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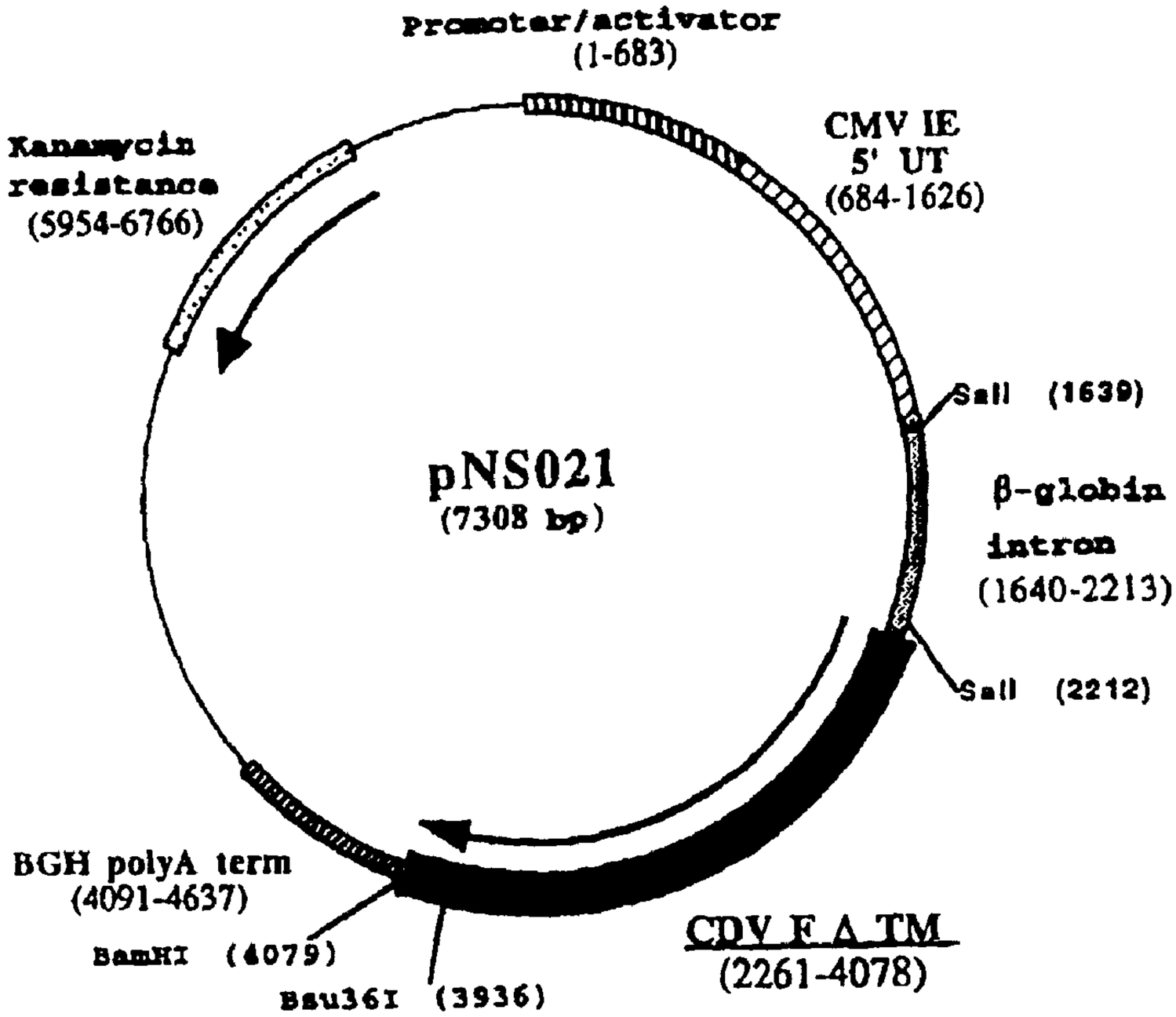

(57) Abrégé/Abstract:
The invention aims at improving the efficacy and protection induced by DNA vaccination against viruses of the family of Paramyxoviridae and against the herpes virus, in pets and sport animals. The improvement of DNA vaccination is achieved either

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(57) Abrégé(suite)/Abstract(continued):
by formulating the vaccine with a cationic lipid containing a quaternary ammonium salt, DMRIE, or by modifications in the nucleotide sequence coding for the antigen of interest in particular of deletions of the fragment of the nucleotide sequence coding for the transmembrane domain of the antigen of interest, and/or insertions of introns and/or insertions of nucleotide sequences coding for the signal peptides, or by adding GM-CSF, or by combinations thereof. The invention also concerns the resulting vaccines.

Abstract

The invention aims at improving the efficacy and protection induced by DNA vaccination against viruses of the family of *Paramyxoviridae* and against the herpes virus, in pets and sport animals. The improvement of DNA vaccination is achieved either by formulating the vaccine with a cationic lipid containing a quaternary ammonium salt, DMRIE, or by modifications in the nucleotide sequence coding for the antigen of interest in particular of deletions of the fragment of the nucleotide sequence coding for the transmembrane domain of the antigen of interest, and/or insertions of introns and/or insertions of nucleotide sequences coding for the signal peptides, or by adding GM-CSF, or by combinations thereof. The invention also concerns the resulting vaccines.

DNA vaccines for pets or for animals used in sports

The present invention relates to improved DNA vaccines for pets or for animals used in sports, in particular dogs, cats and horses.

The use of deoxyribonucleic acid (DNA) molecules for vaccination has been known since the beginning of the 1990s (Wolf *et al.* Science 1990. **247**. 1465-1468). This vaccination technique induces .cellular and humoral immunity after *in vivo* transfection of cells of the subject to be vaccinated with DNA or RNA molecules encoding and expressing immunologically active proteins.

A DNA vaccine is composed of at least one plasmid which may be expressed by the cellular machinery of the subject to be vaccinated and of a pharmaceutically acceptable vehicle or excipient. The nucleotide sequence of this plasmid encodes and expresses, inter alia, one or more immunogens, such as proteins or glycoproteins capable of inducing, in the subject to be vaccinated, a cellular immune response (mobilization of the T lymphocytes) and a humoral immune response (stimulation of the production of antibodies specifically directed against the immunogen) (Davis H.L. Current Opinion Biotech. 1997. **8**. 635-640).

All the immunogens derived from a pathogen are not antigens which are naturally sufficiently effective for inducing an optimum protective immune response in the animal to be vaccinated. It is therefore necessary to improve the immune response.

Each route of administration has its own constraints and difficulties; thus, a DNA vaccine which is effective via one route of administration may be ineffective via another.

The choice of the route of administration should take into account the needs of practitioners and breeders, the difficulties linked to restraining the animals or to the nature of the product.

Although the intramuscular route can be used, the subcutaneous route is of great interest for the vaccination of pets, especially for animals which are small in size and which are difficult to handle.

DNA vaccines must therefore be improved in order to allow their effective administration via various routes.

DNA vaccines have already been used experimentally, in particular a DNA vaccine encoding haemagglutinin (HA) of the measles virus (Etchart *et al*. J. Gen. Virol. 1997. **78**. 1577-1580) whose intranasal administration to mice proved to be more effective than an oral administration. Another example is a DNA vaccine encoding the envelope (Env) protein of the human immunodeficiency virus (HIV) whose subcutaneous administration has not been effective compared to the administration by the intramuscular route (Ishii *et al*. AIDS Res. Hum. Retro. 1997. **13**. 1421-1428).

DNA vaccines have also been used experimentally against animal viruses, in particular against the canine distemper viruses (CDV). Some CDV immunogens are known, in particular the nucleocapsid protein (N), the matrix protein (M), the fusion protein (F) and haemagglutinin (HA) (WO-A-9741236). However, the subcutaneous administration of a DNA vaccine encoding haemagglutinin and the fusion protein of CDV did not make it possible to detect the production of antibodies in mice, and allowed only a small production of antibodies after the intramuscular administration of this DNA vaccine (Sixt *et al*. J. Virol. 1998. **72.** 8472-8476).

The induction of an immune response and the relationships between the various components of the immune system coming into play during this response may be different from one animal species to another. The many teachings taken from experiments carried out on the mouse model have made it possible to better understand the functioning of the immune system in mice, but these teachings are not directly transposable to other species, in particular because it is easier to induce an immune response in mice than in the other species (van Drunen Little-van den Hurk *et al*. J. Gen. Virol. 1998. **79**. 831-839; Böhm *et al*. Vaccine 1998. **16**. 949-954).

Various routes of administration of the DNA vaccine have been proposed (intraperitoneal, intravenous, intramuscular, subcutaneous, intradermal, mucosal, and the like). Various means of administration have also been proposed, in particular gold particles coated with DNA and projected so as to penetrate into the cells of the skin of the subject to be vaccinated (Tang *et al*. Nature 1992. **356**. 152-154) and the liquid jet injectors which make it possible to transfect both skin cells and cells of the underlying tissues (Furth *et al*. Analytical Bioch. 1992. **205**. 365-368).

Chemical compounds have been used for the *in vitro* transfection of DNA:

A/ - cationic lipids.

The cationic lipids are themselves divided into four subgroups.

1) The cationic lipids containing quaternary ammonium salts, such as for example DOTMA (dioleoyloxypropyl-trimethylammonium, produced by Gibco under the name Lipofectine), DOTAP (trimethyl-2,3-(octadec-9-eneoyl-oxy)-1-propaneammonium; Gregoriadis *et al*. FEBS Letters

1997. **402**. 107-110), DMRIE (N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaneammonium; WO-A-9634109), DLRIE (N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propaneammonium; Felgner *et al.* Ann. N Y Acad. Sci. 1995. **772**. 126-139).

These cationic lipids containing quaternary ammonium salts may be combined or otherwise with an additional neutral lipid, such as DOPC (dioleoylphosphatidyl-choline) or DOPE (dioleoylphosphatidylethanolamine) (J.P. Behr, Bioconjugate Chemistry 1994. **5**. 382-389).

2) The lipoamines, such as for example DOGS (dioctadecylamidoglycylspermine, produced by Promega under the name Transfectam; Abdallah *et al.* Biol. Cell. 1995. **85**. 1-7), DC-Chol (dimethylaminoethane-carbamoyl-cholesterol; Gao and Huang, Biochem. Biophys. Res. Commun. 1991. **179**. 280-285), BGSC (bis-guanidine-spermidine-cholesterol), BGTC (bis-guanidine-tren-cholesterol) (Vigneron *et al.* Proc. Natl. Acad. Sci. USA 1996. **93**. 9682-9686).

3) The cationic lipids containing quaternary ammonium salts and lipoamines, such as for example DOSPA (N,N-dimethyl-N-(2-(sperminecarboxamido)ethyl)-2,3-bis-(dioleoyloxy)-1-propaneimidium, pentahydrochloride, marketed by Gibco under the name LipofectAmine®; Hawley-Nelson *et al.* Focus 1993. **15**. 73-79), GAP-DLRIE (N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propaneaminium; Wheeler *et al.* Proc. Natl. Acad. Sci. USA 1996. **93**. 11454-11459; Norman *et al.* Vaccine 1997. **15**. 801-803).

4) The lipids containing amidine salts, such as for example ADPDE, ADODE (Ruysschaert *et al.* Biochem. Biophys. Res. Commun. 1994. **203**. 1622-1628).

B/ - the polymers, such as for example SuperFect™ (molecules of activated dendrimers, produced

by Qiagen; Xu *et al*. Mol. Genet. Metab. 1998. **64**. 193-197), and

C/ - the biochemical agents, such as for example toxins, in particular cholera toxins.

Some of these compounds have also been used in the formulation of DNA vaccines with more than mitigated results. Knowledge in the field of *in vitro* transfection is not transposable to DNA vaccination where the final objective is to ensure a protective immune reaction. Negative effects on the induction of an effective immune protection have even been observed with compounds known to promote transfection *in vitro*. Some formulation chemical compounds are toxic at high doses for the transfected cells.

In the work by Etchart already cited (Etchart *et al*. J. Gen. Virol. 1997. **78**. 1577-1580), the use of DOTAP did not have an adjuvant effect during the administration of the DNA vaccine by the intranasal route, whereas it had an adjuvant effect by the oral route. DOTAP has also been used in DNA vaccines encoding the influenza virus haemagglutinin (HA) on the mouse model which were administered by the intranasal route (Ban *et al*. Vaccine 1997. **15**. 811-813), but the addition of DOTAP inhibited the immune response. The use of DC-Chol or of DOTAP/DOPE in DNA vaccines encoding the hepatitis B virus surface protein (S) on the mouse model which were administered by the intramuscular route made it possible to increase the antibody response, whereas the use of Lipofectine (or DOTMA) did not increase this response (Gregoriadis *et al*. FEBS Letters 1997. **402**. 107-110). DC-Chol/DOPE has also been used in DNA vaccines against the human immunodeficiency virus (HIV, Env protein) on the mouse model, whose administration by the intramuscular route induced a more effective immune response, whereas the administration by the subcutaneous or intradermal route did not increase it

(Ishii *et al*. AIDS Res. Hum. Retro. 1997. **13**. 1421-1428).

Likewise, WO-A-98 40499 proposes preparing nucleic acid + cationic lipid complexes for transfecting the mucosal epithelium in mammals, for gene therapy or the expression of an antigen intended to induce an immune response. This document targets the mucosal route, by inhalation, for example the pulmonary epithelium. It specifies that its results differ from previous studies. It also adds that for the intramuscular (parenteral) route, naked DNA is more effective than a DNA + lipid mixture.

The addition of certain cytokines, in particular interleukins or interferons, can make it possible to enhance the immune response induced in particular by DNA vaccines. Each cytokine triggers a reaction which is specific to it and orients the immune response to a greater or lesser degree towards a cellular response or towards a humoral response (Pasquini *et al*. Immunol. Cell. Biol. 1997. **75**. 397-401; Kim *et al*. J. Interferon Cytokine Res. 1999. **19**. 77-84). The adjuvant effects of a cytokine obtained from a given species are not necessarily the same if the immune context varies, in particular if this cytokine is administered to another species, therefore in a heterologous immune system. The addition of cytokine may also have no adjuvant effect, or may even result in a reversal of the effect sought, that is to say a reduction or an inhibition of the immune response. Thus, a DNA vaccine encoding a single chain of an immunoglobulin fused with GM-CSF does not increase the immune response, whereas direct administration of this fusion protein to mice is effective, in the same way as is the administration of a fusion protein consisting of Fv and of the cytokine IL-1beta or the administration of a DNA vaccine encoding the latter fusion protein (Hakim *et al*. J. Immunol. 1996. **157**. 5503-5511). The use of plasmids co-expressing the

cytokine IL-2 and the hepatitis B virus envelope protein in a fused or nonfused conformation results in an increase in the humoral and cellular immune responses (Chow *et al*. J. Virol. 1997. **71**. 169-78). However, the use of a bicistronic plasmid encoding the human acquired immunodeficiency virus (HIV-1) glycoprotein gp120 and the cytokine IL-2 induced a lower specific anti-gp120 immune response than that obtained by the use of a monocistronic plasmid encoding only gp120 (Barouch *et al*. J. Immunol 1998. **161**. 1875-1882). The co-injection, into mice, of two expression vectors, one coding for the rabies virus G glycoprotein, the other for murine GM-CSF stimulates the activity of the B and T lymphocytes, whereas the co-injection with a plasmid encoding gamma-interferon (in place of murine GM-CSF) results in a decrease in the immune response (Xiang *et al*. Immunity 1995. **2**. 129-135).

Certain modifications in the antigens, such as deletions of part of the nucleotide sequence encoding the antigen, insertions of a DNA fragment into the nucleotide sequence encoding the antigen or into non-translated regions upstream or downstream, can also enhance the efficacy of DNA vaccines, in particular by enhancing the level of expression of the antigen or its presentation.

However in practice, manipulations on the nucleotide sequence encoding the antigen may bring about a reduction or loss of the initial immunological activity. Thus, the deletion of the transmembrane domain from the gene encoding the rabies virus G antigen reduced the level of protection induced in the mouse model after administration by the intramuscular route of a DNA vaccine encoding this modified antigen (Xiang *et al*. Virol. 1995. **209**. 569). The deletion of the transmembrane domain from the gene encoding the bovine herpesvirus (BHV) gD glycoprotein need not make it possible to increase the antibody response and

induced only a partial protection in bovines vaccinated by the intramuscular route (van Drunen Little-van den Hurk *et al.* J. Gen. Virol. 1998. 79. 831-839). The humoral and cellular immune responses and the protection conferred are identical in guinea pigs challenged after having been immunized with the aid of either a DNA vaccine encoding the Ebola virus GP glycoprotein, or of a DNA vaccine encoding this GP glycoprotein but in a secreted form (Xu *et al.* Nature Medicine 1998. 4. 37-42).

The insertion of the signal sequence of the human tissue plasminogen activator (tPA) into the gene encoding the malaria Pf332 antigen did not make it possible to increase the antibody response in mice vaccinated by the intramuscular route (Haddad *et al.* FEMS 1997. 18. 193-202). The addition, in phase, of a tPA sequence to the gene encoding the murine rotavirus VP7 antigen also did not make it possible to increase the antibody response in mice vaccinated by the intradermal route, whereas the fusion protein consisting of the VP4 antigen and tPA allowed this increase, but without inducing an effective protection (Choi *et al.* Virology 1998. 250. 230-240).

The modifications carried out on the nucleotide sequence of one antigen cannot in general be directly transposed to another antigen, because antigens do not always have the same structural arrangements.

The invention desirably provides the enhancement of the efficacy of DNA vaccination. This is in particular to obtain a better immune response and in particular an effective protection in pets and animals used in sports, in particular in dogs, cats and horses, by DNA vaccination, for various routes of administration, and in particular for the subcutaneous route.

The invention desirably provides the production of improved DNA vaccines which induce an effective and protective immune response against the canine distemper virus (CDV), the virus for the respiratory complex or kennel cough (parainfluenza-2 or CPI-2 virus), canine herpesvirus (CHV-1) in dogs.

The invention desirably provides the production of improved DNA vaccines which induce an effective and protective immune response against the feline herpes-virus (FHV-1) in cats.

The invention desirably provides the production of improved DNA vaccines which induce an effective and protective immune response against the equine herpesvirus type 1 (EHV-1), the equine herpesvirus type 4 (EHV-4) in horses.

The invention desirably provides the production of improved DNA vaccines which make it possible to obtain an effective protection in dogs, comprising at least one valency selected from the group consisting of the CDV virus, the CPI-2 virus, the CHV-1 virus, the rabies virus *(rhabdovirus),* the canine parvovirus (CPV), the canine coronavirus (CCV) and *Borrelia burgdorferi.*

The invention desirably provides the production of improved DNA vaccines which make it possible to obtain an effective protection in cats comprising at least one valency selected from the group consisting of the feline herpesvirus (FHV-1), the feline calicivirus (FCV), the rabies virus (rhabdovirus), the feline parvovirus (FPV), the feline infectious peritonitis virus (FIPV), the feline leukemia virus (FeLV), and the feline acquired immunodeficiency syndrome virus (FIV).

The invention desirably provides the production of improved DNA vaccines which make it possible to obtain an effective protection in horses, comprising at least

one valency selected from the group consisting of the equine herpesvirus type 1 (EHV-1), the equine herpesvirus type 4 (EHV-4), the equine influenza virus, the Eastern equine encephalitis virus, the Western equine encephalitis virus, the Venezuelan equine encephalitis virus, the rabies virus, *Clostridium tetani*, and *Borrelia burgdorferi*.

The subject of the invention is improved DNA vaccines which make it possible to obtain an effective protection against at least one pathogen which infects pets or animals used in sports, in particular dogs, cats and horses. The DNA vaccine is improved: either by its formulation, or by the addition of GM-CSF, or by the optimization of the antigen(s), or by combinations of these propositions.

Preferably, the DNA vaccine is improved by its formulation, and optionally either by the addition of GM-CSF, or by the optimization of the antigen(s), or finally by the addition of GM-CSF and by the optimization of the antigen(s).

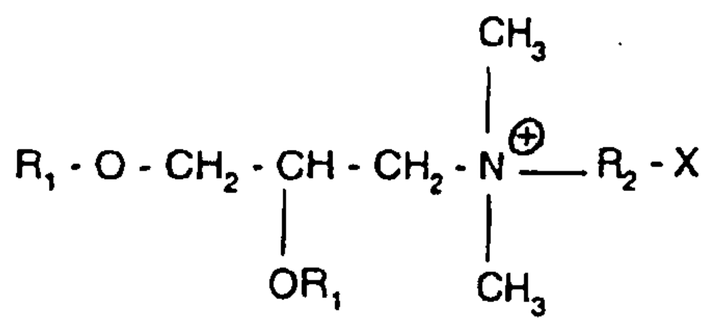
By definition, the DNA vaccine comprises, as active ingredient, a plasmid encoding and expressing a gene or gene fragment. The term plasmid covers a DNA transcription unit comprising a polynucleotide sequence comprising the sequence of the gene to be expressed and the elements necessary for its expression *in vivo*. The circular plasmid form, supercoiled or otherwise, is preferred. The linear form also falls within the scope of this invention.

Each plasmid comprises a promoter capable of ensuring, in the host cells, the expression of the gene inserted under its control. It is in general a strong eukaryotic promoter and in particular a cytomegalovirus early promoter CMV-IE, of human or murine origin, or optionally of other origin such as rat or guinea pig. More

generally, the promoter is either of viral origin or of cellular origin. As a viral promoter other than CMV-IE, there may be mentioned the SV40 virus early or late promoter or the Rous Sarcoma virus LTR promoter. It may also be a promoter from the virus from which the gene is derived, for example the promoter specific to the gene. As cellular promoter, there may be mentioned the promoter of a cytoskeleton gene, such as for example the desmin promoter, or alternatively the actin promoter. When several genes are present in the same plasmid, they may be provided in the same transcription unit or in two different units.

According to a first mode, the DNA vaccines according to the invention are formulated by adding, as adjuvant, cationic lipids containing a quaternary ammonium salt, in particular DMRIE, preferably combined with a neutral lipid, in particular DOPE, to preferably form DMRIE-DOPE.

The subject of the present invention is therefore a DNA vaccine against at least one pathogen affecting pets and animals used in sports, in particular dogs, cats or horses, comprising at least one plasmid containing at least one nucleotide sequence encoding an immunogen of a pathogen of the animal species considered, under conditions allowing the *in vivo* expression of this sequence, and a cationic lipid containing a quaternary ammonium salt, of formula:



in which R_1 is a saturated or unsaturated, linear aliphatic radical having 12 to 18 carbon atoms, R_2 is another aliphatic radical containing 2 or 3 carbon

atoms, and X is a hydroxyl or amine group.

Preferably, it is DMRIE (N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaneammonium; WO-A-9634109), preferably combined with a neutral lipid, in particular DOPE (dioleoylphosphatidylethanol-amine), to form DMRIE-DOPE.

Preferably, the recombinant vector is mixed with this adjuvant immediately before use and it is preferable, before its administration to the animal, to allow the mixture thus prepared to form a complex, for example for a period ranging from 10 to 60 minutes, in particular of the order of 30 minutes.

When DOPE is present, the DMRIE:DOPE molar ratio preferably ranges from 95:5 to 5:95, and is more particularly 1:1.

The plasmid:DMRIE or DMRIE-DOPE adjuvant weight ratio may range in particular from 50:1 to 1:10, in particular from 10:1 to 1:5, preferably from 1:1 to 1:2.

According to a second mode, GM-CSF (granulocyte macrophage-colony stimulating factor; Clark S.C. *et al.* Science 1987. **230**. 1229; Grant S.M. *et al.* Drugs 1992. **53**. 516) is added to the vaccines according to the invention; this may be carried out by incorporating GM-CSF protein directly into the vaccinal composition or preferably by inserting the sequence encoding GM-CSF into an expression vector under conditions allowing its expression *in vivo*. As expression vector, the use of a plasmid, e.g. the plasmid containing the nucleotide sequence encoding the antigen(s) of interest or another plasmid, is preferred. The choice of GM-CSF is made according to the animal species to be vaccinated; thus, for dogs, canine GM-CSF is used; for cats, it is feline GM-CSF, and equine GM-CSF for horses.

According to a third mode, the nucleotide sequence(s) encoding the immunogen are in an optimized form. Optimization is understood to mean any modification of the nucleotide sequence, in particular which manifests itself at least by a higher level of expression of this nucleotide sequence, by an increase in the stability of the messenger RNA encoding this antigen, by the triggered secretion of this antigen into the extracellular medium, and having as direct or indirect consequence an increase in the immune response induced.

In the present invention, the optimization of the antigen of interest preferably consists in the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain of the antigen of interest (deletion is understood to mean the complete deletion or a partial deletion sufficient for the transmembrane domain to no longer, or no longer substantially, be functional), and/or in the addition, in phase, of a nucleotide sequence encoding the tPA (tissue plasminogen activator; Montgomery *et al*. Cell. Mol. Biol. 1997. **43**. 285-292; Harris *et al*. Mol. Biol. Med 1986. **3**. 279-292) signal, and/or in the insertion of a stabilizing intron upstream of the gene to be expressed. The deletion of the DNA fragment encoding the transmembrane domain of the antigen of interest promotes the secretion, into the extracellular medium, of the antigens thus truncated and thus increases the possibilities of their coming into contact with the cells of the immune system. The insertion of the nucleotide sequence encoding the tPA signal facilitates the translatability of the messenger RNA to which the tPA signal is joined, and thus increases the level of expression of this messenger RNA and therefore the production of antigens. The tPA signal also plays a role in the secretion of the antigen synthesized. The insertion of a stabilizing intron into the gene encoding the antigen of interest avoids the aberrant splicings of its messenger RNA and

maintains the physical integrity of the latter.

Preferably, the tPA signal is of human origin. The nucleotide sequence of the human tPA signal is accessible from the GenBank database under the accession number NM_000930. Preferably, the intron is intron II of the rabbit beta-globin gene (van Ooyen *et al.* Science 1979. **206.** 337-344), whose nucleotide sequence is accessible from the GenBank database under the accession number V00882 and designated by a reference under intron No. 2.

The subject of the present invention is an improved DNA vaccine capable of inducing an effective and protective immune response in dogs against canine distemper (Canine Distemper Virus or CDV).

The canine distemper virus is a *Morbillivirus*, a member of the *Paramyxoviridae* family. This virus infects the canine species, but also wild-type felines (Harder *et al.* J. Gen. Virol. 1996. **77**. 397-405).

The present invention makes it possible to obtain an effective and protective DNA vaccine against canine distemper in dogs, in particular by the subcutaneous route which had remained up until now a route inducing a marginal level of protection (Sixt *et al.* J. Virol. 1998. **72**. 8472-8476).

According to the invention, the DNA vaccine against CDV is preferably improved by its formulation with an adjuvant according to the invention, in particular DMRIE, preferably DMRIE-DOPE. Optionally, this may be combined either with the addition of canine GM-CSF (Nash *et al.* Blood 1991. **78**. 50-56), or the optimization of at least one CDV antigen, or finally the addition of canine GM-CSF and the optimization of at least one CDV antigen.

A nucleotide sequence encoding canine GM-CSF is accessible from the GenBank database under the accession number S49738.

The addition of canine GM-CSF may be carried out by the incorporation of the canine GM-CSF polypeptide into the vaccinal composition or preferably by the insertion of the nucleotide sequence encoding the canine GM-CSF into an *in vivo* expression vector, preferably a plasmid. Preferably, the nucleotide sequence encoding canine GM-CSF is inserted into a second expression plasmid, different from that (or those) into which the gene(s) encoding the CDV antigen(s) is(are) inserted.

The optimization of the antigens derived from CDV is carried out by substitution, by a "signal" sequence, in particular that of the tPA signal of human origin (GenBank accession number NM_000930), of the sequence of the signal peptide of haemagglutinin (HA) and/or of the fusion protein (F), and/or by the deletion of the DNA fragment encoding the transmembrane domain of HA and/or of F, and/or by the insertion of an intron, in particular of intron II of the rabbit beta-globin gene (whose nucleotide sequence, noted intron No. 2, is accessible from the GenBank database under the accession number V00882) upstream of the nucleotide sequence encoding HA and/or F. The DNA vaccine against CDV according to the invention can therefore encode and express a single optimized CDV antigen (HA or F) or both, that is to say optimized HA and optimized F.

Optionally, the sequence encoding the CDV matrix protein (M) in its native form (without modification) and/or the nucleotide sequence encoding the CDV nucleoprotein (N) in its native form (without modification) may also be inserted and expressed in a plasmid and combined with the plasmids containing optimized HA and/or optimized F.

Nucleotide sequences encoding the CDV antigens which can be used in the present invention and various constructs of expression vectors are illustrated in the accompanying examples and in WO-A-9803199, in particular in Examples 8 and 9 and Figures 2 and 3.

