

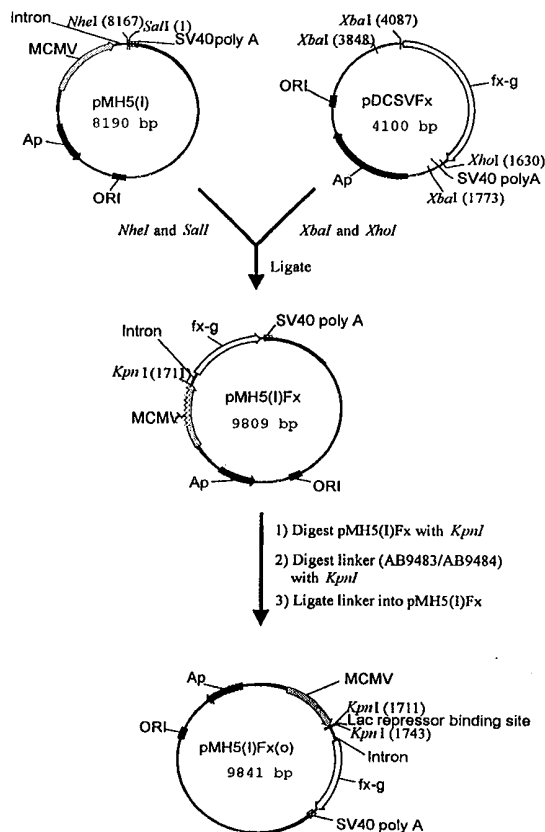


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(54) Title: REPRESSOR PROTEINS PRODUCING CELL LINES FOR THE PROPAGATION OF VECTORS**(57) Abstract**

The present invention provides eukaryotic cells capable of replicating recombinant expression vectors that produce heterologous proteins, wherein the cell comprises a polynucleotide encoding a repressor protein that is capable of repressing production of the heterologous protein. The invention also provides recombinant vectors, such as viruses (for example, adenoviruses) comprising (i) a first polynucleotide sequence encoding a heterologous protein and (ii) a second polynucleotide sequence operably linked to the first polynucleotide in order to permit repression of the expression of the first polynucleotide gene.



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REPRESSOR PROTEINS PRODUCING CELL LINES FOR THE PROPAGATION OF VECTORS

REFERENCE TO RELATED APPLICATION

The subject matter of U.S. provisional patent application serial number 60/094,681 filed July 30, 1998 is incorporated herein by reference. The present application claims priority to provisional patent application serial number 60/094,681.

The present invention relates to various cell lines, including derivatives of the 293 cell line. The present invention also relates to recombinant viruses, such as recombinant adenoviruses.

BACKGROUND OF THE INVENTION

Human adenoviruses have become one of the vectors of choice for the delivery and expression of foreign or exogenous proteins for gene therapy, immunotherapy and vaccination. For a recent review, see Hitt *et al.*, *Adv. Pharmacol.* 40: 137-206 (1997). Human adenoviruses have been shown to be effective vaccines, for example, for the delivery of rabies glycoprotein antigens to non-human hosts. Protective immune responses have been induced in animals immunized with either infectious or defective vectors of this type delivered either by parenteral or oral routes. *Prevec et al.*, *J. Infect. Dis.* 161, 27-30 (1990); Charlton *et al.*, *Arch. Virol.* 123: 169-179 (1992); Yarosh *et al.*, *Vaccine* 14, 1257-1264 (1996); Xiang *et al.*, *Virology* 219, 220-227 (1996).

Replication-defective vectors generally have disruptions/deletions of the early transcription E1 region and optionally the E3 region and inserts of cassettes containing an appropriate exogenous promoter and the gene(s) to be expressed. Vectors of this type are produced and grown on E1

5 complementing cell lines such as the human 293 cell line, which is a human embryonic cell line. Graham *et al.*, *J. Gen. Virol.* 36: 59-72 (1977).

Through established procedures employing plasmid shuttle vectors (Graham & Prevec, *Molec. Biotech.* 3: 207-20 (1995)), the foreign gene (or promoter-gene cassette) is inserted into convenient restriction sites in the shuttle
10 plasmid(s) and co-transfection of 293 cells with the appropriate pair of plasmids results in the rescue of a recombinant adenovirus vector which contains and expresses the desired exogenous protein. This procedure has been successfully used to produce many recombinant viruses expressing different types of proteins from a range of different sources.

15 However, there are instances where exogenous protein expressed by the virus seems to interfere with the subsequent development of viral progeny. As a result, rescue of virus by transfection and viral growth may be severely compromised in some cases.

There have been cases where there is a reduction in glycoprotein
20 expression as a consequence of transcriptional suppression or "squenching." For example, the activity of the Herpes Simplex Virus VP16 transactivation domain on the LAP348 protein. Natesan *et al.*, *Nature* 139: 349-350 (1997)

have shown that transcription of episomal DNA is preferentially inhibited by "squenching".

A number of solutions to the problem of over expression of inactivating genes by viral vectors have been explored. Yoshida and Hamada, *Biochem. and Biophys. Res. Comm.* 230: 426-430 (1997) placed the VSV glycoprotein gene under the control of a minimal HCMV promoter fused to several tetracycline operator sequences. The operator sequences serve as potential binding sites for a chimeric transactivator protein consisting of the C-terminal domain of the herpes simplex virus protein VP16 fused to the tetracycline repressor protein. In the presence of tetracycline, the transactivator cannot bind to the promoter region, while in its absence, expression of transcripts from this promoter is a function of the amount of transactivator present. In order to provide adequate levels of transactivator, Yoshida and Hamada used a second recombinant Ad vector. Massie *et al.*, *J. Virol.* 72: 2289-2296 (1998) also exploited the tetracycline regulatable expression system, developing 293 cells that expressed the tet-regulated transactivator to obtain high level expression of a inactivating protein. In yet another approach, Tomanin *et al.*, *Gene* 193, 129-40 (1997) developed Ad vectors containing cassettes regulated by a T7 promoter in combination with vectors expressing the T7 RNA polymerase to express proteins having an inactivation potential.

These approaches, while potentially useful for the production of proteins in cell culture, do not lend themselves to an *in vivo* expression

system, such as those implicated in genetic therapy and vaccination. In contrast to the efforts of the art, the approach described herein utilizes repression of transgene expression during vector isolation and growth, and has proved to be very successful in producing recombinant adenoviruses capable of expressing heterologous proteins, such as rabies glycoproteins, at high levels in host cells and in animals. Studies in mice indicate that a very high level of rabies neutralizing antibody can be induced by the intron-containing vectors following a single intraperitoneal injection.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a technique for increasing expression of heterologous proteins by recombinant viruses, such as adenoviruses.

It is also an object of the present invention to provide cells for the production of certain viruses.

It is a further object of the present invention to provide cells useful in the production of adenoviruses.

It is a further object of the present invention to provide cells in which certain heterologous foreign protein products from certain adenoviruses is inhibited.

It is a further object of the present invention to provide cells based on human embryonic cell lines, such as embryonic kidney cell lines, that are

capable of producing a protein or other entity capable of binding with a binding site on the genome of certain adenoviruses to inhibit production of certain proteins whose overexpression interferes with the production of those adenoviruses.

5 These and other objects are accomplished by the present invention, which provides, in accordance with one aspect of the invention, a eukaryotic cell capable of replicating a recombinant expression vector that produces a heterologous protein, wherein the cell comprises a polynucleotide encoding a repressor protein that is capable of repressing production of the

10 heterologous protein. The cell can be a mammalian cell. Moreover, the vector can be a plasmid or a virus, such as an adenovirus. The cell can complement a virus, for example, the cell can express the protein product of a defective region of the virus. For example, the cell can express the E1 product (or its functional equivalent) that cannot be expressed by an E1

15 functionally deleted virus. The vector can be a defective virus. For example, the virus can have a functionally disrupted E1 and optionally the E3 region can be functionally disrupted as well. The cells can be derived from embryonic kidney cells, such as 293 cells. The polynucleotide encoding the repressor can be integrated in the genome of the cell. The repressor can

20 be a Lac repressor or a repressor derived therefrom. One source for a repressor-encoding polynucleotide is plasmid pHMCVLAP348. Cells according to the invention include cell line 293Lap10 (ATCC Accession No. CRL-12586) and cell line 293Lap13 (ATCC Accession No. CRL-12587).

In accordance with another aspect of the invention, there are provided recombinant vectors, such as viruses (for example, adenoviruses) comprising (i) a first polynucleotide sequence encoding a heterologous protein and (ii) a second polynucleotide sequence operably linked to the first polynucleotide in order to permit repression of the expression of the first polynucleotide gene. The recombinant vector, such as an adenovirus, includes those wherein the second polynucleotide sequence is an operator that can be bound by a repressor. Representative sequences include a *lac* operator sequence and sequences derived therefrom (all are types of polynucleotides). In the case of a recombinant adenovirus, the adenovirus can have a functionally disrupted E1 and optionally the E3 region can be functionally disrupted as well. A recombinant vector, such as an adenovirus, can encode a variety of heterologous proteins, such as the rabies glycoprotein G. A recombinant vector, such as an adenovirus, also can include an intron sequence to facilitate protein production.

In accordance with still another aspect of the invention, there are provided eukaryotic cells according to the invention that are infected or transfected with vectors according to the invention.

In accordance with yet another aspect of the invention, there are provided methods of propagating vectors encoding a heterologous protein, comprising transfecting or infecting eukaryotic cells according to the invention with vectors according to the invention.

These and other aspects of the present invention will become apparent to the skilled person in view of the teachings contained herein. Modifications may be made without departing from the scope and spirit of the invention.

5

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the construction of plasmids having the rabies glycoprotein gene (Fx) under the control of the MCMV promoter, an intron (I) sequence with or without a copy of the *lac* operator sequence (o) just downstream of the transcription start site. Unlabelled portions of the circles
10 comprise plasmid or Ad5 sequences. The sequences of linkers AB9483 and AB9484, comprising the operator sequence, are set forth below.

FIGURE 2A concerns probing for the inserted genes in 293LAP cells. DNA extracted from 293LAP10 (lanes 1, 4 and 7), 293LAP13 (lanes 2, 5 and 8) or 293 (lanes 3, 6 and 9) cells was digested with Hind III (lanes 1, 2 and 3), Xba I (lanes 4, 5 and 6) or Pst I (lanes 7, 8 and 9) and fragments
15 separated by agarose gel electrophoresis. The DNA was analysed by Southern blot hybridization (as shown in FIGURES 2B and 2C) using respectively, probe A, the BamHI to Bgl II fragment of pMPG2, or probe B, the NdeI to HindIII fragment of pMPG3. The proposed structure of the
20 insert in 293LAP cells is indicated at the bottom of the FIGURE 2D. The markers were generated by digestion of pMPG2 with HindIII and SacI (B) or of pMPG3 with BamHI and SacI (C). FIGURES 2E and 2F are schematic representations of plasmids pMPG2 and pMPG3 respectively.

FIGURE 3 depicts expression of LAP348 protein in 293LAP13 cells and induction following infection with an E1-deleted Ad5 virus. Whole cell extracts, analyzed by Western blot, were prepared from uninfected 293LAP13 cells (lane 1) or at 24 hours (lane 2), 48 hours (lane 3) and 72 hours (lane 4) following infection with Ad dl70-3 at an m.o.i. of 5 pfu/cell. As controls, extracts also were prepared from uninfected 293 cells (lane 5) and from 293 cells at 72 hours following infection with Ad dl70-3 (lane 6). The LAP348 protein was detected with commercial anti-Lac repressor antibody as described in the text. Two exposure times, 15 min.(top) and 1 min (bottom) are shown for comparison.

FIGURE 4A depicts expression of rabies glycoprotein by different vector viruses in 293 and 293LAP13 cells. Monolayer cultures of 293 or 293LAP13 cells were infected at 2 pfu/cell with one of the following viruses: Ad5BHGE3MH5Fx, which lacks intron or operator sequences but is otherwise identical to the other vectors (lanes 1,4,7 and 10); AdMH5(I)E3Fx, which has the intron but no operator sequences (lanes 2,5,8 and 11) and AdMH5(I)E3Fx(o), which has the intron and one operator sequence (lanes 3,6,9 and 12). The infected monolayers were radiolabelled with ³⁵S-methionine from 8 to 10 hours or from 17 to 19 hours post-infection and the cells harvested, cell extracts prepared, immunoprecipitated with anti-rabies glycoprotein antibody and analysed by PAGE, all as described in the text. Lane 13 has extracts from uninfected 293LAP13 cells. Two different exposures (18 hr and 2 hr) are presented for comparison.

FIGURE 4B depicts operator-dependent inhibition of rabies glycoprotein expression in 293LAP13 cells and reversal by IPTG. Monolayer cultures of 293LAP13 cells, without (lanes 3 and 6) or with pretreatment with IPTG (lanes 4 and 7) were infected with AdMH5(I)E3Fx, the vector lacking an operator sequence (lanes 3 and 4) or with AdMH5(I)E3Fx(o), the vector having one operator sequence (lanes 6 and 7). As a control, 293 cells were also infected with AdMH5(I)E3Fx (lane 2) or AdMH5(I)E3Fx(o) (lane 5). All infections were at 2 pfu/cell and all infected cultures were radiolabelled with ³⁵S-methionine between 8 to 10 hours post-infection and analysed as described herein.

FIGURE 5 depicts expression of rabies glycoprotein by different vectors in non-complementing human (MRC5) or canine (MDCK) cell lines. Monolayer cultures of MDCK cells (lanes 1 to 5) or MRC5 cells (lanes 6 to 10) were infected at 10 pfu/cell with one of the following:

Ad5BHGE3MH5Fx (lanes 1 and 6) or 50 pfu/cell (lanes 2 and 7); AdMH5(I)E3Fx (lanes 3 and 8); AdMH5(I)E3Fx(o) (lanes 4 and 9); or were not infected (lanes 5 and 10). The infected cultures were labeled from 8 to 10 hours post-infection and harvested, immunoprecipitated and analyzed on PAGE. The long exposure is 20 hours (top) and the short exposure is 4 hours (bottom).

FIGURE 6 contains growth curves of recombinant adenoviruses expressing rabies glycoprotein in either 293 or 293LAP13 cell lines. Replicate monolayer cultures containing approximately 1×10^6 cells each

(either 293 or 293LAP13 cells) were infected with the recombinant viruses at a moi of 1 pfu/cell. At the indicated times post infection duplicate cells and supernatants were harvested and assayed for pfu on 293LAP13 cells. Titres are plotted on an exponential scale. Open symbols represent yields from 293 cells for AdMH5(I)E3Fx (□) and AdMH5(I)E3Fx(o) (□). Closed symbols represent yields from 293LAP13 cells for AdMH5(I)E3Fx (□) and AdMH5(I)E3Fx(o) (□).

FIGURES 7A-C depict the sequence of plasmid pMPG2.

FIGURES 8A-D depict the sequence of pMPG3 type of plasmid.

FIGURES 9A-B depict the sequence of plasmid pDCSVFx.

DETAILED DESCRIPTION OF THE INVENTION

As will be described below, there are provided cells, including cell lines, for use as hosts for replicating recombinant expression vectors, such as viruses and plasmids, wherein the cells comprise a gene encoding a repressor protein capable of repressing expression of certain heterologous proteins of the vector and which otherwise deleteriously impact replication thereof.

The term “repressor,” particularly in the context of proteins, is intended to refer to entities (*e.g.*, proteins) having the capacity to inhibit, interfere, retard and/or repress the production of heterologous protein product of a recombinant expression vector. For example, by interfering with a binding site at an appropriate location along the expression vector,

such as in an expression cassette. One appropriate repressor for use according to the invention is the repressor that interacts with the lac operator (which is a type of interaction partner for the repressor). See, e.g., Watson *et al.*, MOLECULAR BIOLOGY OF THE GENE (4th ed. 1987)

5 (Benjamin/Cummings Publishing Co.) Other appropriate repressors and interaction partners are available in the field. Thus, examples of repressors include the lac repressor, the trp repressor, the gal repressor and the lambda repressor. Other appropriate repressors can be employed as well.

