-9.

10

In a preferred embodiment, the recombinant baculovirus of the present invention further contains, in addition to the nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof, a recombinant homologous region (*hr*) that can enhance the expression of a recombinant protein by being operably linked to the respective promoter.

15

Homologous regions, *hr*, are comprised of repeated units of about 70-bp with an imperfect 30-bp palindrome near their center. Homologous regions are repeated at eight locations in the *AcMNPV* genome with 2 to 8 repeats at each side. Homologous regions have been implicated as both transcriptional enhancers and origins of baculovirus DNA replication.

20

“Enhancer region” refers to a control sequence, whose binding by transcriptional regulators increases the level of transcription of associated genes.

25

“Recombinant protein” refers to a protein that originates from recombinant DNA. Such proteins can be used for the benefit of humans and animals and may have industrial, commercial or therapeutic application.

30

“Being operably linked” refers to two nucleic acid sequences that are connected in a way that one influences the other in terms of, for example, transcriptional regulation.

“Promoter” refers to a DNA sequence to which RNA polymerase can bind to initiate transcription. The sequence may further contain binding sites for various proteins that regulate transcription, such as transcription factors. The promoter

5 sequence may be composed of different promoter fragments (either different or the same fragments) that are localized closely in the DNA sequence and may be separated by linkers or spacer. Such promoters are referred to as chimeric promoters.

10 The enhancer homologous region sequence *hr* upstream of the promoter/s is preferably *hr1* (SEQ ID NO: 27). The promoters that drive the expression of the recombinant protein are preferably selected from the group comprising SEQ ID NO: 10-16 or a sequence that is able to function as a promoter and has at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at 15 least 85%, more preferably at least 90% and most preferably at least 95% identity with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

20 In a preferred embodiment, the nucleic acid sequence comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

25 As indicated above, the recombinant promoters, sequences encoding transcriptional regulators and enhancer regions of the present invention do not need to form part of a single molecule, instead these sequences may form part of distinct molecules as long as they are operably linked, i.e. contained within the same cells.

30 The recombinant baculovirus of the present invention preferably comprises a nucleic acid sequence encoding a recombinant protein. This nucleic acid sequence is operably linked to the nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof and optionally to a homologous region (*hr*).

In one embodiment, the present invention discloses a host cell that is infected, transfected, transduced or transformed with the recombinant baculovirus, transfer vector, cloning vector or nucleic acid sequence of the present invention. Preferably, the host cell is kept in cell culture. The host cell is preferably an insect cell line, more preferably a cell line derived from an insect belonging to the Lepidoptera or Diptera genus, more preferably the host cell is derived from the group consisting of *Trichoplusia ni*, *Spodoptera frugiperda*, *Ascalapha odorata*, *Bombyx mori*, *Drosophila melanogaster* and *Aedes aegypti* and most preferably it is selected from the group of insect cell lines consisting of Hi-5™, *Sf9*, *Sf21*, BTI-Tn5B-1, Tn368, ExpressSf+®, BTI-TnAo38, ATC-10 and Schneider's *Drosophila* Line 2. The host cell may be cultured in monolayer or in suspension.

In a further aspect the invention discloses methods for producing a recombinant protein using the host cell of the invention. After expression of the recombinant protein, extraction and purification of the recombinant protein is done by conventional means.

In a preferred embodiment for protein production, the host cells are cultured in suspension (bioreactors), at densities between  $2 \times 10^6$  to  $8 \times 10^6$  cells per ml, depending on the cell line and the fermentation procedure used. Furthermore, cells are infected at a MOI of 0.1 to 1.

The recombinant protein that is preferably produced by the methods of the present invention is a protein selected from the group comprising subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone and diagnostic protein reagent.

One aspect of the invention relates to the use of the recombinant baculovirus, transfer vector, cloning vector or nucleic acid sequence of the present invention in a culture medium. In a preferred embodiment the culture medium comprises a baculovirus of the present invention.

The present invention discloses a transfer vector that can be used to produce the recombinant baculovirus of the present invention and comprises said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, in addition to a sequence suitable for integration or 5 transposition in a baculovirus.

Transfer vectors generally permit the insertion of genetic information into a baculovirus.

10 The transfer vector preferably contains in addition to (i) the sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, (ii) a recombinant homologous region (*hr*) linked to (iii) a suitable promoter for driving the expression of a recombinant protein. The preferred combinations of these recombinant DNA elements are as described above for the 15 recombinant baculovirus.

In one preferred aspect, the transfer vector comprises a nucleic acid sequence encoding said recombinant protein, whereas in another preferred embodiment the transfer vector lacks such sequence.

20 In a preferred embodiment, the transfer vector is a bacmid.

"*Bacmid*" refers to a plasmid construct which contains the DNA sequence sufficient for generating a baculovirus when transfected into a cell.

25 In a further preferred embodiment, the transfer vector is derived from any of the commercially available baculovirus expression systems "Bac-to-Bac®" (Invitrogen™), "BacPAK™" (Clontech™), "FlashBAC™" (Oxford Expression Technologies™), "BacuVance™" (GenScript™), "Bac-N-Blue DNA™" (Invitrogen™), "BaculoDirect™" (Invitrogen™), "BacVector®" 1000, 2000, 3000 30 (Novagen®), "DiamondBac™" (Sigma-Aldrich®) or "BaculoGold™" (BD biosciences™).

The present invention discloses a cloning vector that can be used to produce the recombinant baculovirus and/or transfer vector of the present invention and comprises said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, which is further suitable for bacterial 5 replication.

“Cloning vector” refers to any vector that is suitable for cloning, which generally involves the presence of restriction sites, an origin of replication for bacterial propagation and a selectable marker.

10

The cloning vector preferably contains in addition to (i) the sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, (ii) a recombinant homologous region (*hr*) linked to (iii) a suitable promoter for driving the expression of a recombinant protein. The preferred 15 combinations of these recombinant DNA elements are as described above for the recombinant baculovirus.

In one preferred aspect, the cloning vector comprises a nucleic acid sequence 20 encoding said recombinant protein (also referred to as the “donor vector”), whereas in another preferred embodiment the cloning vector lacks such sequence.

The present invention discloses a nucleic acid sequence that can be used to produce the recombinant baculovirus, transfer vector and/or cloning vector of 25 the present invention and comprises said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof.

The nucleic acid sequence preferably contains in addition to (i) the sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments 30 thereof, (ii) a recombinant homologous region (*hr*) linked to (iii) a suitable promoter for driving the expression of a recombinant protein. The preferred combinations of these recombinant DNA elements are as described above for the recombinant baculovirus.

In one preferred aspect, the nucleic acid sequence comprises a nucleic acid sequence encoding said recombinant protein, whereas in another preferred embodiment the nucleic acid sequence lacks such sequence.

5

The transient expression of the *Ac-ie-01* cDNA by a baculovirus system surprisingly confers unique properties of virus resistance and cell proliferation to the cells. This suggests that the overexpression of the transcriptional regulators encoded by this gene activates other virus or cellular genes which 10 may be responsible for such interesting biotechnology applications related to the productivity increase and resistance to different cellular stresses. A potential application derived from this study could be the generation of insect, avian or mammalian transgenic cells which may represent highly productive cell lines to be used for recombinant protein production or virus propagation with potential 15 use in the production of conventional vaccines based on attenuated or inactivated viruses.

Hence, one aspect of the invention relates to a transgenic cell line that carries as a transgene the sequences of SEQ ID NO: 1-5 or variants thereof or sequences 20 that encode the proteins of SEQ ID NO: 6-9 or variants thereof as defined above. The transgenic cell line is preferably of mammalian, avian or insect origin. In a further embodiment, the transgenic cell line of the invention can be used for the production of a recombinant protein, which is extracted and purified by conventional means.

25

“Transgenic cell line” refers to a cell line that contains a gene that was transferred into the cell.

Based on the surprising finding that the recombinant baculovirus promoter *pB2<sub>9</sub>*, 30 (SEQ ID NO: 14) and the chimeric recombinant baculovirus promoters *pB2<sub>9</sub>p10* (SEQ ID NO: 12) and *pB2p10* (SEQ ID NO: 15) allow an improved expression as compared to conventional promoters, such as *pB2*, a further aspect of the present invention relates to nucleic acid sequences comprising such sequence or

sequence variants of SEQ ID NO: 12, 14 or 15 that are at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% identical to the sequences of SEQ ID NO: 12, 14 or 15.

5

## SUMMARY OF SEQUENCES

SEQ ID NO:	Name:
1	Complete <i>Ac-ie-01</i> cDNA
2	Protein coding sequence (CDS) of IE-1
3	CDS of IE-0
4	CDS of the IE-1 N-terminal domain
5	CDS of the IE-0 N-terminal domain
6	IE-1 protein
7	IE-0 protein
8	IE-1 N-terminal domain protein
9	IE-0 N-terminal domain protein
10	<i>polh</i> (promoter)
11	<i>p10</i> (promoter)
12	<i>pB29p10</i> (promoter)
13	<i>p6.9p10</i> (promoter)
14	<i>pB29</i> (promoter)
15	<i>pB2p10</i> (promoter)
16	<i>polhp10</i> (promoter)
17	<i>polhAc-ie-01/hr1p10</i>
18	<i>polhAc-ie-01/hr1pB29p10</i>
19	<i>polhAc-ie-01/hr1p6.9p10</i>
20	<i>pB29Ac-ie-01/hr1p10</i>
21	<i>pB29Ac-ie-01/hr1pB29p10</i>
22	<i>pB29Ac-ie-01/hr1p6.9p10</i>
23	<i>polhAc-ie-01/hr1polh</i>
24	<i>pB29Ac-ie-01/hr1polh</i>

SEQ ID NO:	Name:
25	<i>polhAc-ie-01/hr1polhp10</i>
26	<i>pB29Ac-ie-01/hr1polhp10</i>
27	<i>Homologous region enhancer hr1</i>
28	<i>polhAc-ie-01</i>
29	<i>polhGFP</i>
30	<i>p10pB2 (promoter)</i>
31	<i>polhpB2 (promoter)</i>
32	<i>pB2polh (promoter)</i>
33	<i>pB29polh (promoter)</i>
34	<i>pB2 (promoter)</i>
35	<i>hr1polhpB2</i>
36	<i>hr1pB2polh</i>
37	<i>hr1p10pB2</i>
38	<i>hr1pB2p10</i>
39	<i>hr1pB29polh</i>
40	<i>hr1pB29p10</i>

**DEPOSITION OF MICROORGANISMS ACCORDING TO THE BUDAPEST TREATY**

5 Plasmids were deposited in the Spanish Type Culture Collection (CECT) ([www.cest.org](http://www.cest.org)); University of Valencia, Parc Científic Universitat de València; Catedrático Agustín Escardino, 9; 46980 Paterna (Valencia), Spain, with the accession number CECT 8031, on the date October 4, 2011.

