**Recombinant Infectious Bovine Rhinotracheitis Virus**

The present invention provides recombinant infectious bovine rhinotracheitis (IBR) viruses useful in vaccines to protect bovines from diseases particular to them, including infectious bovine rhinotracheitis and bovine respiratory disease complex. The present invention further provides methods for distinguishing an animal vaccinated with a vaccine of the present invention from an animal infected with a naturally-occurring IBR virus. The present invention also provides isolated DNA encoding the gpE glycoprotein, the gpG glycoprotein, and US2 genes of an IBR virus. The present invention further provides homology vectors for producing recombinant IBR viruses.
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RECOMBINANT INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS

5 Within this application several publications are referenced by arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

15 Field of the Invention

The present invention involves recombinant infectious bovine rhinotracheitis (IBR) viruses useful in vaccines to protect bovines from naturally-occurring infectious bovine rhinotracheitis virus and other bovine diseases.

Background of the Invention

The ability to isolate viral DNA and clone this isolated DNA into bacterial plasmids has greatly expanded the approaches available to make viral vaccines. The methods used to make the present invention involve modifying cloned viral DNA sequences by insertions, deletions and single or multiple base changes. The modified DNA is then reinserted into the viral genome to render the virus non-pathogenic. The resulting live virus may then be used in a vaccine to elicit an immune response in a host animal and to protect the animal against a disease.

35 One group of animal viruses, the herpesviruses or Herpetoviridae, is an example of a class of viruses amenable to this approach. These viruses contain 100,000 to 200,000 base pairs of DNA as their genetic material. Importantly, several regions of the genome have been
identified that are nonessential for the replication of virus in vitro in cell culture. Modifications in these regions of the DNA may lower the pathogenicity of the virus, i.e., attenuate the virus. For example, inactivation of the thymidine kinase gene renders human herpes simplex virus non-pathogenic (28), and pseudorabies virus of swine non-pathogenic (29).

Removal of part of the repeat region renders human herpes simplex virus non-pathogenic (30,31). A repeat region has been identified in Marek's disease virus that is associated with viral oncogenicity (32). A region in herpesvirus saimiri has similarly been correlated with oncogenicity (33). Removal of part of the repeat region renders pseudorabies virus non-pathogenic (U.S. Patent No. 4,877,737, issued October 31, 1989). A region in pseudorabies virus has been shown to be deleted in naturally-occurring vaccine strains (11,3) and it has been shown that these deletions are at least partly responsible for the lack of pathogenicity of these strains.

It is generally agreed that herpesviruses contain non-essential regions of DNA in various parts of the genome, and that modifications of these regions can attenuate the virus, leading to a non-pathogenic strain from which a vaccine may be derived. The degree of attenuation of the virus is important to the utility of the virus as a vaccine. Deletions which cause too much attenuation of the virus will result in a vaccine that fails to elicit an adequate immune response. Although several examples of attenuating deletions are known, the appropriate combination of deletions is not readily apparent.

Infectious bovine rhinotracheitis (IBR) virus, an alphaherpesvirus with a class D genome, is an important pathogen of cattle. It has been associated with
respiratory, ocular, reproductive, central nervous system, enteric, neonatal, and dermal diseases (34). Cattle are the normal hosts of IBR virus, however it also infects goats, swine, water buffalo, wildebeest, mink, and ferrets. Experimental infections have been established in mule deer, goats, swine, ferrets, and rabbits (35).

Conventional modified live virus vaccines have been widely used to control diseases caused by IBR virus. However, these vaccine viruses may revert to virulence. More recently, killed virus IBR vaccines have been used, but their efficacy appears to be marginal.

IBR virus has been analyzed at the molecular level as reviewed in Ludwig (36). A restriction map of the genome is available in this reference, which will aid in the genetic engineering of IBR according to the methods provided by the present invention.

As reported in the current literature, IBR virus has been engineered to contain a thymidine kinase deletion (43,44) and a deletion in the gIII gene (45,46). However, no evidence has been presented for the deletions in the US2, repeat, gpG, or gpE regions. In the subject application, we demonstrate the usefulness of such deletions for both the attenuation of IBR virus and for the development of gene deleted marker vaccines.

As with other herpesviruses, IBR virus can become latent in healthy animals which makes them potential carriers of the virus. For this reason it is clearly advantageous to be able to distinguish animals vaccinated with non-virulent virus from animals infected with disease-causing wild type virus. The development of differential vaccines and companion diagnostic tests has proven valuable in the management of pseudorabies disease (47).
A similar differential marker vaccine would be of great value in the management of IBR disease. The construction of differential diagnostics has focused on the deletion of glycoproteins. Theoretically, the glycoprotein chosen to be the diagnostic marker should have the following characteristics: (1) the glycoprotein and its gene should be non-essential for the production of infectious virus in tissue culture; (2) the glycoprotein should elicit a major serological response in the animal; and (3) the glycoprotein should not be one that makes a significant contribution to the protective immunity. Four major IBR virus glycoproteins (gI, gII, gIII, and gIV) have been described in the literature (48). Three of these genes, gI, gIII, and gIV, have been sequenced and shown to be homologous to the HSV glycoproteins gB, gC, and gD, respectively. Although it has been suggested that the gII protein is analogous to HSV gE, no sequence evidence has been presented to confirm that suggestion (48). The gB and gD homologues are essential genes and would not be appropriate as deletion marker genes. The gC gene of herpesviruses has been shown to make a significant contribution to protective immunity as a target of neutralizing antibody (49) and as a target of cell-mediated immunity (50). Therefore, the gC gene is not desirable as a deletion marker gene. As indicated above, Kim et al. (45) have described the deletion of the IBR virus gIII as a marker gene. It would be expected that such a deletion would compromise the efficacy of an IBR vaccine.

For pseudorabies virus (PRV) the criteria for a deletion marker gene are best met by the glycoprotein X (51). Wirth et al. (52) suggest the existence of a "gX homologue of HSV-1" in the IBR virus. It is not clear what is meant by this because although there is a PRV gX gene, there is no reported HSV-1 gX gene or gX homologous gene. In any case, no sequence evidence is presented to
support this suggestion. We present clear evidence of homologues of PRV gX (HSV-2 gG) and PRV gI (HSV gE) in IBR virus and demonstrate their usefulness as diagnostic markers.

The present invention provides a method of producing a fetal-safe, live recombinant IBR virus which comprises treating viral DNA from a naturally-occurring live IBR virus so as to delete from the virus DNA corresponding to the US2 region of the naturally-occurring IBR virus. The present invention also provides viruses in which (1) DNA corresponding to the US2 region of naturally-occurring IBR virus has been deleted, and (2) DNA encoding gpG and/or gpE has been altered or deleted. Such viruses are useful in vaccines which need diagnostic markers and are safe for use in pregnant animals.

The ability to engineer DNA viruses with large genomes, such as vaccinia virus and the herpesviruses, has led to the finding that these recombinant viruses can be used as vectors to deliver immunogens to animals (53). The herpesviruses are attractive candidates for development as vectors because their host range is primarily limited to a single target species (54), and they have the capacity for establishing a latent infection (55) that could provide for stable in vivo expression of a desired cloned polypeptide. Herpesviruses have been engineered to express a variety of foreign gene products, such as bovine growth hormone (56), human tissue plasminogen activator (57), and E. coli β-galactosidase (58,59). In addition, possible immunogenic polypeptides have been expressed by herpesviruses. Whealy et al. (60) expressed portions of the human immunodeficiency virus type 1 envelope glycoprotein in pseudorabies virus (PRV) as fusions to the PRV glycoprotein III. The hepatitis B virus surface antigen (61) and a hybrid human malaria antigen from Plasmodium falciparum have been expressed in
herpes simplex virus type 1 (HSV-1) (62). The IBR viruses described above may be used as vectors for the insertion of genes encoding antigens from microorganisms causing important cattle diseases. Such recombinant viruses would be multivalent vaccines protecting against IBR as well as other diseases. Kit et al. (63) have described the expression of a Foot and Mouth disease antigen in IBR virus. In some of the prior applications from which the subject application claims priority (which precedes the Kit publication by at least three years), we described the use of IBR virus to express several foreign genes including the E. coli β-galactosidase (lacZ) gene, the Tn5 neomycin resistance gene, and antigens from bovine rota virus, and parainfluenza type 3 virus (see U.S. Serial No. 06/933,107, filed November 20, 1986 and U.S. Serial No. 07/078,519, filed July 27, 1987). These applications precede the Kit publication by at least three years. The viruses described in this application provide a combination of attenuation, differentiation and multivalency. These properties make such viruses useful as vaccines for the management of cattle diseases.
Summary of the Invention

The present invention provides recombinant infectious bovine rhinotracheitis (IBR) viruses useful in vaccines to protect bovines from infectious bovine rhinotracheitis and other bovine diseases. The present invention further provides methods for distinguishing an animal vaccinated with the vaccine of the present invention from an animal infected with a naturally-occurring IBR virus. The present invention also provides isolated DNA encoding the gpE glycoprotein of IBR virus and isolated DNA encoding the gpG glycoprotein of IBR virus. The present invention also provides a method of producing a fetal-safe, live recombinant IBR virus which comprises treating viral DNA from a naturally-occurring live IBR virus so as to delete from the virus DNA corresponding to the US2 region of the naturally-occurring IBR virus.

The present invention also provides isolated DNA encoding the US2 gene of an IBR virus. The present invention further provides a homology vector for producing a recombinant IBR virus by inserting foreign DNA into the genomic DNA of an IBR virus which comprises a double-stranded DNA molecule consisting essentially of double-stranded foreign DNA encoding RNA which does not naturally occur in an animal into which the recombinant IBR is introduced and at one end of the foreign DNA, double-stranded IBR viral DNA homologous to genomic DNA located at one side of a site on the genomic DNA which is not essential for replication of the IBR virus and at the other end of the foreign DNA, double-stranded IBR viral DNA homologous to genomic DNA located at the other side of the same site on the genomic DNA.

The present invention also provides for a homology vector for producing a recombinant IBR virus by deleting DNA which encodes a detectable marker which had been inserted
into the genomic DNA of an IBR virus comprising a double-stranded DNA molecule consisting essentially of double-stranded IBR viral DNA homologous to the genomic DNA which flanks on each side the DNA to be deleted. The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which DNA from the US2 gene, the gpE glycoprotein gene and the gpG glycoprotein gene has been deleted so that upon replication, the recombinant IBR virus produces no gpE glycoprotein and no gpG glycoprotein. The invention also provides a vaccine which comprises an effective immunizing amount of a recombinant virus protective against bovine respiratory disease complex and a suitable carrier.
Brief Description of the Figures

Figure 1 Details of the IBR Cooper Strain. Diagram of IBR genomic DNA showing the unique long, internal repeat, unique short, and Terminal repeat regions. Restriction maps for the enzymes HindIII, EcoRI, and XbaI are indicated (7). Fragments are lettered in order of decreasing size. The unique short region is also expanded for inclusion of more detail. The location of several genes is also indicated, they are unique short 2 (US2), immediate early genes (IE) (20), glycoprotein G (gpG), glycoprotein IV (gpIV) (17), glycoprotein E (gpE). Note that due to the inversion of the short region, which includes the unique short, internal, and terminal repeats, four half molar HindIII fragments are present (HindIII D, C, F, and H).

Figure 2 Details of S-IBR-002. Diagram of S-IBR-002 genomic DNA showing the unique long, internal repeat, unique short, and Terminal repeat regions. Restriction maps for the enzymes HindIII, EcoRI, and XbaI are indicated (7). Fragments are lettered in order of decreasing size. The EcoRI B and F fragments are expanded for inclusion of more detail. The ~800 BP repeat deletions are indicated by deltas. Note that due to the inversion of the short region, which includes the unique short, internal, and terminal repeats, four half molar HindIII fragments are present (HindIII D, C, F, and H).

Figure 3 DNA sequence of the IBR Unique Short 2 gene. The sequence of the first 1080 base pairs of the HindIII K fragment, reading from the
HindIII K/HindIII O junction, are shown. The unique short 2 (US2) gene is transcribed toward the HindIII K/HindIII O junction as indicated in Figure 1. The sequence has been reversed and complemented in order to show the translation start and termination of US2 gene.

Figure 4 Homology between the IBR US2 protein and the US2 proteins of HSV-1, PRV, HSV-2, and MDV. (a) Matrix plot of the amino acid sequence of the IBR US2 protein (309) against the amino acid sequence of the HSV-1 US2 protein (291) (8). (b) Alignment of the conserved region between IBR US2 protein, HSV-1 US2 protein, PRV US2 protein (256 amino acids) (21), HSV-2 US2 protein (291) (9), and MDV US2 protein (270 amino acids) (1).

Figure 5 Details of the Nasalgen deletion. Diagram of IBR genomic DNA showing the unique long, internal repeat, unique short, and terminal repeat regions. A restriction map for the enzyme HindIII is indicated. Fragments are lettered in order of decreasing size. The unique short region is expanded for inclusion of more detail. The location of the deletion in the Nasalgen HindIII K fragment is shown. Three regions of DNA sequence are also shown. The first line shows the first 60 base pairs upstream of the HindIII O/HindIII D junction in the IBR Cooper strain. The second line shows the first 60 base pairs upstream of the HindIII K/HindIII D junction in the Nasalgen strain. The third line shows 60 base pairs flanking the DNA encoding amino acid 59 of the IBR US2 gene in the IBR Cooper strain.
Figure 6 Details of S-IBR-027. Diagram of S-IBR-027 genomic DNA showing the unique long, internal repeat, unique short, and terminal repeat regions. Restriction maps for the enzymes HindIII, EcoRI, and XbaI are indicated (7). Fragments are lettered in order of decreasing size. The unique short region is also expanded for inclusion of more detail. The location of several genes is also indicated, they are unique short 2 (US2), immediate early genes (IE) (20), glycoprotein G (gpG), glycoprotein IV (gpIV) (17), glycoprotein E (gpE). The unique short region and repeat region deletions are indicated by deltas. The location of the approximately 1200 BP deletion of the US2 gene is shown in the expanded region. Note that due to the inversion of the short region, which includes the unique short, internal, and terminal repeats, four half molar HindIII fragments are present (HindIII D, C, P, and H).

Figure 7 Detailed description of the DNA insertion in Homology Vector 129-71.5. Diagram showing the orientation of DNA fragments assembled in plasmid 129-71.5. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments is also shown. The restriction sites used to generate each fragment as well as synthetic linker sequences which were used to join the fragments are described for each junction. The synthetic linker sequences are underlined by a heavy bar. The location of several gene coding regions and regulatory elements is also given. The following two conventions are used: numbers in parentheses, (), refer to amino acids, and restriction sites
in brackets, [ ], indicate the remnants of sites which were destroyed during construction. The following abbreviations are used: polyadenylation signal (pA), infectious bovine rhinotracheitis virus (IBR), Herpes simplex virus type 1 (HSV-1), thymidine kinase (TK), neomycin resistance (NEO), bacterial transposon Tn5 (Tn5).

Figure 8 DNA sequence of the IBR glycoprotein G gene. The sequence of approximately 1400 base pairs of the HindIII K fragment, starting approximately 2800 base pairs downstream of the HindIII K/HindIII O junction, are shown. The glycoprotein G (gpG) gene is transcribed away from the HindIII K/HindIII O junction as indicated in Figure 1. The translational start and termination of the gpG gene are indicated.

Figure 9 Homology between the IBR gpG protein, the gpX protein of PRV and the gpG protein of HSV-2. (a) Matrix plot of the amino acid sequence of the IBR gpG protein (441) against the amino acid sequence of the PRV gpX protein (498) (12). (b) Alignment of the conserved region between IBR gpG protein, PRV gpX protein, and HSV-2 gpG protein (699) (9). Note that IUPAC-IUB Biochemical Nomenclature Commission conventions are used.

Figure 10 Western blot of proteins released into the medium of IBR and PRV infected cells, showing the absence of gpG in S-PRV-013, S-IBR-035, S-IBR-036, S-IBR-037, and S-IBR-038 but its presence in S-PRV-160 and wild type S-IBR-000. Lanes (A) 0.5 µg purified gpG, (B) blank lane, (C) S-PRV-160, (D) S-PRV-013, (E) pre-stained
molecular weight markers, (F) 0.5 µg purified gpG, (G) S-IBR-038, (H) S-IBR-037, (I) S-IBR-036, (J) S-IBR-035, (K) S-IBR-000, (L) uninfected MDBK cells, (M) pre-stained molecular weight markers. Media samples were prepared as described in the PREPARATION OF HERPESVIRUS CELL LYSATES. The concentrated media from the infection of one 6 cm dish of infected cells was loaded in each sample lane except for samples S-PRV-013 and S-PRV-160 for which the media from two 6 cm dishes were loaded.

Figure 11 Detailed description of the DNA insertion in Plasmid 459-12.6. Diagram showing the orientation of DNA fragments assembled in plasmid 459-12.6. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments is also shown. The restriction sites used to generate each fragment as well as synthetic linker sequences which were used to join the fragments are described for each junction. The synthetic linker sequences are underlined by a heavy bar. The location of several gene coding regions and regulatory elements is also given. The following two conventions are used: numbers in parentheses, ( ), refer to amino acids, and restriction sites in brackets, [], indicate the remnants of sites which were destroyed during construction. The following abbreviations are used: unique glycoprotein G (gpG), glycoprotein III (gpIII), glycoprotein X (gpX), polyadenylation signal (pA), infectious bovine rhinotracheitis virus (IBR), pseudorabies virus (PRV), and human cytomegalovirus (HCMV).
Figure 12 Detailed description of the DNA insertion in Homology Vector 439-01.31. Diagram showing the orientation of DNA fragments assembled in plasmid 439-01.31. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments is also shown. The restriction sites used to generate each fragment as well as synthetic linker sequences which were used to join the fragments are described for each junction. The synthetic linker sequences are underlined by a heavy bar. The location of several gene coding regions and regulatory elements is also given. The following two conventions are used: numbers in parentheses, (), refer to amino acids, and restriction sites in brackets, [], indicate the remnants of sites which were destroyed during construction. The following abbreviations are used: unique short 2 (US2), glycoprotein G (gpG), glycoprotein IV (gpIV), polyadenylation signal (pA), infectious bovine rhinotracheitis virus (IBR), pseudorabies virus (PRV), and human cytomegalovirus (HCMV).

Figure 13 Detailed description of the DNA insertion in Homology Vector 439-21.69. Diagram showing the orientation of DNA fragments assembled in plasmid 439-21.69. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments is also shown. The restriction sites used to generate each fragment as well as synthetic linker sequences which were used to join the fragments are described for each junction. The synthetic linker sequences are underlined by a heavy bar. The location of
several gene coding regions and regulatory elements is also given. The following two conventions are used: numbers in parentheses, (), refer to amino acids, and restriction sites in brackets, [], indicate the remnants of sites which were destroyed during construction. The following abbreviations are used: unique short 2 (US2), glycoprotein G (gpG), glycoprotein IV (gpIV), polyadenylation signal (pA), infectious bovine rhinotracheitis virus (IBR), pseudorabies virus (PRV), and human cytomegalovirus (HCMV).