The cell line can be derived from 293 cells and the polynucleotide
10 inserted therein encodes a lac repressor derivative. According to one embodiment, the gene includes a nuclear localization signal from a SV40 large T antigen, along with a transcription activation domain from herpes simplex type 1 VP 16, as well as a portion of plasmid pHMCVLAP348, which is described in U.S. Patent 5,674,730.

15 The vector can be an adenovirus, more preferably a defective adenovirus with a functionally disrupted E1 region and optionally the E3 region can be functionally disrupted as well. In the context of the present invention, the term "heterologous" connotes a difference in origin, source, form, structure, activity or location between two or more entities. The
20 referents and effect of the term "heterologous" depend upon the context in which it is used. For example, in the context of recombinant viruses, a heterologous protein typically is a protein encoded by a recombinant virus wherein that protein does not naturally occur in a native or source virus, or

the protein is changed in a manner that is distinct from the form which naturally or usually occurs in the native or source virus.

In other aspects, the invention provides a system for replicating a recombinant protein expression vector, comprising a cell, such as from a cell line, comprising a gene which encodes a repressor protein capable of repressing certain heterologous protein products of the vector, which vector-encoded proteins otherwise inhibit production of the virus.

The vector can be an adenovirus, such as a defective adenovirus. Defective adenoviruses can have a functionally disrupted E1 region and optionally the E3 region can be functionally disrupted as well. Functional disruptions can be undertaken by insertions and/or deletions in a given region

A particular feature of the vector used in the present system is that it contains a binding site, such as an operator sequence. For example, the vector contains a *lac* operator sequence which serves as a binding site for the repressor protein, preferably within the expression cassette of the vector. The repressor protein can be the *lac* repressor, a derivative thereof, or other repressors available in the art. In the case of defective adenovirus, the binding site may be placed in the E1 region, the E3 region and/or another region where the binding site can be operably linked.

In another aspects, the invention also provides a method of replicating a recombinant protein expression vector.

Repressor proteins for use according to the invention can repress production of the heterologous protein. For example, the vector has an expression cassette containing a gene (a type of polynucleotide) encoding the heterologous protein, the method further comprising the step of locating the binding site within the expression cassette.

In another of its aspects, the present invention provides cells, including cell lines, that produce a repressor protein and a vector for transforming that cell line, the vector comprising an expression cassette in which DNA encoding for a heterologous protein product is operably linked for expression to a promoter sequence or the like that is disfunctional or impaired in the presence of the repressor protein. The phrase "operably linked" in the context of the present invention refers to a first sequence(s) being positioned sufficiently proximal to a second sequence(s) such that the first sequence(s) can exert influence and/or control over the second sequence(s) or another sequence(s) under control of the second sequence(s). Operably linked includes, but is not limited to, sequences that are contiguous.

The vector can be an adenovirus, such as a defective adenovirus, and the cell line complements the defect to allow the adenovirus to replicate.

In still another aspect of the present invention, the invention provides methods of producing cells, such as cell lines, useful as a host for replicating expression vectors, comprising the step of introducing into the host a polynucleotide sequence encoding a repressor protein capable of repressing a

heterologous protein of the vector which otherwise inhibits replication of that vector. Preferably, the introduction comprises integration of the repressor encoding sequence into the genome of the host cell.

The expression vectors can include a promoter sequence and a polynucleotide sequence encoding the heterologous protein product, the binding site being located therebetween, which is one example of operably linked. Alternatively, the expression cassette includes a promoter sequence and a sequence encoding the heterologous protein product, the binding site being located upstream of the promoter sequence, which is another example of being operably linked.

In still another embodiment, the method includes the step of providing a plurality of binding sites.

As will be described below, the present cells, in particular when used in conjunction with expression vectors such as adenovirus, are capable of repressing the production of certain heterologous protein products from such vectors, and thereby minimize the interfering effects of the presence of certain proteins on the further replication of the vector itself. This capability is particularly advantageous in adenovirus-based recombinant vector systems whose protein products, such as rabies glycoprotein, have been shown to interfere with further replication of the adenovirus vector itself.

The present invention also provides a combination of cells that produce a repressor protein and a vector for transforming those cells, the

vector comprising an expression cassette in which DNA (a type of polynucleotide) encoding for a heterologous protein is operably linked to a promoter sequence that is disfunctional or at least substantially impaired in the presence of the repressor protein. In this case, the cell line and the adenovirus can, if desired, be made available in a single package. The package also can include instructions for the user on how to use the combination, such as explaining how to infect and/or transfect the adenovirus into the cell line under conditions suitable for transfection and then to select for and then to culture under suitable conditions for adenovirus replication. The instructions also may instruct the user how to rescue the adenovirus or to recover the protein of interest.

The invention can utilize a cell line which is a derivative of the human 293 cell line, commonly used as a host for the replication of adenovirus-based expression vectors. Other appropriate cells useful according to the invention are disclosed in Fallaux *et al.*, *Hum. Gene Ther.* 7: 215-22 (1996); Fallaux *et al.*, *Hum. Gene Ther.* 9: 1909-17 (1998). In the context of cells, the term "derived" in its various grammatical forms connotes a degree of similarity that is indicative of an archetype. For example, the cell that is derived is based upon or obtained from a naturally occurring cell or other type of cell. Cells that are "derived" can be obtained by screening cells for desired properties, conducting mutations on cells and/or transforming cells with DNA to impart a desired trait or property on the recipient cell. Other approaches also are available.

Two examples of cell lines according to the present inventions are described in more detail below and are 293-based cell lines, designated 293LAP10 and 293LAP13, which contain and express a repressor protein in the form of a derivative of the *lac* repressor protein, viz: LAP348 (Labow *et al.*, 1990). In the context of polynucleotide sequences and regions, genes
5 and plasmids, the term “derived” in its various grammatical forms connotes a degree of similarity that is indicative of an archetype. For example, a DNA sequence that is derived is based upon or obtained from a naturally occurring DNA sequence or other type of source DNA sequence.

10 The *lac* operator sequence, to which the repressor protein binds, is incorporated into a transcription unit, where it is operably linked, controlling rabies glycoprotein expression. In one case, the operator sequence is located immediately downstream of the transcription start site.

Cell lines made according to the invention are provided. Cell line
15 293LAP10 was deposited with the American Type culture Collection pursuant to the Budapest Treaty and was assigned Accession No. CRL-12586. The 293LAP13 cell line was deposited with the American Type culture Collection pursuant to the Budapest Treaty and was assigned Accession No. CRL-12587. The invention is not limited to these cell lines,
20 however. Following the teachings of this application, the skilled person will be able to readily attain other cell lines in accordance with the present invention,

The operator-containing adenovirus of the present invention, can be grown to higher titres in 293LAP10 and 293LAP13 and has been shown to produce plaques more efficiently in 293LAP10 and 293LAP13 cells than in 293 cells not modified in accordance with the teachings of the invention.

5 In accordance with another embodiment, adenovirus expression vectors are provided with a promoter-intron expression cassette for expressing rabies glycoprotein. This vector has been shown to express the rabies glycoprotein with enhanced synthesis kinetics in non-complementing human and canine cell lines compared to vectors lacking the intron.

10 Furthermore, it has been shown that the repressor protein produced by the 293LAP10 and 293LAP13 cell line is effective at repressing the expression of rabies glycoprotein by the operator-containing adenovirus vectors. Moreover, it has also been shown that the repressing effect of the repressor protein is at least partly relieved by isopropylthio- β -galactoside
15 (IPTG).

 Therefore, it is believed that cell lines according to the present invention, including, for example those based on the 293 cell line, still more particularly the 293LAP10 and 293LAP13 cell lines, are useful for the rescue and propagation of other expression vectors in which expression of
20 the desired protein prevents vector rescue in the host cell.

 Although the cell lines disclosed herein are based on 293 cells, other cell lines are contemplated which have utility as a host for adenovirus or

other expression vectors capable of producing a heterologous protein product.

Based on published observations showing that the presence of an intron can significantly increase the expression of some gene sequences (Petitclerc *et al.*, *J. Biotech.* 40, 169-178 (1995); Yew *et al.*, *Gene Therapy* 8: 575-584 (1997)) and observations that expression of a number of glycoprotein antigens in adenovirus vectors can be greatly amplified by the presence of an intron upstream of the gene, attempts to produce defective adenovirus vectors were undertaken in which the expression of a rabies glycoprotein gene was driven by the highly effective murine cytomegalovirus promoter (MCMV) (Dorsch-Hasler *et al.*, *Proc. Nat'l Acad. Sci. USA* 82: 8325-29 (1985); Addison *et al.*, 1997) in combination with an intron sequence previously used in adenovirus vectors (Berkner & Sharp, 1985). The construction detail for plasmid pMH5(I)Fx is shown in Fig. 1.

Numerous attempts, to rescue a defective adenovirus vector containing the rabies glycoprotein gene under the control of this promoter construction were unsuccessful following standard co-transfection protocols in 293 cells. These unsuccessful attempts amounted to over a dozen independent experiments including some with slightly modified constructs and representing over 300 transfected plates of 293 cells in total (data not shown). The only successful rescue of vectors of this type resulted from a plasmid which had a fortuitous deletion of 59 nucleotides from the start of the rabies glycoprotein open reading frame as a result of EcoR1 STAR

endonuclease activity (data not shown). Since these rescued vectors were unable to express the rabies glycoprotein, this suggested that the failure to rescue functional vectors may be due to the inactivation potential of the overexpressed rabies glycoprotein. To overcome this problem, a 293-based cell line was constructed which expressed a repressor protein and could therefore be used to reduce glycoprotein expression, and its attendant interfering capabilities, in transfected, infected and/or transformed cells.

These and other aspects of the invention will become apparent to the skilled person in view of the disclosure contained herein. Modifications may be made to the various teachings contained herein without departing from the scope and spirit of the invention.

EXAMPLE I: Cells, Viruses and Plasmids.

The 293 cells (Graham *et al.*, 1997) and 293 cell derivatives, were maintained as previously described by Graham and Prevec. Isolated colonies of cells transformed to G418 resistance (described below) were maintained in a-MEM supplemented with Fetal Bovine Serum (10% v/v; a supplier), Penicillin (100 U/ml), Streptomycin (100 µg/ml) and G418 (400 µg/ml, Gibco BRL). Control adenovirus Ad dl70-3 is derived from Ad5 but with the E1 region deleted and a deletion and/or substitution in the E3 region (Bett *et al. Proc. Nat'l Acad. Sci. USA* 91: 8802-06 (1994)). In the context of viruses, the term "derived" in its various grammatical forms connotes a degree of similarity that is indicative of an archetype. For example, the virus that is derived is based upon or obtained from a naturally occurring virus or

other type of virus. Viruses that are “derived” can be obtained by screening virally-infected cells for desired properties, conducting mutations on virally-infected cells and/or genetic manipulation of viral genomes with polynucleotides to impart a desired trait or property on the recipient virus.

- 5 Other approaches also are available, including *in vivo* homologous recombination and the the direct *in vitro* insertion of polynucleotide sequences. The term polynucleotide can encompass DNA, RNA and synthetic entities, such as peptide nucleic acids. In the context of the vectors and eukaryotic cells according to the invention, the term polynucleotide can
- 10 be considered synonymous with DNA.

The rescue, isolation, propagation, gradient purification and titration of recombinant viruses described herein can be done according to known techniques. See Hitt *et al.*, *Meth. Molec. Genet.* 7, 13-30 (1995). The virus particle number in gradient purified stocks, was determined from the OD₂₆₀

15 of an aliquot in buffer containing 0.1% SDS using the relationship $OD_{260} = 1.1 \times 10^{12}$ pfu/ml (Mittereder *et al.*, *J. Virol.* 70, 7498-7509. (1996)).

Turning to plasmids, the molecular cloning techniques described herein below are described by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor

20 Laboratory (1989). All DNA fragments were separated from contaminants by agarose gel electrophoresis and eluted from the agarose using GeneClean (Bio 101 Inc.). Plasmid pMPG2 (Figure 2E) contains a neomycin resistance gene under the control of an HCMV promoter all flanked by a Bam HI site

and a Bgl II site as shown in Fig 2A. The sequence of plasmid pMPG2 is depicted in FIGURE 7. Plasmid pHCMVLAP348 (Labow *et al.*, *Molec. Cell. Biol.* 10: 3343-3356 (1990) (NRRL.B-18664)) comprises a modified *lac* repressor gene containing a nuclear localization signal from the SV40 large T antigen and the transcription activation domain from herpes simplex type 1 VP16 (LAP348). This plasmid was cut with Hind III and the 2.5 kbp fragment containing the HCMV promoter and the LAP348 gene was ligated in a series of steps with other plasmids to produce plasmid pMPG3 (figure 2F). Plasmid pMPG3 contains the HCMV promoter, the gene for protein LAP348, an SV40 derived polyA addition sequence and a copy of the Adenovirus inverted terminal repeats (known as "ITRs"), representing the sequences from nt37,628 to nt195 from plasmid pFG140 (Graham, *EMBO J.* 3: 2917-2922 (1984)). This entire insert is flanked on one side by a unique Bam HI site and on the other side a unique Bgl II site as shown in Fig 2B. The sequence of a pMPG3 type of plasmid is depicted in Figure 8.

The gene encoding a rabies glycoprotein, cloned from a red fox isolate (Nadin-Davis *et al.*, *Gen. Virol.* 74: 829-837 (1993)) was modified by removing the ATG sequence immediately preceding the probable protein initiating ATG and cloned into plasmid pDCSVFx. The sequence of plasmid pDCSVFx is depicted in Figure 9. Plasmid pMH5(I) was derived from pMH5 (Addison *et al.*, *J. Gen. Virol.* 78: 1653-1661 (1997)) by insertion into the EcoRI site of an intron sequence obtained by PCR replication of the adenovirus-immunoglobulin hybrid intron present in plasmid pMLPsp1a (Kaufman & Sharp, *Mol. Cell. Biol.* 11, 1304-1319

(1982); Berkner & Sharp, *Nucl. Acids Res.* 13: 841-857 (1985)). As shown in Fig. 1, the rabies glycoprotein gene was cloned into pMH5(I) downstream of the MCMV promoter and intron to produce plasmid pMH5(I)Fx. A derivative of pMH5(I)Fx, namely pMH5(I)Fx(o) contains one copy of the

5 Lac operator inserted at the unique KpnI site located just downstream of the transcription start site (Fig 1). Synthetic primers

C :5' GCGGTACCGTCGGAATTGTGAGCGGA-

TAACAATTGGTACCCG 3' (AB9483) and

D: 5' CGGGTACCAATTGTTATCCGCTCACAATTCCGACGGTACCGC

10 3' (AB9484) were synthesized and annealed together to achieve the desired construction. The annealed primers were then restricted with Kpn I and ligated to similarly restricted pMH5(I)Fx plasmid. Recombinants containing one (pMH5(I)Fx(o)) copy of the repressor binding sequence were identified by restriction enzyme digestion and fragment analysis on agarose gels. The
15 orientation of the near palindromic operator sequence in pMH5(I)Fx(o) was verified by sequencing.

EXAMPLE 2: Transient transfection assays.

Plasmids pMH5(I)Fx, pMH5(I)Fx(o), and pMPG3 were assayed for expression of the appropriate proteins by transient transfection in various cells lines followed by Western blotting analysis of total cell lysates. Briefly, 1 µg of purified plasmid DNA was transfected into approximately 1×10^6 cells using the calcium phosphate technique (Graham and Van der Eb, *Virology* 52, 456-467 (1973)). Two days post transfection, the total cell monolayer was harvested, and the expression of the relevant protein assayed by Western blotting, as discussed herein below. A sample of cells similarly transfected with pE1sp1a (Bett *et al.*, 1994) was used as a control.

EXAMPLE 3: Generation of cell lines expressing the LAP348 protein.

Plasmids pMPG2 (6 µg) and pMPG3 (60 µg) pMPG3 were cut with restriction enzymes Bam HI and Bgl II and the appropriate fragments were isolated. The pMPG3 fragment was then ligated on its own for 30 minutes in a volume of 20 µl at 16°C. The mixture was then restricted again with Bam HI and Bgl II for 60 minutes at 37 C before heat inactivating the mixture at 65°C for 15 minutes. This cycle was repeated once more before adding the pMPG2 fragment and ligating overnight at 16°C. Samples were examined by agarose gel electrophoresis and the remaining mixture was transfected into 60mm diameter dish of confluent 293 cells by the calcium phosphate method (Graham and Van der Eb, 1973). After 24 hours, the cells were

divided amongst 10 similar 60mm diameter dishes and the media supplemented with G418 at a concentration of 400 ug/ml. After 2 weeks of selection, colonies of G418 resistant cells were evident and 12 were isolated and expanded. The procedure was repeated a second time (i.e., fresh DNA
5 was prepared, examined and transfected) and a further 15 colonies were isolated. No more than two colonies from an individual dish thus reducing the possibility that identical clones would be isolated.