## EXAMPLES

**Example 1. Overexpression of baculovirus transcriptional regulators potentiates the enhancer function of a homologous region *hr* functionally**

5 **linked to a promoter increasing the expression of a recombinant protein in a baculovirus vector expression system (BEVS).**

Immediate early viral proteins encoded by the *Ac-ie-01* cDNA, i.e. IE-1 and IE-0, from *AcMNPV* are potent transcriptional regulators in the baculovirus.

10 Transactivation mediated by these proteins is enhanced by their binding as a homodimer to the baculovirus homologous region (*hr*) sequences, which act as transcriptional enhancers. *AcMNPV* IE-1/IE-0 are 67-72 kDa dimeric DNA-binding proteins that stimulate transcription in plasmid transfection assays through the activity of its N-terminal acidic domain (7, 8). Synthesized very early  
15 during infection, IE-1 and IE-0 accumulate within the nucleus, where they are maintained through late times. Using the dual plasmid pFastBac™ (invitrogen™), the *Ac-ie-01* cDNA was cloned under the control of the *polh* promoter. In the same plasmid, but in another locus, the GFP encoding gene was cloned downstream of the *hr1pB29p10* chimeric promoter that was previously  
20 synthesized and contains the homologous region *hr1* fused to the promoters *pB29* and *p10*. Promoter *pB29* is a DNA fragment derived from the promoter *pB2* which drives the expression of the Basic juvenile hormone-suppressible protein 2 (BJHSP-2) in *T. ni* lepidopter. Fragment *pB29*, comprising a sequence consisting of 436 nucleotides derived from the *pB2* promoter, showing higher expression  
25 levels than the full-length insect-derived promoter when incorporated into a baculovirus expression vector. A schematic representation of the resulting baculovirus expression cassette of the present invention and the putative function of the recombinant DNA elements is shown in **Figure 1**. The resulting plasmid was used to generate a recombinant baculovirus by the "Bac-to-Bac®"  
30 system (invitrogen™). In parallel, a conventional baculovirus expressing the GFP protein under the control of *polh* promoter was generated by the same system.

The expression of GFP protein mediated by the different baculoviruses was studied by fluorimetry at different times post-infection in *Sf21* cells cultured in monolayer. Compared to a conventional baculovirus expressing the protein under the control of the *polh* promoter, the expression level of GFP was about 2 times higher with the baculovirus expression cassette containing the transcriptional regulators and enhancer sequence when used at a MOI of 5 (Figure 4A) and 4 times higher when used at a MOI of 0.1 (data not shown). These differences in protein accumulation were also observed in Hi-5™ cells (data not shown), suggesting that the baculovirus expression cassette of the invention could be used to produce recombinant proteins in different insect cell lines used in research and industry.

Importantly, in infected cells observed by fluorescence microscopy, the GFP expression mediated by the baculovirus expression cassette of the present invention was detected earlier than when the conventional promoter *polh* was used for driving GFP expression and, moreover, the fluorescence intensity of infected cells was significantly higher (Figure 4B). Fluorescent cells were detected as early as 16 hours post-infection when infected at a MOI of 5 with the recombinant baculovirus containing the expression cassette of the invention, and said GFP expression was increasing along the time of infection (Figure 4B). These marked differences among novel and control recombinant baculoviruses were also observed at a low MOI of 0.1 (Figure 4C).

To analyze the influence of the promoter used for the overexpression of the transcriptional regulators, the *Ac-ie-01* cDNA was also cloned in the expression cassette described above under the control of *pB2<sub>9</sub>* promoter in substitution of the *polh* promoter. Independently of the promoter used to drive the *Ac-ie-01* cDNA expression, the GFP accumulation was higher than the one observed when using a conventional baculovirus in which the reporter protein was expressed under the control of the *polh* promoter without *Ac-ie-01* and *hr1* elements in the expression cassette (Figure 4A). In a similar way, the absence of *Ac-ie-01* cDNA in an expression cassette containing the *hr1* enhancer linked to *pB2<sub>9</sub>p10* chimeric promoter also resulted in lower expression levels of the GFP with respect to that

containing the *Ac-ie-01* cDNA (data not shown).

**Example 2. Transcriptional regulators encoded by *Ac-ie-01* cDNA potentiate other *hr1* *cis*-linked baculovirus promoters.**

5

To analyze if transcriptional regulators encoded by *Ac-ie-01* in combination with an enhancer sequence increase the expression mediated by other baculovirus promoters, chimeric or not (*p6.9p10* or *p10*), *cis*-linked to the transcription enhancer homologous region 1 (*hr1*), we generated a new set of baculovirus 10 expression cassettes and their corresponding *AcMNPV* recombinant baculoviruses. These expression cassettes contained the *Ac-ie-01* cDNA cloned under the control of the *polh* promoter and the GFP encoding gene downstream of the *hr1p10* or *hr1p6.9p10* promoters. The expression levels were measured by 15 fluorimetric analysis of extracts from insect cells infected with the different baculoviruses. As a control, the conventional recombinant baculovirus expressing the GFP protein under the control of *polh* promoter was used. The results observed demonstrated that transcriptional regulators encoded by *Ac-ie-01* cDNA in combination with a homologous sequence *hr1* were also able to promote unprecedented expression levels of the reporter protein GFP expressed 20 under the control of other baculovirus promoters or combination of promoters (chimeric) (**Figure 4A**). In the case of the expression cassette using the chimeric promoter composed of *p6.9* and *p10*, the recombinant protein production was the highest among different recombinant baculoviruses assayed (about 2.5 times 25 higher compared to the control at 72 hours post-infection).

25

A further quantification of the GFP production in both *Sf21* insect cells in monolayer and *Sf9* insect cells in suspension mediated by a conventional baculovirus or by a baculovirus incorporating the expression cassette of the present invention containing the elements *polhAc-ie-01/hr1pB29p10GFP*, was 30 carried out by microfluidic protein analysis (Experion™; BioRad™, USA). Insect cells in monolayer were infected at a high MOI of 5 and in suspension at a low MOI of 0.1. **Figure 5A** shows the percentages of recombinant GFP produced with respect to the total soluble cell proteins at different times post-infection (24 to

72 hours) and the relative increases of productivity with respect to the conventional baculovirus. At the latest time analyzed, the baculovirus containing the expression cassette of the invention reached levels of the recombinant GFP of more than 40 % of the total cellular protein. Significant differences in the GFP 5 band intensities between the assayed baculoviruses were visible in a Coomassie blue stained SDS-PAGE gel in which cellular extracts from infected cells were resolved (**Figure 5C**). When insect cells were cultured in suspension and infected with each baculovirus, differences in recombinant protein productivity were even higher than those detected in insect cells cultured in monolayer and 10 infected at a high MOI of 5. **Figure 5B** shows such differences found in GFP production between 24 to 120 hours post-infection. Interestingly, when the baculovirus incorporating the expression cassette of the invention was used, recombinant protein productivity increased along time, reaching maximum levels at 120 hours post-infection. In contrast, the recombinant protein produced 15 by a conventional baculovirus reached maximum levels at 72 hours post-infection decreasing at later times post-infection (**Figure 5B**). An increase of productivity of more than 20 times was observed with the baculovirus expression cassette of the present invention at very late times post-infection. Similar results were observed in Hi-5™ cells (data not shown), demonstrating 20 that the expression cassette of the invention can be employed to produce recombinant proteins in different insect cell lines used in research and industry. Significant differences in the GFP band intensities between the assayed baculoviruses from cells cultured in suspension were also visible in a Coomassie blue stained SDS-PAGE gel in which cellular extracts from infected cells were 25 resolved (**Figure 5D**).

**Example 3. The baculovirus expression cassettes of the invention induce cell proliferation and increase cell viability through the transcriptional regulators encoded by the *Ac-ie-01* cDNA**

30

We observed by microscopy that recombinant baculoviruses incorporating the baculovirus expression cassettes of the invention have interesting properties related to a decrease in the virus-induced cytopathic effects and an increase of

the cell density in cultures. To quantify these phenomena and to determine the DNA element/s responsible for such interesting properties, we generated a recombinant baculovirus expressing the transcriptional regulators encoded by the *Ac-ie-01* cDNA under the control of *polh* promoter. This baculovirus jointly 5 with a baculovirus incorporating the elements of expression cassette of the present invention used in example 2 (*polhAc-ie-01/hr1p6.9p10GFP*) and a conventional baculovirus expressing the GFP protein, were used to infect *Sf9* cells in suspension at a low MOI of 0.1. The increase in cell number and cell 10 viability was studied between 24 to 120 hours post-infection. At 24 hours post-infection, insect cells infected by any of the baculoviruses overexpressing the above mentioned transcriptional regulators presented an increase in cell 15 number higher than 10 % with respect to cultures infected by the control recombinant baculovirus (**Figure 6A**). A more detailed analysis by flow cytometry of the time required for these factors to induce the observed differences in cell proliferation revealed an increase of insect cells in S phase at 3 hours post-infection and then at 6 hours post-infection, an increase in the number of insect cells in G1 was observed. These data imply a very early increment of the mitosis in those cultures infected by the baculovirus 20 overexpressing the *Ac-ie-01* cDNA encoding proteins (data not shown).

Fluorescence measurement was performed on a FACSCalibur™ (BD Biosciences™) flow cytometer. Cells were fixed in 70% EtOH, resuspended and 25 incubated in the staining solution (50 µg/ml propidium iodide in PBS, 5 µg /ml RNase). The data were gated to eliminate particles with a distinct size from cells and analyzed by plotting the cell number vs the red fluorescence from propidium iodide. 50,000 cells were counted per assay. Data analysis of the total number of 30 cells per cell cycle phase (G1, S and G2) was made using Modfit software.

