(12) PATENT (11) Application No. AU 199540715 B2
(19) AUSTRALIAN PATENT OFFICE (10) Patent No. 708077

(54) Title
Live recombinant avian vaccine using an avian herpesvirus as vector

(51)[6] International Patent Classification(s)
C12N 015/86 A61K 039/245
A61K 039/12 A61K 039/255

(21) Application No: 199540715 (22) Application Date: 1995.12.28

(30) Priority Data

(31) Number	(32) Date	(33) Country
94/16017	1994.12.30	FR

(43) Publication Date : 1996.07.11
(43) Publication Journal Date : 1996.07.11
(44) Accepted Journal Date : 1999.07.29

(71) Applicant(s)
Merial

(72) Inventor(s)
Jean-Christophe Francis Audonnet; Michel Joseph Marie Bublot; Raphael Jean Darteil ; Carole Veronique Duinat; Eliane Louise Francois Laplace; Michel Albert Emile Riviere

(74) Agent/Attorney
DAVIES COLLISON CAVE,1 Little Collins Street,MELBOURNE VIC 3000

(56) Related Art
J. VIROLOGY 69, PP.2033-2042 (1988)

AU9540715

(12) PATENT ABSTRACT (11) Document No. AU-A-40715/95
(19) AUSTRALIAN PATENT OFFICE

(54) Title
LIVE RECOMBINANT AVIAN VACCINE USING AN AVIAN HERPESVIRUS AS VECTOR

International Patent Classification(s)
(51)[6] C12N 015/86 A61K 039/12 A61K 039/245 A61K 039/255

(21) Application No. : 40715/95 (22) Application Date : 28.12.95

(30) Priority Data

(31) Number (32) Date (33) Country
94 16017 30.12.94 FR FRANCE

(43) Publication Date : 11.07.96

(71) Applicant(s)
RHONE MERIEUX

(72) Inventor(s)
JEAN-CHRISTOPHE FRANCIS AUDONNET; MICHEL JOSEPH MARIE BUBLOT; RAPHAEL JEAN DARTEIL; CAROLE VERONIQUE DUINAT; ELIANE LAPLACE; MICHEL ALBERT EMILE RIVIERE

(74) Attorney or Agent
DAVIES COLLISON CAVE , 1 Little Collins Street, MELBOURNE VIC 3000

(57)

The live recombinant avian vaccine comprises, as vector, an avian herpesvirus comprising at least one nucleotide sequence coding for and expressing an antigenic polypeptide of an avian pathogenic agent, inserted into the region lying between the ATG of ORF UL55 and the junction of U_L with the adjacent repeat region, under the control of the CMV immediate early promoter. The vector is preferably chosen from the group consisting of Marek's disease viruses (MDV and HVT), infectious laryngotracheitis virus ILTV and herpes of ducks. A polyvalent vaccine formula comprises at least two vaccines of this type, with different inserted sequences.

ABSTRACT

Live recombinant avian vaccine using an avian herpesvirus as vector.

The live recombinant avian vaccine comprises, as vector, an avian herpesvirus comprising at least one nucleotide sequence coding for and expressing an antigenic polypeptide of an avian pathogenic agent, inserted into the region lying between the ATG of ORF UL55 and the junction of U_L with the adjacent repeat region, under the control of the CMV immediate early promoter. The vector is preferably chosen from the group consisting of Marek's disease viruses (MDV and HVT), infectious laryngotracheitis virus ILTV and herpes of ducks. A polyvalent vaccine formula comprises at least two vaccines of this type, with different inserted sequences.

Figure 16.

AUSTRALIA

PATENTS ACT 1990

COMPLETE SPECIFICATION

NAME OF APPLICANT(S):

~~Rhone Merieux~~ merial

ADDRESS FOR SERVICE:

DAVIES COLLISON CAVE
Patent Attorneys
1 Little Collins Street, Melbourne, 3000.

INVENTION TITLE:

Live recombinant avian vaccine using an avian herpesvirus as vector

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

The present invention relates to vaccines for avian use based on live recombinant avian herpesviruses, namely, in particular, on Marek's disease virus (MDV) and more especially on HVT virus (herpesvirus of turkeys), into which has been inserted, by genetic recombination, at least one nucleotide sequence coding for and expressing an antigenic polypeptide of an avian pathogenic agent, under conditions affording an immunization leading to an effective protection of the vaccinated animal against the said pathogenic agent. It applies, furthermore, to the infectious laryngotracheitis virus (ILTV) and herpes of ducks.

A number of recombinant avian viral vectors have already been proposed with a view to vaccinating birds against avian pathogenic agents, in particular pathogenic viruses, including the viruses of Marek's disease (MDV), of Newcastle disease (NDV), of infectious laryngotracheitis (ILTV), of Gumboro disease (infectious bursal disease, IBDV), of infectious bronchitis (IBV) and of avian anaemia (CAV).

The viral vectors used comprise avipox viruses, especially fowlpox (EP-A-0,517,292; H.-G. Heine *et al.*, Arch. Virol. 1993. 131. 277-292; D.B. Boyle *et al.*, Veterinary Microbiology 1994. 41. 173-181; C.D. Bayliss *et al.*, Arch. Virol. 1991. 120. 193-205), Marek's virus, in particular serotypes 2 and 3 (HVT) (WO-A-87/04463; WO-A-89/01040; WO-A-93/25665; EP-A-0,513,921; J. McMillen, Poultry Condemnation Meeting, October 1994, 359-363; P.J.A. Sondermeijer *et al.*, Vaccine 1993. 11. 349-357; R.W. Morgan *et al.*, Avian Diseases 1992. 36. 858-870, and 1993. 37. 1032-1040) or alternatively the ILTV and avian adenovirus viruses.

When they are used for vaccination, these recombinant viruses induce variable levels of protection, generally low or partial, even if in special rare cases a substantial protection may be demonstrated.

One of the most difficult protections to be afforded with live recombinant avian vaccines is that against the Gumboro disease virus or IBDV virus. In effect, although traditional inactivated or attenuated live vaccines exist against this disease, no recombinant live vaccine has yet evinced appropriate efficacy.

The genome of the Gumboro disease virus consists of a double-stranded RNA. The largest segment (segment A) codes for a polyprotein of 115 kDa, which is cleaved secondarily into three proteins VP2 (41 kDa), VP4 (28 kDa) and VP3 (32 kDa). VP4 appears to be a protease participating in the maturation of 115 kDa polyprotein. The position of the cleavage site between VP2 and VP4 has been determined only approximately (M. Jagadish, J. Virol. 1988. 62. 1084-1087). The protein VP2 is an immunogen inducing neutralizing antibodies and protection against Gumboro disease.

The proposal has already been made to insert genes coding for immunogenic IBDV proteins into various live vectors: EP-A-0,517,292 (insertion of sequences coding for VP2 or the polyprotein into an avipox); C.D. Bayliss 1991, H.-G. Heine 1993 and D.B. Boyle 1994 *supra* (VP2 into fowlpox).

The Marek's disease viruses have also been proposed in WO-A-90/02802 and WO-A-90/02803 (various insertion sites such as gC, TK, RR1, RR2), in French Patent Applications Nos. 90/03105 (RR2) and 90/11146 (US3), and also, in particular, in Patent Applications WO-A-87/04463 and WO-A-89/01040 (BamHI #16 and #19) and WO-A-93/25655 (US2).

R.J. Isfort *et al.* (Virology 1994. 203. 125-133) have determined a number of sites for integration of retroviruses in the HVT genome, which sites are located in the BamHI restriction fragments F, A and I.

Various promoters, including those generally available on the market, have been used in the different constructions of the prior art, among them the PRV gX, HCMV IE (human CMV immediate early) and herpes simplex alpha-4 promoters, FPV P.E/L (fowlpox promoter) (H. Heine

et al., Arch. Virol. 1993. 131. 277-292), the vaccinia virus P7.5 (C. Bayliss *et al.*, Arch. Virol. 1991. 120. 193-205) and P11 (D. Boyle *et al.*, Vet. Microb. 1994. 41. 173-181) promoters, the promoter originating from the RSV virus (Rous sarcoma virus) LTR sequence, the SV40 early promoter and also MDV or HVT promoters, such as the promoters of the gB, gC, TK, RR2, and the like, genes, without a rule having been discernible, in particular in the case of constructions in HVT. The sequences of some promoters can inhibit the replication of recombinant HVT or MDV vectors (D.R. Marshall *et al.*, J. Vir. Meth. 1992. 40. 195-204 and Virology 1993. 195. 638-648). Among the promoters mentioned, a number, such as, for example, SV40, RSV LTR and PRV gX, have shown some degree of efficacy, as have some promoters belonging to some genes of the Marek viruses, in particular of serotype 3.

The invention has enabled a live recombinant vaccine to be developed, based on an HVT vector into which is inserted at least one sequence coding for an avian immunogen, especially the IBDV protein VP2. Such a vaccine incorporating a sequence coding for VP2 affords satisfactory protection of animals against Gumboro disease, that is to say protection with respect to mortality and with respect to lesions of the bursa of Fabricius.

The subject of the present invention is a live recombinant avian vaccine comprising, as vector, an avian herpesvirus comprising at least one nucleotide sequence coding for and expressing an antigenic polypeptide of an avian pathogenic agent, inserted into the region lying between the ATG of ORF UL55 and the junction of U_L with the adjacent repeat region, under the control of the CMV immediate early promoter. This insertion region corresponds in HVT to the BamHI fragment I and in MDV to the BamHI fragment K + H, as are presented by A.E. Buckmaster in J. Gen. Virol. 1988. 69. 2033-2042.

The avian herpesviruses according to the invention are preferably the Marek's disease viruses, in particular HVT, the infectious laryngotracheitis virus

ILTV and herpes of ducks. The Marek's disease viruses, and more especially the HVT virus, are preferred.

The BamHI restriction fragment I of HVT comprises several ORFs and three intergenic regions and, as an insertion region according to the invention, comprises several preferred insertion regions, namely the three intergenic regions 1, 2 and 3 which are the preferred regions, and ORF UL55.

Insertion into the insertion region is understood to mean, in particular, insertion without deletion or with deletion of a few bases for the intergenic regions, and with total or partial deletion or without deletion for the ORFs.

CMV immediate early (IE) promoter is understood to mean the fragment given in the examples, as well as its subfragments which retain the same promoter activity.

The CMV IE promoter can be the human promoter (HCMV IE) or the murine promoter (MCMV IE), or alternatively a CMV IE promoter of some other origin, for example from rats or from guinea-pigs.

The nucleotide sequence inserted into the Marek vector, in order to be expressed, may be any sequence coding for an antigenic polypeptide of an avian pathogenic agent, capable, when expressed under the favourable conditions achieved by the invention, of affording an immunization leading to an effective protection of the vaccinated animal against the pathogenic agent. The nucleotide sequences coding for the antigens of interest for a given disease may hence be inserted under the conditions of the invention.

The vaccines according to the invention may be used for the vaccination *in ovo* of 1-day or older chicks and of adults.

The invention may be used, in particular, for the insertion of a nucleotide sequence coding appropriately for the polypeptide VP2 of the IBDV virus. A live recombinant vaccine is thereby obtained affording, in addition to protection against Marek's disease, satisfactory protection against Gumboro disease. If so

desired, it is also possible to insert a sequence coding for another IBDV antigen, such as VP3 or alternatively the polyprotein VP2 + VP4 + VP3, these other possibilities not being preferred.

5 The recombinant vaccine against Gumboro disease will preferably be presented at a concentration of 10 to 10^4 pfu/dose.

Other preferred cases of the invention are the insertion of nucleotide sequences coding for antigens of 10 the Marek's disease virus, especially gB, gC, gD and gH + gL genes (WO-A-90/02803), of the Newcastle disease virus, especially F and HN genes, of the infectious bronchitis virus (IBV), especially S and M genes (M. Binns *et al.*, J. Gen. Virol. 1985. 66. 719-726; 15 M. Boursnell *et al.*, Virus Research 1984. 1. 303-313), of the avian anaemia virus (CAV), especially VP1 (52 kDa) + VP2 (24 kDa) (N.H.M. Noteborn *et al.*, J. Virol. 1991. 65. 3131-3139), and of the infectious laryngotracheitis virus (ILTV), especially gB (WO-A-90/02802), gC, gD and gH + 20 gL.

The doses will preferably be the same as those for the Gumboro vaccine.

According to an advantageous development of the invention, the CMV IE promoter is combined with another 25 promoter wherein both promoters direct transcription in opposite directions according to a head-to-tail arrangement, which enables two nucleotide sequences to be inserted into the insertion region, one under the control of the CMV IE promoter, the other under that of the 30 promoter used in combination therewith. This construction is noteworthy for the fact that the presence of the CMV IE promoter, and in particular of its activator portion (enhancer), activates the transcription induced by the promoter used in combination. A preferred promoter used in 35 combination is the Marek 1.8 RNA promoter, the transcriptional activity of which has been shown to be multiplied by approximately 4.4 under these conditions.

An advantageous case of the invention is a vaccine comprising a nucleotide sequence encoding for IBDV VP2 under the control of CMV IE, and a nucleotide

sequence coding for an antigen of another avian disease, in particular the ones mentioned above, under the control of the other promoter.

It is also possible to assemble head to tail two CMV IE promoters of different origins.

The 1.8 RNA promoter may also be used alone in place of the CMV IE promoter, in particular for vaccines against Marek's disease, Newcastle disease, infectious laryngotracheitis, infectious bronchitis and avian anaemia.

The subject of the present invention is also a polyvalent vaccine formula comprising, as a mixture or to be mixed, at least two live recombinant avian vaccines as are defined above, these vaccines comprising different inserted sequences, in particular from different pathogens.

The subject of the present invention is also a method of avian vaccination, comprising the administration of a live recombinant vaccine or of a polyvalent vaccine formula as defined above. Its subject is, in particular, a method of this kind for the vaccination *in ovo* of 1-day or older chicks and of adults.

The invention will now be described in greater detail by means of non-limiting examples of implementation, taken with reference to the drawing, wherein:

Listing of figures and sequences for the constructions in the intergenic sites

Figure 1: Sequence of the HVT BamHI fragment I
Figure 2: plasmid pEL039
Figure 3: plasmid pEL077
Figure 4: plasmid pEL079
Figure 5: plasmid pEL076
Figure 6: plasmid pEL078
Figure 7: plasmid pEL054
Figure 8: plasmid pEL055
Figure 9: plasmid pEL062

Figure 10: plasmid pEL066
Figure 11: plasmid pEL022
Figure 12: plasmid pEL023
Figure 13: plasmid pEL024
5 **Figure 14:** plasmid pCMVβ
Figure 15: plasmid pEL026
Figure 16: plasmid pEL090
Figure 17: plasmid pCD002
Figure 18: plasmid pCD009
10 **Figure 19:** plasmid pEL068
Figure 20: plasmid pEL070
Figure 21: plasmid pEL091
Figure 22: plasmid pCD011
Figure 23: plasmid pCD020
15 **Figure 24:** plasmid pEL092
Figure 25: Sequence of the NDV HN gene
Figure 26: plasmid pEL028
Figure 27: plasmid pEL029bis
Figure 28: plasmid pEL030
20 **Figure 29:** plasmid pEL032
Figure 30: plasmid pEL093
Figure 31: plasmid pEL033
Figure 32: plasmid pEL034
Figure 33: plasmid pEL094
25 **Figure 34:** Sequence of the MDV 1.8-kbp RNA promoter
Figure 35: plasmid pBS002
Figure 36: plasmid pEL069
Figure 37: plasmid pEL080
Figure 38: plasmid pEL081
30 **Figure 39:** plasmid pEL095
Figure 40: plasmid pEL098

SEQ ID sequence listing for the constructions in the intergenic sites

SEQ ID No. 1	Sequence of the HVT BamHI fragment I
SEQ ID No. 2	Oligonucleotide EL102
SEQ ID No. 3	Oligonucleotide EL161
SEQ ID No. 4	Oligonucleotide EL147
SEQ ID No. 5	Oligonucleotide EL162
SEQ ID No. 6	Oligonucleotide EL154
SEQ ID No. 7	Oligonucleotide EL163
SEQ ID No. 8	Oligonucleotide EL164
SEQ ID No. 9	Oligonucleotide EL165
SEQ ID No. 10	Oligonucleotide EL132
SEQ ID No. 11	Oligonucleotide EL133
SEQ ID No. 12	Oligonucleotide MB070
SEQ ID No. 13	Oligonucleotide MB071
SEQ ID No. 14	Oligonucleotide CD001
SEQ ID No. 15	Oligonucleotide CD002
SEQ ID No. 16	Oligonucleotide CD003
SEQ ID No. 17	Oligonucleotide CD004
SEQ ID No. 18	Sequence of the NDV HN gene
SEQ ID No. 19	Oligonucleotide EL071
SEQ ID No. 20	Oligonucleotide EL073
SEQ ID No. 21	Oligonucleotide EL074
SEQ ID No. 22	Oligonucleotide EL075
SEQ ID No. 23	Oligonucleotide EL076
SEQ ID No. 24	Oligonucleotide EL077
SEQ ID No. 25	Sequence of the MDV 1.8-kbp RNA promoter
SEQ ID No. 26	Oligonucleotide MB047
SEQ ID No. 27	Oligonucleotide MB048
SEQ ID No. 28	Oligonucleotide MB072

2. EXAMPLES

All the plasmid constructions were carried out using the standard techniques of molecular biology described by Sambrook J. *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 using the "Geneclean" kit (BIO 101 Inc. La Jolla, CA).

The virus used as parent virus is herpesvirus of turkeys (HVT) strain FC126, isolated by Dr. Witter of the Regional Poultry Research Laboratory (USDA, East Lansing, Michigan) in a flock of 23-week-old turkeys (Witter R.L. *et al.* Am. J. Vet. Res. 1970. 31. 525-538). The conditions of culture of this virus are those described elsewhere (French Patent Application 90/03105).

Example 1: Extraction of the DNA from Marek's disease virus:

The whole blood of a chicken challenged at 7 days with MDV strain RB1B is harvested with a syringe onto anticoagulant (heparin solution at a concentration of 100 IU/ml) 14 days after infection. This blood is then centrifuged at 30 g for 15 minutes at room temperature. The plasma together with the buffy coat is removed and diluted in sterile PBS to have a final volume of 10 ml. After centrifugation for 15 minutes at 150 g, the cell pellet is resuspended in 2 ml of 199 culture medium (Gibco-BRL Cat# 042-01183M) containing 2 % of foetal calf serum (FCS).