EXAMPLE 4: Antibodies, Polyacrylamide gel electrophoresis (PAGE)

10 and Western blotting.

All Western blot analysis and PAGE was performed as previously described (Matthews and Russell, *J. Gen. Virol.* 75: 3365-3374 (1994)). Rabbit anti-Lac repressor serum was purchased from Stratagene and used according to supplier's protocols. Bound primary antibody was detected
15 using a secondary anti-rabbit serum linked to Horse Raddish Peroxidase (HRP) and chemiluminescent detection was performed using ECL (Amersham) according to the manufacturer's instructions.

EXAMPLE 5: DNA preparation and Southern Blotting

Approximately 5×10^7 cells were harvested and treated with SDS
20 (0.5% w/v) and Pronase (0.5 mg/ml) at 37°C overnight in a final volume of 10 ml. The lysed cells were then extracted twice with buffered phenol and once with chloroform before precipitating the DNA with lithium chloride and ethanol. The precipitated DNA was washed twice with 70% ethanol, air

dried and resuspended in 0.5 ml 0.1xSSC. The resuspension of the DNA was facilitated by placing on an end-over-end roller for 20 hours at 4°C. The DNA was quantified by spectroscopy and 10 µg samples digested with appropriate restriction enzymes before agarose electrophoresis on 0.8% (w/v) agarose gels. The agarose gels were prepared for DNA transfer as described by Sambrook *et al.* (1989) then transferred to positive charge nylon membranes (Boehringer Mannheim) using a pressure blotter (Stratagene) over two hours. The nylon membranes were then UV cross-linked using a Stratalinker (Stratagene) and used immediately or stored at -20°C for up to 5 days. DNA fragments were derived from plasmids pSV2neo (SmaI to Bgl II fragment containing the neomycin gene open reading frame) and pHCMVLAP348 (Sac I to Pvu II fragment containing the LAP348 open reading frame) and used to generate 'DIG' labelled probes using the 'DIG HighPrime' system (Boehringer Mannheim) according to the manufacturer's protocols. Detection of DNA bound to the membranes was performed using "High SDS buffer" and the 'DIG detection kit' (Boehringer Mannheim) according to the manufacturer's protocols except that the high temperature stringency wash time was reduced from 2 x 15 minutes to 2 x 10 minutes.

EXAMPLE 6: Isolation and screening of cells

The approach was to produce a 293-based cell line which expressed a *lac* repressor derived protein (LAP348) for use, in one instance, in conjunction both with vector precursors which contained the *lac* operator or other operators or DNA sequences, to which the repressor binds, within the

promoter-transcription region so as to inhibit synthesis of the rabies glycoprotein in this cell line. Low passage (pass 29) 293 cells were transfected with Bam HI - Bgl II fragments from pMPG2 and pMPG3 and resistant clones were selected in the presence of G418. After 3 weeks of selection, 28 resistant colonies were isolated and expanded. All 28 cell lines were assayed for expression of the LAP348 protein by Western blotting and two were identified as suitable for further investigation.

EXAMPLE 7: Characterization of LAP cell lines by Southern

blotting Because the 293LAP cells had been transfected with a ligation mixture containing BglII-BamHI fragments from pMPG2 and pMPG3 it was of some interest to determine the structure of the integrated sequences in these lines. DNA was extracted from 293 cells and 293LAP10 and 293 LAP13 and analyzed by Southern blot hybridization using as probes the Bam HI - Bgl II fragment of pMPG2 (probe A) and the Nde I - Hind III fragment of pMPG3 (probe B) (Fig. 2A). The pattern of fragments detected in both 293LAP10 and 293LAP13 cells corresponded to that predicted by the non-homologous integration of a DNA segment with the structure indicated in Fig 2D. Both probes detected an XbaI fragment of about 8.6 kb in 293LAP10 cells and one of about 7.4 kb in 293LAP13 cells. Since XbaI does not cut within either the pMPG2 or pMPG3 derived fragments these bands suggest a single insertion at distinct sites within the two cell lines. As shown in Fig 2B, probe A primarily detects different fragments in the HindIII digestion products of 293LAP13 and 293LAP10 cells. These are

believed to be the fragments resulting from HindIII cleavage at the site depicted at nt1592 in Fig 2D and a HindIII site, unique to each cell line, in DNA sequences leftward of the insertion site. The fragments at about 2.3 kb in lanes 1 and 2 of Fig. 2B are believed to represent the 2333 kb HindIII fragment (from 1592 to 3925 in Fig 2D) which is detected by the HCMV sequences present in probe A. These HindIII fragments are more strongly detected by probe B (Fig. 2C) as expected. As predicted by the structure indicated in D, probes A and B hybridized to a PstI fragment of approximately 2.7 kb generated by cleavage at 634 bp and 3401 bp in Fig. 2D as well as PstI fragments unique to each cell line due to cleavage at 634 bp and at sites in cell DNA sequences leftward of the insertion site. Probe B consistently detected DNA fragments of 10 kb or larger but these fragments were also detected in 293 DNA.

Both LAP cell lines therefore appeared to contain a single copy of the LAP348 expression cassette (from pMPG3) linked to a single copy of the neomycin resistance gene expression cassette (from pMPG2) as schematically depicted in Fig 2D. This combined cassette is integrated into distinct sites within the 293LAP10 and the 293LAP13 cell lines. The 293LAP13 cell line was chosen for most of the subsequent studies.

EXAMPLE 8: LAP348 protein expression increases after infection of 293LAP cells by adenovirus.

Both 293 and 293LAP13 cell lines were infected with Ad dl70-3 and samples of infected cells were harvested at various times post infection.

Levels of LAP348 protein expression were assayed by Western blotting and compared to protein levels present in uninfected cells. Figure 3 shows that at 24 hours post infection there is significantly more protein LAP348 present than in the uninfected samples. The levels of LAP348 remain high for 3 days post infection. Furthermore, levels of an unknown cellular protein which cross-reacts with the antiserum have dramatically declined as would be expected for a cellular protein with a relatively short half life since adenovirus would shut off mRNA transport.

EXAMPLE 9: Rescue of recombinant viruses in 293LAP 13 cell
line.

Co-transfections of plasmids pMH5(I)Fx or pMH5(I)Fx(o) with either plasmids pBHG10 or pBHGE3 into both 293 and 293LAP13 cell lines were performed to determine if recombinant adenoviruses could be generated containing the expression cassette in the E1 region, along with either a deleted (pBHG10) or wild type (pBHGE3) E3 region (Bett *et al.*, 1994). Table 1 presents the results of one experiment in which the relative efficiency of rescue of recombinant vectors was compared in 293 and 293LAP13 cells employing different plasmid combinations and using standard protocols as outlined in Methods. After 12 days the number of plaques on each dish were recorded. As can be seen from the table, recombinant virus constructed from pMH5(I)Fx(o) in combination with either pBHG10 or pBHGE3 could be efficiently rescued in 293LAP13 cells whereas they could not be rescued in 293 cells. When the operator sequence

was absent from the construct, rescue of virus was very inefficient as evidenced by only one AdMH5(I)E3Fx rescue in combination with pBHGE3 in 293LAP13 cells. These results agree with earlier, failed, attempts to rescue the pMH5(I)Fx construct in 293 cells in combination with either pBHG10 or pBHGE3 as mentioned above. Table 1 presents the results of transfections in 293LAP13 cells alone which again demonstrate the higher efficiency of rescue, in this cell line, of vectors containing an operator sequence compared to that lacking the operator.

In the studies detailed in Table 1, the relative efficiency of rescue of adenovirus recombinants expressing rabies glycoprotein on 293LAP13 cells. Monolayer cultures of 293LAP13 cells were cotransfected with plasmid pBHGE3 and one of pMH5(I)Fx, or pMH5(I)Fx(o) at the total DNA concentrations shown. The control plasmid pFG140 was also included. The total number of plaques observed after 12 days was counted and is presented in column 2 along with the number of dishes represented. The average number of plaques per dish is presented in column 3. Some representative plaques were picked and the virus in these plaques was screened by restriction enzyme analysis to confirm the expected virus DNA structure as shown in column 4.

Table 1: The relative efficiency of rescue of adenovirus recombinants with and without the operator sequence and expressing rabies glycoprotein on 293LAP13 cells.

Plasmid combination	Quantity (μ g) of each plasmid per dish	Number of Plaques observed (number of dishes)	Average number of plaques per dish	Number of plaques analyzed having correct structure
pMH5(I)F x + pBHGE3	5+5	4(5)	0.8	2(2)
pMH5(I)F x(o) + pBHGE3	5+5	68(8)	8.5	4(4)
pMH5(I)F x(o) + pBHGE3	10+10	45(6)	7.5	ND
pFG140	1	129(4)	32.3	ND

- 5 The fact that vectors lacking the operator sequence could be rescued in the 293LAP13 cell line was in contrast to an inability to do so in 293 cells.

In addition to the recombinant plasmids, 293LAP13 cells also were tested for their ability to generate plaques from the infectious plasmid pFG140 which does not require a recombination event to generate E1 deleted adenovirus (Graham, 1984). As shown in Table 2, both 293 and 293LAP13 cell lines are equally capable of generating plaques from this plasmid.

The studies detailed in Table 2 concern the relative efficiency of rescue in 293 and 293LAP13 cells of recombinant adenoviruses expressing rabies glycoprotein. Plasmids pMH5(I)Fx or pMH5(I)Fx(o) were contranfectected onto monolayers of 293 or 293LAP13 cells along with either plasmid pBHG10 or pBHGE3 as described by *Bett et al.*, 1994. All plasmid DNA was used at 5 µg per dish. As a control, cells also were transfected with the infectious plasmid pFG140 but in this case only one µg of DNA was used. The number of plaques observed after 12 days was counted and the total number observed as well as (the number of dishes per experiment) are presented in the table.

Table 2: Relative efficiency of rescue in 293 and 293LAP13 cells of recombinant adenoviruses expressing rabies glycoprotein.

Plasmids used in Transfection	Total # of plaques observed (number of dishes transfected)	
	293 cells	293LAP13 cells
PMH5(I)Fx + pBHG10	0(8)	0(3)
PMH5(I)Fx(o) + pBHG10	0(8)	16(3)
PMH5(I)Fx + pBHGE3	0(10)	1(3)
PMH5(I)Fx(o) + pBHGE3	0(10)	17(3)
pFG140 ^a	42(3)	42(3)

a: all plasmid DNA at 5 µg/dish except pFG140 which is at 1 µg/dish

EXAMPLE 10: Rabies glycoprotein expression in 293 and 293LAP13

5

cell lines

To determine the kinetics of synthesis of rabies glycoprotein by the vectors isolated as described above, 293LAP13 cells or 293 cells, infected at 2 pfu/ml, based on titrations in 293LAP13 cells, with AdMH5(I)E3Fx, AdMH5(I)E3Fx(o) or with the control 'intron-less' virus Ad5BHGE3MH5Fx, were labelled with ³⁵S methionine at 8 to 10 or at 17 to 19 hours post infection. The infected culture was harvested at the end of the labelling period and the expressed rabies glycoprotein was immunoprecipitated and analyzed by SDS-PAGE, and the resultant autoradiograms presented in Figure 4A.

10

To conduct the study, approximately 1×10^6 cells were infected with the indicated recombinant adenovirus (or PBS as a control) and at the indicated time post infection the media was supplemented with ^{35}S labeled methionine. After two hours further incubation the cells were harvested and the radiolabeled rabies glycoprotein immunoprecipitated as previously described (Graham and Prevec). When required, isopropylthio- β -galactoside (IPTG), purchased from Gibco BRL, was used at final concentration of 10 mM in medium and was added to cells 24 hours prior to infection. The anti-rabies glycoprotein mouse monoclonal antibody (Mab) used was kindly provided by Dr Alex Wandeler, Canadian Food Inspection Agency, Napean, Ontario. Other such antibodies are readily attained by the person having skill in the art. The immune precipitated proteins were separated by SDS-PAGE, the gel dried and exposed for various time to Kodak XAR5 X-ray film.

AdMH5(I)E3Fx and AdMH5(I)E3Fx(o) show comparable amounts of radiolabelled rabies glycoprotein in 293 cells at either 8-10 hours or 17-19 hours post infection. However, in 293LAP13 cells the expression of the rabies glycoprotein by AdMH5(I)E3Fx(o) is markedly reduced compared to AdMH5(I)E3Fx at 8-10 hours. Moreover, pre-treatment of the 293LAP13 cells with IPTG relieves this block on expression of rabies glycoprotein by AdMH5(I)E3Fx(o) (Fig. 4B). This is consistent with the results of the transient transfection experiments, described above, using the plasmids from which the recombinant adenoviruses were created. It is possible that the

two, self-annealing palindromic operator sequences in the transcript mRNA encoding the rabies glycoprotein in this construct inhibits normal processing and translation. The other observation apparent from these results is that the presence of the intron sequence in AdMH5(I)E3Fx and AdMH5(I)E3Fx(o) allows increased rabies glycoprotein expression over that produced by the comparable but intron-less vector, Ad5BHGE3MH5Fx. Because the 293LAP13-derived titre was used as a standard in this experiment, as will be seen below, the particle number of AdMH5(I)E3Fx is about six-fold higher than that of the other viruses in this experiment, and direct quantitative comparisons for this virus cannot be made. Nevertheless, comparisons between Ad5BHGE3MH5Fx and AdMH5(I)E3Fx(o), where the particle numbers are not significantly different, show that this conclusion is valid.

EXAMPLE 11: Rabies glycoprotein expression in non-complementing cell lines

To determine if the presence of the intron increased protein expression in non-complementing cell lines as well, purified virions were used to infect human (MRC-5) and canine (MDCK) cells which cannot complement the adenovirus E1 deletion defect present in the recombinant viruses under study. Levels of expression achieved by viruses without (Ad5BHGE3MH5Fx) were compared to those with (AdMH5(I)E3Fx and AdMH5(I)E3Fx(o)) an intron between the transcription start site and the start codon for the glycoprotein. The cells were infected at an m.o.i. of 20 pfu (based on titre in LAP13 cells) and 8 hours later pulsed for 2 hours with ³⁵S. As shown in Fig. 5, a comparison of rabies protein expression kinetics

after infection with approximately equal numbers of virus particles of Ad5BHGE3MH5Fx or AdMH5(I)E3Fx(o), proves that the presence of the intron greatly increases the amount of rabies glycoprotein synthesized.

While the level of rabies glycoprotein produced by the operator containing virus, AdMH5(I)E3Fx(o), appears to be less than that of AdMH5(I)E3Fx, a direct comparison is not possible since there are some six times as many particles of the latter virus per infected cell. Nevertheless, it is clear that expression of rabies glycoprotein can be achieved in non-complementing cells, both human and canine, by the recombinant adenoviruses containing the intron sequence.