Preferably, according to the invention, the DNA vaccine against CDV for administration by the intramuscular route is formulated with DMRIE-DOPE, and is composed of an expression plasmid (e.g. pNS024, Figure 4) encoding the CDV HA antigen optimized by replacement of the HA signal sequence with the human tPA signal peptide sequence, by deletion of the fragment of the nucleotide sequence encoding the transmembrane domain of HA and by insertion of intron II of the rabbit beta-globin gene upstream of the HA gene, and of a second expression plasmid (e.g. pNS021, Figure 3) encoding the CDV F antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain and by the insertion of intron II of the rabbit beta-globin gene upstream of the F gene.

Preferably, according to the invention, the DNA vaccine against CDV for administration by the subcutaneous route is formulated with DMRIE-DOPE, and is composed of an expression plasmid encoding canine GM-CSF and the two plasmids previously defined (e.g. pNS024 and pNS021).

The subject of the present invention is also an improved DNA vaccine capable of inducing an effective and protective immune response in dogs against the respiratory complex or kennel cough (canine para-influenza-2 or CPI-2 virus).

The CPI-2 virus is a *Paramyxovirus*, also a member of the *Paramyxoviridae* family (Bittle *et al*. J. Am. Vet. Med. Assoc. 1970. **156**. 1771-1773; Moloney *et al*. Aust Vet J. 1985. **62**. 285-286).

The DNA vaccine against CPI-2 is preferably formulated with an adjuvant according to the invention, in particular DMRIE, preferably DMRIE-DOPE. This may be optionally combined with either the addition of canine GM-CSF, or the optimization of at least one CPI-2 antigen, or finally the addition of canine GM-CSF and the optimization of at least one CPI-2 antigen.

The addition of canine GM-CSF may be carried out as is described for CDV.

The optimization of the antigens derived from CPI-2 is carried out by substitution, by a "signal" sequence, in particular that of the tPA of human origin, of the signal sequence of the haemagglutinin-neuraminidase (HN) of CPI-2 and/or of the fusion protein (F) of CPI-2, and by the deletion of the DNA fragment encoding the transmembrane domain of HN and/or of F, and by the insertion of an intron, in particular intron II of the rabbit beta-globin gene upstream of the nucleotide sequence encoding HN and/or F. The DNA vaccine against CPI-2 according to the invention may therefore encode and express a single optimized CPI-2 antigen (HN or F) or both (HN and F).

Nucleotide sequences encoding the CPI-2 antigens which can be used in the present invention and various expression vector constructs are given in the accompanying examples.

Preferably, according to the invention, the DNA vaccine against CPI-2 for administration by the intramuscular route is formulated with DMRIE-DOPE, and is composed of an expression plasmid (e.g. pSB034, Figure 6) encoding the HN antigen of CPI-2 optimized by the insertion of the signal sequence of the human tPA in place of the signal sequence of HN, by the deletion of the fragment of the nucleotide sequence of HN encoding the trans-

membrane domain and by the insertion of intron II of the rabbit beta-globin gene upstream of HN, and of a second expression plasmid (e.g. pSB032, Figure 5) encoding the F antigen of CPI-2 optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain of F and by the insertion of intron II of the rabbit beta-globin gene upstream of F.

Preferably, according to the invention, the DNA vaccine against CPI-2 for administration by the subcutaneous route is formulated with DMRIE-DOPE, and is composed of an expression plasmid encoding canine GM-CSF, and of the two plasmids previously defined (e.g. pSB034 and pSB032).

The subject of the present invention is also an improved DNA vaccine capable of inducing an effective and protective immune response in dogs against the canine herpesvirus type 1 (CHV-1).

The CHV-1 virus is a member of the *Alphaherpesvirinae* family. This virus is responsible for canine viral rhinotracheitis. Nucleotide sequences encoding the gB, gC and gD glycoproteins are known (Limbach *et al.* J. Gen. Virol. 1994. **75**. 2029-2039).

The DNA vaccine against CHV-1 is preferably formulated with an adjuvant according to the invention, in particular DMRIE, preferably DMRIE-DOPE. This may be optionally combined with either the addition of canine GM-CSF, or the optimization of at least one CHV-1 antigen, or finally the addition of canine GM-CSF and the optimization of at least one CHV-1 antigen.

The addition of canine GM-CSF may be carried out as is described for CDV.

The optimization of the antigens derived from CHV-1 is

carried out by the deletion of the DNA fragment encoding the transmembranc domain of thc gB glycoprotein and/or of the gC glycoprotein and/or of the gD glycoprotein of CHV-1. The improved DNA vaccine against CHV-1 according to the invention may therefore encode and express a single optimized CHV-1 antigen (gB, gC or gD) or two of them or the three.

Nucleotide sequences encoding the CHV-1 antigens which can be used in the present invention and various expression vector constructs are given in the accompanying examples and in WO-A-98/03199, in particular in Examples 7 and 8, and in Figures 13 and 14.

Preferably, according to the invention, the DNA vaccine against CHV-1 for administration by the intramuscular route is formulated with DMRIE-DOPE, and is composed of an expression plasmid (e.g. pSB016, Figure 7) encoding the CHV-1 gB antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain, of a second expression plasmid (e.g. pSB019, Figure 8) encoding the CHV-1 gC antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain and of a third expression plasmid (e.g. pSB017, Figure 9) encoding the CHV-1 gD antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain.

Preferably, according to the invention, the DNA vaccine against CHV-1 for administration by the subcutaneous route is formulated with DMRIE-DOPE, and is composed of an expression plasmid encoding canine GM-CSF, and the three plasmids previously defined (e.g. pSB016, pSB019 and pSB017).

The subject of the present invention is an improved DNA vaccine capable of inducing an effective and protective immune response in cats against the feline herpesvirus

type 1 (FHV-1).

The FHV-1 virus is a member of the *Alphaherpesvirinae* family, this virus is responsible for feline viral rhinotracheitis (Fargeaud *et al*. Arch. Virol. 1984. **80**. 69-82).

The DNA vaccine against FHV-1 is preferably formulated with an adjuvant according to the invention, in particular DMRIE, preferably DMRIE-DOPE. This may be optionally combined either with the addition of feline GM-CSF, or the optimization of at least one FHV-1 antigen, or finally the addition of feline GM-CSF and the optimization of at least one FHV-1 antigen.

The addition of feline GM-CSF may be carried out by the incorporation of the feline GM-CSF polypeptide into the vaccinal composition or by the insertion of a nucleotide sequence encoding the feline GM-CSF (e.g. accessible from the GenBank database under the accession number AF053007) into an *in vivo* expression vector, preferably a plasmid. Preferably, the nucleotide sequence encoding feline GM-CSF is inserted into a second expression plasmid, different from that (or those) into which the gene(s) encoding the FHV-1 antigen(s) is(are) inserted.

The optimization of the antigens derived from FHV-1 is carried out by the deletion of the DNA fragment encoding the transmembrane domains of the gB glycoprotein and/or of the gC glycoprotein and/or of the gD glycoprotein of FHV-1. The improved DNA vaccine against FHV-1 according to the invention may therefore encode and express a single optimized FHV-1 antigen (gB, gC or gD) or two of them or the three.

Nucleotide sequences encoding the FHV-1 antigens which can be used in the present invention and various expression vector constructs are given in the accom-

panying examples and in WO-A-98/03660, in particular in Examples 14 and 15 and in Figures 11 and 12.

Preferably, according to the invention, the DNA vaccine against FHV-1 for administration by the intramuscular route is formulated with DMRIE-DOPE, and is composed of an expression plasmid (e.g. pSB021, Figure 10) encoding the FHV-1 gB antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain, of a second expression plasmid (e.g. pSB023, Figure 11) encoding the FHV-1 gC antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain and of a third expression plasmid (e.g. pSB024, Figure 12) encoding the FHV-1 gD antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain.

Preferably, according to the invention, the DNA vaccine against FHV-1 for administration by the subcutaneous route is formulated with DMRIE-DOPE, and is composed of an expression plasmid encoding feline GM-CSF, and the three plasmids previously defined (e.g. pSB021, pSB023 and pSB024).

The subject of the present invention is an improved DNA vaccine capable of inducing an effective and protective immune response in horses against the equine herpesvirus type 1 (EHV-1).

The EHV-1 virus is a member of the *Alphaherpesvirinae* family. The EHV-1 virus is responsible for equine viral abortion (Crabb *et al.* Adv. Virus Res. 1995. **45**. 153-190). The complete genome of this virus has been determined (Telford *et al.* Virology 1992. **189**. 304-316).

The DNA vaccine against EHV-1 is preferably formulated with an adjuvant according to the invention, in

particular DMRIE, preferably DMRIE-DOPE. This may be optionally combined with either the addition of equine GM-CSF, or the optimization of at least one EHV-1 antigen, or finally the addition of equine GM-CSF and the optimization of at least one EHV-1 antigen.

The addition of equine GM-CSF may be carried out by the incorporation of the equine GM-CSF polypeptide into the vaccinal composition or by the insertion of the nucleotide sequence (e.g. SEQ ID NO 69, Figure 26) encoding equine GM-CSF into an *in vivo* expression vector, preferably a plasmid. Preferably, the nucleotide sequence encoding equine GM-CSF is inserted into a second expression plasmid, different from that (or those) into which the gene(s) encoding the EHV-1 antigen(s) is(are) inserted.

The optimization of the antigens derived from EHV-1 is carried out by the deletion of the DNA fragment encoding the transmembrane domains of the gB glycoprotein and/or of the gC glycoprotein and/or of the gD glycoprotein of EHV-1. The improved DNA vaccine against EHV-1 according to the invention may therefore encode and express a single optimized EHV-1 antigen (gB, gC or gD) or both of them or the three.

Nucleotide sequences encoding the EHV-1 antigens which can be used in the present invention and various expression vector constructs are given in the accompanying examples and in WO-A-98/03198, in particular in Examples 8 and 10, and in Figures 2 and 4.

The intramuscular route is preferred for horses. Preferably, according to the invention, the DNA vaccine against EHV-1 for administration by the intramuscular route is formulated with DMRIE-DOPE, and is composed of an expression plasmid (e.g. pSB028, Figure 13) encoding the EHV-1 gB antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the

transmembrane domain, of a second expression plasmid (e.g. pSB029, Figure 14) encoding the EHV-1 gC antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain and of a third expression plasmid (e.g. pSB030, Figure 15) encoding the EHV-1 gD antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain.

It is possible, however, to use the subcutaneous route. In this case, the DNA vaccine against EHV-1 is preferably formulated with DMRIE-DOPE, and is composed of an expression plasmid encoding equine GM-CSF, and of the three plasmids previously defined (e.g. pSB028, pSB029 and pSB030).

The subject of the present invention is an improved DNA vaccine capable of inducing an effective and protective immune response in horses against the equine herpesvirus type 4 (EHV-4).

The EHV-4 virus is a member of the *Alphaherpesvirinae* family. The EHV-4 virus is responsible for equine viral rhinopneumonia (Crabb *et al.* Adv. Virus Res. 1995. **45.** 153-190). The complete genome of this virus has been determined (Telford *et al.* J. Gen. Virol. 1998. **79.** 1197-1203).

The DNA vaccine against EHV-4 is preferably formulated with an adjuvant according to the invention, in particular DMRIE, preferably DMRIE-DOPE. This may be optionally combined with either the addition of equine GM-CSF, or the optimization of at least one EHV-4 antigen, or finally the addition of equine GM-CSF and the optimization of at least one EHV-4 antigen.

The addition of equine GM-CSF may be made as is described for EHV-1.

The optimization of the antigens derived from EHV-4 is carried out by the deletion of the DNA fragment encoding the transmembrane domain of the glycoprotein gB and/or the glycoprotein gC and/or the glycoprotein gD of EHV-4. The improved DNA vaccine against EHV-4 according to the invention may therefore encode and express a single optimized EHV-4 antigen (gB, gC or gD) or two of them or the three.

Nucleotide sequences encoding the EHV-4 antigens which can be used in the present invention and various expression vector constructs are given in the accompanying examples and in WO-A-98/03198, in particular in Examples 9 and 11, and in Figures 3 and 5.

Preferably, according to the invention, the DNA vaccine against EHV-4 for administration by the intramuscular route is formulated with DMRIE-DOPE, and is composed of an expression plasmid (e.g. pSB025, Figure 16) encoding the EHV-4 gB antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain, of a second expression plasmid (e.g. pSB026, Figure 17) encoding the EHV-4 gC antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain and of a third expression plasmid (e.g. pSB027, Figure 18) encoding the EHV-4 gD antigen optimized by the deletion of the fragment of the nucleotide sequence encoding the transmembrane domain.

For the subcutaneous route, the DNA vaccine against EHV-4 is preferably formulated with DMRIE-DOPE, and is composed of an expression plasmid encoding equine GM-CSF, and the three plasmids previously defined (e.g. pSB025, pSB026 and pSB027).

Although the invention is described in relation to specific DNA vaccines, the invention also applies to DNA vaccines directed against other pathogens of these

animal species. Thus, the various valencies described in this application may be the subject of an improved vaccine, in particular by the addition of the adjuvant of the invention, or of GM-CSF, or optionally gene optimization, or combinations of these propositions, as is described here in detail for some valencies.

In the same line of thought, the vaccines according to the invention may be, for an animal species, combined with one another and/or with DNA vaccines directed against other pathogens of the same species.

Thus, the subject of the present invention is also improved multivalent DNA vaccines which make it possible to obtain an effective protection in dogs against at least two canine pathogens selected from the group consisting of CDV, CPI-2, CHV-1, rabies virus (*rhabdovirus*), canine parvovirus (CPV), canine coronavirus (CCV), *Borrelia burgdorferi*.

The subject of the present invention is also improved multivalent DNA vaccines which make it possible to obtain an effective protection in cats against at least two feline pathogens selected from the group consisting of FHV-1, feline calicivirus (FCV), rabies virus (rhabdovirus), feline parvovirus (FPV), feline infectious peritonitis virus (FIPV), feline leukaemia virus (FeLV), feline acquired immunodeficiency syndrome virus (FIV).

The subject of the present invention is also improved multivalent DNA vaccines which make it possible to obtain an effective protection in horses against at least two equine pathogens selected from the group consisting of EHV-1, EHV-4, equine influenza virus, Eastern equine encephalitis virus, Western equine encephalitis virus, Venezuelan equine encephalitis virus, rabies virus, *Clostridium tetani*, *Borrelia burgdorferi*.

The multivalent DNA vaccines may be improved by their formulation with an adjuvant according to the invention, in particular with DMRIE, preferably with DMRIE-DOPE. This may be optionally combined either with the addition of GM-CSF as previously described, or with the optimization of at least one antigen of interest as previously described, or finally by the addition of GM-CSF and the optimization of at least one antigen of interest.

The improved multivalent DNA vaccines according to the invention are composed of one or more expression plasmids, such that these vaccines lead to the *in vivo* expression of at least one immunogen of a first pathogen and of at least one immunogen of at least one other pathogen, infecting the same animal species. At least one of these immunogens is preferably selected from the members of the following group:

- F of CDV, HA of CDV, F of CPI-2, HN of CPI-2, gB of CHV-1, gC of CHV-1 and gD of CHV-1 for dogs,
- gB of FHV-1, gC of FHV-1 and gD of FHV-1 for cats, and
- gB of EHV-1, gC of EHV-1, gD of EHV-1, gB of EHV-4, gC of EHV-4 and gD of EHV-4 for horses.

The improved monovalent or multivalent DNA vaccines according to the invention may also be combined with at least one conventional vaccine (inactivated, attenuated live, subunits) or recombinant vaccine using an *in vivo* expression vector (e.g. poxvirus, adenovirus, herpesvirus) directed against at least one, in particular different, pathogen infecting the same species.

Persons skilled in the art may refer to WO-A-9803198 for the methods for constructing the plasmids containing these equine valencies, to WO-A-9803660 for the feline valencies and to WO-A-9803199 for the canine

valencies.

The subject of the present invention is also a method of vaccinating pets and animals used in sports, in particular dogs, cats or horses. This vaccination method comprises the administration of one of the monovalent or multivalent improved DNA vaccines as described above. This vaccination method comprises the administration of one or more doses of the improved DNA vaccine.

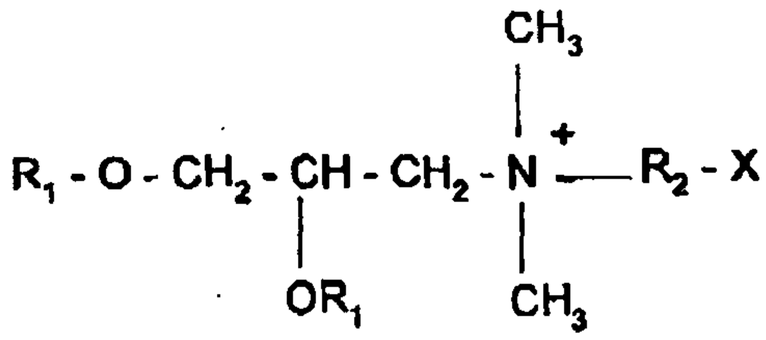
The quantity of DNA used in the vaccines according to the present invention is between about 10 µg and about 1000 µg, and preferably between about 50 µg and about 500 µg, for a given plasmid. Persons skilled in the art possess the competence necessary to precisely define the effective dose of DNA to be used for each vaccination protocol.

The dose volumes may be preferably between 0.5 and 5 ml, preferably between 1 and 3 ml.

The improved DNA vaccines according to the invention may be administered, in the context of this vaccination method, by the various routes of administration proposed in the prior art for polynucleotide vaccination and by means of known techniques of administration.

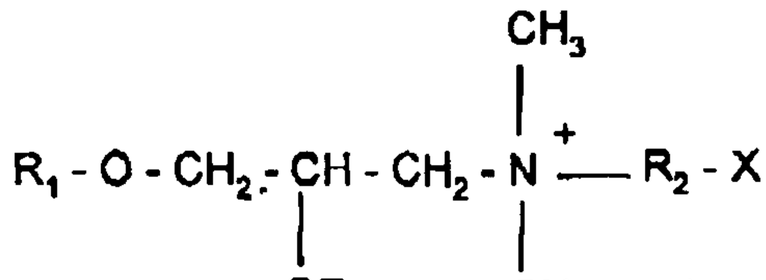
According to the two preferred modes of the invention, the methods of vaccination comprise the administration of the improved DNA vaccines according to the invention by the intramuscular route or by the subcutaneous route.

The present invention provides a DNA vaccine against a pathogen of a host, wherein the host is a dog and the pathogen is canine distemper virus (CDV), comprising at least one DNA plasmid that contains and expresses, in a host cell, a nucleotide sequence encoding an immunogen of the pathogen, and as an adjuvant a cationic lipid containing a quaternary ammonium salt, of formula:



in which R_1 is a saturated or unsaturated linear aliphatic radical having 12 to 18 carbon atoms, R_2 is another aliphatic radical containing 2 or 3 carbon atoms, and X a hydroxyl or amine group.

The present invention also provides an immunogenic composition against a pathogen of a host, wherein the host is a dog and the pathogen is canine distemper virus (CDV), comprising at least one plasmid that contains and expresses, in a host cell, a nucleotide sequence encoding an immunogen of the pathogen, and as an adjuvant a cationic lipid containing a quaternary ammonium salt, of formula:



in which R_1 is a saturated or unsaturated linear aliphatic radical having 12 to 18 carbon atoms, R_2 is another aliphatic radical containing 2 or 3 carbon atoms, and X a hydroxyl or amine group.

The present invention further provides a DNA vaccine against a pathogen of a host, wherein the host is a dog and the pathogen is canine distemper virus (CDV), comprising at least one DNA plasmid that contains and expresses, in a host cell, a nucleotide sequence encoding an immunogen of the pathogen, and as an adjuvant a cationic lipid comprising DMRIE, wherein the vaccine further comprises dioleoylphosphatidylethanolamine (DOPE).

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or

step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

The invention will now be described in greater detail with the aid of embodiments taken as nonlimiting examples and referring to the drawing in which:

Figure No. 1: Plasmid pAB110

Figure No. 2: Plasmid pVR1012
Figure No. 3: Plasmid pNS021
Figure No. 4: Plasmid pNS024
Figure No. 5: Plasmid pSB032
Figure No. 6: Plasmid pSB034
Figure No. 7: Plasmid pSB016
Figure No. 8: Plasmid pSB019
Figure No. 9: Plasmid pSB017
Figure No. 10: Plasmid pSB021
Figure No. 11: Plasmid pSB023
Figure No. 12: Plasmid pSB024
Figure No. 13: Plasmid pSB028
Figure No. 14: Plasmid pSB029
Figure No. 15: Plasmid pSB030
Figure No. 16: Plasmid pSB025
Figure No. 17: Plasmid pSB026
Figure No. 18: Plasmid pSB027
Figure No. 19: Plasmid pJP084
Figure No. 20: sequence of the canine GM-CSF gene
Figure No. 21: Plasmid pJP089
Figure No. 22: sequence of the feline GM-CSF gene 3R3
Figure No. 23: Plasmid pJP090
Figure No. 24: sequence of the feline GM-CSF gene 3R4
Figure No. 25: Plasmid pJP097
Figure No. 26: sequence of the equine GM-CSF gene
Figure No. 27: sequence of the CPI-2 F gene
Figure No. 28: sequence of the CPI-2 HN gene

Sequence listing:

SEQ ID NO 1: oligonucleotide NS030
SEQ ID NO 2: oligonucleotide NS031
SEQ ID NO 3: oligonucleotide NS034
SEQ ID NO 4: oligonucleotide NS035
SEQ ID NO 5: oligonucleotide NS036
SEQ ID NO 6: oligonucleotide NS037
SEQ ID NO 7: oligonucleotide SB090
SEQ ID NO 8: oligonucleotide SB091
SEQ ID NO 9: oligonucleotide PB326
SEQ ID NO 10: oligonucleotide PB329

SEQ ID NO 11: oligonucleotide PB381
SEQ ID NO 12: oligonucleotide PB382
SEQ ID NO 13: oligonucleotide PB383
SEQ ID NO 14: oligonucleotide PB384
SEQ ID NO 15: oligonucleotide SB101
SEQ ID NO 16: oligonucleotide SB102
SEQ ID NO 17: oligonucleotide SB103
SEQ ID NO 18: oligonucleotide SB104
SEQ ID NO 19: oligonucleotide SB105
SEQ ID NO 20: oligonucleotide SB106
SEQ ID NO 21: oligonucleotide SB107
SEQ ID NO 22: oligonucleotide SB108
SEQ ID NO 23: oligonucleotide SB109
SEQ ID NO 24: oligonucleotide SB110
SEQ ID NO 25: oligonucleotide SB111
SEQ ID NO 26: oligonucleotide SB112
SEQ ID NO 27: oligonucleotide SB113
SEQ ID NO 28: oligonucleotide SB114
SEQ ID NO 29: oligonucleotide SB115
SEQ ID NO 30: oligonucleotide SB116
SEQ ID NO 31: oligonucleotide SB117
SEQ ID NO 32: oligonucleotide SB118
SEQ ID NO 33: oligonucleotide AB325
SEQ ID NO 34: oligonucleotide AB326
SEQ ID NO 35: oligonucleotide AB327
SEQ ID NO 36: oligonucleotide AB328
SEQ ID NO 37: oligonucleotide AB329
SEQ ID NO 38: oligonucleotide AB330
SEQ ID NO 39: oligonucleotide NS003
SEQ ID NO 40: oligonucleotide NS004
SEQ ID NO 41: oligonucleotide NS005
SEQ ID NO 42: oligonucleotide NS006
SEQ ID NO 43: oligonucleotide NS007
SEQ ID NO 44: oligonucleotide NS008
SEQ ID NO 45: oligonucleotide SB119
SEQ ID NO 46: oligonucleotide SB120
SEQ ID NO 47: oligonucleotide SB121
SEQ ID NO 48: oligonucleotide SB122
SEQ ID NO 49: oligonucleotide SB123

SEQ ID NO 50: oligonucleotide SB124
SEQ ID NO 51: oligonucleotide SB125
SEQ ID NO 52: oligonucleotide SB126
SEQ ID NO 53: oligonucleotide SB127
SEQ ID NO 54: oligonucleotide SB128
SEQ ID NO 55: oligonucleotide SB129
SEQ ID NO 56: oligonucleotide SB130
SEQ ID NO 57: oligonucleotide SB131
SEQ ID NO 58: oligonucleotide SB132
SEQ ID NO 59: oligonucleotide SB133
SEQ ID NO 60: oligonucleotide SB134
SEQ ID NO 61: oligonucleotide SB135
SEQ ID NO 62: oligonucleotide SB136
SEQ ID NO 63: oligonucleotide SB137
SEQ ID NO 64: oligonucleotide JP578
SEQ ID NO 65: oligonucleotide JP579
SEQ ID NO 66: sequence of the canine GM-CSF gene (see Figure 20)
SEQ ID NO 67: sequence of the feline GM-CSF 3R3 gene (see Figure 22)
SEQ ID NO 68: sequence of the feline GM-CSF 3R4 gene (see Figure 24)
SEQ ID NO 69: sequence of the equine GM-CSF gene (see Figure 26)
SEQ ID NO 70: oligonucleotide JP734
SEQ ID NO 71: oligonucleotide JP735
SEQ ID NO 72: sequence of the CPI-2 F gene (see Figure 27)
SEQ ID NO 73: sequence of the CPI-2 HN gene (see Figure 28)

EXAMPLES:

For each of the pathogens considered, each gene encoding the principal surface antigens (native form and modified form) was the subject of a particular construction in a eukaryotic expression plasmid. The secreted forms of the surface antigens were obtained by deletion of the fragments of genes encoding the

transmembrane and cytoplasmic domains. In all cases, the transmembrane domains of the glycoproteins were identified on the basis of the hydropathy profiles of the corresponding protein sequences. The table given in Example 11 summarizes the sizes of the wild-type proteins (in amino acids), the positions identified in the transmembrane domains, the sizes of the truncated proteins, as well as the names of the corresponding expression plasmids.

Example 1: Basic plasmid constructs

The eukaryotic expression plasmid pVR1020 (C.J. Luke et al. J. of Infectious Diseases 1997, 175: 95-97), derived from the plasmid pVR1012 (Figure 1, Example 7 of WO-A-9803199, repeated in Figure 2 of the present application), contains the coding phase of the signal sequence of the human tissue plasminogen activator (tPA). The plasmid pVR1020 was modified by BamHI-BglII digestion and insertion of a sequence containing several cloning sites (BamHI, NotI, EcoRI, XbaI, PmlI, PstI, BglII) and resulting from the pairing of the following oligonucleotides:

PB326 (40 mer) (SEQ ID NO 9)
5' GATCTGCAGCACGTGTCTAGAGGATATCGAATTCGCGGCC 3' and
PB329 (40 mer) (SEQ ID NO 10)
5' GATCCGCGGCCGCGAATTCGATATCCTCTAGACACGTGCT 3'.

The resulting vector, pAB110 (Figure No. 1), was used for the construction of the plasmids containing the truncated forms of the genes encoding the canine distemper virus (CDV) haemagglutinin (HA) and the parainfluenza virus type 2 (CPI-2) haemagglutinin-neuraminidase (HN).

Intron II of the rabbit β-globin gene was cloned into the vector pCRII (Invitrogen, Carlsbad, CA, USA) after production of the corresponding DNA fragment by PCR

with the aid of the following oligonucleotides:

SB090 (20 mer) (SEQ ID NO 7)
5' TTGGGGACCCTTGATTGTTC 3' and
SB091 (21 mer) (SEQ ID NO 8)
5' CTGTAGGAAAAAGAAGAAGGC 3'
using as template the genomic DNA of rabbit peripheral blood cells. The resulting plasmid was designated pNS050.

Example 2: Plasmids encoding the various forms of the CDV antigens

The genes encoding the fusion protein (F) and the haemagglutinin (HA) of CDV strain Snyder Hill (SH) were obtained by RT-PCR from the viral RNA of the SH strain (accessible from the strain depository American Tissue Culture Collection under the number ATCC VR-526).

2.1. Plasmids encoding the various forms of CDV-F

2.1.1. pPB229: F gene (native form) cloned into the vector pVR1012

The cDNA of the CDV F gene was synthesized with the aid of the primer PB383 and amplified by a PCR reaction with the aid of the following pair of oligonucleotides:

PB383 (26 mer) (SEQ ID NO 13)
5' TTTCTAGACAGCCGAGCCCCATGCAC 3' and
PB384 (30 mer) (SEQ ID NO 14)
5' TTGGATCCGATATATGACCAGAATACTTCA 3'.

The PCR product was digested with BamHI and XbaI and cloned into the expression vector pVR1012 (Example 1) previously digested with BamHI and XbaI, generating the plasmid pPB229 (6925 base pairs or bp). The CDV wild-type F gene encodes a protein of 662 residues.

