This Sheet Has Been Replaced

Recombinant Avipox virus, the culture of cells infected by this virus and vaccines for poultry derived from this virus

The invention relates to recombinant viruses derived from the Avipox virus and in particular from the virus responsible for fowl variola which is commonly called Fowlpox. The invention also relates to a method of production of these viruses by cell culture, to the use of these viruses for the production of vaccines and to vaccines containing these viruses. The invention also relates to the transfer sequences for the insertion of this recombinant virus into the genome, as well as to the plasmids carrying these sequences.

The virus responsible for fowl variola, or Fowlpox virus (FPV), belong to the Avipox genus of the Poxviridae family.

The Fowlpox virus has the characteristics typical of Pox viruses. These are large-sized viruses whose genome is a linear double-stranded DNA of about 300 kb and whose single-stranded ends are covalently linked. About 20 kb of the genome have been sequenced, especially the Terminal Inverted Repeat sequence (TIR) (Campbell et al., 1989; Tomley et al., 1988). The terminal inverted repeats are two identical long sequences which are present at each end of the genome, in the inverse orientation.

The vaccinia virus or Vaccinia was the first Pox developed as live recombinant virus capable of expressing foreign proteins. The list of proteins expressed is very long. In particular, the expression of many foreign antigens has made it possible to obtain immunisations against various diseases.

The use of the Fowlpox virus as a vector for the expression of heterologous proteins for the purpose of preparing a vaccine, that is to say the use of a recombinant virus having integrated a DNA sequence encoding a heterologous protein inside a noncoding intergenic region of its genome, has been proposed in European Patent

This Sheet Has Been Replaced

Application 0,314,569; this region is a short sequence of 32 nucleotides situated between the Open Reading Frames (ORF) ORF 7 and ORF 9 and has been genetically arranged into a nonessential insertion site. In such short intergenic regions, there is a high risk of the insertion separating a transcription signal from its gene and introducing, as a result, a mutation which inactivates this gene. This can be shown to be the case: indeed this 32-nucleotide sequence appears to be an essential region since it has had to be extended to 55 nucleotides in order to avoid possible overlapping between the promoter of ORF 9 and the 3′ coding sequence of ORF 7. In the case of the region which separates ORF 7 from ORF 9, the insertion into the natural sequence is, moreover, unstable (Spehner et al., 1990), and it is essential to arrange it. Furthermore, the proposed manner of arranging a short intergenic region cannot be generalised.

Moreover, the construction of recombinant Fowlpox virus and other recombinant fowl pox viruses characterised by the insertion of foreign DNA into the thymidine kinase gene, has also been proposed in International Patent Application WO88/02022. This insertion creates a mutation in the gene which could attenuate the infectiousness of the virus. This presents a risk of an excessive attenuation of the strain and a reduced immunogenicity of the vaccine which is derived from the recombinant virus.

Moreover, the use of a recombinant Fowlpox virus as vector for the expression of heterologous proteins has also been proposed in European Patent Application 0,353,851. The insertion of a foreign gene into the viral genome is carried out inside the open reading frames (ORF) situated inside the terminal inverted repeats (TIR). The insertion of one or more foreign genes into open reading frames presents the risk of causing the mutation of a gene whose function is not known.

The present invention provides other regions for the insertion of one or more foreign genes into the viral

This Sheet Has Been Replaced

genome, which no longer has the disadvantages of the aforementioned insertion regions.

In this regard, the present invention relates to a recombinant Avipox virus derived from an attenuated strain of Avipox virus and containing, in a nonessential part of its genome, at least one DNA sequence encoding all or part of a protein which is heterologous with respect to the Avipox virus as well as the elements capable of ensuring the expression of this protein inside a cell infected by the said recombinant virus, the non-essential part of the genome being composed of a non-coding intergenic region of the Avipox virus, a region which is situated between two open reading frames including their expression signals.

A nonessential part of the Avipox virus genome is understood to mean a region which may be modified without affecting the functions involved in the development of the virus in vitro and in vivo. A noncoding intergenic region of the genome, that is to say situated between two ORFs, including their expression signals, is chosen as the nonessential part.

The intergenic regions according to the invention are large, such that the risk of an insertion separating the signals for the transcription of their respective genes is minimal and also such that it is unnecessary to arrange them. Moreover, the sequences of these intergenic regions are noncoding and there is therefore no risk of mutation inside an ORF. A large region is understood to mean a region having a sequence of more than 60 nucleotides. Generally, a region having a sequence of more than 100 nucleotides is chosen. Preferably, a region having a sequence of more than 200 nucleotides is chosen. In a particularly preferred manner, a region having a sequence of more than 400 nucleotides is chosen.

Moreover, an intergenic region, in which at least one DNA sequence is cloned, situated inside one of the two TIR regions of the Avipox virus, is generally chosen. Normally, at least one DNA sequence is cloned into at

This Sheet Has Been Replaced

least one of the two TIR regions of the Avipox virus. Preferably, at least one DNA sequence is cloned into the two TIR regions of the virus. Still more preferably, the intergenic region, termed β1 region, and/or the intergenic region, termed β2 region, is chosen; the intergenic region, termed β1 region, being situated between the nucleotides 1675 and 2165 and the intergenic region, termed β2 region, being situated between the nucleotides 2672 and 3605, taking as starting nucleotide the BamH1 restriction site present inside the TIRs. This BamH1 restriction site which is chosen here for positioning the intergenic regions, is the first nucleotide of the sequence published by Tomley et al., 1988. These regions according to the invention may also be defined as follows: the intergenic region, termed β1 region, is situated between the open reading frames ORF 1 and ORF 2, the open reading frame ORF 1 being situated between the nucleotides 416 - the nucleotide A of ATG - and 1674 - the 3rd nucleotide of the stop codon - and the open reading frame ORF 2 being situated between the nucleotides 2166 - the nucleotide A of ATG - and 2671 - the 3rd nucleotide of the stop codon -, taking as starting nucleotide the BamH1 restriction site inside the TIRs, and the intergenic region, termed β2 region, is situated between the open reading frames ORF 2 and ORF 3, the open reading frame ORF 2 being situated between the nucleotides 2166 - the nucleotide A of ATG - and 2671 - the 3rd nucleotide of the stop codon - and the open reading frame ORF 3 being situated between the nucleotides 3606 - the 3rd nucleotide of the stop codon - and 4055 - the nucleotide A of ATG -, taking as starting nucleotide the BamH1 restriction site inside the TIRs. In a particularly preferred manner, there is chosen, inside the β1 region, a part situated between the nucleotides 1775 and 2065 and, inside the β2 region, a part situated between the nucleotides 2772 and 3505. Good results have been obtained by cloning at the level of the nucleotide 1842, termed B1 insertion site, situated inside the β1

This Sheet Has Been Replaced

region, and/or at the level of the nucleotide 3062, termed B2 insertion site, inside the β2 region.

Good results were obtained when at least one DNA sequence is cloned into the β2 region, inside one of the two TIR regions of the Avipox virus. Good results were also obtained when a DNA sequence is cloned into the β1 region, inside each of the two TIRs. This represents another advantage of the invention since it is possible to obtain two copies of the heterologous DNA sequence per genome.

Recombinant Avipox virus derived from an attenuated Avipox virus strain is understood to mean a virus belonging to the Avipox genus, which is attenuated and whose genome contains a heterologous sequence accompanied by expression signals. Generally, a virus of the Avipox genus such as Fowlpox, Pigeonpox or Canarypox, is used as virus. Normally, Fowlpox is used as virus. Preferably, a vaccinal strain of the chicken variola virus or Fowlpox, such as the vaccine produced and marketed by the company SOLVAY under the trademark POXINE or the vaccine produced and marketed by the company SOLVAY under the trademarks CHICK-N-POX, POULVAC CHICK-N-POX, CHICK-N-POX TC, POULVAC CHICK-N-POX TC, POULVAC POXINE and POULVAC P, is used as Fowlpox virus. In a particularly preferred manner, the vaccine marketed by SOLVAY under the trademarks CHICK-N-POX, POULVAC CHICK-N-POX, CHICK-N-POX TC, POULVAC CHICK-N-POX TC, POULVAC POXINE and POULVAC P, abbreviated CNP, is used as Fowlpox virus.

The attenuation is obtained by successive passages on embryo, or by passages on cell cultures if the virus has been adapted to cultured cells.

Elements capable of ensuring the expression of the gene which encodes the heterologous protein in a cell infected by the said recombinant virus are understood to mean the signals of genetic expression, which are recognised by the virus, especially the promoter and the terminator, elements which are known to a person skilled

This Sheet Has Been Replaced

in the art as described especially by Moss, 1990 for example. Generally, a Pox promoter is used. Normally, a Vaccinia or Fowlpox promoter is used. Preferably, the promoter P11 and the promoter P7.5 from Vaccinia, as described by Venkatesan et al., 1981, Cochran et al., 1985 and Bertholet et al., 1985, are used.

At least one DNA sequence encoding all or part of a protein which is heterologous with respect to the Avipox virus is understood to mean any DNA fragment extracted from a genome, or a cDNA copy or alternatively a synthetic DNA, accompanied by the expression signals, and whose sequence encodes all or part of a protein which is foreign with respect to Avipox.

Figures 1 to 11 were prepared in order to allow a better understanding of the invention.

Figure 1 represents the construction of a recombinant Avipox, adapted from Mackett et al., 1984. Cells infected (I: infection) with the Fowlpox virus (FPV) are transfected (T: "transfection") with a transfer plasmid. This plasmid (P) carries a promoter (Pr) which is orientated so as to permit expression of an adjacent heterologous gene (g) which is flanked on either side by viral DNA sequences. The homologous recombination (R) takes place inside the cytoplasm of the cell (C) between the sequences flanking the gene and the sequences present in the viral genome. This results in the insertion of the heterologous gene into the viral genome. This genome may be packaged and may produce recombinant viral particles (VR). N represents the nucleus of the cell (C).

Figure 2 schematically represents the ends of the Fowlpox genome with the right TIR (TIR-R) and the left TIR (TIR-L) symbolised by rectangles and with the unique sequence symbolised by a line. The dashes symbolise the central region of the genome. The EcoR1 restriction fragment of the TIR-L is 6.2 kb while that of TIR-R is 9.0 kb. This schematic representation shows that these two fragments have a common sequence and a unique sequence. A BamH1 site situated downstream of EcoR1 inside

This Sheet Has Been Replaced

the TIRs is indicated.

Figure 3 represents a restriction map of the plasmid pTIRB1 (PTIRB1) for some unique sites and the position of the ORFs. The unique BamH1 site is used for cloning the heterologous genes.
ori (ORI): origin of replication of the plasmid inside Escherichia coli (E. coli); Ap: gene for resistance to ampicillin; ORF 1 to ORF 6: Fowlpox open reading frames.

Figure 4 represents a figure which is similar to Figure 3, but for the plasmid pTIRB2 (PTIRB2).

Figure 5 represents a restriction map of the plasmid p11Lac (P11LAC). This plasmid carries the E. coli LacZ gene under the control of the Vaccinia promoter P11. P11: Vaccinia promoter P11; LACZ: β-galactosidase-encoding gene; ori: origin of replication in E. coli; strand +: strand packaged inside the phage M13; Ap: gene for resistance to ampicillin.

Figure 6 represents an example of a vector for transferring the LacZ gene into the TIRs. This vector is pTIRB1P75Lac (PTIRB1P75LAC). The cassette P7.5-LacZ is cloned into the BamH1 site of pTIRB1 in a clockwise direction. P7.5: Vaccinia promoter P7.5.

Figure 7 is a schematic representation of the inserts of the LacZ gene (represented by a shaded rectangle) inside the CNP genome. The cassettes P7.5-LacZ and P11-LacZ are inserted into the terminal regions (TIR), in either orientation. The right TIR region is not represented. The arrows indicate the direction of transcription of the genes which are represented by rectangles. The scale is not accurate.

Figure 8 represents the segment A from IBDV, the position of the primers and the various fragments obtained by reverse transcription and by amplification of the RNA of the segment A from the Edgar strain.

A : organisation of the ORFs.

p : "primers" used for carrying out the amplification of the segment A.

This Sheet Has Been Replaced

>,< : orientation of the "primers" relative to the coding sequence of the segment A.

fgt PCR : fragments generated by amplification of the RNA of the segment A.

pb : base pairs.

Figure 9 (Figure 9a to Figure 9e) represents the nucleotide sequence of the amplified DNA corresponding to the RNA of the segment A from the Edgar strain and the translation of the ORFs 1, 2 and 3 into amino acids.

Primers 0, 1, 1b, 2, 3, 4, 5 and 6: primers used for generating, by reverse transcription and by amplification, the DNA fragments corresponding to the segment A from the Edgar strain.

Figure 10 represents the schematic representation for the reconstruction of the DNA segment corresponding to the segment A from the Edgar strain using fragments obtained by reverse transcription and by amplification.
*: site-directed mutagenesis.

Figure 11 represents a restriction map of the plasmid pTIR75E1LAC (PTIR75E1LAC).

The portion of the plasmid represented by a thick line corresponds to the sequences which are homologous with respect to the FPV genome. ori (ORI): origin of replication of the plasmid inside E. coli; Ap: gene for resistance to ampicillin; P7.5: Vaccinia promoter P7.5; ORF1-IBDV: ORF 1 of the segment A from the Edgar strain; P11: Vaccinia promoter P11; LACZ: β-galactosidase-encoding gene; pb: base pairs; ORF 1 to ORF 6: open reading frames of Fowlpox.

Figure 12 represents the characterisation, by the ELISA test, of the IBDV proteins expressed in the FPV/IBDV recombinants. The characterisation was carried out as follows: 50 μl of cellular suspensions and two-fold serial dilutions of these suspensions were deposited per well. After incubation of the second anti-IBDV antibody (monoclonal anti-VP3 or anti-VP2), the signal was amplified by means of the mouse anti-IgG system labelled with biotin + streptavidin conjugated to

This Sheet Has Been Replaced

alkaline phosphatase.