The total DNA of the infected lymphocytes is then extracted according to the technique described by R. Morgan *et al.* (Avian Diseases. 1990. 34. 345-351), and may be used directly as template for the PCR experiments. For the cloning of genomic fragments of the MDV virus, the strain RB1B was cultured on CEF and the viral DNA was prepared from purified viral particles as described by Lee Y. *et al.* (J. Gen. Virol. 1980. 51. 245-253).

Example 2: Preparation of MCMV virus (mouse cytomegalovirus) genomic DNA

MCMV virus strain Smith was obtained from the American Type Culture Collection, Rockville, Maryland, USA (ATCC No. VR-194). This virus was cultured on Balb/C mouse embryo cells and the viral DNA of this virus was prepared as described by Ebeling A. *et al.* (J. Virol. 1983. 47. 421-433).

Example 3: Preparation of HVT virus genomic DNA for the transfection experiments:

The viral DNA used for the transfection experiments was prepared according to the technique described by R. Morgan *et al.* (Avian Diseases. 1990. 34. 345-351) from a culture of secondary CEC (CEC II) infected with HVT virus strain FC126.

Example 4: Description of the BamHI fragment I

The 5.8-kbp BamHI fragment I of HVT virus strain FC126 (Igarashi T. *et al.* J. Gen. Virol. 1989. **70**. 1789-1804) was isolated by Geneclean and cloned into the BamHI site of the vector pBS-SK+ to give the plasmid pEL037. The sequence of this fragment was established in its entirety (5838 bp) (Figure 1 and SEQ ID No. 1). 6 open reading frames (ORFs) were identified on this sequence. A study of the proteins potentially encoded by these ORFs revealed that some of these proteins displayed a homology with proteins encoded by ORFs present in other alpha-herpesviruses. The first ORF (ORF1) (position 676 to position 1209 on SEQ ID No. 1) displays a homology with the ORFs HSV-1 UL55, EHV-1 gene 4 and VZV gene 5, and codes for a theoretical protein HVT UL55 of 178 amino acids (aa). ORF 2 is located from position 1941 to position 1387 on the sequence SEQ ID No. 1 and codes for a protein of 185 aa homologous with the protein encoded by the ORF EHV-1 gene 3. ORF 3 is incomplete. It is located from position 5838 to 3573 on SEQ ID No. 1 and displays a homology with ORF 21 of MDV (Ross No. *et al.* Virus Genes. 1993. 7. 33-51). Three other ORFs identified

on this sequence, namely ORF4 (position 1403 to position 1957 (protein of 185 aa)), ORF5 (position 3081 to position 2287 (protein of 265 aa)), and ORF6 (incomplete; position 479 to position 1), do not have homologues in the sequence libraries. The genomic organization of the BamHI fragment I of HVT virus strain FC126 is such that there are 3 intergenic regions which may be used as insertion sites for cassettes for the expression of foreign genes:

An intergenic region (intergenic region 1) exists between ORF UL55 and ORF HVT gene 3. A second intergenic region (intergenic region 2) exists between ORF HVT gene 3 and the 265-aa ORF. A third intergenic region (intergenic region 3) exists between the 265-aa ORF and ORF 21. These three regions are useable for inserting expression cassettes without affecting the *in vivo* replication of the recombinant HVT viruses thereby obtained. Examples of constructions of donor plasmids for these intergenic regions 1, 2 and 3 are described below:

Example 5: Construction of the donor plasmid for intergenic region 1

Plasmid pEL037 was digested with BamHI and EcoRI to isolate 2672-bp and 2163-bp BamHI-EcoRI fragments. These fragments were ligated with the vector pBS-SK+, previously digested with BamHI and EcoRI, to give, respectively, the plasmids pEL039 of 5167 bp and pEL040 of 6104 bp. Plasmid pEL039 (Figure 2) was digested with BamHI and PstI to isolate the 997-bp BamHI-PstI fragment (fragment A). A PCR was carried out with the following oligonucleotides:

EL102 (SEQ ID No. 2) 5' CATTATAAGACCAACGTGCGAGTC 3'

EL161 (SEQ ID No. 3) 5' GTTCACGTCGACAATTATTTTATTTAATAAC 3'

and the template pEL039 to produce a 420-bp fragment. This fragment was digested with PstI and SalI to isolate a 250-bp PstI-SalI fragment (fragment B). Fragments A and B were ligated together with the vector pBSII-SK+ (Stratagene), previously digested with BamHI and SalI, to give the 4160-bp plasmid pEL077 (Figure 3). Plasmid

pEL039 was digested with BstBI and ScaI to isolate a (blunt-ended) 475-bp BstBI-ScaI fragment (fragment C). A PCR was carried out with the following oligonucleotides:
EL147 (SEQ ID No. 4) 5' AAGATAATGGGCTCCCGCTGTTC 3'
5 EL162 (SEQ ID No. 5)
5' TAATTGTCGACCCCGGGGAATTCGTTTAATGTTAGTTTATTC 3'
and the template pEL039 to produce a 715-bp PCR fragment. This fragment was digested with BstBI and SalI to isolate the 465-bp BstBI-SalI fragment (fragment D). Fragments C
10 and D were ligated together with plasmid pEL077, previously digested with ApaI and repaired with Klenow polymerase and digested with SalI, to give the 5082-bp plasmid pEL079 (Figure 4). This plasmid contains an EcoRI-SmaI-SalI polylinker in intergenic site 1.

15 **Example 6: Construction of the donor plasmid for intergenic region 2**

Plasmid pEL039 (Example 5) was digested with BstBI and PstI to isolate the 715-bp BstBI-PstI fragment (fragment A). A PCR was carried out with the following
20 oligonucleotides:
EL154 (SEQ ID No. 6) 5' GAAATGCAAACTAACATTATTGTC 3'
EL163 (SEQ ID No. 7)
5' GTGTAAATAGTCGACAATATAGATAACGGGC 3'
and the template pEL039 to produce a 500-bp PCR fragment.
25 This fragment was digested with BstBI and SalI to isolate the 430-bp BstBI-SalI fragment (fragment B). Fragments A and B were ligated together with the vector pBSII-SK+, previously digested with PstI and SalI, to give the 4081-bp plasmid pEL076 (Figure 5).
30 Another PCR was carried out with the following oligonucleotides:
EL164 (SEQ ID No. 8)
5' CTATATTGTCGACCCCGGGGAATTCATCGACATGATTAAATAC 3'
EL165 (SEQ ID No. 9)
35 5' CAATGAAGAAATATTTTCTTTGTTCCTTGAAATGC 3'
and the template pEL039 to produce a 565-bp PCR fragment. This fragment was digested with SalI and SspI to isolate the 535-bp SalI-SspI fragment. This fragment was ligated

with plasmid pEL076, previously digested with ApaI and repaired with Klenow polymerase and digested with SalI, to give the 4598-bp plasmid pEL078 (Figure 6). This plasmid contains an EcoRI-SmaI-SalI polylinker in intergenic region 2.

Example 7: Construction of the donor plasmid for intergenic region 3

Plasmid pEL040 (see Example 5) was digested with NcoI and SphI to isolate the 1468-bp NcoI-SphI fragment. This fragment was ligated with the plasmid pUC BM20 (Boehringer Mannheim Cat# 1219235), previously digested with NcoI and SphI, to give the 4182-bp plasmid pEL054 (Figure 7). Plasmid pEL040 was digested with EcoRI and SphI to isolate the 614-bp EcoRI-SphI fragment. This fragment was ligated with plasmid pUC BM20, previously digested with EcoRI and SphI, to give the 3263-bp plasmid pEL055 (Figure 8). Plasmid pEL055 was digested with EcoRI, repaired with Klenow polymerase, ligated with itself, digested with HindIII, repaired with Klenow polymerase and lastly ligated with itself to give the 3279-bp plasmid pEL062 (Figure 9). Plasmid pEL054 was digested with NcoI and SalI to isolate the 1492-bp NcoI-SalI fragment (fragment A). The following two oligonucleotides:

EL132 (SEQ ID No. 10) 5' CCGAATTCATATAAGCTTACGTG 3'

EL133 (SEQ ID No. 11)

5' TCGACACGTAAGCTTATATGAATTCGGCATG 3'

were hybridized with one another to produce the 24-bp SalI-SphI fragment (fragment B). Fragments A and B were ligated together with plasmid pEL062, previously digested with NcoI and SphI, to give the 4787-bp plasmid pEL066 (Figure 10). This plasmid contains an EcoRI-HindIII-SalI polylinker in intergenic region 3.

Example 8: Construction of the donor plasmid pEL090 and isolation of vHVT16

The plasmid pEL004 (= plasmid pGH004 described in French Patent Application 92/13109), containing the IBDV

VP2 gene in the form of a BamHI-HindIII cassette, was digested with BamHI and XbaI to isolate the 1104-bp BamHI-XbaI fragment (truncated VP2 gene). This fragment was cloned into the vector pBS-SK+, previously digested with XbaI and BamHI, to give the 4052-bp plasmid pEL022 (Figure 11). The vector pBS-SK+ was digested with EcoRV and XbaI and then ligated with itself to give pBS-SK* (modified). Plasmid pEL004 was digested with KpnI and HindIII to isolate the 1387-bp KpnI-HindIII fragment containing the complete IBDV VP2 gene. This fragment was cloned into the vector pBS-SK*, previously digested with KpnI and HindIII, to give the 4292-bp plasmid pEL023 (Figure 12). Plasmid pEL022 was digested with BamHI and NotI to isolate the 1122-bp BamHI-NotI fragment (fragment A). Plasmid pEL023 was digested with BamHI and NotI to isolate the 333-bp BamHI-NotI fragment (fragment B). Fragments A and B were ligated together with the vector pBS-SK+, previously digested with NotI and treated with alkaline phosphatase, to give the 4369-bp plasmid pEL024 (Figure 13). Plasmid pEL024 was digested with NotI to isolate the 1445-bp NotI-NotI fragment. This fragment was ligated with the plasmid pCMVβ (Clontech Cat# 6177-1) (Figure 14), previously digested with NotI, to give the 5095-bp plasmid pEL026 (Figure 15). Plasmid pEL026 was digested with EcoRI, SalI and XmnI to isolate the 2428-bp EcoRI-SalI fragment. This fragment was ligated with plasmid pEL079 (see Example 5), previously digested with EcoRI and SalI, to give the 7514-bp plasmid pEL090 (Figure 16). This plasmid permits the insertion of the HCMV-IE/IBDV VP2 expression cassette into intergenic site 1 of the HVT virus.

24-hour primary CEF cells were then transfected with the following mixture: 1 μg of linearized plasmid pEL090 + 5 μg of HVT viral DNA in 300 μl of OptiMEM medium (Gibco BRL Cat# 041-01985H) and 100 μg of LipofectAMINE diluted in 300 μl of medium (final volume of mixture = 600 μl). These 600 μl were then diluted in 3 ml (final volume) of medium and plated out on 3×10^6 CEC I. The mixture was left in contact with the cells for

5 hours, then removed and replaced by 5 ml of culture medium. The cells were then left in culture for 3 days at +37°C, and were thereafter pronased, mixed with fresh CEC II (3:1 mixture) and plated out again on 1 96-well plate. This plate was left in culture for 3 days, and the cells were then pronased, mixed with fresh CEF II and plated out again on 2 96-well plates, one initial cup giving 2 sister cups. The 96-well plates were cultured until a cytopathic effect was seen. After 72 hours of culture, one of the two 96-well plates was fixed in 95 % acetone for 30 minutes, and an indirect immuno-fluorescence (IIF) reaction was carried out with an anti-VP2 monoclonal antibody to test for plaques expressing the protein VP2. The "sister" cups of the cups displaying positive plaques in IIF were pronased, mixed with fresh CEF II and applied in limiting dilution to 96-well plates. After 3 days of culture, the cups displaying a cytopathic effect were pronased, mixed with CEF II and plated out again on 96-well plates, one initial cup giving 2 sister cups. 3 days later, the plaques expressing the protein VP2 were tested for again, as before, by IIF on one of the 2 sister plates.

In general, 4 successive cycles of isolation (harvesting of a cup, plating out again, monitoring by IIF, subculturing of a sister cup, etc.) suffice for obtaining recombinant viruses the whole of whose progeny displays a specific fluorescence. One viral plaque which gave 100 % of positive plaques in IIF with an anti-VP2 monoclonal antibody was designated vHVT16. The genomic DNA of this recombinant virus was characterized at molecular level by standard PCR and Southern blot techniques using the appropriate oligonucleotides and DNA probes.

Example 9: Construction of the donor plasmid pEL091 and isolation of vHVT17

Plasmid pCMVβ (Figure 14) was digested with SalI and SmaI to isolate the 3679-bp SalI-SmaI fragment containing the *lacZ* gene as well as the polyadenylation

signal of the SV40 virus late gene. This fragment was inserted into the vector pBS-SK+, previously digested with SalI and EcoRV, to give the 6625-bp plasmid pCD002 (Figure 17). This plasmid contains the *lacZ* reporter gene, but no promoter is located upstream of this gene. The viral genomic DNA of the MCMV virus was prepared as described in Example 2 and digested with PstI to isolate the 2285-bp PstI-PstI fragment. This fragment was cloned into the vector pBS-SK+, previously digested with PstI and treated with alkaline phosphatase, to give the plasmid pCD004. Plasmid pCD004 was digested with HpaI and PstI to isolate the 1389-bp HpaI-PstI fragment, which contains the promoter/activator region of the murine cytomegalovirus (MCMV) immediate early gene (Dorsch-Häsler K. *et al*. Proc. Natl. Acad. Sci. 1985. **82**. 8325-8329, and Patent Application WO-A-87/03905). This fragment was cloned into plasmid pCD002, previously digested with PstI and SmaI, to give the 8007-bp plasmid pCD009 (Figure 18).

A double-stranded oligonucleotide was obtained by hybridization of the following two oligonucleotides:
MB070 (SEQ ID No. 12)
5' CGAATTCACTAGTGTGTGTCTGCAGGCGGCCGCGTGTGTGTCGACGGTAC 3'
MB071 (SEQ ID No. 13)
5' CGTCGACACACACGCGGCCGCCTGCAGACACACACTAGTGAATTCGAGCT 3'
This double-stranded oligonucleotide was ligated in the vector pBS-SK+, previously digested with KpnI and SacI, to give the plasmid pEL067.

Plasmid pCD009 was digested with PstI and SpeI to isolate the 1396-bp PstI-SpeI fragment. This fragment was ligated with plasmid pEL067, previously digested with PstI and SpeI, to give the 4297-bp plasmid pEL068 (Figure 19). Plasmid pEL026 (see Example 8) was digested with HindIII and SalI to isolate the 235-bp HindIII-SalI fragment (fragment B). Fragments A and B were ligated together with plasmid pEL068, previously digested with NotI and SalI, to give the 5908-bp plasmid pEL070 (Figure 20). Plasmid pEL070 was digested with EcoRI, SalI and XmnI to isolate the 3035-bp EcoRI-SalI fragment. This

fragment was ligated with plasmid pEL079 (see Example 5), previously digested with EcoRI and SalI, to give the 8109-bp plasmid pEL091 (Figure 21). This plasmid permits the insertion of the MCMV-IE/IBDV VP2 expression cassette
5 into intergenic site 1 of the HVT virus.

A cotransfection carried out as described in Example 8 with plasmid pEL091 and HVT virus genomic DNA led to the isolation and purification of the recombinant vHVT17.

10 **Example 10: Construction of the donor plasmid pEL092 and isolation of vHVT18**

The 3.9-kbp EcoRI-SalI fragment of MDV virus strain RB1B genomic DNA containing the MDV gB gene (sequence published by Ross N. *et al*. J. Gen. Virol.
15 1989. 70. 1789-1804) was ligated with the vector pUC13, previously digested with EcoRI and SalI, to give the plasmid pCD007. This plasmid was digested with SacI and XhoI to isolate the 2260-bp SacI-XhoI fragment (central portion of the gB gene = fragment A). A PCR was carried
20 out with the following oligonucleotides:
CD001 (SEQ ID No. 14)
5' GACTGGTACCGCGGCCGCATGCACTTTTTAGGCGGAATTG 3'
CD002 (SEQ ID No. 15) 5' TTCGGGACATTTTCGCGG 3'
and the template pCD007 to produce a 222-bp PCR fragment.
25 This fragment was digested with KpnI and XbaI to isolate a 190-bp KpnI-XbaI fragment (5' end of the gB gene = fragment B). Another PCR was carried out with the following oligonucleotides:
CD003 (SEQ ID No. 16) 5' TATATGGCGTTAGTCTCC 3'
30 CD004 (SEQ ID No. 17)
5' TTGCGAGCTCGCGGCCGCTTATTACACAGCATCATCTTCTG 3'
and the template pCD007 to produce a 195-bp PCR fragment. This fragment was digested with SacI and SacII to isolate the 162-bp SacI-SacII fragment (3' end of the gB gene =
35 fragment C). Fragments A, B and C were ligated together with the vector pBS-SK+, previously digested with KpnI and SacI, to give the 5485-bp plasmid pCD011 (Figure 22). Plasmid pCD011 was digested with NotI to isolate the

2608-bp NotI-NotI fragment (whole MDV gB gene). This fragment was ligated with plasmid pCMVβ, previously digested with NotI and treated with alkaline phosphatase, to give the 6299-bp plasmid pCD020 (Figure 23) (in this
5 plasmid, the MDV gB gene replaces the *lacZ* gene). Plasmid pCD020 was digested with EcoRI and SalI to isolate the 3648-bp EcoRI-SalI fragment. This fragment was ligated with plasmid pEL079 (see Example 5), previously digested with EcoRI and SalI, to give the 8718-bp plasmid pEL092
10 (Figure 24). This plasmid permits the insertion of the HCMV-IE/MDV gB expression cassette into intergenic site 1 of the HVT virus.

A cotransfection carried out as described in Example 8 with plasmid pEL092 and HVT virus genomic DNA
15 led to the isolation and purification of the recombinant vHVT18.