2.1.2. pNS021: F gene (form β-globin F ΔTM) cloned into the vector pVR1012

The plasmid pNS013 (6735 bp) containing the F gene truncated of the transmembrane and C-terminal domain was obtained by ligation of a Bsu36I-BamHI fragment (6593 bp) derived from the plasmid pPB229 (Example 2.1.1), and of a 142-bp fragment obtained by PCR from the template pPB229 with the aid of the following oligonucleotides:

NS030 (21 mer) (SEQ ID NO 1)
5' ATGAGCCCACTCTTACAACAA 3' and
NS031 (35 mer) (SEQ ID NO 2)
5' TTTCGCGGATCCATTAAAGGAAGAGCGCCTAACCG 3'
and Bsu36I-BamHI digested. The CDV truncated F gene encodes a protein of 605 residues.

In a second instance, a sequence corresponding to intron II of the rabbit β-globin gene was inserted upstream of the coding sequence of the truncated F gene, into the SalI site of the plasmid pNS013. The DNA fragment corresponding to the intron (573 bp) was obtained by PCR with the aid of the following oligonucleotides:

NS036 (34 mer) (SEQ ID NO 5)
5' TTTACGCGTCGACTTGGGGACCCTTGATTGTTC 3' and
NS037 (36 mer) (SEQ ID NO 6)
5' TTTACGCGTCGACCTGTAGGAAAAAGAAGAAGGCAT 3'
on the template pNS050 (Example 1), followed by SalI digestion to release SalI compatible ends. The derivative of the plasmid pNS013 containing intron II of the β-globin gene is designated pNS021 (7308 bp) (Figure No. 3).

2.2. Plasmids encoding the various forms of CDV-HA

2.2.1. pNS018: HA gene (native form) cloned into the vector pVR1012

The cDNA of the CDV HA gene was synthesized with the aid of the primer PB381 and amplified by PCR reaction with the aid of the following pair of oligonucleotides:

PB381 (30 mer) (SEQ ID NO 11)
5' TTCTGCAGATGCTCTCCTACCAAGAYAAGG 3' and
PB382 (28 mer) (SEQ ID NO 12)
5' TTGTCGACATGTGTATCATCATMCTGTC 3'.

The PCR product was cloned into the vector pCRII (Invitrogen, Carlsbad, CA, USA), generating the plasmid pPB235. The PstI-SalI fragment of 1846 bp of the plasmid pPB235 containing the HA gene was then cloned into the PstI-SalI digested expression vector pVR1012 (Example 1), generating the plasmid pNS018 (6748 bp). The CDV wild-type HA gene encodes a protein of 607 residues.

2.2.2. pNS024: HA gene (form β-globin tPA ΔTM HA) cloned into the vector pVR1012

The truncated form of the CDV HA gene was obtained by deletion of the DNA fragment encoding the first 60 residues of the HA protein. The signal sequence and the transmembrane sequence of this protein being indistinguishable, the secretion of the truncated product is ensured by the production of a fusion in phase between the signal sequence of the human tissue plasminogen activator (tPA) and the truncated HA gene. The plasmid pNS019, the derivative of pNS018 encoding a fusion product with the tPA signal, was obtained by the ligation of the following 3 DNA fragments:

- Fragment A was obtained by BamHI-EcoRV digestion of

pAB110 (Example 1).

- Fragment B was obtained by PCR reaction on the template pNS018 (Example 2.2.1) with the aid of the following oligonucleotides:

NS034 (30 mer) (SEQ ID NO 3)
5' TTTCGCGGATCCCACAAAGTATCAACTAGC 3' and
NS035 (23 mer) (SEQ ID NO 4)
5' GGGATTTGCTGCCGATGCAATAG 3'
followed by a BamHI-SapI digestion of the PCR product.

- Fragment C is a fragment of SapI-EcoRV digestion of pNS018.

The hybrid gene tPA ΔTM HA encodes a protein of 574 residues (1725 bp).

Intron II of the rabbit β-globin gene (Example 2.1.2) was inserted upstream of the coding frame of the HA gene into the SalI site of pNS019 to generate the plasmid pNS024 (Figure No. 4).

Example 3: Plasmids encoding the various forms of the antigens of the canine parainfluenza virus type 2 (CPI-2)

The F and HN genes of CPI-2 strain D008 (MERIAL) were obtained by RT-PCR from the viral RNA.

3.1. Plasmids encoding the various forms of CPI-2 F

3.1.1. pAB115: F gene (native form) cloned into the vector pVR1012

The cDNA of the CPI-2 F gene was synthesized and amplified by RT-PCR with the aid of the following pair of oligonucleotides:

SB131 (38 mer) (SEQ ID NO 57)

5' AAAAACGCGTCGACATGGGTACTATAATTCAATTTCTG 3'

SB132 (38 mer) (SEQ ID NO 58)

5' TTTTCTAGTCTAGATTATTTATGATAAACAAAATTCTC 3'.

The PCR product was digested with SalI and XbaI, generating a fragment of 1594 bp, and cloned into the expression vector pVR1012 (Example 1) previously digested with the same enzymes, generating the plasmid pAB115 (6479 bp). The CPI-2 wild-type F gene (SEQ ID NO 72) (Figure 27) cloned into this plasmid encodes a protein of 529 residues.

3.1.2. pSB032: F gene (form β-globin F ΔTM) cloned into the vector pVR1012

The plasmid pSB031 containing the F gene truncated of the transmembrane and C-terminal cytoplasmic domains was obtained in the following manner. A PCR reaction was carried out with the template pAB115 (Example 3.1.1.) with the aid of the following oligonucleotides:

SB131 (SEQ ID NO 57) and

SB133 (41 mer) (SEQ ID NO 59)

5' TTTTCTAGTCTAGATTAGTATGTGTCACTTTGTGCTAAGTG 3'

to generate a PCR fragment of about 1450 bp. This fragment was digested with SalI and XbaI in order to isolate the SalI-XbaI restriction fragment of 1436 bp. This fragment was ligated into the vector pVR1012 (Example 1) previously digested with SalI and XbaI to give the plasmid pSB031. The CPI-2 truncated F gene encodes a protein of 473 residues.

In a second instance, a sequence corresponding to intron II of the rabbit β-globin gene was inserted upstream of the coding sequence of the CPI-2 truncated F gene, into the SalI site of the plasmid pSB031. The DNA fragment corresponding to the intron (573 bp) was obtained by PCR with the aid of the oligonucleotides

NS036 (SEQ ID NO 5) and NS037 (SEQ ID NO 6) on the template pNS050 (Example 1), followed by SalI digestion to release the SalI compatible ends. This SalI-SalI restriction fragment was ligated into the plasmid pSB031 previously digested with SalI, and then dephosphorylated to give the plasmid pSB032 (6884 bp) (Figure No. 5).

3.2. Plasmids encoding the various forms of CPI-2 HN

3.2.1. pAB114: HN gene (native form) cloned into the vector pVR1012

The cDNA of the CPI-2 HN gene was synthesized and amplified with the aid of the following oligonucleotides:

SB134 (41 mer) (SEQ ID NO 60)
5' AAAAACGCGTCGACATGGTTGCAGAAGATGCCCCTGTTAGG 3'
SB135 (35 mer) (SEQ ID NO 61)
5' TTTTGGAAGATCTTTAGGATAGTGTCACCTGACGG 3'
in order to generate a PCR fragment of about 1720 bp. This fragment was digested with SalI and BglII in order to isolate the SalI-BglI fragment of 1704 bp. This fragment was then ligated into the vector pVR1012 (Example 1), previously digested with SalI and BglII, to give the plasmid pAB114 (6566 bp). The CPI-2 wild-type HN gene (SEQ ID NO 73) (Figure 28) cloned into this plasmid encodes a protein of 565 residues.

3.2.2. pSB034: HN gene (form β-globin HN tPA ΔTM) cloned into the vector pVR1012

The truncated form of the CPI-2 HN gene was obtained by deletion of the DNA fragment encoding the first 40 residues of the HN protein. The signal and trans-membrane sequences of this protein being indistinguishable, the secretion of the truncated protein is ensured by the production of a fusion in phase between

the signal sequence of the human tissue plasminogen activator (tPA) and the truncated HN gene. The plasmid pSB033, derived from pAB114 (Example 3.2.1), encoding a fusion product with tPA, was obtained by ligation of the EcoRI-PmlI fragment of pAB110 (Example 1), a derivative of pVR1012 containing an open reading frame encoding the tPA signal sequence and of a fragment of (1599 bp) obtained by PCR with the aid of the following oligonucleotides:

SB136 (37 mer) (SEQ ID NO 62)
5' TTAAAAGAATTCGACCCAAAAGCAAATCATGAGCCAC 3'
SB137 (33 mer) (SEQ ID NO 63)
5' TTAAAAGGCCTTTAGGATAGTGTCACCTGACGG 3'
on the template pAB114 and digested with EcoRI and EcoRV.

Intron II of the rabbit β-globin gene was inserted upstream of the coding frame of the HN gene into the SalI site of pSB033 to generate the plasmid pSB034 (Figure No. 6). The SalI fragment containing the intron was obtained by PCR with the aid of the oligonucleotides NS036 (SEQ ID NO 5) and NS037 (SEQ ID NO 6) on the template pNS050 (Example 1).

Example 4: Plasmids encoding the various forms of the CHV-1 virus glycoproteins

The genes encoding the glycoproteins gB, gC and gD of the Carmichael strain of the canine herpesvirus type I (CHV-1) were isolated by PCR from the viral genome. The cloning of the genes encoding gB and gD into the vector pVR1012 was previously described in patent application WO-A-9803199 (plasmids pAB037 and pAB038, respectively in Figures 7 and 8 and in Examples 13 and 14). On the other hand, the cloning of the gene encoding gC is described in this document.

4.1. Plasmid encoding the truncated form of CHV-gB

4.1.1. pSB016: gB gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the CHV-1 gB protein (878 amino acids) is positioned between residues 702 and 769. The plasmid containing the truncated form of the gene encoding gB was obtained by ligation of the following three DNA fragments: (a) the vector pVR1012 (Example 1) linearized by a double PstI-XbaI digestion, (b) a fragment of 1997 bp obtained by PstI-NsiI digestion of pAB037 (Example 4) and (c) a fragment of 225 bp obtained by PCR with the aid of the following oligonucleotides:

SB101 (22 mer) (SEQ ID NO 15)
5' TATATTGAAGGACAACTTGGGG 3' and
SB102 (36 mer) (SEQ ID NO 16)
5' CTAGTCTAGATTAATTATTATCAACTTTTACAACAC 3'
using the plasmid pAB037 as template and digested with NsiI and XbaI. The resulting plasmid, pSB016 (6983 bp) (Figure No. 7) contains a truncated gB gene encoding a protein of 701 residues.

4.2. Plasmids encoding the various forms of CHV-gC

4.2.1. pSB018: gC gene (native form) cloned into the vector pVR1012

The DNA fragment containing the open reading frame of the CHV-1 gC gene was obtained by PCR with the aid of the following oligonucleotides:

SB105 (32 mer) (SEQ ID NO 19)
5' AAAACTGCAGATGAGTTTTAAAAATTTTTATC 3' and
SB106 (30 mer) (SEQ ID NO 20)
5' CTAGTCTAGATTAGATCTTATTATTTTTTG 3'
using the viral DNA as template. This PCR product was

digested with PstI and XbaI generating a fragment of 1400 bp, which was then ligated into the vector pVR1012 (Example 1) linearized by the same double digestion. The resulting plasmid, pSB018 (6253 bp), contains the gene encoding the gC glycoprotein of 459 residues.

4.2.2. pSB019: gC gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the gC protein is between residues 422 and 452. The plasmid containing the truncated form of the gene encoding gD was obtained by ligation of the following three DNA fragments: (a) the vector pVR1012 (Example 1) linearized by a double PstI-XbaI digestion, (b) a fragment of 934 bp obtained by PstI-StuI digestion of pSB018 (preceding example) and (c) a fragment of 335 bp obtained by PCR with the aid of the following oligonucleotides:

SB107 (24 mer) (SEQ ID NO 21)
5' TGGATTGACGGTCTTATAACAGGC 3' and
SB108 (37 mer) (SEQ ID NO 22)
5' CTAGTCTAGATTAATTTTCATCCGATGCATCAAACAC 3'

using the plasmid pSB018 as template and digested with StuI and XbaI. The resulting plasmid, pSB019 (6139 bp) (Figure No. 8), contains a truncated gC gene encoding a protein of 421 residues.

4.3. Plasmid encoding the truncated form of CHV-gD

4.3.1. pSB017: gD gene (ΔTM form) cloned into the vector pVR1012

The transmembrane domain of the CHV-1 gD protein (345 amino acids) is between residues 310 and 328. The plasmid containing the truncated form of the gene encoding gD was obtained by ligation of the following three DNA fragments: (a) the vector pVR1012 (Example 1)

linearized by a double PstI-NotI digestion, (b) a fragment of 663 bp obtained by PstI-AvaII digestion of pAB038 (Example 4) and (c) a fragment of 415 bp obtained by PCR with the aid of the following oligonucleotides:

SB103 (25 mer) (SEQ ID NO 17)
5' CGAGAAACTTGTTATTTTTCTAAAG 3' and
SB104 (51 mer) (SEQ ID NO 18)
5'ATAAGAATGCGGCCGCAAAGGCTATATATTTTTTGGGGTATTATTTATTGG 3'
using the plasmid pAB038 as template and digested with AvaII and NotI. The resulting plasmid, pSB017 (5819 bp) (Figure No. 9), contains a truncated gD gene encoding a protein of 309 residues.

Example 5: Plasmids encoding the various forms of the FHV-1 glycoproteins

The genes encoding the glycoproteins gB, gC and gD of the CO strain of the feline herpesvirus type 1 (FHV-1) were isolated by PCR from the viral genome. In the specific case of the gene encoding gD, whose nucleotide sequence is identical to that of the C-27 strain, we used the plasmid pAB029, derived from pVR1012 containing the corresponding gene cloned from strain C-27 and described in patent application WO-A-9803660 (plasmid pAB029, Figure 12 and Example 15).

5.1. Plasmids encoding the various forms of FHV-gB

5.1.1. pSB020: gB gene (native form) cloned into the vector pVR1012

The DNA fragment containing the open reading frame of the FHV-1 gB gene was obtained by PCR with the aid of the following oligonucleotides:

SB113 (34 mer) (SEQ ID NO 27)
5'TTTTCTGCAGATGTCCACTCGTGGCGATCTTGGG 3' and

SB114 (40 mer) (SEQ ID NO 28)
5'ATAGTTTAGCGGCCGCTTAGACAAGATTTGTTTCAGTATC 3'
using the viral DNA as template. The PCR product was digested with PstI and NotI, generating a fragment of 2849 bp, which was then ligated into the vector pVR1012 (Example 1) linearized by the same double digestion. The resulting plasmid, pSB020 (7728 bp), contains the gene encoding the gB glycoprotein of 949 residues.

5.1.2. pSB021: gB gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the FHV-1 gB protein is situated between residues 761 and 834. The plasmid containing the truncated form of the gene encoding gB was obtained by ligation of the following three DNA fragments: (a) the vector pVR1012 (Example 1) linearized by a double PstI-NotI digestion, (b) a fragment of 1839 bp obtained by PstI-HindII digestion of pSB020 (preceding example) and (c) a fragment of 447 bp obtained by PCR with the aid of the following oligonucleotides:

SB109 (24 mer) (SEQ ID NO 23)
5' CTGTGGACAGAGACCCTAAAACTC 3' and
SB110 (50 mer) (SEQ ID NO 24)
5'TTTCCTTTTGCGGCCGCTTATATGCTGTCTATATCATAAAATTTTAAGGC 3'
using the plasmid pSB020 as template and digested with HindII and NotI. The resulting plasmid, pSB021 (7164 bp) (Figure No. 10), contains a truncated gB gene encoding a protein of 760 residues.

5.2. Plasmids encoding the various forms of FHV-gC

5.2.1. pSB022: gC gene (native form) cloned into the vector pVR1012

The DNA fragment containing the open reading frame of the FHV-1 gC gene was obtained by PCR with the aid of

the following oligonucleotides:

SB115 (34 mer) (SEQ ID NO 29)
5'TTTTCTGCAGATGAGACGATATAGGATGGGACGC 3' and
SB116 (34 mer) (SEQ ID NO 30)
5'AGTTTAGCGGCCGCTTATAATCGCCGGGGATGAG 3'
using the viral DNA as template. This PCR product was digested with PstI and NotI, generating a fragment of 1605 bp, which was then ligated into the vector pVR1012 (Example 1) linearized by the same double digestion. The resulting plasmid, pSB022 (6483 bp), contains the gene encoding the gC glycoprotein of 534 residues.

5.2.2. pSB023: gC gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the FHV-1 gC protein is between residues 495 and 526. The plasmid containing the truncated form of the gene encoding gC was obtained by ligation of the following two DNA fragments: (a) a fragment of 6198 bp obtained by BclI and NotI digestion of pSB022 (preceding example) and (b) a fragment of 168 bp obtained by PCR with the aid of the following oligonucleotides:

SB117 (24 mer) (SEQ ID NO 31)
5'GTTAAATGTGTACCACGGGACGGG 3' and
SB118 (41 mer) (SEQ ID NO 32)
5'AGTTTAGCGGCCGCTTATTCAGGGGACGCGTCGTAGACTTG 3'
using the plasmid pSB022 as template and digested with BclI and NotI. The resulting plasmid, pSB023 (6366 bp) (Figure No. 11), contains a truncated gC gene encoding a protein of 494 residues.

5.3. Plasmid encoding the truncated form of FHV-gD

5.3.1. pSB024: gD gene (ΔTM form) cloned into the vector pVR1012

The transmembrane domain of the FHV-1 gD protein (374 amino acids) is between residues 328 and 353. The plasmid containing the truncated form of the gene encoding gD was obtained by ligation of the following two DNA fragments: (a) a fragment of 5712 bp obtained by XbaI-BglII digestion of pAB029 (Example 5) and (b) a fragment of 129 bp obtained by PCR with the aid of the following oligonucleotides:

SB111 (24 mer) (SEQ ID NO 25)
5'GATCGTCCCGCCATACCGTCTGGG 3' and
SB112 (39 mer) (SEQ ID NO 26)
5'TTTGGAAGATCTTTACTGATTATTCATGCCCTTGGGAGG 3'
using the plasmid pAB029 as template and digested with XbaI and BglII. The resulting plasmid, pSB024 (5841 bp) (Figure No. 12), contains a truncated gD gene encoding a protein of 327 residues.

Example 6: Plasmids encoding the various forms of the EHV-1 glycoproteins

The genes encoding the glycoproteins gB, gC and gD of the 2234/88-2 strain of EHV-1 were isolated by PCR from the purified viral DNA.

6.1. Plasmids encoding the various forms of EHV-1 gB

6.1.1. pAB127: gB gene (native form) cloned into the vector pVR1012

The coding frame of the EHV-1 gB gene was amplified by PCR with the aid of the following oligonucleotides:

NS003 (30 mer) (SEQ ID NO 39)

5'TTCTGCAGATGTCCTCTGGTTGCCGTTCGT 3' and
NS004 (30 mer) (SEQ ID NO 40)
5' TTTCTAGATTAAACCATTTTTTCATTTTCC 3',
the DNA fragment obtained was digested with PstI and XbaI and ligated into the vector pVR1012 (Example 1) linearized with PstI and XbaI, generating the plasmid pAB127 (7818 bp). The gB gene encodes a protein of 980 amino acids.

6.1.2. pSB028: gB gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the EHV-1 gB protein is positioned between residues 801 and 875. The plasmid containing the truncated form of the gene encoding gB was obtained by ligation of the following two DNA fragments: (a) the plasmid pAB127 (Example 6.1.1) digested with AfeI and XbaI, and (b) a fragment of 276 bp obtained by PCR with the aid of the following oligonucleotides:

SB125 (24 mer) (SEQ ID NO 51)
5' AACAACAGAGGGTCGATAGAAGGC 3' and
SB126 (39 mer) (SEQ ID NO 52)
5'AATTTTTCTAGATTACACGTTGACCACGCTGTCGATGTC 3'
using the plasmid pAB127 as template and digested with AfeI and XbaI. The resulting plasmid, pSB028 (7279 bp) contains a truncated EHV-1 gB gene encoding a protein of 800 residues.

6.2. Plasmids encoding the various forms of EHV-1 gC

6.2.1. pAB129: gC gene (native form) cloned into the vector pVR1012

The DNA fragment containing the open reading frame of the EHV-1 gC gene was obtained by PCR with the aid of the following oligonucleotides:

NS005 (31 mer) (SEQ ID NO 41)
5' TTGTCGACATGTGGTTGCCTAATCTCGTGAG 3' and
NS006 (33 mer) (SEQ ID NO 42)
5' TTGGATCCCTAAAAGTCAGACTTCTTGTACGGC 3'.

This PCR product was digested with SalI and BamHI generating a fragment of 1412 bp, which was then ligated into the vector pVR1012 (Example 1) linearized by the same double digestion. The resulting plasmid, pAB129 (6281 bp), contains the gene encoding the EHV-1 gC glycoprotein and having a size of 468 residues.

6.2.2. pSB029: gC gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the EHV-1 gC protein is between residues 429 and 455. The plasmid containing the truncated form of the gene encoding gC was obtained by ligation of the following two DNA fragments: (a) the plasmid pAB129 (Example 6.2.1.) linearized by a double AspI-BamHI digestion and (b) a fragment of 287 bp obtained by PCR with the aid of the following oligonucleotides:

SB127 (24 mer) (SEQ ID NO 53)
5' GATCCGGAGGAGGAATACACACCC 3' and
SB128 (39 mer) (SEQ ID NO 54)
5' AATTTTGGATCCCTAAACCGGCCTGTCCTCAACAATCGG 3'
using the plasmid pAB129 as template and digested with AspI and BamHI. The resulting plasmid, pSB029 (6161 bp), contains a truncated gC gene encoding a protein of 428 residues.

6.3. Plasmids encoding the truncated form of EHV-1 gD

6.3.1. pAB131: gD gene (native form) cloned into the vector pVR1012

The DNA fragment containing the open reading frame of

the EHV-1 gD gene was obtained by PCR with the aid of the following oligonucleotides:

NS007 (33 mer) (SEQ ID NO 43)
5' TTGTCGACATGTCTACCTTCAAGCTTATGATGG 3' and
NS008 (32 mer) (SEQ ID NO 44)
5' TTGGATCCTTACGGAAGCTGGGTATATTTAAC 3'.

This PCR product was digested with SalI and BamHI generating a fragment of 1214 bp, which was then ligated into the vector pVR1012 (Example 1) linearized by the same double digestion. The resulting plasmid, pAB138 (6083 bp) contains the gene encoding the gD glycoprotein of 402 residues.

6.3.2. pSB030: gD gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the EHV-1 gD protein is between residues 348 and 371. The plasmid containing the truncated form of the gene encoding gD was obtained by ligation of the following three DNA fragments: (a) the plasmid pVR1012 (Example 1) linearized by a double SalI-BamHI digestion, (b) the fragment of 825 bp derived from the digestion of pAB131 (Example 6.3.1.) with SalI and BsmI and (c) a fragment of 239 bp obtained by PCR with the aid of the following oligonucleotides:

SB129 (24 mer) (SEQ ID NO 55)
5' CGGTTTCTTGGTGAATTCAACTTC 3' and
SB130 (42 mer) (SEQ ID NO 56)
5' AATTTTGGATCCTTACGTAGAGTTGCTCTTAGACGTTTTTGG 3'
using the plasmid pAB131 as template and digested with BsmI and BamHI. The resulting plasmid pSB030 (5921 bp), contains a truncated gD gene encoding a protein of 347 residues.

Example 7: Plasmids encoding the various forms of the EHV-4 glycoproteins

The genes encoding the gB, gC and gD glycoproteins of the KYT445/2 strain of EHV-4 were isolated by PCR from the purified viral DNA.

7.1. Plasmids encoding the various forms of EHV-4 gB

7.1.1. pAB136: gB gene (native form) cloned into the vector pVR1012

The coding frame of the EHV-4 gB gene was amplified by PCR with the aid of the following oligonucleotides:

AB325 (35 mer) (SEQ ID NO 33)
5' TTTCTGCAGATGTCCACTTGTTGCCGTGCTATTTG 3' and
AB326 (31 mer) (SEQ ID NO 34)
5' TTTTCTAGATTAAACCATTTTTTCGCTTTCC 3'
the DNA fragment obtained was digested with PstI and XbaI and ligated into the vector pVR1012 (Example 1) linearized with PstI and XbaI, generating the plasmid pAB136 (7801 bp). The EHV-4 gB gene encodes a protein of 975 amino acids.

7.1.2. pSB025: gB gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the EHV-4 gB protein is positioned between residues 797 and 867. The plasmid containing the truncated form of the gene encoding gB was obtained by the ligation of the following two DNA fragments: (a) the plasmid pAB136 (Example 7.1.1.) digested with SplI and XbaI, and (b) a fragment of 231 bp obtained by PCR with the aid of the following oligonucleotides:

SB119 (36 mer) (SEQ ID NO 45)
5' TTTTGGTCTAGATTAGTCCACGTTGACAACGCTGTC 3' and

SB120 (23 mer) (SEQ ID NO 46)
5' CGCAAGCTTATCGAGCCGTGCGC 3'
using the plasmid pAB136 as template and digested with SplI and XbaI. The resulting plasmid, pSB025 (7264 bp), contains a truncated gB gene encoding a protein of 796 residues.

7.2. Plasmids encoding the various forms of EHV-4 gC

7.2.1. pAB137: gC gene (native form) cloned into the vector pVR1012

The DNA fragment containing the open reading frame of the EHV-4 gC gene was obtained by PCR with the aid of the following oligonucleotides:

AB327 (32 mer) (SEQ ID NO 35)
5' TTTGTCGACATGGGTTTGGTAAATATAATGCG 3' and
AB328 (33 mer) (SEQ ID NO 36)
5' TTTGGATCCTTAGAAGTCTGCTTTCTTGTAGGG 3'.

This PCR product was digested with SalI and BamHI generating a fragment of 1463 bp, which was then ligated into the vector pVR1012 (Example 1) linearized by the same double digestion. The resulting plasmid, pAB137 (6330 bp), contains the gene encoding the gC glycoprotein of 485 residues.

7.2.2. pSB026: gC gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the EHV-4 gC protein is between residues 425 and 472. The plasmid containing the truncated form of the gene encoding gC was obtained by ligation of the following two DNA fragments (a) the plasmid pAB137 (Example 7.2.1.) linearized by a double AclI-BamHI digestion and (b) a fragment of 237 bp obtained by PCR with the aid of the following oligonucleotides:

SB121 (24 mer) (SEQ ID NO 47)
5' GTATCAATCCCAGCTGACCCCGAC 3' and
SB122 (41 mer) (SEQ ID NO 48)
5' AATTTTGGATCCTTAGCCGTCCGGGTAACCCTCTATGATGC 3'
using the plasmid pAB137 as template and digested with AclI and BamHI. The resulting plasmid, pSB026 (6147 bp), contains a truncated gC gene encoding a protein of 424 residues.

7.3. Plasmids encoding the truncated form of EHV-4 gD

7.3.1. pAB138: gD gene (native form) cloned into the vector pVR1012

The DNA fragment containing the open reading frame of the EHV-4 gD gene was obtained by PCR with the aid of the following oligonucleotides:

AB329 (33 mer) (SEQ ID NO 37)
5' TTTGTCGACATGTCTACCTTCAAGCCTATGATG 3' and
AB330 (33 mer) (SEQ ID NO 38)
5' TTTGGATCCTTACGGAAGCTGAGTATATTTGAC 3'.

This PCR product was digested with SalI and BamHI generating a fragment of 1214 bp, which was then ligated into the vector pVR1012 (Example 1) linearized by the same double digestion. The resulting plasmid, pAB138 (6081 bp) contains the gene encoding the gD glycoprotein of 402 residues.

7.3.2. pSB027: gD gene (ΔTM form) cloned into the vector pVR1012

Depending on the hydropathy profile, the transmembrane domain of the EHV-4 gD protein is between residues 348 and 371. The plasmid containing the truncated form of the gene encoding gD was obtained by ligation of the following two DNA fragments: (a) the plasmid pAB138

(Example 7.3.1.) linearized by a double EcoRI-BamHI digestion and (b) a fragment of 310 bp obtained by PCR with the aid of the following oligonucleotides:

SB123 (24 mer) (SEQ ID NO 49)
5' TTTTCCGTAACAATTCCGAGCAGC 3' and
SB124 (39 mer) (SEQ ID NO 50)
5' AATTTTGGATCCTTACGTAGAGTTGCTATTAGACGCTGG 3'
using the plasmid pAB138 as template and digested with EcoRI and BamHI. The resulting plasmid, pSB027 (5919 bp), contains a truncated gD gene encoding a protein of 347 residues.

