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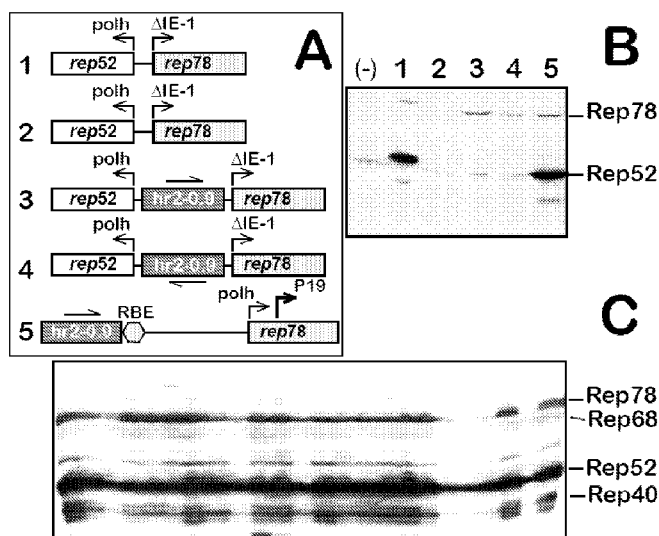
(54) Title: AN INDUCIBLE SYSTEM FOR HIGHLY EFFICIENT PRODUCTION OF RECOMBINANT ADENO-ASSOCIAT-
ED VIRUS (RAAV) VECTORS

FIG. 1

(57) Abstract: Production of clinical grade gene therapy
vectors for human trials remains a major hurdle in advancing
cures for a number of otherwise incurable diseases.
Disclosed herein are systems based on a stably trans-
formed insect cell lines harboring helper genes required
for vector production. Specifically exemplified are system
embodiments that take advantage of DNA regulatory ele-
ments from two unrelated viruses - AcMNPV and AA V2.
System embodiments utilize rep and/or cap genes either
stably transfected in cell lines or which are introduced into
cells as an expression cassette in a vector. Rep and cap
genes that are designed to remain silent until the cell is in-
fected with a viral vector. Infection with viral initiates res-
cue/amplification of integrated AAV helper genes result-
ing in dramatic induction of the expression and assembly
of rAAV. The arrangement of this specific embodiment
provides high levels of Rep and Cap proteins in every cell
thus improving rAAV yields by 10-fold. The described
vectors are modular in design and may be utilized for the
production of other multiprotein complexes.

**An inducible system for highly efficient production of recombinant
Adeno-associated virus (rAAV) vectors**

Related Applications

[1] This application claims the benefit under 35 USC § 119(e) of U. S. Serial No. 61/61/165,994 filed April 2, 2009 and U. S. Serial No. 61/239,775 filed September 3, 2009 which are incorporated herein by reference in their entirety.

Introduction

[2] High production costs of clinical grade gene therapy vectors remain a major impediment preventing many research laboratories from entering the field. This is especially true for replication-deficient rAAV vectors which are produced, for the most part, by plasmid DNA co-transfection. Only recently, alternative scale-up production protocols such as those utilizing baculovirus expression vectors had been developed. Traditionally, BEVs have emerged as one of the most versatile systems for the protein production. They provide high yield combined with the posttranslational modifications of the proteins. In addition to basic protein production BEVs were utilized for more complicated tasks such as the synthesis of heterologous multiprotein complexes (1), production of a variety of virus-like particles, and for the assembly of gene therapy vehicles such as rAAV vectors (2). The latter strategy utilizes insect cells co-infected with three BEVs, a procedure potentially capable of manufacturing rAAV in 'exa-scale' format (3). While extremely promising, the original protocol had not been widely adopted due to several shortcomings including a requirement for the co-infection with three different helpers Bac-Rep, Bac-VP, and Bac-GOI (gene of interest flanked by AAV inverted terminal repeats). Only recently, H. Chen has reported a significantly improved system where *rep* and *cap* helper genes in the respective BEVs incorporated artificial introns (4).

Brief Description of the Drawings

[3] **Fig. 1.** Expression of *rep52/78*-encoding cassettes. A – schematic representation of: 1 – Rep-expression cassettes in recombinant BEV Bac-Rep from Urabe *et al* (2); 2 – transfer plasmid used to derive Bac-Rep; 3-5 – plasmid constructs derived for the current project;

polh – late polyhedrin promoter; Δ IE-1 – attenuated OpMNPV immediate early promoter (33); P19 – wt AAV2 P19 promoter; RBE – Rep-Binding Element (wt AAV2 nt 87-126); leftmost numbers correspond to lanes in panel B. **B** – Western blotting analysis of Rep52/78 proteins in Sf9 cells after transient transfection with various plasmid DNAs. Cells in the lane marked (-) were mocked-transfected, lane 1 - infected with Bac-Rep (MOI of 5), cells in lanes 2-5 were transfected with the respective plasmids shown in panel A and infected with Bac-VP (2) (MOI of 5) to supply transregulator IE-1. **C** – Western blotting analysis of Rep proteins extracted from individual stable BSD^R cell lines after infection with Bac-VP (MOI of 5) and harvested 72 hr post infection.

[4] **Fig. 2** Analysis of AAV2 P19 promoter activation in Sf9 cells. **A** - AAV2 P19 promoter nucleotides are indicated above the sequence. Shaded boxes outline sequences with homology to SP1, GGT, and TATA transcription factor binding elements (9, 25). The P19 transcription initiation sites in mammalian (HeLa) and insect cells (Sf9) are indicated by bent arrows. **B** – EMSA assay. 5'-end-labeled 128 bp PCR fragment of P19 promoter (AAV2 nt 704 to 831, lanes 1 to 8) and 5'-end-labeled IE-1 consensus binding element (34) (lanes 9 to 13) were incubated with crude nuclear extract derived from Sf9 infected with BEV. Samples in the control lanes one and nine contained no nuclear extracts, while in lane 2 the probe was incubated with cell extract from un-infected Sf9. In lanes 4 and 5 ³²P P19 DNA probe was challenged with unlabeled P19 promoter probe (5- and 15-fold excess, respectively); in lanes 6, 7, and 9 – with unlabeled IE-1 probe (5-, 25-, and 100-fold excess); in lanes 11, 12, and 13 – with unlabeled IE-1 probe (5-, 25-, and 100-fold excess). Different DNA-protein complexes are marked by arrows and labeled with letters. Non-specific DNA band, a by-product of PCR reaction in lane 1 is marked with a (*) symbol. The presumed IE-1 mono-, and dimer/DNA-protein complexes are marked at the right edge of a gel.

[5] **Fig. 3.** Expression of AAV2 *cap*-encoding cassettes. **A** – schematic representation of: 1 – *cap* expression cassette in BacVP as described by Urabe et al (2); 2 – same cassette in a shuttle plasmid backbone; 3 – 5 - plasmid constructs derived for the current project. Genetic element designations are the same as in the Fig. 2 legend. The leftmost numbers correspond to lanes in panel B. **B** - Western blotting analysis of VP proteins in Sf9 cells after transient transfection with various plasmid DNAs. Cells in lane 1 were infected with BacVP (MOI of 5), cells in lanes 2-5 were transfected with the respective plasmids (Fig. 3A) and either infected (+), or not infected (-) with Bac-Rep (2) (MOI of 5) to supply transregulator IE-1

and Rep78. **C** – Western blotting analysis of VP proteins extracted from individual stable BSD^R cell lines after infection with Bac-Rep (MOI of 5) and harvested 72 hr post infection.

[6] **Fig. 4.** Characterization of *rep/cap* packaging cell lines F3 and G18. **A** – Average yields of purified rAAV-GFP tabulated in vector genomes per cell. Typical run was conducted in 100 ml suspension culture, cells were infected with BEVs at M.O.I. of 3: for Sf9 cells – with 3 BEV helpers (2); for *cap* stable cell line – with Bac-Rep and Bac-rAAV-GFP; for *rep* stable line – with Bac-VP and Bac-rAAV-GFP; for AAV2 (F3) and AAV1 (G18) *rep/cap* packaging line – with Bac-rAAV-GFP. rAAV-GFP was purified as described earlier (35). Four runs per cell line had been conducted. **B** – Western blotting analysis of AAV2 and AAV1 VP capsid proteins in crude lysates and purified rAAV-GFP. VP protein content in un-infected packaging line F3 (lane F3), F3 infected with Bac-rAAV-GFP (next lane) were compared to Sf9 cells infected with three BEV helpers (Sf9/3x). Crude lysates and purified rAAV-GFP were analyzed. **C** - Western blotting analysis of Rep proteins in crude lysates in F3, F3 – infected with Bac-rAAV-GFP, and Sf9 cells infected with three BEVs.

[7] **Fig. 5** Analysis of the rescue and amplification of the integrated *rep*-, and *cap* genes. **A** - Southern blotting analysis of the integrated *rep* and *cap* genes in F3 (*rep/cap*) and E5 (*cap*) stable lines. The parental plasmids were digested with a single cutter (*Xba*I), per lane amounts loaded were equivalent to 500-, 50-, or 5- copies of the plasmid DNA in 5 µg of chromosomal DNA. Chromosomal DNA samples from uninfected (5 µg, lanes F3 and E5) or BEV-infected (0.5 µg, lanes F3/BacGFP, E5/BacGFP, and E5/BacRep) were digested with *Xba*I (a single cutter, the positions of the *Xba*I sites are schematically shown below the respective panels), separated in 1.2% agarose gel, transferred to nylon filter and hybridized to ³²P-labeled *rep*-, or *cap* ORF DNA probes (left and right panels, respectively). White double arrowhead shows the form co-migrating with a linearized parent plasmid vector; black double arrowhead indicates a position of a DNA fragment hypothetically derived from the Rep-mediated nicking at the RBE. Rep-, and VP-encoding transcripts and their respective ORFs are diagrammed below the integrating cassettes. **B** – Diagram depicting a postulated feed forward loop. The transcription of both integrated *rep*-, and *cap* genes is induced by BEV-encoded IE-1 transregulator. One of the products, most likely Rep68/78 protein, retorts to interact with RBE inducing rescue/amplification and mediating the transcription.