Example 11: Construction of the donor plasmid pEL093 and isolation of vHVT19

The building of a library of DNA complementary to
20 the Newcastle disease virus (NDV), strain Texas, genome was carried out as described by Taylor J. *et al.* (J. Virol. 1990. **64**. 1441-1450). A pBR322 clone containing the end of the fusion gene (F), the whole of the haemagglutinin-neuraminidase (HN) gene and the beginning
25 of the gene for the polymerase was identified as pHN01. The sequence of the NDV HN gene present in this clone is presented in Figure 25 (SEQ ID No. 18). Plasmid pHN01 was digested with SphI and XbaI to isolate the 2520-bp SphI-XbaI fragment. This fragment was ligated with the vector
30 pUC19, previously digested with SphI and XbaI, to give the 5192-bp plasmid pHN02. Plasmid pHN02 was digested with ClaI and PstI to isolate the 700-bp ClaI-PstI fragment (fragment A). A PCR was carried out with the following oligonucleotides:
35 EL071 (SEQ ID No. 19) 5' CAGACCAAGCTTCTTAAATCCC 3'
EL073 (SEQ ID No. 20) 5' GTATTCGGGACAATGC 3'
and the template pHN02 to produce a 270-bp PCR fragment. This fragment was digested with HindIII and PstI to

isolate a 220-bp HindIII-PstI fragment (fragment B). Fragments A and B were ligated together with the vector pBS-SK+, previously digested with ClaI and HindIII, to give the 3872-bp plasmid pEL028 (Figure 26). Plasmid pHN02 was digested with BsphI and ClaI to isolate the 425-bp BsphI-ClaI fragment (fragment C). A PCR was carried out with the following oligonucleotides:

EL074 (SEQ ID No. 21) 5' GTGACATCACTAGCGTCATCC 3'

EL075 (SEQ ID No. 22)

5' CCGCATCATCAGCGGCCGCGATCGGTCATGGACAGT 3'

and the template pHN02 to produce a 425-bp PCR fragment. This fragment was digested with BsphI and NotI to isolate the 390-bp BsphI-NotI fragment (fragment D). Fragments C and D were ligated together with the vector pBS-SK+, previously digested with ClaI and NotI, to give 3727-bp plasmid pEL029bis (Figure 27). Plasmid pEL028 was digested with ClaI and SacII to isolate the 960-bp ClaI-SacII fragment (fragment E). Plasmid pEL029bis was digested with ClaI and NotI to isolate the 820-bp ClaI-NotI fragment (fragment F). Fragments E and F were ligated together with the vector pBS-SK+, previously digested with NotI and SacII, to give the 4745-bp plasmid pEL030 (Figure 28). Plasmid pEL030 was digested with NotI to isolate the 1780-bp NotI-NotI fragment (whole NDV HN gene). This fragment was ligated, in place of the *lacZ* gene, with plasmid pCMVβ, previously digested with NotI and treated with alkaline phosphatase, to give the 5471-bp plasmid pEL032 (Figure 29). Plasmid pEL032 was digested with EcoRI and ClaI to isolate the 1636-bp EcoRI-ClaI fragment (fragment G). Plasmid pEL032 was digested with ClaI and SalI to isolate the 1182-bp ClaI-SalI fragment (fragment H). Fragments G and H were ligated together with plasmid pEL079 (see Example 5), previously digested with EcoRI and SalI, to give the 7890-bp plasmid pEL093 (Figure 30). This plasmid permits the insertion of the HCMV-IE/NDV HN expression cassette into intergenic site 1 of the HVT virus.

A cotransfection carried out as described in Example 8 with plasmid pEL093 and HVT virus genomic DNA

led to the isolation and purification of the recombinant vHVT19.

Example 12: Construction of the donor plasmid pEL094 and isolation of vHVT20

5 A clone originating from the library of DNA complementary to the Newcastle disease virus genome (see Example 11), and containing the whole of the fusion gene (F), was designated pNDV81. This plasmid has been described before, and the sequence of the NDV F gene 10 present in this clone has been published (Taylor J. *et al.* J. Virol. 1990. **64.** 1441-1450). Plasmid pNDV81 was digested with NarI and PstI to isolate the 1870-bp NarI-PstI fragment (fragment A). A PCR was carried out with the following oligonucleotides:

15 EL076 (SEQ ID No. 23) 5' TGACCCTGTCTGGGATGA 3'
EL077 (SEQ ID No. 24)
5' GGATCCCGGTCGACACATTGCGGCCGCAAGATGGGC 3'

and the template pNDV81 to produce a 160-bp fragment. This fragment was digested with PstI and SalI to isolate 20 the 130-bp PstI-SalI fragment (fragment B). Fragments A and B were ligated together with the vector pBS-SK+, previously digested with ClaI and SalI, to give the 4846-bp plasmid pEL033 (Figure 31). Plasmid pEL033 was digested with NotI to isolate the 1935-bp NotI-NotI 25 fragment (whole F gene). This fragment was ligated with plasmid pCMBβ, previously digested with NotI and treated with alkaline phosphatase, to give the 5624-bp plasmid pEL034 (the NDV F gene has replaced the *lacZ* gene) (Figure 32). Plasmid pEL034 was digested with EcoRI and 30 KpnI to isolate the 866-bp EcoRI-KpnI fragment (fragment C). Plasmid pEL034 was digested with KpnI and SalI to isolate the 2114-bp KpnI-SalI fragment (fragment D). Fragments C and D were ligated together with plasmid pEL079 (see Example 5), previously digested with EcoRI 35 and SalI, to give the 8043-bp plasmid pEL094 (Figure 33). This plasmid permits the insertion of the HCMV-IE/NDV F expression cassette into intergenic site 1 of the HVT virus.

A cotransfection carried out as described in Example 8 with plasmid pEL094 and HVT virus genomic DNA led to the isolation and purification of the recombinant vHVT20.

5 **Example 13: Construction of the donor plasmid pEL095 and isolation of vHVT21**

The sequences located upstream of the MDV 1.8-kbp RNA gene are described in Bradley G. *et al.* (J. Virol. 1989. 63. 2534-2542) (Figure 34 and SEQ ID No. 25). A PCR
10 amplification was carried out on DNA extracted from lymphocytes harvested on chickens infected with MDV strain RB1B (see Example 1), with the following oligonucleotides:

MB047 (SEQ ID No. 26)
15 5' GGTCTACTAGTATTGGACTCTGGTGCGAACGC 3'
MB048 (SEQ ID No. 27)
5' GTCCAGAATTCGCGAAGAGAGAAGGAACCTC 3'

The 163-bp PCR fragment thereby obtained was digested with EcoRI and SpeI, and then ligated with plasmid pCD002
20 (see Example 9), previously digested with EcoRI and SpeI, to give the 6774-bp plasmid pBS002 (Figure 35). Plasmid pBS002 contains the promoter of the MDV 1.8-kb RNA gene cloned upstream of the *lacZ* gene.

A PCR was carried out with the oligonucleotides:
25 MB047 (SEQ ID No. 26) and
MB072 (SEQ ID No. 28)
5' GTGTCCTGCAGTCGCGAAGAGAGAAGGAACCTC 3'

and the template pBS002. The PCR fragment thereby obtained was digested with PstI and SpeI to isolate a
30 200-bp PstI-SpeI fragment. This fragment was ligated with plasmid pEL067 (see Example 9), previously digested with PstI and SpeI, to give the plasmid pEL069 (Figure 36). Plasmid pCD007 (see Example 10) was digested with EcoRI and XbaI to isolate the 2670-bp EcoRI-XbaI fragment
35 (fragment A). Plasmid pCD011 (see Example 10) was digested with NotI and XbaI to isolate the 180-bp NotI-XbaI fragment (fragment B). Plasmid pEL069 was digested with NotI and SpeI to isolate the 180-bp NotI-SpeI

fragment (fragment C). Fragments A, B and C were ligated together with plasmid pEL067 (see Example 9), previously digested with EcoRI and SpeI, to give the 5939-bp plasmid pEL080 (Figure 37). Plasmid pEL070 (see Example 9) was digested with KpnI and SpeI to isolate the 1345-bp KpnI-SpeI fragment (fragment D). Plasmid pEL070 was also digested with KpnI and SalI to isolate the 1658-bp KpnI-SalI fragment (fragment E). Fragments D and E were ligated together with plasmid pEL080, previously digested with SalI and SpeI, to give the 8938-bp plasmid pEL081 (Figure 38). Plasmid pEL081 was digested with EcoRI and SalI to isolate the 6066-bp EcoRI-SalI fragment. This fragment was ligated with plasmid pEL079 (see Example 5), previously digested with EcoRI and SalI, to give finally the 11139-bp plasmid pEL095 (Figure 39). This plasmid permits the insertion of the VP2/MCMV-IE//1.8-kbp RNA/MDV gB double expression cassette into intergenic site 1 of the HVT virus.

A cotransfection carried out as described in Example 8 with plasmid pEL095 and HVT virus genomic DNA led to the isolation and purification of the recombinant vHVT21.

Example 14: Construction of the donor plasmid pEL098 and isolation of vHVT24

Plasmid pEL080 (see Example 13) was digested with EcoRI and SalI to isolate the 3040-bp EcoRI-SalI fragment (1.8-kbp RNA/MDV gB cassette). This fragment was ligated with plasmid pEL079 (see Example 5), previously digested with EcoRI and SalI, to give the 8140-bp plasmid pEL098 (Figure 40). This plasmid permits the insertion of the 1.8-kbp RNA/MDV gB cassette into intergenic site 1 of the HVT virus.

A cotransfection carried out as described in Example 8 with plasmid pEL098 and HVT virus genomic DNA led to the isolation and purification of the recombinant vHVT24.

Example 15: Construction of donor plasmids for the insertion of cassettes for the expression of IBV M and S into intergenic site 1 of the HVT virus

According to the same strategy as that described above for the insertion of expression cassettes (genes placed under the control of the HCMV-IE or MCMV-IE promoters or MCMV-IE//1.8-kbp RNA double promoter) into intergenic site 1, it is possible to produce recombinant HVT viruses expressing at a high level the membrane (M) or spike (S) proteins of the avian infectious bronchitis virus (IBV). It is preferable to produce a construction in which the IBV S gene is under the control of the HCMV-IE promoter or the MCMV-IE promoter, or alternatively a construction in which the IBV M and IBV S genes are inserted together with the MCMV-IE/1.8-kbp RNA double promoter into intergenic site 1, the M gene being under the control of the 1.8-kbp RNA promoter and the S gene being under the control of the MCMV-IE promoter. In this arrangement, the 1.8-kbp RNA promoter is activated by the activator region of the MCMV-IE promoter.

Example 16: Construction of recombinant HVT viruses comprising foreign genes inserted into intergenic sites 2 and 3

The obtaining of recombinant HVT viruses which have inserted cassettes for the expression of foreign genes into intergenic sites 2 and 3 is accomplished according to the strategy described for Examples 8 to 14, but using, respectively, plasmids pEL078 (intergenic site 2) and pEL066 (intergenic site 3) in place of plasmid pEL079 in Examples 8 to 14 in order to construct the specific donor plasmids.

Example 17: Preparation of a vaccine according to the invention:

The preparation of the vaccines according to the invention may be accomplished by any standard technique known to a person skilled in the art, for example by culture in roller bottles. Roller bottles (175 cm^2),

seeded with 200x10^{6} primary chick embryo cells, are innoculated after 24 hours of incubation at 37°C with 1 ml of a viral solution of recombinant HVT virus having a titre of 10^{5} pfu/ml. After incubation for 4 days at 37°C, the supernatant is removed and the cells are detached with a trypsin/versene solution and thereafter harvested. The infected cells are then centrifuged. The supernatant is removed and the cells are taken up with 20 ml of a solution containing a lyophilization stabilizer (for example SPGA sucrose, phosphate, glutamate, albumin). This mixture is then sonicated, distributed in vials on the basis of 1 ml fractions and lastly lyophilized.

If necessary, the vaccine may also be distributed and frozen instead of lyophilized.

Example 18:

A recombinant virus obtained according to the invention and expressing the protein VP2 of the Gumboro disease virus was used to immunize 1-day chicks intramuscularly. These chicks were then challenged at the age of 21 days with Gumboro disease virus. The results in respect of protection were evaluated 11 days after challenge by comparing the lesions of the bursa of Fabricius and the mortality between the vaccinated groups and the unvaccinated control group. The results of this vaccination/challenge protocol showed that the vaccine obtained according to the invention enables a good level of protection to be obtained.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Live recombinant avian vaccine comprising, as vector, an avian herpes virus comprising at least one nucleotide sequence coding for and expressing an antigenic polypeptide of an avian pathogenic agent, inserted into the region lying between the ATG of ORF UL55 and the junction of U_L with the adjacent repeat region.

2. Live recombinant avian vaccine according to claim 1 wherein the nucleotide sequence is under the control of the CMV immediate early promoter of RNA 1.8 promoter.

3. Live recombinant avian vaccine according to claim 1, characterized in that the vector is chosen from the group consisting of Marek's disease viruses (MDV and HVT), infectious laryngotracheitis virus ILTV and herpes of ducks.

4. Live recombinant avian vaccine according to claim 3, characterized in that the vector is the HVT virus and the insertion is carried out in the BamHI fragment I.

5. Recombinant avian vaccine according to claim 4, characterized in that the insertion is carried out in one of the intergenic regions 1, 2 and 3 or in ORF UL55 of the BamHI fragment I.

6. Live recombinant avian vaccine according to any one of claims 1 to 5, characterized in that the CMV immediate early promoter is the human promoter HCMV IE or the murine promoter MCMV IE.

7. Live recombinant avian vaccine according to any one of claims 1 to 6, characterized in that the nucleotide sequence inserted under the control of the CMV immediate early promoter is a nucleotide sequence coding for an antigen chosen from the

group of antigens of Gumboro disease, of Marek's disease, of Newcastle disease, of infectious bronchitis, of infectious laryngotracheitis and of avian anaemia.

8. Live recombinant avian vaccine according to any one of claims 1 to 7, characterized in that the nucleotide sequence inserted under the control of the CMV immediate early promoter is a nucleotide sequence coding for the polypeptide VP2 of the IBDV virus.

9. Live recombinant avian vaccine according to any one of claims 1 to 8, characterised in that it comprises another promoter combined with the CMV immediate early promoter wherein the two promoters are linked such that they direct transcription in opposite directions with the latter, two nucleotide sequences being inserted into the insertion region, one under the control of the CMV immediate early promoter, the other under that of the promoter used in combination therewith.

10. Live recombinant avian vaccine according to claim 9, characterized in that the promoter used in combination is the Marek 1.8 RNA promoter.

11. Live recombinant avian vaccine according to claim 9 or 10, characterized in that the nucleotide sequence inserted under the control of the CMV immediate early promoter is a nucleotide sequence coding for the polypeptide VP2 of the IBDV virus, and in that the nucleotide sequence inserted under the control of the promoter used in combination is a nucleotide sequence coding for an antigen of another avian disease.

12. Live recombinant avian vaccine according to claim 11, characterized in that the nucleotide sequence coding for an antigen of another avian disease is chosen from the group of antigens of Marek's disease, of Newcastle disease, of infectious bronchitis, of infectious laryngotracheitis and of avian

anaemia.

13. Live recombinant avian vaccine according to claim 9, characterized in that the promoter used in combination is a CMV immediate early promoter of different origin.

14. Live recombinant avian vaccine according to any one of claims 1 to 13, characterized in that the inserted nucleotide sequence or sequences is/are chosen from the group of sequences coding for the following genes:

- VP2, VP3 and VP2 + VP4 + VP3 of the Gumboro disease virus,
- gB, gC, gD and gH + gL of the Marek's disease viruses,
- VP1 (52 kDa) + VP2 (24 kDa) of the avian anaemia virus,
- S and M of the infectious bronchitis virus, and
- gB, gC, gD and gH + gL of the infectious laryngotracheitis virus.

15. Recombinant avian vaccine according to one of claims 1 to 7, characterized in that the 1.8 RNA promoter is used instead of the CMV immediate early promoter.

16. Polyvalent vaccine formula comprising, as a mixture or to be mixed, at least two live recombinant avian vaccines as are defined in any one of claims 1 to 15, these vaccines comprising different inserted sequences.

17. Polyvalent vaccine formula according to claim 16, characterized in that the different inserted nucleotide sequences originate from different pathogens.