Infected cell cultures were also analyzed by Trypan blue staining to determine 35 cell viability at different times post-infection. Interestingly, at very late times post-infection (96-120 hours), insect cells infected by the viruses overexpressing the transcriptional regulators showed an increase (50-60 % increase) of cell viability and integrity (**Figure 6B**). This suggests that the overexpression of the

transcriptional regulators of the present invention protects the cells from the baculovirus-induced cytopathic effect, allowing long-term expression. Both cell proliferation and increased cell viability after infection have important consequences in the recombinant protein productivity of the BEVS. Similar 5 results were obtained when the overexpression of the transcriptional regulators was driven by both the *pB29* or *polh* promoters (data not shown). Results observed in *Sf9* insect cells infected in suspension were confirmed in *Sf21* cells cultured in monolayer (data not shown) and also in Hi-5™ cells cultured in monolayer (Figure 6C and D). These figures demonstrate how the 10 overexpression of the transcriptional regulators improves the cell integrity at late times post-infection (96 hours).

15 **Example 4. Overexpression in a baculovirus expression system of transcriptional regulators encoded by the *Ac-ie-01* cDNA facilitates the post-translational processing of recombinant proteins.**

Cellular integrity during baculovirus infection is of great importance to ascertain the correct folding or any other post-translational modification of foreign proteins expressed by this system. The baculovirus strong promoters commonly 20 used for research and production, such as *polh* and *p10*, only express the foreign genes at late times post-infection when infected cells already show severe cytopathic effects and the cellular viability decreases. As described above, the overexpression of the transcriptional regulators used in the baculovirus expression cassette of the present invention protects cells from pathogenic 25 effects of the baculovirus infection by a still unknown mechanism, allowing a wide temporal window for recombinant protein production in cells remaining fully viable. We studied the relevance of the elements incorporated into the expression cassette of the invention in relation to post-translational modifications of recombinant proteins. For this purpose, a conventional 30 baculovirus expressing the reporter protein GFP under the control of the *polh* promoter and a baculovirus incorporating the baculovirus cassette of the present invention and also expressing the GFP protein (*polhAc-ie-01/hr1p6.9p10GFP*) were used to infect *Sf9* insect cells in suspension at a MOI of 0.1. Infected cells

were analysed at different times after infection by Western blot using an anti-GFP monoclonal antibody (mab2515; Millipore™) as shown in **Figure 7**. Interestingly, GFP protein expressed by a conventional baculovirus showed several reactive bands at 48 and 72 hours post-infection (suggesting non-proper

5 expression and/or folding of the protein) and at later times a band with a reduced molecular weight (lower than predicted) was observed (suggesting degradation) (**Figure 7A**). In contrast, when the GFP protein expression was

mediated by the baculovirus expression cassette of the invention, only one GFP band was observed at all times post-infection analyzed, showing the expected

10 molecular weight of this protein (**Figure 7A**). The expression of GFP by this vector was not significantly reduced at very late times post-infection (120 hours), confirming that the baculovirus expression cassette of the invention

confers to baculovirus vectors interesting advantages for long-lasting expression.

15 In parallel, the integrity of the cell machinery was measured at different times post-infection by Western blot analysis of the cellular actin protein using a specific antiserum (**Figure 7A**). Infection with a conventional baculovirus

impaired severely the cell integrity at 72 hours post-infection since the actin band detected decreased dramatically after this time point (degradation as a

20 result of a complete loss of cell integrity). Consistent with the cellular protection induced by the recombinant DNA elements contained in the baculovirus

expression cassette of the invention, cellular actin was not equally affected in cells infected by the recombinant baculovirus engineered with the expression cassette.

25

Fluorescence activity of recombinant GFP expressed by the different baculoviruses reflects its correct conformation. As is shown in **Figure 7B**, the GFP expressed in the context of the baculovirus expression cassettes of the invention keep an increased pattern of functionality along infection times. In

30 contrast, the fluorescence of GFP expressed by a conventional baculovirus peaked at 72 hours post-infection and decreased at later times (**Figure 7B**), in parallel to actin degradation (**Figure 7A**) and the observed cell viability

reduction (**Figure 6B**).

**Example 5. Cell culture and viruses.**

The *Spodoptera frugiperda* *Sf21* or *Sf9* cell lines were cultured in 6-well tissue culture plates ( $1 \times 10^6$  cells/well) in TNM-FH insect medium (Pan Biotech™, Germany) containing 10 % heat-inactivated fetal bovine serum (Pan Biotech™, Germany) at 27°C. AcMNPV recombinant baculoviruses were obtained by the “Bac-to-Bac®” Baculovirus Expression System (Invitrogen™, USA). Different transfer vectors containing the recombinant DNA elements of the present invention were generated using the pFastBac™-DUAL plasmid (Invitrogen™). The promoters and regulatory elements incorporated into pFastBac™-DUAL were synthesized (GenScript™, USA) with the adequate flanking restriction sequences to facilitate the cloning. These transfer vectors were used to transfect *Sf21* cells with Cellfectin® (Invitrogen™, USA). The resulting recombinant baculoviruses from the infection of *Sf21* cells were then passaged twice in cells and titered by the plaque assay method. The obtained gene constructs of the baculovirus expression cassettes of the present invention are schematically shown in **Figure 8**, showing different potential combinations of promoters driving the expression of the *Ac-ie-01* cDNA or the foreign gene (e.g. GFP). The different expression cassettes were used to generate the recombinant baculoviruses used in the examples shown in **Figures 4 to 7**.

**Example 6. Generation of the cloning vector**

The cloning vector is a small piece of DNA containing the baculovirus expression cassette of the invention into which a foreign DNA fragment can be inserted by treating the vehicle and the foreign DNA with a restriction enzyme that creates the same overhang, then ligating the fragments together. The essential characteristics of the cloning vector must include a synthetic multiple cloning site (MCS) to facilitate the insertion of foreign genes directed in a chosen orientation, a selectable marker, such as an antibiotic resistance to allow the selection of positively transformed cells and a functional origin of replication (ORI) for propagation in bacteria.

**Example 7. Generation of the donor vector containing the baculovirus expression cassette of the invention**

A donor vector consists of a cloning vector, for example a pUC57 plasmid, 5 containing the baculovirus expression cassette, into which a foreign gene has been cloned using the appropriate restriction enzymes. The baculovirus expression cassette of the invention was synthesized by ligating the following DNA sequences: (i) the baculovirus transcriptional regulator encoding sequence *Ac-ie-01* downstream of a promoter sequence, such as the *polh* or the *pB29*, 10 promoter, and upstream of the HSV TK polyadenylation signal and (ii) in another locus an enhancer sequence, for example, the homologous region *hr1*, upstream of (iii) a promoter sequence, for example, *pB29p10*, *p10*, *p6.9p10* or *polh*, followed by a multiple cloning site (MCS) for cloning the gene of interest and the SV40 15 polyadenylation signal downstream of the MCS (**Figure 1**). The baculovirus expression cassette is flanked by specific restriction sites (for example *Bgl*II and *BstZ17*I at the 5'-terminal end and *Bgl*II and *Sgf*I at the the 3'-terminal end) to facilitate subcloning into a transfer vector of a commercial baculovirus generation system (based on transposition, for example the "Bac-to-Bac®" 20 system (invitrogen™), or based on homologous recombination, for example "flashBAC™" (Oxford Expression Technologies™), "Baculogold™" (BD Biosciences™), "BacPAK6™" (Clontech™), "Bac-N-Blue DNA™" (invitrogen™) (**Figure 2 and 3**).

The encoding gene of the Green Fluorescence Protein (GFP) was cloned into the 25 MCS of the cloning vector using the *Nco* I and *Spe* I restriction sites, generating the donor plasmid vector (**Figure 2**).

**Example 8. Generation of the transfer vector containing the baculovirus expression cassette of the invention**

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The transfer vector was generated by digesting a donor vector with *BstZ17*I of the 5'-flanking site and with *Xba* I and cloning it into the transfer vector pFastBac™1 that was also digested with the same enzymes. In this case, as a

result of the subcloning, the SV40 polyadenylation signal of the baculovirus expression cassette is exchanged by the SV40 polyadenylation signal from the transfer vector. Apart from this, all the elements of the expression cassette are included in the pFastBac transfer vector, substituting the *polh* promoter and MCS of the original commercial transfer vector (Figure 2).

**Example 9. Generation of the baculovirus expression vector containing the baculovirus expression cassette of the present invention using the “Bac-to-Bac®” system**

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The modified transfer vector pFastBac™1 and the individual baculovirus expression cassette were used to generate recombinant baculoviruses by using the “Bac-to-Bac®” Baculovirus Expression System. More specifically, the modified transfer vector was used to transform the *E. coli* host strain DH10Bac™ that 15 contains a baculovirus shuttle vector (bacmid) and a helper plasmid, and allows the generation of the recombinant bacmid following transposition of the expression cassette. The DNA of the recombinant bacmid containing the baculovirus expression cassette of the present invention and the GFP encoding gene was then used to transfect insect cells, for example, *Sf21* cells, using 20 Cellfectin®. 72 hours post-transfection, cells were harvested and the first recombinant baculovirus generation was obtained (Figure 2). This recombinant baculovirus could then be further amplified and/or titered following conventional protocols. Similar procedures can be used to generate recombinant baculoviruses with other transfer vectors provided by commercial BEVSs 25 (Figure 3).

**Example 10. Protein sample preparation.**

Infected cells from each time point ( $1 \times 10^6$ ) were harvested and centrifuged. The 30 supernatants were removed and the cell pellets were resuspended in PBS and subjected to three cycles of freezing (-196 °C) and thawing (37 °C). Cellular debris was removed by centrifugation.

**Example 11. Time-Course Study of Protein Expression.**

*Sf9*, *Sf21* or *Hi-5*<sup>TM</sup> cells were infected with the different recombinant baculoviruses expressing GFP under the control of different combinations of regulatory, enhancer and promoter elements, using a MOI of 5 or 0.1. Cell cultures were harvested at various time points (24, 48, 72, 96 and 120 hours post-infection) and the GFP expression was analyzed by fluorescence microscopy, fluorimetric assay, SDS-PAGE followed by Coomassie blue staining or Western blot and by the microfluidic separation and quantification (Experion<sup>TM</sup> automated electrophoresis system; Bio-Rad<sup>TM</sup>, USA).

10

For quantification of the recombinant GFP, samples were loaded in Pro260 chips (Bio-Rad<sup>TM</sup>) and analyzed by capillary electrophoresis using the Experion<sup>TM</sup> system (Bio-Rad<sup>TM</sup>), following the manufacturer's instructions. The electrophoresis of the samples was made through microchannels by controlling the applied voltage and electric power. The microfluidic chip allowed several sequential procedures including separation, staining, destaining, detection and basic data analysis without any need of user's intervention. The Experion<sup>TM</sup> system resolved and quantified protein samples from 10 to 260 kDa in size, with a high sensitivity, comparable to colloidal Coomassie blue SDS-PAGE gel staining.