Figure 12 A corresponds to the detection by means of the anti-VP3 monoclonal antibody. Figure 12 B corresponds to the detection by means of the anti-VP2 monoclonal antibody.

In these figures, the x-axis represents the 2-fold serial dilutions of the cellular extracts in logarithmic units and the y-axis represents the ratio of the absorbances measured at 690 nm and 405 nm.

Figure 13 represents the characterisation by Western blot analysis of the IBDV proteins expressed in the FPV/IBDV recombinants. This characterisation was carried out as follows: the cellular suspensions are centrifuged at 3,000 g and the supernatants obtained are ultracentrifuged at 185,000 g; the pellets resulting from the two centrifugations are pooled and resuspended in PBS buffer (Phosphate Buffered Saline, Gibco).

A deposit of 20 to 40 μg of proteins for the FPV/IBDV recombinants and 5 μg for the IBDV-infected cells (positive control) is deposited per well, on SDS-acrylamide gel. After transferring onto a membrane, the IBDV proteins are detected by means of a polyclonal serum which recognises all the IBDV proteins, for Figure 13 A, or by means of an anti-VP3 monoclonal antibody, for Figure 13 B. Amplification of the signal is carried out by means of the anti-IgG system labelled with biotin and the conjugate streptavidin-alkaline phosphatase. PM: molecular weight marker proteins.

The method used for implementing the invention is schematically represented in Figure 1 (modified according to Mackett et al., 1984). Cells infected with Avipox or Fowlpox are transfected with a transfer plasmid. This plasmid carries a promoter which is properly orientated so as to permit the expression of an adjacent heterologous gene flanked on either side by viral DNA sequences. The homologous recombination takes place inside the cytoplasm of the cell between the sequences flanking the gene and the sequences present in the viral genome. The

insertion of a heterologous gene into the viral genome results therefrom. This genome may be packaged and may produce recombinant viral particles. This technique of insertion through the infection of cells with Pox followed by a transfer through a plasmid was developed for Vaccinia and is commonly used in many laboratories for the construction of recombinant Pox viruses (Mackett et al., 1985).

The method according to the invention normally comprises the following stages:

- the first stage consists in constructing the transfer plasmids which permit screening,
- the second stage consists in cloning the genes encoding the heterologous antigens into the transfer plasmids,
- the third stage consists in constructing the recombinant Avipox viruses, and
- the fourth stage consists in carrying out vaccination tests using the recombinant viruses.

Preferably, the method comprises the following stages:

a. Construction of the transfer plasmids which permit screening:
   - Cloning of TIR-carrying fragments.
   - Insertion of cloning site into the TIRs.
   - Cloning of the Vaccinia promoters P11 and P7.5.
   - Construction of a cassette carrying the LacZ gene and its cloning downstream of P11 or P7.5.
   - Cloning of the elements P11LacZ or P7.5LacZ into TIR-transfer plasmids.

b. Construction of recombinant CNP viruses (LacZ gene for carrying out a preliminary test):
   - Transfection of the QT35 cells with the CNP virus and a transfer plasmid.
   - Screening and then purification of the recombinants by virtue of the expression of the LacZ gene.

This Sheet Has Been Replaced

This Sheet Has Been Replaced

- Analysis of the genomes of the recombinants and of the expression of LacZ.

c. Vaccination test:
- Vaccination of chicks with recombinant viruses and protection given by the LacZ recombinant virus against fowl variola.

d. Cloning of genes encoding heterologous antigens (heterologous protein) into the transfer plasmids:
- Cloning of the genes encoding heterologous antigens downstream of P11 or P7.5.
- Cloning of the cassettes P11-antigen and P7.5-LacZ or P7.5-antigen and P11-LacZ into the plasmids TIR.

e. Construction of recombinant CNP viruses:
- Transfection of QT35 cells with the CNP virus and a transfer plasmid.
- Screening and subsequent purification of the recombinant viruses by virtue of the expression of the LacZ gene.
- Analysis of the expression of the antigen in cell cultures and analysis of the genome of the recombinant viruses.

f. Vaccination:
- Vaccination with the recombinant CNP viruses.
- Evaluation of the level of protection given to CNP by virtue of the expression of the heterologous antigen.

The invention also relates to transfer sequences which enable the heterologous DNA to be inserted into the genome of the Avipox virus as defined above and especially at the sites B1 or B2. The invention also relates to the plasmids which carry these sequences. Transfer plasmid is understood to mean a plasmid which contains a heterologous gene under the influence of a promoter which is recognised by Avipox, flanked on either side by the sequences situated on either side of the site B1 or of the site B2. The length of these sequences which are homologous with respect to FPV and which flank the

heterologous gene, should be sufficiently long so as to permit recombination on the left and on the right of the heterologous gene. Generally, sequences of at least 1 kb are chosen. Good results are obtained for B1 flanked by a sequence of about 1850 nucleotides upstream and 4321 nucleotides downstream, and for B2, about 3070 nucleotides upstream and 3100 nucleotides downstream. The transfer plasmids play a role in the method for isolating recombinant Avipox viruses, a transfection of the quail cells QT35, previously infected with the Avipox virus, being called into play in the method.

The invention also relates to a culture of eucaryotic cells which are infected with a recombinant Avipox virus derived from an attenuated Avipox virus strain and comprising, in a nonessential part of its genome, at least one DNA sequence encoding all or part of a protein which is heterologous with respect to the Avipox virus, as well as the elements capable of ensuring expression of this protein in a cell infected with the said recombinant virus, the nonessential part being composed of a noncoding intergenic region of the Avipox virus situated between two ORFs, including their expression signals.

Preferably, a culture of fowl cells is used. Good results have been obtained with a culture of quail cells. Excellent results have been obtained with the culture of the quail cells QT35, as described by Cho (1981).

The recombinant Avipox virus according to the invention is developed as vectors which are capable of expressing all or part of the heterologous protein. The present invention therefore also relates to the use of the virus according to the invention, as a vector which is capable of expressing all or part of a heterologous protein such as an antigen in particular.

The invention also relates to a vaccine containing a recombinant Avipox virus derived from an attenuated Avipox virus strain and containing, in a nonessential part of its genome, at least one DNA sequence encoding

This Sheet Has Been Replaced



This Sheet Has Been Replaced

all or part of a protein which is heterologous with respect to the Avipox virus, as well as the elements capable of ensuring the expression of this protein in a cell infected with the said recombinant virus, the non-essential part of the genome being composed of a non-coding intergenic region of the Avipox virus situated between two ORFs, including their expression signals.

Heterologous protein is understood to mean any protein or part of a protein which is foreign with respect to the Avipox virus, or a natural or modified protein. Generally, this protein may be an antigen enabling a vaccine to be developed against a pathogenic organism. Normally, the antigens are in particular proteins of agents which are infectious to fowls, viruses, bacteria, chlamydia, mycoplasmas, protozoa, fungi and worms.

They are in particular the Adenoviridae, such as the virus responsible for aplastic anaemia (AA) and for the egg drop syndrome (EDS), the Birnaviridae, such as the infectious bursal disease virus (IBDV), the Coronaviridae, such as the infectious bronchitis virus (IBV), the Herpesviridae, such as the Marek's disease virus (MDV) and the virus responsible for infectious laryngotrachitis (ILTV), the Orthomyxoviridae, such as the virus responsible for influenza, the Paramyxoviridae, such as the Newcastle disease (NDV), the Picornaviridae, such as the virus responsible for encephalomyelitis (AE), the Reoviridae, responsible for tenosynovitis, the Retrovirus such as the virus responsible for lymphoid leukosis (LL), the virus responsible for chicken anaemia (CAV).

They are in particular chlamydia, such as Chlamydia psittaci.

They are in particular mycoplasmas such as Mycoplasma gallisepticum and M. synoviae.

They are in particular bacteria such as Escherichia coli, Haemophilus including H. paragallinarum which is responsible for coryza, Pasteurella including P. multocida which is responsible for cholera, Salmonella

This Sheet Has Been Replaced

including S. gallinarium responsible for typhus and S. pullorum, Clostridium including C. botulinum which produces a toxin responsible for botulism, C. perfringens and C. septicum responsible for dermatoses, C. colinum, Campilobacter jejuni, Streptococcus aureus.

They are in particular protozoa such as Eimeria which is responsible for coccidiosis and Cryptosporidiosis baileyi which is responsible for cryptosporodiosis.

They are in particular worms such as cestodes and nematodes including Ascaridia galli.

They are in particular fungi such as Aspergillus fumigatus.

The preferred antigens are those of the infectious bursal disease virus (IBDV), the infectious bronchitis virus (IBV), the virus responsible for chicken anaemia (CAV), the protozoa Eimeria which is responsible for coccidiosis, the Newcastle disease virus (NDV) and the Marek's disease virus (MDV).

The antigens which are particularly preferred are those of the infectious bursal disease virus (IBDV), the infectious bronchitis virus (IBV), the virus responsible for chicken anaemia (CAV) and the protozoa Eimeria which is responsible for coccidiosis. Good results have been obtained under the following conditions:

- for IBDV: all or part of the open reading frames comprising polyprotein and parts of polyprotein. Such proteins are in particular VP2, VP3 and VP4. All combinations or modifications thereof are also included. These modifications consist, for example, of the insertion of a cleavage site.
- for IBV: the antigen E2.
- for Eimeria: the natural surface antigen TA4 and the surface antigen TA4 whose proteolytic cleavage site has been modified.
- for CAV: the protein P50.

Excellent results have been obtained with IBDV, the heterologous protein being part of the polyprotein

This Sheet Has Been Replaced

comprising the amino acids 1 to 493 followed by the amino acids 1010 to 1012, in which the protein VP2 is included; as represented in Figure 9.

The DNA sequences may encode the complete protein or, alternatively, fragments of the protein, and may induce the appropriate immune response in the animal. Other molecules, besides antigens, may also be taken into consideration including growth factors and immunomodulatory agents such as interleukins. The present invention is also intended to produce in vivo, in poultry, a factor which plays a role in the metabolism of the animal, such as for example a growth hormone or a growth hormone-inducing factor. The present invention also relates to the preparation of proteins or peptides in cell cultures in vitro or in vivo in the animal. Depending on the heterologous protein introduced, it may be suitable, in particular, as enzyme, as nutritive constituent or, in the field of human or animal health, as pharmaceutical product for human or veterinary use.

Moreover, multiple recombinant viruses, carrying several heterologous genes, may be generated by cloning several genes into the same genome, into the same site, or into different regions or sites. A polyvalent product may also be created by combining different recombinant viruses.

Animal targets for the Avipox virus according to the invention are birds, in particular poultry. The recombinant virus may also be used in species other than fowls.

The vaccine according to the invention may be administered in various forms which are known to a person skilled in the art. Normally, the vaccine is administered by the oral route in feed or in drinking water, by the intranasal route, by subcutaneous injection, by aerosol, by intramuscular injection or by transpiercing the wing according to the so-called wing web method. Preferably, the vaccine is administered by piercing the wing membrane according to the so-called wing web method, by

This Sheet Has Been Replaced

intramuscular injection or by subcutaneous injection.

The vaccine according to the invention is formulated in a manner known to a person skilled in the art. Normally, the vaccinal composition contains stabilisers which are appropriate for the mode of administration of the vaccine. It may be preserved in freeze-dried form.

The present invention is illustrated by the following examples.

Example 1 - Purification of the CNP virus and its DNA

As Fowlpox virus, the vaccinal strain marketed by the company Solvay under the trademark POULVAC CHICK-N-POX, CHICK-N-POX, CHICK-N-POX TC, POULVAC CHICK-N-POX TC, (CNP), is used as attenuated live vaccine. The initial stock is obtained from a field strain passed several times on eggs and adapted to the cells in vitro by 2 passages on CEF (chicken embryo fibroblasts) and 5 passages on the quail cell line QT35 described by Cho (1981). The quail cell line QT35 can be obtained from Dr. Moscovici (6816 Northwest 18th Avenue, Gainesville, Florida 32605, United States) who isolated it and who distributes it.

CNP is cultured on the cell line QT35 whose culture media consists of E199 (Gibco, 500 ml), F12 (Gibco, 500 ml), LAH (Gibco, lactalbumin hydrolysate, 25 ml), FCS (Gibco, foetal calf serum, 25 ml) and fructose (5 ml of a solution at 200 g/l); incubation at 38°C, 3% of CO_2. The normal multiplicity of infection (moi) is 0.01 virus per cell, the cells being 80% confluent.

A stock of virus was purified as follows: the cells, infected with the CNP Fowlpox virus and taken up in PBS phosphate buffer (Phosphate Buffered Saline, Gibco), are centrifuged at 6,000 g (15 min). The pellet is resuspended in Tris (pH 9, 1 mM), trypsinised (trypsin at 0.25 mg/ml final concentration; Gibco) and sonicated. This material is deposited on a 36% (weight/volume) sucrose layer and centrifuged for 45 minutes at 30,000 g. The pellet is taken up in 1 mM Tris, pH 9.0. Finally, the viruses are centrifuged for 30 minutes at 40,000 g. The

This Sheet Has Been Replaced

pellet is taken up in lysis buffer: 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS (Sodium Dodecyl Sulphate), 500 µg/ml proteinase K (Boehringer), 0.1 mg/ml RNase (Boehringer) and incubated for 2 hours at 37°C. The phenol-extracted DNA is precipitated with ethanol. In the order of 20 µg of DNA were purified from 40 150-cm^2 flasks infected.

Example 2 - Construction of an EcoR1 genomic library and a BamH1 genomic library of CNP

The genetic techniques used are described by Maniatis et al., 1982. The restriction enzymes, the polymerases, ligases and phosphatases were provided by the companies Pharmacia, Boehringer and Biolabs. The synthetic DNAs were provided by the company Eurogentec.