DATED this 10th day of June, 1999

Merial

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

Figure 1

```
   1 GGATCCATCAGCAATGCGGGCTGTAGTCCCGATTCCCGTTTCAAATGAAGGTGCTCCAAC
 159◀  AspMetLeuLeuAlaProGlnLeuGlySerGluArgLysLeuHisLeuHisGluLeuV
  61 ACGGTCTTCAAAGCAACCGGCATACCAGCAAACACAGACTGCAACTCCCCGCTGCAATGA
 139◀alThrLysLeuAlaValProMetGlyAlaPheValSerGlnLeuGluGlySerCysHisA
 121 TTGGTTATAAACAGTAATCTGTCTTCTGGAAGTATATTTCGCCCGACAATCCACGGCGCC
 119◀snThrIlePheLeuLeuArgAspGluProLeuIleAsnArgGlyValIleTrpProAlaG
 181 CCCAAAGTTAAAAACCATCCATGTGTATTTGCGTCTTCTCTGTTAAAAGAATATTGACTG
  99◀lyLeuThrLeuPheTrpGlyHisThrAsnAlaAspGluArgAsnPheSerTyrGlnSerA
 241 GCATTTTCCCGTTGACCGCCAGATATCCAAAGTACAGCACGATGTTGCACGGACGACTTT
  79◀laAsnGluArgGlnGlyGlySerIleTrpLeuValAlaArgHisGlnValSerSerLysA
 301 GCAGTCACCAGCCTTCCTTTCCACCCCCCCACCAACAAAATGTTTATCGTAGGACCCATA
  59◀laThrValLeuArgGlyLysTrpGlyGlyValLeuLeuIleAsnIleThrProGlyMetA
 361 TCCGTAATAAGGATGGGTCTGGCAGCAACCCCATAGGCGCCTCGGCGTGGTAGTTCTCGA
  39◀spThrIleLeuIleProArgAlaAlaValGlyTyrAlaGlyArgArgProLeuGluArgP
 421 GGATACATCCAAAGAGGTTGAGTATTCTCTCTACACTTCTTGTTAAATGGAAAGTGCATT
  19◀roTyrMetTrpLeuProGlnThrAsnGluArgCysLysLysAsnPheProPheHisMet
 481 TGCTTGTTCTTACAATCGGCCCGAGTCTCGTTCACAGCGCCTCGTTCACACTTAAACCAC
 541 AAATAGTCTACAGGCTATATGGGAGCCAGACTGAAACTCACATATGACTAATATTCGGGG
 601 GTGTTAGTCACGTGTAGCCCATTGTGTGCATATAACGATGTTGGACGCGTCCTTATTCGC
 661 GGTGTACTTGATACTATGGCAGCGAGCATGGGATATTCATCCTCGTCATCGTTAACATCT
                        1▶MetAlaAlaSerMetGlyTyrSerSerSerSerSerLeuThrSer
 721 CTACGGGTTCAGAATGTTTGGCATGTCGTCGATCCTTTGCCCATCGTTGCAAATTACAAG
  16▶LeuArgValGlnAsnValTrpHisValValAspProLeuProIleValAlaAsnTyrLys
 781 TCCGATCGCCATGACCGCGATAAGCCTGTACCATGTGGCATTAGGGTGACATCTCGATCA
  36▶SerAspArgHisAspArgAspLysProValProCysGlyIleArgValThrSerArgSer
 841 TACATTATAAGACCAACGTGCGAGTCTTCCAAAGACCTGCACGCCTTCTTCTTCGGATTG
  56▶TyrIleIleArgProThrCysGluSerSerLysAspLeuHisAlaPhePhePheGlyLeu
 901 TCAACGGGTTCTTCAGAATCTATGCCCATATCTGGCGTTGAGACCATTGTGCGTTTAATG
  76▶SerThrGlySerSerGluSerMetProIleSerGlyValGluThrIleValArgLeuMet
 961 AACAATAAAGCGGCATGCCATGGAAAGGAGGGCTGCAGATCTCCATTTTCTCACGCCACT
  96▶AsnAsnLysAlaAlaCysHisGlyLysGluGlyCysArgSerProPheSerHisAlaThr
1021 ATCCTGGACGCTGTAGACGATAATTATACCATGAATATAGAGGGGGTATGTTTCCACTGC
 116▶IleLeuAspAlaValAspAspAsnTyrThrMetAsnIleGluGlyValCysPheHisCys
1081 CACTGTGATGATAAGTTTTCTCCAGATTGTTGGATATCTGCATTTTCTGCTGCCGAACAA
 136▶HisCysAspAspLysPheSerProAspCysTrpIleSerAlaPheSerAlaAlaGluGln
1141 ACTTCATCGCTATGCAAAGAGATGCGTGTGTACACGCNGCCGTTGAGTATACGGGAAACT
 156▶ThrSerSerLeuCysLysGluMetArgValTyrThr???ProLeuSerIleArgGluThr
1201 AAATGTTCATAGAGGTCTTTGGGCTATATGTTATTAAATAAAATAATTGACCAGTGAACA
 176▶LysCysSer
1261 ATTTGTTTAATGTTAGTTTATTCAATGCATTGGTTGCAAATATTCATTACTTCTCCAATG
1321 CCAGGTCATTCTTTAGCGAGTGATGTTATGACATTGCTGTGAAAATTACTACAGGATATA
1381 TTTTTAAGATGCAGGAGTAACAATGTGCATAGTAGGCGTAGTTATCGCAGACGTGCAACG
          185◀SerAlaProThrValIleHisMetThrProThrThrIleAlaSerThrCysArg
                             1▶MetCysIleValGlyValValIleAlaAspValGlnAr
1441 CTTCGCATTTGAGTTACCGAAGTGCCCAACAGTGCTGCGGTTATGGTTTATGCGCACAGA
 167◀LysAlaAsnSerAsnGlyPheHisGlyValThrSerArgAsnHisAsnIleArgValSer
  13▶gPheAlaPheGluLeuProLysCysProThrValLeuArgLeuTrpPheMetArgThrGl
1501 ATCCATGCATGTCCTAATTGAACCATCCGATTTTTCTTTTAATCGCGATCGTTGTTTGGG
 147◀AspMetCysThrArgIleSerGlyAspSerLysGluLysLeuArgSerArgGlnLysPro
  33▶uSerMetHisValLeuIleGluProSerAspPheSerPheAsnArgAspArgCysLeuGl
1561 CAACTGCGTTATTTCAGATCTAAAAAATTTACCCTTTATGACCATCACATCTCTCTGGCT
 127◀LeuGlnThrIleGluSerArgPhePheLysGlyLysIleValMetValAspArgGlnSer
  53▶yAsnCysValIleSerAspLeuLysAsnLeuProPheMetThrIleThrSerLeuTrpLe
```

(FIGURE 1)

```
1621 CATACCCCGCTTGGATAAGATATCATGTAGATTCCGCCCTAAGAAATGCAAACTAACATT
 107◀MetGlyArgLysSerLeuIleAspHisLeuAsnArgGlyLeuPheHisLeuSerValAsn
  73▶uIleProArgLeuAspLysIleSerCysArgPheArgProLysLysCysLysLeuThrLe
1681 ATTGTCGGTTCCATATACACTTCCATCTTGTCCTTCGAAAATAACAAACTCGCGCAATAG
  87◀AsnAspThrGlyTyrValSerGlyAspGlnGlyGluPheIleValPheGluArgLeuLeu
  93▶uLeuSerValProTyrThrLeuProSerCysProSerLysIleThrAsnSerArgAsnAr
1741 ACCGTCCGTACATGCATGGCCGATGTGTGTCAACATCATTGGTCTGCTAGATCCCGATGG
  67◀GlyAspThrCysAlaHisGlyIleHisThrLeuMetMetProArgSerSerGlySerPro
 113▶gProSerValHisAlaTrpProMetCysValAsnIleIleGlyLeuLeuAspProAspGl
1801 GACGAATCGTACAGTCGTCGCTCCAGCATTGGCAAAAATCCCCAGATACCCTCCATGCGG
  47◀ValPheArgValThrThrAlaGlyAlaAsnAlaPheIleGlyLeuTyrGlyGlyHisPro
 133▶yThrAsnArgThrValValAlaProAlaLeuAlaLysIleProArgTyrProProCysGl
1861 CAAATCTAAATTGCGACCCCGAAGAGACTGCACCAAAGTCTTATCGACGCACGCTGATTT
  27◀LeuAspLeuAsnArgGlyArgLeuSerGlnValLeuThrLysAspValCysAlaSerLys
 153▶yLysSerLysLeuArgProArgArgAspCysThrLysValLeuSerThrHisAlaAspPh
1921 TTTTGAACAGCGGGAGCCCATTATCTTCAGTGGAGCGTAGACGGGCGAGGCTAATTATGT
   7◀LysSerCysArgSerGlyMet
 173▶ePheGluGlnArgGluProIleIlePheSerGlyAla
1981 GACATAGCAACACTGCATGTATGTTTTTATAAATCAATAAGAGTACATAATTTATTACGT
2041 ATCATTTCCGTTTGTAATATACTGTATACATCATCCACACTATTAGTCAGCACTAGCGCG
2101 CGGGCGCACGTTACAATAGCAGCGTGCCCGTTATCTATATTGTCCGATATTTACACATAA
2161 CATTTCATCGACATGATTAAATACCTAAGTACTGCACACAGATGTTTAATGTATATCGTC
2221 ATATAAATTATATCGCTAGGACAGACCCAAACGACCTTTATCCCAAACAGTCAGATCCTC
2281 TTCTCAAGTGTCGATTTCTGTTATGGAATATGCATACCCTGGCCCAGAAATTGCACGCAC
         265◀ThrAspIleGluThrIleSerTyrAlaTyrGlyProGlySerIleAlaArgVal
2341 GAGCGTAGTGAATGCGTCATTGGTTTTACATTTAAAGGCTAAATGCACAAATTCTTTAGA
 247◀LeuThrThrPheAlaAspAsnThrLysCysLysPheAlaLeuHisValPheGluLysSer
2401 CGACAGCACATCGTTAAATAGCATCTCTAGCGTTCTTATGAATGCTAAGCATTGGAGTCC
 227◀SerLeuValAspAsnPheLeuMetGluLeuThrArgIlePheAlaLeuCysGlnLeuGly
2461 TCCTGGTCGGCCACAATAACAGCTGAGTATCATACCCTGAGCTCCGGGGTTGTCGCACAT
 207◀GlyProArgGlyCysTyrCysSerLeuIleMetGlyGlnAlaGlyProAsnAspCysMet
2521 AGCGGATTCGTATAAACATAGGATTTTCCGCGAATCCATCAGTTGCAAAAATCTGTTAGG
 187◀AlaSerGluTyrLeuCysLeuIleLysArgSerAspMetLeuGlnLeuPheArgAsnPro
2581 CTCCATCAACAACGCTGGATTTACTTCAGATCCACGCGTAAAGTAATGGTGCTCGAATAC
 167◀GluMetLeuLeuAlaProAsnValGluSerGlyArgThrPheTyrHisHisGluPheVal
2641 CGTTTTTAGAGTTGTCGGCATTTCAAGGAACAAAGAATTCATTTCTTCATTGCAACGACG
 147◀ThrLysLeuThrThrProMetGluLeuPheLeuSerAsnMetGluGluAsnCysArgArg
2701 CGCCAGAAATCCCAAGACCTCTTTGGGTAGTATGTTCTTGCCTATAAAACACGGCGTTCC
 127◀AlaLeuPheGlyLeuValGluLysProLeuIleAsnLysGlyIlePheCysProThrGly
2761 AAGTGCCAGGAACCACGCATGTGTTACTGTTGGGGCGTATTCAGAAATAAAGCGGGGTTT
 107◀LeuAlaLeuPheTrpAlaHisThrValThrProAlaTyrGluSerIlePheArgProLys
2821 ATGCGGCTTTTGAAGCTCGGATATCCAAAGTATCGCTTGCTGATGAACGAGCGATGTAGC
  87◀HisProLysGlnLeuGluSerIleTrpLeuIleAlaGlnGlnHisValLeuSerThrAla
2881 TGTTACAAAACCTCCTTTCCATCCTCCAGTCAACATAATATTTATCGGCCTACCTATGTC
  67◀ThrValPheGlyGlyLysTrpGlyGlyThrLeuMetIleAsnIleProArgGlyIleAsp
2941 CGTAATAAGTATTGGTCGGGCAATTATTCCGTATGAGGTCTTGCAGGAATAAGCTCTTAG
  47◀ThrIleLeuIleProArgAlaIleIleGlyTyrSerThrLysCysSerTyrAlaArgLeu
3001 GGACAGCCAGCTTGGATATGGTGCGAAACAGACCTTCTCGGCTTCAGAATGTCGCTCCGC
  27◀SerLeuTrpSerProTyrProAlaPheCysValLysGluAlaGluSerHisArgGluAla
3061 AGTCTCTTCGTGTCGGTGCATCTTAGATCCACCATCAATGTGTGCAGCATTGACTCCCGC
   7◀ThrGluGluHisArgHisMet
```

(FIGURE 1)

```
3121  CCGTCGAATATTCCTTTTGTTACGATGCAGTAATGAGCACGATCATGGGCGGGGCGATGA
3181  CGTTCTATTTGCATGTCTGCGAACAATTTGCGTCAGTCATACAGCTATGGAGTGGGCCAT
3241  TTCTGGCGTCAACTTAAAAACGCGAACCGCAGACATATGTATTTGCATGCAAAGACGTAT
3301  CTTCGTATTTCTGGGCATCTTCAAATGCTCTGGCCAATATGGCAATGAATTTGGATTCGT
3361  TTGACGCCGATGGTATGCAGTGCAAATGTGCCAATAGCCCACATCCGAAAAAGTTATTTG
3421  TCATACAAGCAGGTGTTAAGTAGCAATCACATAAAGGCACCAGACGCCTCATGGCATCAT
3481  AATGAATAGCTCCTTCTCCCCACTGGAACCACTGACAAAATCTGCGAGTATATTCCGCAA
3541  ACCACATTTTATTTCTCATAGAAACTACCCTAAATCCTTTTAACGGGGAAGAAGAATCCT
                                      755◄ IleArgLysValProPhePhePheGlyL
3601  AGATAGTGCTTGAAGTCATGACTGTTACTGCTGCAATAACACTGTATATTATTTATAAAT
 745◄euTyrHisLysPheAspHisSerAsnSerSerCysTyrCysGlnIleAsnAsnIlePheG
3661  TCCGTTTGTCTAGGTATCTGATGTAGGCATTCCGATCCCTTTACTATTGCGTCTTCACGA
 725◄luThrGlnArgProIleGlnHisLeuCysGluSerGlyLysValIleAlaAspGluArgG
3721  CCAAATGGGAATGCGCCAAAATCCCCACACCTCATCACCCTGGAGGCAGATTGTGTATTA
 705◄lyPheProPheAlaGlyPheAspGlyCysArgMetValArgSerAlaSerGlnThrAsnA
3781  TTAATATCCGCCGATTGAAGCACAAAACGGTACGGTACTGTTCCTAATTCTGGTATAGAT
 685◄snIleAspAlaSerGlnLeuValPheArgTyrProValThrGlyLeuGluProIleSerG
3841  TCTATGGTCAAAAGTCTGCATATCCCCGACATTGCCATGAGATCACACAGTCCAAGTAGC
 665◄luIleThrLeuLeuArgCysIleGlySerMetAlaMetLeuAspCysLeuGlyLeuLeuM
3901  ATGTTTATTGAGTCACTCAGACTGTCAACGTCCCTCGCCGCACCACCAATCGAAAATAAA
 645◄etAsnIleSerAspSerLeuSerAspValAspArgAlaAlaGlyGlyIleSerPheLeuT
3961  GTATCTACGCAAGTTATAGCTCCGCATTTTCTATCGCTAGCAGCAATCGCGACGCAAAAC
 625◄hrAspValCysThrIleAlaGlyCysLysArgAspSerAlaAlaIleAlaValCysPheM
4021  ATAAAGGCCATGTTGGGATTTGAACTCTCTGGGGGGCTTGTTATCTTCTGCACCGTCGCA
 605◄etPheAlaMetAsnProAsnSerSerGluProProSerThrIleLysGlnValThrAlaT
4081  GTCGCAGTTTTCCGAAATTTATGTCTAATATATTTTCCGGCCGTGCTCCAATCGGCCGAA
 585◄hrAlaThrLysArgPheLysHisArgIleTyrLysGlyAlaThrSerTrpAspAlaSerP
4141  AAGAATCTGCGTATTACCAGACTCATTGACGGGCCGATAAAGACCATAAAACAAAATTCC
 565◄hePheArgArgIleValLeuSerMetSerProGlyIlePheValMetPheCysPheGluG
4201  TGTGCACTCCCTCCTCCAGTTTTGCCATCGTCCAAGTCCCGTAACTTTTTTTGCGTTTCG
 545◄lnAlaSerGlyGlyGlyThrLysGlyAspAspLeuAspArgLeuLysLysGlnThrGluL
4261  AGGAGCAAGCGTTCGTTATCCCTACCCACACTTGTTTTCCACCGTTTTCTTATTATAAGC
 525◄euLeuLeuArgGluAsnAspArgGlyValSerThrLysTrpArgLysArgIleIleLeuP
4321  GGTTGTATCGCCAACGCGTCACCGCAGGTTGTCACATACAGTGATGGCATACTTGAACGT
 505◄roGlnIleAlaLeuAlaAspGlyCysThrThrValTyrLeuSerProMetSerSerArgA
4381  GCAACAACGCGCTCGCTTTGCAAATCTAAGTCATTGACCATCAAATCGCGTTGAGAGGAT
 485◄laValValArgGluSerGlnLeuAspLeuAspAsnValMetLeuAspArgGlnSerSerL
4441  AGCCAGGCATCTTTTTTCCTAGTATGGTGACGGTGCAGCCACCCCAACTCAGTTCTTGTA
 465◄euTrpAlaAspLysLysArgThrHisHisArgHisLeuTrpGlyLeuGluThrArgThrP
4501  AAAAAAGCTATTGGCGGGAATTTATGTTCTGAGGTGCATTCTATATTTATGAGTCCATCA
 445◄hePheAlaIleProProPheLysHisGluSerThrCysGluIleAsnIleLeuGlyAspP
4561  AATGCCATTAACCAGATTCGTATTTTTTCGCTCGACCCGGCATCACTATGGATACAATAC
 425◄heAlaMetLeuTrpIleArgIleLysGluSerSerGlyAlaAspSerHisIleCysTyrA
4621  CTTTCTATGGCCCATTTCAGCTCTCGAACCAACCACACGGACAATTGACTAACATAAGTA
 405◄rgGluIleAlaTrpLysLeuGluArgValLeuTrpValSerLeuGlnSerValTyrThrH
4681  TGATCTTTATCACAGTCGCACCCATCTGAGTTATATTTATGGCATCCGAGCGCTCTTACT
 385◄isAspLysAspCysAspCysGlyAspSerAsnTyrLysHisCysGlyLeuAlaArgValT
4741  GTACGGTCGGATACACCCATGGTTTTTCCTTTATATAGTCGGGTTATAGTCTGTCGGGTT
 365◄hrArgAspSerValGlyMetThrLysGlyLysTyrLeuArgThrIleThrGlnArgThrG
4801  TGGCGGTAGCACGGAGTAGTTTGATTTTTAAGAATCGAAAACCGGCTTGGAGAGACCACT
 345◄lnArgTyrCysProThrThrGlnAsnLysLeuIleSerPheArgSerProSerValValT
4861  GTCGAATATTTGTCCGTATACTCTACACGTGAGTGTTGTCCATTCCTAGGTATATTCATC
 325◄hrSerTyrLysAspThrTyrGluValArgSerHisGlnGlyAsnArgProIleAsnMetG
```