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For quantification, the Experion<sup>TM</sup> system uses a Pro260 ladder, a modified version of the Precision Plus Protein<sup>TM</sup> standards, that have been optimized for use in that system.

**Example 12. Fluorescence microscopy analysis.**

Infected cells were visualized directly in 6-well cell culture plates using a GFP

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filter on a Leica<sup>TM</sup> DMIL<sup>TM</sup> inverted fluorescence microscope.

**Example 13. Fluorimetric analysis.**

About 20 µg of total soluble proteins derived from infected cells, containing

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different amounts of recombinant GFP protein, were analyzed and quantified by

a Tecan™ GENios™ (CA, USA) fluorescence plate reader (excitation [Ex], 485/emission [Em], 535).

**Example 14. Western blot analysis.**

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Total soluble protein fractions (10 µg) from cells infected with the recombinant baculoviruses were resolved in 15% SDS-PAGE gels. Gels were stained by the Coomassie blue staining method or transferred to nitrocellulose membranes. Western blots were probed with the anti-GFP monoclonal antibody mab2515 (Millipore™, USA) at 1:1000 or actin antiserum (20-33; Sigma-Aldrich™) and the immunocomplexes were revealed with anti-mouse IgG-horseradish peroxidase (HRP)-labeled conjugate (KPL™, UK), diluted 1:2,000 or by an anti-rabbit IgG-horseradish peroxidase (HRP)-labeled conjugate (KPLTM, UK), diluted 1:2,000 respectively as a secondary antibody. Protein bands were detected using an ECL western blotting detection system and analyzed by the ChemiDoc™ XRS Gel Imaging System (Bio-Rad™, USA).

**Example 15. Delimitation of the promoter sequence in the *pB2* DNA fragment.**

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The DNA region upstream of the *BJHSP-2* gene (*pB2*) was PCR amplified from *T. ni* insect DNA based on the previously reported *BJHSP2* sequence (GenBank accession no **U41640**). The amplified DNA region differed in several aspects from the annotated sequence, comprising 2 insertions, 8 deletions as well as 17 mutations (SEQ ID NO: 34). Using different bioinformatic analyses, six putative binding sites related to hormone-response elements were found along the *pB2* sequence, four of them corresponding to putative ecdysone-response elements (EcR) and three of them to putative Broad-Complex sites (Br-C) (**Figure 9A and B**).

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In order to determine essential regulatory regions for transcriptional activity and the relevance of potential hormone-regulated elements, a *pB2* truncated sequence was analyzed (fragment *pB2<sub>9</sub>*) (**Figure 9B**). Both *pB2* and *pB2<sub>9</sub>*

fragments were cloned in a baculovirus vector and tested for their promoter activity by using the GFP reporter protein. **Figure 9B** shows the GFP expression levels obtained with every fragment and quantified by a fluorimetric analysis. Surprisingly, the *pB2<sub>9</sub>* fragment showed a stronger promoter activity than the 5 parental full-length *pB2*, even though it lacked two putative Br-C binding sites (**Figure 9B**).

**Example 16. Synergistic cooperation between *pB2* or *pB2<sub>9</sub>* and conventional baculovirus promoters.**

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Different chimeric promoters comprising the *pB2* or *pB2<sub>9</sub>* sequence and the conventional baculovirus promoters *polh* or *p10* were constructed, resulting in the combinations *pB2polh*, *polhpB2*, *pB2p10*, *p10pB2* and *pB2<sub>9</sub>p10*. All of them were used to generate recombinant baculoviruses and tested for their promoter 15 characteristics. At 24 hours post-infection, the GFP protein expression driven by *polhpB2* and *pB2<sub>9</sub>p10* hybrid promoters was higher than by using the conventional promoters *polh* or *p10* (**Figure 10A**). Interestingly, the GFP expression under the control of the chimeric promoter *polhpB2* dropped at 48 hours post-infection, while the GFP expressed under the control of the chimeric 20 promoter *pB2<sub>9</sub>p10* increased along time at levels even higher than obtained by using the *polh* promoter (**Figure 10B**). The chimeric promoter *pB2p10* showed the maximum GFP expression levels at 48 hours post-infection, but the expression increase was not linear at later times (**Figure 10B**). In conclusion, the hybrid promoter *pB2<sub>9</sub>p10* was earlier and stronger than the *polh* or *p10* 25 promoter alone.

## BIBLIOGRAPHY

1. Nettleship, J.E., Assenberg, R., Diprose, J.M., Rahman-Huq, N., Owens, R.J. 5 Recent advances in the production of proteins in insect and mammalian cells for structural biology. *J. Struct. Biol.* 2010, 172, 55–65.
2. Gomez-Casado E, Gomez-Sebastian S, Núñez MC, Lasa-Covarrubias R, Martínez-Pulgarín S, Escribano JM. Insect larvae biofactories as a platform for influenza vaccine production. *Protein Expr Purif.* 79: 35-43. 2011.
- 10 3. Smith, G.E., M.D. Summers, and M.J. Fraser. 1983. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* 3: 2156-2165.
4. Hitchman RB, Possee RD, King LA. Baculovirus expression systems for recombinant protein production in insect cells. *Recent Pat Biotechnol.* 15 2009;3(1):46-54.
5. Hashimoto Y, Zhang S, Blissard GW. Ao38, a new cell line from eggs of the black witch moth, *Ascalapha odorata* (Lepidoptera: Noctuidae), is permissive for AcMNPV infection and produces high levels of recombinant proteins. *BMC Biotechnol.* 2010;10:50.
- 20 6. Taticek RA, Choi C, Phan SE, Palomares LA, Shuler ML. Comparison of growth and recombinant protein expression in two different insect cell lines in attached and suspension culture. *Biotechnol. Prog.* 2001, 17 (4), 676-684.
7. Hill-Perkins MS, Possee RD. A baculovirus expression vector derived from the basic protein promoter of *Autographa californica* nuclear polyhedrosis virus. *J Gen Virol.* 1990, 71 ( Pt 4):971-6.
- 25 8. Passarelli, A. L., and L. K. Miller. Three baculovirus genes involved in late and very late gene expression: *ie-1*, *ie-n*, and *lef-2*. *J. Virol.* 1993, 67:2149–2158
- 30 9. Rodems, S. M., S. S. Pullen, and P. D. Friesen. DNA-dependent transregulation by IE1 of *Autographa californica* nuclear polyhedrosis virus: IE1 domains required for transactivation and DNA binding. *J. Virol.* 1997, 71: 9270–9277.

10. Lin X, Chen Y, Yi Y, Zhang Z: Baculovirus immediately early 1, a mediator for homologous regions enhancer function in trans. *Virol J* 2010, 7:32.
11. Okano K, Mikhailov VS, Maeda S: Colocalization of baculovirus IE-1 and two DNA-binding proteins, DBP and LEF-3, to viral replication factories. *Journal of virology* 1999, 73(1):110-119.

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**CLAIMS:**

1. Recombinant baculovirus comprising a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators, wherein the nucleic acid is selected from the group consisting of:
  - (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 1-5;
  - (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
  - (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and
  - (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.
- 25 2. Recombinant baculovirus according to claim 1, further comprising at least one recombinant homologous region (*hr*) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein.
- 30 3. Recombinant baculovirus according to claim 2, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 27 (*hr1*).

4. Recombinant baculovirus according to claim 2 or 3, wherein the promoter operably linked to the homologous region (*hr*) is selected from the group of nucleic acids comprising:

5 (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

10 5. Recombinant baculovirus according to any of the claims 1-4, comprising a nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

15 6. Recombinant baculovirus according to any of the claims 1-5, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of claims 1-5.

20 7. Transfer vector suitable for producing a recombinant baculovirus according to any of the claims 1-6, comprising said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof functioning as transcriptional regulators, further comprising a nucleic acid sequence suitable for integration or transposition in a baculovirus.

25 8. Transfer vector according to claim 7, further comprising at least one recombinant homologous region (*hr*) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

9. Transfer vector according to claim 8, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 27 (*hr1*).

5 10. Transfer vector according to claim 8 or 9, wherein the promoter operably linked to the homologous region (*hr*) is selected from the group of nucleic acids comprising:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

10 (b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

15 11. Transfer vector according to any of the claims 7-10, comprising a nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

20 12. Transfer vector according to any of the claims 7-11, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of items 7-11.

25 13. Transfer vector according to any of the claims 7-11, lacking a nucleic acid sequence encoding a recombinant protein.

30 14. Transfer vector according to any of the claims 7-13, characterized in that the transfer vector is a bacmid.

15. Transfer vector according to any of the claims 7-14, characterized in that the transfer vector is derived from any of the baculovirus expression systems "Bac-to-Bac®" (invitrogen™), "BacPAK™" (Clontech™), "FlashBACTM" (Oxford Expression Technologies™), "BacuVance™" (GenScript™), "Bac-N-Blue DNA™" (invitrogen™), "BaculoDirect™" (invitrogen™), "BacVector®" 1000, 2000, 3000 (Novagen®), "DiamondBac™" (Sigma-Aldrich®) or "BaculoGold™" (BD biosciences™).

5

16. Cloning vector suitable for producing a recombinant baculovirus or transfer vector according to any of the claims 1-15, comprising said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof functioning as transcriptional regulators, which is further suitable for bacterial replication.

10

15 17. Cloning vector according to claim 16, further comprising at least one recombinant homologous region (*hr*) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

20 18. Cloning vector according to claim 17, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 27 (*hr1*).

25 19. Cloning vector according to claim 17 or 18, wherein the promoter operably linked to the homologous region (*hr*) is selected from the group of nucleic acids comprising:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 30 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

20. Cloning vector according to any of the claims 16-19, comprising a nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

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21. Cloning vector according to any of the claims 16-20, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of claims 16-20.

22. Cloning vector according to any of the claims 16-20, lacking a nucleic acid sequence encoding a recombinant protein.

23. Nucleic acid sequence suitable for producing a recombinant baculovirus, transfer vector or cloning vector according to any of the claims 1-22, comprising said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof functioning as transcriptional regulators.

24. Nucleic acid sequence according to claim 23, further comprising at least one recombinant homologous region (*hr*) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

25. Nucleic acid sequence according to claim 24, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 27 (*hr1*).

30

26. Nucleic acid sequence according to claim 24 or 25, wherein the promoter operably linked to the homologous region (*hr*) is selected from the group of nucleic acids comprising:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

5 (b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

10 27. Nucleic acid sequence according to any of the claims 23-26, comprising a nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

15 28. Nucleic acid sequence according to any of the claims 23-27, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of claims 23-27.