The EcoR1 or BamH1 restriction fragments of the viral DNA were ligated inside the vector pUC18 described by Messing (1983) and introduced into the bacteria E. coli MC1061 (araD139, (ara, leu)7697, lacX74, galU, galK, hsdR, strA, sold by the company Pharmacia). The two genomic libraries are stored at -70°C in glycerol in the form of two suspensions of transformed cells. Twenty four % of the plasmids contain an insert. Seventeen BamH1 fragments and 45 EcoR1 fragments were differentiated on the basis of their size and their mode of restriction by Bgl2 or HinF1. The inserts are 0.5 to 15 kb in size; the sum of the BamH1 inserts thus characterised is 82 kb and 210 kb for the EcoR1 fragments. The size of the Fowlpox genome is close to 300 kb (Coupar et al., 1990). The fragments isolated therefore represent 25% for BamH1 and 70% for EcoR1 relative to the total genome.

Example 3 - Cloning of the TIR-carrying fragments

The terminal inverted repeats (TIR) are two identical sequences present at the ends of the Pox genome in opposite orientations. A sequence of more than 17 kb at one end of the genome of the strain HP of Fowlpox has been published (Campbell et al., 1989 and Tomley et al., 1988). It comprises about 10 kb of the repetitive sequence and 7.0 kb of its adjacent sequence.

Cloning of the TIR EcoR1 fragments is carried out. The repetitive sequence contains an EcoR1 site. As shown in Figure 2, two EcoR1 fragments are cloned: each carries part of the TIR and a unique adjacent sequence. The unique sequence is delimited by the EcoR1 site which is nearest to the TIRs. These two fragments were isolated by means of an oligonucleotide which is complementary to the sequence situated in the repetitive part. The first fragment is 6.2 kb in size and, by convention, will be denoted TIR-L. The second is 9.0 kb in size and, by convention, will be denoted TIR-R. The sequence of the TIR-L EcoR1 fragment of CNP is identical to the one published.

The junction between the TIR and the unique sequence deduced from comparison of the TIR-L and TIR-R sequences, is situated at nucleotide 4007, the BamH1 site of the sequence published by Tomley et al. (1988) being taken as nucleotide number 1. The junction is situated in the open reading frame denoted ORF 3 by these authors.

Example 4 - Creation by site-directed mutagenesis of BamH1 sites inside the TIR

Open reading frames (ORF) are deduced from the analysis of the nucleotide sequences of the TIRs. In particular, ORF 1 is situated between the nucleotides 416 and 1674, ORF 2 is situated between the nucleotides 2166 and 2671 and, finally, ORF 3 is situated between the nucleotides 3606 and 4055 and coded on the complementary strand. The first nucleotide G of the BamH1 site of the TIRs is taken as nucleotide No. 1. These ORFs delimit two large noncoding intergenic sequences, denoted region $\beta 1$, between ORF 1 and ORF 2 on the one hand, and region $\beta 2$ between ORF 2 and ORF 3 on the other hand. These are two intergenic regions which are chosen as insertion regions.

A unique cloning site was introduced by site-directed mutagenesis (BioRad mutagenesis kit) in $\beta 1$ and in $\beta 2$. The BamH1 site was chosen as cloning site because it is compatible with other restriction sites of which Bcl1 and Bgl2. The location of the cloning site was

This Sheet Has Been Replaced

This Sheet Has Been Replaced

chosen approximately in the middle of each intergenic region so as to avoid any risk of separating the transcription signals from their respective genes, that is to say:

- in the region β1: the site B1 is the sequence 5'GATC 3', at the nucleotide 1842, which is converted to BamH1 GGATCC by insertion of a G in 5' and a C in 3';
- in the region β2: the site B2 is the sequence 5'GGATT 3', at the nucleotide 3062, which is converted to BamH1 by mutation of the second T to CC.

There are two BamH1 sites inside the plasmid carrying the TIR-L fragment (Example 3). One site is derived from the vector pUC18. The second site is derived from the TIR and is situated at 71 nucleotides downstream of the EcoR1 site chosen for the cloning (Campbell et al., 1989; Figure 2). These two BamH1 sites were deleted from the plasmid carrying the TIR-L, by BamH1 restriction followed by filling using Klenow polymerase and ligation. The plasmid resulting therefrom is pTIR1.

To generate a BamH1 site in B1, the 0.9 kb HindII fragment of pTIR1 was cloned into the vector pBSPlus marketed by the company Stratagene. The mutagenesis primer was: 5'TTTCGAAAGACTTTGGATCCGTAGTATAATATTATA 3'. The nucleotides in bold, that is to say GGATCC, are the sequence which is recognised by BamH1.

To generate a BamH1 site in B2, the 1.7 kb XbaI fragment of pTIR1 was cloned into pBSPlus. The primer was:
5'TATATCACGGGATCCAAAAGGTTATTAGTAGTC 3'. The nucleotides in bold, that is to say GGATCC, are the sequence which is recognised by BamH1.

The reinsertion of each mutant fragment into pTIR1 is the result of the ligations:

- for B1: Hind3-Sca1 of the pTIR1 (1491 bp) + Sca1-Aat2 of the pBSPlus after mutagenesis (485 bp) + Aat2-EcoR1 of the pTIR1 (4220 bp) + EcoR1-Hind3 of the pTIR1 (2635 bp);

This Sheet Has Been Replaced

- for B2: Xho1-Spl1 of the pTIR1 (8512 bp) + Spl1-Xho1 of the pBSPlus after mutagenesis (318 bp).

These two ligations generated the vectors pTIRB1 and pTIRB2. Their restriction maps are in Figures 3 and 4.

The plasmid pTIRB1D was generated from the plasmid pTIR1 by deletion of its 2657-bp EcoR1-Hpa1 fragment.

Example 5 - Cloning of the Vaccinia promoters P11 and P7.5

P11 and P7.5 are known Vaccinia promoters. P11 is the promoter of the gene encoding the protein P11 which is transcribed during the late phase of the viral infection. P7.5 is the promoter of the gene encoding the protein P7.5 which is transcribed both during the immediate and late phases.

Construction of vectors with unique Bgl2 and Bcl1 sites

Two cloning vectors containing the unique Bgl2, Bcl1 and BamH1 sites were constructed so as to allow the successive clonings of cassettes which are compatible with BamH1. The sequences of the Bgl2 and Bcl1 sites were introduced into the BamH1 site of pBSPlus by cloning of the following synthetic DNA:

```
                    Bgl2   Bcl1 BamH1
          5' GATCGAGATCTTGATCAG     3'
          3'     CTCTAGAACTAGTCCTAG 5'
```

The vectors are pBSLK1 and pBSLK2. The linkers are in the orientation:

pBSLK1 ... Ava1/Sma1-BamH1-Bcl1-Bgl2-Xba1 ...

pBSLK2 ... Ava1/Sma1-Bgl2-Bcl1-BamH1-Xba1 ...

Cloning of the promoter P7.5

The Bcl1-BamH1 fragment, 143 bp in length, of the plasmid pGS20 contains the P7.5 promoter sequence (Mackett et al, 1984). The sequence is presented below (Venkatesan et al., 1981; Cochran et al., 1985). The late and immediate promoters are underlined. The last BamH1 base is at 10 bp from the initiator ATG of the gene.

5'TGATCACTAATTCCAAACCCACCCGCTTTTTATAGTAAGTTTTTCACCCATAAATAATAAATA

Bcl1

CAATAATTAATTTCTCGTAAAAGTAGAAAATATATTCTAATTTATTGCACGGTAAGGAAGTAGAa

Late

Immediate

TCATAAAGAACAGTGACGGATCC

BamH1

The Bcl1-BamH1 fragment was cloned into the Bcl1 and BamH1 sites of the plasmids pBSLK1 and pBSLK2, generating the plasmids p1P75 and p2P75 respectively. To allow the Bcl1 digestion, pBSLK1 and pBSLK2 are extracted from the strain JM110 which can be obtained from ATCC (Amercian Type Culture Collection) under the reference ATCC 47013.

Cloning of the promoter P11

The sequence of the promoter P11 was cloned in the form of a synthetic DNA using the sequence data from Bertholet et al., 1985. Hanggi et al., (1986) have shown that a 30-nucleotide fragment upstream of the first nucleotide of the mRNA contains the promoter.

The synthetic DNA is a 40-mer fragment carrying a Bgl2 site and a BamH1 site at its ends. This DNA was cloned into pBSLK1 in order to generate p1P11, and into pBSLK2 in order to generate p2P11. The Bgl2-BamH1 fragment of p2P11 was subsequently cloned into the Xho2-BamH1 sites of pACYC184. pACYC184 is a vector described by Chang and Cohen (1978). The ligation between Xho2 (GGATCT) and Bgl2 (AGATCT) restores Bgl2.

The sequence of the P11-carrying synthetic DNA is:

5'GATCAAATTTCATTTTGTTTTTTTCTATGCTATAAATAAG

3' TTTAAAGTAAAACAAAAAAAGATACGATATTTATTCCTAG

Bgl2

BamH1

Example 6 - Construction of an LacZ gene-carrying cassette which is compatible with the BamH1 restriction site

The expression of the LacZ gene will allow screening of the recombinant viruses.

This Sheet Has Been Replaced



A Bgl2-BamH1 cassette carrying the LacZ gene was constructed by site-directed mutagenesis. This cassette was cloned into the plasmid pBSPlus (stratagene) deleted from the fragment LacZα and this in order to avoid recombination between the two homologous sequences, one with LacZ, the other with LacZα. This plasmid is pBSMutLacZ1. The sequence of the ends of the LacZ gene after mutagenesis is given below. The numbers correspond to the numbers of the amino acids of the natural protein.

```
En 5',
    Bgl2    Nco1    8   9   10
... AGATCTCACC ATG GCC GTC GTT  ...
               Met Ala Val Val
               <-     β-gal   -

En 3',
... CAA AAA TAA TAA TAA CCGGGCAGG GGGGATCC ...
... Gln Lys *** *** ***
         ->
```

Example 7 - Cloning of the LacZ gene behind the promoters P7.5 or P11

The LacZ, Bgl2-BamH1, cassette of pBSMutLacZ1 was cloned into the Bgl2-BamH1 sites of p2P75 and p2P11 in order to generate p75Lac and p11Lac respectively. Expression of the LacZ gene is obtained in E. coli when it is placed behind the promoters P7.5 or P11. The transformant colonies have a blue colour on a media containing X-Gal (chromogenic substrate: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) which produces a blue coloration under the action of β-galactosidase. This test proves that the gene is functional. The P11Lac cassette in the form of a Bgl2-Hind3 fragment of the plasmid p11Lac was also cloned into the Xho2 and Hind3 sites of pACYC184, thus generating pACP11Lac. The map of p11Lac is in Figure 5.

This Sheet Has Been Replaced



Example 8 - Cloning of the cassettes P75Lac and P11Lac into the plasmids pTIRB1 and pTIRB2

The Bgl2-BamH1 fragment of p11Lac (or pACP11Lac) and the Bgl2-BamH1 fragment of p75Lac carry the cassette 'Promoter P7.5 or P11 followed by the LacZ gene' (Example 7). These fragments were cloned into the BamH1 sites of pTIRB1 and pTIRB2 (Example 4), in the two possible orientations, using the LacZ phenotype for the screening. Six plasmids were isolated among eight possible recombinants. Their numbering is given below. Let us assume that the '+' orientation is that which places the transcription of the LacZ gene in the ORF1 to ORF2 or ORF2 to ORF3 direction, and the '-' orientation that which corresponds to the reverse orientation. In the plasmid names, 'P75Lac' and 'P11Lac' mean that these cassettes are in the '+' orientation while 'LacP75' and 'LacP11' mean that these cassettes are in the '-' orientation. By way of example, the map of the plasmid pTIRB1P75Lac is in Figure 6.

No.	Prom	Site	Ori	Name	No.	Prom	Site	Ori	Name
3	P7.5	B1	+	pTIRB1P75Lac	8	P11	B1	+	pTIRB1P11Lac
4	P7.5	B2	+	pTIRB2P75Lac	9	P11	B1	-	pTIRB1LacP11
5	P7.5	B2	-	pTIRB2LacP75	10	P11	B2	-	pTIRB2LacP11

No. = number given to the plasmid.
Prom = promoter.
Site B1 or B2 = BamH1 cloning site introduced into TIR.
Ori = orientation. The orientation is + when the direction of transcription of LacZ is in the ORF1 to ORF2 or ORF2 to ORF3 direction. The - orientation is the reverse.
Name = name of the tranfer plasmid.

This Sheet Has Been Replaced



Example 9 - Optimisation of the transfer and screening conditions and isolation of the CNP-LacZ recombinant viruses

The principle for the construction of a recombinant CNP virus is presented in Figure 1. The transfer procedure is as follows:

On day D=1, 2.5×10^6 QT35 cells are inoculated, per 25-cm^2 flask, into 5 ml of growth media (composition described in Example 1).

On day D=2, the following transfer is carried out:

Virus: a 1-ml vial of freeze-dried CNP vaccine is rehydrated with 3 ml of sterile Milli Q water and stored at -70°C. This virus stock is thawed and subjected to gentle sonication for 1 minute. It is then diluted in the culture media labelled E119-F12 and which corresponds to the media E119 described in Example 1, without LAH or serum or fructose, to achieve a multiplicity of infection of 0.05 virus per cell in 2 ml of media. The culture media is then replaced with the viral suspension and the culture is incubated for 2 h at 38°C.