(FIGURE 1.)

```
4921 TGTTCGGATACCTTCAATTGCTGTTCAGGCATAACCTTAAAGCATATGTTATGTTGTACA
 305◄lnGluSerValLysLeuGlnGlnGluProMetValLysPheCysIleAsnHisGlnValA
4981 TCAAAACTTGGTGAGTTATGTTCGATTGCCGCGCATAAAGAATCGTACATGAGCGTTTCT
 285◄spPheSerProSerAsnHisGluIleAlaAlaCysLeuSerAspTyrMetLeuThrGluA
5041 GCTAACATACTATCTATATTCTCACACGCCCCTGCATATACTGTTCCTATTCCAAATTCA
 265◄laLeuMetSerAspIleAsnGluCysAlaGlyAlaTyrValThrGlyIleGlyPheGluA
5101 CGTTTTGCCCCATCGGCTATCTGCTCCCAAAAAGTTGTAATATAGGTGCCGCTGGGTGCG
 245◄rgLysAlaGlyAspAlaIleGlnGluTrpPheThrThrIleTyrThrGlySerProAlaP
5161 AAATTTTCATCAGTTGTATTCCTGATAAACTGAATCACTTTACATAATTTTTGCCACATA
 225◄heAsnGluAspThrThrAsnArgIlePheGlnIleValLysCysLeuLysGlnTrpMetA
5221 TCTGCGTGCAGCCATAGTATCGAACCCGTGGGCTCGGAGACGACAGTGCGTACAATGGGT
 205◄spAlaHisLeuTrpLeuIleSerGlyThrProGluSerValValThrArgValIleProI
5281 ATTTTACCTTTCCCCAACAAAATAATGGTATACAAGTTAGGTCCGTACCTAGACCTTAAT
 185◄leLysGlyLysGlyLeuLeuIleIleThrTyrLeuAsnProGlyTyrArgSerArgLeuT
5341 GTTTCCAATTCTTCTGAATCACTGCACTCTCGTAGGGGAGTAACGGTAATAATTTCGTCT
 165◄hrGluLeuGluGluSerAspSerCysGluArgLeuProThrValThrIleIleGluAspA
5401 CTGAGCCCCGTTTTGCGTTGAAAACTAATCACATTAGATAATGTGCAATCGGTTTCTTTT
 145◄rgLeuGlyThrLysArgGlnPheSerIleValAsnSerLeuThrCysAspThrGluLysI
5461 ATCCGGATACATCTAAGTATTATGACATCGGTGGTCATTGTTTCCATCAACGACCATCTT
 125◄leArgIleCysArgLeuIleIleValAspThrThrMetThrGluMetLeuSerTrpArgL
5521 TTACGATCGCCCATACTACTCATGGACGTTGTCGGTGTTGAAAAATCACCAGAATTGCAA
 105◄ysArgAspGlyMetSerSerMetSerThrThrProThrSerPheAspGlySerAsnCysA
5581 CGGATCTCTGGGTACCATGCTGCTGATGGAATTGGCGGTTTTAATTGTTGTTTCAGTCTA
  85◄rgIleGluProTyrTrpAlaAlaSerProIleProProLysLeuGlnGlnLysLeuArgA
5641 TTATTGCTATCTTTGGCGGGGTTGAATAATGTGGGGGGAGAGTGATTGCAGGAATCCGAA
  65◄snAsnSerAspLysAlaProAsnPheLeuThrProProSerHisAsnCysSerAspSerH
5701 TGGGTCAATAAAACGACCGTGCTCCGTTCTGCCGGCGCCGATCCGATTGAAGCTATATAC
  45◄isThrLeuLeuValValThrSerArgGluAlaProAlaSerGlyIleSerAlaIleTyrL
5761 TTCGCTTCTCTCCCCACTTTTCCAATTTGATCCGGAAATAAAACGGCCCCGGACAACAGT
  25◄ysAlaGluArgGlyValLysGlyIleGlnAspProPheLeuValAlaGlySerLeuLeuI
5821 ATCGTACGATCCGGATCC
   5◄leThrArgAspProAsp
```