[8] FIG. 6 Alignment of nucleotide sequences of AcMNPV *hr2* elements reported in this manuscript (*hr2-0.9* SEQ ID NO. 1) and elsewhere (5, 6). Residues in an alignment are colored according to the following scheme: black on white – non-similar residues; blue on cyan – consensus residue derived from a block of similar residues at a given position; red on yellow – consensus residue derived from a completely conserved residue at a given position. Endonuclease EcoRI recognition sites – axes of symmetry of the imperfect palindromes comprising 28 bp IE-1 transregulator-binding element - are underlined.

[9] FIG. 7 shows a diagram depicting a proposed arrangement of a system embodiment of the invention.

[10] FIG. 8 shows a diagram depicting a proposed arrangement of a system embodiment of the invention.

[11] FIG. 9 shows a diagram depicting a proposed arrangement of a system embodiment of the invention.

Detailed Description

[12] Embodiments of the present invention are based on the inventors' development of a novel, simple and efficient system of rAAV production in insect cells. In one embodiment, the system takes advantage of DNA regulatory elements from two unrelated viruses - AcMNPV and AAV2. In a more specific embodiment, the endpoint design may comprise two components: 1) stable Sf9-based cell line incorporating integrated copies of *rep* and *cap* genes, and 2) Bac-GOI. *Rep* and *cap* genes that are designed to remain silent until the cell is infected with Bac-GOI helper which provides both rAAV transgene cassette and immediate-early (IE-1) transcriptional transregulator. Infection with Bac-GOI initiates rescue/amplification of integrated AAV helper genes resulting in dramatic induction of the expression and assembly of rAAV. The arrangement of this specific embodiment provides high levels of Rep and Cap proteins in every cell thus improving rAAV yields by 10-fold. The described vectors are modular in design and may be utilized for the production of other multiprotein complexes.

[13] According to one embodiment, the invention pertains to a method of producing rAAV in insect cells that includes obtaining a population of cells stably transformed to comprise at

least one copy of a polynucleotide sequence encoding a *rep* gene and at least one copy of a polynucleotide sequence encoding a *cap* gene; and infecting cells of said population with a vector comprising a polynucleotide sequence that encodes an rAAV transgene and a polynucleotide sequence that encodes a transcriptional transregulator that induces expression of said at least one copy of a polynucleotide sequence encoding a *rep* gene and at least one copy of a polynucleotide sequence encoding a *cap* gene.

[14] According to another embodiment, the invention pertains to a population of insect cells transfected with a first polynucleotide sequence with the following components arranged from a 5' to 3' direction: hr2-0.9-RBE-----rep78 and/or a second polynucleotide sequence comprising the following components arranged from a 5' to 3' direction: hr2-0.9-RBE-----cap. The inventors have discovered that the proximity of the hr2-0.9 element to the rep78 component may affect the expression of rep78. Inventors have found that the hr2-0.9 component has a higher up-modulation effect if it is 500 bp or more upstream of the rep78 component. In a more specific embodiment, the hr2-0.9 element is 500-1500 base pairs upstream from the rep78 element. The inventors believe that a similar arrangement will produce a higher up modulation effect on cap expression as well. Furthermore, the placement of the hr2-0.9 element upstream to the rep78 results in the production of Rep78 and Rep52.

[15] It is believed that IE-1 trans-regulator induces expression of the rep78 gene via interaction with the hr2-0.9 upstream element (the hr2-0.9 element may include an origin of replication, which as will be discussed below assist in rescue of the rep78 gene thereby resulting in amplification of Rep78). Expressed Rep78 then binds to the RBE which may cause a cleavage of the rep gene. This cleavage event results in a rescue of the rep78 gene which may then replicate within the cell (Fig. 5). It is believed that a similar phenomenon occurs with the second polynucleotide sequence that includes the cap gene (Fig. 5). In an alternative embodiment, the RBE element is omitted from the polynucleotide sequence. Not intended to be bound by any theory, the inventors believe that the BEV-encoded hr2-0.9 element includes an origin of replication. The wtAAV also includes a RBE which results in the rescue and amplification of AAV upon transfection of the cells with a BEV-rAAV-GOI vector.

[16] Abbreviations: TF – transcription factors; AcMNPV - *Autographa californica* multiple nuclear polyhedrosis virus; BEV - baculovirus expression vector; rAAV -

recombinant Adeno-associated virus; IE-1 - immediate-early transregulator 1; GOI - gene of interest;

[17] Data deposition footnote: GenBank accession # 1143868
AAV2 (Accession No. 043303)

SEQ ID NO. 1 represents a polynucleotide sequence of a rep 78 gene that may be implemented to transform cells of interest as described herein.

SEQ ID NO. 2 represents a polypeptide sequence of a Rep78 protein.

SEQ ID NO. 3 represents a polynucleotide sequence of a rep 52 gene that may be implemented to transform cells of interest as described herein.

SEQ ID NO. 4 represents a polypeptide sequence of a rep52 protein.

SEQ ID NO. 5 represents a polynucleotide sequence of a cap gene.

SEQ ID NO. 6 represents a polynucleotide sequence of a cap protein.

SEQ ID NO. 7 represents a polynucleotide sequence of an IE-1 *trans*-regulator.

SEQ ID NO. 8 represents a polypeptide sequence of an IE-1 *trans*-regulator.

SEQ ID NO. 9 represents an hr2 region from AcMNPV.

[18] In another embodiment, the invention pertains to an inducible expression system that comprises an inducer component and a cell component and utilizes at least two of the following elements: hr element, rep gene and product of rep, RBE and GOI. In a specific embodiment, a virus such as BEV is constructed to include an inducer element and a rep gene. The BEV enters cells (shown as dashed line) that have been transformed to include a construct that has, in 5' to 3' direction, an hr element, a RBE, and a GOI. FIG. 7 shows an example of this embodiment. Not to be bound by any particular theory, the inventors believe when the BEV infects the cell and propagates in the cell, the REP protein induces a cut in one

strand of the cell genome (or plasmid) harboring the expression cassette. The whole cassette including the hr element is rescued from the cell genome, or a plasmid in the cell, and begins replicating in the cell. This leads to an "amplification" of the expression cassette and the expression of the GOI.

[19] In another embodiment, the system includes a cell transformed to include a construct that comprises in 5' to 3' direction an hr element and a GOI. A BEV, upon infecting cell (dashed lines), expresses a transactivator, IE-1, that binds to the hr element, which in turn induces expression of GOI. See FIG 8 for an example of this arrangement.

[20] In a further embodiment, the system includes a cell transformed to include a construct that comprises an hr element, an RBE element, rep gene and GOI. The system also includes a BEV that acts an inducer component. See FIG. 9 for an example of this arrangement. The BEV enters the cell (dashed lines) and expresses a transactivator IE-1, which then interacts with the hr element. This initiates transcription in the expression cassette which involves expression of the rep gene. Expressed REP then binds to the RBE which in turn initiates rescue of the expression cassette from the cell genome and amplification of the expression cassette. While the rep gene is shown upstream of the GOI, the order of the rep gene and GOI should not be critical.

[21] The foregoing proteins and polypeptide sequences, as well as polynucleotides encoding the same, having substantial identity may be used in conjunction with present invention can also be employed in preferred embodiments. Here "substantial identity" means that two sequences, when optimally aligned such as by the programs GAP or BESTFIT (peptides) using default gap weights, or as measured by computer algorithms BLASTX or BLASTP, share at least 50%, preferably 75%, and most preferably 95% sequence identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to effect the properties of a protein. Non-limiting examples include glutamine for asparagine or glutamic acid for aspartic acid.

[22] The term "variant" as used herein refers to nucleotide and polypeptide sequences wherein the nucleotide or amino acid sequence exhibits substantial identity with a specified nucleotide or amino acid sequence SEQ ID NO, preferably 75% sequence identity and most

preferably 90-95% sequence identity to the sequences of the present invention: provided said variant has a biological activity as defined herein. The variant may be arrived at by modification of the native nucleotide or amino acid sequence by such modifications as insertion, substitution or deletion of one or more nucleotides or amino acids or it may be a naturally occurring variant. The term "variant" also includes homologous sequences which hybridise to the sequences of the invention under standard or preferably stringent hybridisation conditions familiar to those skilled in the art. Examples of the in situ hybridisation procedure typically used are described in (Tisdall et al., 1999); (Juengel et al., 2000). Where such a variant is desired, the nucleotide sequence of the native DNA is altered appropriately. This alteration can be made through elective synthesis of the DNA or by modification of the native DNA by, for example, site-specific or cassette mutagenesis. Preferably, where portions of cDNA or genomic DNA require sequence modifications, site-specific primer directed mutagenesis is employed, using techniques standard in the art.