EXAMPLE 12: Comparative plaquing efficiency on 293 cells and 293LAP13 cells

It is believed that high level expression of the rabies glycoprotein results in inhibition of virus replication and hence prevented efficient rescue of vectors. To confirm whether this effect manifests during plaque formation, the relative plaquing efficiency of the recombinant viruses was measured on both 293 cells and 293LAP13 cells. Table 3 shows the results of titrations on 293 and 293LAP13 cells of CsCl gradient banded viruses AdMH5(I)E3Fx, AdMH5(I)E3Fx(o) and a control vector lacking the intron sequence, Ad5BHGE3MH5Fx. The particle to pfu ratios of the control vector and of the vector containing two Lac operons were essentially identical on both 293 and 293LAP13 cells. Interestingly, as predicted, both of the high level glycoprotein expressors, AdMH5(I)E3Fx and

AdMH5(I)E3Fx(o), are an order of magnitude less efficient in inducing
plaques on 293 cells, as measured by the inverse of particle to pfu ratio, than
is the control virus. More importantly, the AdMH5(I)E3Fx(o) vector, which
contains the operator sequence, is as efficient on 293LAP13 cells as is the
5 control virus. Thus decreasing the level of glycoprotein expression by this
vector, through binding of the repressor present in 293LAP13 cells, results
in a plaquing efficiency for this vector equal to that of control virus. Thus,
these results support a correlation between the level of glycoprotein
expression and the level of inactivation potential exhibited by the adenovirus
10 vectors. The vector lacking the operator sequence also had an increased
plaquing efficiency on 293LAP13 cells of approximately three-fold
compared to the result on 293 cells. This suggests that the small reduction in
rabies glycoprotein synthesis in the 293LAP13 cells compared to 293 cells,
as observed in Fig. 4, may be sufficient to allow adenovirus replication to
15 escape some of the inactivation potential associated with rabies glycoprotein
overexpression. Also consistent with this explanation is the finding (Table
3) that the vector containing the operator sequence was almost threefold
more efficient than the vector lacking this sequence when both are plaqued
on 293 cells. The presence of the nucleotides comprising the operator
20 sequence near the transcription start site may reduce the level of expression
of rabies glycoprotein from the former vector and thus allow more
adenovirus synthesis.

The studies detailed in Table 3 concern the relative plaque forming titre and particle to pfu ratios on 293 or 293LAP13 cells of recombinant adenoviruses expressing rabies glycoprotein. The following recombinant adenoviruses: Ad5BHGE3MH5Fx, which lacks intron or operator sequences but is otherwise identical to the other vectors, AdMH5(I)E3Fx, 5 which has the intron but no operator sequences, AdMH5(I)E3Fx(o), were grown in large scale cultures in 293LAP13 cells (except for Ad5BHGE3MH5Fx, which was grown in 293 cells) and were purified on CsCl density gradients. An estimate of the virus particle number in the 10 purified stock was obtained by OD₂₆₀ as described in the text. The virus was titrated in duplicate by standard plaque assay on monolayers of 293 or 293LAP13 cells and the plaques counted after 12 days. The particle to pfu ratio of each recombinant vector was determined for 293 cells and 293LAP13 cells from this data and the ratio of the relative titre on 15 293LAP13 to that on 293 cells is presented in the last column.

Table 3: The relative plaque forming titres and particle to pfu ratios on 293 or 293LAP13 cells of recombinant adenoviruses expressing rabies glycoprotein.

Virus	293 Cells		293LAP13		Ratio of Titres
	Titre (pfu/ml)	particle/pfu ratio	Titre (pfu/ml)	(particle/pfu) ratio	
AdBHGE3-MH5Fx	5.0×10^{10}	120	5.0×10^{10}	120	1
AdBHGE3(I)-MH5Fx	1.3×10^9	2300	3.5×10^{10}	860	2.7
AdBHGE3(I)-MH5Fx(o)	3.5×10^9	1000	2.4×10^{10}	150	6.9

5 EXAMPLE 13: Growth curves on 293LAP13 cells and 293 cells

In view of the effect on transfection and on plaque formation, studies were made to determine whether the growth of these recombinant vectors was substantially different in 293 or 293LAP13 cells. Figure 6 shows one step growth curves for the two viruses AdMH5(I)E3Fx, AdMH5(I)E3Fx(o) grown on 293 and 293LAP13 cells with all virus yields assayed on 293LAP13 cells. As expected, AdMH5(I)E3Fx and AdMH5(I)E3Fx(o), that produce the highest levels of glycoprotein, also produced the lowest titres on 293 cells by 48 hours, a time at which cytopathic effect is essentially

complete in the infected cell monolayer. On 293LAP13 cells the AdMH5(I)E3Fx(o) vector attains a final yield some 20-fold higher than that obtained in 293 cells. The virus lacking the operator sequence, AdMH5(I)E3Fx, also showed a ten fold greater virus yield on 293LAP13 cells compared to the parental 293 cell line. Assuming a comparable particle to pfu ratio as determined for gradient purified viruses, the amount of AdMH5(I)E3Fx virus and AdMH5(I)E3Fx(o) virus synthesized in 293LAP13 cells is approximately the same. It is believed that the enhanced growth on 293LAP13 cells of viruses expressing excess rabies glycoprotein whether the virus does or does not carry the lac operator sequence is due to the relative reduction of rabies glycoprotein expression in the LAP348 expressing cell line.

Inhibition might be most pronounced during transfection with plasmids, permitting the generation of virus in co-transfected 293LAP13 cells and might be sufficiently strong early in virus replication to account for the enhanced replication evident in Figure 6. As a consequence the 293LAP13 cell line may be preferred over 293 cells for rescue of transgenes even without incorporation of the operator, though presence of the latter in the expression cassette clearly results in the highest efficiency of virus rescue and growth.

It is to be understood that the description, specific examples, data, tables and figures, while indicating exemplary embodiments, are given by way of illustration and are not intended to limit the invention in any manner.

Various changes and modifications within the present invention will become apparent to the skilled person from the disclosure contained herein, and thus are considered part of the invention.

WHAT IS CLAIMED IS:

1. A eukaryotic cell capable of replicating a recombinant expression vector that produces a heterologous protein, wherein the cell
5 comprises a polynucleotide encoding a repressor protein that is capable of repressing production of the heterologous protein.
2. The cell according to claim 1, wherein the cell is a mammalian cell.
- 10 3. The cell according to claim 1, wherein the vector is an adenovirus.
4. The cell according to claim 1, wherein the cell produces the protein encoded by an E1 region.
- 15 5. The cell according to claim 4, wherein the vector is a defective adenovirus.
6. The cell according to claim 5, wherein the adenovirus has a functionally disrupted E1 region.
- 20 7. The cell according to claim 5, wherein the cell is derived from 293 cells.

8. The cell according to claim 7, wherein the polynucleotide encodes a lac repressor.
9. The cell according to claim 7, wherein the polynucleotide encodes a lac repressor derivative.
10. The cell according to claim 9, wherein the polynucleotide includes a portion of plasmid pHMCVLAP348.
11. Cell line 293Lap10 (ATCC Accession No. CRL-12586).
12. Cell line 293Lap13 (ATCC Accession No. CRL-12587).
13. A recombinant adenovirus comprising (i) a first polynucleotide sequence encoding a heterologous protein and (ii) a second polynucleotide sequence operably linked to the first polynucleotide in order to permit repression of the expression of the first polynucleotide.
14. The recombinant adenovirus of claim 13, wherein the second polynucleotide sequence is an operator that can be bound by a repressor.
15. The recombinant adenovirus of claim 13, wherein the adenovirus has a functionally disrupted E1 region.

16. The recombinant adenovirus of claim 14, wherein the second polynucleotide sequence is a *lac* operator sequence.
17. The recombinant adenovirus of claim 13, wherein the heterologous protein product is rabies glycoprotein G.
18. The recombinant adenovirus of claim 13, wherein the adenovirus comprises an intron sequence.
19. A eukaryotic cell capable of replicating a recombinant expression vector that produces a heterologous protein, wherein the cell comprises a polynucleotide encoding a repressor protein that is capable of repressing production of the heterologous protein, and wherein the cell is transfected with or infected by a recombinant adenovirus comprising (i) a first polynucleotide sequence encoding a heterologous protein product and (ii) a second polynucleotide sequence operably linked to the first polynucleotide in order to permit repression of the expression of the first polynucleotide.
20. The cell of claim 19, wherein the second polynucleotide sequence is an operator that can be bound by a repressor.
21. The cell of claim 19, wherein the adenovirus has a functionally disrupted E1 region.

22. The cell of claim 19, wherein the second polynucleotide sequence is a *lac* operator sequence.

23. The cell of claim 19, wherein the heterologous protein product is rabies glycoprotein G.

24. A method of propagating an adenovirus vector encoding a heterologous protein, comprising
transfecting or infecting eukaryotic cell with a recombinant
adenovirus comprising (i) a first polynucleotide sequence encoding a
heterologous protein and (ii) a second polynucleotide sequence operably
linked to the first polynucleotide in order to permit repression of the
expression of the first polynucleotide, wherein the eukaryotic cell comprises
a polynucleotide encoding a repressor protein that is capable of repressing
production of the heterologous protein.

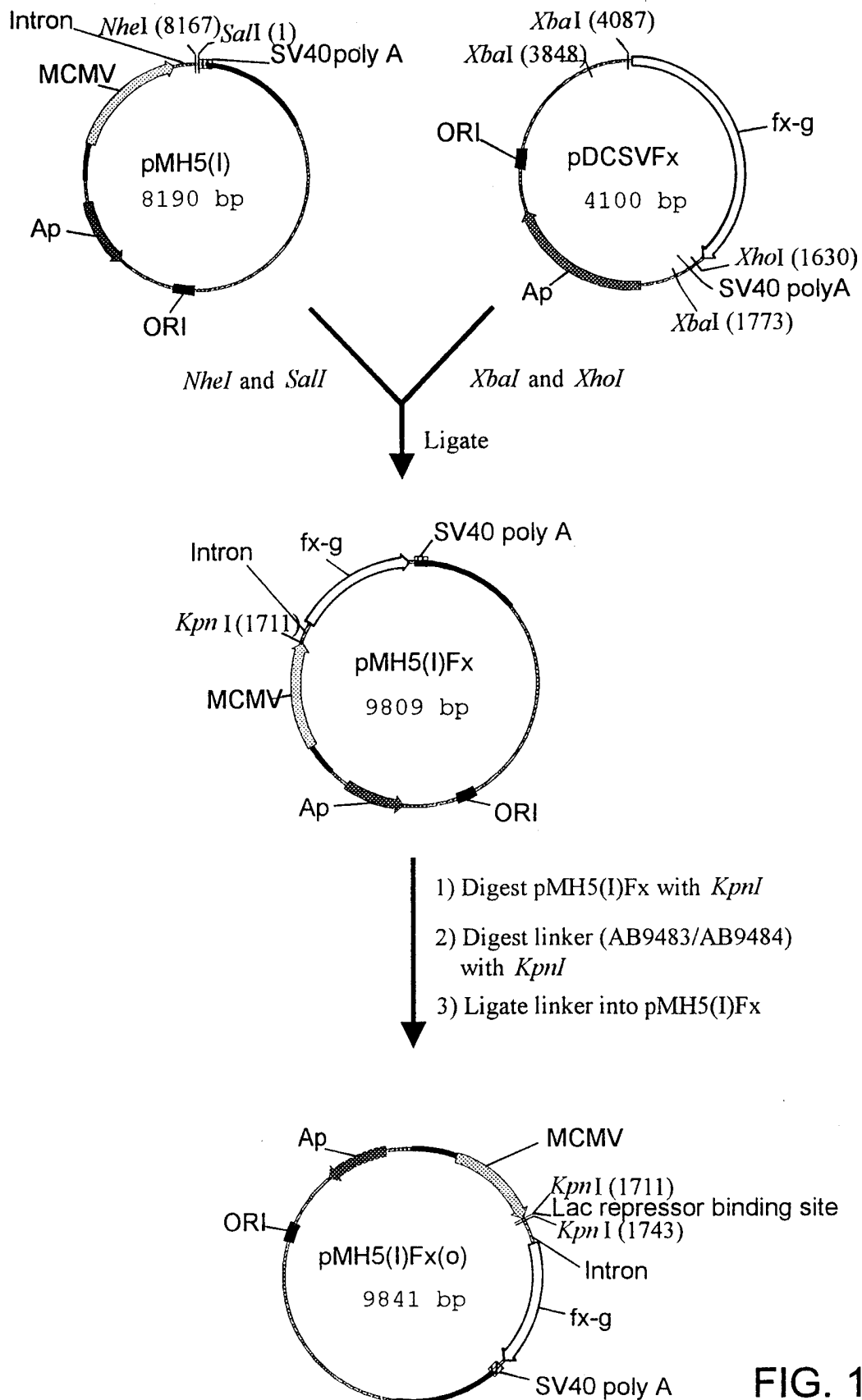
harvesting recombinant adenovirus propagated in the eukaryotic
cells