Figure 14 Detailed description of the DNA insertion in Homology Vector 439-70.4. Diagram showing the orientation of DNA fragments assembled in plasmid 439-70.4. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments is also shown. The restriction sites used to generate each fragment as well as synthetic linker sequences which were used to join the fragments are described for each junction. The synthetic linker sequences are underlined by a heavy bar. The location of several gene coding regions and regulatory elements is also given. The following two conventions are used: numbers in parentheses, (), refer to amino acids, and restriction sites in brackets, [], indicate the remnants of sites which were destroyed during construction. The following abbreviations are used: glycoprotein G (gpG), glycoprotein IV (gpIV), and infectious bovine rhinotracheitis virus (IBR).

Figure 15 DNA sequence of the IBR glycoprotein E gene. The sequence of 2038 base pairs of the IBR
unique short region, starting approximately
1325 base pairs upstream in the HindIII
K/HindIII F junction in the HindIII K fragment,
are shown. The glycoprotein E (gpE) gene is
transcribed toward the HindIII K/HindIII F
junction as indicated in Figure 1. The
translation start and termination of the gpE
gene are indicated. Note that IUPAC-IUB
Biochemical Nomenclature Commission conventions
are used.

Figure 16 Homology between the IBR gpE protein and the
gpE protein of HSV-1, the gpI protein of VZV,
and the gI protein of PRV. (a) Matrix plot of
the amino acid sequence of the IBR gpE protein
(617) against the amino acid sequence of the
PRV gI protein (577) (64). (b) Alignment of
the conserved region between IBR gpE protein,
PRV gI protein, and VZV gpI protein (37).

Figure 17 Detailed description of a plasmid containing
the gpE gene. Diagram showing the orientation
of DNA fragments to be assembled in the gpE-
containing plasmid. The origin of each
fragment is indicated in the table. The
sequences located at each of the junctions
between fragments are also shown. The
restriction sites used to generate each
fragment are described for each junction. The
location of several gene coding regions and
regulatory elements is also given. The
following two conventions are used: numbers in
parentheses, ( ), refer to amino acids, and
restriction sites in brackets, [], indicate the
remnants of sites which were destroyed during
construction. The following abbreviations are
used: unique glycoprotein E (gpE),
glycoprotein IV (gpIV), and infectious bovine rhinotracheitis virus (IBR).

Figure 18 Detailed description of the DNA insertion in the homology vector 536-03.5. Diagram showing the orientation of DNA fragments to be assembled in the homology vector. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments is also shown. The restriction sites used to generate each fragment as well as synthetic linker sequences which were used to join the fragments are described for each junction. The synthetic linker sequences are underlined by a heavy bar. The location of several gene coding regions and regulatory elements is also given. The following two conventions are used: numbers in parentheses, (), refer to amino acids, and restriction sites in brackets, [], indicate the remnants of sites which were destroyed during construction. The following abbreviations are used: glycoprotein E (gpE), immediate early promoter (IE), infectious bovine rhinotracheitis virus (IBR), and pseudorabies virus (PRV).

Figure 19 Construction of Recombinant S-IBR-004 Virus. S-IBR-004 is an IBR recombinant virus carrying an inserted foreign gene (NEO) under the control of the PRV gpX promoter. A new XbaI site was created at the short unique region and the original SacI site was deleted.

Figure 20 Construction of Recombinant S-IBR-008 Virus. S-IBR-008 is a recombinant IBR virus that has a bovine rotavirus glycoprotein gene and the
plasmid vector inserted in the XbaI site on the unique long region. A site specific deletion was created at the [SacI] site due to the loss of NEO gene in the small unique region.

Figure 21 Sequence of the PI-3 (SF-4 Strain) HN Gene. Note that IUPAC-IUB Biochemical Nomenclature Commission conventions are used.

Figure 22 Details of S-IBR-018 Construction.

A. First line shows the IBR (Cooper Strain) BamHI-C fragment map. Second line shows the construction of the alpha-4 promoter on the PI-3 HN gene and its insertion into the HindIII site in BamHI-C. Also shown are the beta-gal and neomycin (NEO) gene constructions under the control of the gX promoter that were put into the XbaI site and used as selectable markers to purify the recombinant virus.

B. The BamHI-C fragment map of S-IBR-018 after insertion of the PI-3 HN, beta-gal, and neomycin genes.

C. The S-IBR-018 genome showing the location of the three inserted foreign genes.

Legend: B = BamHI; H = HindIII; X = XbaI; S = StuI; UL = unique long region; US = unique short region; IR = internal repeat region; TR = terminal repeat region.

Figure 23 Details of S-IBR-019 Construction.

A. First line shows the IBR (Cooper Strain) BamHI-C fragment map. Second line shows the
construction of the alpha-4 promoter on the PI-3 F gene and its insertion into the HindIII site in BamHI-C. Also shown are the beta-gal and neomycin (NEO) gene constructions under the control of the gX promoter that were put into the XbaI site and used as selectable markers to purify the recombinant virus.

B. The BamHI-C fragment map of S-IBR-019 after insertion of the PI-3 F, beta gal, and neomycin genes.

C. The S-IBR-019 genome showing the location of the three inserted foreign genes.

Figure 24 Detailed description of the DNA insertion in Homology Vector 591-21.20. The diagram shows the orientation of DNA fragments assembled in plasmid 591-21.20. The origin of each fragment is described in the MATERIALS AND METHODS section. The sequences located at the junctions between each fragment are shown. The restriction sites used to generate each fragment as well as synthetic linker sequences which were used to join the fragments are described for each junction. The synthetic linker sequences are underlined by a double bar. The location of the Tk gene coding region is also given. The following two conventions are used: numbers in parenthesis () refer to amino acids, and restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction. The following abbreviation is used, infectious bovine rhinotracheitis virus (IBR).
Figure 25 Detailed description of the marker gene insertion in Homology Vector 591-46.12. The diagram shows the orientation of DNA fragments assembled in the marker gene. The origin of each fragment is described in the MATERIALS AND METHODS section. The sequences located at the junctions between each fragment and at the ends of the marker gene are shown. The restriction sites used to generate each fragment are indicated at the appropriate junction. The location of the uidA gene coding region is also given. The following two conventions are used: numbers in parenthesis () refer to amino acids, and restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction. The following abbreviations are used, pseudorabies virus (PRV), uronidase A gene (uidA), Escherichia coli (E. coli), herpes simplex virus type 1 (HSV-1), poly adenylation signal (pA), and glycoprotein X (gpX).

Legend:  B = BamHI;  H = HindIII;  X = XbaI;  S = StuI;  UL = unique long region;  US = unique short region;  IR = internal repeat region;  TR = terminal repeat region.
Detailed Description of the Invention

The present invention provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein. The DNA encoding gpG glycoprotein may be deleted or foreign DNA may be inserted into the DNA encoding gpG glycoprotein. The DNA encoding gpG glycoprotein may be deleted and foreign DNA may be inserted in place of the deleted DNA encoding gpG glycoprotein.

The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted and DNA encoding the gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein and no gpE glycoprotein. The DNA encoding gpE glycoprotein may be deleted or foreign DNA may be inserted into the DNA encoding gpE glycoprotein. The DNA encoding gpE glycoprotein may be deleted and foreign DNA may be inserted in place of the deleted DNA encoding gpE glycoprotein.

The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein, DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and DNA encoding the gpE glycoprotein has been altered or deleted.

The present invention also provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR
virus in which (1) DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and (2) DNA encoding gpG glycoprotein has been altered or deleted. The DNA encoding the gpG glycoprotein may be deleted or foreign DNA may be inserted in place of the deleted DNA encoding gpG glycoprotein. Foreign DNA may be inserted in place of the deleted DNA corresponding to the US2 region of the naturally-occurring IBR virus.

The present invention also provides S-IBR-037, a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which (1) DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and (2) DNA encoding gpG glycoprotein has been deleted. S-IBR-037 was deposited on April 16, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2320.

The present invention also provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which (1) DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted and a foreign DNA sequence which encodes Escherichia coli β-galactosidase has been inserted in place of the deleted DNA encoding gpG glycoprotein, and (2) DNA encoding gpG glycoprotein has been altered or deleted. The present invention also provides two examples of such viruses, S-IBR-035 and S-IBR-036.

The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpE glycoprotein has been altered or deleted so that upon replication the
recombinant IBR virus produces no gpE glycoprotein. The DNA encoding gpE glycoprotein may be deleted or foreign DNA may be inserted in the DNA encoding gpE glycoprotein. The DNA encoding gpE glycoprotein may be deleted and foreign DNA may be inserted in place of the deleted DNA encoding gpE glycoprotein.

The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpE glycoprotein and DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted.

The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which DNA in the unique short region of the naturally-occurring IBR virus has been deleted. Foreign DNA may be inserted into the DNA of the recombinant IBR virus. The foreign DNA may be inserted into the XbaI site in the long unique region. The foreign DNA may be a sequence which encodes bovine rotavirus glycoprotein 38; this sequence may be inserted into the XbaI site in the long unique region.

The present invention provides S-IBR-008, a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted and in which a foreign DNA sequence which encodes bovine rotavirus glycoprotein 38 has been inserted into the XbaI site in the long unique region. S-IBR-008 was deposited on June 18, 1986 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn
The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which (1) DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted and (2) at least a portion of both repeat sequences has been deleted. The present invention further provides an example of such a recombinant virus, designated S-IBR-027. S-IBR-027 was deposited on April 17, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2322.

The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which at least a portion of both repeat sequences has been deleted.

The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which (1) at least a portion of both repeat sequences has been deleted and (2) DNA encoding one or more EcoRV restriction sites has been deleted. The present invention further provides an example of such a recombinant virus, designated S-IBR-002. S-IBR-002 was deposited on June 18, 1986 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2140.
The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which (1) at least a portion of both repeat sequences has been deleted and (2) wherein foreign DNA has been inserted into the DNA of the recombinant IBR virus. The foreign DNA may be a sequence which encodes the Tn5 NEO gene.

The present invention further provides S-IBR-020, a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which (1) at least a portion of both repeat sequences has been deleted and (2) wherein a foreign DNA sequence which encodes the Tn5 NEO gene has been inserted into the DNA of the recombinant IBR virus.

The present invention also provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which (1) at least a portion of both repeat sequences has been deleted, (2) wherein a foreign DNA sequence which encodes the Tn5 NEO gene has been inserted into the DNA of the recombinant IBR virus, and (3) wherein at least a portion of the thymidine kinase gene has been deleted.

The present invention also provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which (1) at least a portion of both repeat sequences has been deleted, (2) wherein a foreign DNA sequence which encodes the Tn5 NEO gene has been inserted into the DNA of the recombinant IBR virus, and (3) wherein at least a portion of the thymidine kinase gene has been deleted. The subject invention provides an example of such a recombinant virus, designated S-IBR-028. S-IBR-028 was deposited on May 14, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with
The present invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which a foreign DNA sequence which encodes the Tn5 NEO gene has been inserted into the viral DNA. The Tn5 NEO gene may be under the control of an inserted, upstream, pseudorabies virus glycoprotein X promoter. The subject invention further provides an example of a recombinant virus wherein the Tn5 NEO gene is under the control of an inserted, upstream, pseudorabies virus glycoprotein X promoter, designated S-IBR-004. S-IBR-004 was deposited on May 23, 1986 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2134.

The subject invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which a foreign DNA sequence which encodes the Escherichia coli β-galactosidase and Tn5 NEO genes, and the parainfluenza type 3 virus hemagglutinin gene, HN, has been inserted into the viral DNA. The subject invention provides an example of such a recombinant virus, designated S-IBR-018.

The subject invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which a foreign DNA sequence which encodes the Escherichia coli β-galactosidase and Tn5 NEO genes, and the parainfluenza type 3 virus fusion gene, F, has been inserted into the viral DNA. The subject invention
provides an example of such a recombinant virus, designated S-IBR-019.

The recombinant viruses of the subject invention were derived from the Cooper Strain. However, other IBR viruses, such as the LA strain or the 3156 strain, may also be used.

The subject invention also provides a vaccine which comprises a suitable carrier and an effective immunizing amount of any of the recombinant viruses of the present invention. The vaccine may contain either inactivated or live recombinant virus.

Suitable carriers for the recombinant virus are well known in the art and include proteins, sugars, etc. One example of such a suitable carrier is a physiologically balanced culture medium containing one or more stabilizing agents such as hydrolyzed proteins, lactose, etc. Preferably, the live vaccine is created by taking tissue culture fluids and adding stabilizing agents such as stabilized, hydrolyzed proteins. Preferably, the inactivated vaccine uses tissue culture fluids directly after inactivation of the virus.

The subject invention also provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein.

The subject invention provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG
glycoprotein has been altered or deleted and DNA encoding the gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein and no gpE glycoprotein.

The subject invention also provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein, DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and DNA encoding the gpE glycoprotein has been altered or deleted.

The subject invention further provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which (1) DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and (2) DNA encoding gpG glycoprotein has been altered or deleted.

The subject invention also provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpE glycoprotein.

The subject invention provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpE glycoprotein has been altered or deleted so that upon
replication the recombinant IBR virus produces no gpE
glycoprotein and DNA corresponding to the US2 region of
the naturally-occurring IBR virus has been deleted.

5 The subject invention also provides a vaccine which
comprises a suitable carrier and an effective immunizing
amount of a recombinant IBR virus comprising viral DNA
from a naturally-occurring IBR virus from which DNA
corresponding to the US2 region of the naturally-
occuring IBR virus has been deleted.

10 The subject invention provides a vaccine which comprises
a suitable carrier and an effective immunizing amount of
a recombinant IBR virus comprising viral DNA from a
naturally-occurring IBR virus from which at least a
portion of both repeat sequences has been deleted.

15 The subject invention further provides a vaccine which
comprises a suitable carrier and an effective immunizing
amount of a recombinant IBR virus comprising viral DNA
from a naturally-occurring IBR virus in which a foreign
DNA sequence which encodes the Tn5 NEO gene has been
inserted into the viral DNA.

20 The subject invention also provides a vaccine which
comprises a suitable carrier and an effective immunizing
amount of a recombinant IBR virus comprising viral DNA
from a naturally-occurring IBR virus in which a foreign
DNA sequence which encodes the Escherichia coli B-
galactosidase and Tn5 NEO genes, and the parainfluenza
type 3 virus hemagglutinin gene, HN, has been inserted
into the viral DNA.

25 The subject invention also provides a vaccine which
comprises a suitable carrier and an effective immunizing
amount of a recombinant IBR virus comprising viral DNA
from a naturally-occurring IBR virus in which a foreign

30 The subject invention also provides a vaccine which
comprises a suitable carrier and an effective immunizing
amount of a recombinant IBR virus comprising viral DNA
from a naturally-occurring IBR virus in which a foreign

35
DNA sequence which encodes the *Escherichia coli* B-galactosidase and Tn5 NEO genes, and the parainfluenza type 3 virus fusion gene, F, has been inserted into the viral DNA.

All of the vaccines described hereinabove and hereinbelow may contain either inactivated or live recombinant virus. The vaccines may be administered by any of the methods well known to those skilled in the art, for example, by intramuscular, subcutaneous, intraperitoneal, or intravenous injection. Alternatively, the vaccine may be administered intranasally or orally.

The present invention also provides a method of immunizing an animal against infectious bovine rhinotracheitis virus which comprises administering to the animal an effective immunizing dose of any of the vaccines of the present invention. The animal may be a bovine.

The subject invention also provides a method for distinguishing an animal vaccinated with a vaccine which comprises an effective immunizing amount of a recombinant virus of the present invention from an animal infected with a naturally-occurring IBR virus which comprises analyzing a sample of a body fluid from the animal for the presence of gpG glycoprotein of IBR virus and at least one other antigen normally expressed in an animal infected by a naturally-occurring IBR virus, identifying antigens which are present in the body fluid, and determining whether gpG glycoprotein is present in the body fluid. The presence of antigens which are normally expressed in an animal by a naturally-occurring IBR virus and the absence of gpG glycoprotein in the body fluid is indicative of an animal vaccinated with the vaccine and not infected with a naturally-occurring IBR virus. The presence of antigens and gpG glycoprotein in the body
fluid may be determined by detecting in the body fluid antibodies specific for the antigens and gpG glycoprotein.

One of the vaccines that is useful in this method is a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein. Another vaccine that is useful in this method is a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted and DNA encoding the gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein and no gpE glycoprotein. Yet another vaccine that is useful in this method is a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein, DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and DNA encoding the gpE glycoprotein has been altered or deleted. Still another vaccine that is useful in this method is a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which (1) DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and (2) DNA encoding gpG glycoprotein has been altered or deleted.
The present invention also provides a method for distinguishing an animal vaccinated with a vaccine which comprises an effective immunizing amount of a recombinant virus of the present invention from an animal infected with a naturally-occurring IBR virus which comprises analyzing a sample of a body fluid from the animal for the presence of gpE glycoprotein of IBR virus and at least one other antigen normally expressed in an animal infected by a naturally-occurring IBR virus, identifying antigens which are present in the body fluid and determining whether gpE glycoprotein is present in the body fluid. The presence of antigens which are normally expressed in an animal by a naturally-occurring IBR virus and the absence of gpE glycoprotein in the body fluid is indicative of an animal vaccinated with the vaccine and not infected with a naturally-occurring IBR virus. The presence of antigens and gpE glycoprotein in the body fluid may be determined by detecting in the body fluid antibodies specific for the antigens and gpE glycoprotein.

One of the vaccines useful in this method is a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted and DNA encoding the gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein and no gpE glycoprotein. Another vaccine that is useful in this method is a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein, DNA corresponding to the US2 region of the naturally-
occurring IBR virus has been deleted, and DNA encoding the gpE glycoprotein has been altered or deleted. Yet another vaccine that is useful in this method is a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpE glycoprotein. Still another vaccine that is useful in this method is a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpE glycoprotein and DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted.

The present invention also provides isolated DNA encoding the gpG glycoprotein of IBR virus. The subject invention also provides purified recombinant gpG glycoprotein encoded by the DNA encoding the gpG glycoprotein of IBR virus. The subject invention further provides a recombinant cloning vector which comprises the DNA encoding the gpG glycoprotein of IBR virus. The subject invention also provides a recombinant expression vector which comprises the DNA encoding the gpG glycoprotein of IBR virus. The subject invention provides a host cell which comprises the recombinant expression vector which comprises the DNA encoding the gpG glycoprotein of IBR virus.