[23] In specific embodiments, a variant of a polypeptide is one having at least about 80% amino acid sequence identity with the amino acid sequence of a native sequence full length sequence of satiation gut peptides as taught herein and known in the art. Such variant polypeptides include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N- and/or C-terminus, as well as within one or more internal domains, of the full-length amino acid sequence. Fragments of the peptides are also contemplated. Ordinarily, a variant polypeptide will have at least about 80% amino acid sequence identity, more preferably at least about 81% amino acid sequence identity, more preferably at least about 82% amino acid sequence identity, more preferably at least about 83% amino acid sequence identity, more preferably at least about 84% amino acid sequence identity, more preferably at least about 85% amino acid sequence identity, more preferably at least about 86% amino acid sequence identity, more preferably at least about 87% amino acid sequence identity, more preferably at least about 88% amino acid sequence identity, more preferably at least about 89% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, more preferably at least about 91% amino acid sequence identity, more preferably at least about 92% amino acid sequence identity, more preferably at least about 93% amino acid sequence identity, more preferably at least about 94% amino acid sequence identity, more preferably at least about 95% amino acid sequence identity, more preferably at least about 96% amino acid sequence identity, more preferably at least about 97% amino acid sequence identity, more preferably at least about 98% amino acid sequence

identity and yet more preferably at least about 99% amino acid sequence identity with a polypeptide encoded by a nucleic acid molecule shown in Attachment B or a specified fragment thereof. Ordinarily, variant polypeptides are at least about 10 amino acids in length, often at least about 20 amino acids in length, more often at least about 30 amino acids in length, more often at least about 40 amino acids in length, more often at least about 50 amino acids in length, more often at least about 60 amino acids in length, more often at least about 70 amino acids in length, more often at least about 80 amino acids in length, more often at least about 90 amino acids in length, more often at least about 100 amino acids in length, or more.

[24] "Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to re-anneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired identity between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995).

[25] "Stringent conditions" or "high stringency conditions", as defined herein, are identified by those that: (1) employ low ionic strength and high temperature for washing, 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50° C.; (2) employ during hybridization a denaturing agent, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42 degrees C.; or (3) employ 50% formamide, 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5.times. Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42 degrees C, with washes at 42 degrees C in 0.2X SSC (sodium chloride/sodium citrate) and 50% formamide at 55 degrees

C, followed by a high-stringency wash consisting of 0.1XSSC containing EDTA at 55 degrees C.

[26] "Moderately stringent conditions" are identified as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and % SDS) less stringent than those described above. An example of moderately stringent conditions is overnight incubation at 37.degree. C. in a solution comprising: 20% formamide, 5XSSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5.times. Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1.times.SSC at about 37-50 degrees C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

[27] Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20⁰ C below the calculated T_m of the hybrid under study. The T_m of a hybrid between a polynucleotide having a nucleotide sequence shown in SEQ ID NO: 1 or the complement thereof and a polynucleotide sequence which is at least about 50, preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences can be calculated, for example, using the equation of Bolton and McCarthy, Proc. Natl. Acad. Sci. U.S.A. 48, 1390 (1962):

$$T_m = 81.5^{\circ} - C - 16.6(\log_{10} [Na^+]) + 0.41(\% G+C) - 0.63(\% \text{formamide}) - 600/l,$$

where l = the length of the hybrid in basepairs.

[28] In a specific embodiment, stringent wash conditions include, for example, 4 X SSC at 65⁰ C, or 50% formamide, 4 X SSC at 42⁰ C, or 0.5 X SSC, 0.1% SDS at 65⁰ C. Highly stringent wash conditions include, for example, 0.2 X SSC at 65⁰ C.

Examples

[29] Example 1: Cloning of AcMNPV (Accession No. NC_001623) homologous region 2 (hr2). In order to simplify this system embodiment and reduce the number of components, the inventors sought to derive Sf9-based stable lines expressing AAV *rep* and *cap* genes. The

challenge of expressing AAV helper genes in the heterologous environment of an insect cell necessitates the use of baculovirus-derived promoters (e.g. *polh*) which are fully functional only in the context of the whole genome, i.e. next to other viral regulatory elements. Simple cassettes with *rep* and *cap* ORFs placed downstream of baculovirus promoters and integrated into the host chromosome, will not achieve similar expression levels as compared to the same modules in the context of a BEVs. Therefore, the inventors set forward to develop a novel modular cassette capable of highest levels of expression when remotely separated from BEV. An additional challenge in constructing such cassettes is that in the wt AAV genome, genes encoded by collinear ORFs within one DNA sequence are transcribed into separate mRNAs from the P5, P19, and P40 promoters. Making three independent integrating modules, each driven by its own promoter, makes the selection process technically complicated.

[30] It was hypothesized that the *rep78* ORF could be designed to express both Rep78 and Rep52 hence eliminating the need for a separate vector encoding Rep52 ORF. It was inferred that a BEV-derived enhancer, such as homologous region sequence (*hr*), could be utilized to increase the transcription rate from wt AAV P19 promoter thus improving the stoichiometry of Rep52/78.

[31] To test the hypothesis, *hr2* was cloned from the wt AcMNPV and its sequence was compared to the other two previously published *hr2* sequences (6, 7) (Fig. 6). Our *hr2* isolate (hereafter referred as *hr2-0.9*), although homologous to both reported sequences, showed significant variability, including multiple single nucleotide deletions and insertions, as well as longer stretches such as 72 bp insertion incorporating an additional 28 bp IE-1 binding element.

[32] Example 2: Rep-expressing cassettes. To test whether *hr2-0.9* could enhance the transcription from wt AAV2 P19 promoter, a series of plasmid vectors (Fig. 1A) were constructed and the expression of *rep52* and *rep78* genes were tested in a transient transfection assay in insect Sf9 cells. As reported earlier (2), infection Sf9 cells with Bac-Rep harboring head-to-head *rep78* and *rep52* genes resulted in the expression of both Rep52 and Rep78 proteins (Fig. 1B, lane 1). The same cassette in the context of a transfer plasmid pFBD-LSR (2) did not produce any Rep proteins (Fig. 2B, lane 2) suggesting a requirement for an enhancer apparently present in a context of Bac-Rep BEV vector carrying Rep-expression cassettes. Insertion of *hr2-0.9* between head-to-head *polh* and Δ IE-1 promoters

created a cassette reminiscent of a transcription control element described earlier (8), with the exception of a *hr2-0.9* substituted for *hr5*. As expected, in the presence of BEV-derived IE-1 transregulator (supplied by helper Bac-VP BEV), the enhancer mediated an expression of both Rep78 and Rep52, the latter one expressed at a lower rate in spite of the fact that it was driven by a considerably stronger *polh* promoter (Fig. 2B, lane 3). The orientation of *hr2-0.9* did not appear to significantly change the expression of either gene (Fig. 2B, lane 4). Because of a limited success in expressing Rep52, we re-designed the vector placing *rep78* ORF under control of the late *polh* promoter while completely removing *polh/rep52* cassette. *hr2-0.9* was also moved further upstream in order to emulate the context of BEV genome with more distantly positioned *hr* elements. The inventors discovered that if the *hr* element is too close to introduce an additional regulatory element upstream of the *rep78* ORF, the DNA sequence containing wt AAV2 Rep-binding element (RBE) was inserted. It was shown earlier that either AAV ITR or P5 RBE modulate the expression from P19 promoter (9). In addition, P5 RBE mediated the amplification of integrated adeno-associated virus sequences in mammalian cells (10) thus dramatically improving yields of rAAV production in HeLa-based packaging cell lines. The combination of introduced changes resulted in a dramatic upregulation of the expression of *rep52* gene driven by AAV2 P19 promoter (Fig. 2B, lane 5). Therefore, combining regulatory elements from both AAV and BEV in one vector allowed us to achieve an efficient expression of both Rep78 and Rep52 from a single *rep78* ORF cassette.

[33] Example 3: Rep-expressing stable cell lines. For the purpose of rAAV production, stable mammalian cell lines expressing AAV Rep78/52 are notoriously difficult to generate due to the genotoxic effect of the *rep* component (11). To make a stable line, a complete shutoff of the integrated *rep* ORF is therefore required. Until now, no similar stable insect cell-based lines expressing AAV *rep/cap* functions have been reported. Having designed the vector with *hr2-0.9*-mediated robust expression of both Rep78 and Rep52 in Sf9 cells, the inventors wondered whether the Rep-expression cassette could be utilized to derive stable cell lines.

[34] The plasmid pIR-*rep78-hr2-RBE* (#5 in Fig. 1A), in addition to Rep expression cassette was constructed to harbor OpIE1 viral promoter-driven *bsd* (blasticidin S deaminase) gene conferring resistance to blasticidin S. This plasmid had been used to transfect Sf9 cells and select for BS-resistance to derive 24 individual stable cell lines. Remarkably, all analyzed

BS^R cell lines, upon infection with Bac-VP BEV, showed expression of both Rep proteins albeit at different levels and at variable stoichiometric ratios (Fig. 1C).

[35] Example 4: Analysis of AAV2 P19 promoter in Sf9 cells. Without being limited to any theory, induced expression of *rep52* suggested two possibilities: 1) a read-through activation from the upstream *polh* promoter similar to Adenovirus type 5 early region 1 transcription (12); and 2) activation by elements present within the P19 promoter itself. To distinguish between these two mechanisms, we mapped the transcription initiation site of integrated *rep* gene from a *rep/cap* stable BS^R line F3. Using RLM-RACE protocol, the transcription of *rep52* gene was determined to be initiated at AAV2 nt 874 (Fig. 2A), i.e. exactly at the same position as in mammalian cells. Therefore, it appeared that the upstream *hr2-0.9* directed host cell and BEV-encoded factors to initiate strong transcription from the heterologous P19 promoter.