20 29. Nucleic acid sequence according to any of the claims 23-27, lacking a nucleic acid sequence encoding a recombinant protein.

25 30. A host cell infected, transfected, transduced or transformed with the recombinant baculovirus, transfer vector, cloning vector or nucleic acid sequence of any of the claims 1-29.

31. Infected, transfected, transduced or transformed host cell according to claim 30, characterized in that it is an insect cell line.

30 32. Infected, transfected, transduced or transformed host cell according to claim 30 or 31, characterized in that it is derived from an insect belonging to the Lepidoptera or Diptera genus.

33. Infected, transfected, transduced or transformed host cell according to any of the claims 30-32, characterized in that it is derived from

*Trichoplusia ni, Spodoptera frugiperda, Ascalapha odorata, Bombyx mori, Drosophila melanogaster or Aedes aegypti.*

34. Infected, transfected, transduced or transformed host cell according to

5 any of the claims 30-33, characterized in that it is a cell line selected from the group consisting of Hi-5™, Sf9, Sf21, BTI-Tn5B-1, Tn368, ExpressSf+®, BTI-TnAo38, ATC-10 and Schneider's *Drosophila* Line 2.

35. Culture medium comprising the recombinant baculovirus, transfer vector,

10 cloning vector or nucleic acid sequence of any of the claims 1-29.

36. Method for producing a recombinant protein that comprises the infected,

transfected, transduced or transformed host cell according to claims 30-

15 34 and the extraction and purification of the recombinant protein by conventional means.

37. Method for producing a recombinant protein according to claim 36,

wherein the recombinant protein is selected from the group comprising subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone and diagnostic protein reagent.

38. Use of the transfer vector according to any of the claims 7-15 for

producing a recombinant baculovirus according to any of the claims 1-6.

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39. Use of the cloning vector according to any of the claims 16-22 for producing a recombinant baculovirus or transfer vector according to any of the claims 1-15.

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40. Use of the nucleic acid sequence according to any of the claims 23-29 for producing a recombinant baculovirus, transfer vector or cloning vector according to any of the claims 1-22.

35

41. Transgenic cell line comprising a transgene, which is a nucleic acid selected from the group consisting of:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 1-5;

(b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;

(c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and

(d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.

42. Transgenic cell line according to claim 41, which is of insect, avian or mammalian origin.

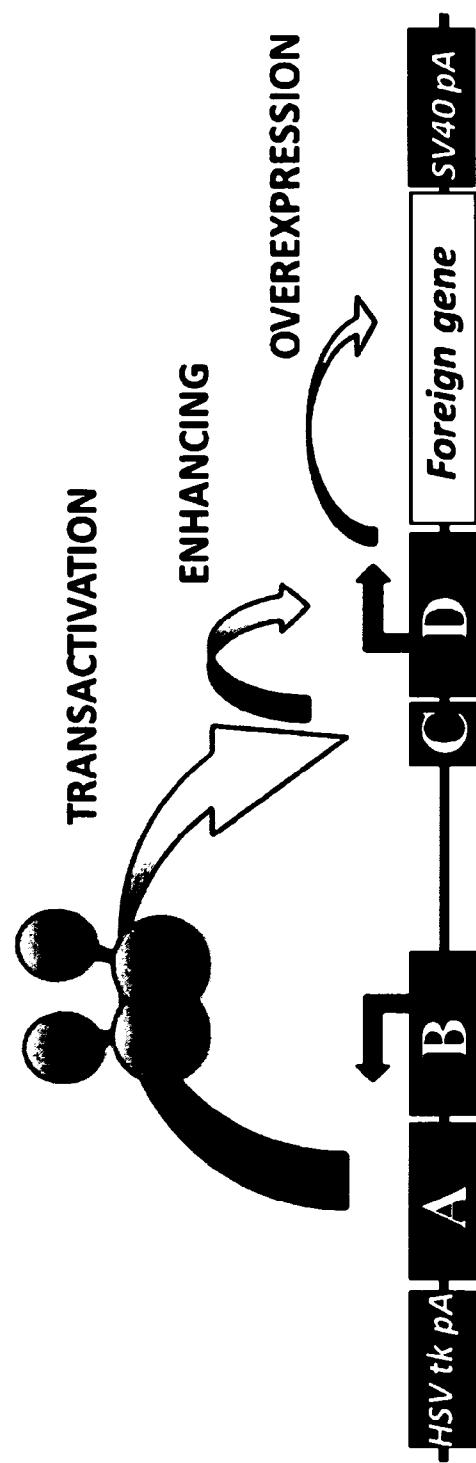
43. Method for producing a recombinant protein that comprises growth of a transgenic cell line according to claim 41 or 42 and the extraction and purification of the recombinant protein by conventional means.

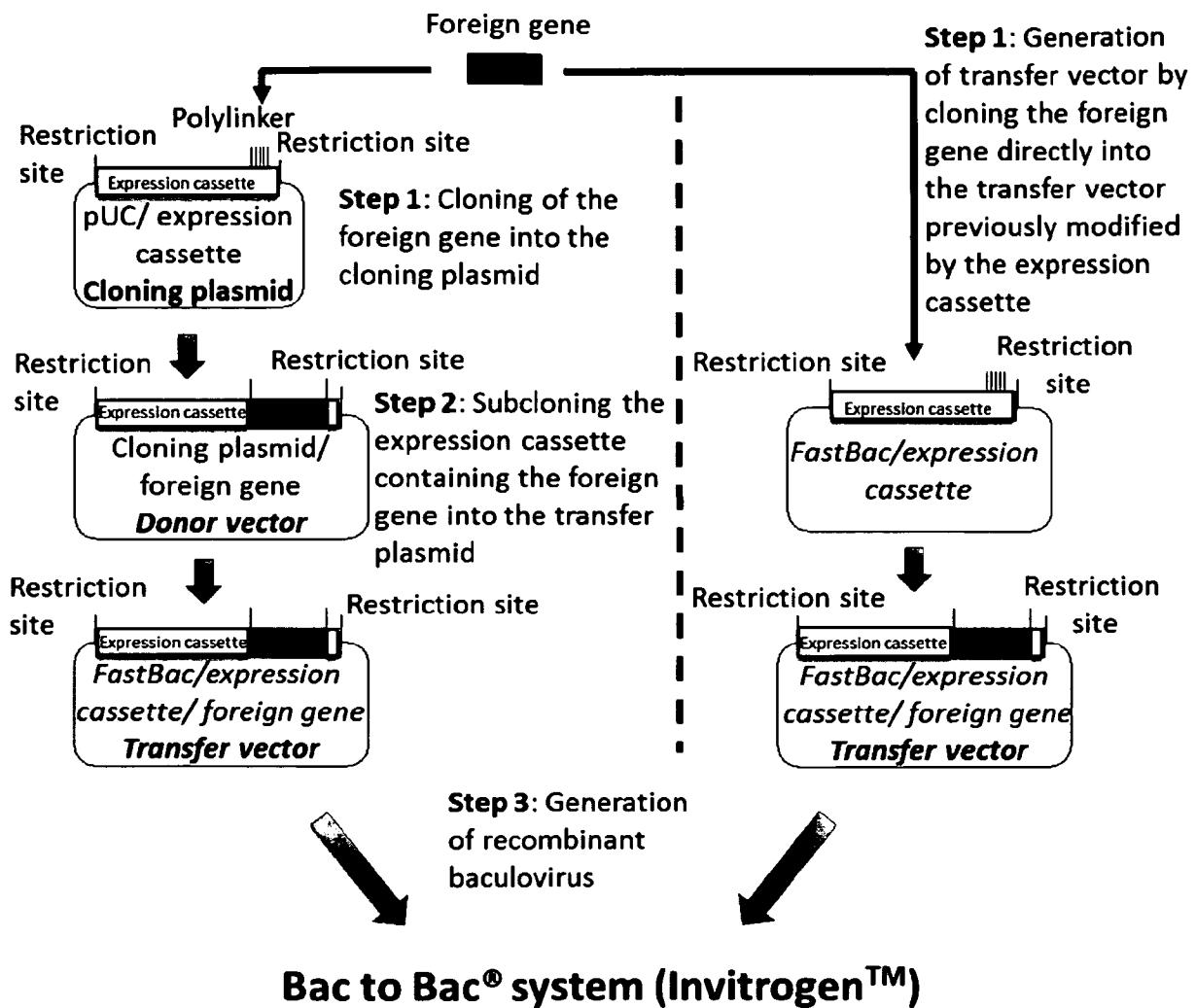
44. Nucleic acid sequence comprising a nucleic acid sequence functioning as a promoter in a recombinant baculovirus, wherein the nucleic acid sequence is selected from the group consisting of:

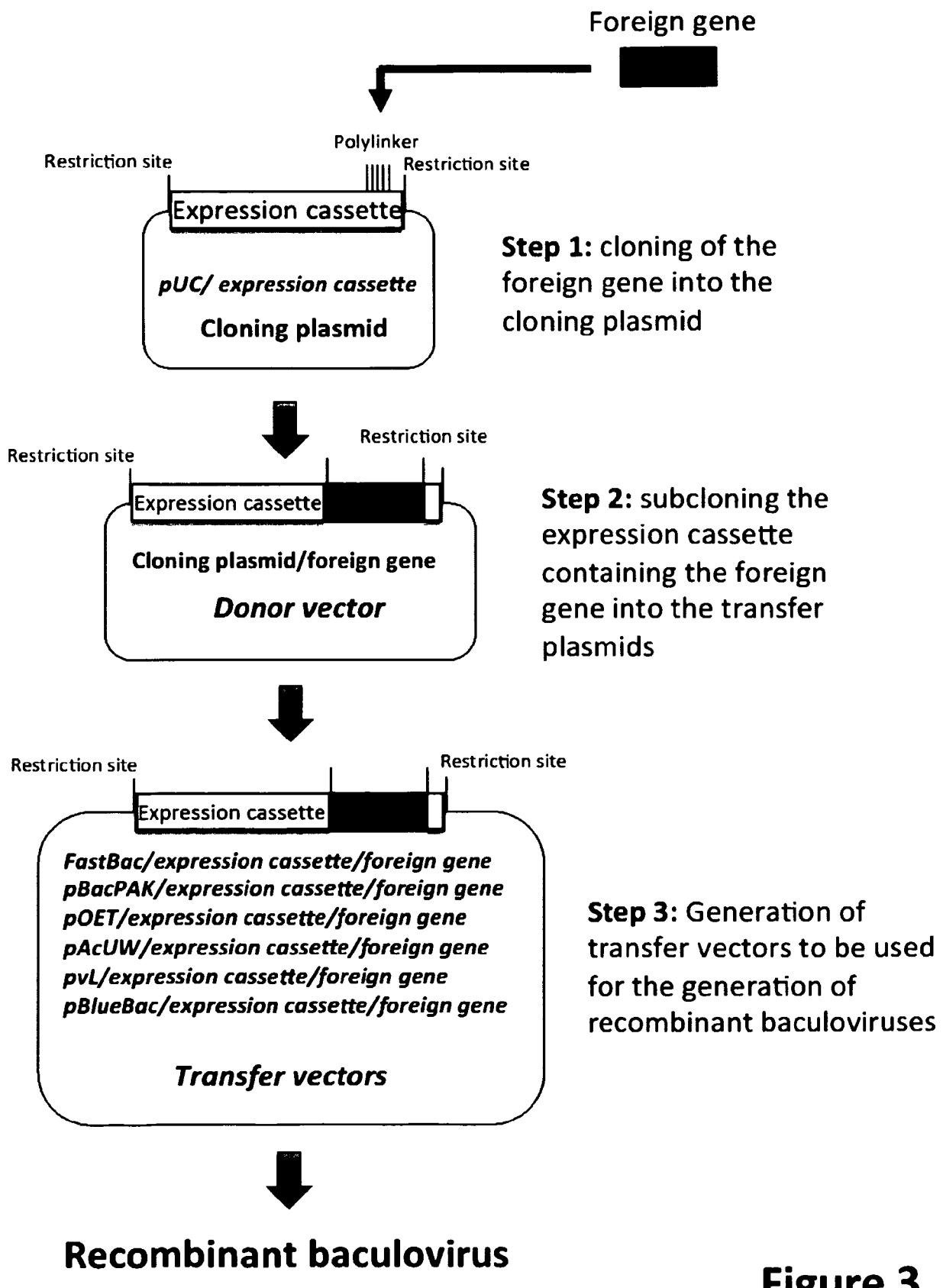
(a) a nucleic acid containing the nucleotide sequence indicated in SEQ ID NO: 12, 14 or 15; and

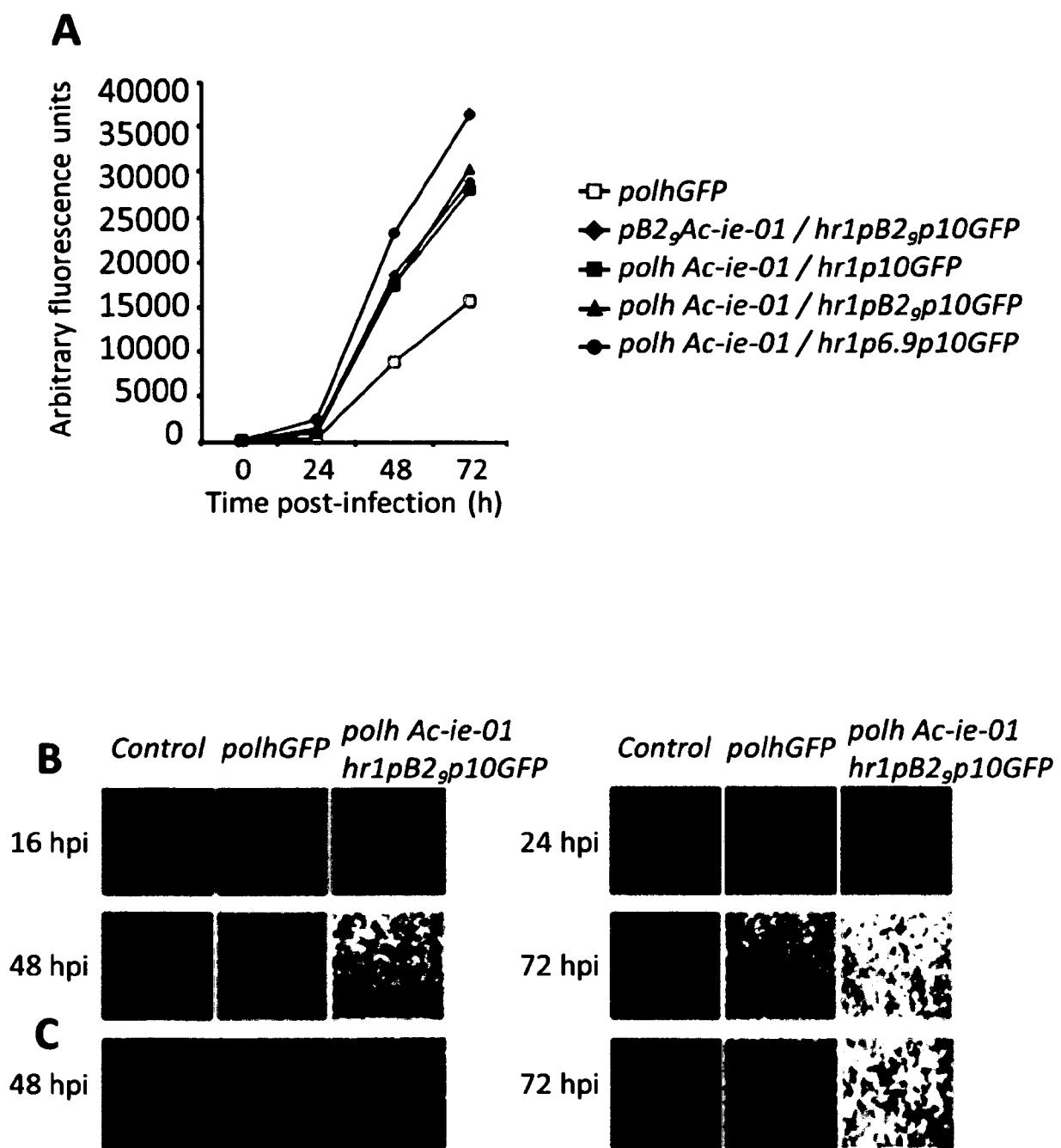
(b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most

preferably at least 95% with the nucleotide sequence indicated in SEQ ID NO: 12, 14 or 15.