25. The method of claim 24, wherein the adenovirus has a functionally
disrupted E1 region.

26. The method of claim 24, wherein the second polynucleotide
sequence is a *lac* operator sequence.

27. The method of claim 24, wherein the heterologous protein product is rabies glycoprotein G.

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**FIG. 1**

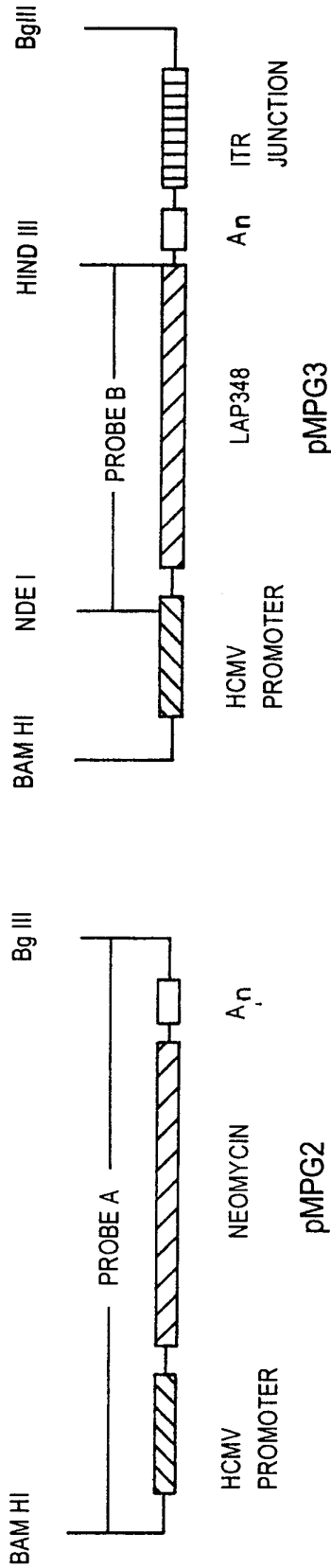


FIG. 2A

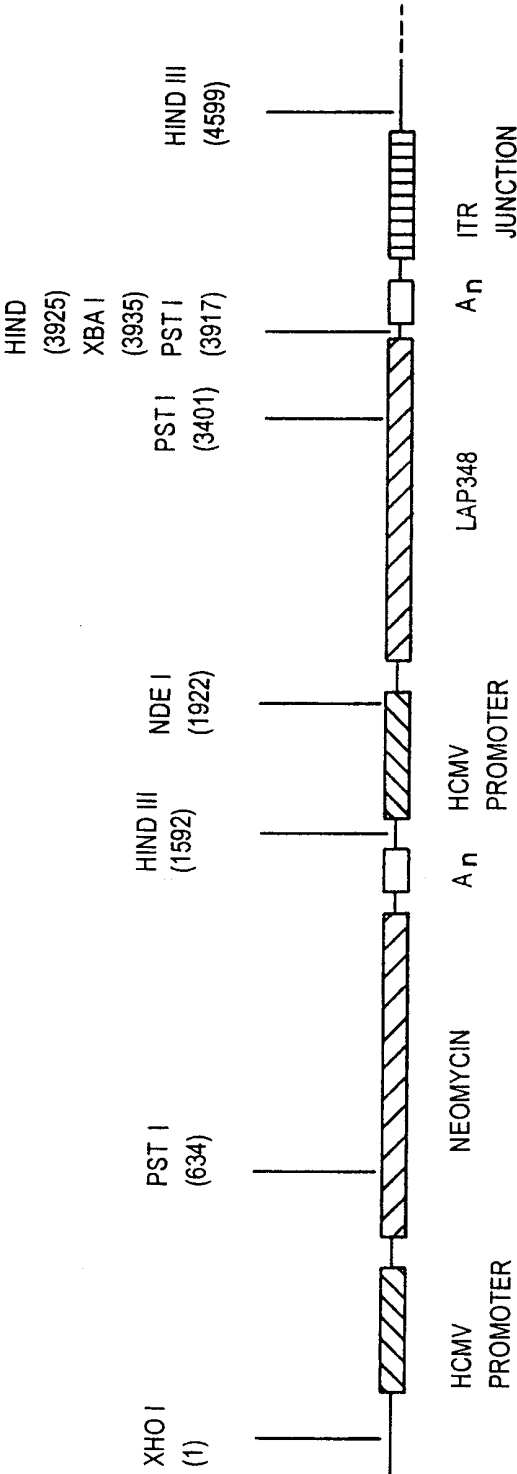


FIG. 2D

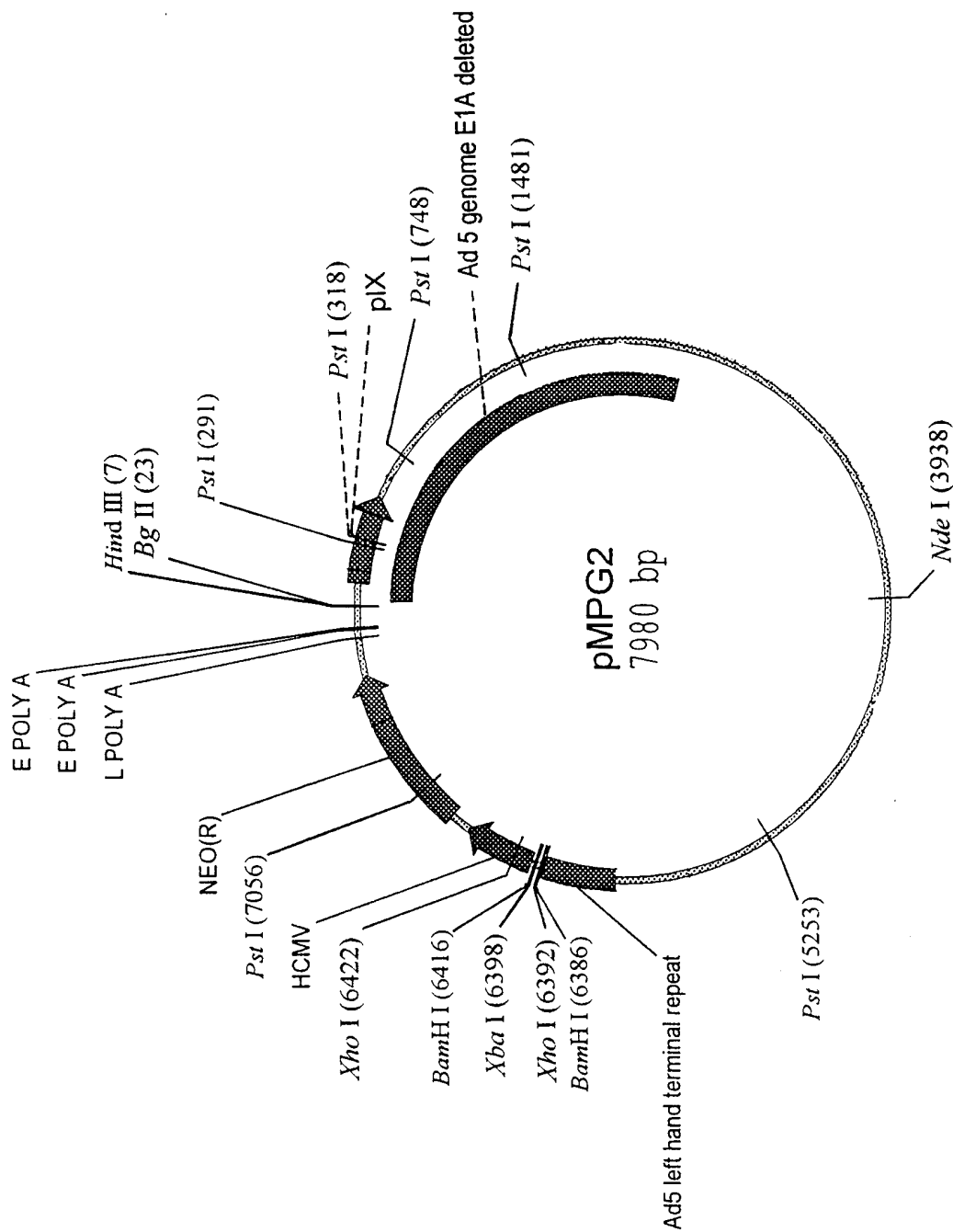
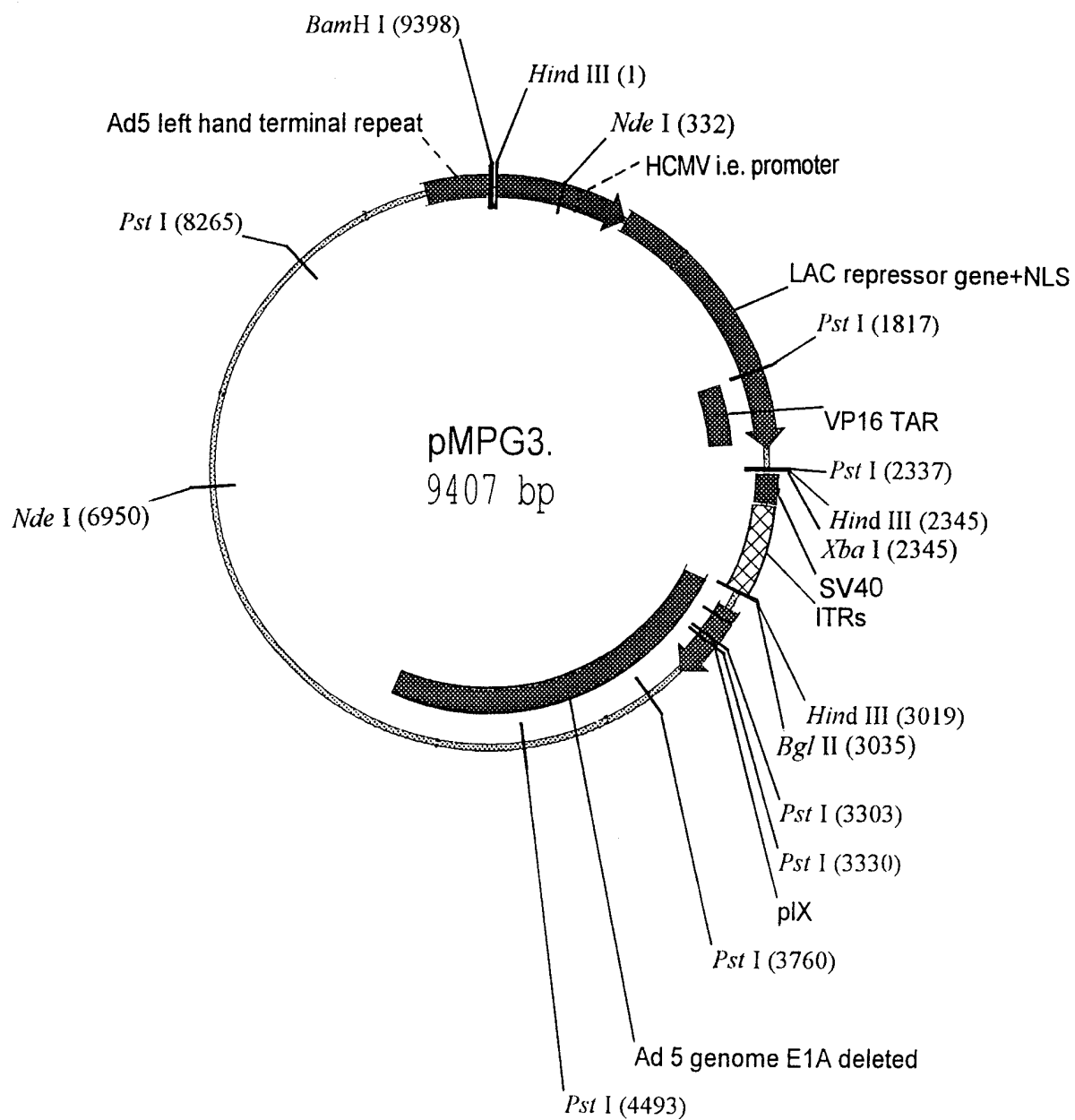


FIG. 2E

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**FIG. 2F**

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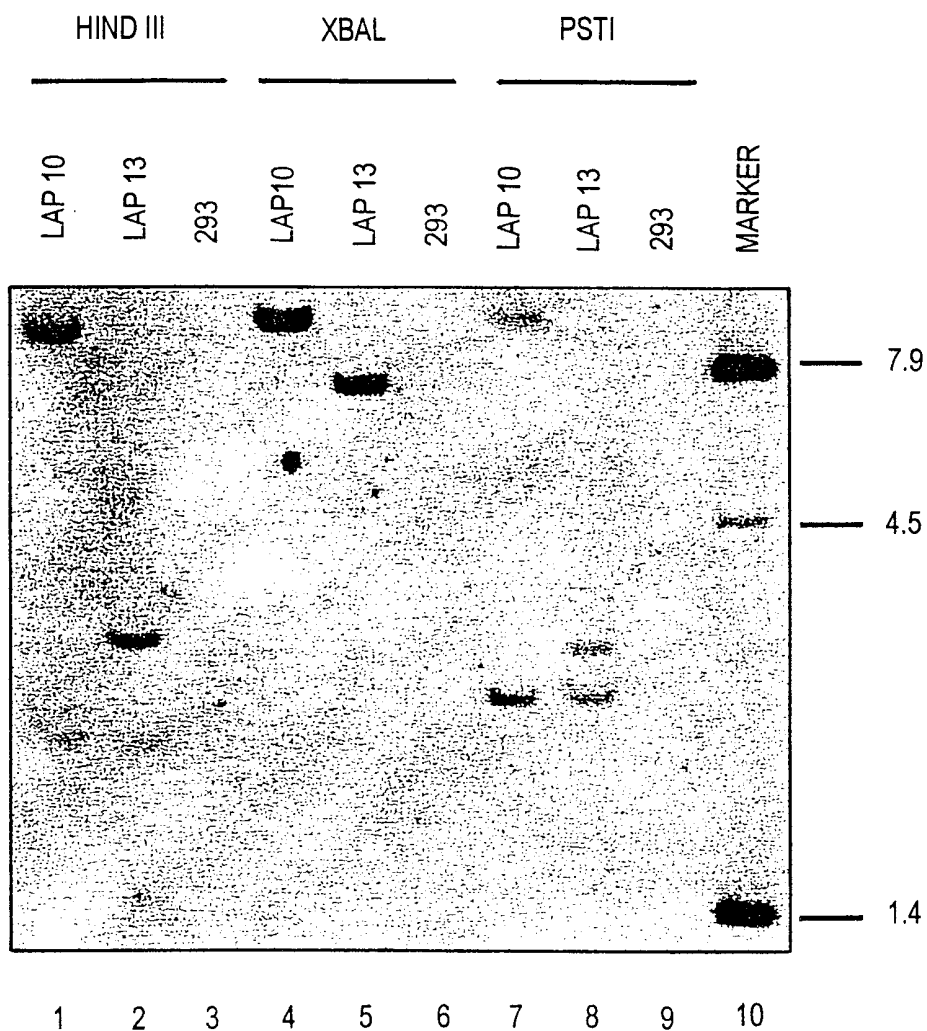
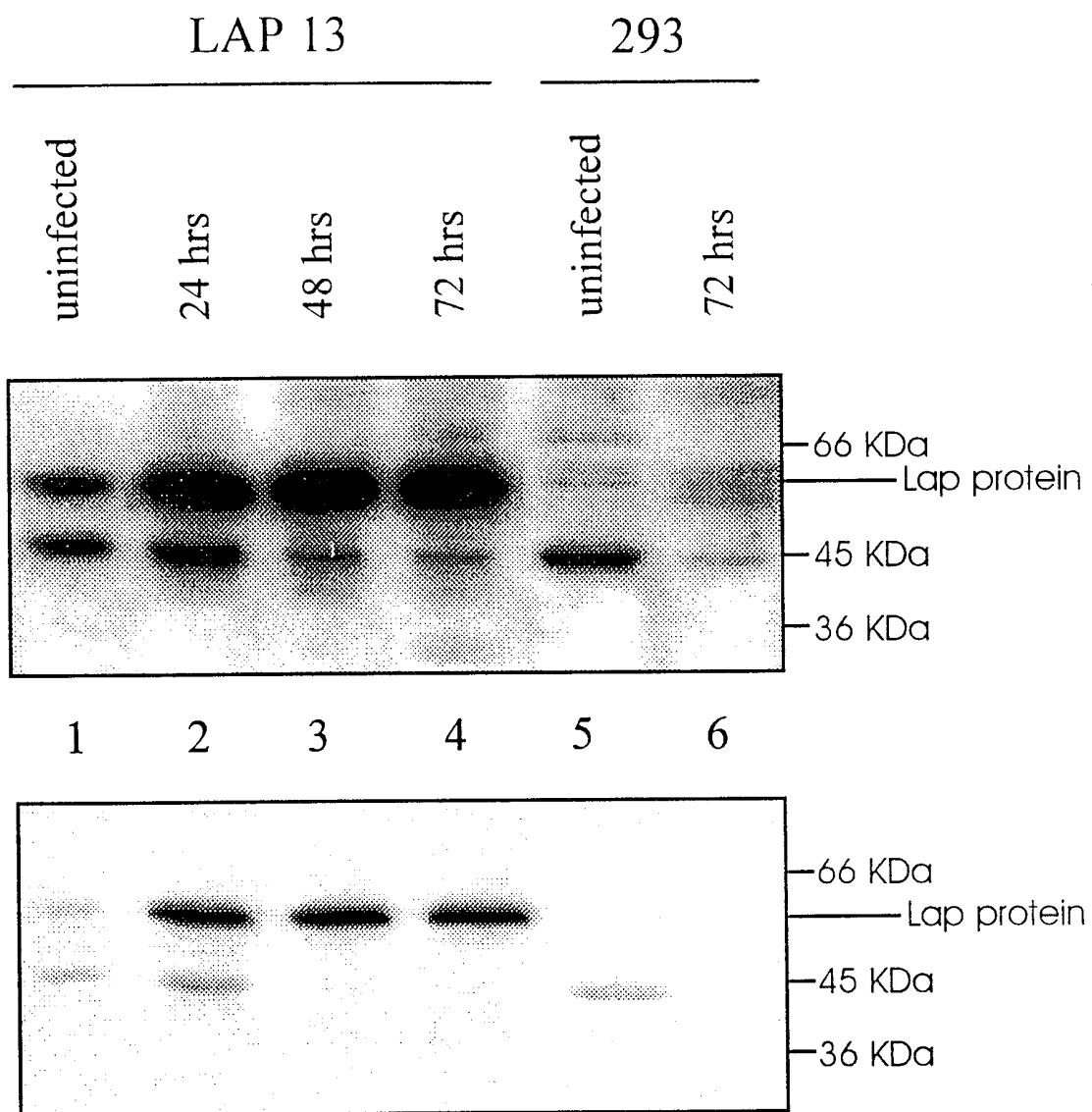


FIG. 2B

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**FIG. 3**

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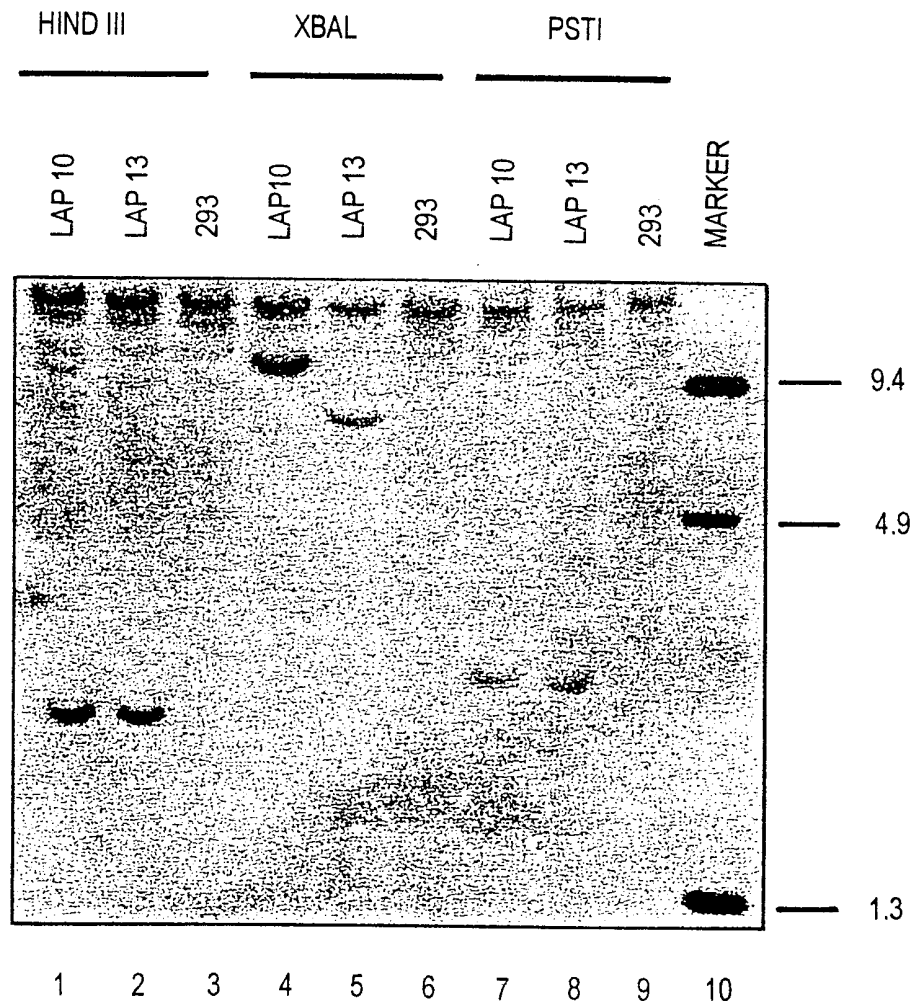