Example 8: Plasmid encoding the canine GM-CSF

8.1. Preparation of the total RNA of dog lymphocytes stimulated *in vitro* by mitogens

Dog blood was collected over a tube containing EDTA by a blood collection made on a Beagle dog. The mononucleated cells were harvested by centrifugation on a Ficoll gradient, and then cultured on a Petri dish 60 mm in diameter. The dog mononucleated cells were then stimulated with concanavalin A (ConA) (final concentration of about 4 µg/ml) and with phyto-haemagglutinin (PHA) (final concentration of about 10 µg/ml). After stimulation, the "ConA" and "PHA" lymphoblasts were harvested by scraping the culture dishes, and the total RNA of these cells was extracted using the kit "mRNA isolation kit for White Blood Cells" (Boehringer Mannheim/Roche Cat # 1 934 325).

8.2. Isolation of the gene encoding canine GM-CSF and construction of the plasmid pJP074

The total RNA extracted from the dog lymphoblasts stimulated by ConA or by PHA (Example 8.1.) served as template for the synthesis of the complementary DNA first strand. This complementary DNA first strand was

produced by extension of the oligonucleotide p(dT) 15 (Boehringer Mannheim/Roche Cat # 814 270). The single-stranded complementary DNA obtained was then used as template for a PCR reaction with the following oligonucleotides:

JP578 (SEQ ID NO 64) (33 mer)
5' TATGCGGCCGCCACCATGTGGCTGCAGAACCTG 3'
and JP579 (SEQ ID NO 65) (36 mer)
5' TATGCGGCCGCTACGTATCACTTCTTGACTGGTTTC 3'
in order to amplify a PCR fragment of about 450 base pairs (bp). This fragment was purified by agarose gel electrophoresis, and then ligated into the vector pCR2.1 (InVitrogen, Carlsbad, CA, USA) to give the plasmid pJP074. The sequence of the canine GM-CSF gene cloned in the plasmid pJP074 was found to be equivalent to that of the canine GM-CSF sequence available on GenBank (accession number # S49738).

8.3. Construction of the plasmid pJP084 and sequence of the canine GM-CSF gene

The plasmid pJP074 (Example 8.2) was digested with NotI in order to isolate, after agarose gel electrophoresis, the NotI-NotI fragment of about 450 bp containing the canine GM-CSF gene. This fragment was ligated into the plasmid pVR1012 (Example 1). The clone containing the canine GM-CSF sequence (SEQ ID NO 66, Figure No. 20) in the correct orientation relative to the hCMV/IE promoter was identified pJP084. This plasmid has a size of 5364 bp (Figure No. 19).

Example 9: Plasmid encoding feline GM-CSF

9.1. Preparation of the total RNA of cat lymphocytes stimulated *in vitro* by mitogens

Cat blood was collected over a tube containing EDTA by a blood collection. The mononucleated cells were

harvested by centrifugation on a Ficoll gradient, and then cultured on a Petri dish 60 mm in diameter. The cat mononucleated cells were then stimulated with concanavalin A (ConA) (final concentration of about 4 µg/ml) and with phytohaemagglutinin (PHA) (final concentration of about 10 µg/ml). After stimulation, the "ConA" and "PHA" lymphoblasts were harvested by scraping the culture dishes, and the total RNA of these cells was extracted using the kit "mRNA isolation kit for White Blood Cells" (Boehringer Mannheim/Roche Cat # 1 934 325).

9.2. Isolation of the gene encoding feline GM-CSF and construction of the plasmids pJP089 and pJP090

The total RNA extracted from the cat lymphoblasts stimulated by ConA or by PHA (Example 9.1.) served as template for the synthesis of the complementary DNA first strand. This complementary DNA first strand was produced by extension of the oligonucleotide p(dT) 15 (Boehringer Mannheim/Roche Cat #814 270). The single-stranded complementary DNA obtained was then used as template for a PCR reaction with the following oligonucleotides:

JP578 (SEQ ID NO 64) (33 mer)
5' TATGCGGCCGCCACCATGTGGCTGCAGAACCTG 3'
and JP579 (SEQ ID NO 65) (36 mer)
5' TATGCGGCCGCTACGTATCACTTCTTGACTGGTTTC 3'
in order to amplify a PCR fragment of about 450 base pairs (bp). This fragment was digested with NotI in order to isolate, after agarose gel electrophoresis, the NotI-NotI fragment of 450 bp. This fragment was then ligated into the plasmid pVR1012 (Example 1). Two clones containing the feline GM-CSF sequence (SEQ ID NO 67 and SEQ ID NO 68), in the correct orientation relative to the hCMV/IE promoter were identified pJP089 and pJP090 respectively. These two plasmids have a size of 5364 bp (Figures No. 21 and 23).

The sequence of the feline GM-CSF gene cloned into the plasmid pJP089 contains 13 differences at the nucleotide level with the feline GM-CSF sequence available on GenBank (accession number AF053007). The most important change is a C → T change which causes a Leucine → Phenylalanine change for the amino acid (first base of the amino acid codon # 107; Figure No. 22). The sequence of the feline GM-CSF gene cloned into the plasmid pJP090 is equivalent to that contained in the plasmid pJP089, except that the Leucine → Phenylalanine change does not exist for the amino acid # 107 (Figure No. 24). Verification of the 3' sequence of the feline GM-CSF gene by means of the 3' RACE kit showed that, at this position 107, it is possible to have, in the same cat, the amino acid Leucine or the amino acid Phenylalanine.

Example 10: Plasmid encoding equine GM-CSF

10.1 Preparation of the total RNA of horse lymphocytes stimulated *in vitro* by mitogens

Horse blood was collected over a tube containing EDTA by a blood collection from the jugular vein. The mononucleated cells were harvested by centrifugation on a Ficoll gradient, and then cultured on a Petri dish 60 mm in diameter. The horse mononucleated cells were then stimulated either with concanavalin A (ConA) (final concentration of about 5 µg/ml) or with phytohaemagglutinin (PHA) (final concentration of about 10 µg/ml). After stimulation, the "ConA" and "PHA" lymphoblasts were harvested by scraping the culture dishes, and the total RNA of these cells was extracted using the kit "mRNA isolation kit for White Blood Cells" (Boehringer Mannheim/Roche Cat # 1 934 325).

10.2. Isolation of the gene encoding equine GM-CSF

The total RNA extracted from the horse lymphoblasts stimulated by ConA or by PHA (Example 10.1.) served as template for the synthesis of the complementary DNA first strand. This complementary DNA first strand was produced by extension of the oligonucleotide p(dT) 15 (Boehringer Mannheim/Roche Cat # 814 270). The single-stranded complementary DNA obtained was then used as template for a PCR reaction with the following oligonucleotides:

JP734 (SEQ ID NO 70) (44 mer)
5' CATCATCATGTCGACGCCACCATGTGGCTGCAGAACCTGCTTCT 3'
and JP735 (SEQ ID NO 71) (41 mer)
5' CATCATCATGCGGCCGCTACTTCTGGGCTGCTGGCTTCCAG 3'
in order to amplify a PCR fragment of about 500 base pairs (bp). This fragment was purified by agarose gel electrophoresis.

10.3. Construction of the plasmid pJP097 and sequence of the equine GM-CSF gene

The purified PCR fragment obtained in Example 10.2. was digested with NotI in order to isolate, after agarose gel electrophoresis, the NotI-NotI fragment of about 450 bp containing the equine GM-CSF gene. This fragment was ligated into the plasmid pVR1012 (Example 1). The clone containing the equine GM-CSF sequence (SEQ ID NO 69, Figure No. 26) in the correct orientation relative to the hCMV/IE promoter was identified pJP097. This plasmid has a size of 5334 bp (Figure No. 25).

Example 11: Summary table for the plasmids

Pathogen	Antigen	No. of residues native form	TM domain	No. of residues truncated form	Expression plasmids
CDV	F	662	606-626	605	pPB229 and pNS021*
	HA	607	1-60**	574	pNS018 and pNS024*
CPI-2	F	529	474-517	473	pAB115 and pSB032*
	HN	565	1-40**	557	pAB114 and pSB034*
CHV-1	gB	878	702-769	701	pAB037 and pSB016*
	gC	459	422-452	421	pSB018 and pSB019*
	gD	345	310-328	309	pAB038 and pSB017*
FHV-1	gB	949	761-834	760	pSB020 and pSB021*
	gC	534	495-526	494	pSB022 and pSB023*
	gD	374	328-353	327	pAB029 and pSB024*
EHV-1	gB	980	801-875	800	pAB127 and pSB028*
	gC	468	429-455	428	pAB129 and pSB029*
	gD	402	348-371	347	pAB131 and pSB030*
EHV-4	gB	975	797-867	796	pAB136 and pSB025*
	gC	485	425-472	424	pAB137 and pSB026*
	gD	402	348-371	347	pAB138 and pSB027*

** Plasmids encoding the truncated forms of the antigens

** Specific case of the CDV HA and CPI-2 HN genes: transmembrane domain and signal sequence indistinguishable

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Example 12: Molecular biology methods

Culture and purification of the viruses

The viruses were cultured on appropriate cellular systems until a cytopathic effect was obtained. The cellular systems to be used for each virus are well known to persons skilled in the art. Briefly, the appropriate cells were infected with the viral strain studied at a multiplicity of infection of one and were incubated at 37°C for the time necessary to obtain a cytopathic effect (on average 36 hours).

In the case of the DNA viruses, after the culture, the supernatant and the lysed cells were harvested and the cellular debris was removed by centrifugation at 1000 g and at 4°C for 10 minutes. The viral particles were harvested by ultracentrifugation at 400,000 g and 4°C for 1 hour. The pellets were taken up in a minimum volume of buffer (10 mM Tris, 1 mM EDTA).

The RNA viruses were purified according to standard purification techniques well known to persons skilled in the art.

Extraction of the viral genomic DNA

The concentrated viral suspensions were treated with proteinase K (100 mg/ml final) in the presence of sodium dodecyl sulphate (SDS) (0.5% final) for 2 hours at 37°C. The viral DNA was then extracted with the aid of a phenol/chloroform mixture, and then precipitated with two volumes of absolute ethanol at -20°C for 16 hours and then centrifuged at 10,000 g for 15 minutes at 4°C. The DNA pellets were dried, and then taken up in a minimum volume of sterile ultrapure water.

Isolation of viral genomic RNA

The genomic RNA of each virus was extracted using the "guanidinium thiocyanate/phenol-chloroform" technique described by P. Chomczynski and N. Sacchi (Anal. Biochem. 1987. **162**. 156-159).

Molecular biology techniques

All the constructions of plasmids were carried out using the standard molecular biology techniques described by Sambrook et al. (Molecular Cloning: A Laboratory Manual. 2nd Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). All the restriction fragments used for the present invention were isolated with the aid of the "Geneclean" kit (BIO101 Inc., La Jolla, CA). For all the constructs, the cloned DNA fragments, as well as the junctions with the expression vector, were sequenced by the Sanger method (Sambrook et al., 1989).

PCR and RT-PCR

The oligonucleotides specific to the genes or gene fragments cloned were synthesized, some of them containing, in some cases, at their 5' end, restriction sites facilitating the cloning of the amplified fragments. The reverse transcription (RT) reactions and the polymerase chain reaction (PCR) were carried out according to standard techniques (Sambrook et al., 1989).

Large-scale purification of plasmids

The production, on the scale of about ten mg, of purified plasmids entering into the vaccinal compositions was carried out by the caesium chloride-ethidium bromide gradient method (Sambrook et al., 1989).

Example 13: Formulation of the vaccinal plasmids

The DNA solution containing one or more plasmids according to Examples 2 to 10 is concentrated by ethanolic precipitation as described in Sambrook et al. (1989). The DNA pellet is taken up in a 0.9% NaCl solution so as to obtain a concentration of 1 mg/ml. A 0.75 mM DMRIE-DOPE solution is prepared by taking up a lyophilisate of DMRIE-DOPE with an appropriate volume of sterile H_2O.

The formation of the plasmid DNA-lipid complexes is achieved by diluting, in equal parts, the 0.75 mM DMRIE-DOPE solution with the DNA solution at 1 mg/ml in 0.9% NaCl. The DNA solution is gradually introduced, with the aid of a seamed 26G needle, along the wall of the vial containing the cationic lipid solution so as to avoid the formation of foam. Gentle shaking is carried out as soon as the two solutions have been mixed. A composition comprising 0.375 mM of DMRIE-DOPE and 500 μg/ml of plasmid is finally obtained.

It is desirable for all the solutions used to be at room temperature for all the operations described above. The DNA/DMRIE-DOPE complex formation is allowed to take place at room temperature for 30 minutes before immunizing the animals.

Example 14: Immunization of dogs against CDV

An injection of 2 ml by the subcutaneous or intramuscular route repeated 28 days later. The total mass of plasmid used during each immunization is 100, 500, 1000 or 2000 μg according to the vaccines. Persons skilled in the art possess the knowledge necessary to adjust the volume or the concentration as a function of the plasmid dose required.

14.1. Virulent challenge

The challenge strain used corresponds to a ground product of the spleen removed from a dog infected with the canine distemper virus, strain Snyder Hill, and diluted one hundred fold in PBS buffer. The dilution is kept on crushed ice until used.

After general anaesthetic, the challenge strain, diluted one hundred fold, is administered by the intracranial route in a volume of 0.5 ml, 49 days after the first injection.

14.2. Post-challenge clinical monitoring

This daily clinical monitoring was carried out for 21 days following the challenge and included, for each dog, a clinical examination to detect possible clinical signs of canine distemper and taking of rectal temperature.

The clinical examination comprises:

- An observation of the general state of the animal on a 4-point scale:

"good" with a score of 0, "apathy" with a score of 1, "depression" with a score of 2 and "prostration" with a score of 3. The death of the animal is equivalent to a clinical score of 10.

- An evaluation of the oculonasal symptoms (test for serous or purulent discharge, rhinitis and/or conjunctivitis). Conjunctivitis/rhinitis with a serous nasal discharge is equivalent to a score of 1, that with a purulent nasal discharge is equivalent to a score of 2.

- An evaluation of the digestive symptoms (test for signs of gastroenteritis). A mild gastroenteritis is

equivalent to a score of 1, a severe gastroenteritis to a score of 2.

- An evaluation of the nervous symptoms (test for myoclonias, convulsions and/or paralysis). Myoclonia is equivalent to a score of 1, convulsion to a score of 2 and paralysis to a score of 3.

- The monitoring of the body temperature of the animal.

A temperature of less than or equal to 37.5°C is equivalent to a score of 3, a temperature of between 37.5°C and 39.5°C is equivalent to a score of 0, a temperature equal to 39.5°C or between 39.5°C and 40°C is equivalent to a score of 1, a temperature equal to 40°C or between 40°C and 40.5°C is equivalent to a score of 2, and finally a temperature greater than 40.5°C is equivalent to a score of 3.

Of the animals that succumbed to the challenge, the spleen was removed in order to identify the canine distemper virus by immunofluorescence.

An overall clinical score was calculated in the following manner: for each clinical sign, a mean score was established per group of animals over the observation period and the total score is the sum of the mean scores for the five clinical signs considered.

Results of the challenge experiments after immunization of dogs by the intramuscular route:

Plasmid	Formulation	Antigens	Dose	Cytokine	Mortality	Clinical score	Antibody response*
control	---	---	---	---	4/4	26.1	0.3 ± 0.3
pNS018 pPB229	--	Native HA Native F	50 µg 50 µg	---	4/5	21.6	0.3 ± 0.2
PNS018 pPB229	DMRIE-DOPE	Native HA Native F	50 µg 50 µg	---	1/5	7.8	0.8 ± 0.3
PNS024 pNS021	DMRIE-DOPE	Optimized HA Optimized F	50 µg 50 µg	---	1/5	7.2	1.9 ± 0.8
pNS024 pNS021	DMRIE-DOPE	Optimized HA Optimized F	500 µg 500 µg	---	0/5	0.6	1.5 ± 0.3

* mean titre ± standard deviation

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Plasmid	Formulation	Antigens	Dose	Cytokine	Mortality	Clinical score	Antibody response*
control	---	---	---	---	4/4	25.0	0.2 ± 0.0
pNS018 pPB229	DMRIE-DOPE	Native HA Native F	50 µg 50 µg	---	5/5	24.4	0.2 ± 0.0
pNS018 pPB229	DMRIE-DOPE	Native HA Native F	500 µg 500 µg	---	3/5	19.0	0.4 ± 0.2
pNS024 pNS021	DMRIE-DOPE	Optimized HA Optimized F	500 µg 500 µg	---	1/5	9.2	0.6 ± 0.7
pNS024 pNS021 pJP084	DMRIE-DOPE	Optimized HA Optimized F	500 µg 500 µg 200 µg	GM-CSF	0/5	2.4	1.7 ± 0.9
pNS024	DMRIE-DOPE	Optimized HA	500 µg	---	0/5	0.8	1.4 ± 1.0
pNS024 pNS021 pNS016 pNS017	DMRIE-DOPE	Optimized HA Optimized F Native M Native N	500 µg 500 µg 500 µg 500 µg	---	0/5	5	1.4 ± 1.3

* mean titre ± standard deviation

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The plasmids pNS016 and pNS017 inserting the native gene M and the native gene N, respectively, were constructed in the same manner as the plasmid pNS018.

It is surprising to observe that the protection result obtained with optimized HA alone is greater than or equal to the results obtained with optimized HA and F or with optimized HA and F + native M and N.

It should be clearly understood that the invention defined by the appended claims is not limited to the specific embodiments indicated in the description above, but encompasses the variants which depart neither from the scope nor the spirit of the present invention.

SEQUENCE LISTING

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BARZU-LE ROUX, Simona
AUDONNET, Jean-Christophe Francis

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caggtgacac tatcctaa 1698

CLAIMS:

1. A DNA vaccine against the canine distemper virus (CDV) affecting dogs, comprising

a plasmid comprising a nucleotide sequence coding for a CDV immunogen, and the elements necessary for its expression in vivo, a cationic lipid, (N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1 propane ammonium (DMRIE) and dioleolyphosphatidylethanol amine (DOPE),

wherein the DNA vaccine when administered intramuscularly or subcutaneously induces an effective immune response protective in a dog against CDV.

2. A vaccine according to claim 1 further comprising a canine granulocyte-macrophage colony stimulating factor (GM-CSF) protein.

3. A vaccine according to claim 2, further comprising an expression vector comprising a nucleotide sequence coding for the canine GM-CSF protein under conditions permitting in vivo expression of said sequence.

4. A vaccine according to claim 1, further comprising an expression vector containing a nucleotide sequence coding for a canine granulocyte-macrophage colony stimulating factor (GM-CSF) protein under conditions permitting in vivo expression of said sequence.

5. A vaccine according to claim 3 or claim 4, wherein the expression vector is a plasmid.

6. A vaccine according to any one of claims 1 to 5, wherein the nucleotide sequence coding for the CDV immunogen is a sequence of a gene from which a part coding for a transmembrane domain has been deleted.

7. A vaccine according to any one of claims 1 to 6, wherein the plasmid comprising the nucleotide sequence coding for the CDV immunogen further comprises a nucleotide sequence coding for a tissue plasminogen activator (tPA) signal.

8. A vaccine according to any one of claims 1 to 7, wherein the plasmid comprising the nucleotide sequence coding for the CDV immunogen further comprises a stabilizing intron.

9. A vaccine according to claim 8, wherein the stabilizing intron is intron II of rabbit beta-globin gene.

10. A vaccine according to any one of claims 1 to 5, wherein the CDV immunogen is haemagglutinin (HA) antigen.

11. A vaccine according to claim 10, wherein the nucleotide sequence coding for the HA antigen is a sequence from which: (i) a signal peptide of the HA antigen is substituted with a nucleotide sequence coding for a tissue plasminogen activator (tPA) signal, (ii) a part encoding the HA transmembrane domain is deleted, (iii) an intron is inserted upstream of the nucleotide sequence coding for HA, (iv) or any combinations thereof.

12. A vaccine according to any one of claims 1 to 5, wherein the CDV immunogen is F antigen.

13. A vaccine according to claim 12, wherein the nucleotide sequence coding for the F antigen is a sequence from which: (i) a signal peptide of the F antigen is substituted with a nucleotide sequence coding for a tissue plasminogen activator (tPA) signal, (ii) a part encoding the F transmembrane domain is deleted, (iii) an intron is inserted upstream of the nucleotide sequence coding for the F antigen, or (iv) any combinations thereof.

14. A vaccine according to claim 11 or claim 13 wherein the tPA signal is the human tPA signal.

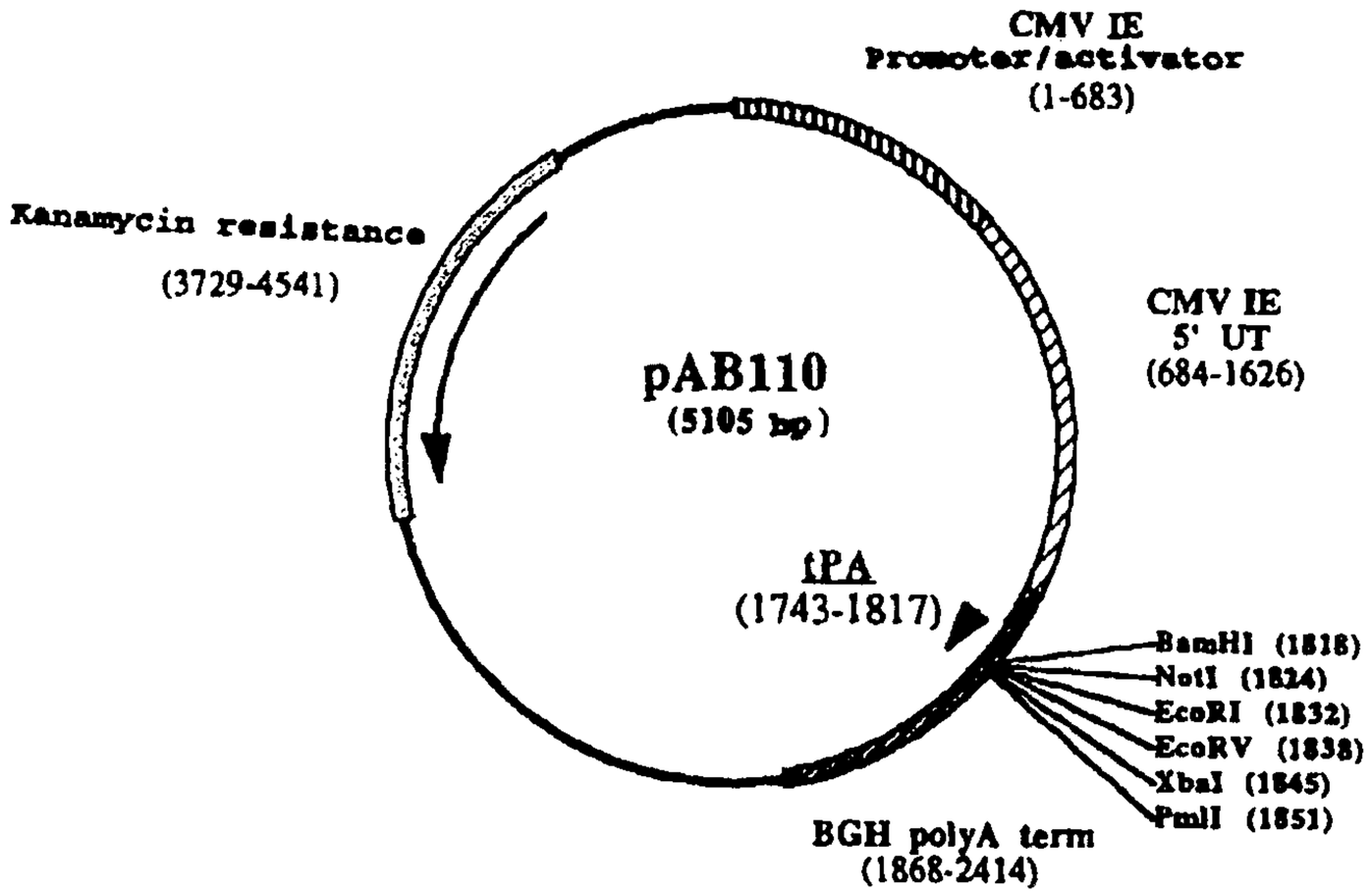
15. A vaccine according to claim 11 or claim 13 wherein the intron is intron II of rabbit beta-globin gene.

16. A vaccine according to any one of claims 10 to 15 further comprising in the same plasmid or in another plasmid a nucleotide sequence coding for protein M or protein N of CDV.

17. A vaccine according to any one of claims 1 to 5, wherein said plasmid encoding for the CDV immunogen is a first expression plasmid, wherein the immunogen is the haemagglutinin (HA) antigen of CDV, and wherein the nucleotide sequence coding for a peptide signal of the HA antigen is substituted with a nucleotide sequence coding for the human tissue plasminogen activator (tPA) signal, a nucleotide sequence coding for the HA transmembrane domain is deleted, and intron II of the rabbit beta-globin gene is inserted upstream of the nucleotide sequence coding for the HA antigen; and wherein said vaccine further comprises a second expression plasmid comprising a nucleotide sequence coding for the F antigen of CDV, wherein the nucleotide sequence coding for the F transmembrane domain is deleted and intron II of rabbit beta-globin gene is inserted upstream of the nucleotide sequence coding for the F antigen.

18. A vaccine according to claim 17, further comprising a nucleotide sequence coding for protein M or protein N of CDV present in (i) said first expression plasmid, (ii) said second expression plasmid, or (iii) another plasmid.