**Figure 1**

**Figure 2**



**Figure 4**

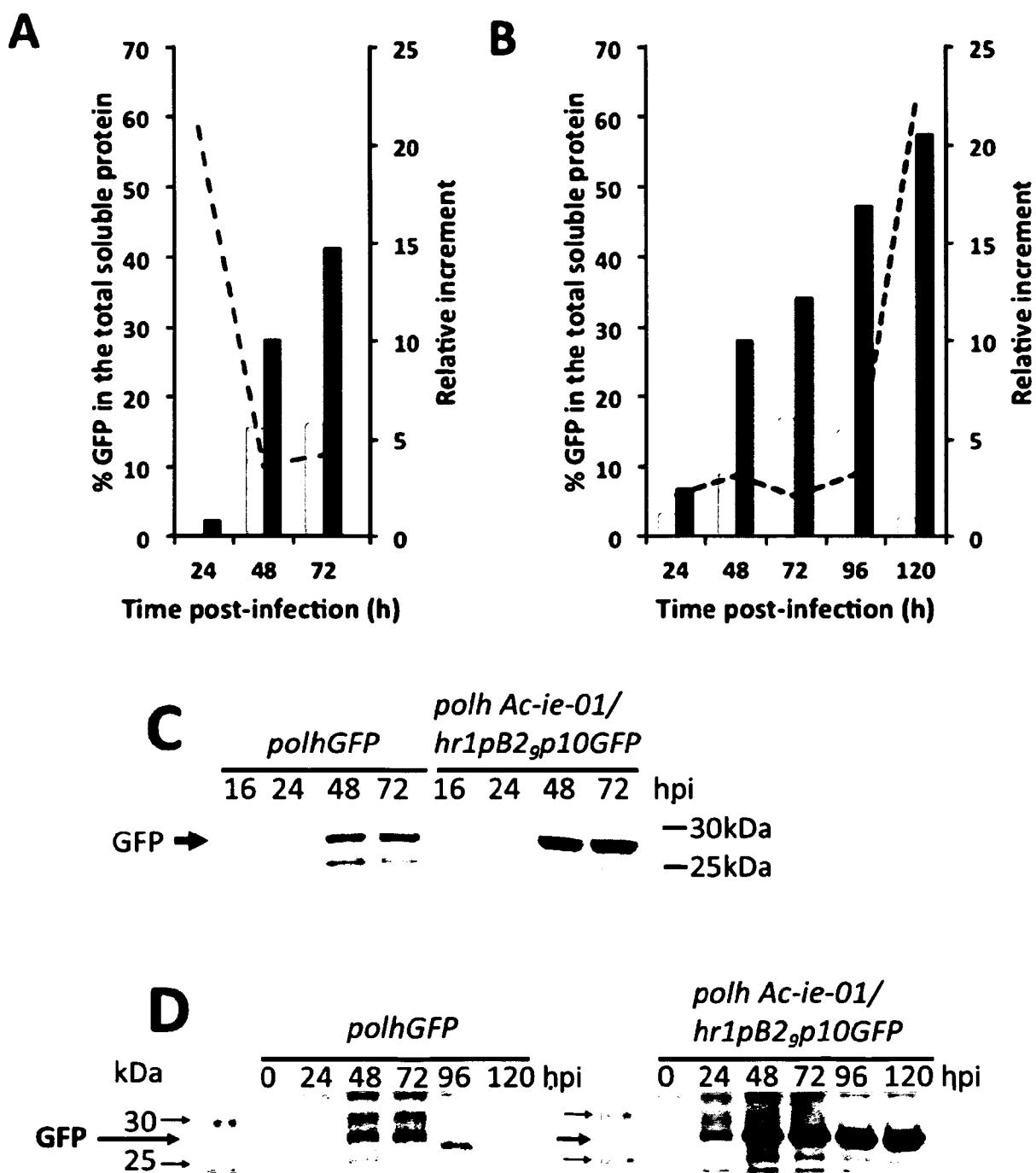
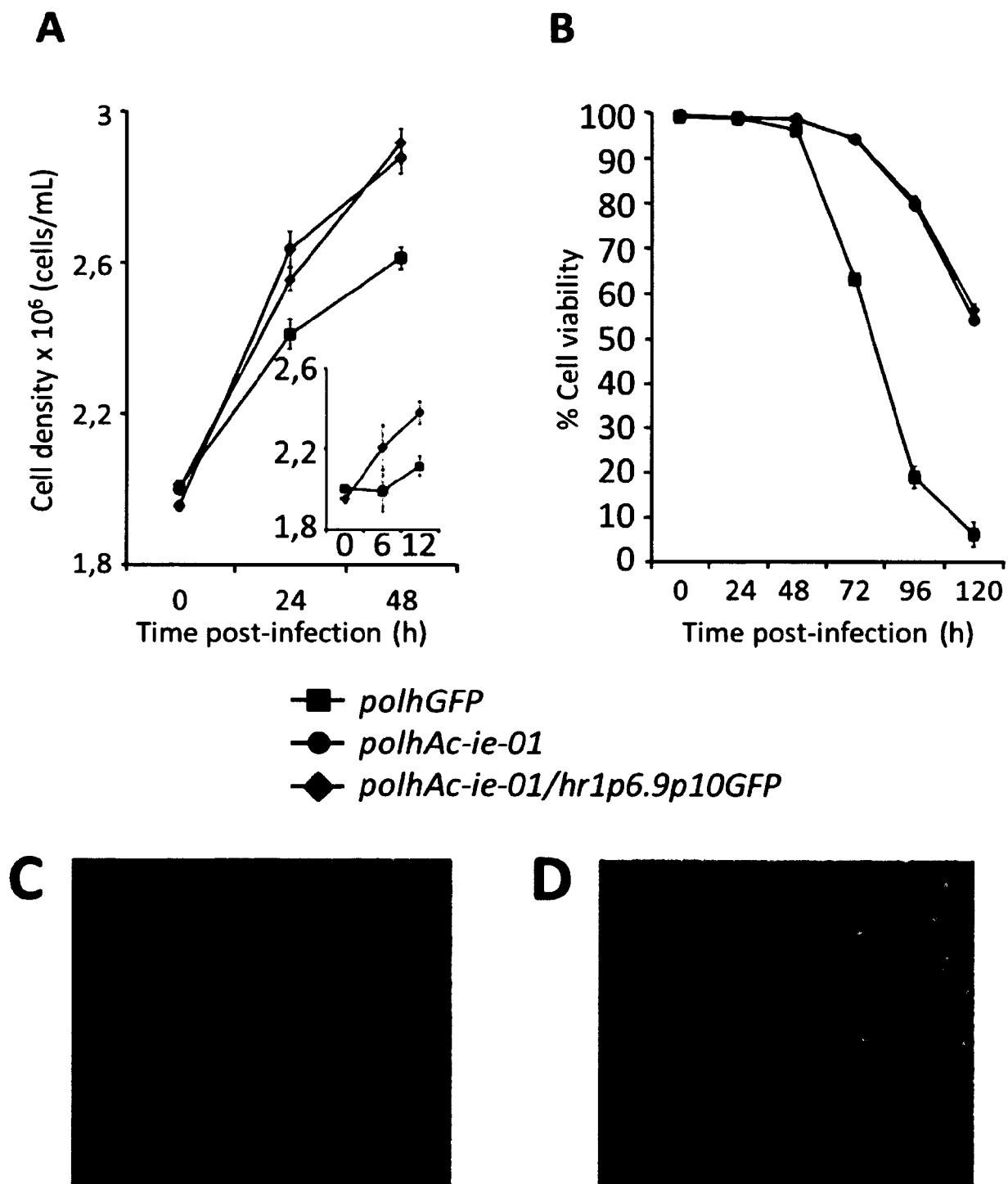
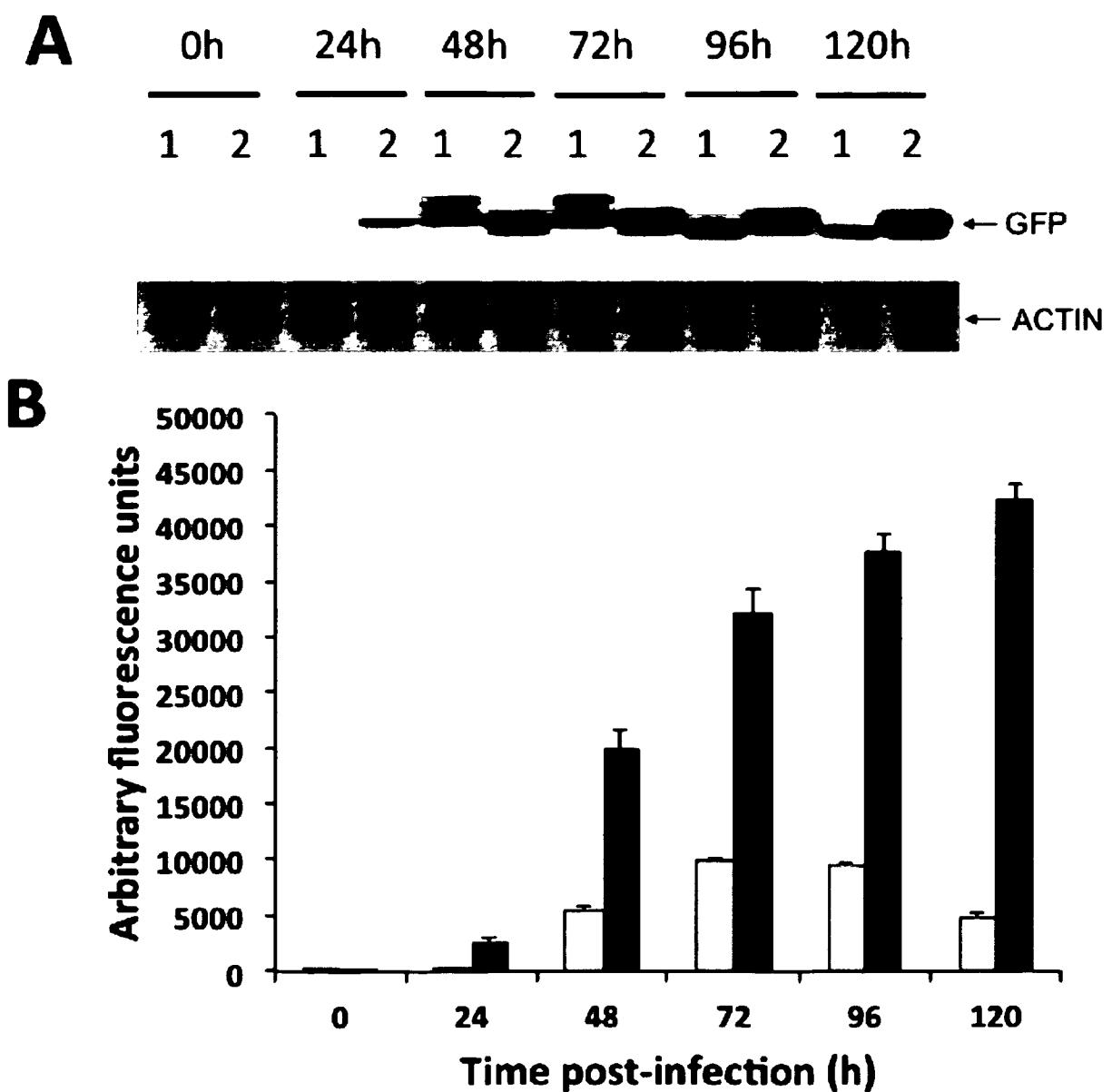
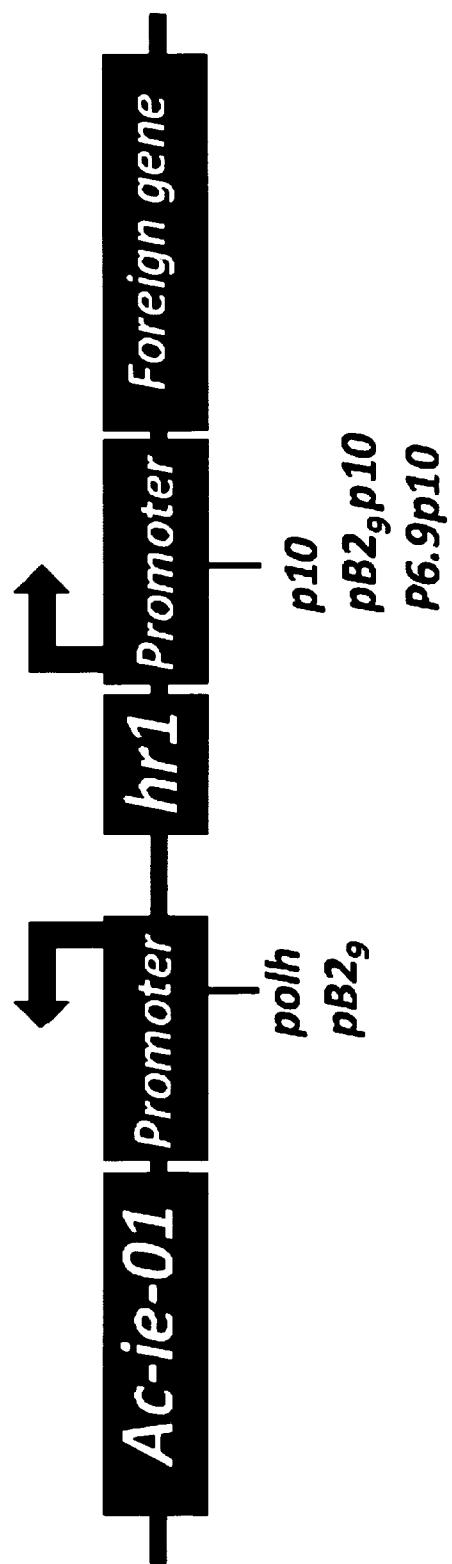