[36] It was further hypothesized that BEV-encoded immediate early transactivator IE-1 mediates the induction of P19 transcription by interacting with other transcription factors (TF) upstream of P19. To elucidate the mechanism, an EMSA using 128 bp P19 PCR fragment was performed which excluded both TATA-35 and TATA-20 sites. Little if any binding was detected in extracts from uninfected Sf9 cells (Fig. 2B, lane 2). On the contrary, extracts from BEV-infected Sf9 cells formed multiple DNA-protein complexes with P19 promoter (Fig. 2B, lane 3). Some of these complexes (for example, doublet band marked 'A') were not associated with IE-1, as demonstrated by competitive binding to unlabeled P19 probe (lanes 4 and 5) as opposed to the absence of such competition by IE-1 consensus binding element (lanes 6 to 8). The other complexes, such as band 'D', were clearly IE-1-specific as they were outcompeted by both with P19 and IE-1 probes. Complexes 'B' and 'C' were partially competed with 100-fold excess of IE-1 (lane 8) while 15-fold excess of p19 in lane 5 might not have been sufficient to significantly reduce the intensity of these bands.

[37] Lanes 9-13 demonstrate the specificity of IE-1 consensus element in binding: three DNA-protein complexes ('E', 'F', and 'G') are specifically competed with un-labeled IE-1. Two lower bands presumably consist of mono-, and dimer forms of IE-1 (13) while the upper complex 'G' might represent an IE-1 dimer bound to another TF. Therefore, the Rep-expressing integrating cassette provides multiple TF binding sites thus emulating the regulation of the transcription in wt AAV2 genome expressing Rep78/52 and Rep68/40 from a single *rep78* ORF.

[38] Example 5: Cap-expressing cassettes. Using the same plasmid backbone and same regulatory elements, a series of vectors were constructed to express AAV2 *cap* gene encoding structural proteins VP1, VP2, and VP3 (Fig. 3A). Similarly to *rep* cassette, the presence of *hr* enhancer element was required for the *cap* gene to be expressed: same *polh*-driven *cap* ORF was either strongly expressed in the context of BEV Bac-VP (2) (Fig. 3B, lane 1) or was completely silent in the plasmid backbone, regardless whether IE-1 is supplied *in trans* by BEV (Fig. 3B, lanes 2+/-). Adding *hr2-0.9* element upstream resulted in the induction of VPs expression but only in the presence of BEV infection (Fig. 3A, B, lanes 3+/-). The requirement for IE-1 appears to be absolute as we were unable to detect the expression of VPs in either of the constructs if transiently transfected cells were not infected with Bac-Rep helper (Fig. 3B, all lanes marked by “-“ symbol). We have also observed a slight orientational effect of *hr2-0.9* (compare lanes 3+ and 4+ in Fig. 3B). In the presence of RBE the VP expression appears to be also enhanced (lanes 3+ vs 5+).

[39] pIR-VPm11-hr2-RBS (vector #5 in Fig. 3A) was used to derive twenty BS^R stable cell lines selected and propagated similar to Rep-expressing lines. Upon induction of *cap* gene with BacRep BEV infection, the majority (18 out of 20) of lines expressed VP1, VP2, and VP3 structural proteins of AAV2 (Fig. 3C).

[40] Example 6: *rep/cap*-packaging stable cell lines. To derive Rep/VP-expressing lines, cells were co-transfected with the respective Rep-, and Cap-expressing plasmids and BS^R clones were derived. Twenty individual *rep/cap* packaging cell lines were screened for their capacity to produce rAAV-GFP upon infection with Bac-rAAV-GFP helper (data not shown). One (designated as F3) was selected for the further analysis and for the production of rAAV. The yields of the purified vectors derived using F3 packaging cells were on average, 1.4×10^5 drp per cell. This yield exceeded the yield of a triple infection protocol by 10-fold (Fig. 4A).

[41] Because the described vectors are modular in design, the same control elements could be utilized for the expression of other AAV serotypes capsid proteins. In particular, we have also constructed AAV2 *rep*-AAV1 *cap* stable cell line dubbed G18. The analysis of the purified GFP-expressing AAV1 vector capsid composition and the yield is shown in Fig. 4B and 4A, respectively.

[42] To characterize the mechanism of the higher yields, the amount of Rep and VP proteins synthesized in F3 cell line were analyzed. There was no detectable expression of VP (Fig. 4B, lane F3) or Rep (Fig. 4C, lane F3) proteins in uninfected F3 cells followed by the dramatic increase of the expression of both genes induced by Bac-GFP infection. At the time of harvest, the concentration of Cap proteins in F3 was 2-3 times higher than in triple-infected Sf9 cells (Fig. 4B, lane F3/Bac-GFP vs Sf9/3x), while the amounts of Rep proteins, especially Rep78 were overwhelmingly higher compared to SF9 cells co-infected with three BEV helpers (Fig. 4C).

[43] Example 7: Integrated *rep* and *cap* genes are amplified by BEV infection. The mechanism of the induction of the expression of the integrated helper genes was investigated by analyzing total DNA isolated from stable F3 (*rep/cap*) and E5 (*cap*) cell lines (Fig. 5). Southern blotting in conjunction with RT-PCR assays were used to estimate the number of integrated copies. In uninfected cells from *rep/cap* line F3, two copies of *rep* and four copies of *cap* genes were documented (Table 1, Fig. 5, lanes marked F3). Although RT-PCR data indicated less than two *rep* copies (1.47 copies) per cell, the DNA hybridization pattern revealed that at least two copies of the *rep* gene were integrated as a head-to-tail concatemer producing, upon digestion with a single cutter *Xba*I, a monomer fragment of the exact size of the linearized vector (Fig. 5). At 72 hrs post infection with Bac-rAAV-GFP (MOI of 3), the number of copies of *rep* and *cap* genes increased up to 57 and 211, respectively (Table 1, Fig. 5, lanes marked F3/BacGFP). This further exhibits that a portion of the amplified molecular structures apparently incorporated a head-to-tail concatemers (Fig. 5, white double arrowhead). A lower discrete band of about 3.6 Kbp (Fig. 5, black double arrowhead) is equimolar to the upper band, suggesting that it could be junction fragment of the integrated concatemers in the amplified structure. It should be noted that the size of this fragment is equivalent to the distance between *Xba*I recognition site and an RBE site upstream which might indicate a Rep-dependent mechanism of the rescue and amplification. Indeed, in the absence of Rep (Fig. 5, lane E5/BacGFP), when *cap*-containing E5 cells are infected with Bac-rAAV-GFP, the lower band no longer appears in the rescued structure. When Rep was driven by the immediate early promoter Δ IE-1 (as in Bac-Rep helper), the effect of total and Rep-mediated amplification was much stronger boosting *cap* copy number per cell almost up to 1,200 (Table 1, Fig. 5, lane E5/BacRep). The precise molecular mechanism of the rescue/amplification is currently under investigation.

Discussion related to Examples 1-7

[44] While extremely promising, the original protocol of rAAV production in Baculovirus system had not been widely adopted. One of the main reasons is the complexity of the system involving three independent BEV helpers. According to the Poisson distribution (14), at the optimal multiplicity of infection (MOI) of 3 (15), 95% of cells are infected with at least one BEV, but only 22.4% of cells are infected with 3 particles. At the higher MOIs this ratio reduced even further: for example, at MOI of 9 only 12.5 % cells are infected with 9 particles. For rAAV production, cells are infected with the combination of three different BEVs, and the fraction of cells infected with all three helpers at the optimal stoichiometric ratio apparently is even lower (16). For example, solving the Poisson distribution for a particular combination of individual BEV helpers (e.g., 3:3:3) predicts the ratio of the respectively infected cells to be only 1.1%. The specifics of rAAV production also requires a coordinated and timely expression of seven helper Rep and Cap proteins at the optimal stoichiometry (2, 5, 16, 17). Adding to the complexity of the system is the fact of the apparent instability of the recombinant BEVs, especially Bac-Rep helper (5). In essence, rAAV production in insect cells poses technical challenges that are quite different from the basic protein manufacturing. We sought to reduce the complexity by deriving packaging stable cell line incorporating *rep* and *cap* helper genes. To this end, we utilized one of the critical genetic elements of the AcMNPV – homologous region. Earlier Habib *et al.* have reported that AcMNPV *hr1* enhances transcription from the polyhedrin promoter in a classic enhancer-like manner (18). Other BEV *hrs* have also been shown to display a potent enhancer function on exogenous and endogenous promoters in the absence of any viral transactivator suggesting that the binding of host factors might be involved in the enhancer mechanism (19). Considering the enhancing propensity of *hrs* to mediate transcription even without BEV-encoded IE-1 factor, one would predict some basal level of transcription from the integrated *rep* genes. However, it appears that we have achieved a complete expression shutoff of both *rep* and *cap* ORFs positioned downstream of *hr2-0.9* in the absence of BEV infection.

[45] The mechanism by which DNA binding promotes IE-1 transactivation is unknown. Olson *et al.*, hypothesized that DNA binding is required for conformational changes in IE-1, a pre-requisite to subsequent interaction with other transcription factors and *trans* stimulation (13). However, binding to *hr* alone is insufficient for IE-1-mediated enhancer activity (20-22). Here, we show that upon infection with BEV, several proteins form complexes with P19

promoter, and some of these complexes apparently incorporate IE-1. It is unlikely that IE-1 directly binds to P19 as we were unable to identify canonical IE-1 binding element within the tested P19 fragment. More likely, *hr*-bound IE-1 interacts with Sf9 host cell factors such as SP1 described recently in Sf9 cells, a transcription factor that was also shown to be capable of binding to the canonical SP1-response element from mammalian cells (23, 24). Incidentally, two SP1-binding sites (SP1-130 and SP1-50, Fig. 2A) were shown to be involved in regulation of transcription from AAV2 P19 promoter (9, 25). Hence, our working model of P19 activation includes insect host cell SP1 and BEV IE-1 bound to their respective response elements within P19 and *hr2-0.9*. Bound factors subsequently form a *trans*-activation complex via bent and looped-out DNA similar to what was described for AAV2 P5/P19 interaction (9, 26).