The subject invention also provides a method of producing a polypeptide which comprises growing the host cell which comprises the recombinant expression vector which comprises the DNA encoding the gpG glycoprotein of IBR
virus under conditions such that the recombinant expression vector expresses gpG glycoprotein and recovering the gpG glycoprotein so expressed.

The subject invention also provides an antibody directed to an epitope of the purified gpG glycoprotein of IBR virus encoded by the DNA encoding the gpG glycoprotein of IBR virus. The antibody may be a monoclonal antibody.

The subject invention also provides a method of detecting the presence or absence of gpG glycoprotein of IBR virus in a sample which comprises contacting the sample with an antibody directed to an epitope of the purified gpG glycoprotein of IBR virus encoded by the DNA encoding the gpG glycoprotein of IBR virus under conditions such that the antibody forms a complex with any gpG glycoprotein present in the sample and detecting the presence or absence of such complex. The sample may be bovine-derived.

The subject invention also provides isolated DNA encoding the gpE glycoprotein of IBR virus. The subject invention also provides purified recombinant gpE glycoprotein encoded by the DNA encoding the gpE glycoprotein of IBR virus. The subject invention further provides a recombinant cloning vector which comprises the DNA encoding the gpE glycoprotein of IBR virus. The subject invention provides a recombinant expression vector which comprises the DNA encoding the gpE glycoprotein of IBR virus. The subject invention also provides a host cell which comprises the recombinant expression vector which comprises the DNA encoding the gpE glycoprotein of IBR virus.

The subject invention also provides a method of producing a polypeptide which comprises growing the host cell which comprises the recombinant expression vector which
comprises the DNA encoding the gpE glycoprotein of IBR virus under conditions such that the recombinant expression vector expresses gpE glycoprotein and recovering the gpE glycoprotein so expressed.

The subject invention also provides an antibody directed to an epitope of the purified gpE glycoprotein of IBR virus encoded by the DNA encoding the gpE glycoprotein of IBR virus. The antibody may be a monoclonal antibody.

The subject invention also provides a method of detecting the presence or absence of gpE glycoprotein of IBR virus in a sample which comprises contacting the sample with an antibody directed to an epitope of the purified gpE glycoprotein of IBR virus encoded by the DNA encoding the gpE glycoprotein of IBR virus under conditions such that the antibody forms a complex with any gpE glycoprotein present in the sample and detecting the presence or absence of such complex. The sample may be bovine-derived.

The subject invention also provides a method of producing a fetal-safe, live recombinant IBR virus which comprises treating viral DNA from a naturally-occurring live IBR virus so as to delete from the virus DNA corresponding to the US2 region of the naturally-occurring IBR virus.

The subject invention also provides a recombinant pseudorabies virus designated S-PRV-160. The subject invention also provides an antibody which directed to an epitope of the recombinant pseudorabies virus designated S-PRV-160.

The subject invention also provides isolated DNA encoding the US2 gene of an IBR virus. The present invention further provides a homology vector for producing a recombinant IBR virus by inserting foreign DNA into the
genomic DNA of an IBR virus which comprises a double-stranded DNA molecule consisting essentially of double-stranded foreign DNA encoding RNA which does not naturally occur in an animal into which the recombinant IBR is introduced, with at one end of the foreign DNA, double-stranded IBR viral DNA homologous to genomic DNA located at one side of a site on the genomic DNA which is not essential for replication of the IBR virus and at the other end of the foreign DNA, double-stranded IBR viral DNA homologous to genomic DNA located at the other side of the same site on the genomic DNA. The double-stranded foreign DNA may further comprise a promoter. The promoter can be from HSV-1 α 4 immediate early gene, Human cytomegalovirus immediate early gene or pseudorabies virus glycoprotein X gene. The double-stranded foreign DNA may further comprise a polyadenylation signal. The polyadenylation signal may be from HSV-1 thymidine kinase gene or pseudorabies virus glycoprotein X gene. The subject invention also provides a homology vector wherein the RNA encodes a polypeptide. The polypeptide may be a detectable marker such as *Escherichia coli* β-galactosidase or bacterial transposon neomycin resistance protein. The DNA which encodes the polypeptide may be flanked on each side by restriction sites permitting said DNA to be cut out with a restriction endonuclease which cuts at a limited number of sites on the genome. The subject invention further provides for a homology vector wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 860 bp NcoI to BamHI subfragment of the *HindIII* A fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 1741 bp *BglII* to *SstI* subfragment of the *HindIII* A fragment of IBR virus.
The subject invention further provides a homology vector wherein upstream double-stranded foreign DNA which comprises a promoter and downstream double-stranded foreign DNA which comprises a polyadenylation signal flank on each side double-stranded foreign DNA which encodes a detectable marker. The invention further a homology vector wherein the upstream promoter is homologous to genomic DNA present within the approximately 490 bp PvuII to BamHI subfragment of the BamHI N fragment of HSV-1 and the downstream polyadenylation signal is homologous to genomic DNA present within the approximately 784 bp SmaI to SmaI subfragment of the BamHI Q fragment of HSV-1. The invention further provides a homology vector wherein the DNA which encodes a detectable marker is homologous to the approximately 1541 bp BglII to BamHI fragment of Tn5.

The subject invention also provides a homology vector wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 3593 bp HindIII to XhoI subfragment of the HindIII K fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 785 bp XhoI to NdeI subfragment of the HindIII K fragment of IBR virus. The invention further provides a homology vector wherein upstream double-stranded foreign DNA which comprises a promoter and downstream double-stranded foreign DNA which comprises a polyadenylation signal flank on each side double-stranded foreign DNA which encodes a detectable marker. This upstream promoter is homologous to genomic DNA present within the approximately 1191 bp AvaII to PstI subfragment of the XbaI B fragment of HCMV and the downstream polyadenylation sequence is homologous to genomic DNA present within the approximately 753 bp SalI to NdeI subfragment of the BamHI #7 fragment of PRV. The DNA which encodes a detectable marker is homologous to
the approximately 3347 bp BalI to BamHI fragment of pJF751.

The invention further provides a homology vector wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 888 bp MluI to SmaI subfragment of the HindIII K fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 785 bp XhoI to NdeI subfragment of the HindIII K fragment of IBR virus. The upstream double-stranded foreign DNA may comprise a promoter and double-stranded foreign DNA which comprise a polyadenylation signal flank on each side double-stranded foreign DNA which encodes a detectable marker. The subject invention also provides a homology vector wherein the upstream promoter is homologous to genomic DNA present within the approximately 1191 bp AvaII to PstI subfragment of the XbaI B fragment of HCMV and the downstream polyadenylation signal is homologous to genomic DNA present within the approximately 753 bp SalI to NdeI subfragment of the BamHI #7 fragment of PRV. The DNA which encodes a detectable marker is homologous to the approximately 3347 bp BalI to BamHI fragment of pJF571.

The present invention further provides a homology vector wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 1704 bp SmaI to SmaI subfragment of the HindIII K fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 742 bp NheI to BglII subfragment of the SmaI 2.5KB fragment of IBR virus. The present invention further provides a homology vector wherein upstream double-stranded foreign DNA which comprises a promoter and downstream double-stranded foreign DNA which comprises a polyadenylation signal...
flank on each side double-stranded foreign DNA which encodes a detectable marker. The upstream promoter is homologous to genomic DNA present within the approximately 413 bp SalI to BamHI subfragment of the BamHI #10 fragment of PRV and the downstream polyadenylation signal is homologous to genomic DNA present within the approximately 754 bp NdeI to SalI subfragment of the BamHI #7 fragment of PRV. The detectable marker is homologous to the approximately 3010 bp BamHI to PvuII fragment of pJF751.

The present invention provides for a homology vector for producing a recombinant IBR virus by deleting DNA which encodes a detectable marker which had been inserted into the genomic DNA of an IBR virus comprising a double-stranded DNA molecule consisting essentially of double-stranded IBR viral DNA homologous to the genomic DNA which flank on each side the DNA to be deleted. The subject invention further provides a homology vector wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 888 bp MluI to SmaI subfragment of the HindIII K fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 785 bp XhoI to NdeI subfragment of the HindIII K fragment of IBR virus.

The present invention also provides a method of immunizing an animal against infectious bovine rhinotracheitis virus which comprises administering to the animal an effective immunizing dose of any of the vaccines of the present invention. The animal may be a bovine. The subject invention also provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which at least a portion of both repeat sequences have been deleted, specifically, wherein DNA encoding one or more EcoRV
restriction sites has been deleted, and wherein foreign DNA has been inserted into the DNA of the recombinant virus. The foreign DNA may be a DNA sequence which encodes bovine viral diarrhea virus glycoprotein gp53. The subject invention provides an example of such a recombinant IBR virus, designated S-IBR-032.

The subject invention provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which DNA from the US2 gene, the gpE glycoprotein gene and the gpG glycoprotein gene have been deleted so that upon replication, the recombinant IBR virus produces no gpE glycoprotein and no gpG glycoprotein. A foreign DNA sequence may be inserted in place of the deleted DNA which encodes gpE glycoprotein. The foreign DNA sequence that may be inserted can be a foreign DNA sequence which encodes *Escherichia coli* β-galactosidase. The subject invention provides an example of such a recombinant virus, designated S-IBR-039.

The subject invention further provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA from the US2, gpE glycoprotein gene, the gpG glycoprotein gene and the thymidine kinase gene has been deleted so that upon replication, the recombinant IBR virus produces no gpE glycoprotein, no gpG glycoprotein and no thymidine kinase. The subject invention provides an example of such a recombinant virus, designated S-IBR-045. A foreign DNA sequence may be inserted in place of the deleted DNA encoding gpE glycoprotein. The foreign DNA sequence may encode *Escherichia coli* β-galactosidase. The subject invention provides an example of such a recombinant virus, designated S-IBR-044. The foreign DNA sequence may encode bovine viral diarrhea virus gp53 glycoprotein. The subject invention provides an example of such a recombinant virus, designated S-IBR-046. The foreign DNA
sequence may encode Parainfluenza virus type 3 fusion protein and Parainfluenza virus type 3 hemagglutinin protein. The subject application provides an example of such a virus, designated S-IBR-047. The foreign DNA sequence may encode Bovine respiratory syncytial virus fusion protein, Bovine respiratory syncytial virus attachment protein and Bovine respiratory syncytial virus nucleocapsid protein. The subject invention provides an example of such a recombinant virus, designated S-IBR-049. The foreign DNA sequence may encode Pasteurella haemolytica leukotoxin and Pasteurella haemolytica iron regulated outer membrane proteins. The subject invention provides an example of such a recombinant virus, designated S-IBR-051.

The subject invention also provides a recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which DNA from the US2 gene, the gpE glycoprotein gene, the gpG glycoprotein gene and the thymidine kinase gene have been deleted so that upon replication, the recombinant IBR virus produces no gpE glycoprotein, no gpG glycoprotein and no thymidine kinase. The subject invention provides for a foreign DNA sequence inserted in place of the DNA which encodes thymidine kinase. The foreign DNA sequence may encode Escherichia coli β-glucuronidase. The present invention further provides a recombinant virus wherein a foreign DNA sequence is inserted in place of the DNA encoding gpE glycoprotein. The foreign DNA sequence may encode Escherichia coli β-galactosidase. The present invention further provides an example of such a recombinant virus, designated S-IBR-043.

The subject invention also provides a vaccine which comprises an effective immunizing amount of any of the recombinant viruses of the present invention and a
suitable carrier. The vaccine may contain either inactivated or live recombinant virus.

The present invention provides a vaccine which comprises an effective immunizing amount of recombinant virus protective against bovine respiratory disease complex and a suitable carrier. A recombinant virus may be a recombinant IBR virus and the recombinant virus can consist essentially of any or all of the recombinant viruses of the present invention.

The subject invention also provides for a vaccine which comprises an effective immunizing amount of a recombinant virus and non-recombinant virus protective against bovine respiratory disease complex and a suitable carrier.

The subject invention further provides a vaccine which comprises an effective immunizing amount of a recombinant IBR virus and non-recombinant virus protective against bovine respiratory disease complex and a suitable carrier. The recombinant IBR virus can consist essentially of any or all of the recombinant viruses of the subject invention.

For purposes of this invention, the infectious diseases that contribute to bovine respiratory disease complex include infectious bovine rhinotracheitis, parainfluenza type 3 virus, bovine viral diarrhea virus, bovine respiratory syncytial virus and Pasteurella haemolytica.

For purposes of the present invention, non-recombinant viruses can include, but are not limited to, conventionally derived viruses which include killed virus, inactivated bacterins, and modified live viruses.

The subject invention further provides for a method of immunizing an animal against infectious bovine
rhinotracheitis which comprises administering to the animal an immunizing dose of any of the vaccines of the present invention. The subject invention further provides a method of immunizing an animal against Parainfluenza type 3 which comprises administering to the animal an immunizing dose of the vaccine of the present invention that contains the IBR virus encoding antigens for Parainfluenza type 3 virus. The subject invention further provides a method of immunizing an animal against bovine viral diarrhea which comprises administering to the animal an immunizing dose of the vaccine of the present invention that contains the IBR virus encoding antigens for bovine viral diarrhea virus. The subject invention further provides a method of immunizing an animal against bovine respiratory syncytial virus disease which comprises administering to the animal an immunizing dose of the vaccine of the present invention that contains the IBR virus encoding antigens for bovine respiratory syncytial virus. The subject invention further provides for a method of immunizing an animal against Pneumonic pasteurellosis which comprises administering to the animal an immunizing dose of the vaccine of the present invention that contains the IBR virus encoding antigens for Pasteurella haemolytica.

The invention further provides a method of immunizing an animal against bovine respiratory disease complex which comprises administering to an animal an immunizing dose of the vaccine containing the recombinant IBR viruses of the present invention or the recombinant viruses of the present invention and non-recombinant viruses. For purposes of this invention, the animal may be a bovine. The invention further provides a method for distinguishing an animal vaccinated with a vaccine which comprises an effective immunizing amount of a recombinant virus of the present invention from an animal infected with a naturally-occurring IBR virus which comprises
analyzing a sample of a body fluid from the animal for the presence of gpE glycoprotein of IBR virus and at least one other antigen normally expressed in an animal infected by a naturally-occurring IBR virus, identifying antigens which are present in the body fluid and determining whether gpE glycoprotein is present in the body fluid, the presence of antigens which are normally expressed in an animal by a naturally-occurring IBR virus and the absence of gpE glycoprotein in the body fluid being indicative of an animal vaccinated with the vaccine and not infected with a naturally-occurring IBR virus.
Materials and Methods

PREPARATION OF IBR VIRUS STOCK SAMPLES. IBR virus stock samples were prepared by infecting MDBK cells at a multiplicity of infection of 0.01 PFU/cell in Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin (these components were obtained from Irvine Scientific or an equivalent supplier, and hereafter are referred to as complete DME medium) plus 1% fetal bovine serum. After cytopathic effect was complete, the medium and cells were harvested and the cells were pelleted at 3000 rpm for 5 minutes in a clinical centrifuge. Cells were resuspended in 1/10 the original volume of medium, and an equal volume of skim milk (9% skim milk powder in H₂O weight/volume) was added. The virus sample was frozen at -70°C. The titers were usually about 10^8 PFU/ml.

PREPARATION OF HERPESVIRUS DNA. For herpesvirus DNA preparation, a confluent monolayer of cells (MDBK for IBR virus or Vero for PRV) in a 25 cm² flask or 60 mm petri dish was infected with 100 µl of virus sample. After overnight incubation, or when the cells were showing 100% cytopathic effect, the cells were scraped into the medium. The cells and medium were centrifuged at 3000 rpm for 5 minutes in a clinical centrifuge. The medium was decanted, and the cell pellet was gently resuspended in 0.5 ml of solution containing 0.5% NONIDET P-40™ (NP-40, purchased from Sigma Chemical Co., St. Louis, MO). The sample was incubated at room temperature for 10 minutes. Ten µl of a stock solution of RNase A (Sigma) was added (stock was 10 mg/ml, boiled for 10 minutes to inactivate DNase). The sample was centrifuged to pellet nuclei. The DNA pellet was removed with a pasteur pipette or wooden stick and discarded. The supernatant fluid was decanted into a 1.5 ml Eppendorf tube containing 25 µl of 20% sodium dodecyl sulfate (Sigma)
and 25 μl proteinase-K (10 mg/ml; Boehringer Mannheim). The sample was mixed and incubated at 37°C for 30-60 minutes. An equal volume of water-saturated phenol was added and the sample was mixed briefly. The sample was centrifuged in an Eppendorf minifuge for 5 minutes at full speed. The upper aqueous phase was removed to a new Eppendorf tube, and two volumes of absolute ethanol were added and the tube put at -20°C for 30 minutes to precipitate nucleic acid. The sample was centrifuged in an Eppendorf minifuge for 5 minutes. The supernatant was decanted, and the pellet was washed with ~300 μl of 80% ethanol, followed by centrifugation in an Eppendorf minifuge for 5 minutes. The supernatant was decanted, and the pellet was air dried and rehydrated in ~16 μl H₂O. For the preparation of larger amounts of DNA, the procedure was scaled up to start with a 850 cm² roller bottle of MDBK cells. The DNA was stored in 0.01 M tris pH 7.5, 1 mM EDTA at 4°C.

PREPARATION OF HERPESVIRUS CELL LYSATES. For cell lysate preparation, serum free medium was used. A confluent monolayer of cells (MDBK for IBR virus or Vero for PRV) in a 25 cm² flask or a 60 mm petri dish was infected with 100 μl of virus sample. After cytopathic effect was complete, the medium and cells were harvested and the cells were pelleted at 3000 rpm for 5 minutes in a clinical centrifuge. For media samples medium was concentrated approximately 10-fold by filtration with a centricon-10 microconcentrator (Amicon). For cell samples the cell pellet was resuspended in 250 μl of disruption buffer (2% sodium dodecyl sulfate, 2% β-mercaptoethanol). The samples were sonicated for 30 seconds on ice and stored at -20°C.

WESTERN BLOTTING PROCEDURE. Samples of lysates, controls and protein standards were run on a polyacrylamide gel according to the procedure of Laemmli (2). After gel
electrophoresis the proteins were transferred according to Sambrook (14). The primary antibody was a mouse hyper-immune serum raised against chemically-synthesized gpG peptides (amino acids 232-252 and 267-287) linked to keyhole limpet hemocyanin. The secondary antibody was a goat anti-mouse alkaline phosphatase coupled antibody.