Figure 1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CMV IE
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pAB110
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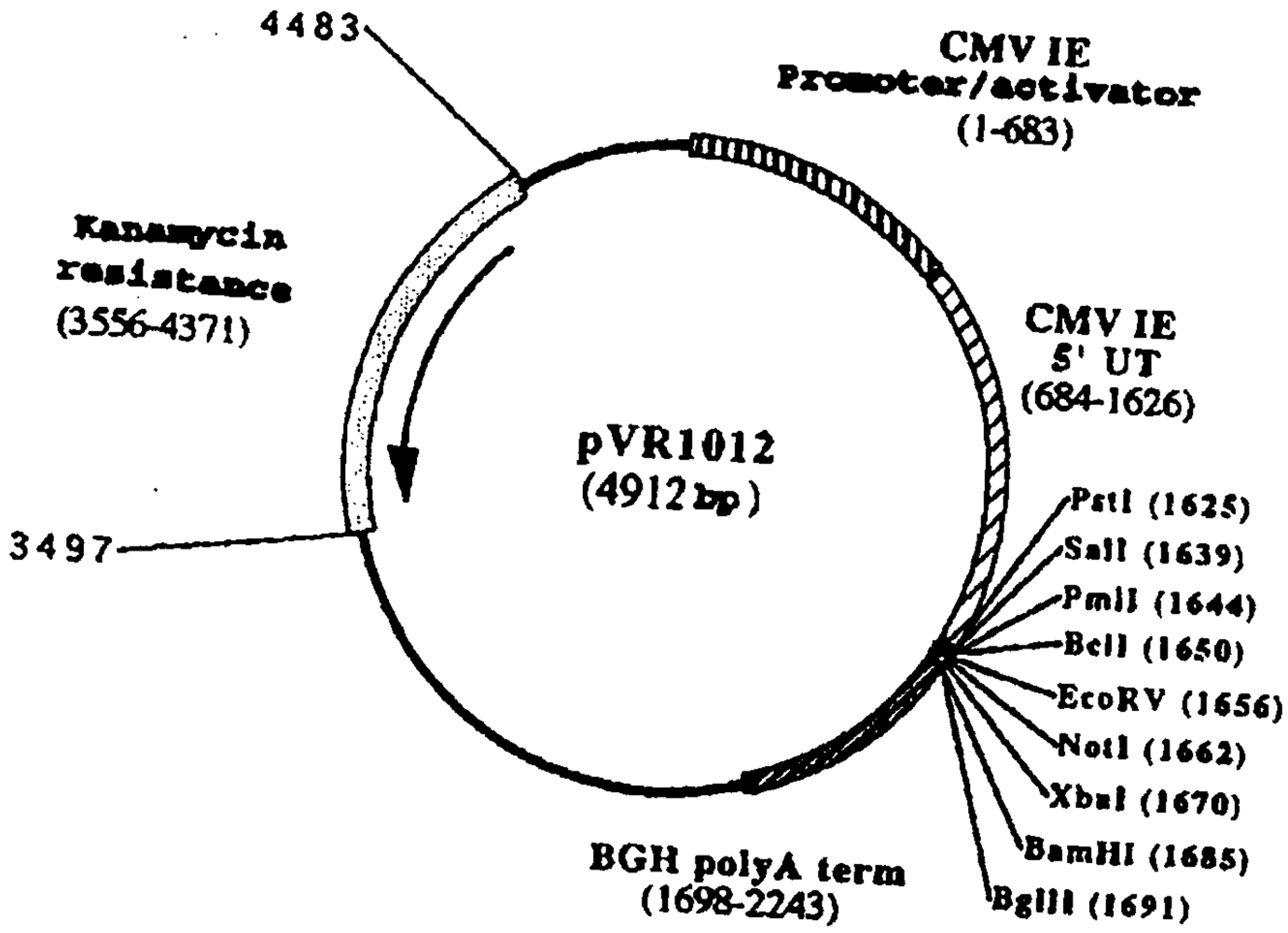
tPA
(1743-1817)
BamHI (1818)
NotI (1824)
EcoRI (1832)
EcoRV (1838)
XbaI (1845)
PmlI (1851)
BGH polyA term
(1868-2414)

Figure 2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(684-1626)
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PstI (1625)
SalI (1639)
PmlI (1644)
BclI (1650)
EcoRV (1656)
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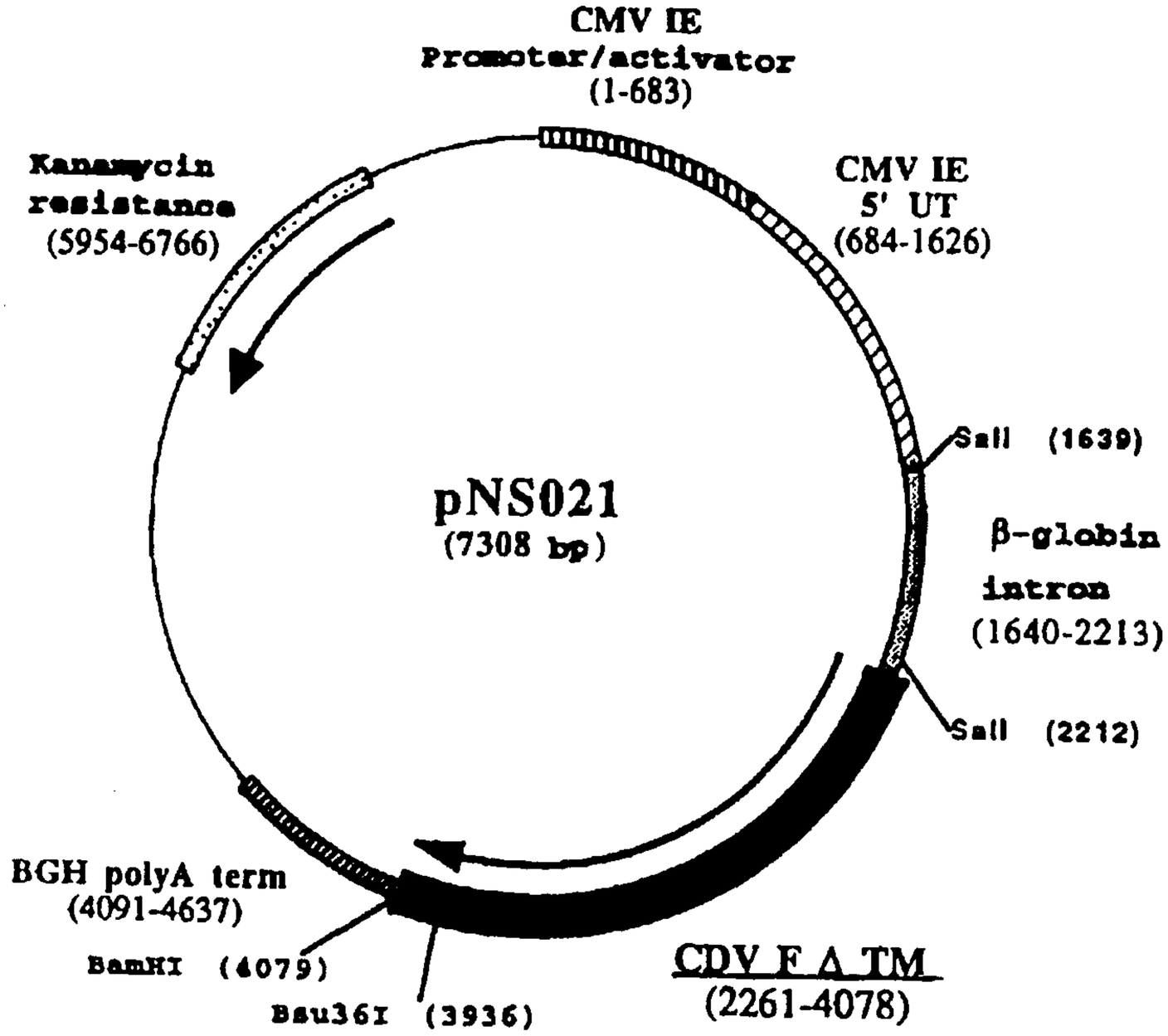
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β-globin
intron
(1640-2213)
SalI (2212)
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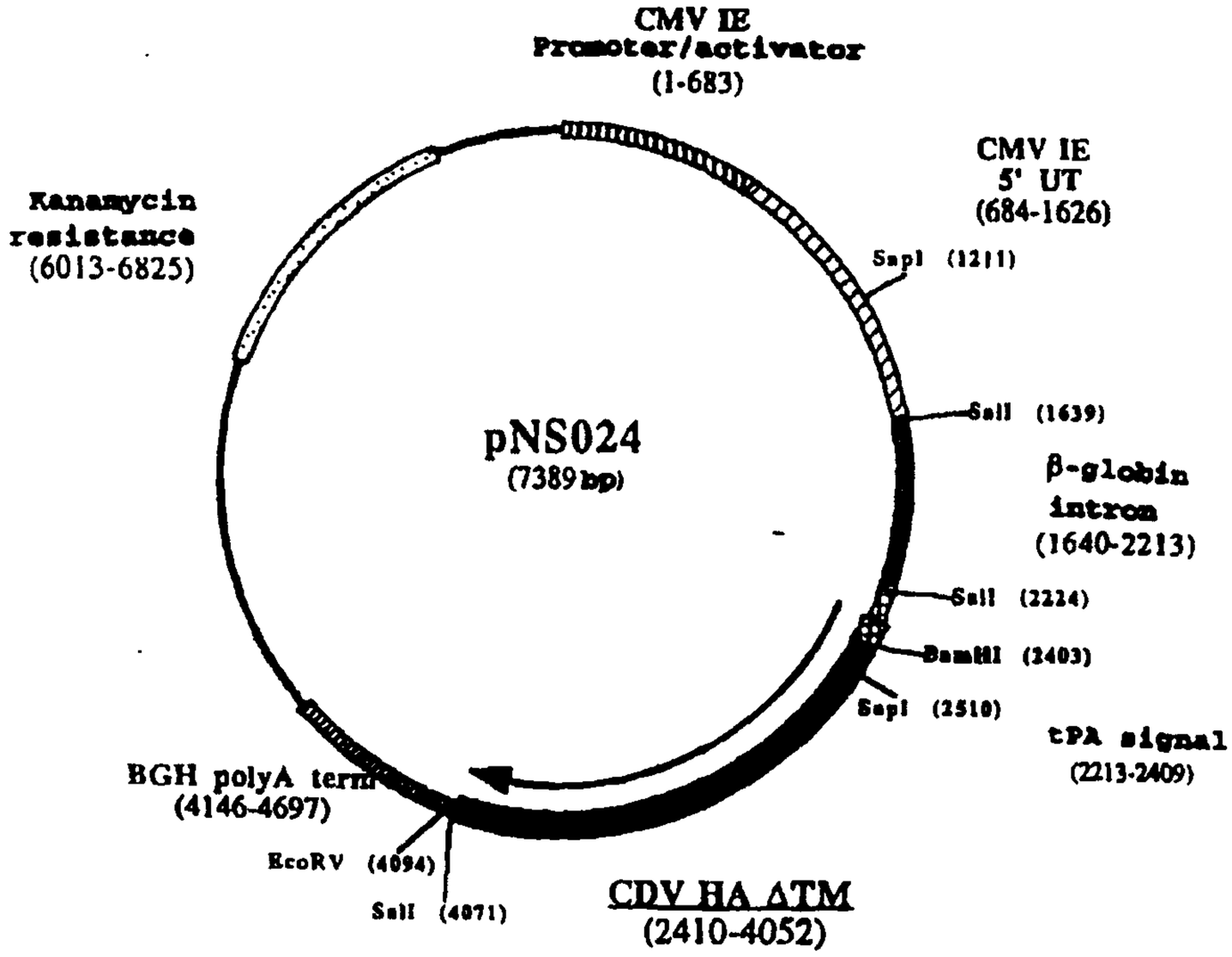
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SapI (1211)
SalI (1639)
β-globin
intron
(1640-2213)
SalI (2224)
BamHI (2403)
SapI (2510)
tPA signal
(2213-2409)
CDV HA ΔTM
(2410-4052)
Kanamycin
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(7389 bp)
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(4146-4697)
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SalI (4071)

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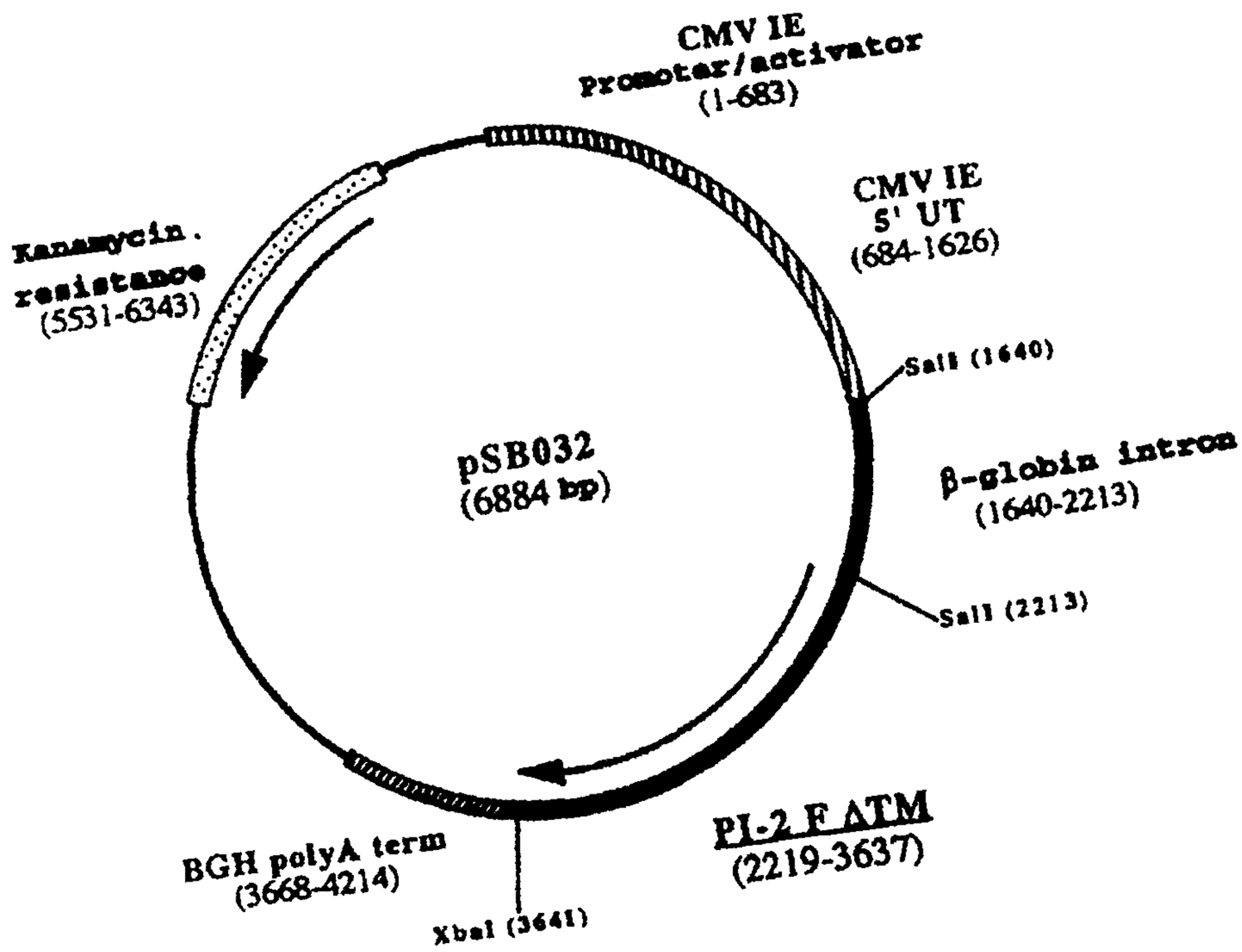
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PI-2 F ΔTM
(2219-3637)
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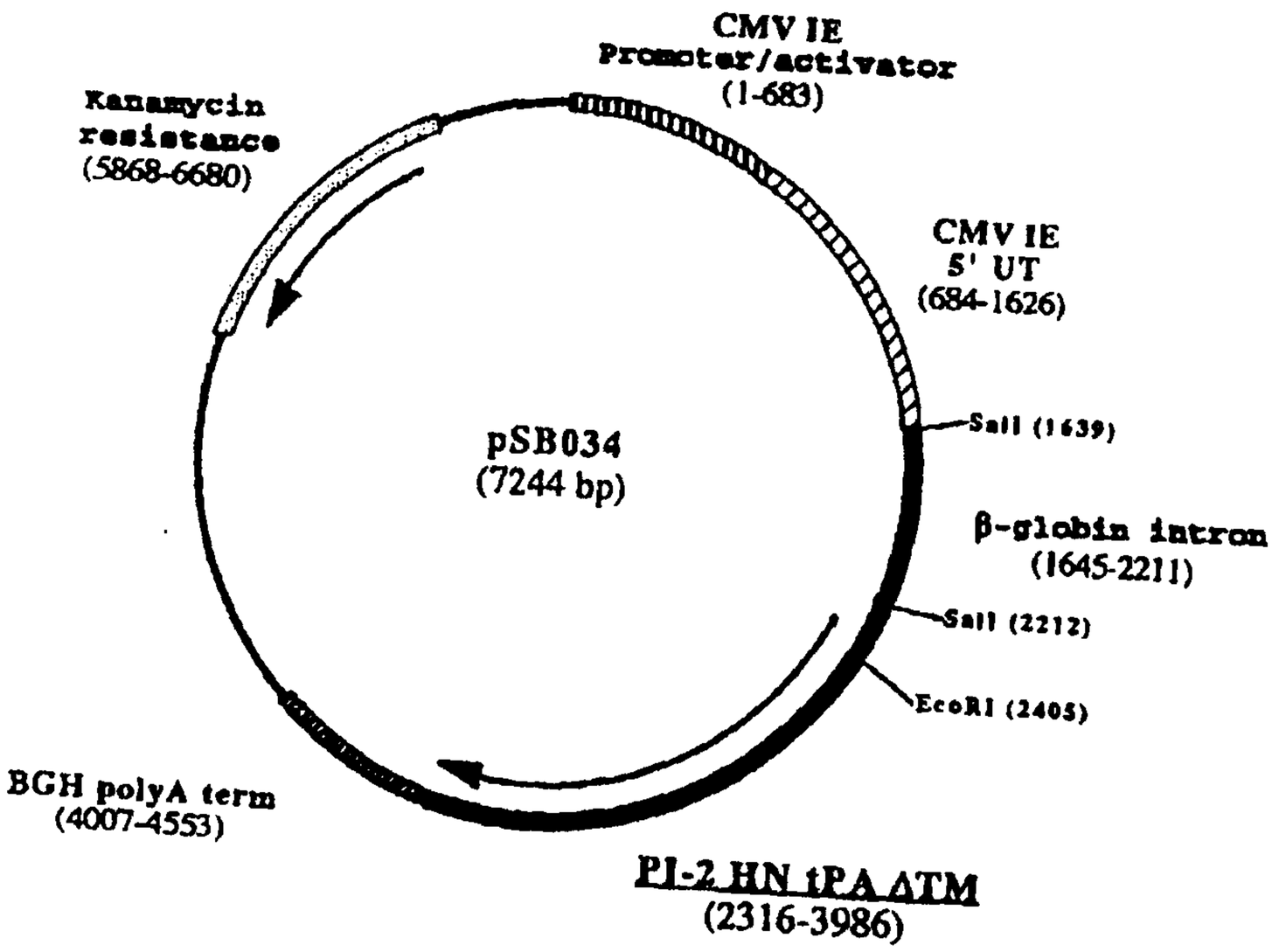
Figure 6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β-globin intron
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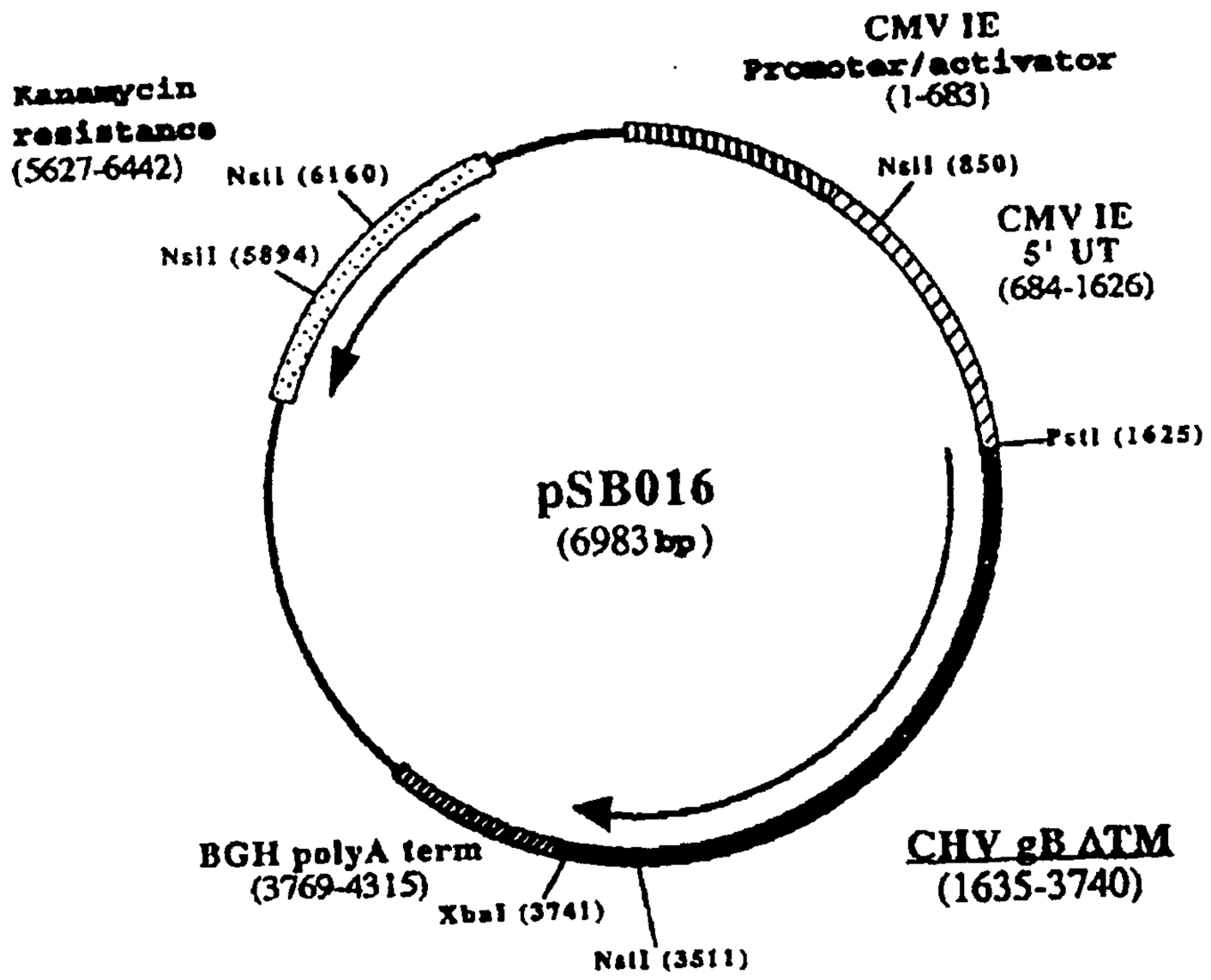
Figure 7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NsiI (5894)
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pSB016
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Figure 8.

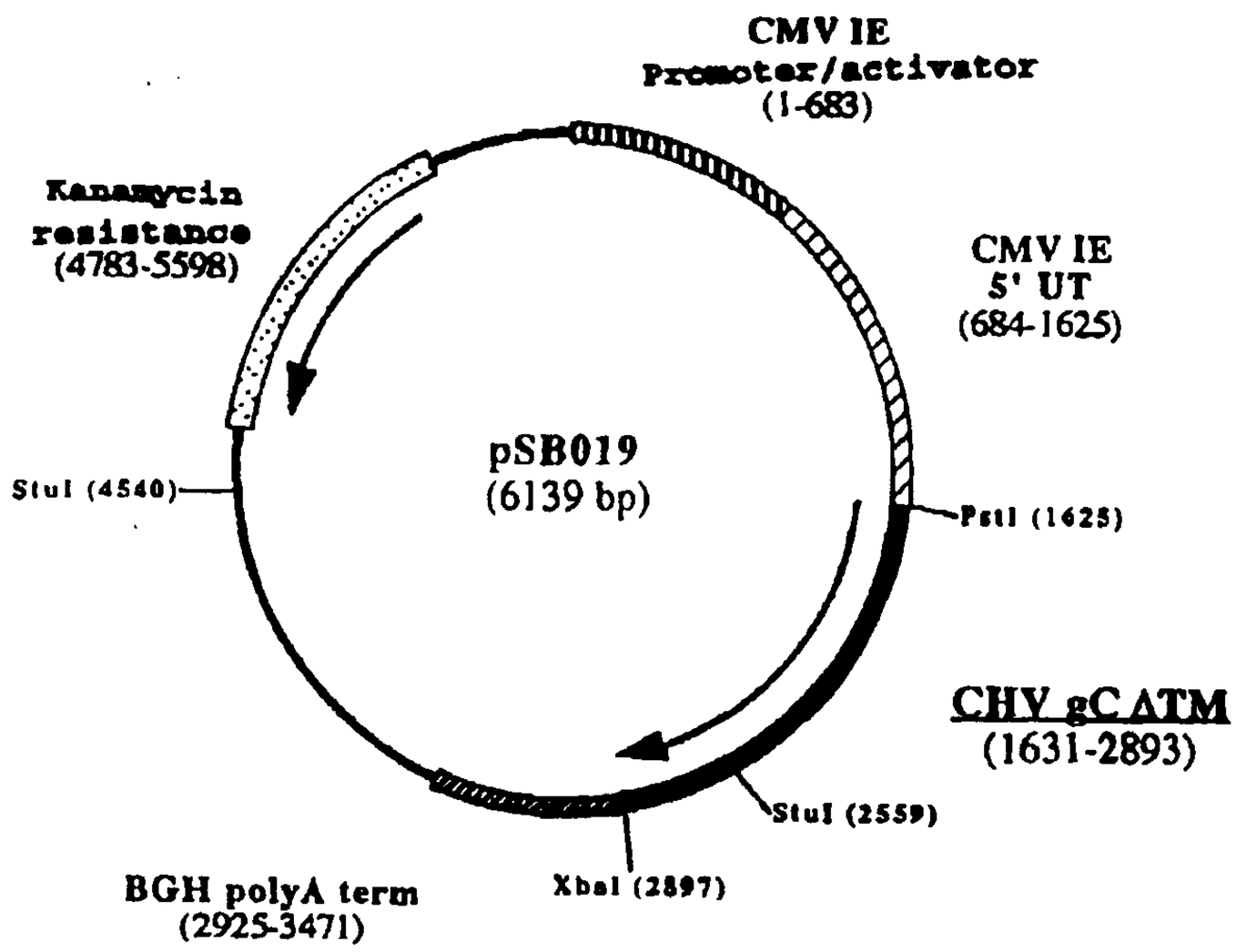
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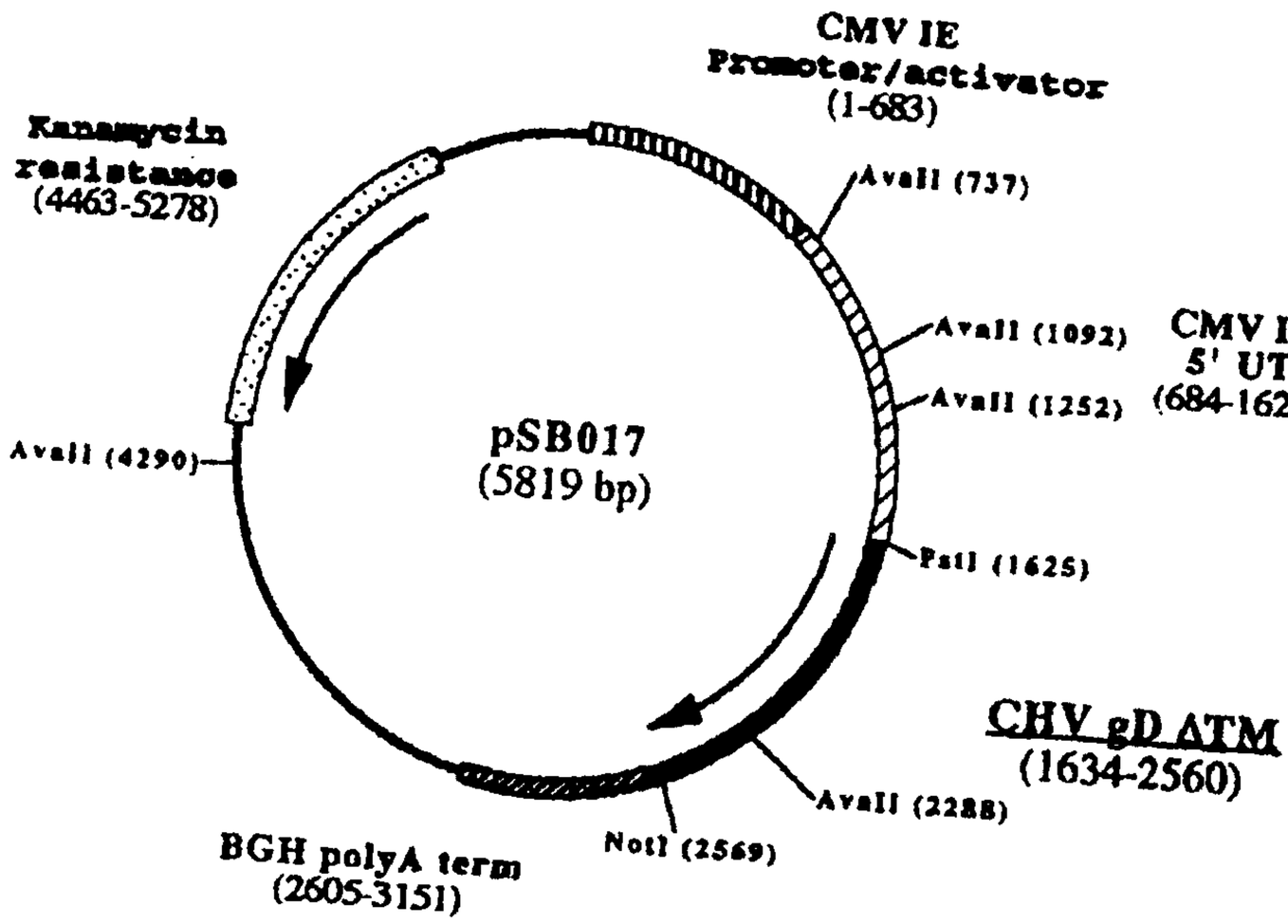
Figure 9.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(684-1626)
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(5819 bp)
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BGH polyA term
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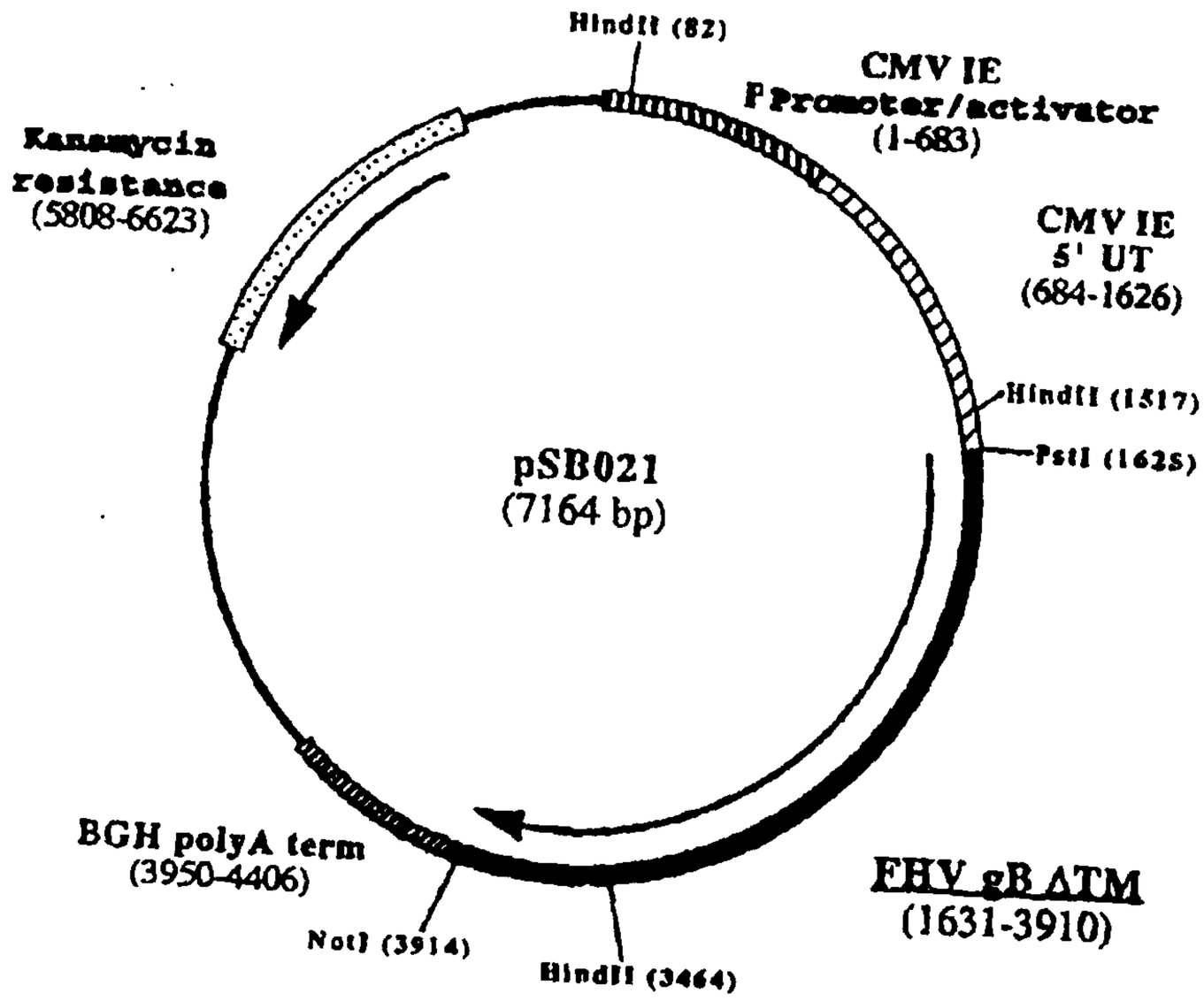
Figure 10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(1631-3910)