[46] Interestingly, it appeared that the induced *rep* gene generated a complete set of Rep proteins including smaller Rep68 and Rep40, products of the spliced P5- and P19-derived transcripts. This seems to be a plausible scenario as splicing does occur for BEV-encoded *ie-1* transcripts generating another immediate-early transregulator IE-0 (27). Moreover, even AAV2 *rep*-, and *cap*-derived transcripts undergo splicing in Sf9 cells infected with BEVs (4). The Rep- and VP-expressing cassettes, therefore, emulate wt AAV genome utilizing *hr* and RBE DNA elements to upregulate the internal P19 promoter allowing high level expression of the smaller Rep isoforms while still relying on non-canonical ACG start codon for VP1 initiation (Fig. 5A).

[47] One of the advantageous features of the described system is its propensity to rescue and amplify the integrated genes up to 1200 copies per cell. The precise molecular mechanism of such amplification is a subject of a separate study. Nevertheless, it is clear that a feed forward loop is initiated whereby Rep protein encoded by the integrated *rep* genes interacts with RBE to further enhance the helper cassette amplification and expression (Fig. 5B). Moreover, Rep might take part in the transcription initiation complex mediating P19 promoter activity in conjunction with IE-1. Although Rep is an integral part of rAAV production, the system can efficiently function without Rep protein (Fig. 5, Table 1) suggesting that its modular design could be utilized not only for the production of other AAV serotypes but for unrelated multiprotein complexes as well.

[48] In conclusion, we have designed a simple inducible expression system consisting of only two components: stable Sf9-based cell line and a single BEV. To this end, we utilized, in unconventional way, two genetic elements - *hr2* from AcMNPV and RBE from AAV2 providing inducible expression of *polh*-driven *rep78/68* and *cap* helper genes as well as P19-driven *rep52/40*. The arrangement provided 10-fold higher yield of rAAV vectors compared to the original triple infection protocol.

Materials and Methods related to Examples 1-7

[49] Cloning of AcMNPV homologous region 2 (*hr2*). Wild type AcMNPV was prepared as described previously (28) and the DNA sequence of *hr2* [AcMNPV complete genome, nt 26293-26961 GenBank accession # NC_001623, (7)] was amplified using PCR-mediated protocol (Suppl. 2). The band was cloned into pGEM-TEasy and sequence-verified (GenBank accession # 1143868).

Mapping 5'-end of *rep52* transcript. We utilized RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) kit (Ambion). Cells from *rep/cap* BS^R line F3 were propagated at 2x10⁶ cells/ml and infected with recombinant Bac-rAAV-GFP (MOI of 5). Seventy-two hrs post-infection, cells were harvested and total RNA was isolated. AAV-specific primers (Suppl. 2) were used in conjunction with 5'RACE Outer and Inner Control primers were provided with the kit. The resulting PCR fragment was subcloned into pGEM-TEasy plasmid (Promega) and 10 random clones were sequenced.

[50] Baculovirus titering. BEV titers were determined by qPCR assay developed in our laboratory. The assay is an adaptation of an alkaline PEG-based method for direct PCR (29). Briefly, 5 µl of baculovirus stock is added to 95 µl of alkaline PEG solution (PEG 200, pH 13.5) prepared as described earlier (29). After vortexing, the sample is incubated at room temperature for 15 min and then diluted 5-fold by adding 0.4 ml H₂O. Five µl of this diluted mixture was used directly in RT-PCR 25 µl reaction mixture containing 12.5 µl SybrGreenER and 1.5 µl of 5 µM primers (Suppl. 2). The sample is assayed side-by-side with a serially diluted reference standard, a BEV of a known titer. The amplified sequence is part of AcMNPV gene *Ac-IE-01*, locus tag - *ACNVgpl42*, a putative early gene transactivator; the size of the amplified DNA fragment is 103 bp.

[51] Construction of Sf9 stable cell lines. To select and propagate cell lines procedures previously described were followed (30). Blasticidin selection (25 µg/ml) was used for three weeks after which antibiotics were omitted from the media and cells were maintained in regular SFM. To construct cell lines expressing all wt AAV2 proteins, Sf9 cells were co-transfected with undigested pIR-rep78-hr2-RBE and pIR-VPm11-hr2-RBE at the molar ratio of 1:2.5. In-house liposomes were used for the transfection. Screening for the most efficient packaging *rep/cap* cell line had been performed with 10⁶ cells from each clonal line infected with Bac-rAAV-GFP. At 72 hr post-infection, cell were harvested and subjected to two freeze/thaw cycles. Aliquots of the lysates were used to infect 293 cells. *Rep*-, and *cap* lines were picked randomly and the protein expression had been analyzed by Western blotting analysis in cells infected with BEV (Bac-rAAV-GFP for *rep*- and Bac-Rep for *cap* lines).

RNA Isolation. Total RNA from Sf9 cells was isolated by using TRIzol reagent (Invitrogen,) following on-column of DNA digestion and concentration by using RNase-Free DNase Set and RNeasy Mini Elute Cleanup Kit (Qiagen Inc, Valencia, CA). RNA integrity was verified by agarose gel (1.2%) electrophoresis with EtBr staining.

[52] Western blot analysis. Sf9 cells growing in SFM media in suspension were harvested by centrifugation, washed with ice-cold PBS and resuspended in lysis buffer containing 50 mM Tris HCl, pH7.5, 120 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM Na₄P₂O₇, 1 mM phenyl-methylsulfonyl fluoride (PMSF), 1 mM EDTA, and 1 mM EGTA supplemented with Protease Inhibitor Cocktail (Set 3) (Calbiochem, San Diego, CA). The suspension was incubated on ice for one hr and clarified by centrifugation for 30 min at 14000 rpm, 4°C. Normalized for protein concentration samples were separated using 12% PAAG/SDS electrophoresis, transferred to a nitrocellulose membrane, and probed with the anti-cap B1 monoclonal antibodies (1:4000, generously donated by Dr. Muzyczka or anti-Rep 11F monoclonal antibodies (1:4000, a gift from Dr. Muzyczka), following by ECL Anti-mouse IgG, horse radish peroxidase- linked, secondary antibodies (1:1000, Amersham Biosciences, Little Chalfont Buckinghamshire, UK).

[53] Electrophoretic Mobility Shift Assay (EMSA). EMSA was carried out as described previously (31). In brief, un-infected, or BEV-infected (72 hr post infection) Sf9 cells were harvested at 2x10⁶ cells/ml and washed with ice-cold PBS. Packed cells were resuspended in 5 volumes of hypotonic buffer (10 mM HEPES, pH7.9; 1.5 mM MgCl₂; 10 mM KCl; 0.2 mM PMSF; 0.5 mM DTT) and allowed to swell on ice for 10 min. Following homogenization in a

glass Dounce, nuclei were collected by centrifugation 15 min at 3300xg. The nuclei were then resuspended in high salt buffer containing 20 mM HEPES, pH7.9; 1.5 mM MgCl₂; 0.7 M KCl; 0.2 mM EDTA; 0.2 mM PMSF; 0.5 mM DTT; 25% glycerol. Nuclei were allowed to extract for 30 min on ice and pelleted for 30 min at 25,000xg. Aliquots of the supernatant were flash-frozen in liquid nitrogen. Binding reactions (20 µl) containing 50 fmol ³²P-labeled DNA probe; 1 µg poly(dI-dC); 20 mM HEPES, pH7.9; 100 mM KCl; 1 mM EDTA; 1 mM DTT, 12 % glycerol and 60 µg nuclear extract were incubated for 30 min at 27°C. In some instances, unlabeled DNA fragments were added into the assay for competition binding. Non-denaturing 4% PAAG (40:1 acrylamide/bisacrylamide) containing 2.5% glycerol was ran in 0.5xTBE at 30 mA for 2 hr, the gel was transferred on DEAE filter paper, dried, and exposed to X-ray film.

[54] DNA Isolation and RT PCR. Total DNA from approximately 5x10⁶ cells was isolated using DNeasy Blood & Tissue Kit (Qiagen Inc, Valencia, CA). One hundred ng of DNA was used for the quantification analysis by RT-PCR. DNA was amplified by using SYBR GreenER qPCR Supermix (Invitrogen, Carlsbad, CA) and the specific primers (Suppl.2). For the calculations of the integrated gene copy number, the size of Sf9 genome was assumed to be 400 Mb (32).

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Baculovirus and Insect Cell Expression Protocols (chapter 6) By David W. Murhammer Contributor David W. Murhammer, Edition: 2, illustrated, Published by Humana Press, 2007, ISBN 1588295370, 9781588295378 discussing alternative cell lines that can be utilized in accordance with the teachings herein. The subject matter referenced in this cite is incorporated by reference.

[55] In reviewing the detailed disclosure which follows, and the specification more generally, it should be borne in mind that all patents, patent applications, patent publications, technical publications, scientific publications, and other references referenced herein are hereby incorporated by reference in this application in order to more fully describe the state of the art to which the present invention pertains.

[56] Reference to particular buffers, media, reagents, cells, culture conditions and the like, or to some subclass of same, is not intended to be limiting, but should be read to include all such related materials that one of ordinary skill in the art would recognize as being of interest or value in the particular context in which that discussion is presented. For example, it is often possible to substitute one buffer system or culture medium for another, such that a different but known way is used to achieve the same goals as those to which the use of a suggested method, material or composition is directed.

[57] It is important to an understanding of the present invention to note that all technical and scientific terms used herein, unless defined herein, are intended to have the same meaning as commonly understood by one of ordinary skill in the art. The techniques employed herein are also those that are known to one of ordinary skill in the art, unless stated otherwise. For purposes of more clearly facilitating an understanding the invention as disclosed and claimed herein, the following definitions are provided.