MOLECULAR BIOLOGICAL TECHNIQUES. Techniques for the manipulation of bacteria and DNA, including such procedures as digestion with restriction endonucleases, gel electrophoresis, extraction of DNA from gels, ligation, phosphorylation with kinase, treatment with phosphatase, growth of bacterial cultures, transformation of bacteria with DNA, and other molecular biological methods are described by Maniatis (6). Except as noted, these were used with minor variation.

LIGATION. DNA was joined together by the action of the enzyme T4 DNA ligase (BRL). Ligation reactions contained various amounts of DNA (from 0.2 to 20µg), 20mM Tris pH 7.5, 10mM MgCl₂, 10mM dithiothreitol (DTT), 200 µM ATP and 20 units T4 DNA ligase in 10-20 µl final reaction volume. The ligation proceeded for 3-16 hours at 15°C.

DNA SEQUENCING. Sequencing was performed using the BRL Sequenase Kit and ³⁵S-dATP (NEN). Reactions using both the dGTP mixes and the dITP mixes were performed to clarify areas of compression. Alternatively, compressed areas were resolved on formamide gels. Templates were double-stranded plasmid subclones or single stranded M13 subclones, and primers were either made to the vector just outside the insert to be sequenced, or to previously obtained sequence. Sequence obtained was assembled and compared using Dnastar software. Manipulation and comparison of sequences obtained was performed with Superclene and Supersee programs from Coral Software.
SOUTHERN BLOTTING OF DNA. The general procedure for Southern blotting was taken from Maniatis (6). DNA was blotted to nitrocellulose filters and hybridized to appropriate, labeled DNA probes. Probes for southern blots were prepared using either the Nonradioactive DNA Labeling and Detection Kit of Boehringer Mannheim or the nick translation kit of Bethesda Research Laboratories (BRL). In both cases the manufacturers' recommended procedures were followed.

DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS. The method is based upon the calcium phosphate procedure of Graham and Van der Eb (24) with the following modifications. Virus and/or plasmid DNA were diluted to 298 µl in 0.01 M Tris pH 7.5, 1mM EDTA. Forty µl 2M CaCl₂ was added followed by an equal volume of 2X HEPES buffered saline (10g N-2-hydroxyethyl piperazine N'-2-ethanesulfonic acid (HEPES), 16g NaCl, 0.74g KCl, 0.25g Na₂HPO₄·2H₂O, 2g dextrose per liter H₂O and buffered with NaOH to pH 7.4). The mixture was then incubated on ice for 10 minutes, and then added dropwise to an 80% confluent monolayer of MDBK or rabbit skin (RS) cells growing in a 60 mm petri dish under 5 ml of medium (DME plus 2% fetal bovine serum). The cells were incubated 4 hours at 37°C in a humidified incubator containing 5% CO₂. The cells were then washed with three 5 ml aliquots of 1XPBS (1.15g Na₂HPO₄, 0.2g KH₂PO₄, 0.8g NaCl, 0.2g KCl per liter H₂O), and fed with 5 ml of medium (DME plus 2% fetal bovine serum). The cells were incubated at 37°C as above for 3-7 days until cytopathic effect from the virus was 50-100%. Virus was harvested as described above for the preparation of virus stocks. This stock was referred to as a transfection stock and was subsequently screened for recombinant virus by the BLUOGAL™ SCREEN FOR RECOMBINANT IBR VIRUS.
HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. This method relies upon the homologous recombination between herpesvirus DNA and plasmid homology vector DNA which occurs in tissue culture cells co-transfected with these elements. From 0.1-1.0 μg of plasmid DNA containing foreign DNA flanked by appropriate herpesvirus cloned sequences (the homology vector) were mixed with approximately 0.3 μg of intact herpesvirus DNA. The DNAs were diluted to 298 μl in 0.01 M Tris pH 7.5, 1mM EDTA and transfected into MDBG cells according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS (see above).

DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. Rather than using homology vectors and relying upon homologous recombination to generate recombinant virus, we have also developed the technique of direct ligation to engineer herpesviruses. In this instance, a cloned foreign gene did not require flanking herpesvirus DNA sequences but only required that it have restriction sites available to cut out the foreign gene fragment from the plasmid vector. A compatible restriction enzyme was used to cut herpesvirus DNA. A requirement of the technique was that the restriction enzyme used to cut the herpesvirus DNA must cut at a limited number of sites. We have used XbaI, which cuts IBR virus DNA in one place. We have also used EcoRV which cuts IBR virus DNA in two places. For PRV we have used XbaI and HindIII, both of which cut in two places.

Restriction sites previously introduced into herpesviruses by other methods may also be used. The herpesvirus DNA was mixed with a 30-fold molar excess of plasmid DNA (typically 5μg of virus DNA to 10μg of plasmid DNA), and the mixture was cut with the appropriate restriction enzyme. The DNA mixture was phenol extracted and ethanol precipitated to remove restriction enzymes, and ligated together according to
the ligation procedure detailed above. The ligated DNA mixture was then resuspended in 298 μL 0.01 M Tris pH 7.5, 1mM EDTA and transfected into cells (MDBK or RS for IBR virus and Vero for PRV) according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS (see above). The direct ligation procedure may also be used to delete DNA from herpesviruses. Non-essential DNA which is flanked by appropriate restriction enzyme sites may be deleted by digesting the virus DNA with such enzymes and religation. The frequency of engineered viruses generated by the direct ligation procedure is high enough that screening can be accomplished by restriction enzyme analysis of randomly picked plaques from the transfection stock.

BLUOGAL® SCREEN FOR RECOMBINANT HERPESVIRUS. When the E.coli β-galactosidase (lacZ) marker gene was incorporated into a recombinant virus the plaques containing recombinants were visualized by a simple assay. The chemical BLUOGAL® (GIBCO-Bethesda Research Labs) was incorporated (200 μg/ml) into the agarose overlay during the plaque assay, and plaques that expressed active β-galactosidase turned blue. The blue plaques were then picked onto fresh cells (MDBK for IBR virus and Vero for PRV) and purified by further blue plaque isolations. In recombinant virus strategies in which the E.coli β-galactosidase marker gene is removed, the assay involves plaque purifying white plaques from a background of parental blue plaques. In both cases viruses were typically purified with three rounds of plaque purification.

SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES. When the E. coli β-galactosidase (lacZ) or β-glucuronidase (uidA) marker gene was incorporated into a recombinant virus the plaques containing recombinants were visualized by a simple assay. The enzymatic
substrate was incorporated (300 μg/ml) into the agarose overlay during the plaque assay. For the lacZ marker gene the substrate BLUGAL™ (halogenated indolyl-B-D-galactosidase, Bethesda Research Labs) was used. For the uidA marker gene the substrate X-Glucuro Chx (5-bromo-4-chloro-3-indolyl-B-D-glucuronic acid Cyclohexylammonium salt, Biosynth AG) was used. Plaques that expressed active marker enzyme turned blue. The blue plaques were then picked onto fresh cells and purified by further blue plaque isolation. In recombinant virus strategies in which the enzymatic marker gene is removed the assay involves plaque purifying white plaques from a background of parental blue plaques. In both cases viruses were typically purified with three rounds of plaque purification.

ANTIBODY SCREEN FOR RECOMBINANT HERPESVIRUS. A third method for screening the recombinant virus stock was to look directly for the expression of the foreign gene with antibodies. Herpesvirus plaques were spotted and picked by inserting a toothpick through the agarose above the plaque and scraping the plaque area on the dish. Viruses were then rinsed from the toothpick by inserting the toothpick into a well of a 96-well micro-titer dish (Falcon Plastics) containing a confluent monolayer of tissue culture cells that had been washed 3 times in DME medium without serum. It was important for the virus to grow without serum at this stage to allow the immunological procedure to work. After cytopathic effect was complete, the plates were put at -70°C to freeze and lyse the cells. The medium was thawed, and the freeze/thaw procedure was repeated a second time. Then 50-100 microliters of medium were removed from each well and filtered under vacuum through a nitrocellulose membrane (S&S BA85) using a DotBlot- apparatus (BRL). The filter blots were soaked in a blocking solution of 0.01 M Tris pH 7.5, 0.1 M NaCl, 3% bovine serum albumin
at room temperature for two hours with shaking. The filter blots were then placed in a sealable bag (Sears SEAL-A-MEAL™ or equivalent), and 10 mls of the blocking solution that contained 10 microliters of antibody specific for the foreign protein were added. After overnight incubation at room temperature with shaking, the blot was washed 3 times with 100 mls 0.01 M Tris, pH 7.5, 0.1 M NaCl, 0.05% Tween 20 detergent (Sigma). The blot was put in another sealable bag and 10 mls blocking solution containing $10^6$ counts per minute of $^{125}$I-protein A (New England Nuclear) were added. After allowing the protein A to bind to the antibody for 2 hours at room temperature with shaking, the blot was washed as above, dried, and overlayed with an X-ray film and an intensifying screen (Dupont) and autoradiographed for 1-3 days at $-70^\circ$C. The film was developed by standard procedures. Virus from the positive wells which contained the recombinant virus was further purified.

SELECTION OF G418 RESISTANT IBR VIRUS. The antibiotic G418 (GIBCO) has a wide range of inhibitory activity on protein synthesis. However, recombinant viruses expressing the aminoglycosidase 3′-phosphotransferase, encoded by the NEO gene of the transposable element Tn5, are resistant to G418. The transfection stocks of recombinant viruses were grown on MDBK cells in the presence of 500 µg/ml G418 in complete DME medium plus 1% fetal bovine serum. After one or two days at 37°C, plaques from the dishes inoculated with the highest dilution of virus were picked for virus stocks. The selection was repeated a second or third time. The virus stocks generated from the G418 selection were tested for NEO gene insertion by the SOUTHERN BLOTTING OF DNA hybridization procedure described above.

CONSTRUCTION OF DELETION VIRUSES. The strategy used to construct deletion viruses involved the use of either
homologous recombination and/or direct ligation techniques. Initially a virus was constructed via homologous recombination, in which the DNA to be deleted was replaced with a marker gene such as *E. coli* β-galactosidase (lacZ) or β-glucuronidase (uidA). A second virus was then constructed in which the marker gene was deleted either by homologous recombination or via direct ligation. The advantage of this strategy is that both viruses may be purified by the SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES. The first virus is purified by picking blue plaques from a white plaque background, the second virus is purified by picking white plaques from a blue plaque background.

Several homology vectors were constructed for the purpose of deleting the gpG, gpE and Tk gene coding regions. A detailed description of these homology vectors follows.

**HOMOLOGY VECTOR 129-71.5.** The plasmid 129-71.5 was constructed for the purpose of deleting a portion of the TK gene coding region from the IBR virus. It incorporates a selectable marker, the bacterial transposon neomycin resistance gene, flanked by IBR virus DNA. Upstream of the marker gene is an approximately 860 base pair fragment of IBR virus DNA which ends with sequences encoding amino acids 1-62 of the TK primary translation product. Downstream of the marker gene is an approximately 1741 base pair fragment of IBR virus DNA which begins with sequences encoding amino acids 156-367 of the TK primary translation product. When this plasmid is used according to the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS, it will replace the DNA coding for amino acids 63-155 of the TK primary translation product with DNA coding for the marker gene. Note that the marker gene will be under the control of the herpes simplex type 1 alpha-4 immediate early gene promoter (5). A detailed description of the
plasmid is given in Figure 7. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (6). It may be constructed by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in Figure 7. The plasmid vector is derived from an approximately 2975 base pair Smal to HindIII restriction fragment of pSP65 (Promega). Fragment 1 is an approximately 860 base pair NcoI to BamHI restriction fragment of the IBR virus HindIII restriction fragment A (7). This fragment is located on an approximately 5500 base pair ClaI to NruI fragment contained in the IBR virus HindIII A fragment. Fragment 2 is an approximately 490 base pair PvuII to BamHI restriction sub-fragment of the HSV-1 BamHI restriction fragment N (5). Note that the HSV-1 oriS region has been removed from this fragment by deletion of the sequences between the Smal sites located 1483 and 128 base pairs away from the PvuII end (10). Fragment 3 is an approximately 1541 base pair BglII to BamHI restriction fragment of plasmid pNEO (P.L. Biochemicals, Inc.). Fragment 4 is an approximately 784 base pair Smal to SmaI restriction sub-fragment of the HSV-1 BamHI restriction fragment Q (10). Note that this fragment is oriented such that the polyadenylation sequence (AATAAA) is located closest to junction D. Fragment 5 is an approximately 1741 base pair BglII to StuI restriction sub-fragment from the IBR HindIII restriction fragment A (7).

PLASMID 459-12.6. The plasmid 459-12.6 was generated for the purpose of constructing a recombinant cloning vector which expresses the IBR virus glycoprotein G. This was accomplished by inserting the IBR virus gpG gene into S-PRV-013 (U.S. Serial No. 07/823,102 filed January 27, 1986). Plasmid 459-12.6 contains a chimeric gene under the control of the IBR virus gpG promoter. The chimeric gene expresses a fusion protein consisting of the first
362 amino acids of IBR virus gpG fused to amino acids 421-467 of the PRV gpIII (13) followed by amino acids 480-498 of the PRV gpX (12). The chimeric gene is flanked by HindIII restriction sites. When this plasmid is used with S-PRV-013 and the restriction enzyme HindIII according to the DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS the resulting recombinant will express the IBR virus gpG. A detailed description of the plasmid is given in Figure 11. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (6). It may be constructed by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in Figure 11. The plasmid vector is derived from an approximately 2999 base pair XbaI to XbaI restriction fragment of a hybrid cloning vector derived from pSP64 and pSP65 (Promega). The hybrid cloning vector was constructed by joining approximately 1369 base pair PvulI to SmaI fragment from pSP64 with the approximately 1652 base pair PvulI to SmaI fragment from pSP65. Fragment 1 is an approximately 182 base pair PstI to EcoRV restriction sub-fragment of the HCMV XbaI restriction fragment B (16). Fragment 2 is an approximately 2121 base pair MluI to XhoI restriction sub-fragment of the IBR virus HindIII restriction fragment K (7). Fragment 3 is an approximately 121 base pair XhoI to BamHI restriction sub-fragment of the PRV BamHI restriction fragment #2 (3). Fragment 4 is an approximately 760 base pair NdeI to SalI restriction sub-fragment of the PRV BamHI restriction fragment #7 (3).

HOMOLOGY VECTOR 439-01.31. The plasmid 439-01.31 was constructed for the purpose of deleting a portion of the gpG gene coding region from the IBR virus. It incorporates an E.coli β-galactosidase marker gene flanked by IBR virus DNA. Downstream of the marker gene is an approximately 3593 base pair fragment of IBR virus DNA which ends with sequences encoding the first 262
amino acids of the gpG primary translation product. Upstream of the marker gene is an approximately 785 base pair fragment of IBR virus DNA which begins with sequences encoding the last 80 amino acids of the gpG primary translation product. When this plasmid is used according to the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS it will replace the DNA coding for amino acids 263-361 of the gpG primary translation product with DNA coding for the marker gene. Note that the β-galactosidase (lacZ) marker gene will be under the control of the human cytomegalovirus immediate early gene promoter. A detailed description of the plasmid is given in Figure 12. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (6). It may be constructed by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in Figure 12. The plasmid vector is derived from an approximately 2965 base pair HindIII to SmaI restriction fragment of pSP64 (Promega). Fragment 1 is an approximately 3593 base pair HindIII to XhoI restriction fragment of the IBR HindIII restriction fragment K (7). Fragment 2 is an approximately 753 base pair SalI to NdeI restriction fragment of the PRV BamHI restriction fragment #7 (3). Note that this fragment was resected with Exonuclease III/S1 nuclease digestion such that approximately 57 base pairs were removed from the NdeI end. Fragment 3 is an approximately 3347 base pair BalI to BamHI restriction fragment of plasmid pJF751 (38). Fragment 4 is an approximately 1191 base pair AvaI to PstI restriction fragment from the HCMV XbaI restriction fragment E (16). Fragment 5 is an approximately 785 base pair XhoI to NdeI restriction fragment from the IBR HindIII restriction fragment K (7). Note that the lacZ marker gene is flanked by XbaI sites located at Junction B and Junction E in this plasmid permitting the marker gene to be cut out with XbaI.
HOMOLOGY VECTOR 439-21.69. The plasmid 439-21.69 was constructed for the purpose of deleting a portion of the gpG gene coding region from the IBR virus. It incorporates an *E. coli* β-galactosidase (*lacZ*) marker gene flanked by IBR virus DNA. Downstream of the marker gene is an approximately 888 base pair fragment of IBR virus DNA which begins approximately 1042 base pairs upstream of the initiation codon of the gpG gene and ends approximately 154 base pairs upstream of the initiation codon of the gpG gene. Upstream of the marker gene is an approximately 785 base pair fragment of IBR virus DNA which begins with sequences encoding the last 80 amino acids of the gpG primary translation product. When this plasmid is used according to the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS it will replace the DNA coding for amino acids 1-361 of the gpG primary translation product with DNA coding for the marker gene. Note that the β-galactosidase (*lacZ*) marker gene will be under the control of the human cytomegalovirus immediate early gene promoter. A detailed description of the plasmid is given in Figure 13. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (6). It may be constructed by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in Figure 13. The plasmid vector is derived from an approximately 2965 base pair *HindIII* to *SmaI* restriction fragment of pSP64 (Promega). Fragment 1 is an approximately 3593 base pair *HindIII* to *XhoI* restriction fragment of the IBR *HindIII* restriction fragment K (7). Fragment 2 is an approximately 753 base pair *SalI* to *NdeI* restriction fragment of the PRV *BamHI* restriction fragment #7 (3). Note that this fragment was resected with Exonuclease III/S1 nuclease digestion such that approximately 57 base pairs were removed from the *NdeI* end. Fragment 3 is an approximately 3347 base pair *BaiI* to *BamHI* restriction fragment of plasmid pJF751
-58-

(38). Fragment 4 is an approximately 1191 base pair AvaI to PstI restriction fragment from the HCMV XbaI restriction fragment E (16). Fragment 5 is an approximately 785 base pair XhoI to NdeI restriction fragment from the IBR HindIII restriction fragment K (7). Note that the lacZ marker gene is flanked by XbaI sites located at Junction B and Junction E in this plasmid permitting the marker gene to be cut out with XbaI.