Plasmids: 20 μg of plasmid are mixed with 400 μl of water and 100 μl of 1.25 M $CaCl_2$, 500 μl of 2× BBS buffer (BES buffer saline) (50 mM of BES (Sigma), 280 mM NaCl, 1.5 mM Na_2HPO_4; pH 7.0) are added dropwise and the mixture is incubated for 15 to 30 minutes at 25°C. One ml of the plasmid preparation is then added to the cells after removing the media. The mixture is then incubated for 30 minutes at 38°C. 4 ml of LAH-free media supplemented with 5% FCS and 15 mM Hepes (Sigma) pH 7.2, are then added. The mixture is again incubated for 4 hours at 38°C. The media is then replaced with 5 ml of the growth media labelled E199 (composition described in Example 1).

On day D=0, the viral cells are harvested as described in Example 1.

The screening is based on the production of β-galactosidase which is detected by a "plaque assay" test on a Petri dish. The procedure on a 6-cm Petri dish

This Sheet Has Been Replaced



is as follows:

On day D=1: 2.5 x 10^6 cells are inoculated into 5 ml of growth media.

On day D=2: this culture is infected with 1 ml of virus diluted 1:10 and 1:100, the mixture is incubated for 4 h at 38°C and 4 ml of growth media (composition described in Example 1) are then added.

On day D=3: the media is replaced with 5 ml of an agarose layer consisting of 1 volume of 2% agarose [see plaque agarose (FMC) dissolved in water] and 1 volume of the following mixture: 9 ml of 2 x 199 media (Gibco), 0.5 ml of LAH (Gibco) and 0.1 ml of Hepes (pH 7.2, 15 mM final) and 1.0 ml of FCS. The melted agarose and the mixture are maintained at 38°C before being mixed. The agarose is allowed to gel for 30 minutes at room temperature and the mixture is then incubated at 38°C.

On day D=6: the culture is layered with 2 ml of 1% agarose in PBS (Gibco) containing 0.3 mg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Boehringer). The X-Gal is dissolved at 30 mg/ml in DMSO (dimethyl sulphoxide, Fluka).

On day D=7: the number and proportion of blue plaques relative to the number of colourless plaques are counted. Five plaques are then individually removed by means of a Pasteur pipette. The viral cells are stored in 500 μl of growth media at -70°C.

The recombination frequencies for each plasmid are presented in the table below. The notation of the recombinant viruses is 'V' followed by the number of the transfer plasmid (as defined in Example 8). The percentage of recombinants ranges from 0.1% to 0.5%, which corresponds to the literature data for Pox viruses. A representation of the recombinant genomes is schematically presented in Figure 7.

This Sheet Has Been Replaced



Virus	Blue/total	%	Virus	Blue/total	%
V3	7/1400	0.5	V8	1/1000	0.1
V4	10/2000	0.5	V9	60/25000	0.25
V5	15/5000	0.3	V10	50/30000	0.17

Blue/total = number of blue plaques/total number of plaques

% = percentage of blue plaques.

Example 10 - Purification of the CNP/LacZ recombinant viruses

A clone is obtained by successive purification of a blue plaque on a Petri dish (as described in Example 9). In principle, when all the plaques are blue, all the viruses express the LacZ gene and are no longer contaminated with the wild virus. Three to four passages are required in order to achieve this homogeneity. By way of example, the variation of the proportion of blue plaques during the passages was:

	Number of passages							
	1st		2nd		3rd		4th	
Virus	Blue/total	%	Blue/total	%	Blue/total	%	Blue/total	%
V3	20/200	10	40/50	80	22/22	100		
V4	1/20	20	20/30	70	95/100	95	100/100	100
V5	50/500	10	2/5	40	30/30	100		
V8	50/200	25	4/5	80	8/8	100		
V9	1/5	20	1/1	100				
V10	1/100	1	3/20	15	12/12	100		

Blue/total = number of blue plaques/total number of plaques

% = percentage of blue plaques.

This Sheet Has Been Replaced



This Sheet Has Been Replaced

Structures which are typical of the Pox viruses, in particular the inner structure which has the form of a dog bone, are clearly visible on an electronic microscope photograph of a section of QT35 cells infected with the recombinant virus V8.

Example 11 - Production of a large quantity of each recombinant

Six recombinants were amplified by three successive passages in flasks. The titre of the second passage ranges from 4×10^5 to 1.1×10^7 TCID50 (tissue culture infectious dose) per ml. Only V10 has a titre of less than 10^4. The other recombinants, for which the titres are normally between 10^5 and 10^7, indeed multiply as well as the wild strain.

Example 12 - "Plaque assay" analysis of the stability of the LacZ gene inserted into TIR

The LacZ gene inserted into the viral genome may be stable or unstable depending on the type of recombination which occurred (this is stated explicitly by Shuman et al., 1989). Thus, if there are two recombinations on either side of the insert, the LacZ gene inserted is stable. On the other hand, a simple recombination results in the insertion of the complete transfer plasmid and therefore of LacZ. This virus gives blue plaques. However, the viral genome carries homologous sequences in a direct orientation, whose recombination may result in the loss of the insert and the appearance of non-blue plaques.

Moreover, a change in the sequence of a TIR, in Pox viruses, may be transferred to the other TIR during the viral infection and replication cycles. A double recombination may insert LacZ into TIR-L; the plaque of this virus appears blue. During the following infections, a recombination between this recombinant TIR-L and a wild TIR-R generates a mixed population of TIR-L/LacZ, TIR-L/LacZ-TIR-R/LacZ and WT (wild) virus. Other recombinations may, on the other hand, generate the last possible type which is TIR-R/LacZ. Consequently, a blue

This Sheet Has Been Replaced

plaque may contain three types of virus: viruses wich have lost the LacZ gene, viruses which carry a single copy of the LacZ gene and viruses which carry two copies of the LacZ gene.

The homogeneity of a viral preparation is tested using a "plaque assay" after a few successive infections in liquid media (Example 9). The "plaque assays" of the 3rd passages of the viruses V3, V4, V5, V8, V9 and V10 produce the following proportions of blue:

Virus	P3 : Blue/total	%	P6 : Blue/total	%
V3	60/70	85	-	
V4	80/90	88	70/80	88
V5	95/100	95	-	
V8	50/50	100	40/40	100
V9	70/70	100	-	
V10	3/10	30	-	

P3 and P6 = 3rd and 6th passage of the virus.
Blue/total = Number of blue plaques/total number of plaques.
% = Percentage of blue plaques.
- = Not carried out.

In conclusion, the preparations of the recombinant viruses V8 and V9 are homogeneous. The recombinant V8 is stable up to the 6th passage. On the other hand, the preparations V3, V4 and V5 are not homogeneous. They contain a proportion of 5 to 20% of wild-type virus. This proportion is even higher for V10.

Example 13 - Analysis of the CNP/LacZ genomes by Southern blotting

The homogeneity of a viral preparation is tested by Southern blotting (Maniatis et al., 1982). The restriction fragments of the genomes of the recombinant viruses and of the non-recombinant viruses are

This Sheet Has Been Replaced

differentiated on the basis of their size.

The procedure for the preparation of the viral DNA is as follows:

On day D=1: a 25-cm^2 flask containing 5 ml of growth media is inoculated with 2×10^6 QT35 cells.

On day D=2: the cells are infected at a multiplicity of infection of 0.01.

On day D=5: the cells are detached using a scraper (Costar). The mixture is centrifuged for 10 minutes at 6,000 g. 500 μl of lysis buffer consisting of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1% SDS, 0.1 mg/ml RNase, 0.1 mg/ml proteinase K are added to the centrifugation pellet. The cells are transferred into an Eppendorf tube. The suspension obtained is then stirred and incubated for 1 hour at 50°C. The aqueous phase is then extracted 3 times with phenol chloroform. The DNA is precipitated with 0.3 M sodium acetate and 2 volumes of ethanol at -20°C for 15 minutes. The mixture is centrifuged for 10 minutes at 18,300 g. The centrifugation pellet is suspended in 500 μl of water. In the order of 5 μl of this total DNA suspension are sufficient for a Southern blotting.

For the B1 site

The genomes are digested with EcoR1. The probe is the plasmid pTIRB1LacP75 digested with EcoR1. This probe differentiates the recombinant and parental TIR-L and TIR-R fragments. The sizes of the fragments are 9.3 and 6.2 kb for the wild virus, 7.4, 5.1 (doublet) and 4.4 for V3, 7.4, 4.9 and 4.4 kb for V8 and finally for Vp, 10.4, 7.3 and 2.0 kb (doublet). The viruses V8 and V9 acquired two copies of the LacZ gene, one in each TIR, and do not contain any parental-type TIR fragment. This is, on the other hand, the case for the virus V3.

Since a copy of LacZ can be cloned into each TIR, it can be concluded that the insertion site B1 is nonessential for the development of the virus and for its growth on cultured cells. Moreover, given that the LacZ gene is about 3 kb in size, the isolation of these double

This Sheet Has Been Replaced

recombinants proves that 6 kb can be inserted per genome of the CNP virus.

For the B2 site

The Southern blots show the presence of recombinant TIR-L and TIR-R fragments, but also of the parental fragments in the preparation of V4, V5 and V10. These three preparations are therefore not homogeneous.

Example 14 - Alternative for isolating a double recombinant in the B1 insertion site

The development of recombinations in the TIRs foretells the appearance of stable double recombinants in the progeny of a virus carrying a wild TIR and a recombinant TIR. Viral plaques are reisolated from a blue plaque using a Petri dish "plaque assay", either by a new "plaque assay" or on 96-well microtitre plates. The genome of each new plaque may be analysed either by Southern blotting or by PCR. These different possibilities were exploited.

The Southern blotting carried out on the virus genomes of 10 different blue plaques obtained from a "plaque assay" for the 3rd passage of V3, showed that, of the 10 plaques, one preparation is homogeneous and contains a LacZ insert in each of the TIRs.

For the microtitre plate, the dilution is such that there is zero or one viral plaque per well. This optimal dilution is assessed by prior titration. Good results are obtained by infecting a microtitre plate (200 μl per well, 20 ml per plate) with 50 μl of the viral suspension obtained from a Petri dish "plaque assay" taken up in 1 ml. When the plaques are clearly visible, the wells containing a plaque are noted. After freezing and thawing, 100 μl of media are taken per well.

For the PCR, the total DNA is rapidly extracted under the following conditions: the mixture is centrifuged for 10 minutes at 10,000 g, the pellet is taken up in 200 μl of lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1% SDS), supplemented with RNase (0.1 mg/ml) and proteinase K (0.1 mg/ml) and the mixture thus obtained is

incubated for 1 hour at 50°C. The aqueous phase is then extracted three times with phenol/chloroform. The DNA is precipitated from this aqueous phase with ethanol. The pellet obtained is taken up in 50 µl of water. 10 µl of this suspension are used per PCR. The PCR conditions used are those recommended by Perkin Elmer (GeneAmp DNA Amplification Reagent Kit). The amplified fragments are analysed on agarose gel.

The primers 5168 and 5169 hybridise on either side of the B1 insertion site, downstream and upstream of the site respectively. Their sequence is as follows.

Number	5′ to 3′ sequence	Complementary to
5169	5′ GCTTTACTGTTAGTTCTACAATCGAAATTATGC 3′	FPV
5168	5′ CATGTAGATTTTAATCCGTTAACACGCGCAG 3′	FPV
3254	5′ GCCGGAAAACCTACCGGATTGATGG 3′	LacZ

A 220-bp fragment is amplified on the wild TIRs and a fragment of more than 3 kb for the recombinant TIR containing the LacZ gene. For the detection of the LacZ gene, a second amplification uses the primer 5168 situated downstream of the B1 site and the primer 3254 situated inside the LacZ gene. A 674-bp fragment is amplified. This judicious choice of primers therefore enables the recombinant TIRs and the non-recombinant TIRs to be differentiated. A viral suspension is considered to be homogeneous, double recombinant, if the WT (wild) fragment is not amplified. The controls are the genome of the WT virus, the plasmids pTIRB1 and pTIRB1Lac.

Primers	Wild fragment	Recombinant fragment
5169 + 5168	220 bp	3 kb
3254 + 5168	--	674 bp

-- = no amplified fragment.

This Sheet Has Been Replaced



This Sheet Has Been Replaced

Example 15 - Expression of the LacZ gene in tissue culture by the CNP/LacZ recombinant viruses

The β-galactosidase (β-gal) activity is measured using o-nitrophenyl-β-D-galatopyranoside (ONPG) as substrate. The enzyme converts ONPG to galactose and o-nitrophenol which is yellow in colour and the quantity of which is measured by absorption at 420 nm. This absorption is converted to β-gal units using a calibration curve according to the rule "1 βgal unit = 1 μmol of ONP produced/3 × 10^6 cells/60 minutes of incubation of the extracts at 28°C".

This "β-gal" test is carried out as follows, for a flask with a surface area of 25 cm^2. Immediate and late phases of infection can be differentiated in Pox viruses. The shortest is the immediate phase; it lasts for about 6 hours. It ends at the time of the replication of the genome. The late phase starts at the replication and ends at the end of the infectious cycle with the release of viral particles, that is to say three days later. For sufficient expression of LacZ during the immediate phase, the immediate phase is artificially prolonged using cytosine β-D-arabinofuranoside (AraC) which is an inhibitor of replication.

a. Cells

On day D=1, 3 × 10^6 QT35 cells are inoculated, per flask, into 5 ml of growth media.

On day D=2: the cells are infected at a multiplicity of infection of 3 viruses per cell. The mixture is incubated for 1 hour at 38°C. The viral inoculum is then removed by means of a pipette. 5 ml of maintenance media, supplemented or not with AraC (Sigma) at 40 μg/ml, are added per flask. The cultures are incubated for 16 hours at 38°C.

b. Harvest on day D=3

The cells are scraped from the flask.

The culture is centrifuged for 10 minutes at 6,000 g. The centrifugation pellet is resuspended in 500 μl of PBS, this suspension is mixed and it is

transferred into a 1.4-ml Eppendorf tube.