[58] While a number of embodiments of the present invention have been shown and described herein in the present context, such embodiments are provided by way of example only, and not of limitation. Numerous variations, changes and substitutions will occur to those of skilled in the art without materially departing from the invention herein. For example, the present invention need not be limited to best mode disclosed herein, since other applications can equally benefit from the teachings of the present invention. Also, in the claims, means-plus-function and step-plus-function clauses are intended to cover the structures and acts, respectively, described herein as performing the recited function and not only structural equivalents or act equivalents, but also equivalent structures or equivalent acts, respectively. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the following claims, in accordance with relevant law as to their interpretation.

Claims

1. An inducible protein expression system, said system comprising
a viral vector component that comprises an inducer element component and a rep gene; and
a cell component, said cell component comprising at least one cell stably transformed with an expression cassette comprising, in a 5' to 3' direction
an hr element, a RBE element, and a gene of interest,
wherein expression of said gene of interest is induced upon infection of said cell with said BEV component.
2. The system of claim 1, further comprising a promoter upstream of said gene of interest and downstream of said RBE element.
3. The system of claim 1, wherein said expression cassette is integrated into a genome of said cell component and is rescued therefrom and amplified upon expression of said rep gene.
4. The system of claim 1, wherein said viral vector component is BEV
5. The system of claim 1, wherein said cell component comprises an insect cell.
6. The system of claim 1, wherein said cell component comprises an insect sf9 cell.
7. An inducible protein expression system, said system comprising a
a viral vector component that comprises an inducer element; and
a cell component, said cell component comprising at least one cell stably transformed with an expression cassette comprising an hr element upstream of a gene of interest;
wherein expression of gene of interest induced upon infection of said cell component with said viral vector component.
8. The system of claim 7, wherein said viral vector component is BEV
9. The system of claim 7, wherein said cell component comprises an insect cell.

10. The system of claim 7, wherein said cell component comprises an insect sf9 cell.
11. An inducible protein expression system, said system comprising
a viral vector component that comprises an inducer element; and
a cell component comprising at least one cell stably transformed with an expression cassette comprising, an hr element, a RBE element, a rep gene and a gene of interest, wherein expression of said rep gene and said gene of interest is induced upon infection of said cell component with said viral vector component; and rescue and amplification of said expression cassette is induced upon expression of said rep gene.
12. The system of claim 11, wherein said viral vector component is BEV.
13. The system of claim 11, wherein said rep gene is upstream of said gene of interest or vice versa.
14. The system of claim 11, wherein said expression cassette is integrated into a genome of said cell component and is rescued therefrom and amplified upon expression of said rep gene.