HOMOLOGY VECTOR 439-70.4. The plasmid 439-70.4 was constructed for the purpose of deleting the E.coli β-galactosidase (lacZ) marker gene from S-IBR-035 virus. It incorporates two regions of IBR viral DNA which flank the marker gene in S-IBR-035. The first region is an approximately 888 base pair fragment of IBR virus DNA which begins approximately 1042 base pairs upstream of the initiation codon of the gpG gene and ends approximately 154 base pairs upstream of the initiation codon of the gpG gene. The second region is an approximately 785 base pair fragment of IBR virus DNA which begins with sequences encoding the last 80 amino acids of the gpG primary translation product. When this plasmid is used in conjunction with S-IBR-035 DNA according to the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS it will delete the DNA coding for the E.coli β-galactosidase (lacZ) marker gene. A detailed description of the plasmid is given in Figure 14. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (6). It may be constructed by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in Figure 14. The plasmid vector is derived from an approximately 2965 base pair HindIII to SmaI restriction fragment of pSP64 (Promega). Fragment 1 is an approximately 3593 base pair HindIII to XhoI restriction fragment of the IBR HindIII restriction fragment K (7). Fragment 2 is an approximately 785 base
pair XhoI to NdeI restriction fragment from the IBR HindIII restriction fragment K (7).

IBR VIRUS gpE PLASMID. A plasmid may be generated for the purpose of constructing a recombinant cloning vector which expresses the IBR virus glycoprotein E (gpE). This plasmid may be used to insert the IBR virus gpE gene into S-PRV-002 (U.S. Patent No. 4,877,737). The plasmid will contain the gpE gene flanked by XbaI restriction sites. When this plasmid is used with S-PRV-002 and the restriction enzyme XbaI according to the DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS the resulting recombinant will express the IBR virus gpE. A detailed description of the plasmid is given in Figure 17. It may be constructed, utilizing standard recombinant DNA techniques (6), by joining restriction fragments from the following sources. The plasmid vector is derived from an approximately 2999 base pair XbaI to XbaI restriction fragment of a hybrid cloning vector derived from pSP64 and pSP65 (Promega). The hybrid cloning vector was constructed by joining an approximately 1369 base pair PvuI to SmaI fragment from pSP64 with the approximately 1652 base pair PvuI to SmaI fragment from pSP65. Fragment 1 is an approximately 3647 base pair NdeI to HindIII restriction sub-fragment of the IBR virus HindIII restriction fragment K (7). Fragment 2 is an approximately 832 base pair HindIII to SacI restriction sub-fragment of an IBR virus 2400 base pair SmaI restriction fragment. This SmaI fragment has been cloned into the SmaI site of the plasmid pSP64 (Promega). This plasmid is designated PSY1645. PSY1645 was deposited on July 16, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 68650. Note that the lacZ marker gene
is flanked by XbaI sites located at Junction B and Junction E in this plasmid permitting the marker gene to be cut out with XbaI.

HOMOLOGY VECTOR 536-03.5. The plasmid 536-03.5 was constructed for the purpose of deleting a portion of the gpE gene coding region from the IBR virus. It incorporates an E.coli β-galactosidase (lacZ) marker gene flanked by IBR virus DNA. Upstream of the marker gene is an approximately 1704 base pair fragment of IBR virus DNA which ends with sequences encoding amino acids 1-76 of the gpE primary translation product. Downstream of the marker gene is an approximately 742 base pair fragment of IBR virus DNA which begins with sequences encoding amino acids 548-617 of the gpE primary translation product. When this plasmid is used according to the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS, it will replace the DNA coding for amino acids 77-547 of the gpE primary translation product with DNA coding for the marker gene. Note that the β-galactosidase (lacZ) marker gene will be under the control of the PRV gpX. A detailed description of the plasmid is given in Figure 18. It may be constructed utilizing standard recombinant DNA techniques (6), by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in Figure 18. The plasmid vector is derived from an approximately 2975 base pair Smal to HindIII restriction fragment of pSP65 (Promega). Fragment 1 is an approximately 1704 base pair Smal to Smal restriction sub-fragment of the IBR HindIII restriction fragment K (7). Fragment 2 is an approximately 413 base pair SalI to BamHI restriction fragment #7 (3). Fragment 5 is an approximately 742 base pair NheI to BglII sub-fragment of an IBR virus 2400 base pair Smal fragment. This Smal fragment has been cloned into the Smal site of the plasmid pSP64 (Promega). This plasmid is designated PSY1645. PSY1645 was deposited on
July 16, 1991 with the American Type Culture Collection. Note that the lacZ marker gene is flanked by XbaI sites located at Junction B and Junction E in this plasmid permitting the marker gene to be cut out with XbaI.

VACCINATION STUDIES IN CALVES WITH INACTIVATED IBR VIRUS. Calves, seronegative to IBR virus, were housed in facilities secure from IBR virus exposure. Groups of four calves were vaccinated intramuscularly with vaccines containing $10^{7.3}$ or $10^{8.0}$ plaque forming units of inactivated IBR virus formulated with an oil adjuvant. A second vaccination was given 21 days later; four calves were maintained as unvaccinated controls. At 21 days after the second vaccination, animals were challenged intranasally with virulent wild-type IBR virus. After vaccination and challenge, animals were observed and the injection site was palpated weekly. Blood samples were taken on days 0, 7, 21, 28, and 42 post vaccination. After challenge, animals were observed daily for clinical signs of IBR. Blood samples were taken on days 7 and 13 post challenge. Nasal swabs were collected on days 3, 6, 9, and 12 post challenge.

PURIFICATION OF IBR VIRUS gpG. gpG was purified from the tissue culture medium of infected MDBK cells. Confluent MDBK cells in serum-free medium were infected at a multiplicity of infection equal to 5, with wild-type, Cooper strain of IBR virus. The cells and media were harvested at approximately twenty-two hours after infection, when the cells showed considerable cytopathic effect and the fluids were centrifuged at 5000 rpm for 15 minutes.

The supernatant fluid was concentrated approximately 10-fold by ultrafiltration through an Amicon ym-30 membrane, and dialyzed against 10mM NaPO$_4$, pH 7.2. The dialysate was treated for 20 minutes at 0°C with 70% perchloric acid to
a final concentration of 0.2M perchloric acid, then centrifuged at 12,000 rpm for 20 minutes. The supernatant fluid was then dialyzed against 20mM Tris pH 9.5.

The acid-soluble proteins were separated by column chromatography on a DEAE-Sephael anion exchange column using a liner gradient elution: 0 to 100% A to B where A = 20mM Tris pH 9.5 and B = 20mM Tris pH 9.5/800 mM NaCl. The gpG eluted at approximately 35-40% B. Peak fractions were assayed by Western blot using anti gpG peptide sera. Reactive fractions were combined and dialyzed against 5 mM Tris pH 7.0. The sample was then concentrated 10-fold by lyophilization and stored at -20°C.

ELISA ASSAY. A standard enzyme-linked immunosorbent assay (ELISA) protocol was used to determine the immune status of cattle following vaccination and challenge.

A purified gpG antigen solution (100 µl at 1 ng/µl in PBS) was allowed to absorb to the wells of microtiter dishes for 18 hours at 4°C. The coated wells were rinsed one time with PBS. Wells were blocked by adding 250 µl of PBS containing 1% BSA (Sigma) and incubating 1 hour at 37°C. The blocked wells were rinsed one time with PBS containing 0.02% Tween 20. 50 µl of test serum (previously diluted 1:2 in PBS containing 1% BSA) were added to the wells and incubated 1 hour at 37°C. The antiserum was removed and the wells were washed 3 times with PBS containing 0.02% Tween 20. 50 µl of a solution containing anti-bovine IgG coupled to horseradish peroxidase (diluted 1:500 in PBS containing 1% BSA, Kirkegaard and Perry Laboratories, Inc.) was added to visualize the wells containing antibody against the specific antigen. The solution was incubated 1 hour at 37°C, then removed and the wells were washed 3 times with
PBS containing 0.02% Tween 20. 100 μl of substrate solution (ATBS, Kirkegaard and Perry Laboratories, Inc.) were added to each well and color was allowed to develop for 15 minutes. The reaction was terminated by addition of 0.1M oxalic acid. The color was read at absorbance 410nm on an automatic plate reader.

PROCEDURE FOR GENERATING MONOCLONAL ANTIBODIES. To produce monoclonal antibodies, 8 to 10 week old BALB/c female mice were vaccinated intraperitoneally seven times at two to four week intervals with 10⁷ PFU of S-PRV-160. Three weeks after the last vaccination, mice were injected intraperitoneally with 40 μg of purified gpG. Spleens were removed from the mice three days after the last antigen dose.

Splenocytes were fused with mouse NS1/Ag4 plasmacytoma cells by the procedure modified from Oi and Herzenberg, (39). Splenocytes and plasmacytoma cells were pelleted together by centrifugation at 300 x g for 10 minutes. One ml of a 50% solution of polyethylene glycol (m.w. 1300-1600) was added to the cell pellet with stirring over one minute. Dulbecco’s modified Eagles’s medium (5 ml) was added to the cells over three minutes. Cells were pelleted by centrifugation at 300 x g for 10 minutes and resuspended in medium with 10% fetal bovine serum and containing 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine (HAT). Cells (100 μl) were added to the wells of eight to ten 96-well tissue culture plates containing 100 μl of normal spleen feeder layer cells and incubated at 37°C. Cells were fed with fresh HAT medium every three to four days.

Hybridoma culture supernatants were tested by the ELISA ASSAY in 96-well microtiter plates coated with 100 ng of purified gpG. Supernatants from reactive hybridomas were further analyzed by black-plaque assay and by Western
Blot. Selected hybridomas were cloned twice by limiting dilution. Ascetic fluid was produced by intraperitoneal injection of 5 X 10^6 hybridoma cells into pristane-treated BALB/c mice.

METHOD FOR cDNA CLONING BOVINE ROTAVIRUS gp38 GENE. The Calf Nebraska strain of bovine rotavirus (USDA) was propagated on MA-104 cells (Rhesus monkey kidney cells from MA Bioproducts). Confluent monolayers were infected at a multiplicity of infection of greater than 10 in DMEM containing 5 micrograms/ml trypsin. Cells were incubated with virus for 48 hours or until a cytopathic effect was obtained. Media and cell debris were collected and centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatant containing the rotavirus was then centrifuged at 10,000 x g in a preparative Beckman Ti45 rotor at 4°C. Virus pellets were resuspended in SM medium (50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM MgCl_2) and homogenized lightly in a Dounce-type homogenizer. The resuspended virus was centrifuged at 10,000 x g for 10 minutes then loaded onto 25-50% CsCl gradients in SM buffer. Gradients were centrifuged at 100,000 x g for 4 hours at 20°C. The two blue-white bands representing intact virions and cores of rotavirus were collected, diluted, and the CsCl gradient procedure was repeated a second time. Virus obtained from the second gradient was dialyzed overnight against SM buffer at 4°C.

Dialyzed bovine rotavirus was twice extracted with an equal volume of SDS/phenol, then twice more with chloroform: isoamylalcohol (24:1). The double stranded RNA was precipitated with ethanol in the presence of 0.2 M sodium acetate, centrifuged and resuspended in water. The yield was typically 100 micrograms from 1,000 cm^2 of infected cells.
160 micrograms of double-stranded bovine rotavirus RNA obtained from the above procedure was mixed with one microgram each of two synthetic oligonucleotide primers in a volume of 160 microliter (sequences of primers were: 5’-GGGAATTCGTCAGTACATCATAATCTAATCTAAG-3’ and 5’-GGGAATTCGTCAGTACATCATACATAATCTAATCTAAG-3’), derived from the published sequence of bovine rotavirus (40). The RNA-primer mixture was boiled for 3 minutes in a water bath then chilled on ice. Additions of 25 microliters of 1 M Tris-HCl pH 8.3, 35 microliters of 1 M KCl, 10 microliters of 0.25 M MgCl₂, 7 microliters of 0.7 M 2-mercaptoethanol, 7 microliters of 20 mM dNTP’s, and 6 microliters of reverse transcriptase (100 units) were made sequentially. The reaction was incubated at 42°C for 1.5 hours then 10 microliters of 0.5 M EDTA pH 8.0 was added and the solution was extracted once with chloroform:phenol (1:1). The aqueous layer was removed and to it 250 microliters of 4 M ammonium acetate and 1.0 ml of 95% ethanol was added, the mixture was frozen in dry ice and centrifuged in the cold. The resulting pellet was resuspended in 100 microliters of 10 mM Tris-HCl pH 7.5 and the ammonium acetate precipitation procedure was repeated. The pellet was resuspended in 100 microliters of 0.3 M KOH and incubated at room temperature overnight, then at 37°C for 2 hours. The solution was brought to neutral pH by addition of 10 microliters of 3.0 M HCl and 25 microliters of 1.0 M Tris-HCl pH 7.5. The resulting single-stranded cDNA was then precipitated two times by the above-described ammonium acetate-ethanol procedure. The pellet obtained was resuspended in 50 microliters of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, boiled in a water bath for 2 minutes, then incubated at 59°C for 16 hours. The solution was lyophilized to a volume of 15 microliters and the resulting double-stranded cDNA was run on a 1.0% agarose gel (Sigma agarose Type II). The ethidium bromide-stained DNA migrating at 1,000-1,100 base pair
length was excised from the gel and electroeluted in a CBS electroleuter device. The solution was lyophilized, and the cDNA was resuspended in 25 microliters of water. To this solution was added 2 microliters of 1.0 M Tris-HCl pH 7.5, 2 microliters of 1 M KCl, 1 microliter of 0.25 M MgCl$_2$, 1 microliter of 20 mM dNTP's and 5 units of E. coli DNA polymerase I. The reaction was incubated at room temperature for 15 minutes, then chloroform/phenol extracted and ammonium acetate-ethanol precipitated as described above. The resulting cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase (BRL buffer and enzyme used). The reaction was stopped with 2 microliters of 0.5 M EDTA, chloroform/phenol extracted and precipitated with sodium acetate in the presence of 10 micrograms of carrier tRNA. The resuspended cDNA was mixed with 200 ng of dGMP-tailed Pst I cut pBR322 (BRL catalog #5355SA) in 200 microliters of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, heated to 65°C for 5 minutes, then 57°C for 2 hours. The annealed cDNA-vector pBR322 was transformed onto E. coli DH-1 cells prepared for high efficiency transformation. Colonies that showed sensitivity to ampicillin and tetracycline resistance were grown and DNA was prepared and cut with Pst I to determine the size of the cDNA insert. Several clones having Pst I inserts of 1,050-1,100 base pairs were analyzed and found to have identical restriction enzyme digest patterns. For one of these clones, the 1,100 base pair PstI insert was subcloned into a M13 phage sequencing vector. Part of the DNA sequence of this clone was determined and was found to be identical to the published sequence (40).

cDNA CLONING. cDNA cloning refers to the methods used to convert RNA molecules into DNA molecules following state of the art procedures. Applicants' methods are described in Gubler and Hoffman (23). Bethesda Research Laboratories (Gaithersburg, MD) have designed a cDNA
Cloning Kit that is very similar to the procedures used by applicants and contains the best set of reagents and protocols to duplicate our results.

For cloning virus mRNA species, a host cell line sensitive to infection by the virus was infected at 5-10 plaque forming units per cell. When cytopathic effect was evident, but before total destruction, the medium was removed and the cells were lysed in 10 ml lysis buffer (4 M guanidine thiocyanate, 0.1% antifoam A, 25 mM sodium citrate pH 7.0, 0.5% N-lauroyl sarcosine, 0.1 M beta-mercaptoethanol). The cell lysate was poured into a sterilized Dounce homogenizer and homogenized on ice 8-10 times until the solution was homogenous. For RNA purification, 8 ml of cell lysate were gently layered over 3.5 ml of CsCl solution (5.7 M CsCl, 25 mM sodium citrate pH 7.0) in a Beckman SW41 centrifuge tube. The samples were centrifuged for 18 hours at 20°C at 36,000 rpm in a Beckman SW41 rotor. The tubes were put on ice and the supernatants from the tubes were carefully removed by aspiration to leave the RNA pellet undisturbed. The pellet was resuspended in 400 microliters glass distilled water, and 2.6 ml of guanidine solution (7.5 M guanidine·HCl, 25 mM sodium citrate pH 7.0, 5 mM dithiothreitol) were added. Then 0.37 volumes of 1 M acetic acid were added, followed by 0.75 volumes of cold ethanol and the sample was put at -20°C for 18 hours to precipitate RNA. The precipitate was collected by centrifugation in a Sorvall centrifuge for 10 min at 4°C at 10,000 rpm in an SS34 rotor. The pellet was dissolved in 1.0 ml distilled water, recentrifuged at 13,000 rpm, and the supernatant saved. RNA was reextracted from the pellet 2 more times as above with 0.5 ml distilled water, and the supernatants were pooled. A 0.1 volume of 2 M potassium acetate solution was added to the sample followed by 2 volumes of cold ethanol and the sample was put at -20°C for 18 hours.
The precipitated RNA was collected by centrifugation in the SS34 rotor at 4°C for 10 minutes at 10,000 rpm. The pellet was dissolved in 1 ml distilled water and the concentration taken by absorption at A260/280. The RNA was stored at -70°C.

mRNA containing polyadenylate tails (poly-A) was selected using oligo-dT cellulose (Pharmacia #27 5543-0). Three milligrams of total RNA was boiled and chilled and applied to a 100 mg oligo-dT cellulose column in binding buffer (0.1 M Tris pH 7.5, 0.5 M LiCl, 5 mM EDTA pH 8.0, 0.1% lithium dodecyl sulfate). The retained poly-A⁺ RNA was eluted from the column with elution buffer (5 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 0.1% sodium dodecyl sulfate). This mRNA was reapplied to an oligo-dT column in binding buffer and eluted again in elution buffer. The sample was precipitated with 200 mM sodium acetate and 2 volumes cold ethanol at -20°C for 18 hours. The RNA was resuspended in 50 microliters distilled water.

Ten micrograms poly-A⁺ RNA was denatured in 20 mM methyl mercury hydroxide for 6 minutes at 22°C. Beta-mercaptoethanol was added to 75 mM and the sample was incubated for 5 min at 22°C. The reaction mixture for first strand cDNA synthesis in 0.25 ml contained 1 microgram oligo-dT primer (P-L Biochemicals) or 1 microgram synthetic primer, 28 units placental ribonuclease inhibitor (Bethesda Research Labs #5518SA), 100 mM Tris pH 8.3, 140 mM KCl, 10 mM MgCl₂, 0.8 mM dATP, dCTP, dGTP, and dTTP (Pharmacia), 100 microcuries ³²P-labelled dCTP (New England Nuclear #NEG-013H), and 180 units AMV reverse transcriptase (Molecular Genetics Resources #MG 101). The reaction was incubated at 42°C for 90 minutes, and then was terminated with 20 mM EDTA pH 8.0. The sample was extracted with an equal volume of phenol/chloroform (1:1) and precipitated with 2 M ammonium acetate and 2 volumes of cold ethanol -20°C for
3 hours. After precipitation and centrifugation, the pellet was dissolved in 100 microliters distilled water. The sample was loaded onto a 15 ml G-100 Sephadex column (Pharmacia) in buffer (100 mM Tris pH 7.5, 1 mM EDTA pH 8.90, 100 mM NaCl). The leading edge of the eluted DNA fractions were pooled, and DNA was concentrated by lyophilization until the volume was about 100 microliters, then the DNA was precipitated with ammonium acetate plus ethanol as above.