FIG. 2C

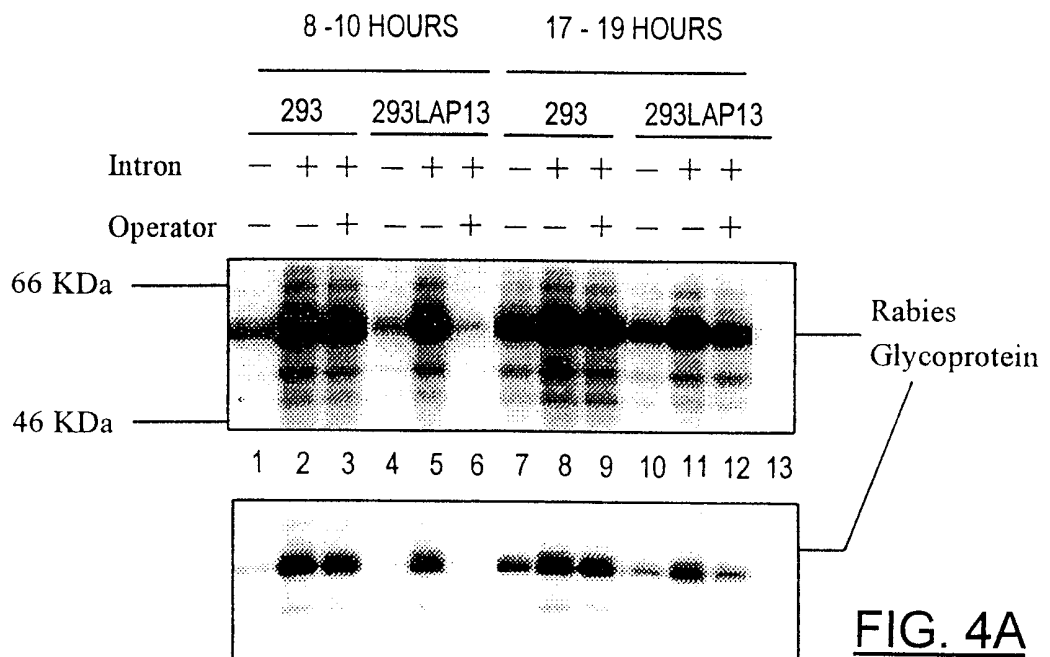
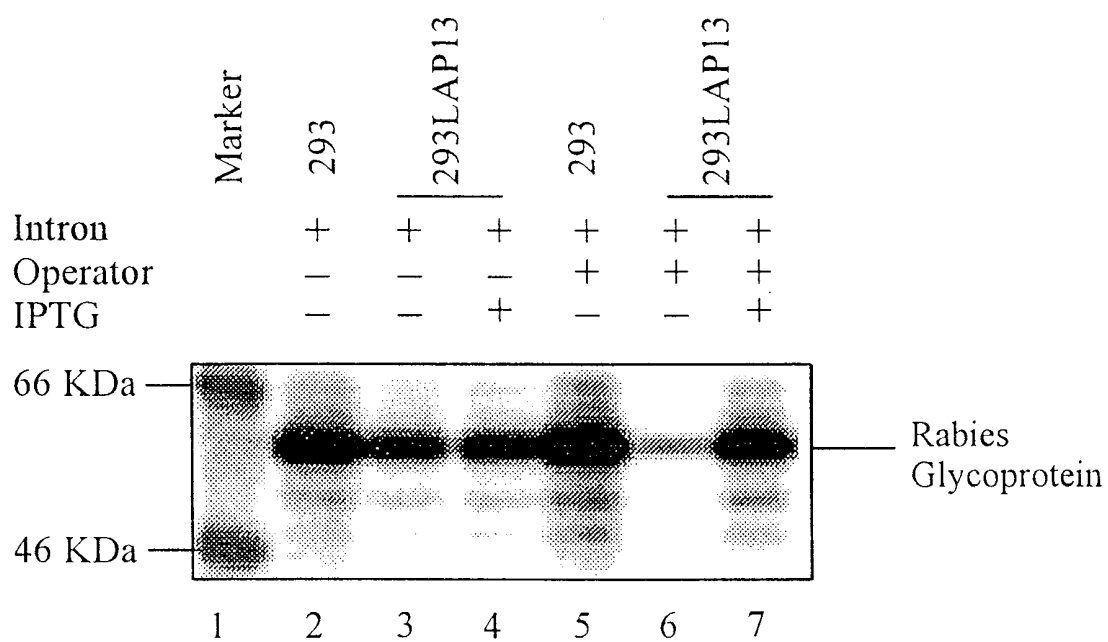
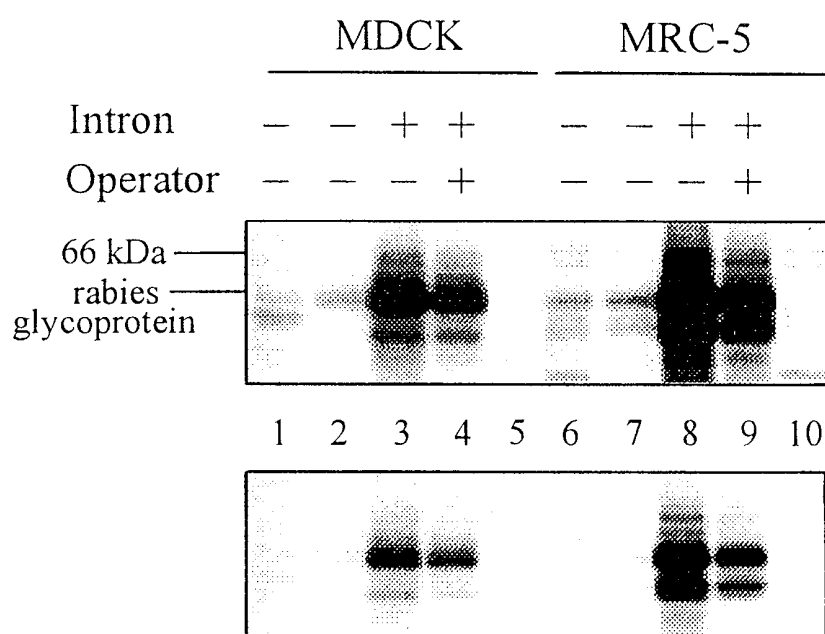


FIG. 4A

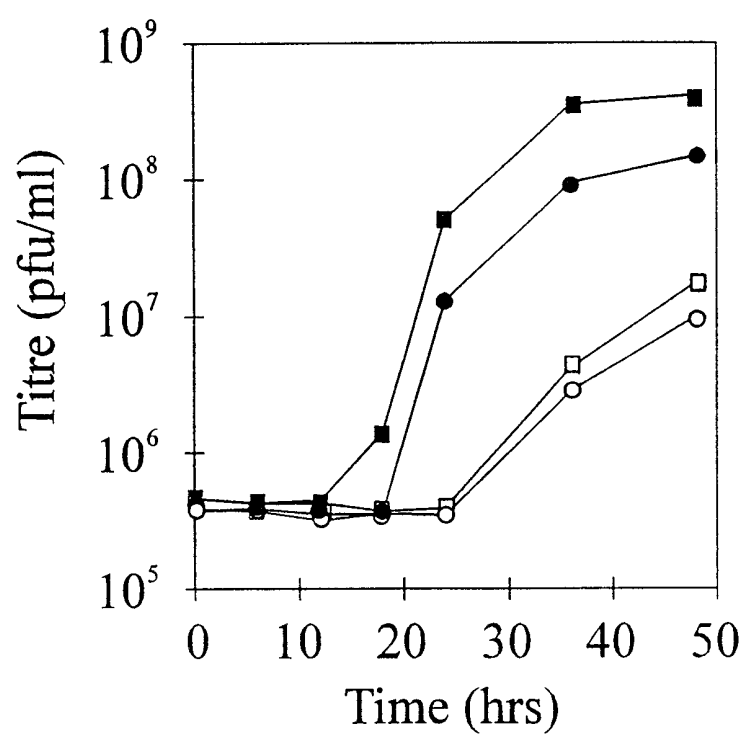
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FIG. 4B

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FIG. 5

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FIG. 6

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Plasmid pMPG2

1 aattcaagct tgcgactcg aagatctggg cgtggtaag ggtgggaaag aatatataag
 61 gtgggggtct tatgtattt tttatctgt ttgcagcagc cgccgcccgc atgagcacca
 121 actcgtttga tggaagcatt gtgagctcat attgacaac gcgcatgccc ccatgggccc
 181 ggggtcgtca gaatgtgat ggctccagca ttgatggcg ccccgctctg cccgcaaaact
 241 ctactacctt gacctacgag accgtgtctg gaacgccgtt ggagactgca gcctccgccc
 301 ccgcttcagc cgtgcagcc accgcccgcg ggattgtgac tgactttgct ttctgagcc
 361 cgcttgcaag cagtgcagct tcccggtcat ccgcccgcga tgacaagtgt acggctcttt
 421 tggcacaatt ggattctttg acccggaac ttaatgtct ttctcagcag ctgttgatc
 481 tgcgccagca ggttctgcc ctgaaggct cctcccctcc caatgcggt taaaacataa
 541 ataaaaaacc agactctgt tggattgga tcaagcaagt gtctgtctgt cttatttag
 601 gggtttgcg cgcgcggtag gcccgggacc agcggctctg gtcttgagg gtctgtgta
 661 tttttccag gacgtgtaa agtgactct ggaattcag atacatggc ataagccgt
 721 ctctgggtg gaggtagcac cactgcagag ctcatgctg cgggtgtgtg tttagatga
 781 tccagtcga gcaggagcgc tggcggtgt gcctaaaaat gtcttcagt agcaagctga
 841 ttgccagggg caggcccttg gtgtaagtgt ttacaaagcg gtaagctgg gatgggtgca
 901 tacgtgggga tatgagatgc atctggact gtatttttag gtggctatg ttccagcca
 961 tatccctccg gggatcatg ttgtcagaa ccaccagcac agtgtatccg gtgcacttg
 1021 gaaattgtc atgtagcta gaaggaaatg cgtggaagaa ctggagacg ccctgtgac
 1081 ctccaagatt ttcatgcat tctccataa tgatggcaat gggccacagc gcgcgccct
 1141 gggcgaagat atttctggga tctaactgt catagttgt ttccaggatg agatcgtcat
 1201 aggccatttt tacaagcgc gggcgagggtg tgcagactg cgtataatg gtccatccg
 1261 gccaggggc gtagttacc tcacagattt gcatttccca cgtttgagt tcagatggg
 1321 ggaatcatg tacctgcgg gcgatgaaga aaacgggttc cgggtaggg gagatcagct
 1381 gggaagaaag caggttctg agcagctgc actaccgca gccggtggc ccgtaaatca
 1441 cactattac cgggtgcaac tggtagtaa gagagctgca gtcgctgca tccctgagca
 1501 ggggggccac ttcttaagc atgtccctga ctgcagtt ttccctgacc aaatccgcca
 1561 gaaggcgtc gccgccagc gatagcagtt ctgcaagga agcaaagtt ttaacgggt
 1621 tgagaccgtc gccgtaggc atgctttga gcgttgacc aagcagttcc aggcgtccc
 1681 acagctcgt cactgtctc acggcatct gatccagcat atctcctgt ttgcgggtt
 1741 gggggtggt tgcgtgacg gcagtagtc gtgctctcc agacgggcca gggcatgct
 1801 ttccacggg cgagggtcc tgcagcgt agtctgggt acggtgaag ggtgcgtcc
 1861 gggctgcgcg ctggccaggg tgcgttag gctggtctg ctggtgctga agcgtgcg
 1921 gtctcgcgc tgcgctcg ccaggtagca ttgaccatg gtgtcatagt ccagccctc
 1981 cgcggcgtg ccctgggcg gcagctgcc ctggaggag gcgcgcacg aggggcagt
 2041 cagactttg agggcgtaga gctgggcg gagaaatacc gattccggg agtaggcac
 2101 cgcgcgcag gccccgcaga cgtctcgca ttccagagc cagtgagct ctggccgtt
 2161 ggggtcaaaa accaggttc cccatgctt ttgatgct ttctacctc tggttccat
 2221 gagccgtgt ccacgtcgg tgacgaaaag gctgtccgt tccccgata cagactgag
 2281 aggcctgtc tcgaccgat cccttgagag cctcaacc agtcagctcc ttccggtgg
 2341 cgcggggcat gactatgct gccgcacta tgactgtct cttatcatg caactgtag
 2401 gacagggtcc ggcagcgtc tgggtcatt tggcgagga ccgcttcgc tggagcgga
 2461 cgaatgcgg cgtgtcgtt gcgtattc gaattctga cgcctcgt caagccttc
 2521 tcactgttc gccacaaaa cgtttggcg agaagcagg cattatgcc ggcattggg
 2581 ccgacgcgt gggctacgt ttgctggct tcgcagcgc aggcgtgat gcttcccca
 2641 ttatgattc tctgcttc gccggcatg ggaatccgc gttcaggcc atgctgtca
 2701 ggcaggtaga tgacgacct caggacagc ttcaaggatc gctcgggt cttaccagcc