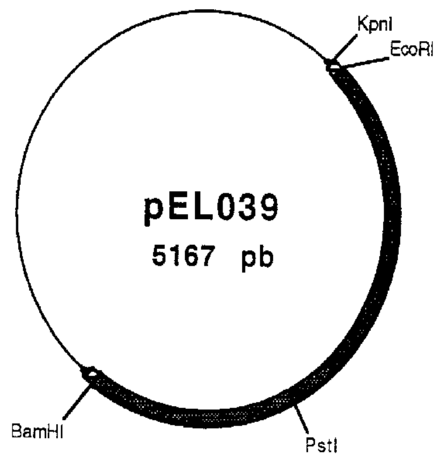


Figure 2

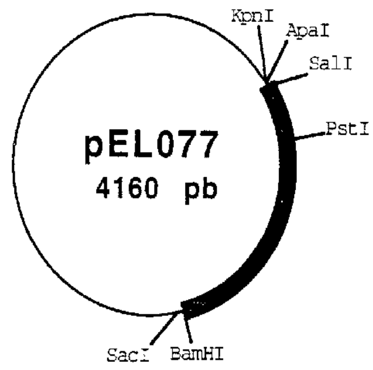


Figure 3

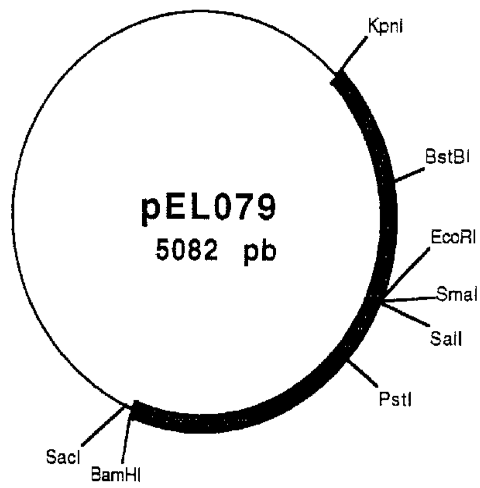


Figure 4

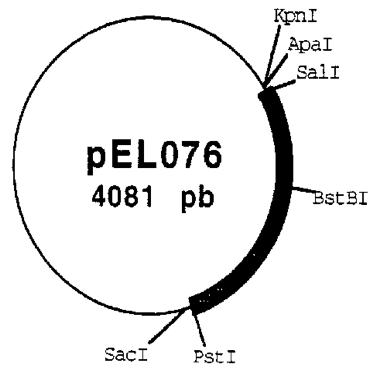


Figure 5

2 0 2 3 6 4 0 7 1 5

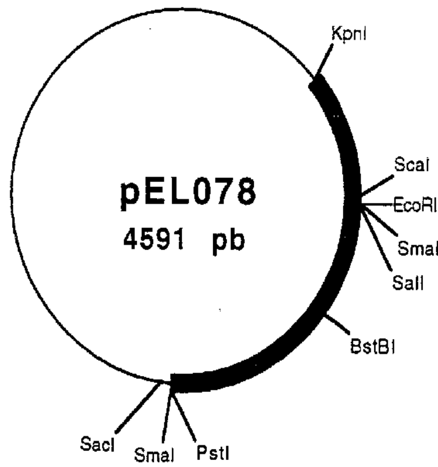


Figure 6

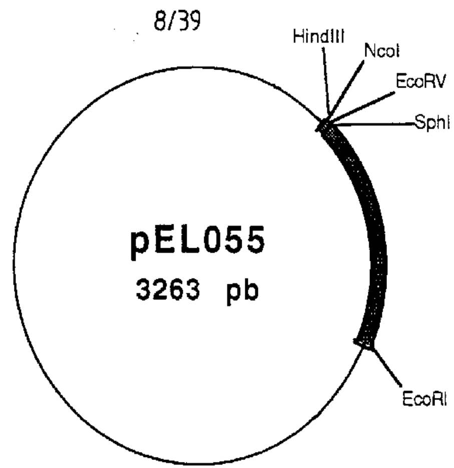


Figure 8

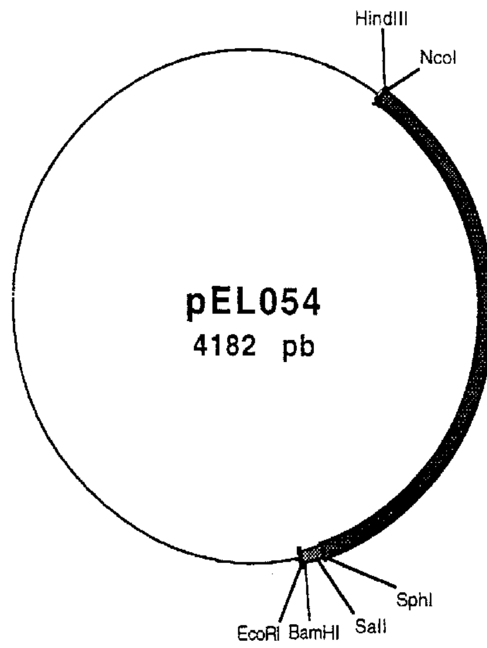


Figure 7

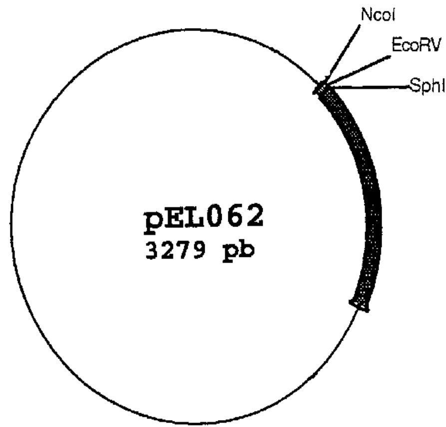


Figure 9

20 2315 40715

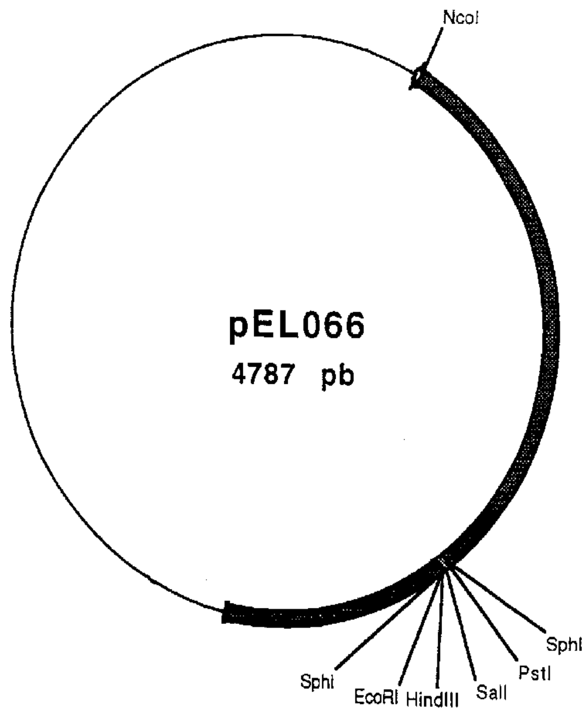


Figure 10

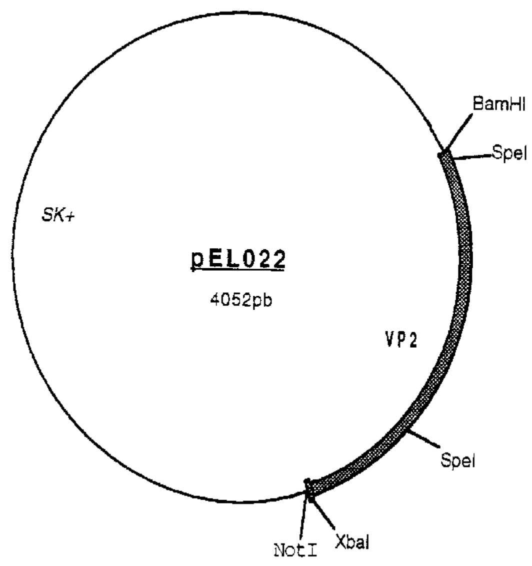


Figure 11

2 836 407 15

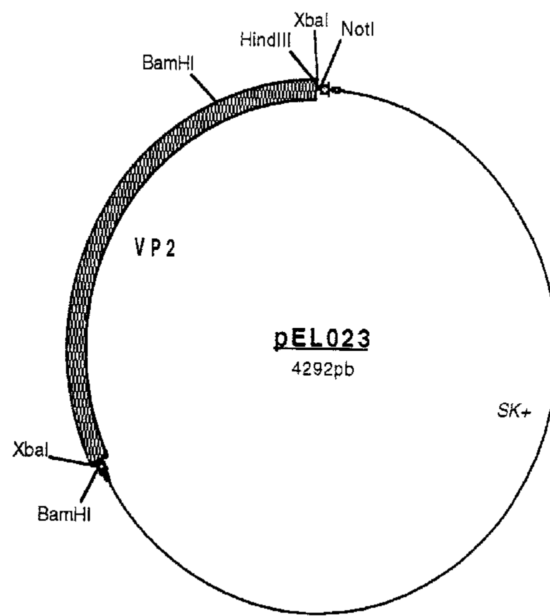


Figure 12

20 236 4075

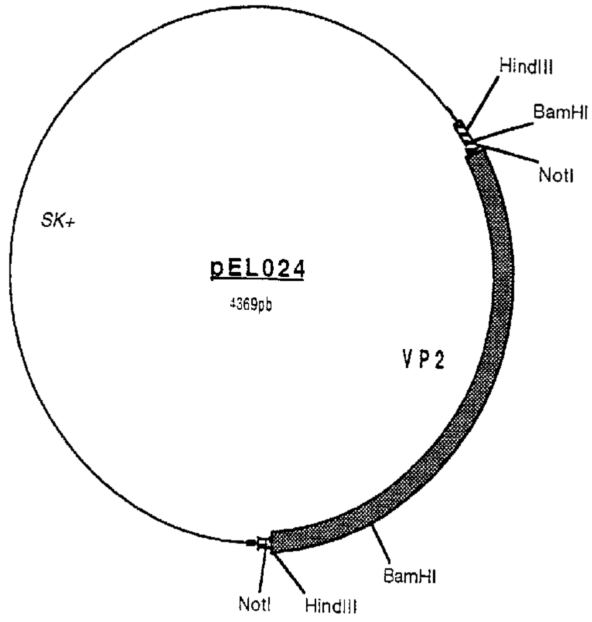


Figure 13

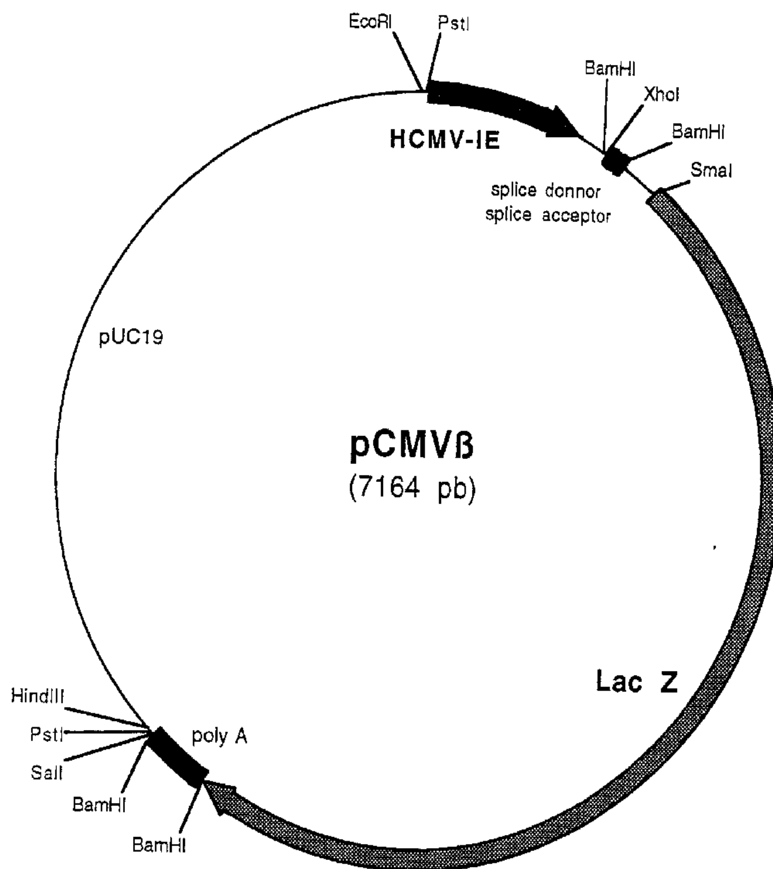


Figure 14

20 2385 407 15

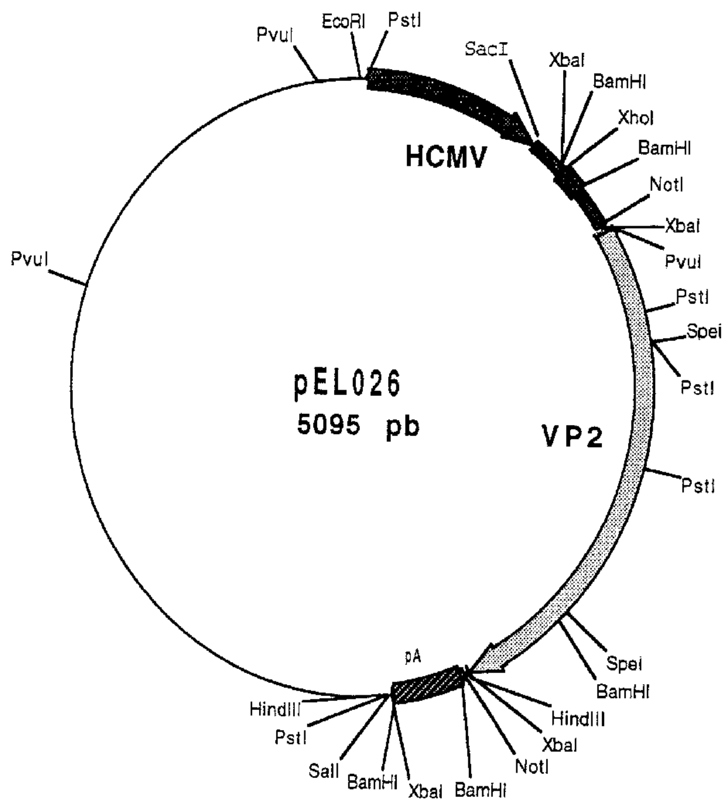


Figure 15

2 736 407 15

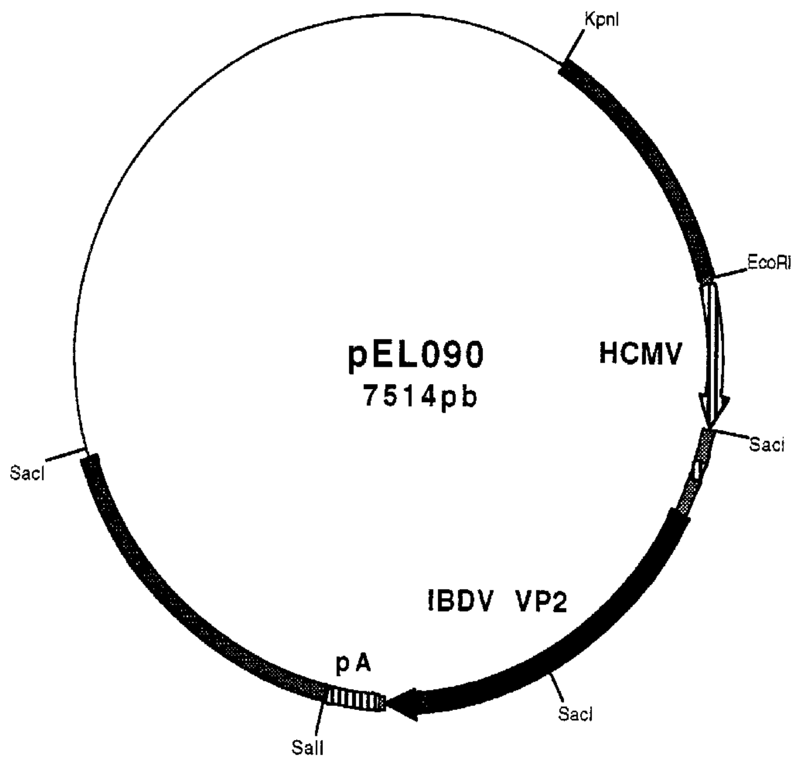


Figure 16

2 2 35 4 0 7 15

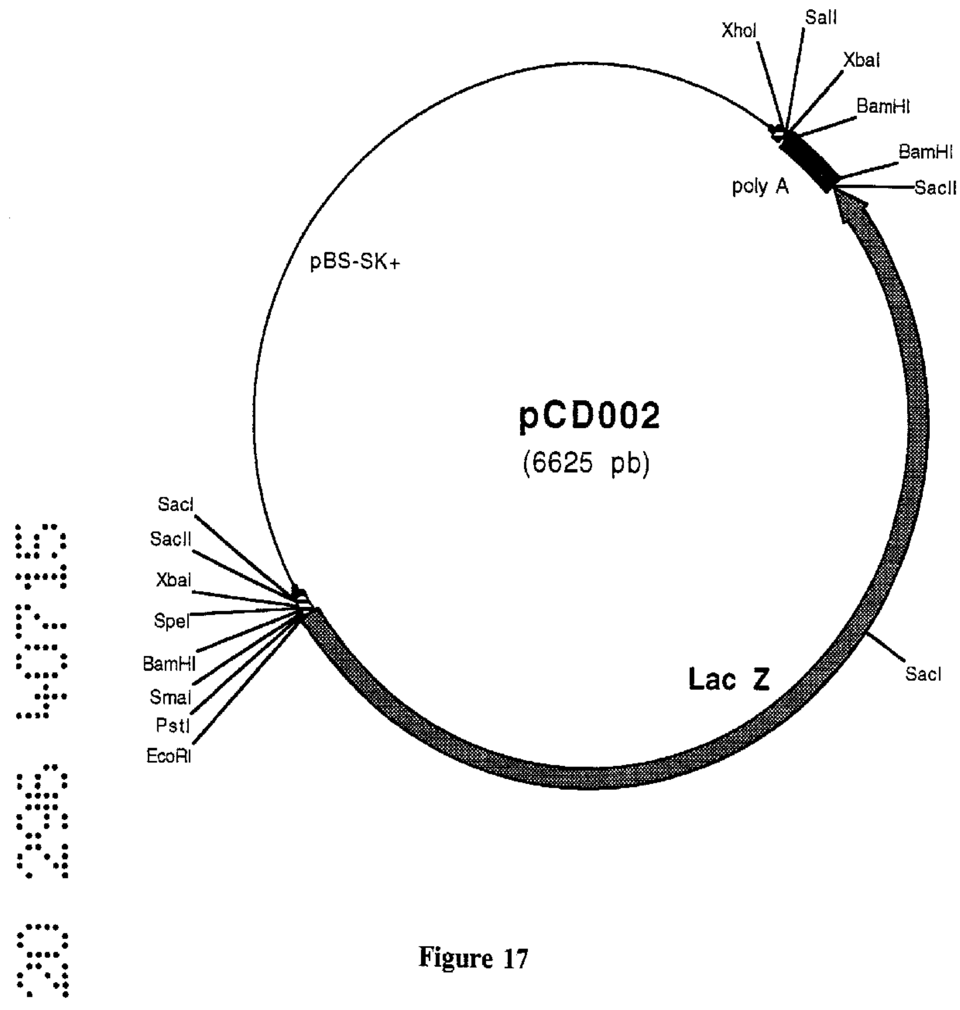


Figure 17

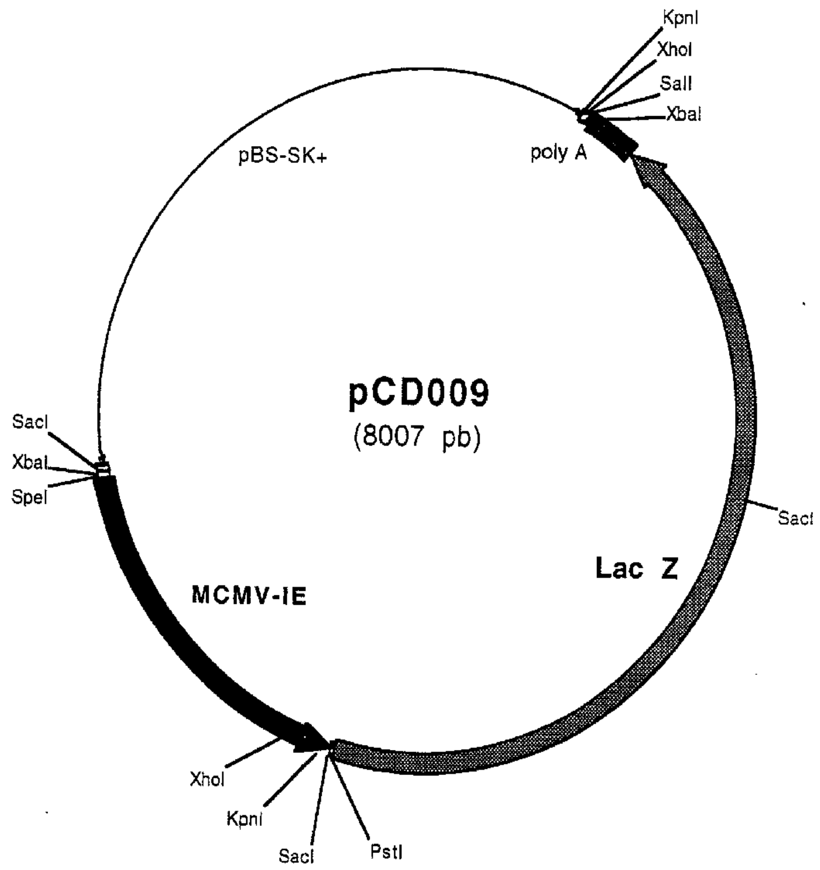


Figure 18

20 2315 40715

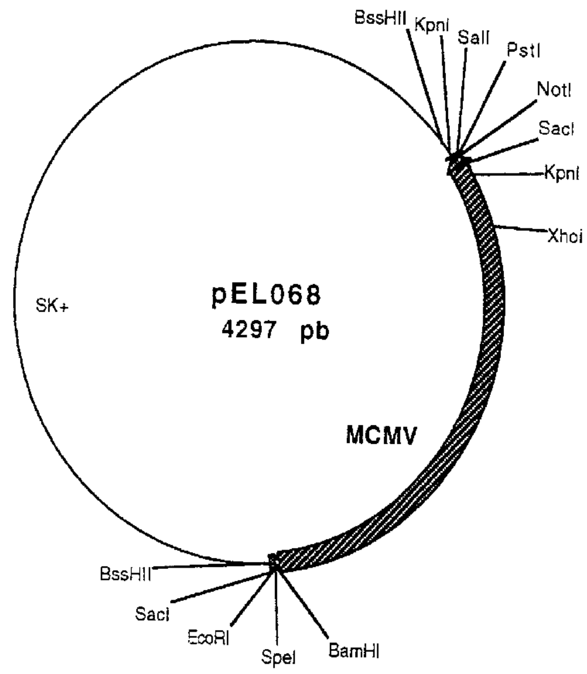


Figure 19

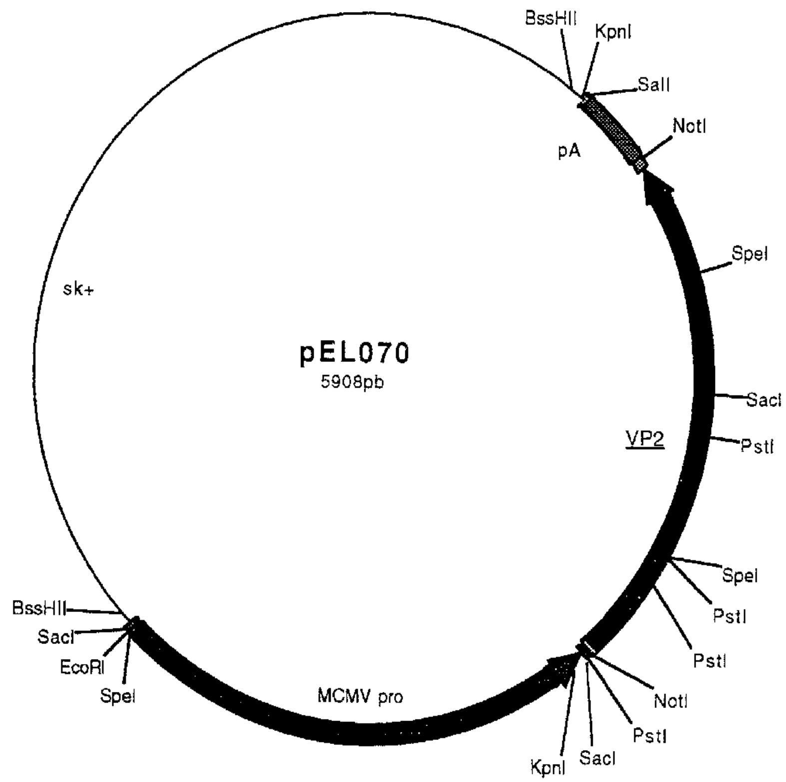


Figure 20

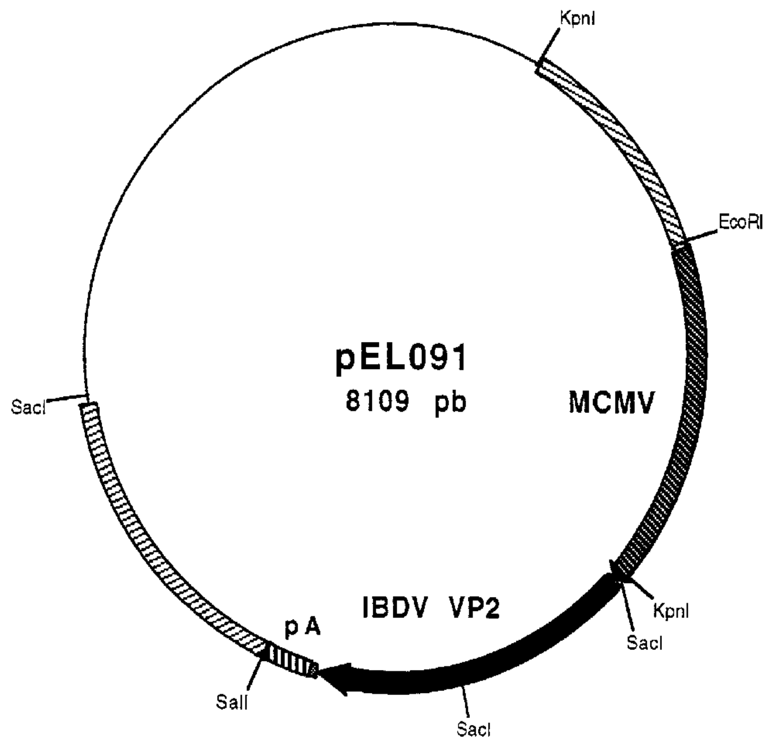


Figure 21

2 0 2 3 6 1 4 0 7 1 5

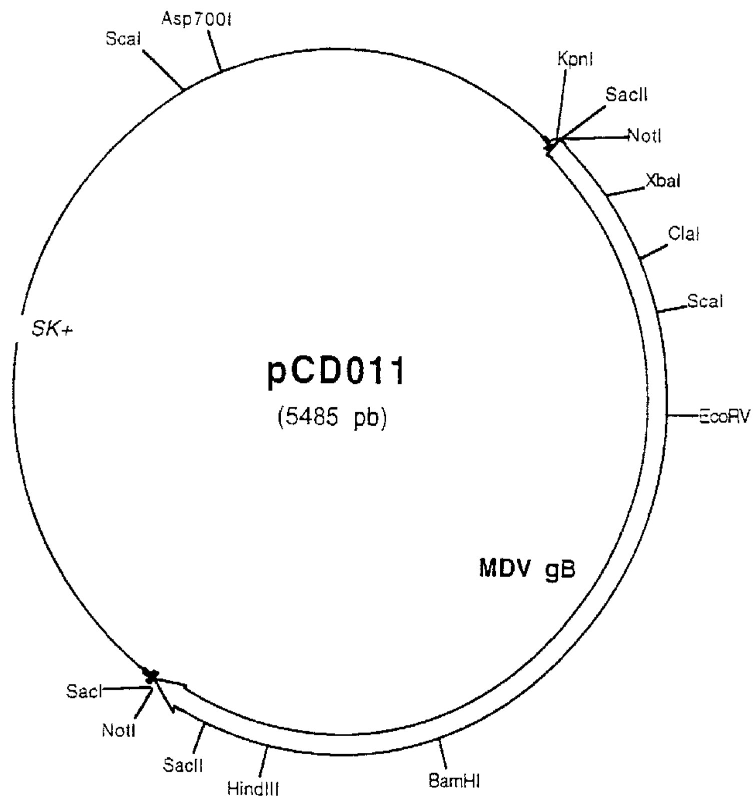


Figure 22

20 2216 140715

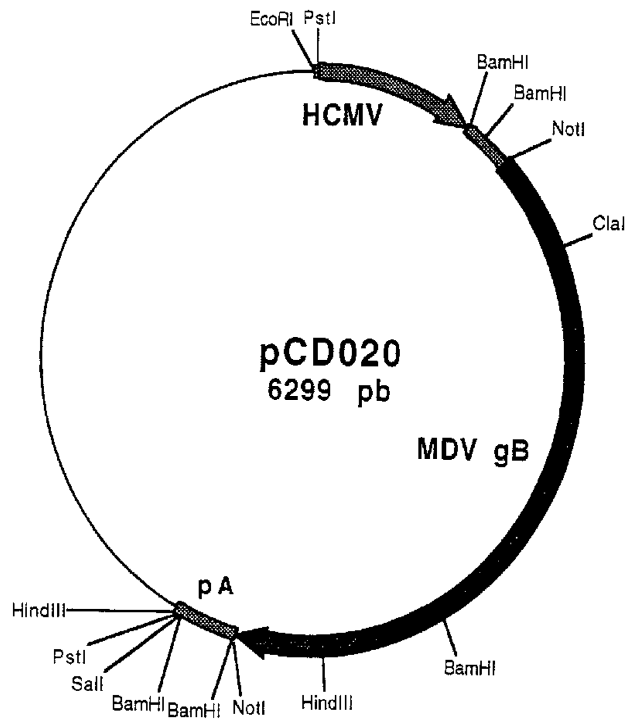
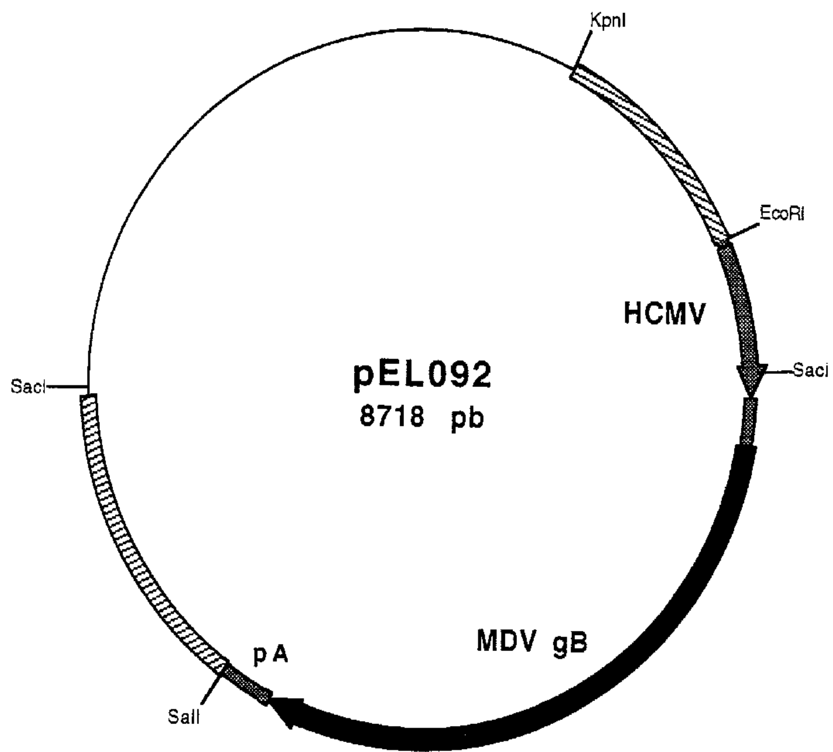