The entire first strand sample was used for second strand reaction which follow the Gubler and Hoffman (23) method except that 50 micrograms/ml dNTP’s, 5.4 units DNA polymerase I (Boehringer Mannheim #642-711), and 100 units/ml E. coli DNA ligase (New England Biolabs #205) in a total volume of 50 microliters were used. After second strand synthesis, the cDNA was phenol/chloroform extracted and precipitated. The DNA was resuspended in 10 microliters distilled water, treated with 1 microgram RNase A for 10 minutes at 22°C, and electrophoresed through a 1% agarose gel (Sigma Type II agarose) in 40 mM Tris-acetate buffer pH 6.85. The gel was stained with ethidium bromide, and DNA in the expected size range was excised from the gel and electroeluted in 8 mM Tris-acetate pH 6.85. Electroeluted DNA was lyophilized to about 100 microliters, and precipitated with ammonium acetate and ethanol as above. The DNA was resuspended in 20 microliters water.

Oligo-dC tails were added to the DNA to facilitate cloning. The reaction contained the DNA, 100 mM potassium cacodylate pH 7.2, 0.2 mM dithiothreitol, 2 mM CaCl₂, 80 micromoles dCTP, and 25 units terminal deoxynucleotidyl transferase (Molecular Genetic Resources #S1001) in 50 microliters. After 30 minutes at 37°C, the reaction was terminated with 10 mM EDTA, and the sample
was phenol/chloroform extracted and precipitated as above.

The dC-tailed DNA sample was annealed to 200 ng plasmid vector pBR322 that contained oligo-dG tails (Bethesda Research Labs #5355 SA/SB) in 200 microliters of 0.01 M Tris pH 7.5, 0.1 M NaCl, 1 mM EDTA pH 8.0 at 65°C for 2 minutes and then 57°C for 2 hours. Fresh competent E. coli DH-1 cells were prepared and transformed as described by Hanahan (41) using half the annealed cDNA sample in twenty 200 microliter aliquots of cells. Transformed cells were plated on L-broth agar plates plus 10 micrograms/ml tetracycline. Colonies were screened for the presence of inserts into the ampicillin gene using Ampscreen (Bethesda Research Labs #5537 UA), and the positive colonies were picked for analysis.

POLYMERASE FILL-IN REACTION. DNA was resuspended in buffer containing 50 mM Tris pH 7.4, 50 mM KCl, 5 mM MgCl₂, and 400 micromolar each of the four deoxynucleotides. Ten units of Klenow DNA polymerase (BRL) were added and the reaction was allowed to proceed for 15 minutes at room temperature. The DNA was then phenol extracted and ethanol precipitated as above.

HOMOLOGY VECTOR 523-78.72. The plasmid 523-78.72 was constructed for the purpose of deleting a portion of the gpE gene coding region from the IBR virus. It may also be used to insert foreign DNA into IBR. Plasmid 523-78.72 may be constructed by digestion of the plasmid 536-03.5 with the enzyme XbaI followed by religation to remove the lacZ marker gene.

HOMOLOGY VECTOR 591-21.20. The plasmid 591-21.20 was constructed for the purpose of deleting a portion of the IBR thymidine kinase gene. It may also be used to insert
foreign DNA into IBR. It contains a unique BglII restriction enzyme site into which foreign DNA may be inserted. It may be constructed utilizing standard recombinant DNA techniques (6, 14) by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in figure 24. The plasmid vector is derived from an approximately 2999 base pair SalI to SalI restriction fragment of pSP64 (Promega). Fragment 1 is an approximately 1400 base pair SalI to NalI restriction subfragment contained on the approximately 2700 base pair SalI-SalI restriction subfragment of the IBR HindIII restriction fragment A (72). Fragment 2 is an approximately 1215 base pair BglIII to SalI restriction subfragment contained on the approximately 2700 base pair SalI-SalI restriction subfragment of the IBR HindIII restriction fragment A (72).

HOMOLOGY VECTOR 552-46.12. The plasmid 591-46.12 was constructed for the purpose of deleting a portion of the Tk gene coding region from the IBR virus. It incorporates an E. coli β-glucuronidase (uidA) marker gene flanked by IBR virus DNA. The uidA marker gene was inserted into the homology vector 591-21.20 at the unique BglIII site. The marker gene is oriented in the same direction as the Tk gene in the homology vector. A detailed description of the marker gene is given in figure 25. It may be constructed utilizing standard recombinant DNA techniques (6, 14) by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in figure 25. Fragment 1 is an approximately 404 base pair SalI to EcoRI restriction subfragment of the PRV BamHI restriction fragment #10 (3). Note that the EcoRI site was introduced at the location indicated in figure 12 by PCR cloning. Fragment 2 is an approximately 1823 base pair EcoRI to SmaI fragment of the plasmid pRAJ260 (Clonetech). Note that the EcoRI and SmaI sites were introduced at the locations
indicated in figure 25 by PCR cloning. Fragment 3 is an approximately 784 base pair SmaI to SmaI restriction subfragment of the HSV-1 BamHI restriction fragment Q (10). Note that this fragment is oriented such that the polyadenylation sequence (AATAAA) is located closest to junction C.

CLONING OF BOVINE VIRAL DIARRHEA VIRUS gp53 GENE. The bovine viral diarrhea (BVDV) gp53 gene was cloned essentially as described earlier (see cDNA CLONING) using the random priming method (6). Viral RNA prepared from BVDV Singer strain grown in MADIN-DARBY bovine kidney (MDBK) cells was converted to cDNA using the random priming method. The cDNA was used for second strand reaction (23) and the resulting double stranded DNA was used cloned as described in the cDNA CLONING procedure. From this procedure a series of clones were obtained that comprised parts of the genome of BVDV. The location of the gene for gp53 gene has been published (66) and this sequence information was used to locate and isolate the gp53 encoding region from the 449 kilodalton primary translation product open reading frame contained in the complete cDNA clone.

The gp53 encoding gene of BVDV was also cloned essentially as described by Katz et al. for the HA gene of human influenza virus. Viral RNA prepared from the Singer strain of BVDV virus grown in MDBK cells was first converted to cDNA utilizing an oligo nucleotide primer specific for the target gene. The cDNA was then used as a template or polymerase chain reaction (PCR) cloning (67) of the gp53 gene. The PCR primers were designed to incorporate restriction endonuclease enzyme sites that permit the cloning of the amplified coding region into vectors that contain the appropriate signals for gene expression in IBR. The gp53 gene of the Singer strain of BVDV was cloned using the following oligo nucleotide
primers: 5’-CATAGATCTTTGTGTTGCTCTCGGACTTCGCA-3’ for cDNA priming and was combined with 5’-CGTGGATCCTCAATTACAGAGGTATCGTCTAC-3’ for PCR amplification. Note that this general strategy may be used to clone the gp53 coding region from BVDV strains other than Singer.

CLONING OF BOVINE RESPIRATORY SYNCYTIAL VIRUS FUSION PROTEIN AND NUCLEOCAPSID PROTEIN GENES. The bovine respiratory virus (BSRV) fusion (F), attachment (G), an nucleocapsid protein (N) genes may be cloned essentially as described by Katz et al. for the HA gene of human influenza. Viral RNA prepared from virus grown in bovine nasal turbinate (BT) cells is first converted to cDNA utilizing an oligo nucleotide primer specific for the target gene. The cDNA is then used as a template for polymerase chain reaction (PCR) cloning (67) of the targeted region. The PCR primers are designed to incorporate restriction sites which permit the cloning of the amplified coding regions into vectors containing the appropriate signals for expression in IBR. One pair of oligo nucleotides will be required for each coding region. The N gene coding region from the BRSV strain A51908 (or 391-2???) would be cloned utilizing the following primers: 5’-AAAAGATCTTGGCAAGGCTAAACTAAATGACACTTTCAAC-3’ for cDNA priming and combined with 5’-CGTGGATCCTCAATTCCACATCATATCTTTGGGATT-3’ for PCR. The G gene coding region from the BRSV strain A51908 (or 391-2???) would be cloned utilizing the following primers: 5’-TATAGATCTTTCATACCCATCATCTTTAATTCAAGACATTA-3’ for cDNA priming and combined with 5’-CGTGGATCCTAATTAGATTTATATGGGAGGTGTGTTG3’ for PCR. The F gene from strain A51908 (or 391-2???) of BRSV would be cloned utilizing the following primers: 5’-TATAGATCTAACGCGGATGATCATCAGCCATTATC-3’ for cDNA priming and combined with 5’-
CGTGGATCCTCTGAGGTAGATTGTAAACATTATGCA-3' for PCR. Note that this general strategy may be used to clone the coding region of F and N genes from other strains of BRSV.

CLONING OF PASTEURELLA HAEMOLYTICA LEUKOTOXIN AND IRON REGULATED OUTER MEMBRANE PROTEIN(S). The Pasteurella haemolytica strain A1 leukotoxin gene may be cloned from a genomic DNA sample. Genomic DNA is prepared from P. haemolytica A1 cells grown in culture (68) by the methods described in Maniatis et al. (1982). The purified P. haemolytica DNA is then used as a template for polymerase chain reaction (PCR) cloning (67) of the targeted leukotoxin gene. The PCR primers are designed so that restriction endonuclease sites are incorporated that allow the cloning of the 102 kilodalton toxin portion of the gene into vectors containing the appropriate signals for expression in IBR. The P. haemolytica A1 (ATCC 43279 biotype A, serotype 1) leukotoxin gene would be cloned utilizing the following primers: 5'-TATAGATCTTAGACTACCAACCTAAAC-3' and 5'-CGTGGATCCTCTTATATAATGTGTGAAACATATAG-3' for PCR. Note that this general strategy may be used to clone the coding regions for the leukotoxin gene of all P. haemolytica serotypes.

The P. haemolytica A1 iron regulated outer membrane proteins (IRP) of 3 major polypeptides with molecular weights of 35, 70 and 100 kilodaltons. The DNA coding for the array of P. haemolytica genes can be cloned in Escherichia coli using plasmid vectors essentially as described in Maniatis et al. (1982). The clone library is constructed by partial digestion of the genomic DNA. The IRP genes can be isolated from this library of P. haemolytica clones by screening for the production of iron regulated outer membrane antigens by a colony enzyme-linked immunosorbent assay blot method with
antiserum that is specific to the IRPs. This antiserum may be obtained by eluting antibodies derived from polyclonal antiserum raised against whole *P. haemolytica* or membrane enriched fractions but selectively bound to the IRPs on Western blots (69). The specificity of the antibodies can be verified by immunoblot screening of *P. haemolytica* polypeptides from iron restricted and iron induced cultures.
EXAMPLES

Example 1

S-IBR-002

S-IBR-002 is an IBR virus that has a deletion of approximately 800 bp in the repeat region of the genome. This deletion removes the only two EcoRV restriction sites on the virus genome and an adjacent BglII site (Figure 2).

To construct this virus, the DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS was performed. Purified IBR virus DNA (Cooper strain) digested with EcoRV restriction enzyme was mixed with DraI-restriction enzyme-digested plasmid DNA containing the E.coli β-galactosidase (lacZ) gene under the control of the HSV-1 TK promoter. After ligation the mixture was used to transfect animal cells and the transfection stock was screened for recombinant IBR virus by the SOUTHERN BLOTTING OF DNA procedure. The final result of the purification was the recombinant IBR virus designated S-IBR-002. It was shown by Southern hybridization that this virus does not carry any foreign genes. Restriction enzyme analysis also showed that the insertion sites (EcoRV) in both repeats were deleted. Figure 2 shows the restriction map of the EcoRI B fragment which contains the EcoRV restriction sites and the map of S-IBR-002 which lacks the EcoRV sites. S-IBR-002 was deposited on June 18, 1986 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2140.
A study was conducted to determine the safety and serological response of young calves following intramuscular administration of S-IBR-002. These results are presented in Table 1. Three calves were inoculated intramuscularly with $10^7$ PFU of S-IBR-002. Clinical signs of IBR and febrile response were absent in these calves, as well as in the contact control calf. All three calves developed significant neutralizing antibody to IBR virus but the contact control remained seronegative. These results suggest that S-IBR-002 is useful as a vaccine against IBR disease.
Table 1. Serologic and Clinical Response of Young Calves Following Vaccination with S-IBR-002

<table>
<thead>
<tr>
<th>Virus Construct</th>
<th>Calf #</th>
<th>Clinical and Febrile response</th>
<th>Virus Isolation(^a)</th>
<th>Antibody Titer Days Post Inoculation</th>
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<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td>S-IBR-002</td>
<td>28</td>
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<td>(-)</td>
<td>&lt;2</td>
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<tr>
<td>Control</td>
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<td>NONE</td>
<td>(-)</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

\(^a\)From nasal swabs and peripheral blood leukocytes.
Example 2

Unique Short 2 gene

The unique short region of IBR virus contains a gene homologous to the US2 gene of several other herpesviruses. In the studies described below we show that deletion of the IBR unique short 2 gene (US2) may render the virus safe for use in pregnant cows, as determined by direct fetal inoculation.

Observing that the Nasalgen IBR vaccine strain will not cause abortion when used in IBR-susceptible pregnant cows at various stages of gestation (18,65), we attempted to determine the genomic lesion responsible for this property. We characterized the genome of this virus by restriction mapping and DNA sequence analysis. It was determined that a major portion of the IBR virus US2 gene was deleted from the Nasalgen virus. Restriction mapping of the Nasalgen virus indicated that the HindIII K fragment contained an approximately 800 base pair deletion. The deletion was localized to the end of the HindIII K Fragment located next to the HindIII O fragment (see Figure 1). Therefore, the HindIII K fragment from the Cooper strain was subcloned and this region was sequenced. The first 1080 base pairs of the fragment were found to contain an open reading frame (ORF) coding for 309 amino acids (see Figure 3). The ORF is 68% G+C and encodes a protein with a predicted molecular weight of 46,094. Comparison of the sequence of the predicted protein with sequences of gene products of HSV-1, PRV, HSV-2, and MDV in the unique short region indicated that this ORF is homologous to the herpesvirus US2 gene (see Figure 4). Although the function of the herpesvirus US2 gene is not known, the gene has been shown to be nonessential for growth of HSV in cell culture (4,19).
The US2 gene has also been shown to be deleted in the PRV vaccine strains Norden and Bartha (11).

The *HindIII* K fragment from the Nasalgen virus was subcloned and the deletion region was sequenced. When the sequence obtained from the Nasalgen strain was compared to the sequence obtained from the Cooper strain (see Figure 5), it was possible to determine that amino acids 59 to 309 of the US2 gene had been deleted. It was also determined that most of the *HindIII* O fragment had also been deleted.

Cattle studies have shown that the Nasalgen virus will not cause abortion when used in IBR-susceptible pregnant cows at various stages of gestation (18). Since the only major difference between the wild-type IBR strain and the Nasalgen strain resides in the deletion of the US2 gene, this gene may be involved in the fetal virulence observed for the wild type virus.
Example 3

S-IBR-027

S-IBR-027 is an IBR virus that has a deletion of approximately 800 bp in the repeat regions and approximately 1200 bp in the short unique region of the genome. The deletion in the short unique region removes the US2 gene (Figure 6). The repeat deletion was derived from the parental virus S-IBR-002 and is described in Example 2.

To construct this virus, the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS was performed. A homology vector containing the bacterial transposon Tn5 NEO (aminoglycosidase 3'-phosphotransferase) gene under the control of the HSV α4 promoter flanked by sequences from the IBR virus TK gene was constructed. The IBR virus homology regions were derived from the TK gene. The upstream homology included the first amino acid of the TK gene (15) and extended approximately 800 base pairs upstream of the TK coding region. The downstream homology included amino acids 156 to 357 and extended downstream of the TK coding region approximately 60 base pairs. S-IBR-002 DNA was mixed with the homology vector and transfected into rabbit skin cells as indicated in the methods. The transfection stock was selected according to the SELECTION OF G418 RESISTANT IBR VIRUS. Individual clones were picked after one round of selection and analyzed by the SOUTHERN BLOTTING OF DNA procedure. When a probe derived from the NEO gene was used in this analysis, one clone was found which did not hybridize to the NEO probe but had a HindIII restriction digestion pattern clearly distinct from the parental S-IBR-002. Further analysis indicated that the NEO had not been inserted into the TK region,
however an approximately 1200 base pair deletion had occurred in the HindIII K fragment.

In order to characterize the HindIII K deletion, that fragment was subcloned and subjected to restriction mapping. Utilizing a series of oligonucleotide probes derived from the wild type sequence it was determined that approximately 1200 base pairs were deleted from the end of the HindIII K fragment adjacent to the HindIII K/HindIII O junction (see Figure 6). This deletion removes the entire coding region of the US2 gene. S-IBR-027 was deposited on April 17, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2322.

Direct fetal inoculation is the most sensitive test for determining the safety of live, IBR vaccines as regards their use in pregnant cows or in calves nursing pregnant cows. Three virus constructs were tested for fetal safety by inoculating directly into the bovine fetus, following laparotomy to expose the uterus. Abortion occurring within seven days after inoculation was considered to be surgically-induced. If fetuses aborted after this time, tissue samples were removed and cultured for the presence of the IBR construct. Caesarean sections were performed on cows with fetuses surviving for greater than 30 days post-inoculation. Fetal tissue was removed for virus culturing and blood samples were taken for evaluation of serum antibody to IBR virus.

The S-IBR-027 construct described above was tested, as well as two other constructs, S-IBR-020 and S-IBR-028. The S-IBR-020 construct was derived from the Cooper
strain of IBR virus by making deletions in the repeat regions of the DNA and by inserting the Tn5 NEO gene. The S-IBR-028 construct was derived from the Cooper strain of IBR virus by making deletions in the repeat region of the DNA and in the TK gene. The Tn5 NEO gene was also inserted into the TK deletion.

The following results were obtained from studies with the three virus constructs. In the studies with S-IBR-020, two fetuses were inoculated, one at approximately 130-140 days gestation and the other at approximately 170-180 days gestation. The younger fetus aborted twenty days after inoculation, but virus could not be recovered from tissue samples of this fetus (Table 2). The other fetus was live and appeared normal when it was surgically removed 60 days post-inoculation. In studies with S-IBR-027, four fetuses, ranging in age from 125 days to >250 days, were inoculated (Table 2). All fetuses survived and appeared normal. In studies with S-IBR-028, three fetuses, ranging in age from 140 days to >250 days, were inoculated. The youngest and eldest fetuses survived and appeared normal, however the fetus inoculated at 160-170 days gestation aborted nine days after inoculation.