FIG. 7A

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2761 taacttcgat cactggaccg ctgatctca cggcgattta tgcgcctcg ggcagcacat
 2821 ggaacgggtt ggcattgatt gtagcgccg cctatacct tgcctgctc cccgcgttc
 2881 gtcgcgttc atggagccg gccacctga cctgaatga agccggcggc acctcgctaa
 2941 cggattcacc actccaagaa ttggagccaa tcaattctg cggagaactg tgaatgcga
 3001 aaccaaccct tggcagaaca tatcatcgc gtccgccatc tccagcagcc gcacgcggcg
 3061 catctcggc agcgttggg cctggccacg ggtgcgatg atcgtgtcc tgcgttgag
 3121 gacccggcta ggctggcgg gttgcctac tggtagcag aatgaatcac cgatacgcga
 3181 gcgaacgtga agcgactgt gtcgcaaac gtctgcgacc tgagcaaca catgaatgtt
 3241 ctccggttc cgtgttcgt aaagtctga aacgcggaag tcagcgccct gcaccattat
 3301 gttccgcatc tgcctgcag gatgtgtgt gctaccctgt ggaacaccta catctgtatt
 3361 aacgaagcgc tggcattgac cctgagtgt tttctctgg tccgcgcga tccataccg
 3421 cagttgtta cctcacaac gtccagtaa cggggcatgt tcatcatcag taaccggtat
 3481 cgtgagcatc ctctctgtt tcatcgtat cattacccc atgaacagaa attccccct
 3541 acacggaggc atcaagtac caaacaggaa aaaaccgccc ttaacatggc ccgcttlatc
 3601 agaagccaga catlaacgt tctggagaaa ctcaacgagc tggacgcgga tgaacaggca
 3661 gacatctgt aatcgctca cgaccagct gatgagctt accgcagctg cctcgcgct
 3721 ttcgtgatg acggtgaaaa cctctgacac atgcagctcc cggagacggt cacagctgt
 3781 ctgtaagcgg atccgggag cagacaagcc cgtcaggcgc cgtcagcggg tgtggcggg
 3841 tgcggggcg cagccatgac ccagtcacgt agcagatagc gagtgtatac tggcttaact
 3901 atcgccatc agagcagatt gtactgagag tgcaccatat gcggtgtgaa ataccgcaca
 3961 gatgcgaag gagaaaatac cgcacagc gctctccgc ttcctgctc actgactgc
 4021 tgcgtcggg cgttcggctg cggcgagcgg tatcagctca ctcaaaggcg gtaatacgt
 4081 tatccacaga atcaggggat aacgcaggaa agaactgtg agcaaaaggc cagcaaaagg
 4141 ccaggaaccg taaaaggcc gcgtgtctg cgttttcca taggctccg cccctgacg
 4201 agcatcaca aatcgacgc tcaagtaca ggtggcgaac cccgacagga ctataaagt
 4261 accaggcggt tccccctga agtccctcg tgcgtctcc tgtccgacc ctgcgctta
 4321 ccggatacct gtccgcttt ctccctcg gaagcgtggc gcttctcaa tgcacgct
 4381 gtaggtatct cagtcggtg taggtcttc gctcaagct gggctgtgt cacgaacccc
 4441 ccgttcagcc cgaccgctgc gcctatccg gtaactatc tctgagtc aaccggtaa
 4501 gacacgact atcgccactg gcagcagcca ctgtaacag gattagcaga gcgaggtatg
 4561 taggcgtgc tacagagttc ttgaagtgt ggcctaacta cggctacact agaaggacag
 4621 tatgtgtat ctgcgtctg ctgaagccag ttacctcgg aaaaagagtt ggtagctct
 4681 gatccggcaa acaaacacc gctggtagc gtggttttt tgttgcaag cagcagatta
 4741 cgcgcagaaa aaaaggatc caagaagatc cttgatctt ttctacggg tctgacgctc
 4801 agtgaacga aaactcacgt taagggaatt tggcatgag attatcaaaa aggatcttca
 4861 cctagatcct tttaaattaa aatgaagtt taaatcaat cttaaagata tatgagtaaa
 4921 ctggtctga cagttacaa tgcctaatca gtgaggcacc tatctcagc atctgtctat
 4981 ttcgtcatc catagttgcc tgactcccc tctgtagat aactacgata cgggagggct
 5041 taccatctgg cccagtgct gcaatgata cgcgagacc acgctcacc gctccagatt
 5101 tatcagcaat aaaccagcca gccggaagg cgcagcgag aagtgtcct gcaactttat
 5161 ccgcctccat ccagcttatt aattgtgcc gggaagctag agtaagtagt tcgccagtta
 5221 atagtttgc caacgttgt gccattgctg caggcatcgt ggtgtcacgc tgcgtttg
 5281 gtatggctc attcagctcc gttcccaac gatcaaggcg agttacatga tccccatgt
 5341 tgtcaaaaa agcgttagc tcttcggtc ctccgatcgt tgcagaagt aagtggccg
 5401 cagtttatc actcatggt atggcagcac tgcataatc tctactgtc atgccatccg
 5461 taagatgctt ttctgtgact ggtgagtact caaccaagtc attctgagaa tagtgtatgc
 5521 ggcgaccgag tigtcttgc ccggcgtaa cccgggataa taccgcgcca catagcagaa
 5581 cttaaaagt gctcatcatt ggaaaacgt ctcggggcg aaaacttca aggatcttac

FIG. 7B

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5641 cgctgtgag atccagttcg atgtaaccca ctctgcacc caactgatct tcagcatctt
 5701 ttactttcac cagcgtttct gggtagcaa aaacaggaag gcaaaatgcc gcaaaaaagg
 5761 gaataagggc gacacggaaa tgtgaatac tcatactctt ccttttcaa tattattgaa
 5821 gcattatca gggttattgt ctcatgagcg gatacatatt tgaatgtatt tagaaaaata
 5881 aacaaatagg ggttcgcgc acatttcccc gaaaagtgc acctgacgtc taagaaacca
 5941 ttattatcat gacattaacc tataaaaaata ggcgtatcac gaggcctttt cgtctcaag
 6001 aattgatccg ggccccatt tccccctct ccagctctc tgcccccttt ggattgaagc
 6061 caatatgata atgagggggt ggagtttgt acgtggcgcg gggcgtggga acggggcggg
 6121 tgacgtagta gtgtggcgga agtgtgatgt tgcaagtgtg gcggaacaca tgaagcgac
 6181 ggatgtggca aaagtacgt ttttggtgt cgcgggtga cacaggaagt gacaatttc
 6241 gcgcggttt aggcggatgt ttagtaaat ttggcgtaa ccgagtaaga ttggccatt
 6301 ttgcgggaa aactgaataa gaggaagtga aatctgaata attttgtt atcatagcg
 6361 cgtaatctct agcatgatg tcgaggatcc ctgagtcta gagatatga atcgcatcc
 6421 ctgagtcta ggaatcaatt cctggcatt atgcccagta catgacctta tgggacttc
 6481 ctactggca gtacatctac gtattagtca tcgtattac catggtgatg cggtttggc
 6541 agtacatcaa tggcggtgga tagcggttg actcacgggg attccaagt ctccaccca
 6601 ttgacgtcaa tgggagttg tttggcacc aaaatcaacg ggactttcca aaatgtcgta
 6661 acaactccgc cccattgacg caaatggcg gtaggcgtg acggtgggag gtctatataa
 6721 cgagagctcg ttagtgaac cgtcagatcg cctggagacg ccatccacgc tgtttgacc
 6781 tccatagaag acaccgggac cgtaccagcc tggggatcag tcttcgagtc gacaagtga
 6841 tctgatcaag agacaggatg aggatcgtt cgtatgatt aacaagatgg attgcacga
 6901 ggttcccg cgcgtgggt ggagaggcta ttccgctatg actgggcaca acagacaatc
 6961 ggctgctctg atgcccggt gtccggctg tcagcgcagg ggcgcccgt tctttgtc
 7021 aagaccgacc tgcgggtgc cctgaatgaa ctgcaggacg aggcagcgc gctatcgtg
 7081 ctggccacga cggcggttc ttgcgcagct gtgctcgacg ttgtcactga agcgggaagg
 7141 gactggctgc tatgggcga agtgccgggg caggatctcc tgtcatctca ccttgctct
 7201 gccgagaaag tatccatcat ggctgatgca atgcggcggc tgcatacgt tgatccggct
 7261 acctgccc atcgaccacca agcgaacat cgcacgagc gagcacgtac tcggatggaa
 7321 gccggtctg tcgatcagga tgatctggac gaagagcatc aggggctcg gccagccgaa
 7381 ctgttcgcca ggctcaaggc gcgcatgcc gacggcgagg atctcgtgt gacctatgg
 7441 gatgcctgt tccgaatat catggtggaa aatggccgt tttctgatt catcgactgt
 7501 ggccggctg gtgtggcgga ccgctatcag gacatagcgt tggctacccg tgatattgt
 7561 gaagagcttg gcggcgaatg ggctgaccgc ttctcgtgc ttacggtat cgccgtccc
 7621 gattcgcagc gcatgcctt ctatgcctt ctgacgagt tctctgagc gggactctg
 7681 ggttcgaaat gaccgaccaa gcgacgcca acctgccatc acgagattc gattccaccg
 7741 ccgcctcta tgaagggtg ggctcggaa tcgtttccg ggacgcccg tggatgatcc
 7801 tccagcggg ggaatcatg ctggagtct tcgcccacc caactgttt atgcagctt
 7861 ataaggtta caaataaagc aatagcatca caaatttcac aaataaagca tttttcac
 7921 tgcattctag ttgtggttg tccaaactca tcaatgtatc ttatcatgtc tggatcatg

FIG. 7C

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pMPG3-type plasmid

1 agcttgccc attgcatcg ttgtatccat atcataatat gtacatttat attggctcat
 61 gtccaacatt accgccatgt tgacattgat tattgactag ttattaatag taatcaatta
 121 cggggtcatt agttcatagc ccatatatgg agttccgctg tacataactt acggtaaatg
 181 gcccgctgg ctgaccgccc aacgaccccc gccattgac gtcaataatg acgtatgttc
 241 ccatagtaac gccaataggg actttccatt gacgtcaatg ggtggagtat ttacggtaaa
 301 ctgcccactt ggcagtacat caagtgtatc atatgccaag tacgccccct attgacgtca
 361 atgacggtaa atggcccgcc tggcattatg cccagtacat gaccttatgg gactttccta
 421 ctggcagta catctacgta ttatcatcgt ctattacat ggtgatgcgg ttitggcagt
 481 acatcaatgg gcgtggatag cgtttgact cacggggatt tccaagtctc caccacattg
 541 acgtcaatgg gagtttgtt tggcaccaaa atcaacggga ctttccaaaa tgcgttaaca
 601 actcgcgccc attgacgcaa atgggcggtg ggcgtgtacg gtgggaggtc tatataagca
 661 gagctcgtt agtgaaccgt cagatcgctt ggagacgcca tccacgctgt ttgacctcc
 721 atagaagaca cgttatcatg ccaaaaaaga agagaaaggt aaacatgaaa ccagtaacgt
 781 tatacagatg cgcagagtat gccggtgtct cttatcagac cgtttccgcg gtggtgaacc
 841 aggccagcca cgtttctcg aaaacgcggg aaaaagtga agcggcgatg gcggagctga
 901 attacattcc caaccgctg gcacaacaac tggcgggcaa acagtcgtt ctgattggcg
 961 ttgccacctc cagctggcc ctgcacgcg cgtcgcaaat tgcgcggcg attaaatctc
 1021 gcgccgatca actgggtgcc agcgtggtg tgcgatggt agaacgaagc ggcgtcgaag
 1081 cctgtaaagc ggcgggtcac aatcttctg cgcaacgcgt cagtgggctg atcattaact
 1141 atccgctgga tgaccaggat gccattgctg tgaagctgc ctgcactaat gtccggcgt
 1201 tatttctga tctctctgac cagacacca tcaacagtat tatttctcc catgaagacg
 1261 gtacgcgact gggcggtgag catctggtc cattgggtca ccagcaaatc gcgctgttag
 1321 cgggcccatt aagtctctc tggcgcgctc tgcgtctggc tggctggcat aaatatctca
 1381 ctgcgaatca aattcagccg atagcggaac ggaaggcga ctggagtgc atgtccggt
 1441 ttcaacaac catgcaaatg ctgaatgagg gcatcgctcc cactgcgatg ctggtgcc
 1501 acgatcagat ggcgtctggc gcaatgcgcg ccattaccga gtccgggctg cgcgttggg
 1561 cggatatctc ggtagtggga tacgacgata ccgaagacag ctcatgttat atccgcgct
 1621 caaccacat caaacaggat ttgcgctgc tggggcaaac cagcgtggac cgttctctc
 1681 aactctctca gggccaggcg gtgaaggga atcagctgtt gcccgctca ctggtgaaaa
 1741 gaaaaaccac cctggcgccc aatacgcaaa ccgcctctcc ccgcgcgtg gccgattcat
 1801 taatgcaggg gctgcaggaa ttgatacga aaaacaatta cgggtctacc atcgagggcc
 1861 tgcctgatct cccggacgac gacgccccg aagaggcggg gctggcggt cgcgcctgt
 1921 ccttctccc cgcgggacac acgcgcagac tgcgacggc ccccccacc gatgcagcc
 1981 tgggggacga gctccacta gacggcgagg acgtggcgat ggcgcatgcc gacgcgctag
 2041 acgatttca tctggacatg ttgggggacg gggattcccc ggggccggga ttacccccc
 2101 acgactccgc cccctacgac gctctggata tggccgactt cgagtttag cagatgtta
 2161 ccgatgccct tgaattgac gagtatcaag ctctggcacg acaggttcc cgactgaaa
 2221 gcgggcagt agcgcaacgc aattaatgt agttagctca ctattaggc acccaggct
 2281 ttacacttta tgcctcggc tctatgtga ccatgattac gaatggtga cctgcaggca
 2341 tgcaagcttc gatctagaga tgactcag caactgttt attgcagctt ataattgta
 2401 caaataaagc aatagcatca caaattcac aaataaagca tttttcac tgcattctag
 2461 ttgtgtttg tcaaaactca tcaatgtatc ttatcatgct tggatgctc agcatcgaag
 2521 atccggcgcg gtacaccgac gcacacaaa aacgtcactt ttgccacac cgtcgcttac
 2581 atgtgttccg ccacactgac aacatcacac ttccgccaca ctactacgct acccgccccg
 2641 ttccacgccc ccgcgccacg tcacaaactc caccctctca ttatcatatt ggttcaatc

FIG. 8A

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2701 caaataagg tatattatg atgaatatac ctattttgg attgaagcca atatgataat
 2761 gagggggtgg agttgtgac gtggcgcggt gctggggaac gggcggggtg acgtagggtt
 2821 tagggcgag taactgtat gtgtgggaa ttgtagttt cttaaatgg gaagtacgt
 2881 aacgtgggaa aacggaagt acgattgag gaagtgtg gtttttggc ttctgttct
 2941 gggcgtaggt tcgctgctg tttctgggt gtttttgg gacttaacc gtacgtcat
 3001 ttttagtct cgaatcaag ctgtcgact cgaagatcg ggcgtggtta aggggtggaa
 3061 agaataata aggtgggggt ctatgtagt ttgtatctg tttgcagca gccgcgcgc
 3121 ccatgagcac caactcgtt gatggaagca ttgtgagtc atattgaca acgcgcgtc
 3181 ccccatgggc cggggtgctg cagaatgta tgggtccag cattgatgt cgccccgtc
 3241 tgcccgcaaa ctctactacc ttgacctag agaccgtgc tggaaacgcg ttggagactg
 3301 cagcctcgc cgccgttca gccgtgcag ccaccgccc cggtattgt actgacttg
 3361 cttctctgag ccgcttga agcagtgca cttccggtc atccgcccgc gatgacaagt
 3421 tgacggctt tttgcacaa ttgattct tgaccggga actaatgtc gttctcagc
 3481 agctgttga tctgcgcag caggtttct cctgaaggc ttctccct ccaatgcg
 3541 ttaaaacat aaataaaaa ccagactct tttgattg gatcaagca gtgtctgt
 3601 gtcttatt aggggtttg cgcgcgcgt agggccggga ccagcgctc cggtcgtga
 3661 ggtcctgt tattttcc aggacgtgt aaaggtgact ctggtgtc agatactgg
 3721 gcataagccc gtctctggg tggaggtag accactgag agctcatgc tgcgggtgg
 3781 tttgtatg gatccagtc tagcaggagc gctggcggt gtgcctaaa atgtcttca
 3841 gtgcaagct gattgccag ggcaggccct tgggtgaagt gttacaaag cggtaagct
 3901 gggatgggtg catagctgg gatagagat gcatcttga ctgtatttt aggttgcta
 3961 tttccagc catatccct cggtgattca tttgtgag aaccaccag acagtgtatc
 4021 cgtgcactt gggaaattg tcatgtagt tagaagaaa tgcgtggaag aacttgaga
 4081 cgccctgt accccaaga tttccatgc attcgtccat aatgatggca atggccac
 4141 gggcgcggc ctggcggaag atattctg gatcactaac gcatagttg tgtccagga
 4201 tgagatgct ataggccatt ttacaaagc gcggcgag ggtgccagac tgcgtataa
 4261 tggttccat cgcccgagg gcgtagttc ctcacagat ttgcattcc cagccttga
 4321 gttcagatg ggggatcat tctacctgc ggcgatgaa gaaacggt tccgggtgag
 4381 gggagatcag ctgggaagaa agcaggttc tgagcagct cgaactacc cagccggtg
 4441 gcccgtaat cacacctat accgggtgca actggtagt aagagagct cagctccgt
 4501 catccctgag cagggggcc actcgtta gcatgtcct gactgcag tttccctga
 4561 ccaaatccg cagaaggcg tcgcccga gcatagcag ttctgcaag gaagcaaagt
 4621 tttcaacg tttagaccg tccgctgag gcatgttt gagcgttga ccaagcgtt
 4681 ccaggcggt ccacagctg gtcacctgt ctacggcatc tcatccagc atctctctc
 4741 gttcgcgg ttggggcgg ttcgctgt cggcagtagt cgtgtcgt ccagacggg
 4801 cagggtcatg tcttcacg ggcgcaggt cctcgtcag gtagctgtg tccggtgaa
 4861 ggggtgct cgggctgc cgtgcccag ggtgcgctt aggtgtgtc tctgtgtct
 4921 gaagcgtgc cgttctgc cctgcgct ggcaggtag cattgacca tgggtcata
 4981 gtccagccc tccggcgt ggccttgc gcgcagctt ccttggagg aggcgcgca
 5041 cgaggggag tgcagactt tgaggcgta gagctgggc gcgagaaata ccgattccg
 5101 ggagtaggca tccgcgcgc agggccgca gacggtctg cattccagca gccagtgag
 5161 ctctggcgt tgggggtcaa aaaccaggt tccccatgc ttttgaic gtttctacc
 5221 tctgtttcc atgagccgt gtccagctc ggtgacgaa aggtgtccg tgtcccgta
 5281 tacagactg agaggcctt cctgaccga tgcctttag agcctcaac ccagtcagct
 5341 cttccggt ggcgcgggc atgactatc tgcgcgact tatgactgc ttttatca
 5401 tgcaactct aggcaggtg ccggcagcg tctgggtcat ttgggcgag gaccgcttc
 5461 gctggagcg gacgatgac ggcctgtgc tgcggtatt cggaatctt cagccctcg
 5521 ctcaagcct cgtcactgt cccgccacca aacgttctg cgagaagcag gccattatc
 5581 ccggcatgg gccgcagcg ctgggtacg tctgtctgc gttcgcagc cgaggctga