Figure 23

20 2000 4075



2 2 5 6 4 0 7 15

Figure 24

```
   1 TGCTACCTGATGTACAAGCAAAAGGCACAACAAAAGACCTTGTTATGGCTTGGGAATAAT
  61 ACCCTTGATCAGATGAGAGCCACTACAAAAATATGAATACAAACGAGAGGCGGAGGTATC
 121 CCCAATAGCAATTTGCGTGTAAATTCTGGCAACCTGTTAATTAGAAGAATTAAGAAAAAA
 181 CCACTGGATGTAAGTGACAAACAAGCAATACACGGGTAGAACGGTCGGAGAAGCCACCCC
 241 TCAATCGGGAATCAGGCCTCACAACGTCCTTTCTACCGCATCATCAATAGCAGACTTCGG
 301 TCATGGACCGTGCAGTTAGCAGAGTTGCGCTAGAGAATGAAGAAAGAGAAGCAAAGAATA
   1▶MetAspArgAlaValSerArgValAlaLeuGluAsnGluGluArgGluAlaLysAsnT
 361 CATGGCGCTTTGTATTCCGGATTGCAATCTTACTTTTAATAGTAACAACCTTAGCCATCT
  20▶hrTrpArgPheValPheArgIleAlaIleLeuLeuLeuIleValThrThrLeuAlaIleS
 421 CTGCAACCGCCCTGGTATATAGCATGGAGGCTAGCACGCCTGGCGACCTTGTTGGCATAC
  40▶erAlaThrAlaLeuValTyrSerMetGluAlaSerThrProGlyAspLeuValGlyIleP
 481 CGACTATGATCTCTAAGGCAGAAGAAAAGATTACATCTGCACTCAGTTCTAATCAAGATG
  60▶roThrMetIleSerLysAlaGluGluLysIleThrSerAlaLeuSerSerAsnGlnAspV
 541 TAGTAGATAGGATATATAAGCAGGTGGCCCTTGAGTCTCCATTGGCGTTGCTAAACACTG
  80▶alValAspArgIleTyrLysGlnValAlaLeuGluSerProLeuAlaLeuLeuAsnThrG
 601 AATCTGTAATTATGAATGCAATAACGTCTCTCTCTTATCAAATCAATGGAGCTGCAAATA
 100▶luSerValIleMetAsnAlaIleThrSerLeuSerTyrGlnIleAsnGlyAlaAlaAsnA
                              BspHI
 661 ATAGCGGGTGTGGGGCACCTGTTCATGACCCAGATTATATCGGGGGGATAGGCAAAGAAC
 120▶snSerGlyCysGlyAlaProValHisAspProAspTyrIleGlyGlyIleGlyLysGluL
 721 TTATTGTGGATGACGCTAGTGATGTCACATCATTCTATCCCTCTGCGTTCCAAGAACACC
 140▶euIleValAspAspAlaSerAspValThrSerPheTyrProSerAlaPheGlnGluHisL
 781 TGAACTTTATCCCGGCACCTACTACAGGATCAGGTTGCACTCGGATACCCTCATTCGACA
 160▶euAsnPheIleProAlaProThrThrGlySerGlyCysThrArgIleProSerPheAspI
 841 TAAGCGCTACCCACTACTGTTACACTCACAATGTGATATTATCTGGTTGCAGAGATCACT
 180▶leSerAlaThrHisTyrCysTyrThrHisAsnValIleLeuSerGlyCysArgAspHisS
 901 CACACTCATATCAGTACTTAGCACTTGGCGTGCTTCGGACATCTGCAACAGGGAGGGTAT
 200▶erHisSerTyrGlnTyrLeuAlaLeuGlyValLeuArgThrSerAlaThrGlyArgValP
 961 TCTTTTCTACTCTGCGTTCCATCAATTTGGATGACAGCCAAAATCGGAAGTCTTGCAGTG
 220▶hePheSerThrLeuArgSerIleAsnLeuAspAspSerGlnAsnArgLysSerCysSerV
1021 TGAGTGCAACTCCCTTAGGTTGTGATATGCTGTGCTCTAAAATCACAGAGACTGAGGAAG
 240▶alSerAlaThrProLeuGlyCysAspMetLeuCysSerLysIleThrGluThrGluGluG
                                   ClaI
1081 AGGATTATAGTTCAATTACGCCTACATCGATGGTGCACGGAAGGTTAGGGTTTGACGGTC
 260▶luAspTyrSerSerIleThrProThrSerMetValHisGlyArgLeuGlyPheAspGlyG
1141 AATACCATGAGAAGGACTTAGACGTCATAACTTTATTTAAGGATTGGGTGGCAAATTACC
 280▶lnTyrHisGluLysAspLeuAspValIleThrLeuPheLysAspTrpValAlaAsnTyrP
1201 CAGGAGTGGGGGGTGGGTCTTTTATTAACAACCGCGTATGGTTCCCAGTCTACGGAGGGC
 300▶roGlyValGlyGlyGlySerPheIleAsnAsnArgValTrpPheProValTyrGlyGlyL
1261 TAAAACCCAATTCGCCTAGTGACACCGCACAAGAAGGGAGATATGTAATATACAAGCGCT
 320▶euLysProAsnSerProSerAspThrAlaGlnGluGlyArgTyrValIleTyrLysArgT
1321 ACAATGACACATGCCCAGATGAACAAGATTACCAGATTCGGATGGCTAAGTCTTCATATA
 340▶yrAsnAspThrCysProAspGluGlnAspTyrGlnIleArgMetAlaLysSerSerTyrL
1381 AGCCTGGGCGGTTTGGTGGAAAACGCGTACAGCAGGCCATCTTATCTATCAAGGTGTCAA
 360▶ysProGlyArgPheGlyGlyLysArgValGlnGlnAlaIleLeuSerIleLysValSerT
1441 CATCTTTGGGCGAGGACCCGGTGCTGACTGTACCGCCTAATACAATCACACTCATGGGGG
 380▶hrSerLeuGlyGluAspProValLeuThrValProProAsnThrIleThrLeuMetGlyA
1501 CCGAACGGAGAGTTCTCACAGTAGGGACATCTCATTTCTTGTACCAGCGAGGGTCTTCAT
 400▶laGluArgArgValLeuThrValGlyThrSerHisPheLeuTyrGlnArgGlySerSerT
```

Figure 25

(FIGURE 25)

```
1561 ACTTCTCTCCTGCTTTATTATACCCTATGACAGTCAACAACAAAACGGCTACTCTTCATA
 420▸yrPheSerProAlaLeuLeuTyrProMetThrValAsnAsnLysThrAlaThrLeuHisS
1621 GTCCTTACACATTCAATGCTTTCACTAGGCCAGGTAGTGTCCCTTGTCAGGCATCAGCAA
 440▸erProTyrThrPheAsnAlaPheThrArgProGlySerValProCysGlnAlaSerAlaA
1681 GATGCCCCAACTCATGTGTCACTGGAGTTTATACTGATCCGTATCCCTTAGTCTTCCATA
 460▸rgCysProAsnSerCysValThrGlyValTyrThrAspProTyrProLeuValPheHisA

1741 GGAACCATACCTTGCGGGGGGTATTCGGACAATGCTTGATGATGAACAAGCAAGACTTA
 480▸rgAsnHisThrLeuArgGlyValPheGlyThrMetLeuAspAspGluGlnAlaArgLeuA
              PstI
1801 ACCCTGTATCTGCAGTATTTGATAACATATCCCGCAGTCGCATAACCCGGGTAAGTTCAA
 500▸snProValSerAlaValPheAspAsnIleSerArgSerArgIleThrArgValSerSerS
1861 GCCGTACTAAGGCAGCATACACGACATCGACATGTTTTAAAGTTGTCAAGACCAATAAAA
 520▸erArgThrLysAlaAlaTyrThrThrSerThrCysPheLysValValLysThrAsnLysT
1921 CATATTGCCTCAGCATTGCAGAAATATCCAATACCCTCTTCGGGGAATTCAGGATCGTTC
 540▸hrTyrCysLeuSerIleAlaGluIleSerAsnThrLeuPheGlyGluPheArgIleValP
1981 CTTTACTAGTTGAGATTCTCAAGGATGATGGGATTTAAGAAGCCAGGTCTGGCCAGTTGA
 560▸roLeuLeuValGluIleLeuLysAsp
2041 GTCAACTGCGAGAGGGTCGGAAAGATGACATTGTGTCACCTTTTTTTTGTAATGCCAAGG
2101 ATCAAACTGGATACCGGCGCGAGCCCGAATCCTATGCTGCCAGTCAGCCATAATCAGATA
2161 GTACTAATATGATTAGTCTTAATCTTGTCGATAGTAACTTGGTTAAGAAAAAATATGAGT
2221 GGTAGTGAGATACACAGCTAAACAACTCACGAGAGATAGCACGGGTAGGACATGGCGAGC
2281 TCCGGTCCCGAAAGGGCAG..GCATCAGATTATCCTACCAGAGTCACATCTGTCCTCACCA
2341 TTGGTCAAGCACAAACTGCTCTATTACTGGAAATTAACTGGCGTACCGCTTCCTGACGAA
2401 TGTGACTTCGACCACCTCATTATCAGCCGACAATGGAAGAAAATACTTGAATCGGCCACT
2461 CCTGACACTGAGAGGATGATAAAGCTCGGGCGGGCAGTACACCAGACTCTCGACCACCGC
2521 C
```