Figure 5

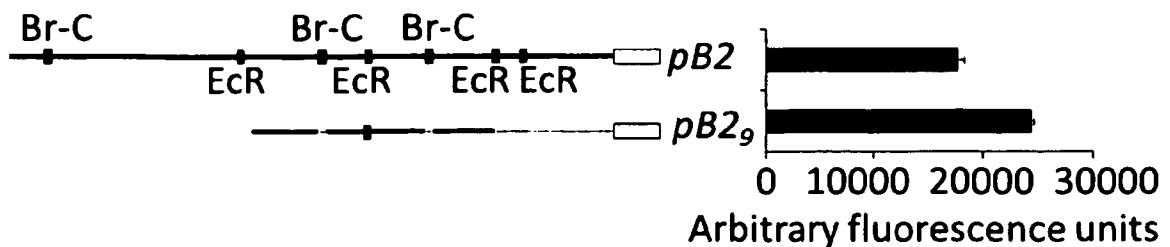
**Figure 6**

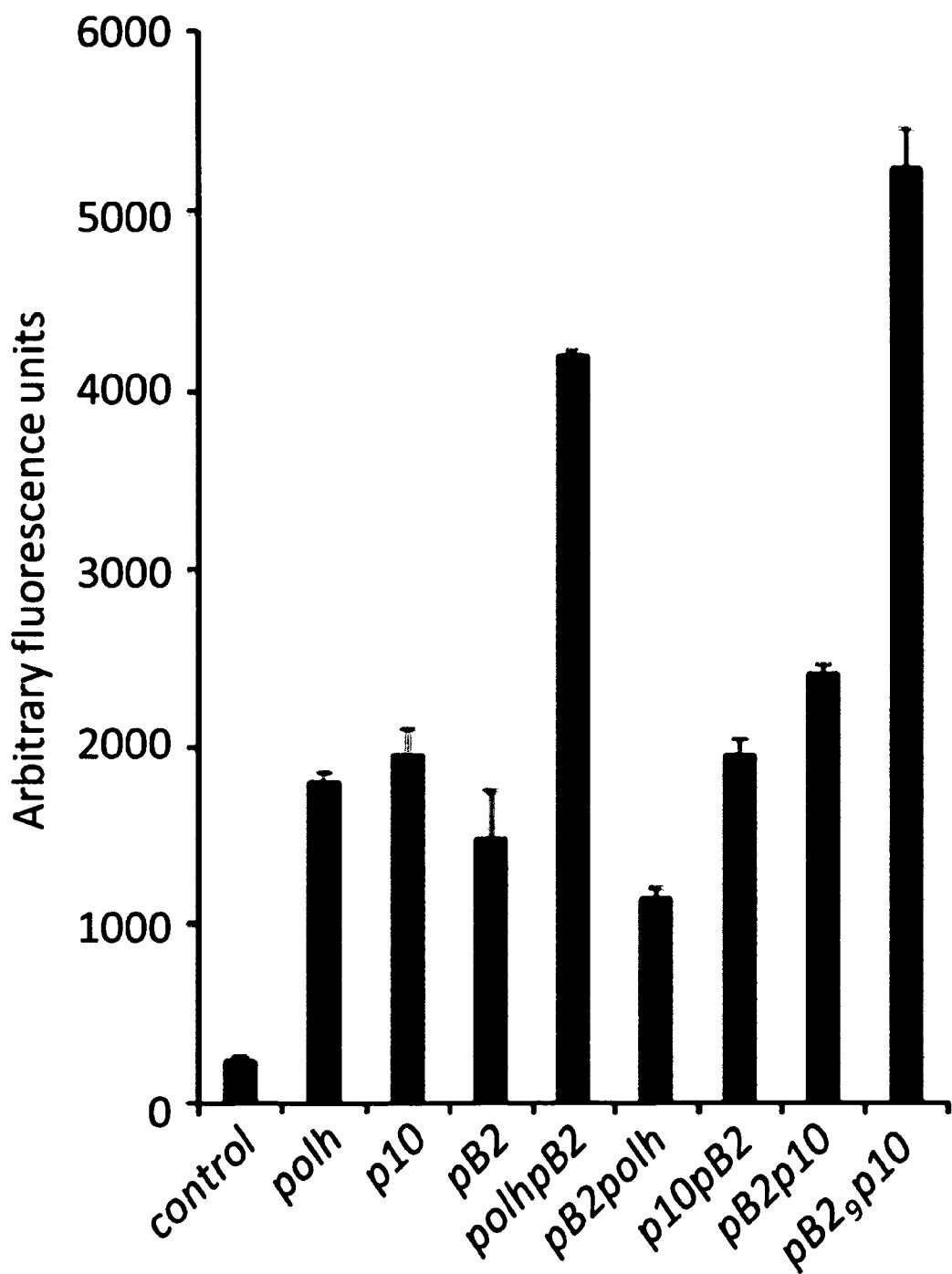
**Figure 7**

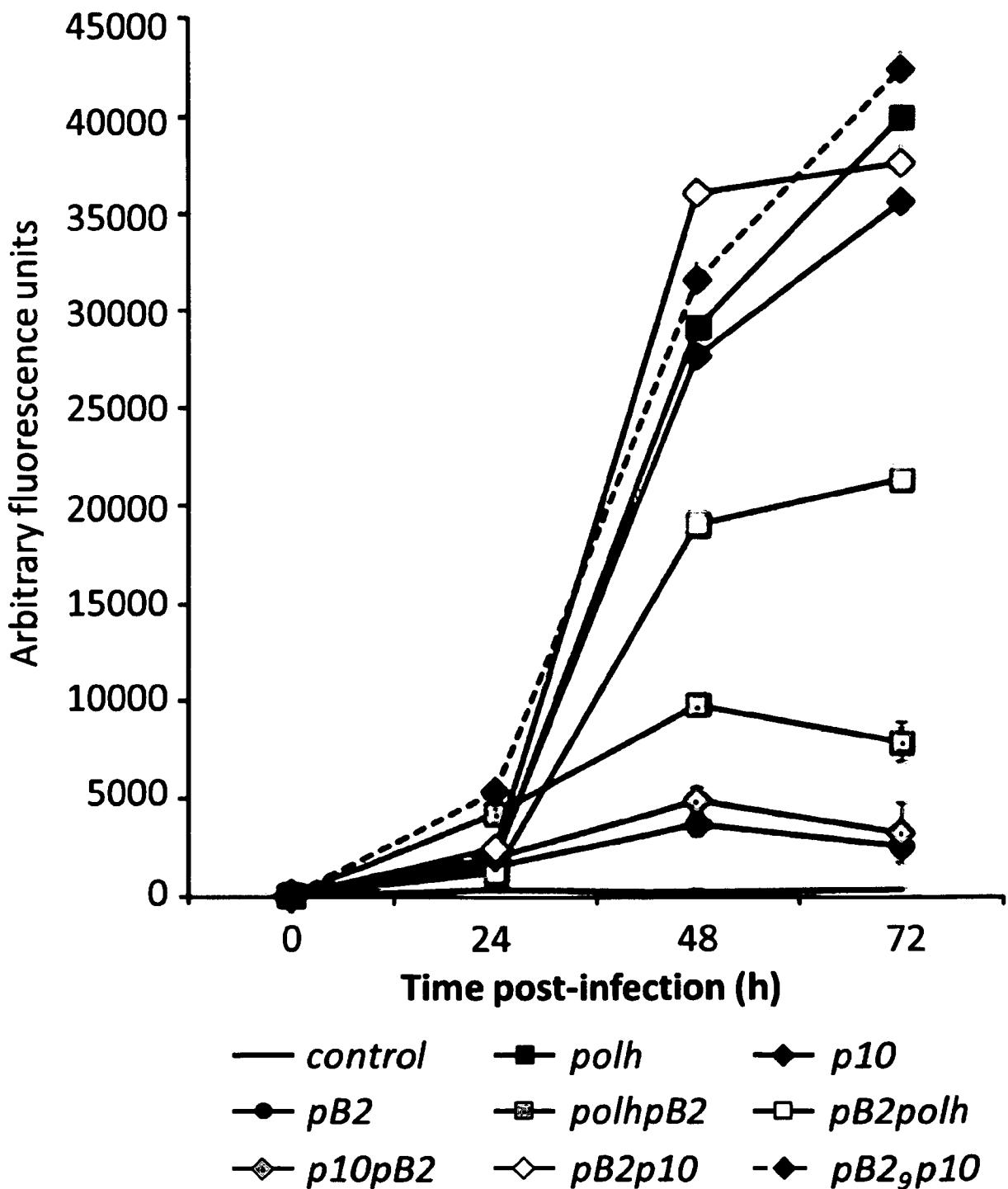
**Figure 8**

**A**

CTTGAATGTTAGTGAACACCCCTGCACACAAGTATTACATTCCCTAGTGCTGAATCCTT **TTAGGAAAGAAAAGCCAATTT**  
 Br-C  
 CAAAATCTTAGCACTTGTAACTCGCGAAAAGACCAACAGATTCCCATACTACAATTGACATTAGAAATGAAACCA  
 TTATCATTATTACGCCTCATTCATCCAATAATAAGTTAAGTACGTTGAGATAAAACTGGCTTACCTAGAACTTGACAT  
 GGCGACCTTTGCACTCTGTATCTCAAGTCACCTCTATCCAAATATTGATAACATTGACATGATATTGAAGTAAGA  
 TTGTTACTAAGGCTTACATTGTAATATTACTGACGCAAGTTCTT **[ATCAATAAAA]AGCTGAAAACAAAAAAACATC**  
 EcR  
**GATTAGGGTACTGAAGGTTACATTGGGGTAGGTTATGGTTAACGTAATGGTTAACACCAAAACGATATCATGGATT**  
 GACT **TTATAAAATTTATATAAGGTGTAAATAATTTTTAATGAGTGGACGCGTCG** **GGTCAATGTCC]GCCTATTGACGTCA**  
 Br-C EcR  
**AACATATTAGGTGATTATATTTAAACTCAAATATTACTTGCAAGTTAACGTTACATCATAATCTGATCATAAAGTTTCACC**  
**CAAACAGAAACCAAAAGCATAACTA** **[CTGCTATTGAAATATCTTACGTTCCATGAAGAAAGATTACCGTAACCATCACT**  
 Br-C  
**AGGATTTTACGATTGTAGAAAATAAGTATTCTCAGTCTCTT** **CAGTTAAAATCTGCTGGCATTTACAAGTCGCTG**  
 EcR  
 TATCAGTCATGTTATAACAAT **[GTCAATGTAC]TTCGTATTAAATCAGAAAAAAATATTCTACTAGTTTGATAAGCTATCA**  
 EcR  
 CTTTGTACATTGTACTGCCCTTACAGTTACAGGTATTATGAATGACATATTGGAGAAACATCGTAATCAGTCCAGT  
**ATAAAAAAGGGGTGCATTCTCGTAAGAGTACAGTTGAACTCACATCGAGTTAACCTCCACG**  
 ▲

**B****Figure 9**

**A****Figure 10**

**B****Figure 10**