FIG. 8B

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5641 tggccttccc cattatgatt ctctcgtt cggcgggcat cgggatgccc gcggtgcagg
 5701 ccatgctgtc caggcaggta gatgacgacc atcagggaca gcttcaagga tcgctcgccg
 5761 ctcttaccag cctaacttcg atcactggac cgctgatcgt cagggcgatt tatccgcct
 5821 cggcgagcac atggaacggg ttggcatgga ttgtaggcgc cgccctatac ctgtctgcc
 5881 tcccccggtt gcgctcggtt gcatggagcc gggccacctc gacctgaatg gaagccggcg
 5941 gcacctcgtt aacggattca ccactccaag aattggagcc aatcaattct tgcggagaac
 6001 tgtgaatgcg caaaccaacc ctggcagaa catatccatc gcgctcgcca tctccagcag
 6061 ccgcacgcgg cgcactcgg gcagcgttgg gtcttgccca cgggtgcgca tgatcgtgt
 6121 cctgtcgttg aggaccggc taggctggcg ggggtgcctt actggttagc agaataatc
 6181 accgatacgc gagcgaacgt gaagcgactg ctgctgcaaa acgtctgcga cctgagcaac
 6241 aacatgaatg gtcttcggtt tccgtgttc gtaaagtctg gaaacgcgga agtcagcgcc
 6301 ctgcaccatt atgtccgga tctgcatgc aggatgtgc tggctacctt gtggaacacc
 6361 tacatctgta ttaacgaagc gctggcattg accctgagt attttctct ggtcccgccg
 6421 catccatacc gccagtgtt taccctcaca acgttccagt aaccgggcat gtccatcatc
 6481 agtaaccgt atcgtgagca tctctctcg ttcatcgtt atcattacc ccatgaacg
 6541 aaattccccc ttacacggag gcatcaagt accaaacagg aaaaaaccgc ccttaacatg
 6601 gcccgcttta tcagaagcca gacattaacg ctctggaga aactcaacga gctggacgag
 6661 gatgaacagg cagacatctg tgaatcgct cagcaccacg ctgatgagct ttaccgcagc
 6721 tgcctcgcgc gtttcggtga tgacggtgaa aacctctgac acatgcagct cccggagacg
 6781 gtcacagctt gctgtgaagc ggtatgcggg agcagacaag cccgtcaggg cgcgtcagcg
 6841 ggtgttgccg ggtgtcgagg cgagccatg acccagtcac gtacgcatag cggagtgtat
 6901 actggcttaa ctatgcgga tcagagcaga ttgtactgag agtgcacat atcggtgtg
 6961 aaataccgca cagatgcga aggagaaaa accgcatcag gcgctctcc gcttctcgc
 7021 tctactgact gctgcgctcg gctgtcggc tgcggcgagc ggtatcagct cactcaaagg
 7081 cggtaatacg gttatccaca gaatcagggg ataacgcagg aaagaacatg tgagcaaaaag
 7141 gccagcaaaa ggccaggaac cgtaaaaagg ccgctgtgtt ggcgttttc cataggctcc
 7201 gccccctga cgagcatcac aaaaatcgac gctcaagtca gaggtggcga aaccgcagac
 7261 gactataaag ataccaggcg ttccccctg gaagctccct cgtgcgctt cctgttccga
 7321 ccctgccgtt taccggatac ctgtccgct ttctccctc gggaagcggt gcgcttctc
 7381 aatgctcagc ctgtaggat ctcatgtcg tttaggtcgt tgcctccaag ctgggctgtg
 7441 tgacgaacc ccccgctcag cccgaccgct gcgcctatc cggtaactat cgtctgagt
 7501 ccaaccgggt aagacacgac ttatcgccac tggcagcagc cactggtaac aggattagca
 7561 gagcgaggta ttagggcgtt gctacagagt tctgaagtg gtggcctaac tacggctaca
 7621 ctagaaggac agtatgtgt atctgcgctc tctgaagcc agttacctc ggaaaaagag
 7681 ttgtagctc ttgatccggc aaacaaacca ccgctggtag cgggtgttt ttgtttgca
 7741 agcagcagat tacgcgcaga aaaaaggat ctcaagaaga tctttgac ttcttacg
 7801 ggtctgacgc tcagtgaac gaaaactcac gtaagggt ttgtgtcatg agattatcaa
 7861 aaaggatctt cactagatc ctttaaat aaaaatgaag ttttaata atctaaagta
 7921 tatatgagta aacttggtct gacagtacc aatgctaat cagttaggca cctatctcag
 7981 cgatctgtct atttcttca tccatagtt cctgactccc cgtcgtgtag ataactacga
 8041 tacgggaggg ctaccatct ggccccagtg ctgcaatgat accgcgagac ccacgctcac
 8101 cggctccaga ttatcagca ataaaccagc cagccggaag ggccgagcgc agaagtggc
 8161 ctgcaacttt atccgctcc atccagtcta ttaattgtt cgggaagct agagtaagta
 8221 gttccagct taatgtttg cgcaacgtt ttgccattg tgcaggcatc gtggtgtcac
 8281 gctcgtcgtt tggatggct tcatcagct ccggttcca acgatcaagg cgagttacat
 8341 gatccccat gttgtgcaaa aaagcggtta gctcctcgg tctccgac gtgtcagaa
 8401 gtaagtggc cgagtgta tcatcatgg ttatggcagc actgcataat tctctactg
 8461 tcatgccatc cgtaatatgc tttctgtga ctggtgagta ctcaaccaag tcatctgag

FIG. 8C

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8521 aatagtgtat gggcgaccg agttgctctt gcccggcgtc aacacgggat aataccgcg
8581 cacatagcag aactttaaaa gtgctcatca ttggaaaacg ttcttgggg cgaaaactct
8641 caaggatctt accgctgttg agatccagtt cgatgtaacc cactcgtgca cccaactgat
8701 cttcagcatc ttctacttc accagcgttt ctgggtgagc aaaaacagga aggcaaaatg
8761 ccgcaaaaaa ggaataagg gcgacacgga aatgtgaat actcatactc ttctttttc
8821 aatattattg aagcatttat cagggttatt gtctcatgag cggatacata ttgaatga
8881 tttagaaaaa taaacaata ggggttccgc gcacatttcc ccgaaaagtg ccacctgacg
8941 tctaagaaac cattattatc atgacattaa cctataaaaa taggcgtatc acgaggccct
9001 ttctgtctca agaattgatc cgggccccca ttccccctcc ctccagctc tctgcccctt
9061 ttggattgaa gccaatatga taatgagggg gtggagtgtg tgacgtggcg cggggcggtg
9121 gaacggggcg ggtgacgtag tagtggtggc gaagtgtgat gtgcaagtg tggcggaaca
9181 catgtaagcg acggatgtgg caaaagtgac gttttggtg tgcgccggtg tacacaggaa
9241 gtgacaattt tcgcgcggtt ttaggcggat gtgtagtaa atttggcgt aaccgagtaa
9301 gatttgcca ttctgcggg aaaactgaat aagaggaagt gaaatctgaa taattttgtg
9361 ttactcatag cgcgtaatct ctagcatcga tgcgaggat ccctcga

FIG. 8D

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Plasmid pDCSVx

1 atggttcctc aggcctcttt gttgtgccc ctctggctt ttccaatgtg ttccgggaaa
 61 ttcccatct acacaatacc agacaaactt ggtccctgga gcccgattga catacatcat
 121 ctacgtgcc caaataactt ggtgtggaa gacgaaggat gcaccaactt gtctggttc
 181 tctacatgg aacttaaagt gggatacatt tcagccataa aagtgaacgg gttcacgtgc
 241 acaggtgtgg tgacagaggc tgagacttac actaactttg tcggttatgt caccactacg
 301 ttcaagagaa agcatttccg cccaacacca gatgcattgc gtgtgctta caactggaaa
 361 atggccggcg accccagata tgaagaatct ctccataatc cgtaccctga ctaccattgg
 421 ctccaacca taaaaccac gaaggagtc ctcatcatca tatccctag ttagcggac
 481 ctgacccat atgacaaatc cctcattcg aggtctttc ctgacgggaa gtgcttgga
 541 ataacaatat catctaccta ctgctgact aaccatgatt acaccatctg gatgcccgag
 601 aatctgagat tagggacatc ttgtgacatc ttaccaata gtagaggga gagagcatcc
 661 aaaggagca agactgcgg attgttgac gaaaggggct tgataagtc ttgaagggg
 721 gcatgcaagc tcaaatatg tggggtctg ggacttagac ttatggacgg aacgtgggtc
 781 gcgacgcaa catcgatga gaccaagtgg tgcctccctg atcagttggt gaatctacac
 841 gactttcgt cggacgaaat cgagcatctt gtctggagg agttggtcaa gaaaagagag
 901 gagtgttgg atgccctaga gtccatcatg actaccaagt ctgtgagttt tagacgtctc
 961 agccactga gaaaactgt ccttgggttc ggaaaagcat acaccatatt caacaaaacc
 1021 ctgatggagg ctgatgctca ctacaagtca gtccggacct ggaatgagat catccctca
 1081 aaggggtgct tgagagtgg aggaagtgt catcccatg taaacggagt gtttttaac
 1141 ggcataatac tggggcctga cggccatgtt ctgatccag agatgcaatc atccctctt
 1201 cagcagcata tggagtgtt ggaatcatca gtatccctc tgatgcatcc tttagcagac
 1261 ccgtctacag tctcaagga cgggtatgaa gcagaggatt ttgtgaggt tcaccttccc
 1321 gatgtgcaca acaaatctc aggggtgac ctgggtctcc ctaactgggg aaagtatgtc
 1381 ttaataattg caggggtcct gattgccatg atattgaca tctcttgat gacatgtgt
 1441 ggaagaggta atcgaccaa gtccacaaa cacagtcttg gaggatagg aaggaagggt
 1501 tcagccactt ccaaaagcgg gaaggcata tctcgtggg agtcatataa aagcgggggt
 1561 gagaccagac tgtgaaggcc ggttatccct tccaaactc aagtcocgaa gatgcctcc
 1621 ctcgggggct cgagcaactt gttattgca gttataatg gttacaaata aagcaatagc
 1681 atcaaaatt tcacaaataa agcattttt tcatgcatt ctagtgtgg ttgtccaaa
 1741 ctcatcaatg tatctatca tgtctggatc gtctagacaa ttctgaaga cgaaagggcc
 1801 tcgtgatacg cctatttta taggttaatg tcatgataat aatggtttct tagacgtcag
 1861 gtggcacttt tcggggaaat gtgcgcggaa ccctatttg ttatttttc taaatcatt
 1921 caaatatgta tccgtcatg agacaataac cctgataaat gctcaataa tattgaaaaa
 1981 ggaagagtat gattattcaa catttccgtg tcgcccttat tcccttttt gcggcatttt
 2041 gccttctgt tttgtcac ccagaaacgc tggtgaaagt aaaagatgt gaagatcagt
 2101 tgggtgcacg agtgggttac atcgaactgg atctcaacag cggtaagatc cttagaggtt
 2161 ttgccccga agaactgtt ccaatgatga gcactttta agttctgta tgtggcggg
 2221 tattatcccg tttgacgcc gggcaagagc aactcggctc ccgcatacac tatttcaga
 2281 atgacttgtg tgagtactca ccagtcacag aaaagcatct tacggatggc atgacagtaa
 2341 gagaattatg cagtgtgcc ataaccatga gtgataacac tgcggccaac ttactctga
 2401 caacgatcgg aggaccgaag gagctaaccg ctttttgca caacatgggg gatcatgtaa
 2461 ctgccttga tctgtggaa ccggagctga atgaagccat accaaacgac gagcgtgaca
 2521 ccacgatgcc tgcagcaatg gcaacaacgt tgcgcaaact attactggc gaactactta
 2581 ctctagcttc ccggcaaaa ttaatagact ggaaggaggc ggataaagt gcaggaccac
 2641 ttctgcgtc ggccctccg gctggctggt ttattgtga taaatctgga gccggtgagc
 2701 gtgggtctcg cggatcatt gcagcactgg gccagatgg taagccctcc cgtatcgtag
 2761 ttatctacac gacggggagt caggcaacta tggatgaacg aaatagacag atcgtgaga
 2821 taggtgctc actgattaag cattggaac tgcagacca agttactca tatatactt
 2881 agatgatft aaaacttcat tttaattta aaaggatcta ggtgaagatc cttttgata

FIG. 9A

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2941 atctcatgac caaaatccct taacgtgagt ttctgtcca ctgagcgta gaccccgtag
3001 aaaagatcaa aggatcttct tgagatcctt ttttctgog cgtaatctgc tcttgcaaa
3061 caaaaaaacc accgctacca gcggtgggtt gttgcccga tcaagagcta ccaactctt
3121 ttccgaaggt aactggcttc agcagagcgc agataccaaa tactgtcctt ctagttagc
3181 cgtagttagg ccaccacttc aagaactctg tagcaccgcc tacatacctc gctctgctaa
3241 tctgttacc agtggctgct gccagtggcg ataagtcgtg tcttaccggg ttggactcaa
3301 gacgatagtt accggataag gcgcagcggc cgggctgaac gggggggtcg tgcacacagc
3361 ccagcttga gcaacgacc tacaccgaac tgagatacct acagcgtgag cattgagaaa
3421 gcgccacgct tcccgaaggg agaaaggcgg acaggatatcc ggtaagcggc agggtcggaa
3481 caggagagcg cagcaggag cttccagggg gaaacgcctg gtatctttat agtctgtcg
3541 ggttgcgcca cctctgact gagcgtcgat tttgtgatg ctgctcaggg gggcggagcc
3601 tatggaaaaa cgccagcaac gcggccttt tacggttcct ggccttttg tggcctttg
3661 ctacatggt ctttctgog ttatcccctg attctgtgga taaccgtatt accgccttg
3721 agtgagctga taccgctcgc cgcagccgaa cgaccgagcg cagcgagta gtgagcgagg
3781 aagcggaaga gcgcctgatg cggatatttc tccttacgca tctgtcggg atttcacacc
3841 gcatagtcta gactatggtg cactctcagt acaatctgct ctgatgccg atagttaagc
3901 cagtatacac tccgctatcg ctacgtgact gggcatggc tgcgccccga caccgccaa
3961 caccgctga cgcgccctga cgggctgtc tgctcccggc atccgcttac agacaagctg
4021 tgaccgtctc cgggagctgc atgtgtcaga ggtttcacc gtcacaccg aaacgcgcga
4081 ggcagtctag acttaagacc

FIG. 9B