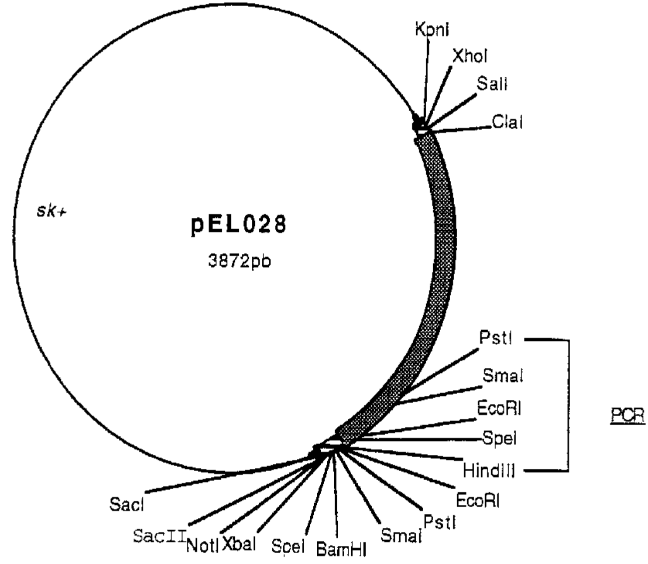


Figure 26

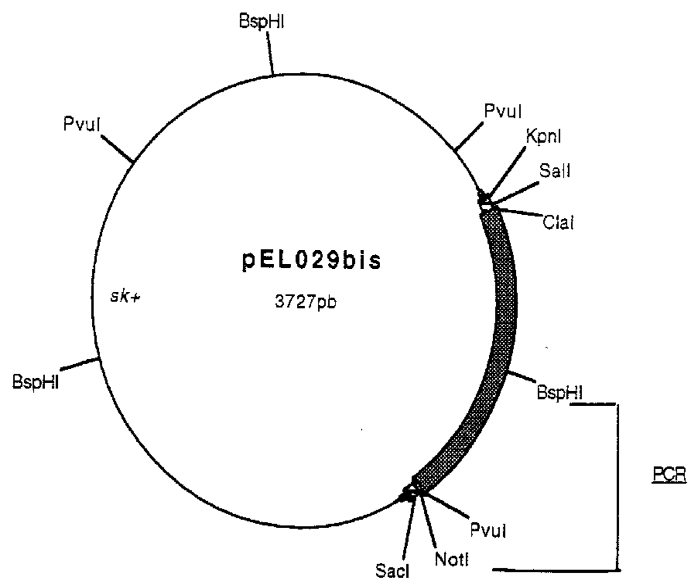


Figure 27

2 826 407 15

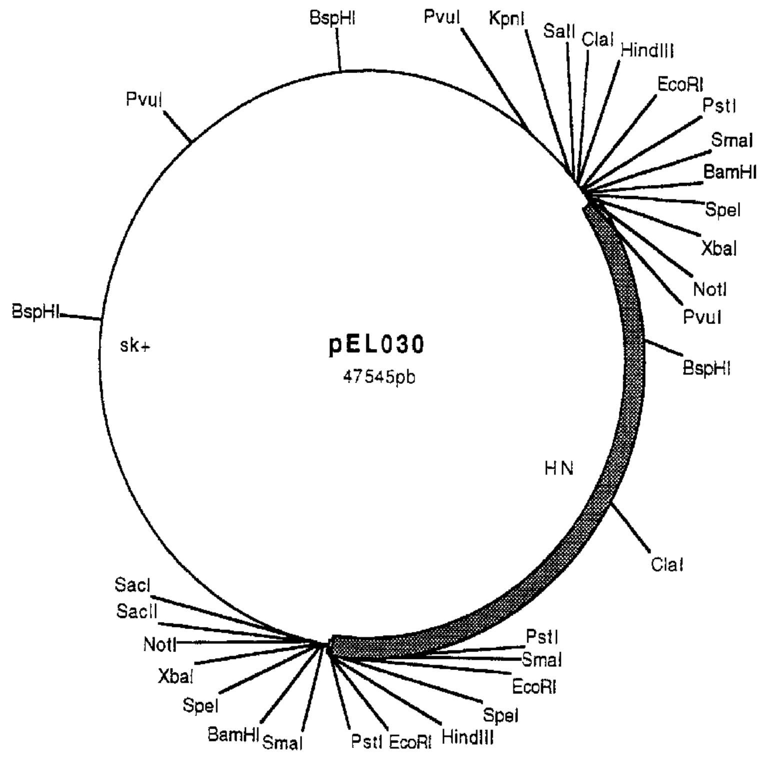


Figure 28

20 2396 4079 15

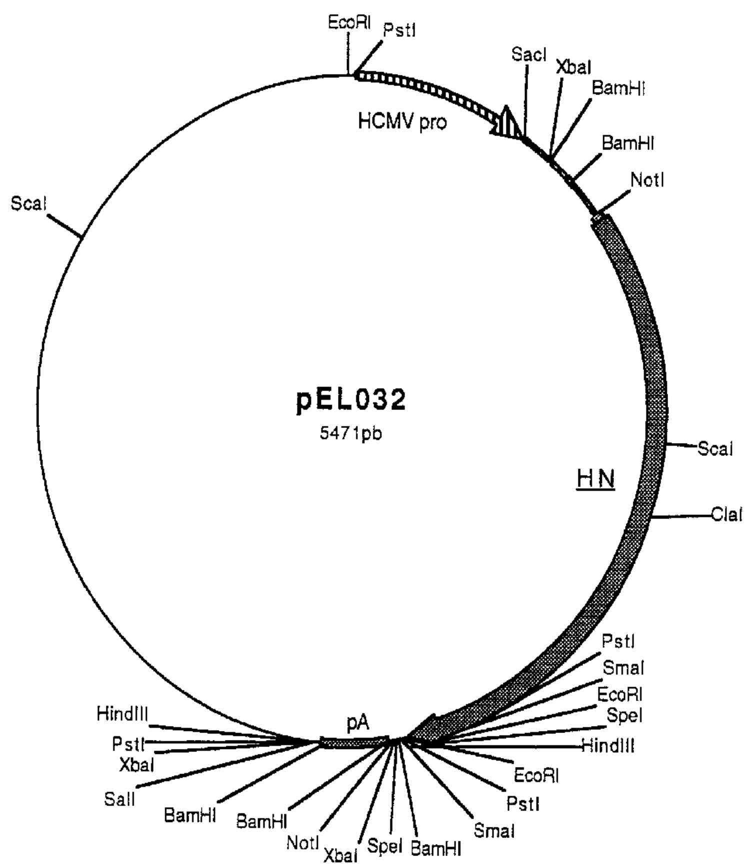


Figure 29

20 2365 4071 15

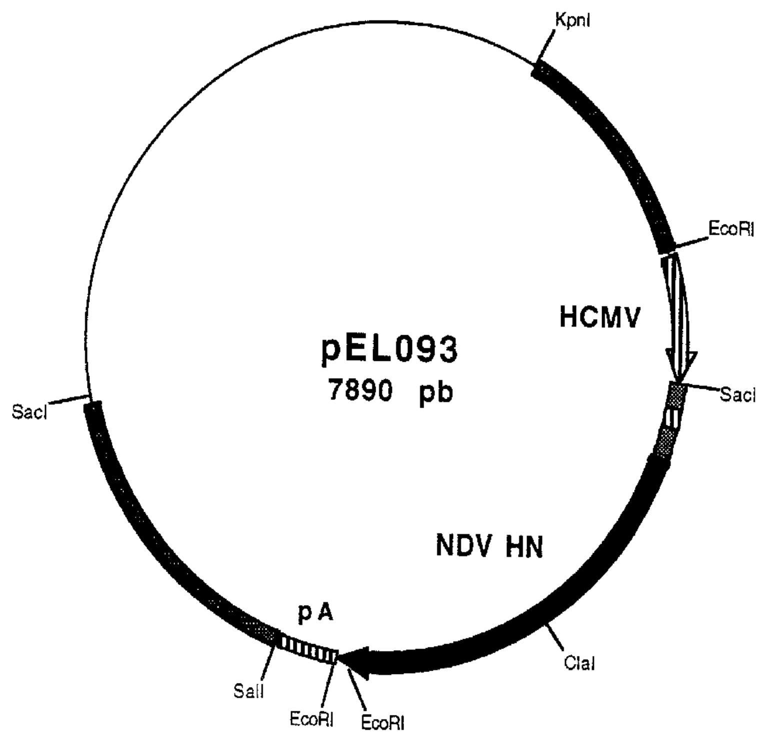


Figure 30

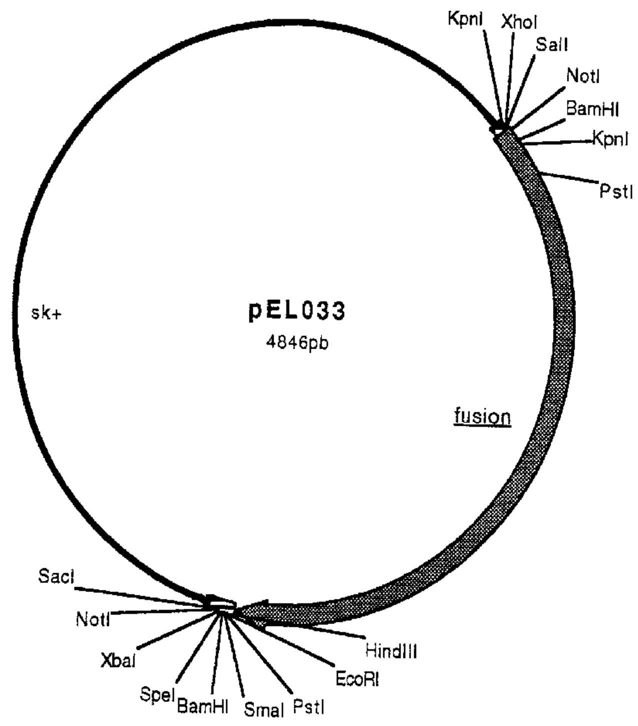


Figure 31

2 0 2 3 6 4 0 7 1 5

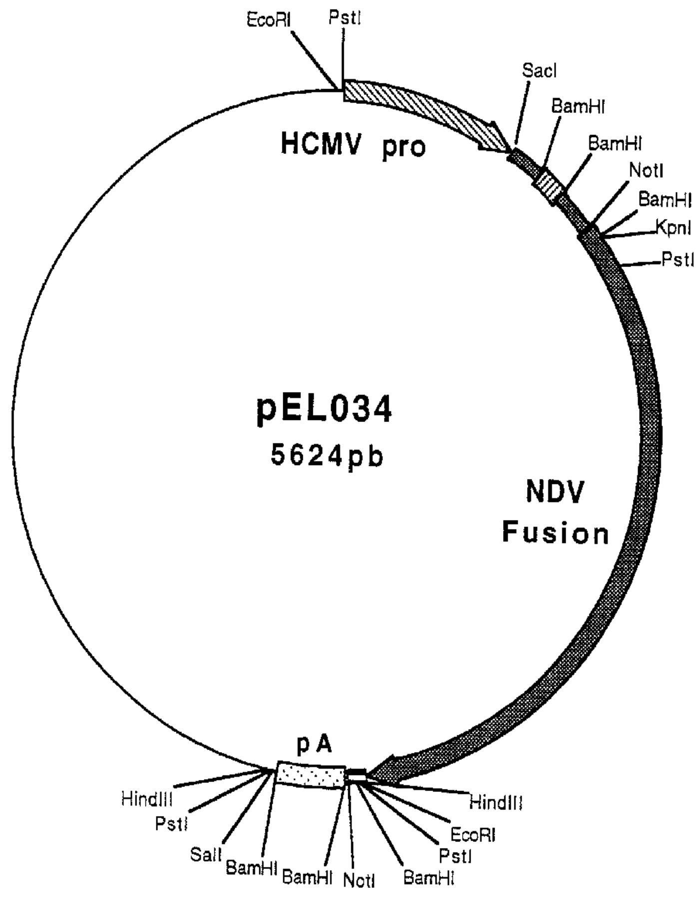


Figure 32

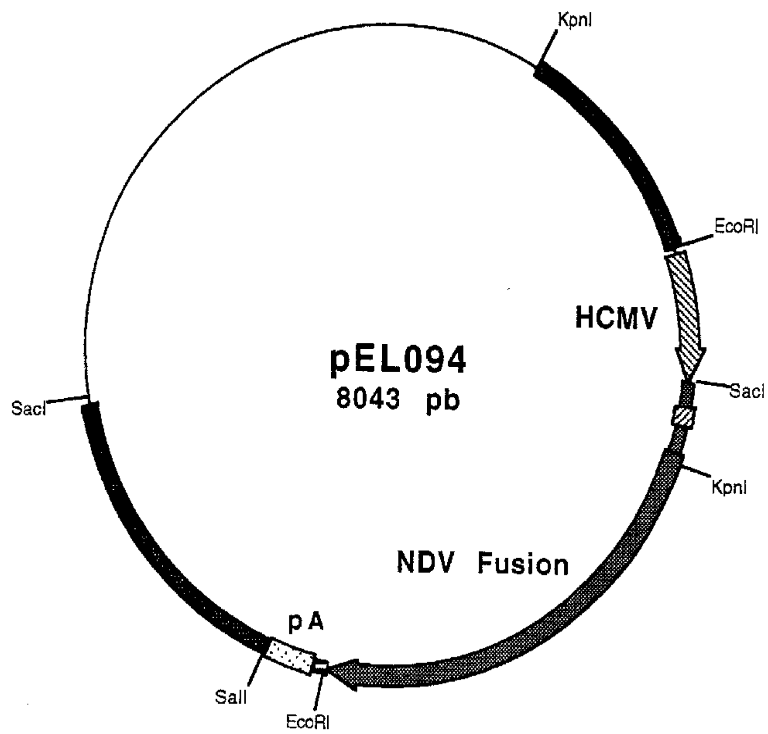


Figure 33

30 2000 407015

```
                10        20        30        40        50        60
                 |         |         |         |         |         |
  1 GAATTCCATCACCCCCTGCCGATCTTGCACGCGGGGACGAGCAAAGCGTGCGGTGCGGGC
 61 AGAAAGACAAGGATGGCTGTGGGTTGAAAGATGAAAAACAAATCGCGGTTGTGGGTCATG
121 AGTGGAGGGAGGGTGCCATCTGTGATGCCGAGAGGTCAAACTATGTTATAAAGAAAAACG
181 ATGGGTGGGAAATATAATAAAGCAACCGAAATGGTACATAAAAACTAAAAATACCTACAC
241 GGTTACACCACCGATCAGGCGAAGAAGTTCCAAACGATTAACAACCGGGACGAGACGTTG
301 CCGTTCGATCCAGGTCTCTGCTTTTTTGTATCTCTTATCCTATACCGCCGCCTCCCGTCC
361 GACGAGAGCAAGTCGCACCGCCACTCGAGGCCACAAGAAATTACGATTCTTATACGGGTG
421 GGCGTACCGCCTACTCGAACTATCACGTGATGTGTATGCAAATGAGCAGTGCGAACGCGT
481 CAGCGTTCGCACTGCGAACCAATAATATATTATATTATATTATATTATTGGACTCTGGTG
541 CGAACGCCGAGGTGAGCCAATCGGATATGGCGATATGTTATCACGTGACATGTACCGCCC
601 CAAATTCGCACTTGAGTGTTGGGGGTACATGTGGGGGCGGCTCGGCTCTTGTGTATAAAA
661 GAGCGGCGGTTGCGAGGTTCCTTCTCTCTTCGCGATGCTCTCTCAGAATGGCACGGCCGA
721 TCCCCCATATATTTCCTGAAGGAACGCATAGCTAGGCGACGAACGAGCTGAATTTCTCCC
781 TTCATCAAATAAGTAATAAA
```

Figure 34

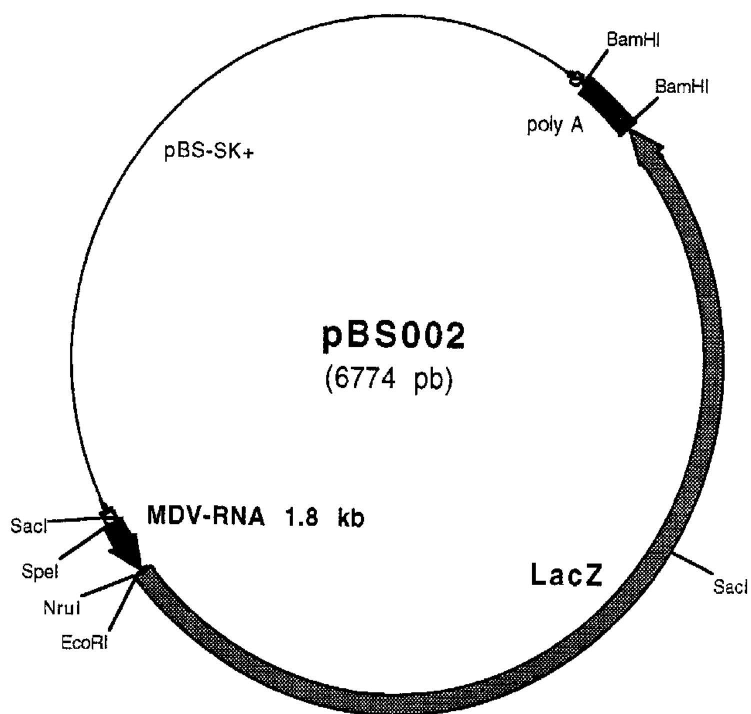


Figure 35

2 725 407 15

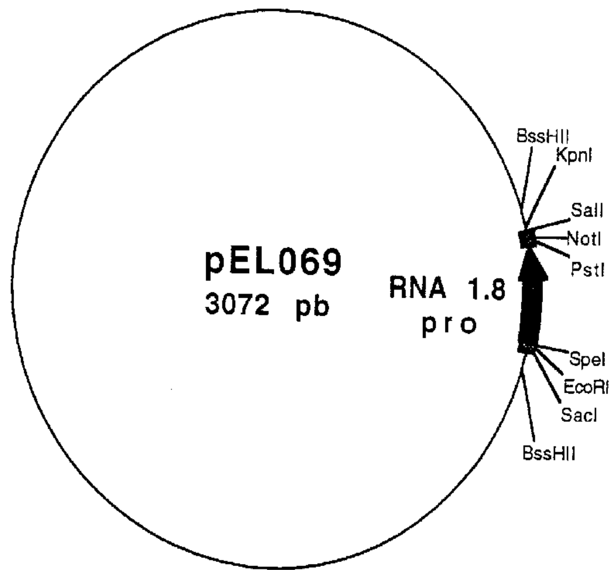


Figure 36

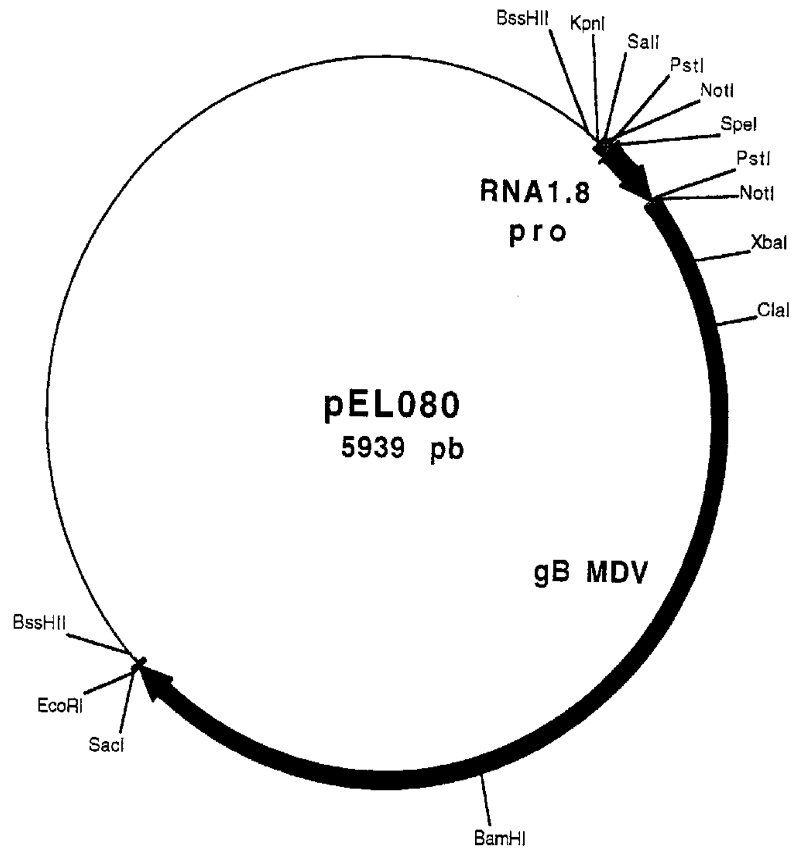


Figure 37

20 2385 4075

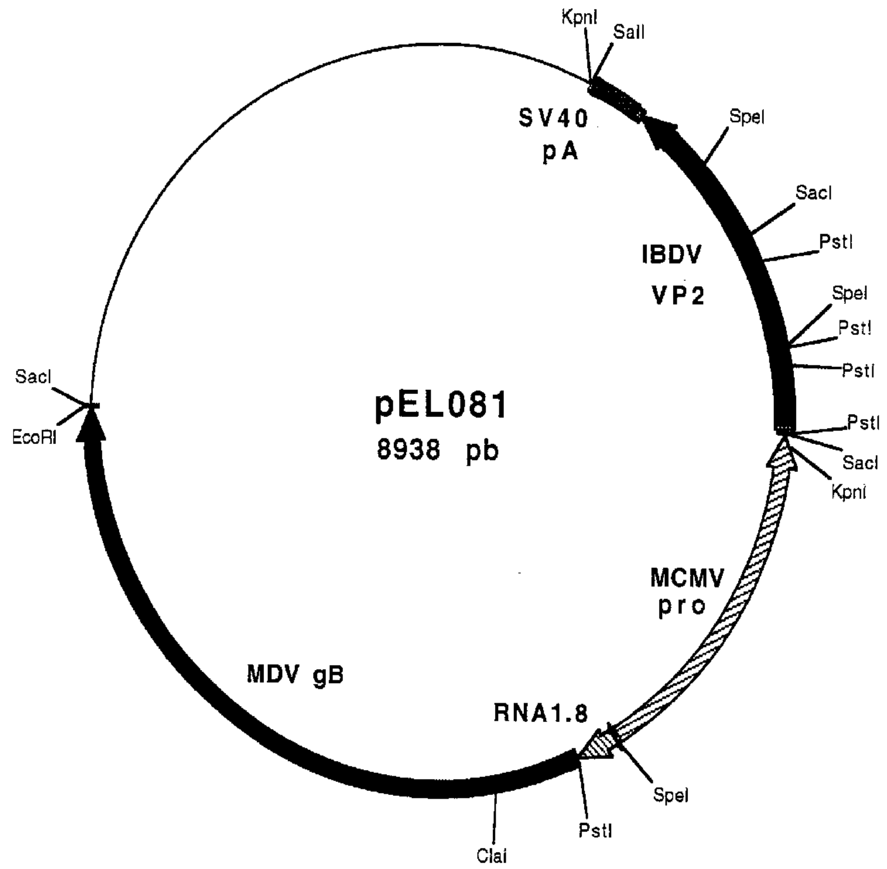


Figure 38

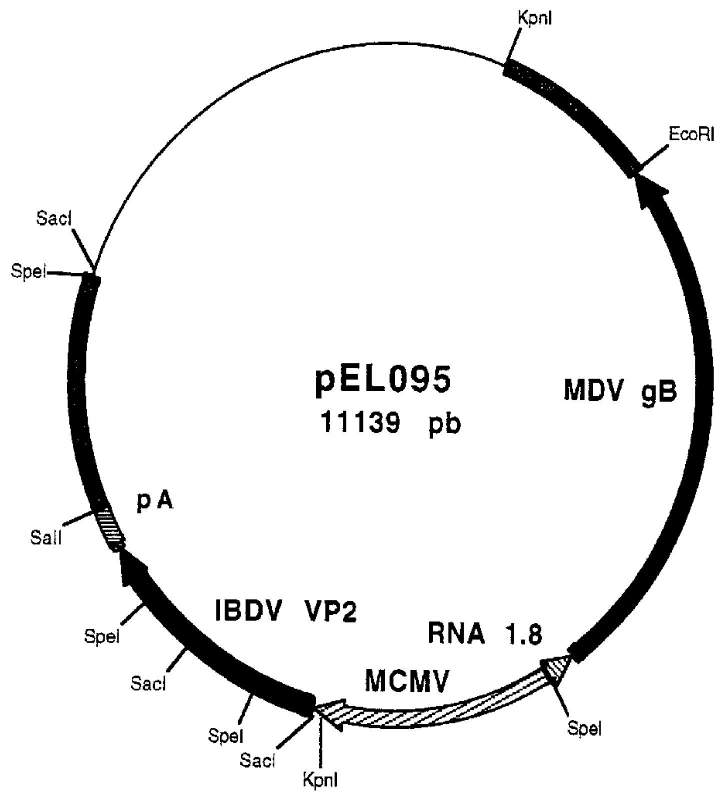


Figure 39

2 2 2 2 3 5 4 0 7 1 5

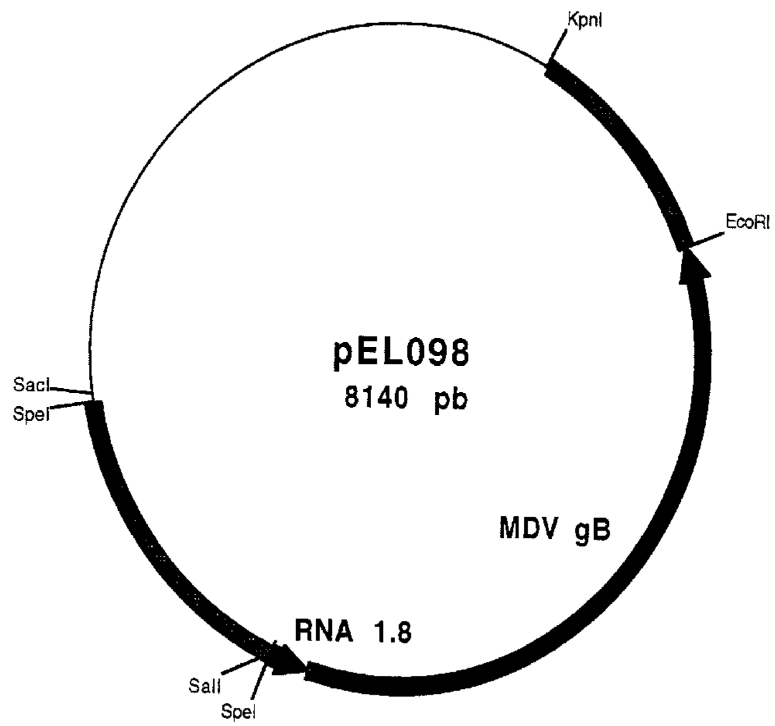


Figure 40

2 236 407 15