The cells are then lysed by the addition of 50 µl of $CHCl_3$ and 5 µl of 10% SDS. The cell suspension is briefly mixed. This suspension is then centrifuged for 5 minutes at 9,000 g.

c. Enzymatic reaction

The ONPG substrate for 25 samples is prepared as follows: 27.7 mg of ONPG (Sigma) and 50 ml of diluent consisting of 1 ml of 0.1 M $MgSO_4$ and 1 ml of 2-mercaptoethanol (Merck) in a final volume of 100 ml of buffer (90 ml of 0.1 M $NaHPO_4$, pH 7.0, 0.1 M $MgSO_4$, 1 ml of mercaptoethanol).

1.95 ml of the ONPG solution and 50 µl of the cellular suspension are mixed and the mixture is incubated for 1 hour at 28°C. 2 ml of 1 M Na_2CO_3 are added to this suspension and the absorbance of this suspension is measured at 420 nm.

Comparison of the β-gal activity for the recombinant viruses is given below in β-gal units.

Virus	Immediate (AraC)		Immediate + late (-AraC)	
	Abs	u β-gal	Abs	u β-gal
wt	0	0	0	0
V3	0.28	57	0.58	120
V4	0.34	71	0.58	120
V5	0.26	52	0.49	101
V8	0.05	10	0.65(1:5)	670
V9	0.05	10	0.31(1:10)	640

wt = non-recombinant wild type.

Immediate (AraC) = immediate growth phase artificially prolonged for 16 hours by AraC.

This Sheet Has Been Replaced



Immediate + late (-AraC) = natural immediate and late phases without AraC.

Abs = absorbance.

u β-gal = β-galactosidase unit measured.

1:5 and 1:10 = prior 5-fold and 10-fold dilutions of the extracts.

The Vaccinia promoter P7.5 functions during the immediate and late phases of the infection (V3, V4 and V5); the promoter P11 has only a late activity (V8 and V9); the promoter P11 is stronger than the promoter P7.5, as in Vaccinia. The temporal regulation as well as the relative strength of the two promoters are therefore preserved in the CNP virus, whether the insertion is into B1 or B2 or whether it is of the LacZ orientation.

Example 16 - Vaccination of chicks with the CNP/LacZ recombinant virus. Protection against the fowl variola. Immune response against β-gal

The vaccinating power of the CNP/LacZ recombinants is tested. The two criteria selected are the protection against fowl variola and the immune response against β-galatosidase.

a. Administration by transpiercing the wing

A suspension of the recombinant viruses V4 and V8 or a suspension of the vaccinal CNP strain were injected into one-day-old chicks, certified specific-pathogen-free (SPF), by transpiercing the wing membrane (so-called "wing web" method) (WW).

The tests are carried out on three groups of 28 to 30 chicks which are vaccinated with V4 or with V8 or with CNP, and on a group of 15 non-vaccinated chicks, as negative control.

Each vaccinated chick received 10 µl of a suspension at 5×10^5 TCID50 (Tissue Culture Infectious Dose)/ml of virus, which is eqivalent to 5×10^3 TCID50 per bird. At 29 days, the chicks were brought into contact with the virulent virus (Fowl Pox Challenge Virus strain obtained from Aphis, USA).

This Sheet Has Been Replaced



This Sheet Has Been Replaced

The absence of lesions, evaluated ten days later, shows a protection of all the chicks vaccinated against fowl variola. On the other hand, half of the non-vaccinated chicks had lesions. Analysis of the antibody titres against β-galactosidase by direct ELISA showed that 21 of the 29 (72%) chicks vaccinated with the V4 viruses and 20 of the 28 (71%) chicks vaccinated with the V8 virus exhibit a seroconversion, that is to say are seropositive.

The ELISA titre being the last dilution which gives an optical density greater than 100, the mean anti-β-galactosidase ELISA titre in the sera of these chicks is 1:800.

b. <u>Administration by the intramuscular and subcutaneous routes</u>

One-day-old chicks are vaccinated on the first day with $10^{4.4}$ TCID50 of virus V8 by the intramuscular route (1M, 27 chicks) or subcutaneous route (SC, 34 chicks). They are subjected to a challenge 27 days later. They are protected against the fowl variola (100%). Seroconversion against β-galactosidase is observed in 24 of the 27 (88%) chicks vaccinated by the IM route and in 27 of the 34 (79%) chicks vaccinated by the SC route.

In conclusion, the immunisation results show:

- that the recombinant viruses containing the LacZ gene in the TIR regions of their genome, at the B1 or B2 site, preserved their immunogenicity;
- that the P7.5 and P11 promoters function in the animal;
- that an antibody response is induced against β-galactosidase, which is considered here as a heterologous protein which is expressed by the CNP recombinant;
- that the intramuscular (IM), subcutaneous (SC) and wing membrane transpiercing (WW) routes are equivalent both in terms of protection against the fowl variola and of the percentage of seroconversion

This Sheet Has Been Replaced

against β-galactosidase.

Example 17 - Insertion of the genes, encoding the glycoprotein E2 of the fowl bronchitis virus, into the transfer vectors

The virus responsible for the infectious fowl bronchitis (IBV) is a coronavirus. The most important surface antigen of this virus is the protein E2 which consists of two sub-units S1 and S2 (Cavanagh, 1983, Cavanagh et al., 1988). Many serotypes exist including Massachusetts, denoted M41, and Dutch serotypes, especially D1466 and D274 (Kusters et al., 1987).

A recombinant CNP vaccine expressing the E2 protein of an IBV is developed so as to obtain an effective vaccine against the virus responsible for the infectious fowl bronchitis (IBV).

The gene for the protein E2, of the serotype M41, was obtained from Dr. Kusters of the University of Utrecht (NL), as well as large fragments of the E2 genes, of the serotypes D1466, D207 and D274.

A BamH1 cassette of the E2 gene of the serotype M41 was constructed by site-directed mutagenesis. The complete gene of the strain D1466 and a complete hybrid gene D207/D274 were constructed from fragments, also on cassettes which were compatible with BamH1. These three cassettes were cloned downstream of the promoter P7.5, thus generating the three plasmids p75M41, p75D1466 and p75D207.

The cassettes P7.5-E2 were cloned into the BamH1 site of the transfer vector pTIRB1, and the cassette P11Lac is then cloned, downstream of E2, into the BamH1 site. These transfer plasmids are pTIRB1P75M41Lac, pTIRB1P75D1466Lac and pTIRB1P75D207Lac. The recombinant viruses are constructed by transfer and purified as described in Examples 9, 10 and 11. The genomes are analysed by Southern blotting as described in Example 13. The expression of the E2 antigen is detected by the following immunological techniques: ELISA, Western blotting or immunofluorescence, using specific

antibodies. A stock of recombinant viruses is produced. Poultry is vaccinated with a dose of these recombinants. The efficacy of the vaccine is evaluated after infection with pathogenic viruses using the conventional methods for infectious bronchitis, and by evaluation of the level of antibodies against the virus.

Example 18 - Insertion of an Eimeria gene into the transfer plasmids - construction of CNP/TA4 recombinants

The Eimeria genus comprises parasites of poultry, which are responsible for coccidiosis. Surface antigens have been described for the E. tenella, E. necatrix, E. maxima species in particular (European Patent Applications 0,164,176 and 0,231,537).

An effective recombinant vaccine against coccidiosis is developed.

The E. tenella antigen, denoted TA4 or A4, is a 25-kd protein consisting of two sub-units of 17 kd and 8 kd linked by a disulphide bridge. The gene was isolated from a genomic library and also from mRNA. The complete description of the gene has been published in the above-mentioned patent applications.

A BamH1 cassette of the TA4 gene was constructed by subcloning. The plasmid pTA406 contains the coding sequence of TA4. The plasmid pTA410 carries the TA4 gene with a modification in the proteolytic sequence which separates the two sub-units. The native sequence Arg-Arg-Leu was replaced by the sequence Arg-Glu-Lys-Arg (described by Kieny et al., 1988), by site-directed mutagenesis. These two BamH1 cassettes were cloned downstream of the promoter P7.5 of the plasmid p1P7.5, in the orientation which places the TA4 gene under the control of P7.5.

The cassettes P7.5-TA406 and P7.5-TA410 are cloned into the BamH1 site of the transfer vector pTIRB1D. The cassette P11-Lac is then cloned into the BamH1 site downstream of the TA4 gene. The transfer plasmids are pTIRTA406Lac and pTIRTA410Lac.

This Sheet Has Been Replaced



This Sheet Has Been Replaced

The recombinant viruses were obtained by transfer as described in Examples 9, 10 and 11. Blue plaques are obtained. The recombinant which carries the TA406 gene is V20; the recombinant which carries the TA410 gene is V21.

A double recombinant V21 virus, that is to say which carries a copy of TA410 and LacZ in each TIR, is purified on each microtitre plate and its genome analysed by PCR as described in Example 14. The primers used and the size of the amplified fragments are presented in the table below. The primer 1871 is complementary to the LacZ gene.

Primers	Wild fragments	Recombinant fragments
5169 + 5168	220 bp	4 kb
5169 + 1871	--	1.2 kp

The expression of the TA4 antigen is detected by the following immunological techniques: ELISA, Western blotting or immunofluorescence, using specific antibodies.

A stock of recombinant viruses is produced. Poultry is vaccinated with a dose of these recombinants. The efficacy of the vaccine is evaluated after a challenge with virulent Eimeria using conventional methods for coccidiosis including analysis of sera, rate of weight gain and clinical signs.

Example 19 - Insertion of the polyprotein gene of the infectious bursal disease virus into the transfer vectors

The causal agent of the infectious bursal disease virus is a virus of the Birnaviridae family. The virus, called IBDV (Infectious Bursal Disease Virus), causes a highly contagious disease (infectious bursal disease) which affects young chickens and is characterised by the destruction of the lymphoid cells of the bursa of Fabricius. The virus possesses a genome consisting of 2

segments of double-stranded RNA, called segment A (about 3,400 base pairs; bp) and segment B (about 2,900 bp). The viral particles are without envelopes and are of icosahedral form of about 60 nanometres in diameter. Four viral proteins were clearly identified: VP1 with a molecular weight (MW) of 90 K (K: kilodalton); VP2, MW of 37 K to 40 K; VP3, MW of 32 K to 35 K and VP4, MW of 24 K to 29 K (Dobos 1979, Fahey et al., 1985). VP2 is derived from a precursor VPX with an MW of 41 K to 54 K.

The segment B encodes VP1 which is probably the viral polymerase. The segment A encodes 3 other proteins. These proteins are generated, by proteolytic cleavage, from a precursor with an MW of about 110 K corresponding to a large open reading frame of the segment A. The protein VP4 is involved in this proteolytic cleavage (Jagadish et al., 1988). The proteins VP2 and VP3 constitute the viral capsid. VP2 contains the antigenic determinants which are capable of inducing the synthesis of antibodies which neutralise the virus (Becht et al., 1988; Fahey et al., 1989).

A recombinant Fowlpox vaccine against the infectious bursal disease is developed.

The stages of this development are as follows:

1. Isolation of the genetic material of the selected IBDV strain, namely the EDGAR strain. The EDGAR virus strain can be obtained from the United States Department of Agriculture (USDA, APHIS 6505 Belcrest Road, Hyattsville, MD 20782, United States).
2. Synthesis, cloning and determination of the nucleotide sequence of the cDNA corresponding to the segment A.
3. Insertion of the cDNA as well as the sequences required for the expression of this genetic material in animal cells infected with Fowlpox, and the sequences permitting screening of the recombinant Fowlpox viruses, into the transfer plasmid pTIRB1 described in Example 4.

This Sheet Has Been Replaced

This Sheet Has Been Replaced

4. Isolation and purification of the recombinant viruses.
5. Genetic analysis of the recombinant viruses.
6. Analysis of the expression in vitro, in cell culture, of the IBDV genes carried by these recombinant viruses.
7. Vaccination of chicks with the recombinant viruses and analysis of the protection against infectious bursal disease.

Stage 1

The viral RNA of the EDGAR strain was isolated from the bursas of chickens infected with the virus.

About 40 g of bursas, harvested 7 days after infection with the virus, are ground in 40 ml of TNE buffer (TNE: 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8). The ground product is centrifuged at 17,000 g and the aqueous phase obtained is deposited on a preformed sucrose gradient consisting of 2 layers, containing 40% and 60% sucrose (% by weight/volume, in the TNE buffer). The gradient is centrifuged at 134,000 g for 2 hours 30 minutes. The 40%-60% interphase of the sucrose gradient is harvested and it contains the partially purified virus. 5 ml of this phase are added to 5 ml of buffer containing 10 mM Tris-HCl, 100 mM NaCl, 0.5% SDS, 10 mM EDTA, 2 mg/ml proteinase K, pH 7.5. The mixture is incubated for 1 hour at 37°C. The aqueous phase is then extracted with phenol/chloroform. The nucleic acids of the aqueous phase are precipitated with ethanol in the presence of 0.8 M LiCl and taken up in 500 μl of water.

Stage 2

The synthesis and the amplification of the cDNA corresponding to the segment A were carried out according to the method described in "GENEAMP RNA PCR KIT, PERKIN ELMER CETUS" and using synthetic oligonucleotides or primers, which are complementary to the sequence of the segment A, as primer for the synthesis of the first DNA strand by reverse transcriptase. The choice of these synthetic oligonucleotides was determined by analysis of

the published sequences of the segments A of other IBDV strains: the Australian strain 002-73 (Hudson et al., 1986), the German strain CU-1 (Spies et al., 1989) and the British strain 52/70 (Bayliss et al., 1990). The sequence of the primers and their position relative to the segment A of the EDGAR strain are presented in Figures 8 and 9.