Figure 11

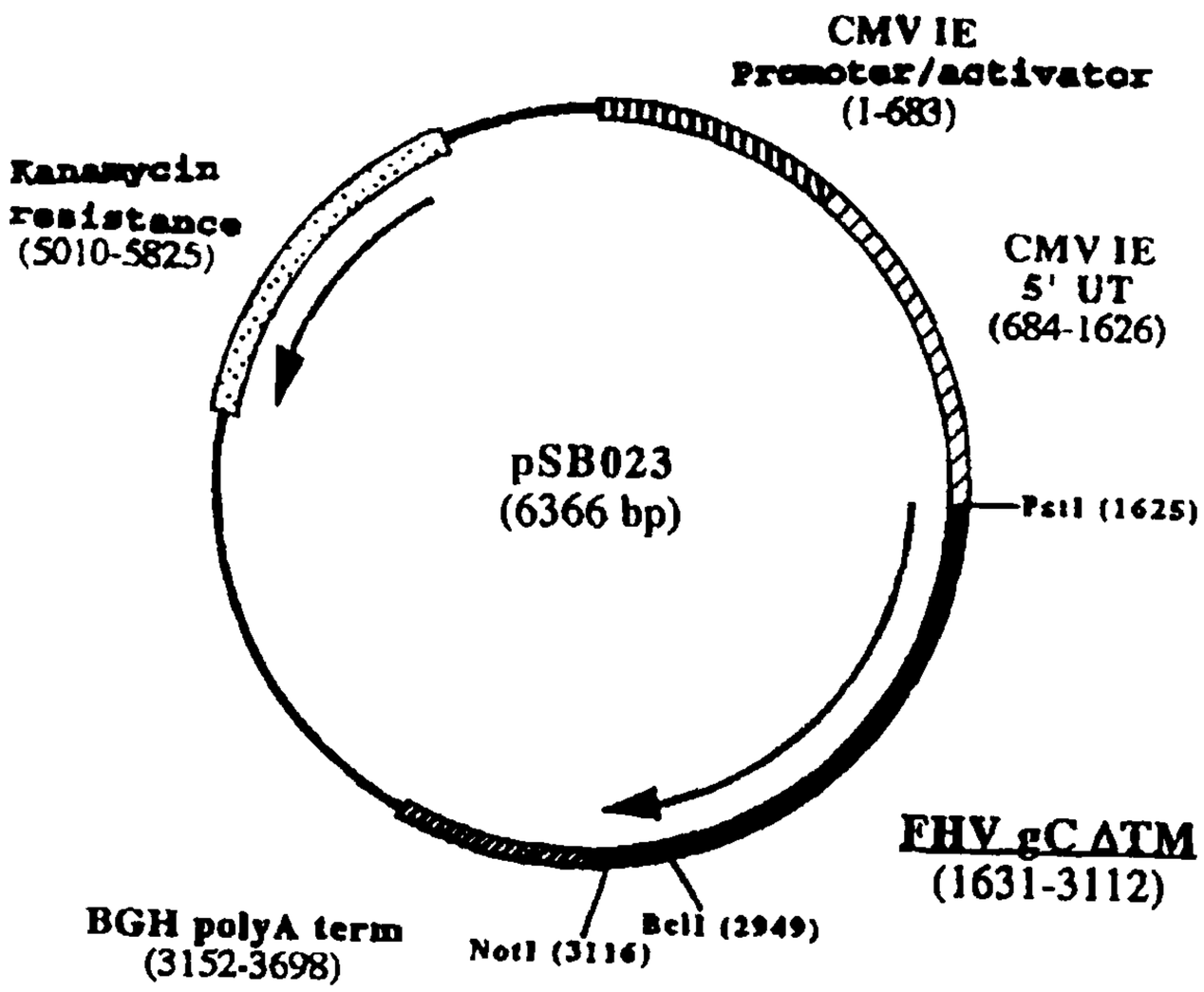
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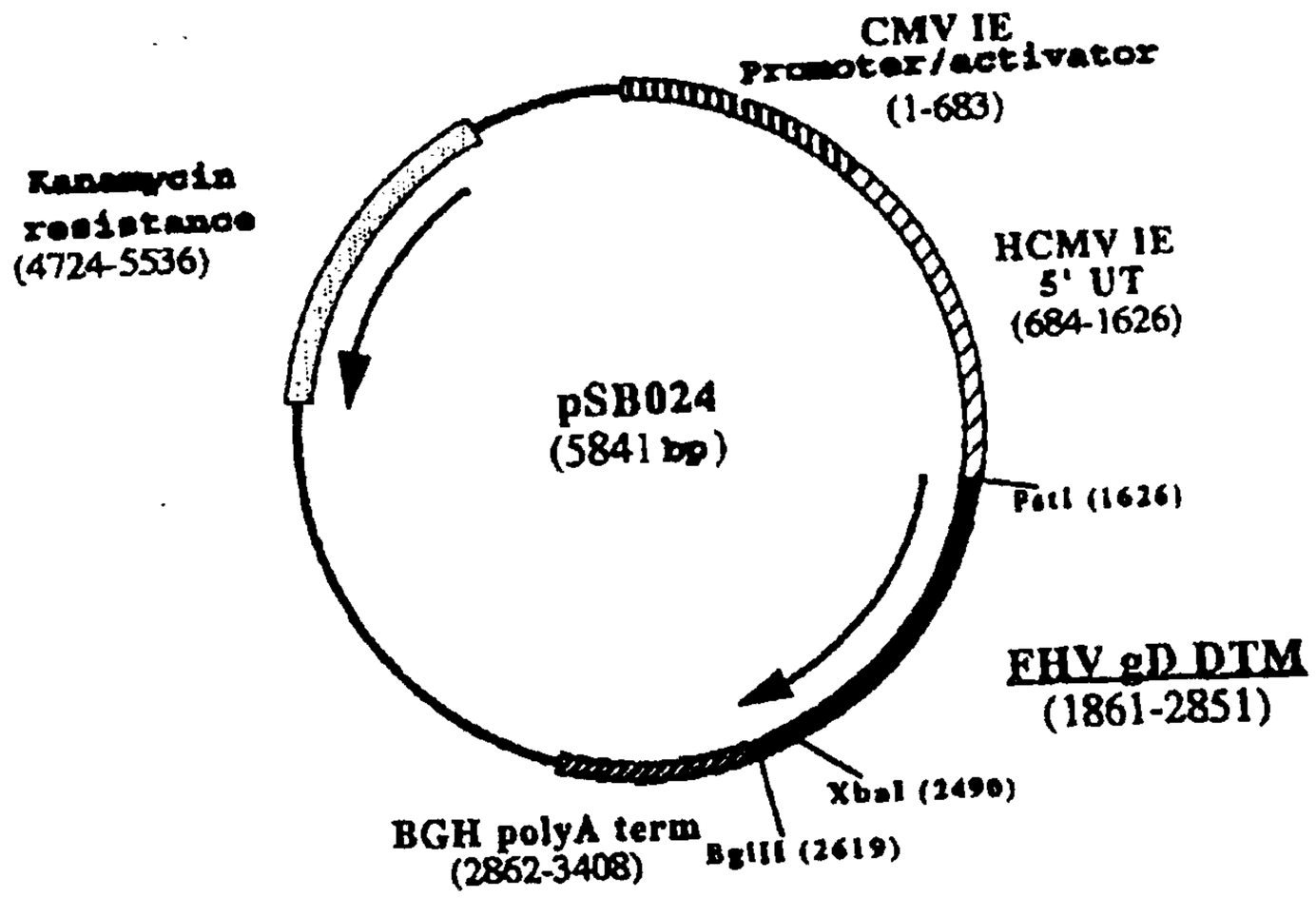
Figure 12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(684-1626)
pSB024
(5841 bp)
PstI (1626)
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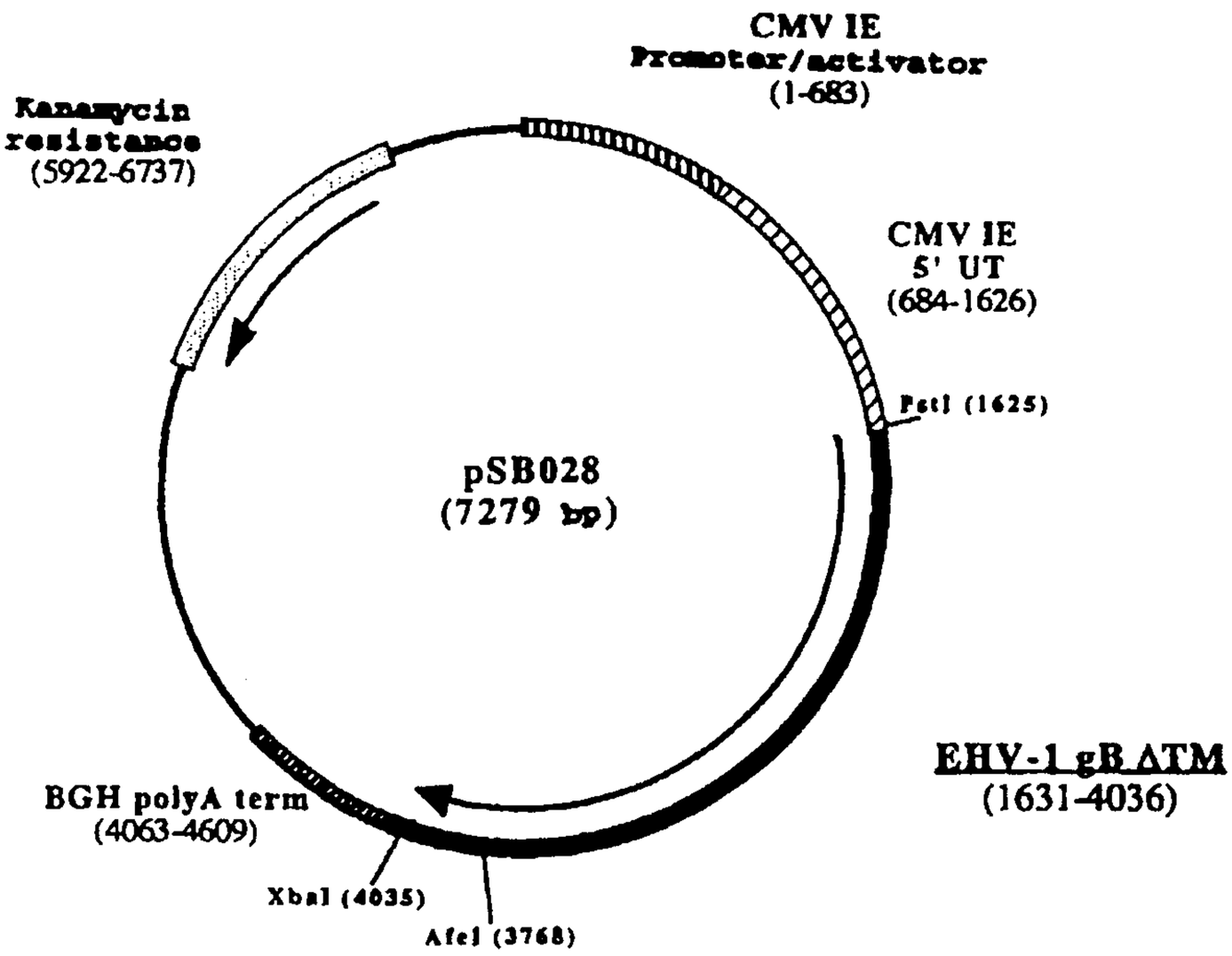
XbaI (2490)
BGH polyA term
(2862-3408)
BglII (2619)

Figure 13.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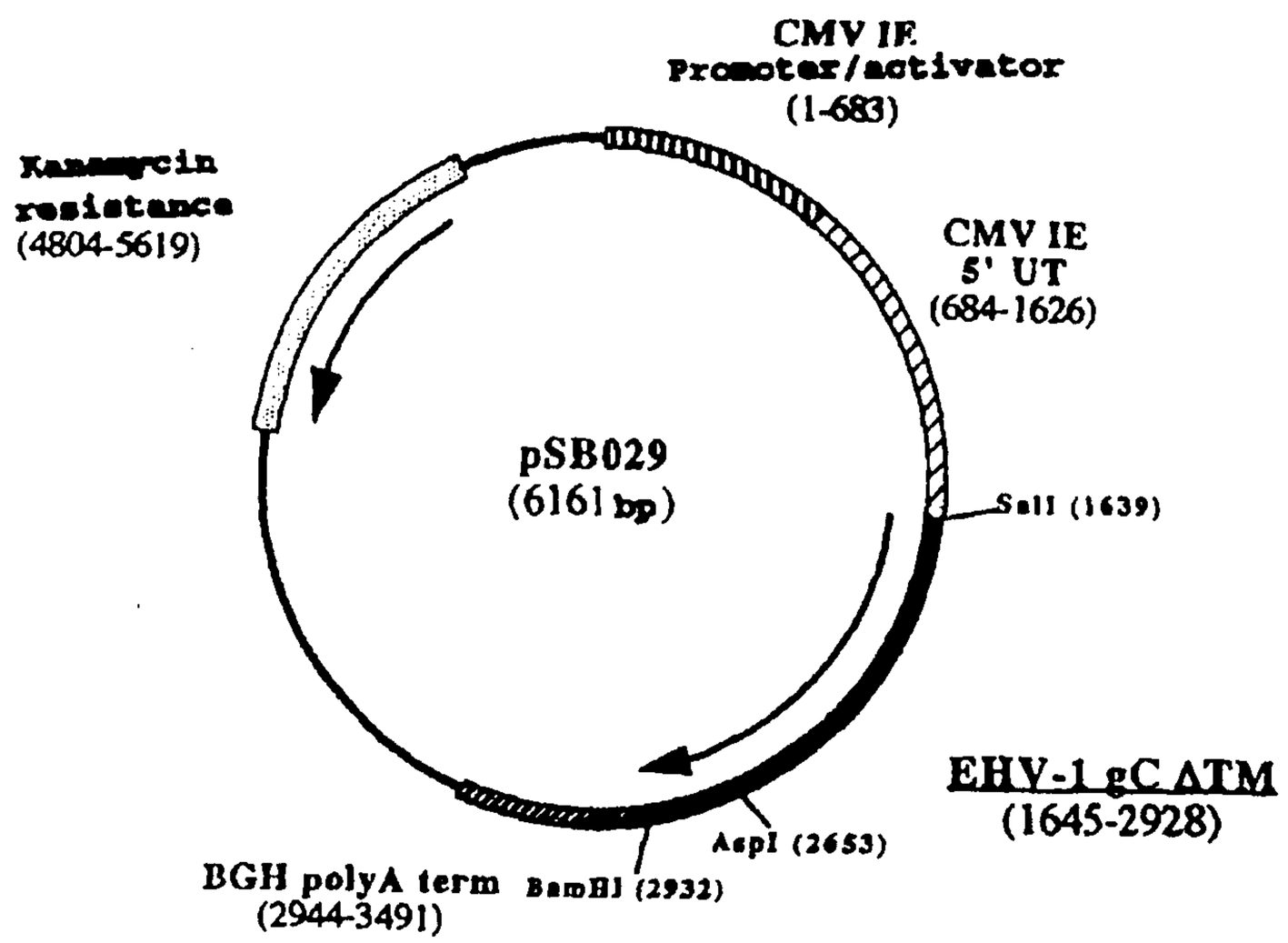
(1631-4036)
BGH polyA term
(4063-4609)
XbaI (4035)
AflI (3768)

Figure 14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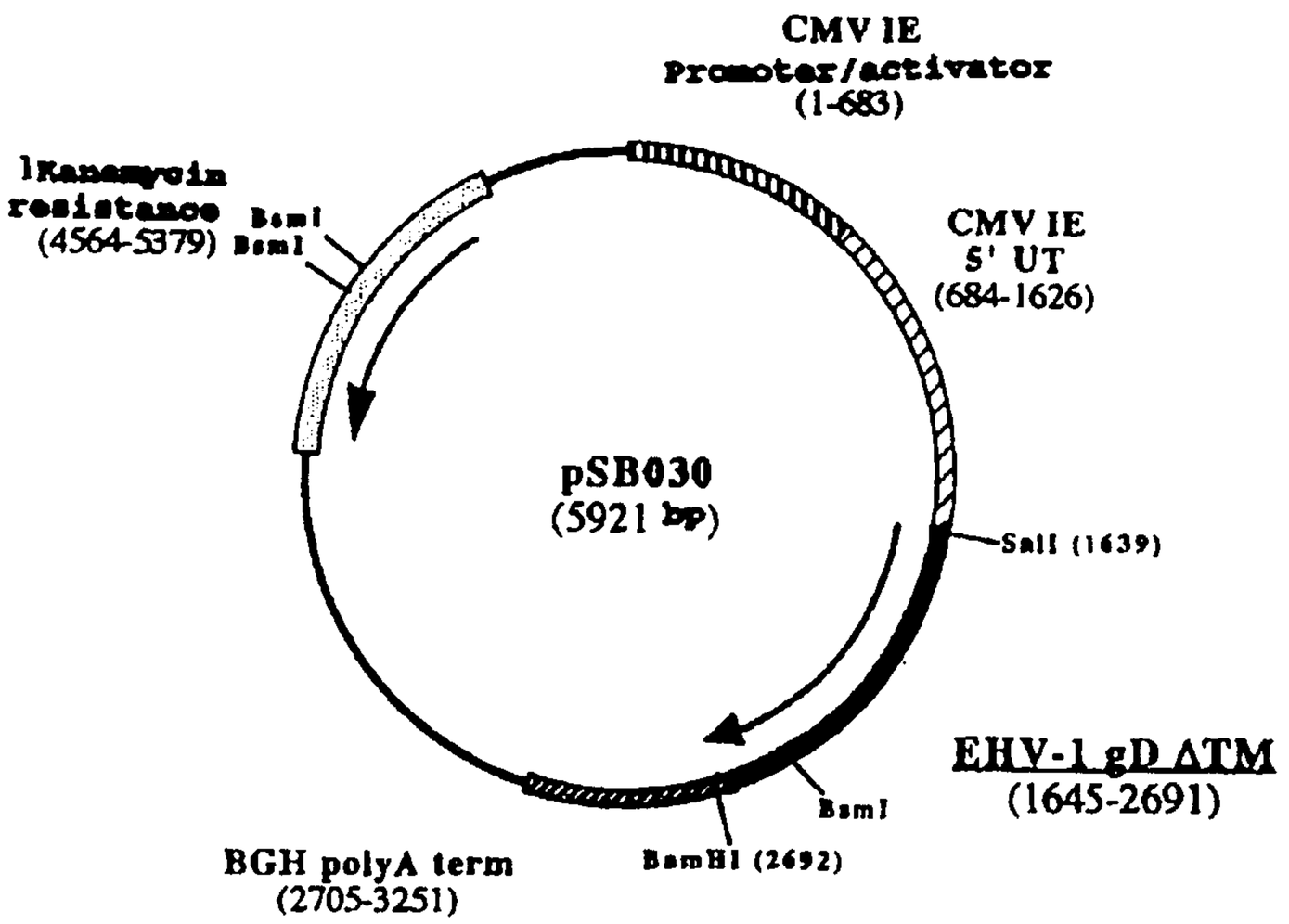
Promoter/activator
(1-683)
Kanamycin
resistance
(4804-5619)
CMV IE
5' UT
(684-1626)
pSB029
(6161 bp)
SalI (1639)
EHV-1 gC ΔTM
(1645-2928)
AspI (2653)
BGH polyA term
(2944-3491)
BamHI (2932)

Figure 15.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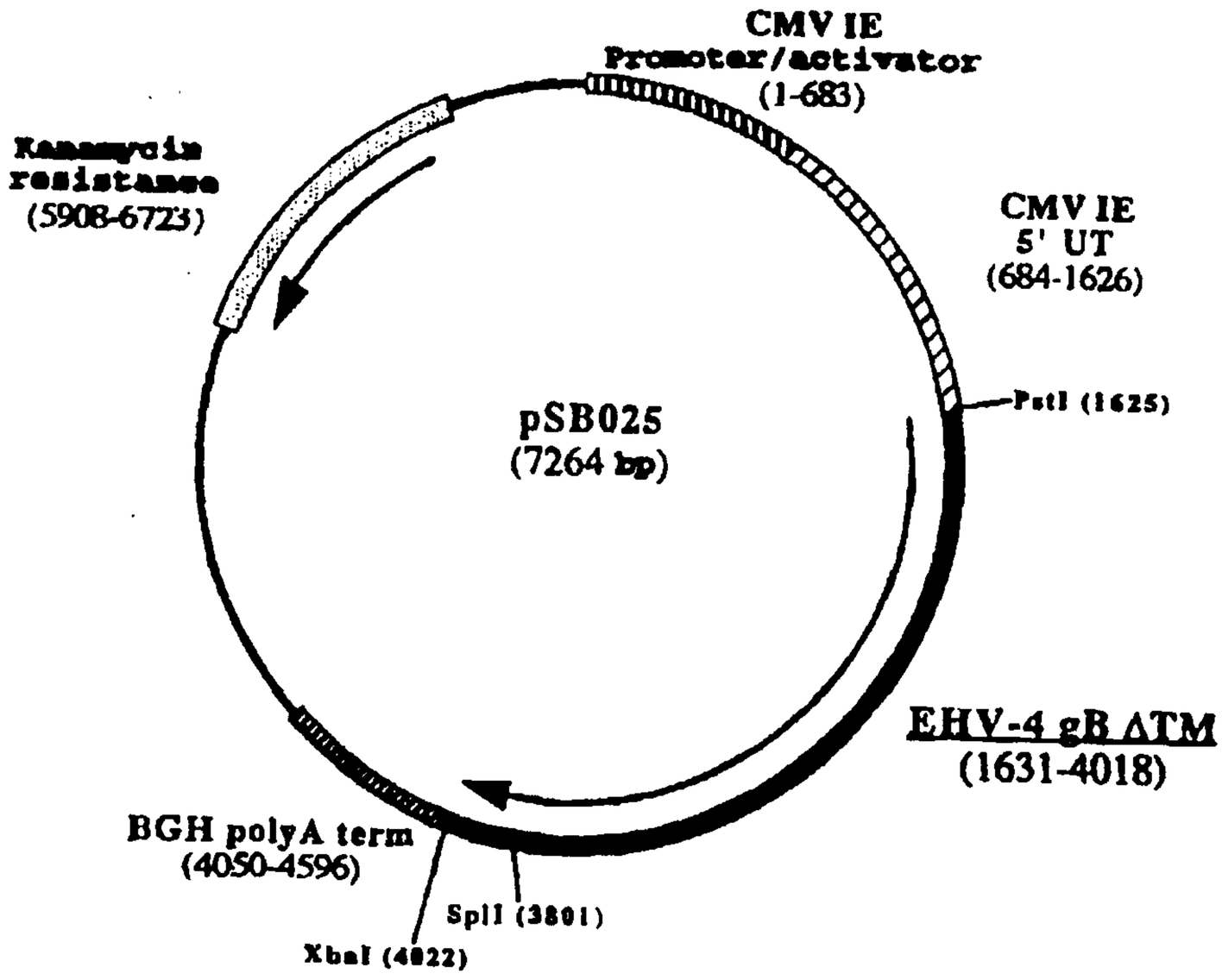
Promoter/activator
(1-683)
lKanamycin
resistance
(4564-5379)
BsmI
BsmI
CMV IE
5' UT
(684-1626)
pSB030
(5921 bp)
SalI (1639)
BsmI
EHV-1 gD ΔTM
(1645-2691)
BamHI (2692)
BGH polyA term
(2705-3251)

Figure 16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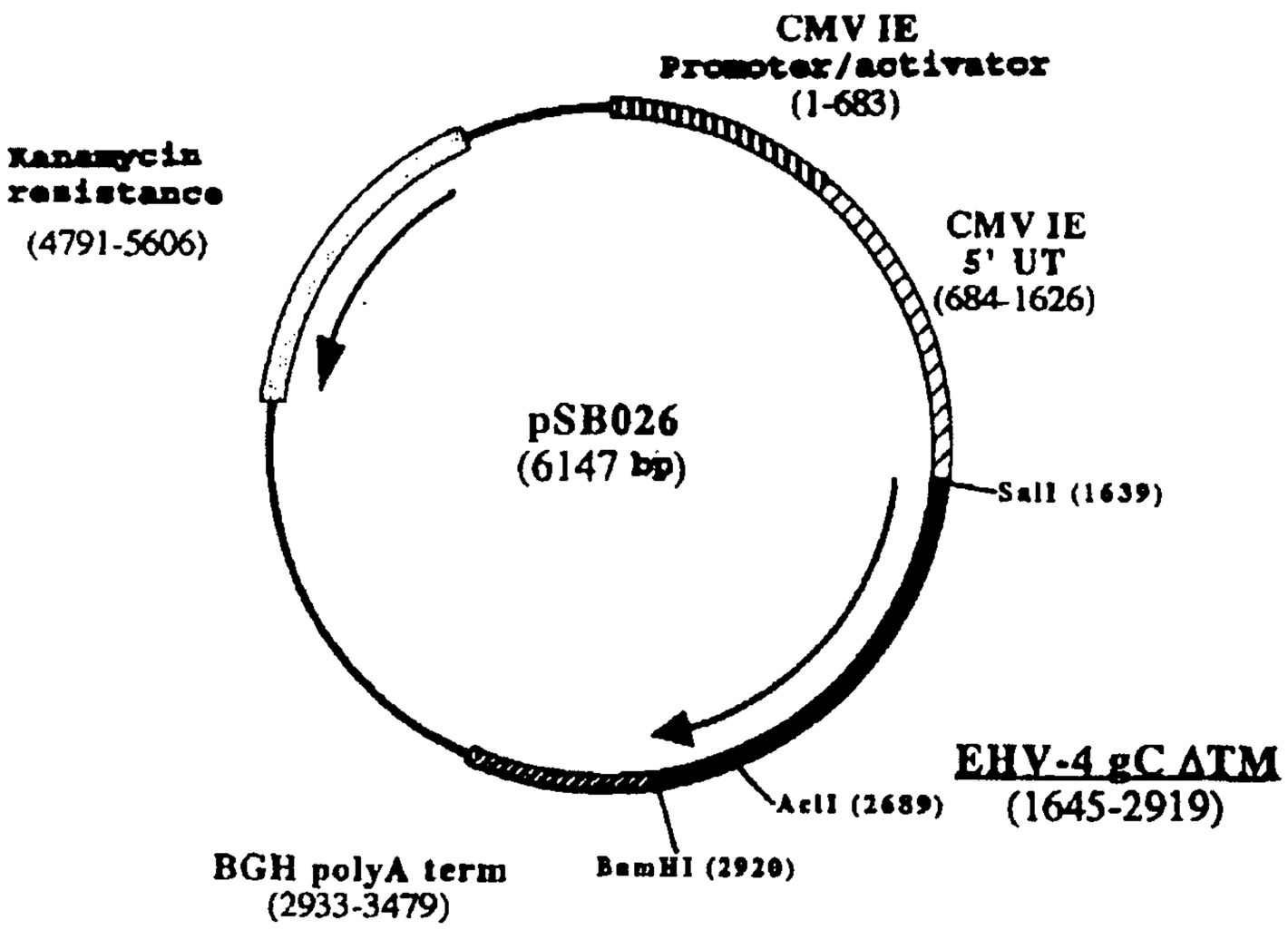
Promoter/activator
(1-683)
Kanamycin
resistance
(5908-6723)
CMV IE
5' UT
(684-1626)
PstI (1625)
pSB025
(7264 bp)
EHV-4 gB ΔTM
(1631-4018)
BGH polyA term
(4050-4596)
SpII (3801)
XbaI (4822)