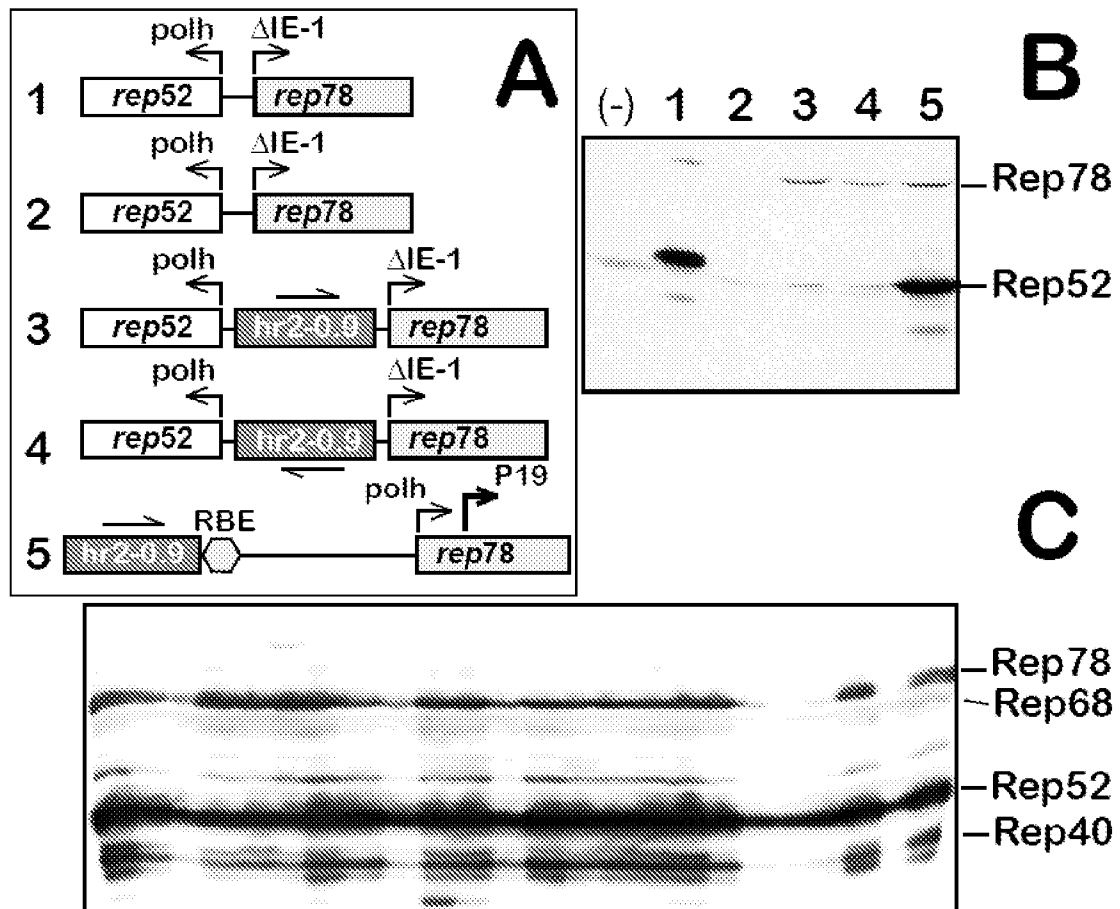


FIG. 1

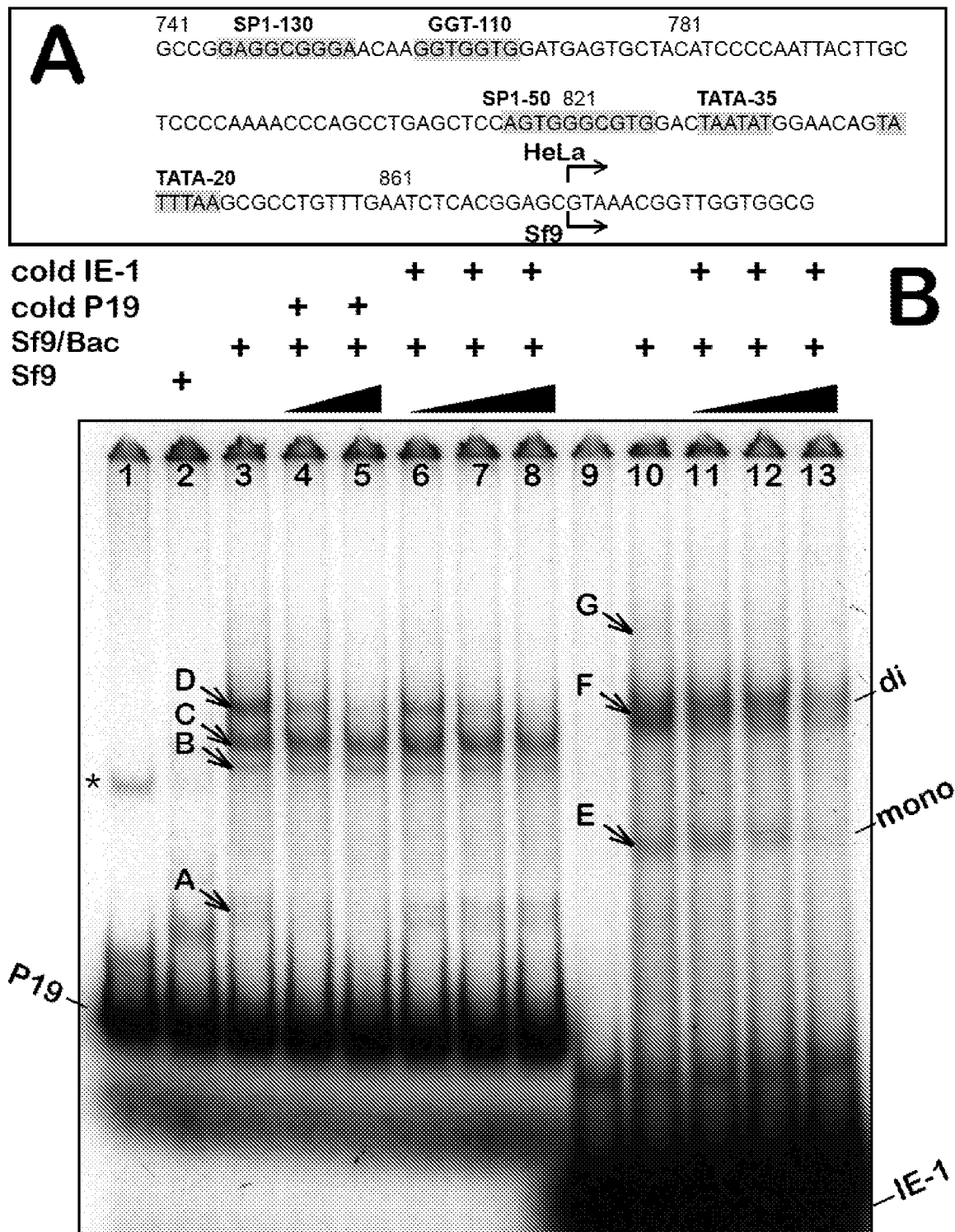


FIG. 2

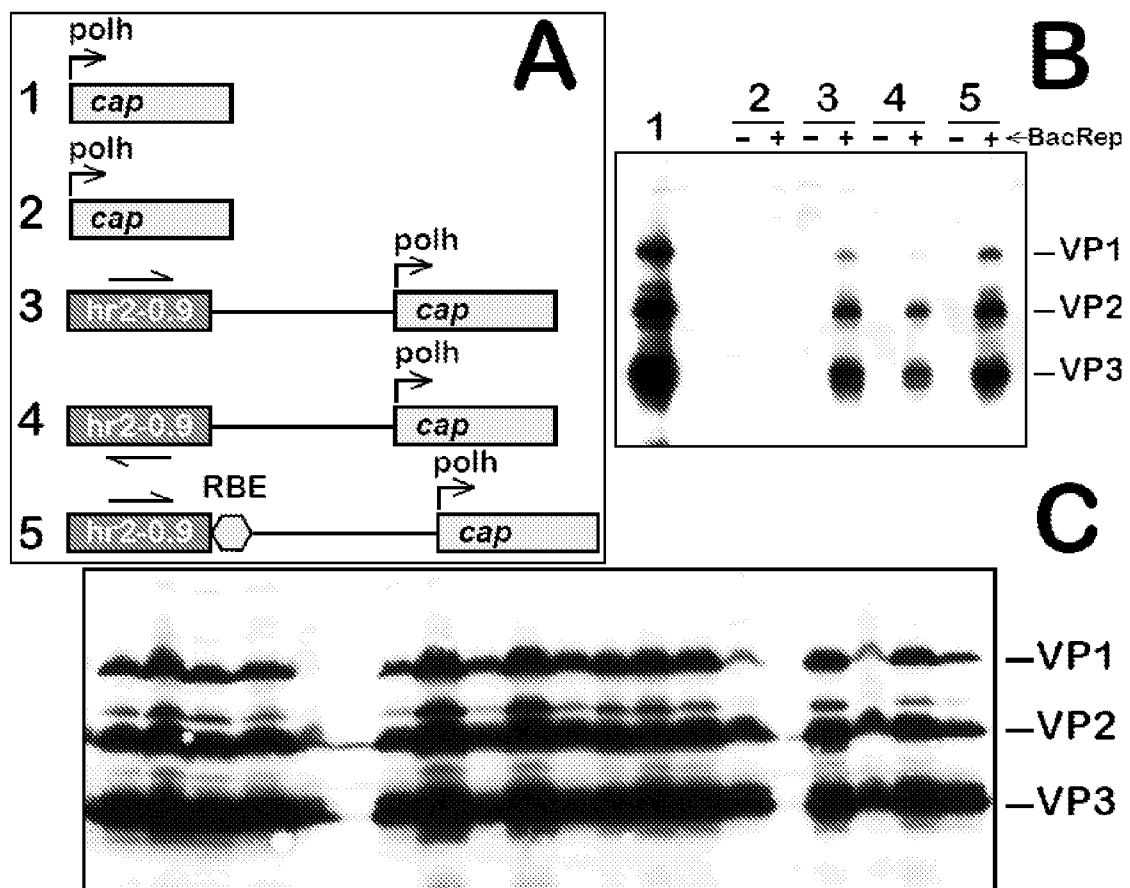


FIG. 3

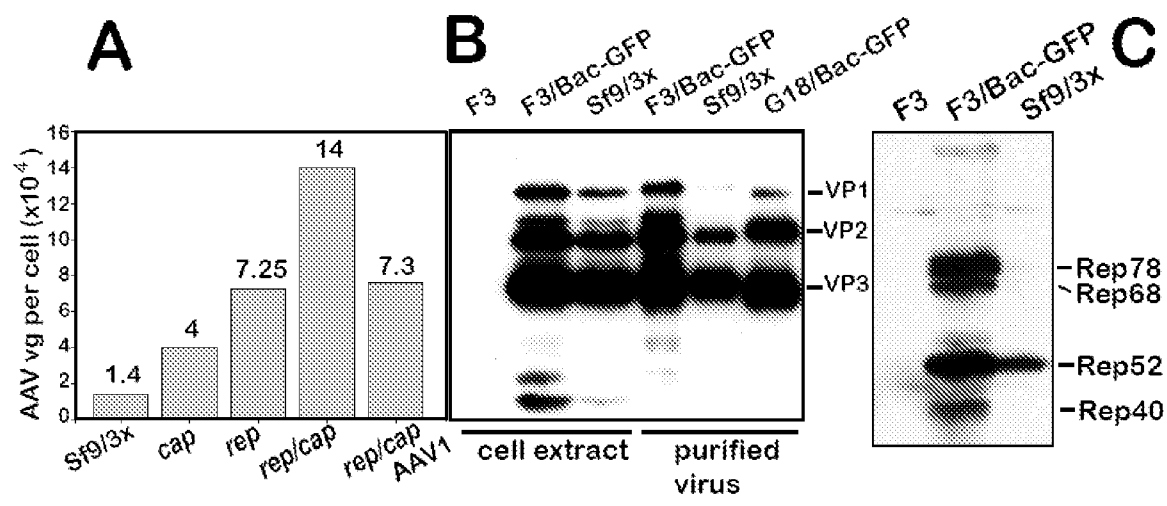


FIG. 4

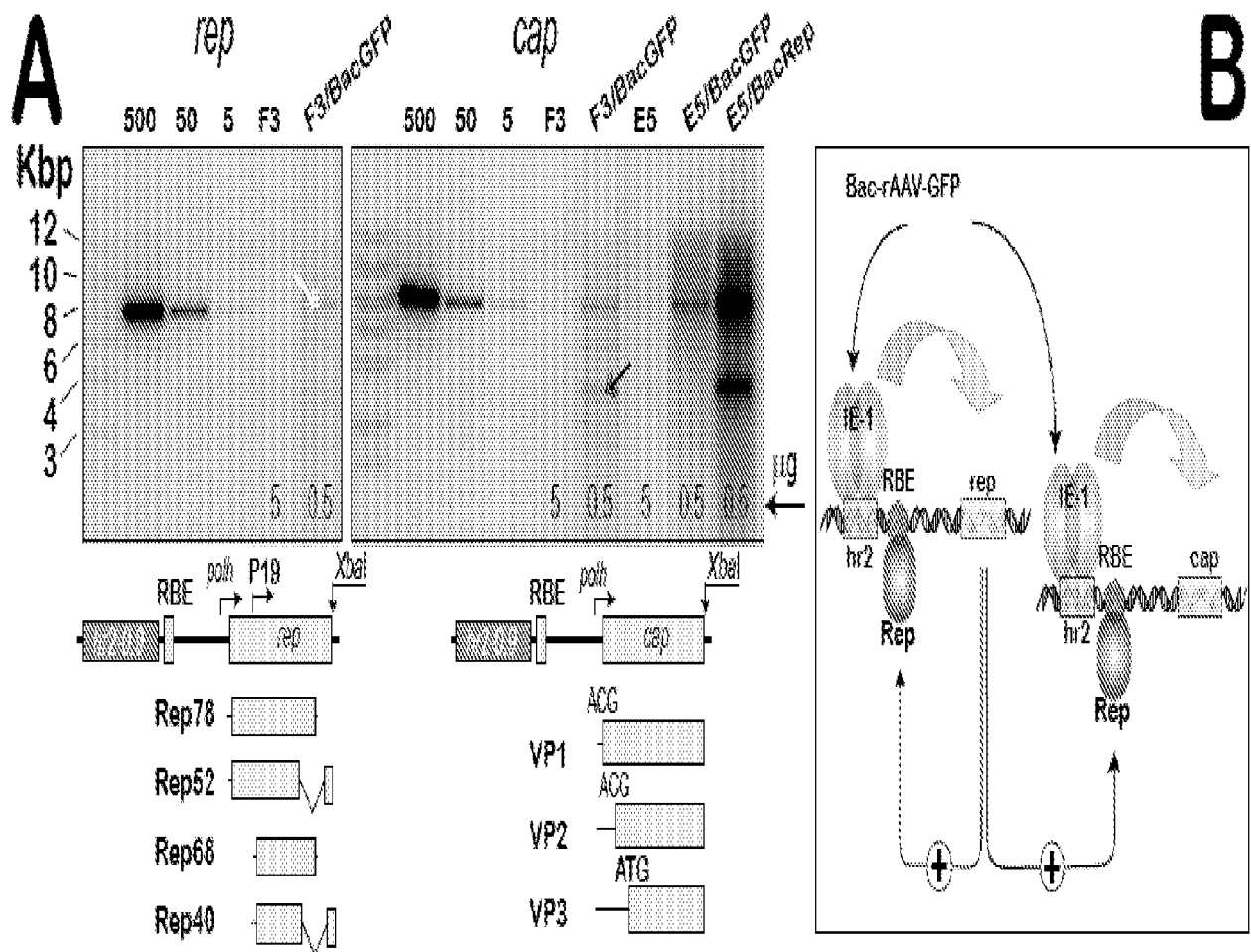


FIG. 5

FIG. 6

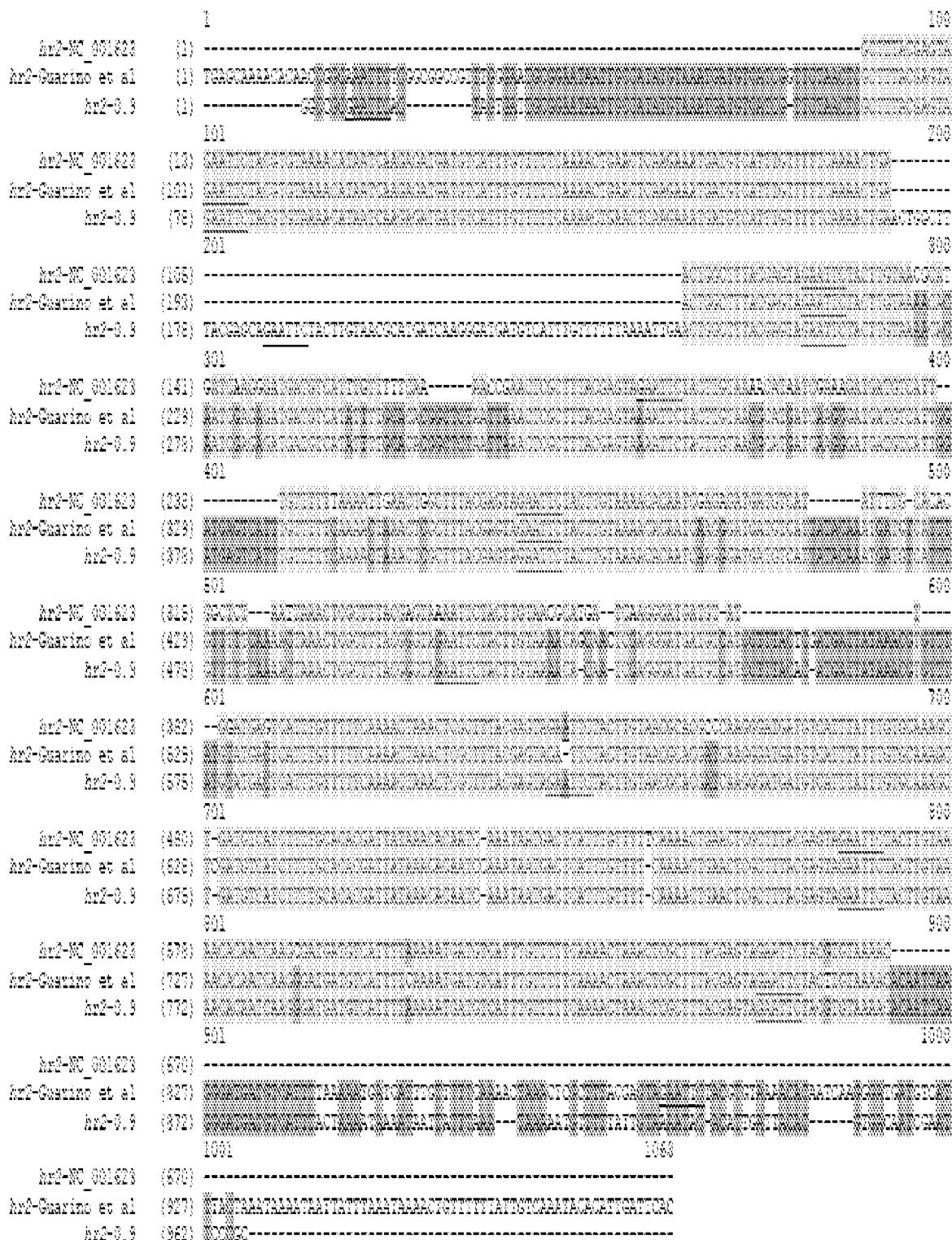


FIG.6 (cont)

Consensus IE-1 binding site ACBYGTAA (Leisy et al, Virology, 2000)