Analysis of the published sequences for the IBDV strains indicates that two other open reading frames exist on the same coding strand of the segment A, in addition to the large open reading frame ORF1 which encodes the proteins VP2, VP4 and VP3. One of them, ORF2, which is initiated 34 bp upstream of the ATG of ORF1, overlaps the latter and is 435 bp in length. The other, ORF3, is initiated 32 bp upstream of ORF2 and is 31 bp in length. The possible role of these ORFs in the biology of the virus is currently undefined.

Four double-stranded cDNA fragments, delimited by the pairs of primers, 0-1b (585 bp), 1-2 (1129 bp), 3-4 (670 bp), 5-6 (1301 bp) respectively and spanning ORF1 and ORF2 of the segment A (see Figure 8), were generated.

These fragments were cloned into the plasmids pBSPlus or pBSLK1; six plasmids were thus constructed (see Figures 8 and 10):

This Sheet Has Been Replaced



This Sheet Has Been Replaced

Name of plasmid	Size (bp)	Construction scheme
pBSIBDV0	3478	pBSPlus (Pst1 + Hinc2) + 277-bp Pst1 fragment of the 0-1b fragment
pBS1A2	3932	pBSLK1 (Pst1 + T4 DNA polymerase, Sac1) + 760-bp Sac1 fragment of the 1-2 fragment
pBS1B	3538	pBSLK1 (Pst1 + T4 DNA polymerase, Sac1) + 369-bp Sac1 fragment of the 1-2 fragment
pEDGAR34i	3887	pBSLK1 (Pst1 + T4 DNA polymerase, Hinc2) + 670-bp 3-4 fragment
pBS3A	4175	pBSLK1 (Pst1 + T4 DNA polymerase, Sph1) + 956-bp Sph1 fragment of the 5-6 fragment
pBS3B	3509	pBSLK1 (Pst1 + T4 DNA polymerase, Sph1) + 345-bp Sph1 fragment of the 5-6 fragment

The nucleotide sequences of the IBDV fragments of these plasmids were determined and aligned so as to reconstitute the sequence of the segment A of the EDGAR strain, which is amplified by PCR (see Figure 9). The original sequences of the EDGAR strain, which were

replaced by the use of primers which were defined on the basis of published sequences of other IBDV strains, were also determined from the amplification products of these regions using primers situated outside them.

The reconstruction of the segment A from the clones obtained is schematically represented in Figure 10.

The stages are as follows:

1. Construction of pEDGAR12:

pBS1A2 (EcoR1 + T4 DNA polymerase, Sac1)
+ pBS1B (Sph1 + T4 DNA polymerase, Sac1)

2. Construction of pEDGARM12 by site-directed mutagenesis on pEDGAR12:

a. deletion of the Sph1 site of pEDGAR12 situated upstream of the IBDV sequence:

Original sequence

Sph1

5′ GTCTGATCTCTACGCATGCAAGCTTTTGTTCCCTTTAGTGAGGG 3′

Mutagenesis primer

5′ GTCTGATCTCTACGGTTCCCTTTAGTGAGGG 3′

b. deletion of the EcoR1 site of pEDGAR12 and introduction of an Sph1 site downstream of the IBDV sequence;

Original sequence:

EcoR1

5′ CGACTCACTATAGGGCGAATTCCCCCCAGCGACCGTAACGACTG 3′

<- IBDV -

Mutagenesis primer

Sph1

5′ CGACTCACTATAGGGCGGATCCCGCATGCCCCAGCGACCGTAACGACTG 3′

<- IBDV -

3. Construction of pACEDGARM12:

Insertion of the Bcl1-Sph1 fragment of pEDGARM12 into the Bcl1-Sph1 sites of pACYC184

4. Construction of pEDGARM34i by site-directed mutagenesis on pEDGAR34i:

Replacement of the Sph1 site by an Nsi1 site;

This Sheet Has Been Replaced



This Sheet Has Been Replaced

Sph1

Original sequence: 5′ GCTCGAAGTTGCTCAGGCATGCAAGCTTTTG 3′

- IBDV ->

mutagenesis Nsi1

primer 5′ GCTCGAAGTTGCTCATGCATGCAAGCTTTTG 3′

- IBDV ->

5. Construction of pEDGAR45:

pBS3A (Hinc2 + Pst1) + Hinc2-Pst1 fragment of pEDGAR34i

6. Construction of pACEDGAR14:

pACEDGARM12 (Sph1 + T4 DNA polymerase, BamH1) + pEDGARM34i (Nsi1 + T4 DNA polymerase, BamH1)

7. Construction of pACEDGAR15:

pACEDGAR14 (BamH1 + T4 DNA polymerase, Sal1) + pEDGAR45 (Pvu2-Sal1)

8. Construction of pACEDGAR1:

pACEDGAR15 (Sph1 + EcoRV) + pBS3B (Sph1 + Pvu2).

This plasmid contains the complete sequence of ORF1 of the segment A on the Bcl1-BamH1 fragment.

9. Construction of pBSIBDV0b by site-directed mutagenesis of pBSIBDV0:

Introduction of ORF3 into pBSIBDV0 which carries the beginning of ORF1 and ORF2;

Original sequence:

- pBSPlus -> --ORF2-->

5′ CCCGGGGATCCTCTAGAGTCCCTCCTTCTACAACGTGATCATTGATGG 3′

Bcl1

Mutagenesis primer:

--ORF2-->

- pBSPlus -> ----------ORF3---------->

5′ CCCGGGGATCCTCTAGAGTCTGATCAGGATGGAACTCCTCCTTCTACAACGCTATCATTGATGG 3′

Bcl1

10. Construction of pACEDGAR2 and pACEDGAR3:

The plasmids pACEDGAR2 and pACEDGAR3 were obtained by replacing the Bcl1-Rsr2 region of the plasmid pACEDGAR1 with the Bcl1-Rsr2 fragment of the plasmids pBSIBDV0 and pBSIBDV0b respectively.

The 3 plasmids pACEDGAR1, pACEDGAR2 and pACEDGAR3 therefore enable ORF1, ORF1-ORF2 and ORF1-ORF2-ORF3,

respectively, of the segment A of the EDGAR strain, to be isolated on a Bcl1-BamH1 fragment.

The sequences of these 3 plasmids, on the ATG side of the ORFs, are represented below:

```
                Bcl1
pACEDGAR1: 5' TGATCACAGCGATGACA ... 3'
                  ---ORF1---->

                Bcl1
pACEDGAR2: 5' TGATCATTGATGGTTAGTAGAGATCAGACAAACCATCGCAGCGATGACA ... 3'
                       ------------------ORF2---------------------->
                                                        ---ORF1---->

                Bcl1
pACEDGAR3: 5' TGATCAGGATGGAACTCCTCCTTCTACAACGCTATCATTGATGGTTAGT ... 3'
                       -------------ORF3------------->
                                                    ------ORF2----->
```

Stage 3

These Bcl1-BamH1 fragments were then cloned into the BamH1 site of the plasmid p2P75, thus generating the plasmids p75EDGAR1, p75EDGAR2 and p75EDGAR3 and placing the coding sequence(s) of the segment A under the control of the promoter P7.5.

These coding sequences were also placed under the control of the promoter P11. The Bcl1-BamH1 fragment of the plasmid pACP11, carrying the promoter P11, was inserted into the BamH1 site of the plasmids pACEDGAR1, pACEDGAR2 and pACEDGAR3 so as to generate the plasmids p11EDGAR1, p11EDGAR2 and p11EDGAR3 respectively.

Bcl1-BamH1 cassettes, P75-EDGAR or P11-EDGAR, were isolated from these different plasmids and inserted into the BamH1 site of the plasmid pTIRB1, thus producing the plasmids pTIR75EDGAR1, pTIR75EDGAR2, pTIR75EDGAR3, pTIR11EDGAR1, pTIR11EDGAR2 and pTIR11EDGAR3. The orientation of these Bcl1-BamH1 cassettes inside the plasmid pTIRB1, which was conserved, is such that the direction of transcription, initiated from the promoters P7.5 and P11, coincides with that of ORF1 and ORF2 which are present inside the plasmid pTIRB1.

This Sheet Has Been Replaced



The Bgl2-BamH1 fragment of the plasmid pACP11LAC, carrying the P11-LACZ cassette, was inserted into the BamH1 site of the plasmids pTIR75EDGAR1 and pTIR75EDGAR2 so as to generate the plasmids pTIR75E1LAC and pTIR75E2LAC respectively. The orientation of P11-LACZ inside these plasmids is such that the direction of transcription of the LACZ gene coincides with that of ORF1 and ORF2.

In a similar manner, the plasmids pTIR11E1LAC, pTIR11E2LAC and pTIR11E3LAC were generated by insertion of the p75-LACZ cassette, isolated in the form of a Bgl2-BamH1 fragment of the plasmid p75LAC, into the BamH1 site of the plasmids pTIR11EDGAR1, pTIR11EDGAR2 and pTIR11EDGAR3 respectively.

Finally, the plasmid pTIR11VP2LAC was generated by inserting the Xho1 (treated with T4 DNA polymerase)-Bgl2 fragment of the plasmid p11EDGAR1, into the PpuM1 (treated with T4 DNA polymerase) and Bgl2 sites of the plasmid pTIR11E1LAC. The transfer plasmid pTIR11VP2LAC contains, under the control of the promoter P11, the complete sequence of the protein VP2 as well as the amino-terminal part of the protein VP4; by virtue of the construction, the last three amino acids (Asp, Leu and Glu; carboxy-terminal part of VP3) and the stop codon for translation (TGA) of the polyprotein are fused in phase. It can be seen in Figure 9 that this heterologous protein therefore corresponds to a part of the polyprotein containing the amino acids 1 to 493, followed by the amino acids 1010 to 1012, in which the protein VP2 is included.

By way of example, the plasmid pTIR75E1LAC is represented in Figure 11.

Stage 4 and stage 5

The recombinant Fowlpox viruses, in which the ORFs of the EDGAR strain have been integrated, were isolated, purified and characterised according to the methods described in Examples 9, 10, 11, 12, 13 and 14.

The notation of the recombinant viruses is:

This Sheet Has Been Replaced



V11 = P7.5E1 V12 = P7.5E2
V14 = P11E1 V15 = P11E2
V17 = P11VP2 V16 = P11E3

The primers used for selecting the double recombinant TIRs by PCR, differentiate the WT TIRs and the TIRs containing LacZ, E1, E2, E3 or VP2. The combinations of primers and the size of the amplified fragments are given in the table below.

The primers IBDV16, 3, 1b and 22 hybridise to IBDV. The primers IBDV16, 1b and 3 hybridise to the coding part of VP2. The primer IBDV22 hybridises to the coding part of VP3 and therefore not to the V17 genome. The primers 5168 and 5169 hybridise to FPV. The primer 1871 hybridises to the β-galactosidase gene. The sequence of these primers is defined in the table "List and sequences of the primers".

Primers	Virus	Fragments (nt)
5169 + 5168	WT	220
	recombinants	6.0 kb
5169 + 1871	V8	310
	recombinants	3.6 kb
5169 + IBDV16	V11	341
	V14, V17	339
	V12	373
	V15	371
	V16	402
5169 + IBDV1b	V17	766
IBDV22 + 1871	V11, V12	379
	V14, V15, V16	479
	V17	--
IBDV3 + 1871	V14, V15, V16	2280
	V17	732

Stage 6

The expression of the IBDV antigens of the recombinant viruses inside the QT35 cells was compared.

This Sheet Has Been Replaced



This Sheet Has Been Replaced

The antibodies used, which were raised against the IBDV strain Cu-1, were kindly provided by Professor H. Müller, Institüt für virologie, Justus-Liebig-Universität, Giessen, Germany.

They are: 1, a polyclonal serum (No. B22) of a rabbit which was hyperimmunised against the Cu-1 strain

2, an anti-VP3 mouse monoclonal antibody (No. I/A10) which recognises the protein VP3 in native and denatured form

3, an anti-VP2 mouse monoclonal antibody (No. B1) which recognises a conformational epitope of the protein VP2; the B1 antibody is an antibody which is capable of neutralising the viral strain Cu-1.

a. Experimental conditions for the infection

On day D=1, 2.5×10^6 QT35 cells are inoculated, per 25-cm^2 flask, into 5 ml of growth media (composition described in Example 1). Eight flasks are prepared.

On day D=2, the cells are infected at a multiplicity of infection of 0.01 virus per cell with each recombinant virus, V11, V12, V14, V15, V16, V17, V8 (negative control) and IBDV (bursine strain 2, positive control).

On day D=5, the infected cells are harvested and [lacuna] the media after freezing followed by thawing.

b. Analysis of the expression products by sandwich type ELISA

The ELISA test is carried out using, as first antibody, the polyclonal antibody B22 and, as second antibody, either the anti-VP3 monoclonal antibody (No. I/A10) or the anti-VP2 neutralising monoclonal antibody (No. B1). The ELISA curves are presented in Figure 12A for anti-VP3, and in Figure 12B for anti-VP2.

The results of these analyses show that:

1 - Figure 12A: the protein VP3 is expressed in all the recombinants (V11, V12, V14, V15 and V16), which contain at least the sequence encoding the polyprotein (ORF1, VP2-VP4-VP3 proteins), but not in the recombinant

This Sheet Has Been Replaced

V17 since it contains only the region encoding VP2 and the amino-terminal part of VP4, nor in the recombinant V8 since it contains no IBDV sequence.

The level of expression of the protein VP3 is lower in the recombinants V11 and V12 than in the recombinants V14, V15 and V16, which is probably correlated with the strength of the promoters which are present in the various recombinants: the activity of the promoter p7.5 (recombinants V11 and V12) being less strong than that of the promoter p11 (recombinants V14, V15 and V16) (see Example 15).

No difference is observed in the level of expression of VP3 as a function of the reading frames of the segment A, which are introduced into the various recombinants: ORF1 for the recombinants V11 and V14, ORF1 + ORF2 for the recombinants V12 and V15 and ORF1 + ORF2 + ORF3 for the recombinant V16.