Direct fetal inoculation is the most sensitive test for measuring the safety of live, IBR viruses used in pregnant cows. To date, the gene(s) involved in fetal virulence has not been reported. We have engineered IBR viruses with deletions in three different regions of IBR virus DNA and then determined the effect of the gene deletion. All three virus constructs tested have a deletion in the repeat region of the DNA and two constructs do not have TK activity. One fetus inoculated with each of the TK- constructs has aborted. In contrast, the construct with deletions in the repeat regions and the US2 gene (S-IBR-027) has been inoculated into four fetuses with no adverse reactions.
Table 2. Safety of IBR Viruses for Bovine Fetuses

<table>
<thead>
<tr>
<th>Construct</th>
<th>Fetal Age*</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-IBR-020</td>
<td>130-140 Days</td>
<td>Fetus aborted Day 20 post-inoculation; no virus isolated</td>
</tr>
<tr>
<td></td>
<td>170-180 Days</td>
<td>Normal, live fetus 60 days post-inoculation</td>
</tr>
<tr>
<td>S-IBR-027</td>
<td>125-135 Days</td>
<td>Normal, live fetus 60 days post-inoculation</td>
</tr>
<tr>
<td></td>
<td>150-160 Days</td>
<td>Normal, live calf born 56 days post-inoculation</td>
</tr>
<tr>
<td></td>
<td>220-240 Days</td>
<td>Normal, live calf born 30 days post-inoculation</td>
</tr>
<tr>
<td></td>
<td>&gt;250 Days</td>
<td>Normal, live calf born 30 days post-inoculation</td>
</tr>
<tr>
<td>S-IBR-028</td>
<td>140-150 Days</td>
<td>Normal, live fetus 60 days post-inoculation</td>
</tr>
<tr>
<td></td>
<td>160-170 Days</td>
<td>Fetus aborted Day 9 post-inoculation; no virus isolated</td>
</tr>
<tr>
<td></td>
<td>&gt;250 Days</td>
<td>Normal, live calf born 12 days post-inoculation</td>
</tr>
</tbody>
</table>

*Approximate age at time of virus inoculation
We have shown that S-IBR-027 is safe for fetal inoculation in contrast to S-IBR-020 and S-IBR-028 which are not. Although all three viruses were engineered by similar approaches, the distinguishing difference of S-IBR-027 is the deletion of the US2 gene. We have also shown that the Nasalgen virus, which was generated by independent methods and is also safe for use in IBR-susceptible pregnant cows, has been deleted in the US2 gene.

Although the S-IBR-027 and Nasalgen have the similar property of fetal safety, S-IBR-027 offers additional advantages. The major portion of the US2 gene (251 out of 309 amino acids) has been deleted in the Nasalgen virus. This deletion would clearly inactivate the gene, however the remaining portion of the gene may make it more likely to revert to virulence via recombination with other viruses. The complete coding region of the US2 has been deleted from S-IBR-027 making it less likely that this gene could be restored and revert the virus to virulence. The S-IBR-027 construct also carries an important deletion in the repeat region, which is not present in the Nasalgen virus. A deletion in the analogous region of the pseudorabies virus (PRV) has been shown to be valuable in attenuating PRV for swine (see U.S. Patent No. 4,877,737). This deletion has also been shown to attenuate IBR for cattle as seen in the testing of S-IBR-002 (see Example 1).
Example 4

S-IBR-028

S-IBR-028 is an IBR virus that has a deletion of approximately 800 bp in the repeat regions and approximately 250 bp in the TK region of the genome. The deletion in the TK region inactivates the TK gene. The repeat deletion was derived from the parental virus S-IBR-002 and is described in Example 2.

To construct this virus, the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS was performed. A homology vector containing the bacterial transposon Tn5 NEO (aminoglycosidase 3'-phosphotransferase) gene under the control of the HSV-1 α4 gene promoter flanked by sequences from the IBR virus TK gene was constructed. The IBR virus homology regions were derived from the TK gene. The upstream homology included amino acids 1 to 62 of the TK gene (15) and extended approximately 674 base pairs upstream of the TK coding region. The downstream homology included amino acids 156 to 357 and extended downstream of the TK coding region approximately 1138 base pairs. S-IBR-002 DNA was mixed with the homology vector 129-71.5 and transfected into rabbit skin cells as indicated in the methods. The transfection stock was selected according to the SELECTION OF G418 RESISTANT IBR VIRUS.

Individual clones were picked after two rounds of selection and analyzed by the SOUTHERN BLOTTING OF DNA procedure. Several clones were assayed for TK activity by a 14C-thymidine incorporation assay (29). One clone which was negative for TK activity was chosen and characterized by digestion with HindIII and XbaI. The restriction endonuclease analysis confirmed that the NEO
gene had been inserted into the TK gene. This clone, designated S-IBR-028, was deposited on May 14, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2326.
Example 5

Glycoprotein G gene

Deletion of the PRV gpX gene has been shown to be valuable both as an attenuating lesion and as a negative serological marker (see U.S. Serial No. 192,866, filed May 11, 1988 now U.S. Patent No. 5,047,237 issued September 10, 1991). In the studies described below we show that the unique short region of IBR virus contains a gene homologous to the gpX gene of PRV.

The sequence of an approximately 1400 base pair region of the IBR HindIII K fragment (see Figure 8), located approximately 2800 base pairs downstream of the HindIII K/HindIII O junction was determined. This region was found to contain an ORF coding for 441 amino acids translated in the direction away from the HindIII K/HindIII O junction (see Figure 1). The ORF is 69% G+C and encodes a protein with a predicted molecular weight of 58,683. Comparison of the sequence of the predicted protein with sequences of gene products of HSV-2 and PRV in the unique short region indicated that this ORF is homologous to the herpesvirus gpG gene (see Figure 9).

The complete gpG gene resides on an approximately 2800 base pair MluI to NdeI sub-fragment of the IBR virus HindIII K fragment. This subfragment has been cloned as a blunt ended fragment into the plasmid pSP64. This plasmid is designated PSY1643. PSY1643 was deposited on July 16, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 68652. This plasmid may be used to confirm the sequence of the gpG gene. The sequence of the gpG gene may also be confirmed by comparing the appropriate
DNA sequence of the wild type virus S-IBR-000 (Cooper strain with the sequence of the gpG deleted virus S-IBR-037 (ATCC Accession No. 2320).

5 To confirm the expression of the IBR virus gpG gene product, cells were infected with IBR virus and samples of media from infected cultures were subjected to SDS-polyacrylamide gel electrophoresis. The gel was blotted and analyzed using the WESTERN BLOTTING PROCEDURE. The anti-serum used was a mouse hyper-immune serum raised against chemically-synthesized gpG peptides (amino acids 242-254 and 269-289) linked to keyhole limpet hemocyanin. As shown in Figure 10, gpG is prominent in the media of cells infected with wild type virus (S-IBR-000), but is not detected in media of mock infected cells.
Example 6

S-PRV-160

S-PRV-160 is a pseudorabies virus that has a deletion in the TK gene in the long unique region, a deletion in the repeat region, and an approximately 1414 base pair deletion in the gpX coding region. The gene for E.coli β-galactosidase (lacZ gene) was inserted in the place of the gpX gene and is under the control of the gpX promoter. A chimeric gene coding for an IBR virus gpG, PRV gpIII and PRV gpX fusion protein was inserted at the HindIII sites located in each repeat.

S-PRV-160 was constructed utilizing plasmid 459-12.6, pseudorabies virus S-PRV-013 (see U.S. Serial No. 823,102, filed January 27, 1986 now U.S. Patent No. 5,068,192 issued November 26, 1991 and U.S. Serial No. 07/192,866, filed May 11, 1988 now U.S. Patent No. 5,047,237 issued September 10, 1991) and the restriction enzyme HindIII in the DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. Several clones were screened by digestion with HindIII for the presence of the HindIII band containing the chimeric gene insert from plasmid 459-12.6. One clone exhibiting the correct HindIII insert band was chosen and designated S-PRV-160.

S-PRV-160 was constructed so that it would express precisely the gpG specific amino acids that were deleted in S-IBR-037. This allows the gpG fusion protein expressed in S-PRV-160 to be used as an antigen to identify antibodies directed against the wild type virus as opposed to antibodies directed against S-IBR-037. Note that gpX, the PRV homologue of IBR virus gpG, has been deleted from S-PRV-160 to prevent any confusion resulting from cross reactivity that might exist between the two proteins. To confirm that S-PRV-160 does express
IBR virus gpG, a Western blot analysis was performed. As can be seen in Figure 10, gpG specific antibody does react with an appropriately sized media protein from S-PRV-160.

S-PRV-160 may also be utilized as an antigen for the production of gpG specific monoclonal antibodies. Such antibodies are useful in the development of diagnostic tests specific for the gpG protein. Monoclonal antibodies were generated in mice utilizing S-PRV-160 according to the PROCEDURE FOR GENERATING MONOCLONAL ANTIBODIES. One of these antibodies’ clone 3-1G7 was shown to react specifically with purified gpG in the gpG ELISA assay.
Example 7

S-IBR-035

S-IBR-035 is an IBR virus that has two deletions in the short unique region of the genome. The first deletion is approximately 2500 base pairs and begins in the HindIII K fragment approximately 1750 base pairs downstream of the HindIII O/HindIII K junction and extends back through that junction. This deletion removes the US2 gene. The second deletion is approximately 294 base pairs and begins in the HindIII K fragment approximately 3900 base pairs downstream of the HindIII K/HindIII O junction and extends back toward that junction. This deletion removes amino acids 263 to 361 of the gpg gene. The gene for E.coli β-galactosidase (lacZ gene) was inserted into the deletion in the gpg gene and is under the control of the HCMV immediate early promoter.

S-IBR-035 was derived from S-IBR-000 (Cooper strain). This was accomplished utilizing the homology vector 439-01.31 (see Materials and Methods) and virus S-IBR-000 in the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. The transfection stock was screened by the BLUOGAL™ SCREEN FOR RECOMBINANT HERPESVIRUS. The final result of blue plaque purification was the recombinant virus designated S-IBR-035. This virus was characterized by restriction mapping and the SOUTHERN BLOTTING DNA procedure. This analysis confirmed the insertion of the β-galactosidase (lacZ) marker gene and the deletion of approximately 294 base pairs of the gpg gene. It was also confirmed that an approximately 2500 base pair deletion had occurred in the region of the US2 gene.
Example 8

S-IBR-036

S-IBR-036 is an IBR virus that has two deletions in the short unique region of the genome. The first deletion is approximately 2500 base pairs and is similar to the deletion in S-IBR-035 (see Example 7) which removes the US2 gene. The second deletion is approximately 1230 base pairs and begins in the HindIII K fragment approximately 3900 base pairs downstream of the HindIII O/HindIII K junction and extends back toward that junction. This deletion removes amino acids 1 to 361 of the gpG gene. The gene for E.coli β-galactosidase (lacZ gene) was inserted into the deletion in the gpG gene and is under the control of the HCMV immediate early promoter.

S-IBR-036 was derived from S-IBR-000 (Cooper strain). This was accomplished utilizing the homology vector 439-21.69 (see Materials and Methods) and virus S-IBR-000 in the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. The transfection stock was screened by the BLUOGAL SCREEN FOR RECOMBINANT HERPESVIRUS. The final result of blue plaque purification was the recombinant virus designated S-IBR-036. This virus was characterized by restriction mapping and the SOUTHERN BLOTTING DNA procedure. This analysis confirmed the insertion of the β-galactosidase (lacZ) marker gene and the deletion of approximately 1230 base pairs of the gpG gene. It was also confirmed that an approximately 2500 base pair deletion had occurred in the region of the US2 gene (see above).
Example 9

S-IBR-037

S-IBR-037 is an IBR virus that has two deletions in the short unique region of the genome. The first deletion is approximately 2500 base pairs and begins in the HindIII K fragment approximately 1750 base pairs downstream of the HindIII O/HindIII K junction and extends back through that junction. This deletion removes the US2 gene. The second deletion is approximately 1230 base pairs and begins in the HindIII K fragment approximately 3900 base pairs downstream of the HindIII O/HindIII K junction and extends back toward that junction. This deletion removes amino acids 1 to 361 of the gplG gene.

S-IBR-037 was derived from S-IBR-035. This was accomplished utilizing the homology vector 439-70.4 (see Materials and Methods) and virus S-IBR-035 in the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. The transfection stock was screened by the BLUGAL SCREEN FOR RECOMBINANT HERPESVIRUS. The result of white plaque purification was the recombinant virus designated S-IBR-037. This virus was characterized by restriction mapping and the SOUTHERN BLOTTING DNA procedure. This analysis confirmed the deletion of the β-galactosidase (lacZ) marker gene and the deletion of approximately 1230 base pairs of the gpG gene. It was also confirmed that an approximately 2500 base pair deletion had occurred in the region of the US2 gene (see above). S-IBR-037 was deposited on April 16, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2320.
To test the efficacy of S-IBR-037 as an inactivated IBR virus vaccine in protecting susceptible calves against virulent IBR virus challenge, a study was performed according to the VACCINATION STUDIES IN CALVES WITH INACTIVATED IBR VIRUS. The following results were observed.

Virus neutralization antibody titers were elicited in animals after the first vaccination (see Table 3). Antibody titers were not significantly different between animals that received a vaccine dose of $10^{7.3}$ virus and animals vaccinated with $10^{8.0}$ virus. After the second vaccination, mean antibody titers increased to 1:19 and 1:32, respectively, for the $10^{7.3}$ and $10^{8.0}$ vaccine groups. Control animals remained seronegative to IBR virus throughout the vaccination period. Antibody titers in both vaccinate groups showed an increase typical of an anamnestic response after challenge with virulent IBR virus. By 13 days post challenge, mean antibody titers were 1:152 and 1:215 for the $10^{7.3}$ and $10^{8.0}$ vaccinate groups respectively. In contrast, mean antibody titers in challenged control animals were 1:4 at 7 days and 1:8 at 13 days post challenge.

Nasal swabs were collected from challenged animals to determine whether vaccination decreased the time of virus shedding (Table 4). The most dramatic difference between vaccinates and control animals was observed at 12 days post challenge. At this time, seventy-five percent of control animals continue to shed, whereas, only twenty-five percent of both vaccinate groups shed virus. Virus was not isolated from control or vaccinated groups at 15 days post challenge.
Table 3. Generation of virus neutralizing antibody in animals vaccinated with inactivated S-IBR-037 vaccine.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Antigen dose 10^{7.3}</th>
<th>Post Vaccination</th>
<th>Post Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>Controls</td>
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</tr>
<tr>
<td>9</td>
<td>≤2</td>
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<td>32</td>
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</tr>
<tr>
<td>64</td>
<td>≤2</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td>GMT</td>
<td>≤2</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td></td>
<td>1</td>
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<td>8</td>
</tr>
<tr>
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<td>20</td>
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</tr>
<tr>
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<td>36</td>
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<tr>
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</tr>
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<tr>
<td>GMT</td>
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<td>6.7</td>
<td>32*</td>
</tr>
</tbody>
</table>

*Statistically greater than controls (p≤0.05)

*Expressed as reciprocal of dilution.
Table 4. Isolation of IBR virus from vaccinated and unvaccinated control animals after challenge with virulent IBR virus.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>IBR virus isolated (+/-) from animals on days post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
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<td>Controls</td>
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<td>-</td>
</tr>
<tr>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>Vaccinates dose $10^{7.3}$</td>
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</tr>
<tr>
<td>1</td>
<td>-</td>
</tr>
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<td>20</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>36</td>
<td>-</td>
</tr>
<tr>
<td>Vaccinates dose $10^{8.0}$</td>
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</tr>
<tr>
<td>7</td>
<td>-</td>
</tr>
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<tr>
<td>33</td>
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</tr>
<tr>
<td>69</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 5. Vaccinated animals demonstrate reduced clinical signs of IBR.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Clinical scores post challenge</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Attitude&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ulcers&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Serious Discharge&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Mucopurulent Discharge&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Temperature&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Controls</td>
<td>5</td>
<td>3</td>
<td>11</td>
<td>5</td>
<td>3</td>
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<tr>
<td>9</td>
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<td>3</td>
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<td>64</td>
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<td>2</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Days with depressed attitude.
<sup>b</sup> Number of ulcers.
<sup>c</sup> Days with serous discharge.
<sup>d</sup> Days with mucopurulent discharge.
<sup>e</sup> Days with ≥ 2°F above baseline temperature.
<sup>f</sup> Animal exhibited mucopurulent discharge on the day of challenge and for 13 days post challenge.
<sup>*</sup> Statistically greater than controls (p ≤ 0.05)
Animals were observed daily for 13 days post challenge for clinical signs of IBR infection. Clinical disease was evaluated with respect to attitude, the number of ulcers, extent of serious and mucopurulent discharge and the number of days with elevated temperature. The results presented in Table 5 show that vaccinated animals exhibited less severe disease than did unvaccinated control animals. Control animals showed clinical depression ("Attitude" in Table 5) for 4.5 days compared with 1 to 1.5 days for vaccinated animals. The amount and extent of serous discharge was substantially reduced in both vaccinate groups compared with controls. The extent of mucopurulent discharge was also reduced in vaccinated animals, although to a lesser degree. However, vaccinate animal #36 did have mucopurulent discharge on the day of challenge and is not consistent with the results for other vaccinates. None of the vaccinates exhibited temperatures of ≥2°F above baseline.

In contrast, all control animals exhibited elevated temperatures of ≥2°F over baseline and 2 of 4 control animals had temperatures of 104°F and above.

Vaccination of calves with inactivated S-IBR-037 vaccine protected the animals against virulent wild-type IBR virus challenge. Virus neutralization titers were statistically greater in vaccinated than in control animals. An anamnestic response in antibody titer was observed 7 days post challenge, indicating the development of humoral memory response. Except for 7 days post challenge, neutralization titers between the $10^{7.3}$ and $10^{8.0}$ vaccinate groups were not statistically different. Fewer vaccinated animals shed virulent challenge virus than control animals. These results suggest that virulent IBR virus is cleared more rapidly in vaccinated than in unvaccinated animals. Clinical symptoms of IBR virus infection were also reduced in
vaccinated animals. After challenge, both vaccine groups exhibited fewer days of depressed attitude, reduced serous discharge, and no elevated temperature compared with controls.