Site-directed mutagenesis

NsiI

hr-Nsi-F	GATGATGTCATCCTTTAT <u>GCATG</u> ATTATAAAAGTG
hr-Nsi-R	CACGTTTATAATCATGCATAAAGGATGACATCATC

ie-1 NsiI

hr-Nsi345-F	GCAGAAFTCTACTTGTAA <u>TGCATG</u> ATCAAGGGATG
hr-Nsi345-R	CATCCCTTGATCATCGGTTACAAGTAGAATCTCTGC

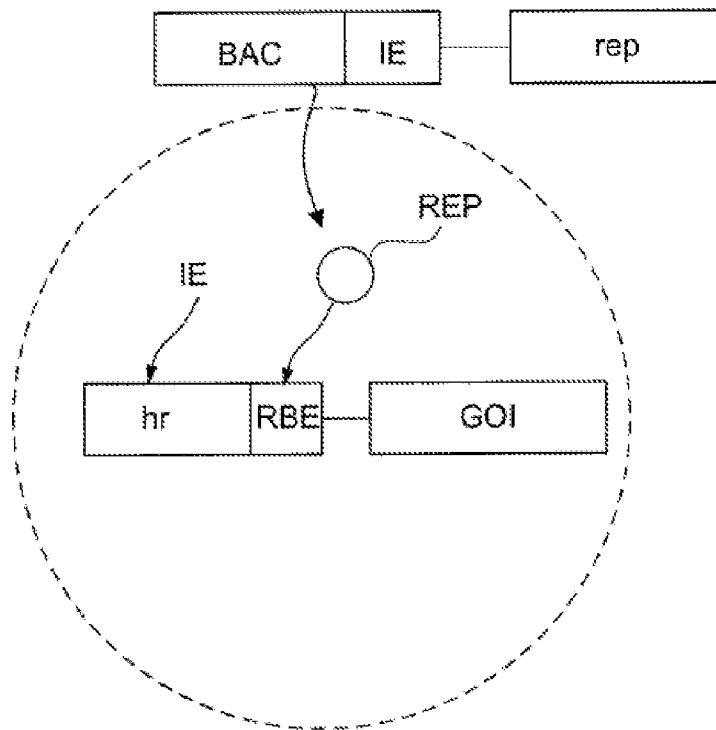


FIG. 7

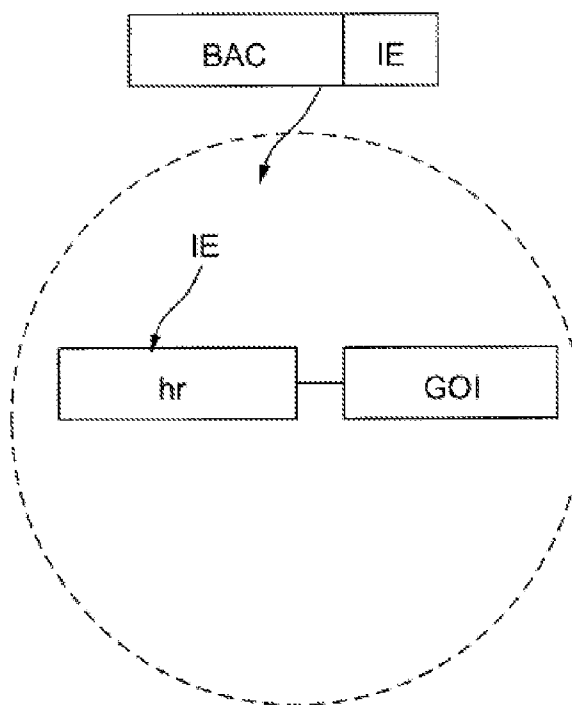


FIG. 8

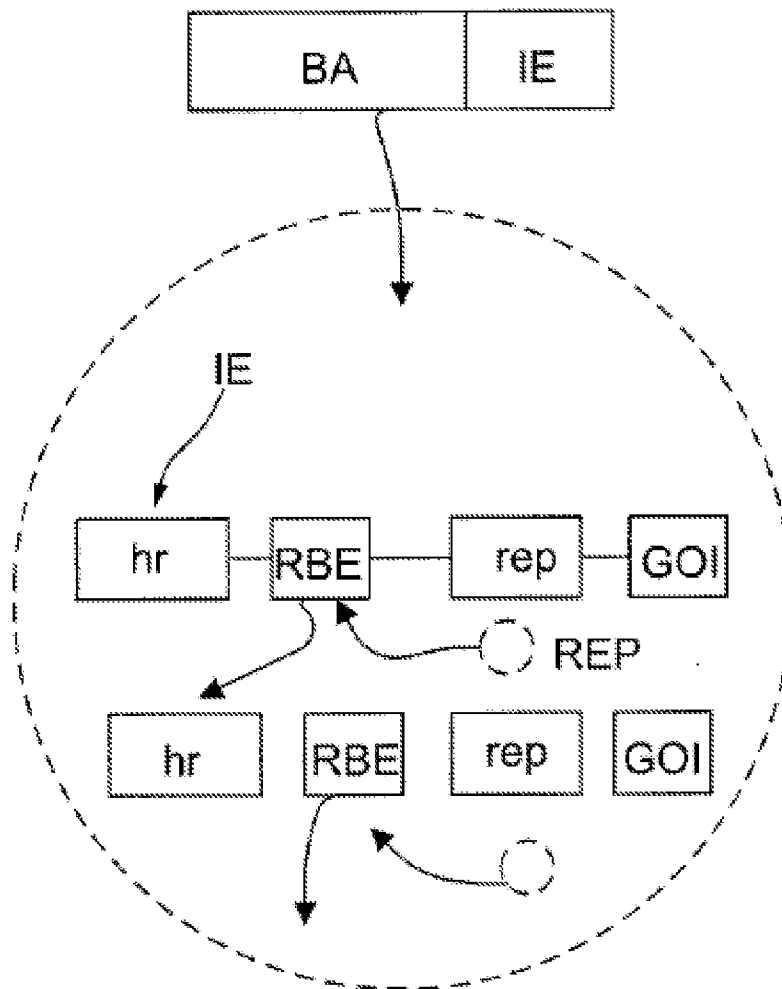


FIG. 9