2 - Figure 12B: the protein VP2 is expressed in all the recombinants except for V8 which does not contain IBDV sequences.

The level of expression is highest for the recombinant V17; in this case, it is close to that of the protein VP2 which is expressed in the cells infected by the IBDV virus.

A correlation is again oberved between the strength of the promoters present in the various recombinants and the level of expression of the protein VP2.

These results show, on the one hand, that the epitope of the protein VP2 which is recognised by the neutralising antibody B1 raised against the strain Cu-1, is also present in the protein VP2 of the American strain Edgar, and on the other hand, that the original conformation of the protein VP2 is preserved, at least in this region, in all the recombinants.

c. <u>Analysis of the expressidon products by Western blotting</u>

Western blot analysis of the proteins produced by the various recombinants was carried out, on the one

hand, using the polyclonal serum (B22) which recognises all of the IBDV proteins and, on the other hand, using the anti-VP3 monoclonal antibody (I/A10).

The results, which are illustrated in Figure 13A, show that the polyclonal serum recognises, in the extracts of the cells infected by IBDV, essentially 3 proteins corresponding to VP2 (~ 40 kd), VP3 (~ 32 kd) and VP4 (~ 28 kd). A protein of about 46 kd can also be observed and it may constitute the VP2 precursor (VPX, ~ 48-49 kd).

For all the recombinants (V11, V12, V14, V15 and V16), which contain at least the sequence encoding the polyprotein (ORF1, VP2-VP3-VP4), proteins with a molecular weight (MW) corresponding to VP3, VP4 and probably VPX respectively are detected with the anti-IBDV polyclonal serum (Figure 13A). For the recombinant V17, only one protein, whose molecular weight is close to that of the fusion protein VP2 + amino-terminal region of VP4 (~ 52 kd), is detected.

The anti-VP3 monoclonal antibody (Figure 13B), recognises essentially two proteins, VP3 and probably a degradation product of VP3, in the cells infected by IBDV and all the recombinants expressing the polyprotein. No IBDV protein is recognised in the recombinant V17 expressing the fusion protein VP2-VP4.

All these results indicate that the reading frame encoding the polyprotein (ORF1) is translated in all the recombinants irrespective of the presence of the additional reading frames ORF2 (recombinants V12 and V15) or ORF2 and ORF3 (recombinant V16). The cleavage of the precursor polyprotein is carried out accurately but appears to be incomplete in the case of VP2; it has been suggested that the complete maturation of the precursor VPX would take place during or after transportation of the IBDV viral particles to the surface of the cells (Müller et al., 1982).

This Sheet Has Been Replaced



Stage 7

The vaccinating power of the recombinants FPV/IBDV is tested.

a. Seroconversion with V11

A first vaccination campaign with a recombinant FPV/IBDV compared the seroconversion against β-galactosidase and against the IBDV virus for three different administration routes: by the intramuscular route (IM), by the subcutaneous route (SC) and by piercing the wing (WW or wing web).

One-day-old chicks are vaccinated with $10^{4.4}$ V11 recombinant viruses using the three administration routes, IM, SC or WW. They are killed by euthanasia 11 days later.

Analysis of the sera by direct ELISA showed that 2/14 (14%) of the chicks vaccinated by the intramuscular route, 3/6 (50%) of the chicks vaccinated by the subcutaneous route and 8/15 (53%) of the chicks vaccinated by transpiercing the wing, had antibodies against β-galactosidase, while 5/14 (36%) of the chicks vaccinated by the intramuscular route, 1/6 (17%) of the chicks vaccinated by the subcutaneous route and 1/15 (7%) of the chicks vaccinated by transpiercing the wing, had antibodies against the IBDV virus.

In conclusion, the IBDV proteins are expressed by the recombinant FPV/IBDV11 in the vaccinated and they induce an antibody response in a few chicks. Of the three administration routes, the intramuscular route induces a higher response than the other two.

b. Early protection with V11, V15 and V16

One-day-old chicks received a dose of $10^{4.4}$ V8 (negative control), V11, V15 or V16 virus by the intramuscular route and were challenged 10 days later with 100 LD50 (100 times the lethal dose) of the virus 849VB administered by the ocular route. The 849VB strain is described by Van Den Berg et al., 1991.

The chicks are killed by euthanasia 20 days later.

This Sheet Has Been Replaced



The ratio, multiplied by 100, between the weight of the bursa and the total weight is a protection index.

In the control group, which is vaccinated with V8 and not subjected to the IBDV challenge, this ratio is 0.57 (mean for 10 chicks).

In the V8 group subjected to the challenge, this ratio becomes 0.11 (9 chicks).

In the chicks vaccinated with V11, V15 and V16, these ratios are 0.11 (20 chicks), 0.12 (18 chicks) and 0.12 (18 chicks) respectively.

On the basis of this criterion, there is no early protection against the IBDV virus by the recombinants V11, V15 and V16.

c. Late protection with V11, V15 and V16

Chicks vaccinated with V8, V11, V15 and V16, as described in the preceding section, were subjected to the IBDV challenge 42 days after the injection.

The sera collected before the challenge are analysed by the ELISA method in order to evaluate the antibody response against β-galactosidase and the IBDV virus.

The anti-β-galactosidase response shows that 100% seroconversion is obtained in all the vaccinated chicks, that is to say 70 chicks in total. The titres range from 1:200 to 1:51,200. There is no significant difference between the titres obtained with V11, whose LacZ gene is under the control of P7.5, and V8, V15 and V16, whose LacZ genes are under the control of the promoter P11.

The anti-IBDV response in the 10 chicks vaccinated with V8 is zero; it is positive in all the chicks vaccinated with V11, V15 and V16. In detail, the titres of the responses in the 20 chicks vaccinated with V11 range from 1:800 to 1:51,200, with a mean of 1:6,400. They are from 1:6,400 to 1:102,400, with a mean of 1:25,600 for the 20 chicks vaccinated with V15. They are between 1:6,400 and 1:204,800, with a mean of 1:25,600 in the 20 chicks vaccinated with V16. The antibody levels are substantially higher for the groups vaccinated with

This Sheet Has Been Replaced



V15 or V16 than for those vaccinated with V11.

The seroneutralisation titres for the vaccinal strain PBG68, adapted to cultured cells, are 1:10 for V11, as negative control; 4 sera out of 20 for V15 and 1 serum out of 20 for V16 have seroneutralisation titres of 1:20. This titre is therefore low and is observed in only a few of the animals.

The ratio (weight of bursas/total weight) x 100 has a mean of 0.66 in the unchallenged V8 control group (10 chicks).

It becomes 0.1 in the challenged group V8 (1 survival).It is 0.1, 0.11, 0.11 for the three groups V11 (3 chicks), V15 (11 chicks) and V16 (8 chicks) respectively. There is no protection against atrophy of the bursas.

The mortality during the four days which followed the challenge was almost total for the control group (9 chicks out of 10). In the group V11, 3 chicks out of 20 survived while 11 chicks out of 20 which were vaccinated with V15 and 8 chicks out of 20 which were vaccinated with V16 survived. A protection of the order of 50% of the animals vaccinated with V15 or V16 is therefore observed.

d. <u>Protection with V14, V15 and V17</u>

Four groups of three-week old poultry received 0.2 ml of the recombinant viruses V8 (negative control), V14, V15 or V17. The amount of virus per chicken was $10^{6.4}$ TCID50. The administration route is intramuscular.

Twenty-one days later, serum is collected from each animal in order to evaluate the anti-IBDV antibody titre. One hundred times the lethal dose of IBDV is injected into the animals of groups V14, V15 and V17 and into some of the animals of the group V8 (that is to say the group of poultry which received the recombinant virus V8).

11 days later, that is to say 32 days after the vaccination, the control animals of the group V8 and those which survived the challenge, are killed by

This Sheet Has Been Replaced



This Sheet Has Been Replaced

euthanasia and the ratio (weight of the bursas/weight of the poultry) × 100 is determined.

The results are:

- Mortality: the number of poultry animals which succumbed to the challenge can be divided as follows: for V8: 12 poultry animals/14 (85%), for V14: 21/24 (87%), for V15: 17/25 (68%) and for V17: 0/25 (0%).

In other words, all the poultry vaccinated with the recombinant V17 resisted the challenge. The protection is total in this group. Furthermore, none of the poultry showed the slightest clinical sign of disease. The 32% protection obtained with the recombinant V15 is close to the 50% protection obtained in the preceding experiment.

- ELISA: the mean titres estimated against the virus are: for V8, titres less than 1:100 and considered as negative; for V14, titres of 1:12800 (from 1:6400 to 1:51200), for V15, titres of 1:6400 (from 1:800 to 1:25600), for V17, titres less than 1:100 except for 3 poultry animals (1:800, 1:6400 and 1:51200).

Consequently, the antibody response against IBDV, which is induced by the expression of the polyprotein of the recombinant FPV/IBDV, is very high. In contrast, the response against the complete virus, which is induced by V17, is low.

- Seroneutralisation: the seroneutralisation titres against the PBG68 strain have a mean value of: for V8, V14 and V15, titres less than 1:10 and considered as negative; for V17, 5 poultry animals out of 20 have a titre above 1:20, that is to say, in detail, a titre of 1:20 (twice), 1:40, 1:80 and 1:320.

Consequently, a seroneutralisation is observed only in the sera of poultry which were vaccinated with V17.

- Bursas: the mean of the ratio (weight of the bursas/total weight) × 100 is as follows: for unchallenged V8, a ratio of 0.68 (± 0.13), for challenged V8, a ratio of 0.09 (± 0.01), for V14, a ratio of 0.10 (±

0.02), for V15, a ratio of 0.13 (± 0.04) and for V17, a ratio of 0.34 (± 0.23).

Consequently, a partial protection of the bursas of Fabricius is observed only in the group V17. Indeed, 8 poultry animals out of 24 have a ratio which is similar to that of the unchallenged controls. The other sixteen have a higher ratio than the challenged controls.

Conclusions

The main conclusions of these vaccination campaigns are:

- The recombinants which express the IBDV polyprotein induce a strong ELISA response in poultry, with high titres against the virus.
- The recombinant which expresses the VP2 portion of the polyprotein completely protects the poultry against mortality and partially protects them against atrophy of the bursa.

This Sheet Has Been Replaced



List of the plasmids

Name	Characteristics
Vectors for cloning into E. coli	
pUC18	Cloning vector (Messing 1983)
pBSPlus	Cloning vector (Stratagene)
pBSLK1	pBSPlus with a Bcl1 and Bgl2 site inside the polylinker
pBSLK2	Identical to pBSLK1 but the Bcl1 and Bgl2 sites are in the inverse order
pACYC184	Cloning vector (Chang, 1978)
Vaccinia promoters	
pGS20	Transfer vector for Vaccinia (Mackett 1984)
p1P75	pBSLK1 + Bcl1-BamH1 fragment of pGS20 with the Vaccinia promoter P7.5 inside Bcl1-BamH1
p2P75	Identical to P1P75 but inside pBSLK2
p1P11	pBSLK1 + 40-mer synthetic DNA with the Vaccinia promoter P11 inside Bgl2-BamHI
p2P11	Identical to p1P11 but inside pBSLK2
pACP11	Identical to p1P11 but inside Xho2-BamHI of pACYC184
LacZ gene	
pBSMutLacZ1	pBSPlus with LacZ in a Bgl2-BamH1 cassette
p75lac	p2P75 + Bgl2-BamH1 cassette with LacZ in Bgl2-BamH1
p11Lac	Identical to p75Lac but in p2P11
pACP11Lac	pACYC184 + P11-Lac cassette in a Bgl2-Hind3 fragment of p11Lac in Xho2-Hind3
Transfer vectors for CNP	
pTIR1	pUC18 with the EcoRI TIR-L fragment of CNP. The fragment which separates the BamH1 sites of the pUC18 linker of the CNP BamH1 was deleted.
pTIRB1	Insertion of a BamH1 into pTIR1, between ORF1 and ORF2.

This Sheet Has Been Replaced



pTIRB2	Identical to pTIRB1 but between ORF2 and ORF3.
pTIRB1D	Identical to pTIRB1, but deletion of the EcoR1-Hpa1 fragment.

Transfer vectors with LacZ

pTIRB1P75Lac	pTIRB1 + P75-Lac cassette of p75Lac in a Bgl2-BamH1 fragment cloned into BamH1.
pTIRB2P75Lac	As above but inside pTIRB2
pTIRB2LacP75	As above but in the opposite orientation
pTIRB1P11Lac	pTIRB1 + P11-Lac cassette of p11Lac in a Bgl2-BamH1 fragment cloned into BamH1
pTIRB1LacP11	As above but in the opposite orientation
pTIRB2LacP11	As above but inside pTIRB2

Plasmids which carry the E2 of IBV

p75M41	E2 gene of the serotype M41, cloned downstream of P7.5
p75D1466	As above but of the serotype D1466
p75D207	As above but of the serotype D207/D274

Transfer vectors with the E2 of IBV

pTIRB1P75M41Lac	P7.5-E2 cassettes of M41 and P11-Lac cloned into the BamH1 site of TIRB1.
pTIRB1P75D1466Lac	As above but E2 of D1466.
pTIRB1P75D207Lac	As above but E2 of D207/D274.