Figure 17.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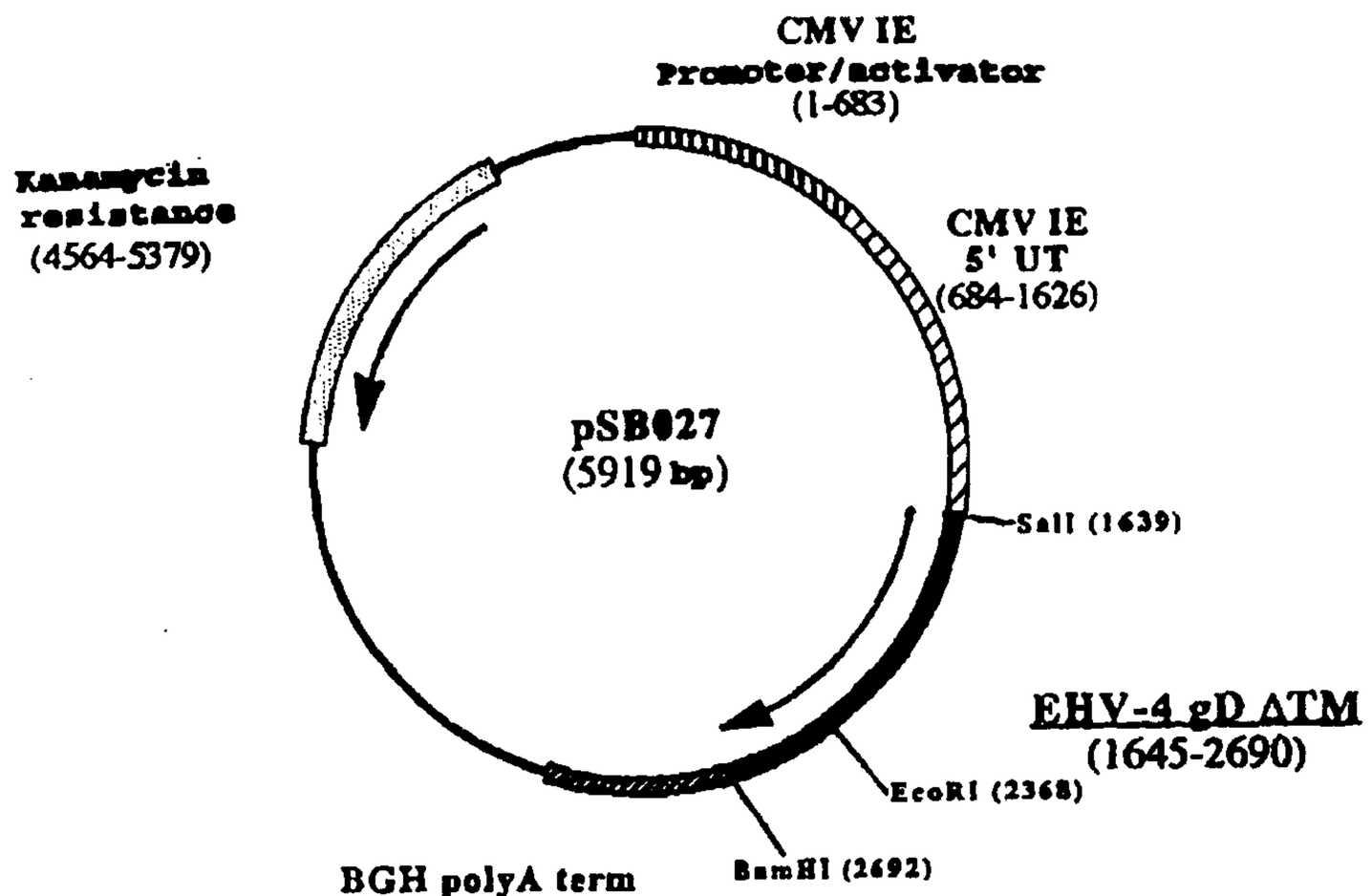
Promoter/activator
(1-683)
Kanamycin
resistance
(4791-5606)
CMV IE
5' UT
(684-1626)
pSB026
(6147 bp)
SalI (1639)
EHV-4 gC ΔTM
(1645-2919)
AclI (2689)
BGH polyA term
(2933-3479)
BamHI (2920)

Figure 18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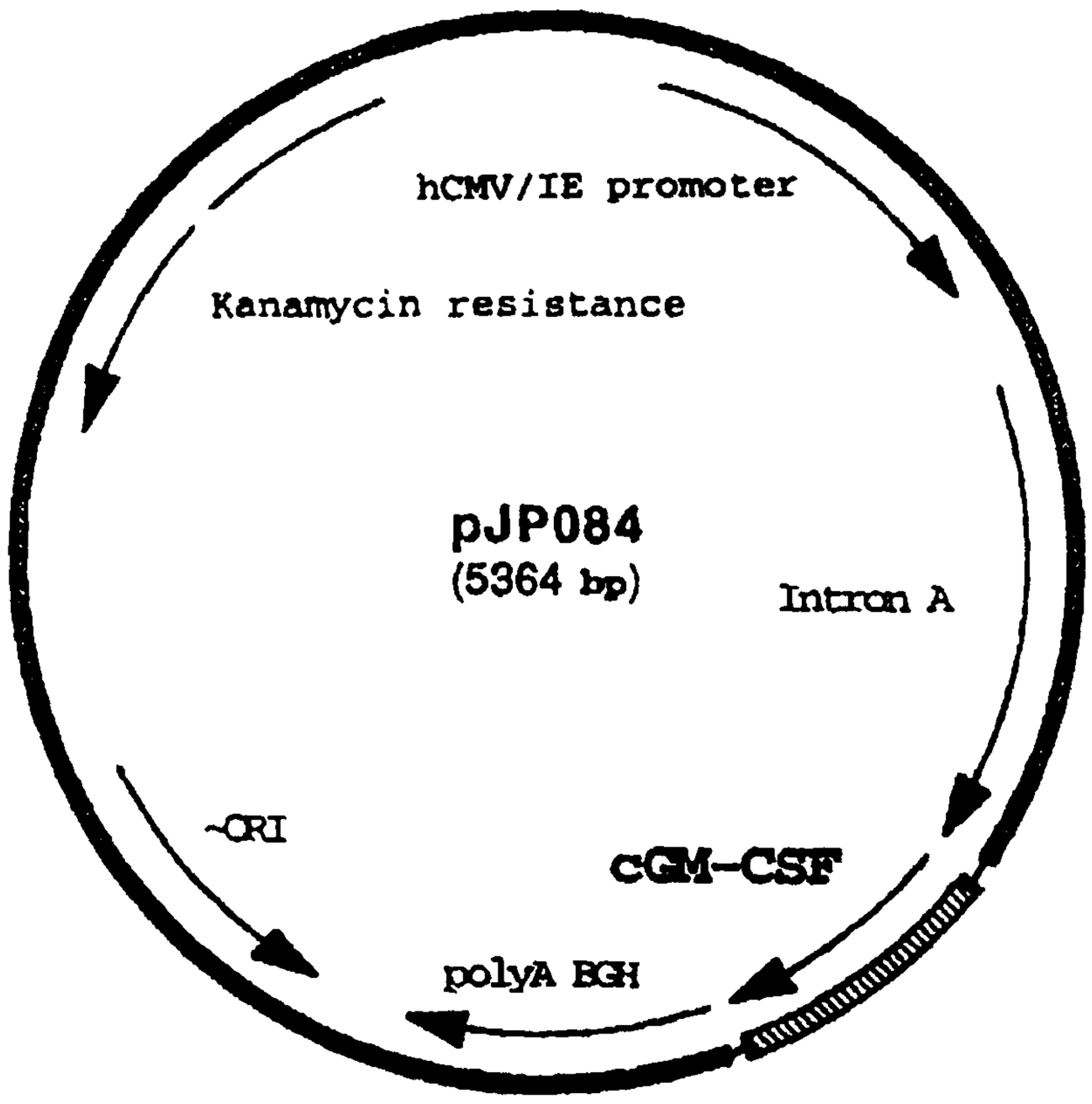
Promoter/activator
(1-683)
Kanamycin
resistance
(4564-5379)
CMV IE
5' UT
(684-1626)
pSB027
(5919 bp)
SalI (1639)
EHV-4 gD ΔTM
(1645-2690)
EcoRI (2368)
BamHI (2692)
BGH polyA term
(2705-3251)

Figure 19.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Kanamycin resistance
pJP084
(5364 bp)
Intron A
-ORI
cGM-CSF
polyA BGH

Figure 20

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      TAC ACC GAC GTC TTG GAC GAA AAG AAC CCG TGA CAC CAG ACG TCG TAG AGA CGT GGG
    1 Met Trp Leu Gln Asn Leu Leu Phe Leu Gly Thr Val Val Cys Ser Ile Ser Ala Pro

1966  ACC CGC TCA CCC ACC CTT GTC ACT CGG CCC TCT CAG CAC GTG GAT GCC ATC CAG GAA
      TGG GCG AGT GGG TGG GAA CAG TGA GCC GGG AGA GTC GTG CAC CTA CGG TAG GTC CTT
   20 Thr Arg Ser Pro Thr Leu Val Thr Arg Pro Ser Gln His Val Asp Ala Ile Gln Glu

2023  GCC CTG AGC CTT TTG AAC AAC AGT AAT GAC GTG ACT GCT GTG ATG AAT AAA GCA GTA
      CGG GAC TCG GAA AAC TTG TTG TCA TTA CTG CAC TGA CGA CAC TAC TTA TTT CGT CAT
   39 Ala Leu Ser Leu Leu Asn Asn Ser Asn Asp Val Thr Ala Val Met Asn Lys Ala Val

2080  AAA GTG GTC TCT GAA GTG TTT GAC CCT GAG GGG CCA ACA TGC CTG GAG ACC CGC CTA
      TTT CAC CAG AGA CTT CAC AAA CTG GGA CTC CCC GGT TGT ACG GAC CTC TGG GCG GAT
   58 Lys Val Val Ser Glu Val Phe Asp Pro Glu Gly Pro Thr Cys Leu Glu Thr Arg Leu

2137  CAG CTG TAC AAG GAG GGC CTG CAG GGC AGC CTC ACC AGC CTC AAG AAT CCC TTA ACC
      GTC GAC ATG TTC CTC CCG GAC GTC CCG TCG GAG TGG TCG GAG TTC TTA GGG AAT TGG
   77 Gln Leu Tyr Lys Glu Gly Leu Gln Gly Ser Leu Thr Ser Leu Lys Asn Pro Leu Thr

2194  ATG ATG GCC AAT CAC TAT AAG CAG CAC TGT CCC CCT ACC CCG GAA TCT CCC TGT GCA
      TAC TAC CGG TTA GTG ATA TTC GTC GTG ACA GGG GGA TGG GGC CTT AGA GGG ACA CGT
   96 Met Met Ala Asn His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu Ser Pro Cys Ala

2251  ACC CAG AAT ATT AAC TTC AAA AGT TTC AAA GAG AAC CTG AAG GAT TTT CTG TTT AAC
      TGG GTC TTA TAA TTG AAG TTT TCA AAG TTT CTC TTG GAC TTC CTA AAA GAC AAA TTG
  115 Thr Gln Asn Ile Asn Phe Lys Ser Phe Lys Glu Asn Leu Lys Asp Phe Leu Phe Asn

2308  ATC CCC TTT GAC TGC TGG AAA CCA GTC AAG AAG TGA
      TAG GGG AAA CTG ACG ACC TTT GGT CAG TTC TTC ACT
  134 Ile Pro Phe Asp Cys Trp Lys Pro Val Lys Lys •••
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Figure 21.

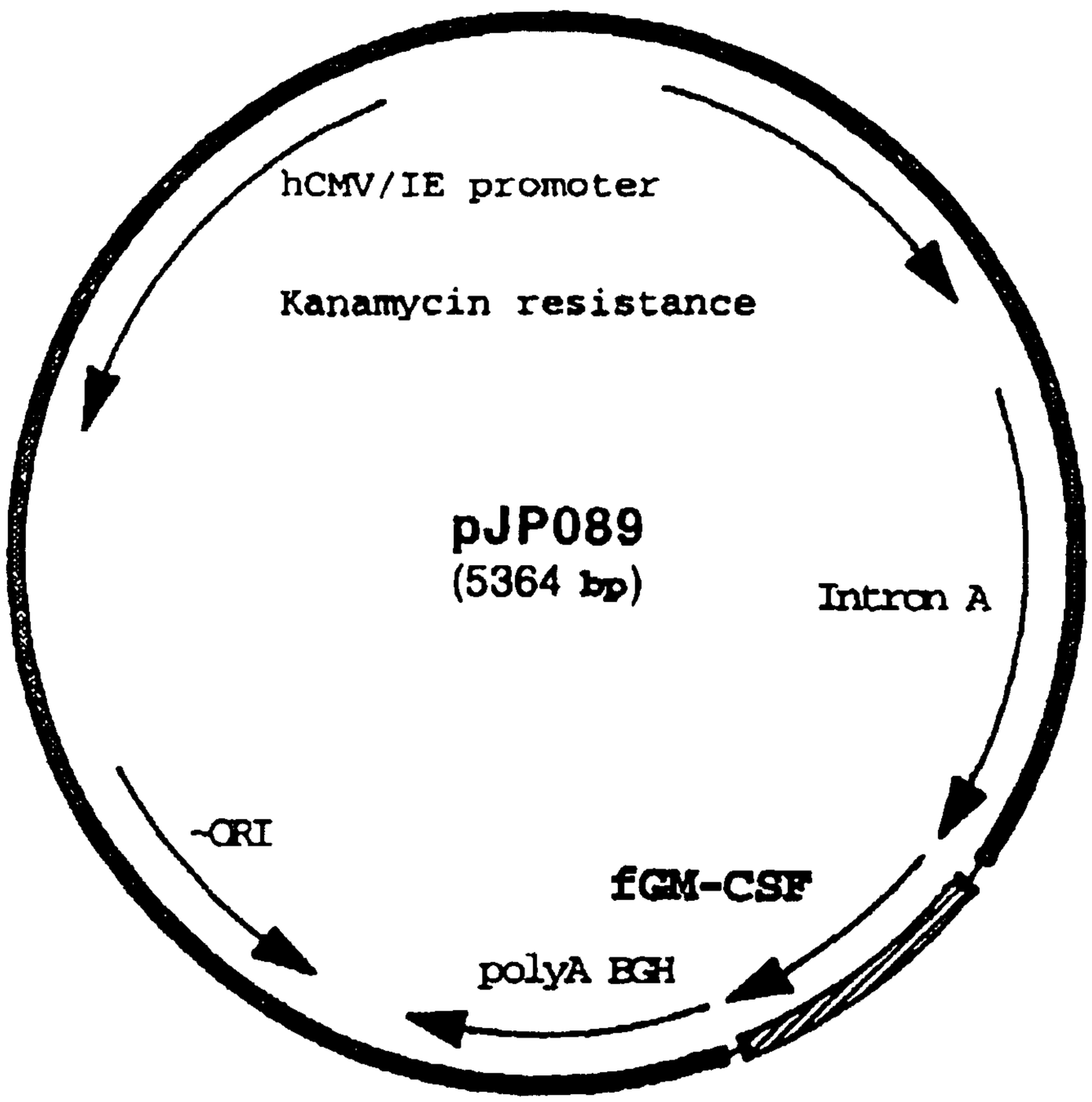
hCMV/IE promoter
Kanamycin resistance
pJP089
(5364 bp)
Intron A
-ORI
fGM-CSF
polyA BGH

Figure 22

```
1909  ATG TGG CTG CAG AAC CTG CTT TTC CTG GGC ACT GTG GTC TGC AGC ATC TCT GCA CCC
        TAC ACC GAC GTC TTG GAC GAA AAG GAC CCG TGA CAC CAG ACG TCG TAG AGA CGT GGG
    1▶Met Trp Leu Gln Asn Leu Leu Phe Leu Gly Thr Val Val Cys Ser Ile Ser Ala Pro

1966  ACC AGT TCA CCC AGC TCT GTC ACT CGG CCC TGG CAA CAC GTG GAT GCC ATC AAG GAG
        TGG TCA AGT GGG TCG AGA CAG TGA GCC GGG ACC GTT GTG CAC CTA CGG TAG TTC CTC
   20▶Thr Ser Ser Pro Ser Ser Val Thr Arg Pro Trp Gln His Val Asp Ala Ile Lys Glu

2023  GCT CTG AGC CTT CTG AAC AAC AGT AGT GAA ATA ACT GCT GTG ATG AAT GAA GCA GTA
        CGA GAC TCG GAA GAC TTG TTG TCA TCA CTT TAT TGA CGA CAC TAC TTA CTT CGT CAT
   39▶Ala Leu Ser Leu Leu Asn Asn Ser Ser Glu Ile Thr Ala Val Met Asn Glu Ala Val

2080  GAA GTC GTC TCT GAA ATG TTT GAC CCT GAG GAG CCG AAA TGC CTG CAG ACT CAC CTA
        CTT CAG CAG AGA CTT TAC AAA CTG GGA CTC CTC GGC TTT ACG GAC GTC TGA GTG GAT
   58▶Glu Val Val Ser Glu Met Phe Asp Pro Glu Glu Pro Lys Cys Leu Gln Thr His Leu

2137  AAG CTG TAC GAG CAG GGC CTA CGG GGC AGC CTC ATC AGC CTC AAG GAG CCT CTG AGA
        TTC GAC ATG CTC GTC CCG GAT GCC CCG TCG GAG TAG TCG GAG TTC CTC GGA GAC TCT
   77▶Lys Leu Tyr Glu Gln Gly Leu Arg Gly Ser Leu Ile Ser Leu Lys Glu Pro Leu Arg

2194  ATG ATG GCC AAC CAT TAC AAG CAG CAC TGC CCC TTT ACT CCG GAA ACG CCC TGT GAA
        TAC TAC CGG TTG GTA ATG TTC GTC GTG ACG GGG AAA TGA GGC CTT TGC GGG ACA CTT
   96▶Met Met Ala Asn His Tyr Lys Gln His Cys Pro Phe Thr Pro Glu Thr Pro Cys Glu

2251  ACC CAG ACT ATC ACC TTC AAA AAT TTC AAA GAG AAT CTG AAG GAT TTT CTG TTT AAC
        TGG GTC TGA TAG TGG AAG TTT TTA AAG TTT CTC TTA GAC TTC CTA AAA GAC AAA TTG
  115▶Thr Gln Thr Ile Thr Phe Lys Asn Phe Lys Glu Asn Leu Lys Asp Phe Leu Phe Asn

2308  ATC CCC TTT GAC TGC TGG AAA CCA GTC AAG AAG TGA
        TAG GGG AAA CTG ACG ACC TTT GGT CAG TTC TTC ACT
  134▶Ile Pro Phe Asp Cys Trp Lys Pro Val Lys Lys •••
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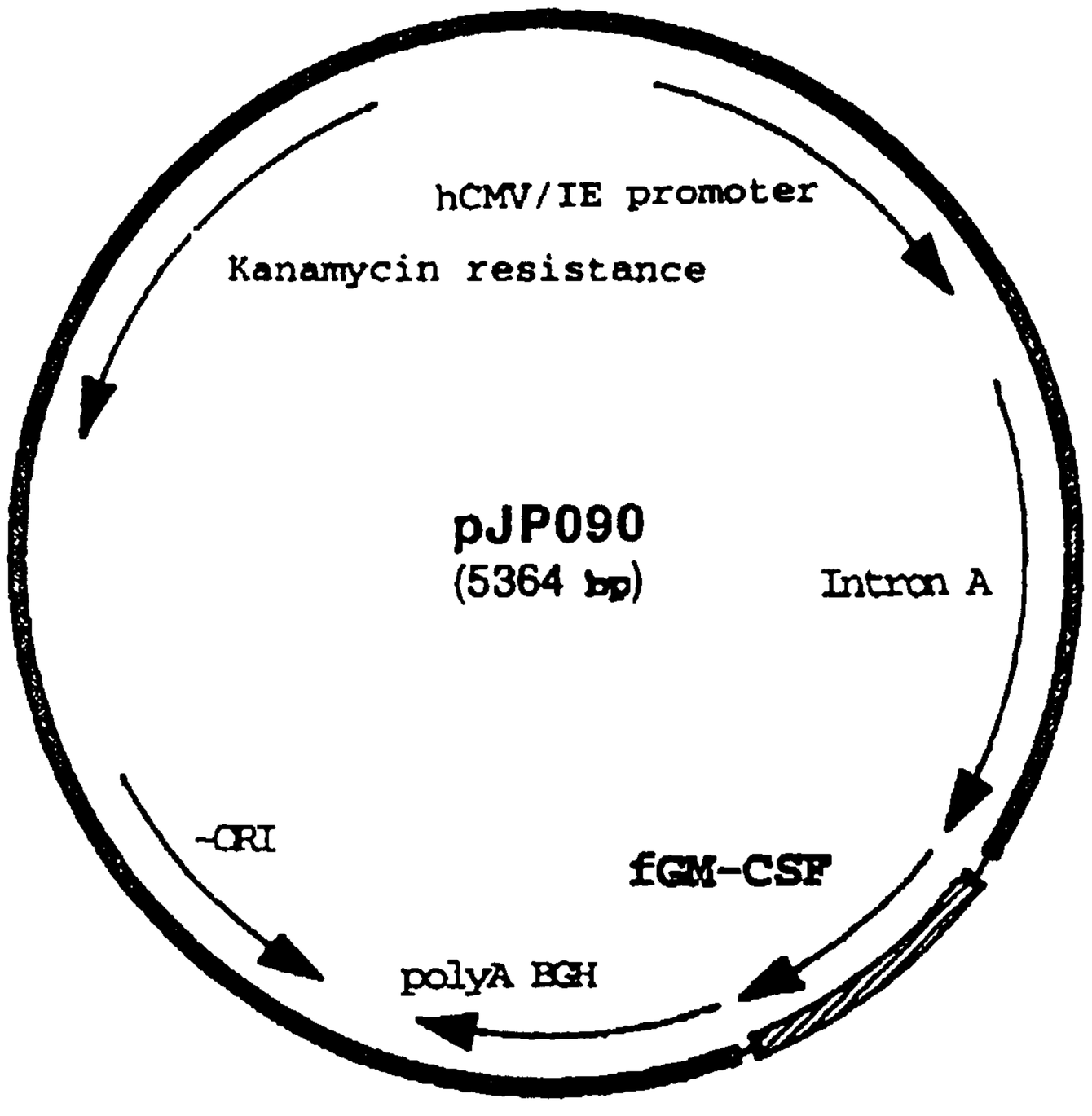
Figure 23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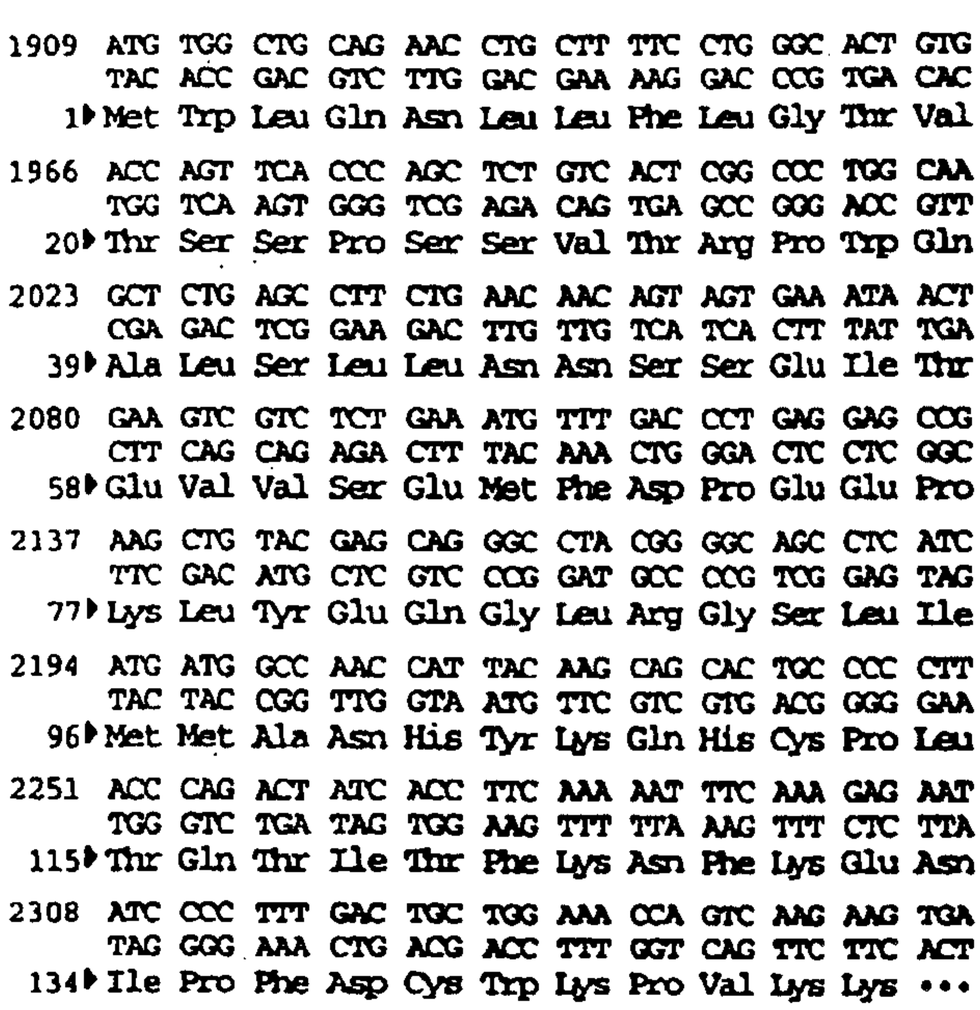
Kanamycin resistance
pJP090
(5364 bp)
Intron A
-ORI
fGM-CSF
polyA BGH

Figure 24



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      TAC ACC GAC GTC TTG GAC GAA AAG GAC CCG TGA CAC CAG ACG TCG TAG AGA CGT GGG
   1▶ Met Trp Leu Gln Asn Leu Leu Phe Leu Gly Thr Val Val Cys Ser Ile Ser Ala Pro

1966  ACC AGT TCA CCC AGC TCT GTC ACT CGG CCC TGG CAA CAC GTG GAT GCC ATC AAG GAG
      TGG TCA AGT GGG TCG AGA CAG TGA GCC GGG ACC GTT GTG CAC CTA CGG TAG TTC CTC
  20▶ Thr Ser Ser Pro Ser Ser Val Thr Arg Pro Trp Gln His Val Asp Ala Ile Lys Glu

2023  GCT CTG AGC CTT CTG AAC AAC AGT AGT GAA ATA ACT GCT GTG ATG AAT GAA GCA GTA
      CGA GAC TCG GAA GAC TTG TTG TCA TCA CTT TAT TGA CGA CAC TAC TTA CTT CGT CAT
  39▶ Ala Leu Ser Leu Leu Asn Asn Ser Ser Glu Ile Thr Ala Val Met Asn Glu Ala Val

2080  GAA GTC GTC TCT GAA ATG TTT GAC CCT GAG GAG CCG AAA TGC CTG CAG ACT CAC CTA
      CTT CAG CAG AGA CTT TAC AAA CTG GGA CTC CTC GGC TTT ACG GAC GTC TGA GTG GAT
  58▶ Glu Val Val Ser Glu Met Phe Asp Pro Glu Glu Pro Lys Cys Leu Gln Thr His Leu

2137  AAG CTG TAC GAG CAG GGC CTA CGG GGC AGC CTC ATC AGC CTC AAG GAG CCT CTG AGG
      TTC GAC ATG CTC GTC CCG GAT GCC CCG TCG GAG TAG TCG GAG TTC CTC GGA GAC TCC
  77▶ Lys Leu Tyr Glu Gln Gly Leu Arg Gly Ser Leu Ile Ser Leu Lys Glu Pro Leu Arg

2194  ATG ATG GCC AAC CAT TAC AAG CAG CAC TGC CCC CTT ACT CCG GAA ACG CCC TGT GAA
      TAC TAC CGG TTG GTA ATG TTC GTC GTG ACG GGG GAA TGA GGC CTT TGC GGG ACA CTT
  96▶ Met Met Ala Asn His Tyr Lys Gln His Cys Pro Leu Thr Pro Glu Thr Pro Cys Glu

2251  ACC CAG ACT ATC ACC TTC AAA AAT TTC AAA GAG AAT CTG AAG GAT TTT CTG TTT AAC
      TGG GTC TGA TAG TGG AAG TTT TTA AAG TTT CTC TTA GAC TTC CTA AAA GAC AAA TTG
 115▶ Thr Gln Thr Ile Thr Phe Lys Asn Phe Lys Glu Asn Leu Lys Asp Phe Leu Phe Asn

2308  ATC CCC TTT GAC TGC TGG AAA CCA GTC AAG AAG TGA
      TAG GGG AAA CTG ACG ACC TTT GGT CAG TTC TTC ACT
 134▶ Ile Pro Phe Asp Cys Trp Lys Pro Val Lys Lys •••
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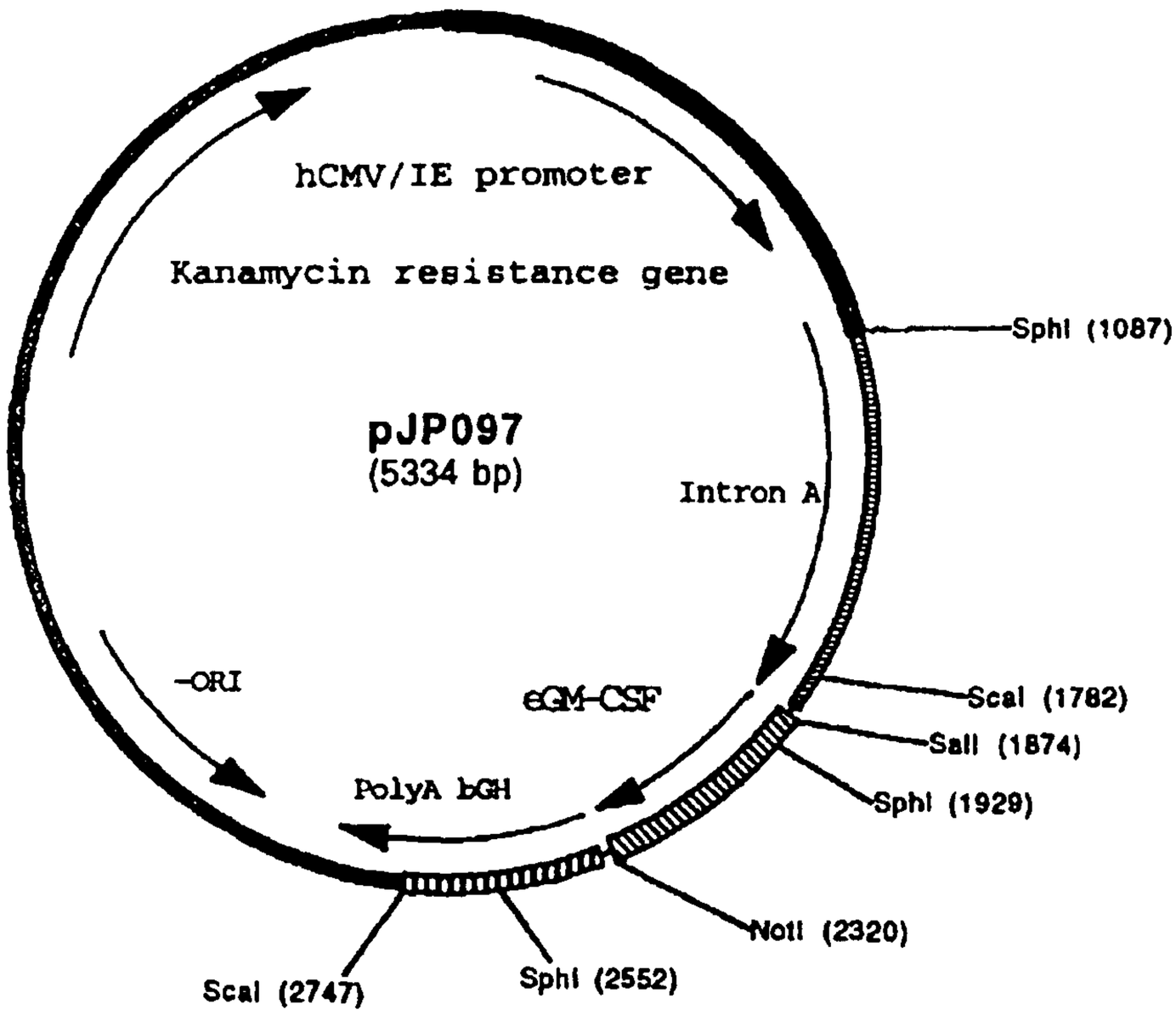
Figure 25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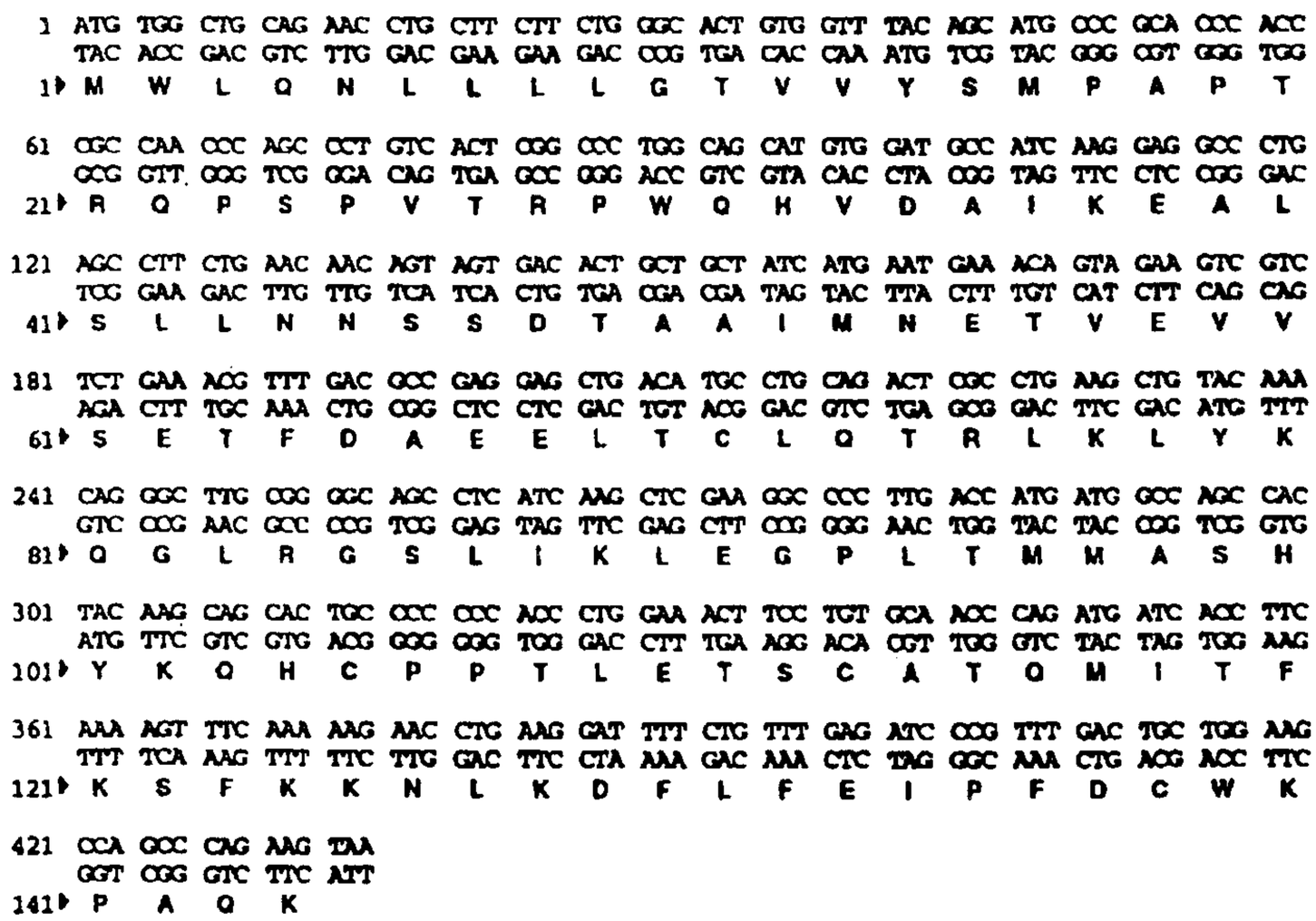
Kanamycin resistance gene
pJP097
(5334 bp)
Intron A
SphI (1087)
-ORI
eGM-CSF
ScaI (1782)
SalI (1874)
SphI (1929)
PolyA bGH
NotI (2320)
ScaI (2747)
SphI (2552)

Figure 26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TAC ACC GAC GTC TTG GAC GAA GAA GAC CCG TGA CAC CAA ATG TCG TAC GGG CGT GGG TGG
1 M W L Q N L L L L G T V V Y S M P A P T
61 CGC CAA CCC AGC CCT GTC ACT CGG CCC TGG CAG CAT GTG GAT GCC ATC AAG GAG GCC CTG
GCG GTT GGG TCG GGA CAG TGA GCC GGG ACC GTC GTA CAC CTA CGG TAG TTC CTC CGG GAC
21 R Q P S P V T R P W Q H V D A I K E A L
121 AGC CTT CTG AAC AAC AGT AGT GAC ACT GCT GCT ATC ATG AAT GAA ACA GTA GAA GTC GTC
TCG GAA GAC TTG TTG TCA TCA CTG TGA CGA CGA TAG TAC TTA CTT TGT CAT CTT CAG CAG
41 S L L N N S S D T A A I M N E T V E V V
181 TCT GAA ACG TTT GAC GCC GAG GAG CTG ACA TGC CTG CAG ACT CGC CTG AAG CTG TAC AAA
AGA CTT TGC AAA CTG CGG CTC CTC GAC TGT ACG GAC GTC TGA GCG GAC TTC GAC ATG TTT
61 S E T F D A E E L T C L Q T R L K L Y K
241 CAG GGC TTG CGG GGC AGC CTC ATC AAG CTC GAA GGC CCC TTG ACC ATG ATG GCC AGC CAC
GTC CCG AAC GCC CCG TCG GAG TAG TTC GAG CTT CCG GGG AAC TGG TAC TAC CGG TCG GTG
81 Q G L R G S L I K L E G P L T M M A S H
301 TAC AAG CAG CAC TGC CCC CCC ACC CTG GAA ACT TCC TGT GCA ACC CAG ATG ATC ACC TTC
ATG TTC GTC GTG ACG GGG GGG TGG GAC CTT TGA AGG ACA CGT TGG GTC TAC TAG TGG AAG
101 Y K Q H C P P T L E T S C A T Q M I T F
361 AAA AGT TTC AAA AAG AAC CTG AAG GAT TTT CTG TTT GAG ATC CCG TTT GAC TGC TGG AAG
TTT TCA AAG TTT TTC TTG GAC TTC CTA AAA GAC AAA CTC TAG GGC AAA CTG ACG ACC TTC
121 K S F K K N L K D F L F E I P F D C W K
421 CCA GCC CAG AAG TAA
GGT CGG GTC TTC ATT
141 P A Q K

Figure 27