Plasmids which carry the TA4 of Eimeria

pTA406	cDNA of the TA4 antigen of Eimeria tenella cloned into a BamH1 cassette
pTA410	As above but the proteolytic site is different
p75TA406	TA406 gene cloned downstream of P7.5
p75TA410	As above but TA410 gene
pTIRTA406	P7.5-TA406 cassette cloned into BamH1 of pTIRB1D
pTIRTA410	As above but P7.5-TA410 cassette

Transfer vectors with the TA4 of Eimeria

pTIRTA406Lac	P11Lac cassette cloned into pTIRTA406

This Sheet Has Been Replaced



pTIRTA410Lac	As above but inside pTIRTA410

Plasmids for the cloning of IBDV

pBSIBDV0	pBSPlus (Pst1-Hinc2), this work + 277-bp Pst1 fragment of the fragment EDGAR 0-1b obtained by PCR.
pBSIBDV0b	Derived from pBSIBDV0 by site-directed mutagenesis
pBS1A2	pBSLK1 (Pst1 + T4 DNA polymerase, Sac1) + 760-bp Sac1 fragment of the fragment EDGAR 1-2 obtained by PCR.
pBS1B	pBSLK1 (Pst1 + T4 DNA polymerase, Sac1) + 369-bp Sac1 fragment of the fragment EDGAR 1-2 obtained by PCR.
pEDGAR34i	pBSLK1 (Pst1 + T4 DNA polymerase, Hinc2) + 670-bp EDGAR 3-4 fragment obtained by PCR.
pEDGARM341	Derived from pEDGAR34i by site-directed mutagenesis
pBS3A	pBSLK1 (Pst1 + T4 DNA polymerase, Sph1) + 956-bp Sph1 fragment of the fragment EDGAR 5-6 obtained by PCR.
pBS3B	pBSLK1 (Pst1 + T4 DNA polymerase, Sph1) + 345-bp Sph1 fragment of the fragment EDGAR 5-6 obtained by PCR.
pEDGAR12	pBS1A2 (EcoR1 + T4 DNA polymerase, Sac1) + pBS1B fragment (Sph1 + T4 DNA polymerase, Sac1)
pEDGARM12	Derived from pEDGAR12 by site-directed mutagenesis
pACEDGARM12	Insertion of the Bcl1-Sph1 fragment of pEDGARM12 into the Bcl1-Sph1 sites of pACYC184
pEDGAR45	pBS3A (Hinc2-Pst1) + Hinc2-Pst1 fragment of pEDGAR34i
pACEDGAR14	pACEDGARM12 (Sph1 + T4 DNA polymerase, BamH1) + pEDGARM34i fragment (Nsi1 + T4 DNA polymerase, BamH1)

This Sheet Has Been Replaced



pACEDGAR15	pACEDGAR14 (BamH1 + T4 DNA polymerase, Sal1) + pEDGAR45 fragment (Pvu2-Sal1)
pACEDGAR	pACEDGAR15 (Sph1-EcoRV) + pBS3B fragment (Sph1-Pvu2)
pACEDGAR2	Replacement of the Bcl1-Rsr2 fragment of pACEDGAR1 by the Bcl1-Rsr2 fragment of pBSIBDV0
pACEDGAR3	Identical to the Bcl1-Rsr2 fragment of pBSIBDV0b

Plasmids with IBDV and a Vaccinia promoter

p75EDGAR1	Bcl1-BamH1 fragment of pACEDGAR1 inserted into the BamH1 site of p2P75
p75EDGAR2	Identical to the Bcl1-BamH1 fragment of pACEDGAR2
p75EDGAR3	Identical to the Bcl1-BamH1 fragment of pACEDGAR3
p11EDGAR1	Bcl1-BamH1 fragment carrying P11 of pACP11 inserted into the BamH1 site of pACEDGAR1
p11EDGAR2	As above but inside the plasmid pACEDGAR2
p11EDGAR3	As above but inside the plasmid pACEDGAR3

Transfer vectors with IBDV alone

pTIR75EDGAR1	Bcl1-BamH1 fragment of p75EDGAR1 inserted into the BamH1 site of pTIRB1
pTIR75EDGAR2	Identical to the plasmid p75EDGAR2
pTIR75EDGAR3	Identical to the plasmid p75EDGAR3
pTIR11EDGAR1	Identical to the plasmid p11EDGAR1
pTIR11EDGAR2	Identical to the plasmid p11EDGAR2
pTIR11EDGAR3	Identical to the plasmid p11EDGAR3

Transfer vectors with IBDV and Lac

pTIR75E1LAC	Bgl2-BamH1 fragment of pACP11LAC inserted into the BamH1 site of pTIR75EDGAR1
pTIR75E2LAC	Identical to the plasmid pTIR75EDGAR2
pTIR11E1LAC	Bgl2-BamH1 fragment of p75LAC inserted into the BamH1 site of pTIR11EDGAR1

This Sheet Has Been Replaced



pTIR11E2LAC	Identical to the plasmid pTIR11EDGAR2
pTIR11E3LAC	Identical to the plasmid pTIR11EDGAR3
pTIR11VP2LAC	Xho1 (T4 DNA polymerase)-Bgl2 fragment of p11EDGAR1 cloned into the PpuM1 (T4 DNA polymerase)-Bgl2 sites of pTIR11E1LAC

This Sheet Has Been Replaced



REFERENCES

- Bayliss C.D., Spies U., Shaw K., Peters R.W., Papageorgiou A., Müller H. and Boursnell M.E.G., J. gen. Virology 71, 1303-1312 (1990).
- Becht H., Müller H. and Müller H.K., J. gen. Virology 69, 631-640 (1988).
- Bertholet C., R. Drillien, R. Wittek. 1985. Proc. Natl. Acad. Sci. USA, 82:2096-2100.
- Campbell J.A., M.M. Binns, F.M. Tomley, M.E.G. Boursnell. 1989. J. Gen. Virol., 70:145-154.
- Cavanagh, D. 1983. J. Gen. Virol., 64:2577-2583.
- Cavanagh, D. et al. 1988. Virus research, 11:141-150.
- Chang, A.C.Y. et Cohen, S.N. 1978. J. Bacteriol. 134:1141-1156.
- Cho B.R., Avian Diseases vol. 25, n° 4, 839-846 (1981).
- Cochran et al, 1985. J. of Virol., 54 (1), 30-37.
- Coupar et al. 1990. Virology, 179:159-167.
- Dobos P., Journal of Virology 32, 1046-1050 (1979).
- Fahey K.J., O'Donnell I.J. and Azad A.A., J. gen. Virology 66, 1479-1488 (1985).
- Fahey K.J., Erny K. and Crooks J., J. gen. Virology 70, 1473-1481 (1989).
- Hanggi et al, 1986. EMBO Journal, 5 (5), 1071-1076.
- Hudson P.J., McKern N.M., Power B.E. and Azad A.A., Nucleic acids research 14, 5001-5012 (1986).
- Jagadish M.N., Staton V.J., Hudson P.J. and Azad A.A., J. Virology 62, 1084-1087 (1988).
- Kieny et al, 1988. Protein engineering, 2 (3):219-225.
- Kusters, J.G. et al. 1987. J. Gen. Virol. 68:343-352.
- Mackett et al, 1984. J. of virol., 49 (3), 857-864.
- Mackett et al, 1985. DNA cloning. A practical approach, Vol. 2, 191-211.
- Maniatis, T., E.F. Fritisch, and J. Sambrook. 1982. Molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Messing, J. 1983. Methods Enzymol. 101:20-78.

This Sheet Has Been Replaced



- Moss, B. 1990. Annu. Rev. Biochem. 59:661-88.
- Müller H. and Becht H., 1982. Journal of Virology 44 (1), 384-392
- Spehner et al, 1990. J. of Virol., 64:527-533.
- Spies U., Müller H. and Becht H., Nucleic acids research 17, 7982 (1989).
- Shuman et al, 1989. Virology, 170:302-306.
- Tomley et al, 1988. J. Gen. Virol., 69, 1025-1040.
- Venkatesan et al, 1981. Cell, 125, 805-813.
- Van Den Berg, Gonze et Meulemans, 1991. Avian Pathology, 20, 133-143

x x x

This Sheet Has Been Replaced



List of organisms

Name	Characteristics
1. E. COLI BACTERIA	
MC1061	araD139, (ara, leu)7697, lacX74, galU, galK, hsdR, strA (Pharmacia)
JM1110	Used for its dam- phenotype (N3837dam4)
2. CNP VIRUS	
LVCO	Standard Fowlpox challenge strain of the USDA.
CNP	Fowlpox vaccinal strain (SOLVAY)
V3	Recombinant CNP virus with the P7.5-LacZ cassette in TIRB1
V4	Identical to V3, but the cassette is in TIRB2
V5	Identical to V4, but the cassette is in the opposite orientation
V8	Identical to V3, but the cassette is P11-Lac
V9	Identical to V8, but the cassette is in the opposite orientation
V10	Identical to V9, but the cassette is in TIRB2
3. IBDV VIRUS	
EDGAR	EDGAR strain, American isolate from S.A. EDGAR, Auburn University, propagated 3 times on chicken and once on embryonic eggs. USDA standard challenge.
849VB	Pathogenic strain described by Van Den Berg et al., 1991.
PBG98	Strain adapted to CEF cells.
Bursine2	Vaccinal strain adapted to QT35 cells.
4. CELLS	
QT35	Quail cell line.
CEF	Chicken embryo fibroblasts

This Sheet Has Been Replaced



5. CNP/IBD VIRUS

V11	Carries the cassettes P11Lac and P7.5E1
V12	Carries the cassettes P11Lac and P7.5E2
V14	Carries the cassettes P7.5E1 and P11Lac
V15	Carries the cassettes P7.5E2 and P11Lac
V16	Carries the cassettes P7.5E3 anbd P11Lac
V17	Carries the cassettes P7.5VP2 and P11Lac

6. CNP/TA4 VIRUS

V21	Carries the cassettes P11Lac and P7.5TA410

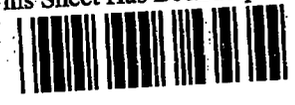
This Sheet Has Been Replaced



LIST AND SEQUENCES OF THE PRIMERS

Number	Sequence from 5′ to 3′	Complementary to
5169	5′ CCTTTACTGTTAGTTCTACAATCGAAATTATGC 3′	FPV
5168	5′ CATGTAGATTTTAATCCGTTAACACGCGCAG 3′	FPV
3254	5′ GCCGGAAAACCTACCGGATTGATGG 3′	Lac
1871	5′ GAAACCAGGCAAAGCGCC 3′	Lac
IBDV16	5′ CCAGGGTGTCGTCCGGAATGG 3′	IBDV
IBDV1b	5′ CCCAAGATCATATGATGTGGGTAAGCTGAGG 3′	IBDV
IBDV3	5′ TGAGCAACTTCGAGCTGATCCCAAATCCTG 3′	IBDV
IBDV22	5′ CTGCTCTTGACTGCGATGGAG 3′	IBDV

This Sheet Has Been Replaced



This Sheet Has Been Replaced



CLAIMS

1. Recombinant Avipox virus derived from an attenuated strain of Avipox virus and containing, in a nonessential part of its genome, at least one DNA sequence encoding all or part of a protein which is heterologous with respect to the Avipox virus as well as the elements capable of ensuring the expression of this protein inside a cell infected by the said recombinant virus, characterised in that the nonessential part of the genome is composed of a noncoding intergenic region situated between two open reading frames (ORF) including their expression signals.

2. Virus according to Claim 1, characterised in that it is derived from an attenuated strain of Fowlpox virus.

3. Virus according to Claim 1 or 2, characterised in that the nonessential part is composed of an intergenic region having a sequence of more than 60 nucleotides.

4. Virus according to any one of Claims 1 to 3, characterised in that the intergenic region is situated in one of the two TIR regions of the virus.

5. Virus according to Claim 4, characterised in that at least one DNA sequence is cloned into the two TIR regions of the virus.

6. Virus according to Claim 4 or 5, characterised in that the intergenic region is the region, termed region $\beta 1$, situated between the nucleotides 1675 and 2165, taking as starting nucleotide the BamH1 restriction site present inside the TIRs.

7. Virus according to Claim 4 or 5, characterised in that the intergenic region is the region, termed region $\beta 2$, situated between the nucleotides 2672 and 3605, taking as starting nucleotide the BamH1 restriction site present inside the TIRs.

8. Virus according to Claim 5 and 6, characterised in that a DNA sequence is cloned into the $\beta 1$ region, inside each of the two TIRs.

9. Virus according to any one of Claims 1 to 8, characterised in that the heterologous protein is chosen



This Sheet Has Been Replaced

from the antigens of the infectious bursal disease virus (IBDV), the infectious bronchitis virus (IBV), the virus responsible for chicken anaemia (CAV), the protozoa Eimeria which is responsible for coccidiosis, the Newcastle disease virus (NDV) and the Marek's disease virus (MDV).

10. Virus according to Claim 9, characterised in that the heterologous protein is chosen from the antigens of the infectious bursal disease virus (IBDV), the infectious bronchitis virus (IBV), the virus responsible for chicken anaemia (CAV) and the protozoa Eimeria.

11. Virus according to Claim 10, characterised in that the heterologous protein is chosen from all or part of the open reading frames comprising polyprotein and parts of polyprotein for IBDV, the antigen E2 for IBV, the surface antigen TA4 for Eimeria and the protein P50 for CAV.

12. Virus according to Claim 11, characterised in that the heterologous protein is part of the polyprotein containing the amino acids 1 to 493 followed by the amino acids 1010 to 1012.

13. Culture of eukaryotic cells which are infected with a recombinant Avipox virus according to any one of Claims 1 to 12.

14. Culture of cells according to Claim 13, characterised in that it relates to fowl cells.

15. Vaccine characterised in that it contains an Avipox virus according to any one of Claims 1 to 12.

16. Use of an Avipox virus according to any one of Claims 1 to 12 as a vector which is capable of expressing all or part of a protein which is heterologous with respect to the virus.

17. Transfer sequence which enables a DNA sequence, encoding all or part of a heterologous protein, to be inserted into the genome of the Avipox virus according to any one of Claims 1 to 12.

18. Plasmid carrying a sequence according to Claim 17.

* C L M S *