```
   1 ATGGGTACTATAATTCAATTTCTGGTGGTCTCCTGTCTATTGGCAGGAGCAGGCAGCCTTGATCTAGCAGCGCTC
   1 MetGlyThrIleIleGlnPheLeuValValSerCysLeuLeuAlaGlyAlaGlySerLeuAspLeuAlaAlaLeu

  76 ATGCAAATCGGTGTCATTCCAACAAATGTCCGGCAACTTATGTATTATACTGAGGCCTCATCAGCATTCATTGTT
  26 MetGlnIleGlyValIleProThrAsnValArgGlnLeuMetTyrTyrThrGluAlaSerSerAlaPheIleVal

 151 GTGAAGTTAATGCCTACAATTGACTCGCCGATTAGTGGATGTAATATAACATCAATTTCAAGCTATAATGCAACA
  51 ValLysLeuMetProThrIleAspSerProIleSerGlyCysAsnIleThrSerIleSerSerTyrAsnAlaThr

 226 GTGACAAAACTCCTACAGCCGATCGGTGAGAATTTGGAAACGATTAGGAACCAGTTGATTCCAACTCGGAGGAGA
  76 ValThrLysLeuLeuGlnProIleGlyGluAsnLeuGluThrIleArgAsnGlnLeuIleProThrArgArgArg

 301 CGCCGGTTTGCAGGGGTGGTGATTGGATTAGCTGCATTAGGAGTAGCTACTGCCGCACAGGTCACTGCCGCAGTA
 101 ArgArgPheAlaGlyValValIleGlyLeuAlaAlaLeuGlyValAlaThrAlaAlaGlnValThrAlaAlaVal

 376 GCACTAGTAAAGGCAAATAAAAATGCTGCGGCTATACTCAATCTCAAAAATGCAATCCAAAAAACAAATACAGCA
 126 AlaLeuValLysAlaAsnLysAsnAlaAlaAlaIleLeuAsnLeuLysAsnAlaIleGlnLysThrAsnThrAla

 451 GTTGCAGATGTGGTCCAGGCCACACAATCACTAGGAACGGCAGTTCAAGCAGTTCAAGATCACATAAACAGTGTG
 151 ValAlaAspValValGlnAlaThrGlnSerLeuGlyThrAlaValGlnAlaValGlnAspHisIleAsnSerVal

 526 GTAAGTCCAGCAATTACAGCAGCCAATTGTAAGGCCCAAGATGCTATCATTGGCTCAATCCTCAATCTCTATTTG
 176 ValSerProAlaIleThrAlaAlaAsnCysLysAlaGlnAspAlaIleIleGlySerIleLeuAsnLeuTyrLeu

 601 ACCGAGTTGACAACTATCTTCCACAATCAAATTACAAACCCTGCATTGAGTCCTATTACAATTCAAGCTTTAAGG
 201 ThrGluLeuThrThrIlePheHisAsnGlnIleThrAsnProAlaLeuSerProIleThrIleGlnAlaLeuArg

 676 ATCCTACTGGGGAGTACCTTGCCGACTGTGGTCGAAAAATCTTTCAATACCCAGATAAGTGCAGCTGAGCTTCTC
 226 IleLeuLeuGlySerThrLeuProThrValValGluLysSerPheAsnThrGlnIleSerAlaAlaGluLeuLeu

 751 TCATCAGGGTTATTGACAGGCCAGATTGTGGGATTAGATTTGACCTATATGCAGATGGTCATAAAAATTGAGCTG
 251 SerSerGlyLeuLeuThrGlyGlnIleValGlyLeuAspLeuThrTyrMetGlnMetValIleLysIleGluLeu

 826 CCAACTTTAACTGTACAACCTGCAACCCAGATCATAGATCTGGCCACCATTTCTGCATTCATTAACAATCAAGAA
 276 ProThrLeuThrValGlnProAlaThrGlnIleIleAspLeuAlaThrIleSerAlaPheIleAsnAsnGlnGlu

 901 GTCATGGCCCAATTACCAACACGTGTTATTGTGACTGGCAGCTTGATCCAAGCCTATCCCGCATCGCAATGCACT
 301 ValMetAlaGlnLeuProThrArgValIleValThrGlySerLeuIleGlnAlaTyrProAlaSerGlnCysThr

 976 ATTACACCCAACACTGTGTACTGTAGGTATAATGATGCCCAAGTACTCTCAGATGATACGATGGCTTGCCTCCAA
 326 IleThrProAsnThrValTyrCysArgTyrAsnAspAlaGlnValLeuSerAspAspThrMetAlaCysLeuGln

1051 GGTAACTTGACAAGATGCACCTTCTCTCCAGTGGTTGGGAGCTTTCTCACTCGATTCGTGCTGTTCGATGGAATA
 351 GlyAsnLeuThrArgCysThrPheSerProValValGlySerPheLeuThrArgPheValLeuPheAspGlyIle

1126 GTTTATGCAAATTGCAGGTCGATGTTATGCAAGTGCATGCAGCCTGCTGCTGTGATCCTACAGCCGAGTTCATCC
 376 ValTyrAlaAsnCysArgSerMetLeuCysLysCysMetGlnProAlaAlaValIleLeuGlnProSerSerSer

1201 CCTGTAACTGTCATTGACATGTACAAATGTGTGAGTCTGCAGCTTGACAATCTCAGATTCACCATCACTCAATTG
 401 ProValThrValIleAspMetTyrLysCysValSerLeuGlnLeuAspAsnLeuArgPheThrIleThrGlnLeu
```

(Fig. 27)

```
1276 GCCAATGTAACCTACAATAGCACCATCAAGCTTGAAACATCCCAGATCTTGCCTATTGATCCGTTGGATATATCC
 426 AlaAsnValThrTyrAsnSerThrIleLysLeuGluThrSerGlnIleLeuProIleAspProLeuAspIleSer

1351 CAGAATCTAGCTGCGGTGAATAAGAGTCTAAGTGATGCACTACAACACTTAGCACAAAGTGACACATACCTTTCT
 451 GlnAsnLeuAlaAlaValAsnLysSerLeuSerAspAlaLeuGlnHisLeuAlaGlnSerAspThrTyrLeuSer

1426 GCAATCACATCAGCTACGACTACAAGTGTATTATCCATAATGGCAATCTGTCTTGGATCGTTAGGTTTAATATTA
 476 AlaIleThrSerAlaThrThrThrSerValLeuSerIleMetAlaIleCysLeuGlySerLeuGlyLeuIleLeu

1501 ATAATCTTGCTCAGTGTAGTTGTGTGGAAGTTATTGACCATTGTCACTGCTAATCGAAATAGAATGGAGAATTTT
 501 IleIleLeuLeuSerValValValTrpLysLeuLeuThrIleValThrAlaAsnArgAsnArgMetGluAsnPhe

1576 GTTTATCATAAATAA
 526 ValTyrHisLys***
```

Figure 28

```
   1 ATGGTTGCAGAAGATGCCCCTGTTAGGGGCACTTGCCGAGTATTATTTCGAACAACAACTTTAATTTTTCT
   1 MetValAlaGluAspAlaProValArgGlyThrCysArgValLeuPheArgThrThrThrLeuIlePheLe

  72 ATGCACACTACTAGCATTAAGCATCTCTATCCTTTATGAGAGTTTAATAACCCAAAAGCAAATCATGAGCC
  24 uCysThrLeuLeuAlaLeuSerIleSerIleLeuTyrGluSerLeuIleThrGlnLysGlnIleMetSerH

 143 ACGCAGGCTCAACTGGATCTAATTCTAGATTAGGAAGTATCACTGATCTTCTTAATAATATTCTCTCTGTC
  48 isAlaGlySerThrGlySerAsnSerArgLeuGlySerIleThrAspLeuLeuAsnAsnIleLeuSerVal

 214 GCAAATCAGATTATATATAACTCTGCAGTCGCTCTACCTCTACAATTGGACACTCTTGAATCAACACTCCT
  72 AlaAsnGlnIleIleTyrAsnSerAlaValAlaLeuProLeuGlnLeuAspThrLeuGluSerThrLeuLe

 285 TACAGCCATTAAGTCTCTTCAAACCAGTGACAAGCTAGAACAGAACTGCTCGTGGGGTGCTGCACTGATTA
  95 uThrAlaIleLysSerLeuGlnThrSerAspLysLeuGluGlnAsnCysSerTrpGlyAlaAlaLeuIleA

 356 ATAATAATAGATACATTAATGGCATCAATCAGTTCTATTTCTCAATTGCTGAGGGTCGCAATCTGACACTT
 119 snAsnAsnArgTyrIleAsnGlyIleAsnGlnPheTyrPheSerIleAlaGluGlyArgAsnLeuThrLeu

 427 GGCCCACTTCTTAATATACCTAGTTTCATTCCAACTGCCACGACACCAGAGGGCTGCACCAGGATCCCATC
 143 GlyProLeuLeuAsnIleProSerPheIleProThrAlaThrThrProGluGlyCysThrArgIleProSe

 498 ATTCTCGCTCACCAAGACACACTGGTGTTATACACACAATGTTATCCTGAATGGATGCCAGGATCATGTAT
 166 rPheSerLeuThrLysThrHisTrpCysTyrThrHisAsnValIleLeuAsnGlyCysGlnAspHisVals

 569 CCTCAAATCAATTTGTTTCCATGGGAATCATTGAACCCACTTCTGCCGGGTTTCCATCCTTTCGAACCCTA
 190 erSerAsnGlnPheValSerMetGlyIleIleGluProThrSerAlaGlyPheProSerPheArgThrLeu

 640 AAGACTCTATATCTCAGCGATGGGGTCAATCGTAAGAGCTGCTCTATCAGTACAGTTCCGGGGGGTTGTAT
 214 LysThrLeuTyrLeuSerAspGlyValAsnArgLysSerCysSerIleSerThrValProGlyGlyCysMe

 711 GATGTACTGTTTTGTCTCTACTCAACCAGAGAGGGATGACTACTTTTCTACCGCTCCTCCAGAACAACGAA
 237 tMetTyrCysPheValSerThrGlnProGluArgAspAspTyrPheSerThrAlaProProGluGlnArgI

 782 TTATTATAATGTACTATAATGATACAATCGTGGAGCGCATAATTAATCCACCCGGGGTACTAGATGTATGG
 261 leIleIleMetTyrTyrAsnAspThrIleValGluArgIleIleAsnProProGlyValLeuAspValTrp

 853 GCAACATTGACCCCAGGAACAGGAAGCGGGGTATATTATTTAGGTTGGGTGCTCTTTCCAATATATGGCGG
 285 AlaThrLeuThrProGlyThrGlySerGlyValTyrTyrLeuGlyTrpValLeuPheProIleTyrGlyGl

 924 CGTGATTAAAGATACGAGTTTATGGAATAATCAAGCAAATAAATACTTTATCCCCCAGATGGTTGCTGCTC
 308 yValIleLysAspThrSerLeuTrpAsnAsnGlnAlaAsnLysTyrPheIleProGlnMetValAlaAlaL

 995 TCTGCTCACAAAACCAGGCAACTCAAGTCCAAAATGCTAAGTCATCATACTATAGCAGCTGGTTTGGCAAT
 332 euCysSerGlnAsnGlnAlaThrGlnValGlnAsnAlaLysSerSerTyrTyrSerSerTrpPheGlyAsn

1066 CGAATGATTCAGTCTGGGATCCTGGCATGTCCTCTTCAACAGGATCTAACCAATGAGTGTTTAGTTCTGCC
 356 ArgMetIleGlnSerGlyIleLeuAlaCysProLeuGlnGlnAspLeuThrAsnGluCysLeuValLeuPr

1137 CTTTTCTAATGATCAGGTGCTTATGGGTGCTGAAGGGAGATTATACATGTATGGTGACTCGGTGTATTACT
 379 oPheSerAsnAspGlnValLeuMetGlyAlaGluGlyArgLeuTyrMetTyrGlyAspSerValTyrTyrT
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(Fig. 28)

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1208 ACCAAAGAAGCACTAGTTGGTGGCCTATGACCATGCTGTATAAGGTAACCATAACATTCACTAATGGTCAG
 403 yrGlnArgSerThrSerTrpTrpProMetThrMetLeuTyrLysValThrIleThrPheThrAsnGlyGln

1279 CCATCTGCTATATCAGCTCAGAATGTGCCCACACAGCAGGTCCCTAGACCTGGGACAGGAGGCTGCTCTGC
 427 ProSerAlaIleSerAlaGlnAsnValProThrGlnGlnValProArgProGlyThrGlyGlyCysSerAl

1350 AACAAATAGATGTCCCGGTTTTTGCTTGAAAGGAGTGTATGCTGATGCCTGGTTACTGACCAACCCTTCGT
 450 aThrAsnArgCysProGlyPheCysLeuLysGlyValTyrAlaAspAlaTrpLeuLeuThrAsnProSerS

1421 CTACCAGTACATTTGGATCAGAAGCAACCTTCACTGGTTCTTATCTCAACGCAGCAACTCAGCGTATCAAT
 474 erThrSerThrPheGlySerGluAlaThrPheThrGlySerTyrLeuAsnAlaAlaThrGlnArgIleAsn

1492 CCGACGATGTATATCGCGAACAACACACAGATCATAAGCTCACAGCAATTTGGATCAAGCGGTCAAGAAGC
 498 ProThrMetTyrIleAlaAsnAsnThrGlnIleIleSerSerGlnGlnPheGlySerSerGlyGlnGluAl

1563 AGCATATAGCCACACAACTTGTTTTAGGGACACAGGCTCTGTTATGGTATACTGTATCTATATTATTGAAT
 521 aAlaTyrSerHisThrThrCysPheArgAspThrGlySerValMetValTyrCysIleTyrIleIleGluL

1634 TGTCCTCATCTCTCTTAGGACAATTTCAGATTGTCCCATTTATCCGTCAGGTGACACTATCCTAA
 545 euSerSerSerLeuLeuGlyGlnPheGlnIleValProPheIleArgGlnValThrLeuSer•••
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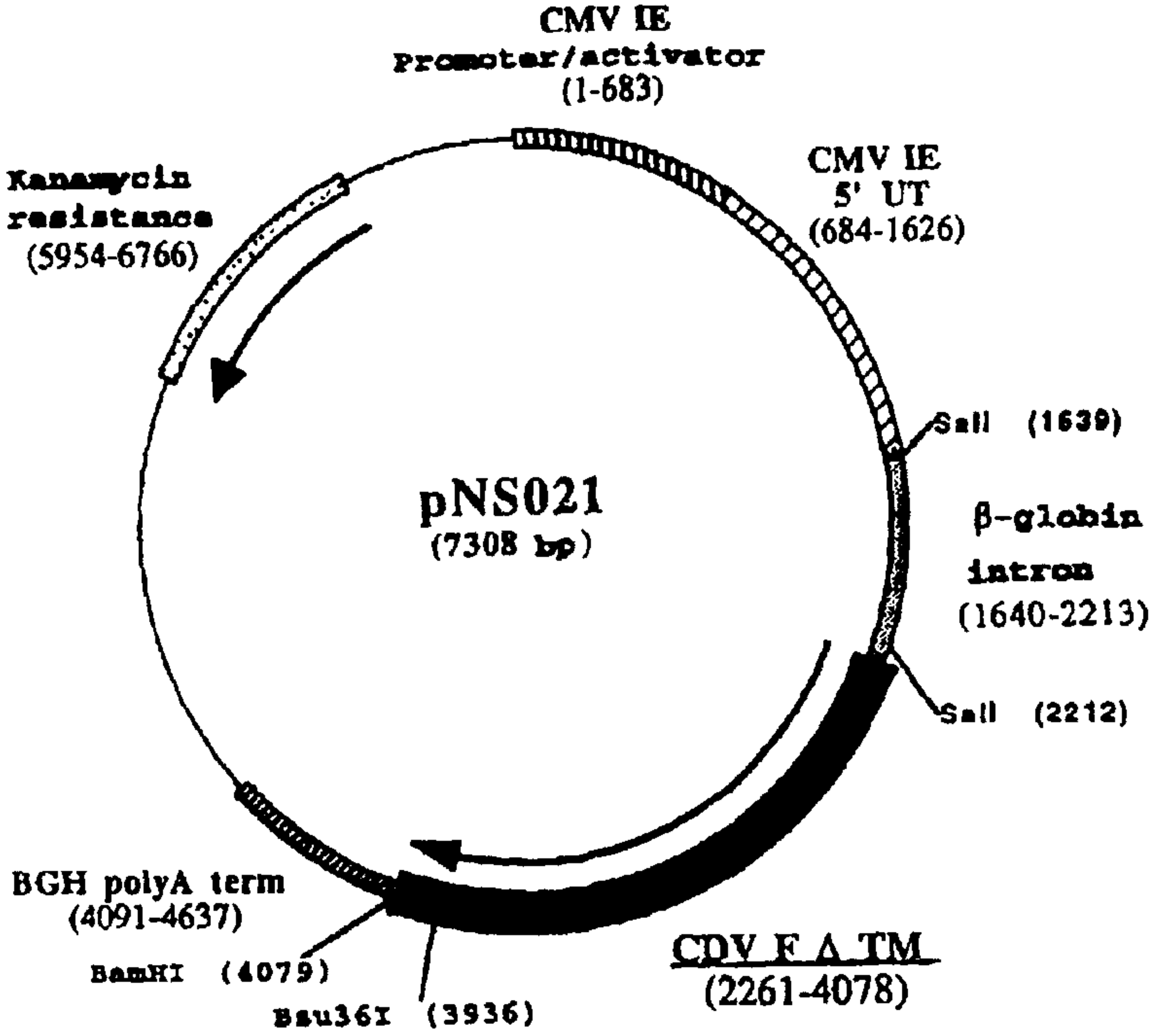
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