In order to show that gpG antibody is produced in vaccinated calves following exposure to wild-type virus, serum samples taken pre- and post-exposure to wild-type viruses were subjected to the ELISA assay. Samples taken at the day of challenge and at 13 days post-challenge were analyzed. As seen in Table 6, the post-challenge absorbance readings for gpG increase for each animal (ratio of > 1.0), indicating that within 13 days of infection a detectable immune response to gpG is present.
Table 6. Detection of antibody to gpG in serum of animals vaccinated with S-IBR-037 and challenged with wild type.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Ratio of pre- vs. post challenge¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.22</td>
</tr>
<tr>
<td>22</td>
<td>1.96</td>
</tr>
<tr>
<td>32</td>
<td>1.87</td>
</tr>
<tr>
<td>64</td>
<td>2.19</td>
</tr>
<tr>
<td>Vaccinates dose</td>
<td></td>
</tr>
<tr>
<td>10⁷.³</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.39</td>
</tr>
<tr>
<td>20</td>
<td>1.40</td>
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<tr>
<td>25</td>
<td>1.84</td>
</tr>
<tr>
<td>36</td>
<td>1.18</td>
</tr>
<tr>
<td>Vaccinates dose</td>
<td></td>
</tr>
<tr>
<td>10⁸.⁰</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.19</td>
</tr>
<tr>
<td>30</td>
<td>1.29</td>
</tr>
<tr>
<td>33</td>
<td>1.52</td>
</tr>
<tr>
<td>69</td>
<td>2.66</td>
</tr>
</tbody>
</table>

¹Animals were challenged with 10⁷.⁶ PFU of wild type IBR virus. Pre-challenge serum from day of challenge, post-challenge serum from 13 days post challenge. Data reflects the average of the ratio of absorbance readings for three independent ELISA determinations.
Example 10

S-IBR-038

S-IBR-038 is an IBR virus that has two deletions in the short unique region of the genome. The first deletion is approximately 2500 base pairs and begins in the HindIII K fragment approximately 1750 base pairs downstream of the HindIII O/HindIII K junction and extends back through that junction. This deletion removes the US2 gene. The second deletion is approximately 294 base pairs and begins in the HindIII K fragment approximately 3900 base pairs downstream of the HindIII K/HindIII O junction and extends back toward that junction. This deletion removes amino acids 261 to 359 of the gpg gene.

S-IBR-038 resulted from the removal of the marker gene from S-IBR-035 (see above). This was accomplished by digestion of S-IBR-035 with XbaI as described in the DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. The structure of S-IBR-035 was confirmed by restriction enzyme analysis with HindIII, BamHI and XbaI.
Example 11

Glycoprotein E gene

Deletion of the PRV gI gene has been shown to be valuable both as an attenuating lesion and a negative serological marker (3,42). In the studies described below we show that the unique short region of IBV virus contains a gene homologous to the gI gene of PRV.

The sequence of 2038 base pairs of the IBR unique short region, starting approximately 1325 base pairs upstream of the HindIII K/HindIII F junction in the HindIII K fragment was determined. This region was found to contain an ORF coding for 617 amino acids translated in the direction away from the HindIII K/HindIII O junction (see Figure 1). The ORF is 70.5% G+C and encodes a protein with a predicted molecular weight of approximately 88,980. Comparison of the sequence of the predicted protein with sequences of gene products of HSV-1, VZV, and PRV in the unique short region indicated that this ORF is homologous to the herpesvirus gpE gene (see Figure 16).

The DNA encoding the gpE gene has been cloned in two plasmids, PSY1644 and PSY1645. The amino-terminal half of the gene (encoding amino acids 1-276) was cloned as an approximately 2300 base pair fragment resulting from a partial Smal digest of wild type S-IBR-000 (Cooper Strain) DNA. This fragment was inserted into the plasmid pSP64 to yield PSY1644. This plasmid, designated PSY1644, was deposited on July 16, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 68651.
The carboxyl-terminal half of the gene (encoding amino acids 277-617) was cloned as an approximately 2400 base pair SmaI fragment. The fragment was inserted into the plasmid pSP64 to yield PSY1645. This plasmid, designated PSY1645, was deposited on July 16, 1991 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 68650. These plasmids may be used to confirm the sequence of the gpE gene.
Example 12

**Pseudorabies virus expressing IBR virus gpE**

A pseudorabies virus analogous to S-PRV-160 may be constructed for the purpose of expressing the IBR virus gpE. This may be accomplished by inserting the gene coding for IBR virus gpE into S-PRV-002 (U.S. Patent No. 4,877,737).

Such an expression vector may be constructed utilizing the IBR virus gpE plasmid described in the methods section, pseudorabies virus S-PRV-002 and the restriction enzyme XbaI in the **DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS**. Viruses resulting from this procedure may be screened by digestion with XbaI for the presence of the XbaI band containing the IBR virus gpE gene.

The gpE protein expressed from this vector may be used as an antigen to identify antibodies directed against the wild type virus as opposed to antibodies directed against gpE deleted viruses. This virus may also be utilized as an antigen for the production of gpE specific monoclonal antibodies. Such antibodies are useful in the development of diagnostic tests specific for the gpE protein. Monoclonal antibodies may be generated in mice utilizing this virus according to the **PROCEDURE FOR GENERATING MONOCLONAL ANTIBODIES.**
Example 13

Glycoprotein E deleted IBR viruses

The HOMOLOGY VECTOR 536-03.5 was used to generate various gpE-deleted IBR viruses. Utilizing the general strategy described in CONSTRUCTION OF DELETION VIRUSES, a gpE deletion of approximately 1410 base pairs (amino acids 77-547) was introduced into two different IBR virus backbones, S-IBR-000 (Cooper Strain) and S-IBR-037. The virus resulting from the S-IBR-000 parent contains the gpE deletion alone. The virus resulting from the S-IBR-037 parent contains the gpE deletion in conjunction with the US2 and gpG deletions. The lacZ marker gene may be removed from these viruses utilizing the procedures outlined in the methods section.

These gpE-deleted viruses are of great value as IBR vaccines. Their combination of different deletions will provide the varying degrees of attenuation which are required for a superior vaccine. These viruses will also provide a negative serological marker which may be used to distinguish vaccinated from infected animals. The virus containing both gpG and gpE deletions should be of even greater value by having two negative markers. The availability of two negative markers permits one marker to be used as a confirmatory test, greatly increasing the reliability of such a diagnostic determination.
Example 14

S-IBR-004

S-IBR-004 is an IBR recombinant virus carrying an inserted foreign gene, Tn5 NEO (aminoglycoside 3′-phosphotransferase) gene, under the control of the pseudorabies virus (PRV) glycoprotein X promoter.

To construct this virus, the HindIII K DNA fragment from wild type IBR virus was cloned into the plasmid pSP64 at the HindIII site. This plasmid was designated pSY524. A map of the HindIII K fragment is shown in Figure 19.

The DNA from the XhoI site to the HindIII site and containing the NdeI site from pSY524 was cloned into plasmid pSP65 and called pSY846. The NdeI to EcoRI fragment was removed from pSY846 by digestion with NdeI and EcoRI restriction enzymes, followed by POLYMERASE FILL-IN REACTION and LIGATION. The resulting plasmid was called pSY862. The plasmid pNEO (P.L. Biochemicals, Inc.) contains the aminoglycoside 3′-phosphotransferase (NEO) gene and confers resistance to ampicillin and neomycin on E. coli hosts. The coding region of this gene (BglII-BamHI fragment) was isolated and cloned between the PRV gpX promoter and the HSV-TK poly A sequence in a plasmid called pSY845.

The NEO gene construct in pSY845 was excised with HindIII, made blunt ended by the POLYMERASE FILL-IN REACTION, and cloned into the SacI site of plasmid pSY862. The final product was called pSY868.

Wild type IBR DNA was mixed with pSY868 DNA and the mixture was transfected into rabbit skin cells to generate recombinant IBR. The recombinant IBR virus carrying a functional NEO gene was then isolated and
purified according to the SELECTION OF G418 RESISTANT IBR VIRUS method.

S-IBR-004 recombinant IBR was shown to express the NEO gene by the fact that cells infected with this virus were resistant to the toxicity of G418. A detailed map of the plasmid construction is shown in Figure 19. The structure of S-IBR-004 is also shown in Figure 19. S-IBR-004 was deposited on May 23, 1986 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2134.
Example 15

S-IBR-008

S-IBR-008 is an IBR virus that has a deletion in the short unique region, and an insertion of the bovine rotavirus glycoprotein 38 (gp38) gene in the XbaI site in the long unique region.

The bovine rotavirus gp38 gene was cloned utilizing the METHOD FOR cDNA CLONING BOVINE ROTAVIRUS gp38 GENE. The bovine rotavirus gp38 gene was then engineered to contain herpesvirus regulatory signals as shown in Figure 20. This was accomplished by cloning the gp38 gene BamHI fragment contained in pSY1053 between the BamHI and BgIII sites in pSY1052. The resulting plasmid, pSY1023, contained the PRV gpX promoter in front of the gp38 gene, and the HSV-1 TK polyadenylation signal behind the gp38 gene. The entire construct was flanked by XbaI sites to allow for the insertion of the XbaI fragment into IBR by direct ligation.

S-IBR-004 was the starting virus for the generation of S-IBR-008. S-IBR-004 DNA and pSY1023 DNA were mixed together, cut with XbaI, and transfected into rabbit skin cells according to the DIRECT LIGATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. The transfection stock was screened for recombinant virus by the ANTIBODY SCREEN FOR RECOMBINANT HERPESVIRUS procedure using antibodies prepared against the rotavirus gp38 protein.

One of the viruses purified by this screen was S-IBR-008, which has the following characteristics. It contains the rotavirus gp38 gene plus the plasmid DNA inserted into the XbaI site in the long unique region of the virus genome, but no longer contains the NEO gene of parent S-IBR-004 in the unique short region. In fact, a small
deletion was created in the unique short region at the location of the NEO gene, as evidenced by the absence of an XbaI site at this location in S-IBR-008.

5 S-IBR-008 was shown to be expressing the rotavirus gp38 gene by analysis of RNA transcription in infected cells, and by the ANTIBODY SCREEN FOR RECOMBINANT HERPESVIRUS procedure using antibodies specific for the gp38 gene. S-IBR-008 was deposited on June 18, 1986 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2141. The structure of S-IBR-008 is shown in Figure 20.
Example 16

S-IBR-018

S-IBR-018 is an IBR virus that has three foreign genes inserted: the E.coli beta-galactosidase gene and the neomycin resistance gene in the XbaI site in the unique long region, and the parainfluenza type 3 (PI-3) virus hemagglutinin gene (HN) in the HindIII site in the unique long region immediately adjacent to the XbaI site.

For cloning the PI-3 HN gene, the SF-4 strain of PI-3 was grown in MADIN-DARBY bovine kidney (MDBK) cells in culture and RNA was extracted from infected cells. The RNA was used in a reverse transcription protocol as outlined in the cDNA CLONING procedure using poly-dT as primer for reverse transcriptase. From this procedure, a series of clones was obtained that comprised parts of the genome of the PI-3 virus. The location of the gene for the human PI-3 HN gene has been published (25,26) and this information was used to locate the gene in applicants' bovine PI-3 clones. The entire open reading frame of the bovine PI-3 HN gene was sequenced by applicants and is given in Figure 21.

The HSV ICP4 promoter was used to express the PI-3 HN gene and the HSV TK poly-A signal was used to terminate transcription. The engineering of this construct was done as shown in Figure 22 A and B. The construct contained (5' to 3') the HSV ICP4 promoter, the ICP4 TATA box, the ICP4 cap site, a fusion within the ICP4 5' untranslated region to the PI-3 HN gene at the HhaI site, the HN gene start codon, the HN structural gene, the HN stop codon, a fusion within the HN 3' untranslated region to the HSV TK untranslated 3' region, and the HSV TK poly-A signal sequence.
This plasmid also contained the beta-galactosidase (lacZ) gene under the control of the PRV gpX promoter with the gpX poly-A termination signal, as well as the neomycin resistance gene under the control of the gpX promoter with the TK poly-A termination signal. These latter two genes were cloned in tandem at the XbaI site in BamHI-C fragment (Figure 22 A and B). This BamHI-C fragment contained the homology regions for use in the DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure. After the transfection step in the procedure, the resulting recombinant virus from the transfection stock was selected for by the SELECTION of G418 RESISTANT IBR VIRUS procedure, followed by the BLUOGAL™ SCREEN FOR RECOMBINANT HERPESVIRUS procedure, and subsequently analyzed for the insertion of the PI-3 HN gene by the SOUTHERN BLOTTING OF DNA procedure. The virus that resulted from this screening was designated S-IBR-018.

S-IBR-018 was deposited on July 21, 1987 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. VR 2180. The structure of S-IBR-018 is shown in Figure 22 C.
**Example 17**

**S-IBR-019**

5 S-IBR-019 is an IBR virus that has three foreign genes inserted: the *E.coli* beta-galactosidase (lacZ) gene and the neomycin resistance gene in the *XbaI* site in the unique long region, and the parainfluenza type 3 (PI-3) virus fusion gene (F) in the *HindIII* site in the long unique region adjacent to the *XbaI* site.

For cloning the PI-3 F gene, the SF-4 strain of PI-3 was grown in MDBK cells in culture and RNA was extracted from infected cells. The RNA was used in a reverse transcription protocol as outlined in the cDNA CLONING procedure using poly-dT as primer for reverse transcriptase. From this procedure, a series of clones was obtained that comprised parts of the genome of the PI-3 virus. The location of the gene for the Sendai virus F gene has been published (27) and this comparative sequence information was used to locate the homologous gene in applicants' bovine PI-3 clones.

The HSV alpha-4 promoter was used to express the PI-3 F gene and the HSV TK poly-A signal was used to terminate transcription. The construct contained (5' to 3') the HSV alpha-4 promoter, the alpha-4 TATA box, the alpha-4 cap site, a fusion in the alpha-4 5' untranslated region to the PI-3 F gene, the F start codon, the F structural gene, the F stop codon, a fusion in the F 3' untranslated region to the HSV TK 3' untranslated region, and the TK poly-A signal sequence.

This plasmid also contained the beta-galactosidase (lacZ) gene under the control of the PRV gpX promoter with the gpX poly-A termination signal, as well as the neomycin resistance gene under the control of the gpX promoter
with the TK poly-A termination signal. These latter two
genesis were cloned in tandem at the XbaI site in BamHI-C
fragment (Figure 23 A and B). This BamHI-C fragment
contained the homology regions for use in the DNA
TRANSFECTION FOR GENERATING RECOMBINANT VIRUS procedure.
After the transfection step in the procedure, the
resulting recombinant virus from the transfection stock
was selected for by the SELECTION OF G418 RESISTANT
HERPESVIRUS procedure, followed by the BLUGALY SCREEN
FOR RECOMBINANT HERPESVIRUS procedure, and subsequently
analyzed for the insertion of the PI-3 F gene by SOUTHERN
BLOTTING OF DNA procedure. The virus that resulted from
this screening was designated S-IBR-019.

The structure of S-IBR-019 is shown in Figure 23 C.
Example 18

S-IBR-032

S-IBR-032 is an IBR virus that has two foreign genes inserted: the Escherichia coli beta-galactosidase (lacZ) gene with the bovine viral diarrhea virus (BVDV) gp53 gene fused to the lacZ C-terminus and inserted in the long unique region at the XbaI restriction endonuclease site.

For cloning the BVDV gp53 gene, the Singer strain of BVDV was grown in MADIN-DARBY bovine kidney (MDBK) cells in culture and the RNA was extracted from infected cells. The RNA was used in a reverse transcriptase procedure as outlined in the cDNA CLONING procedure using random primers for reverse transcriptase. From this procedure, a series of clones was obtained that comprised parts of the genome of BVDV. The location of the gene for BVDV gp53 has been published (66) and this comparative sequence information was used to locate the homologous gene in the applicant’s BVDV clones.

The PRV gpX promoter was used to express lacZ with a region of BVDV gp53 fused to the C-terminus, and the PRV poly-A signal was used to terminate transcription. A plasmid construct was engineered that contained (5' to 3') the PRV gpX promoter and then the coding region consisting of amino acid codons 1-7 of the PRV gpX gene, 10-1024 of the Escherichia coli lacZ gene, and 684-758 of the BVDV major open reading frame, and the PRV poly-A sequence. This lacZ fusion gene cassette was then excised from the plasmid vector at the flanking XbaI sites and cloned into the unique XbaI site in IBR-002 using the in vitro ligation method described in CONSTRUCTION OF DELETION VIRUSES. After the transfection step in DNA TRANSFECTION FOR GENERATING RECOMBINANT VIRA
procedure, the resulting recombinant virus was screened and isolated from the transfection stock using the BLUGAL™ SCREEN FOR RECOMBINANT HERPESVIRUS procedure, and subsequently analyzed for the insertion of the BVDV gp53 region by SOUTHERN BLOTTING OF DNA procedure. The virus that resulted from this screening was designated S-IBR-032.
Example 19

S-IBR-039

S-IBR-039 is an IBR virus that has three deletions in the short unique region of the genome. The first deletion is approximately 2500 base pairs and begins in the HindIII K fragment approximately 1750 base pairs downstream of the HindIII O/HindIII K junction and extends back through that junction. This deletion removes the US2 gene. The second deletion is approximately 1230 base pairs and begins in the HindIII K fragment approximately 3900 base pairs downstream of the HindIII O/Hind K junction and extends back toward that junction. This deletion removes amino acids 1 to 361 of the gpG gene. The third deletion is approximately 1410 base pairs and removes amino acids 77-547 of the gpE gene.

S-IBR-039 was derived from S-IBR-037. This was accomplished utilizing the homology vector 536-03.5 (see MATERIALS AND METHODS) and virus S-IBR-037 in the HOMOLOGOUS RECOMBINATION PROCEDURE FOR Generating RECOMBINANT HERPESVIRUS. The transfection stock was screened by the SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES. The result of blue plaque purification was the recombinant virus designated S-IBR-039. This virus was characterized by restriction mapping and the SOUTHERN BLOTTING DNA procedure. This analysis confirmed the insertion of the β-galactosidase (lacZ) marker gene and the deletion of approximately 1230 base pairs of the gpG gene. It was also confirmed that an approximately 1410 base pair deletion has occurred in the region of the gpE gene (see above). This virus will be useful as a vaccine to protect cattle from infection with IBR virus. The deletions of the glycoproteins G and E genes from this virus also provides two negative
serological markers for differentiating it from wild type IBR.
Example 20

S-IBR-045

S-IBR-045, a recombinant IBR virus with deletions in the Tk, US2, gpG and gpE genes may be constructed in the following manner. S-IBR-045 would be derived from S-IBR-039 (see example 19) through the construction of two intermediate viruses. The first intermediate virus, S-IBR-043, would be constructed utilizing the homology vector 591-46.12 (see MATERIALS AND METHODS) and virus S-IBR-039 in the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. The transfection stock would be screened by the SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES for a white plaque recombinant virus (uidA substrate). The resulting virus would have deletions of the Tk, US2, gpG and gpE genes and insertion of lacZ gene in the gE gene deletion. Finally, S-IBR-045 would be constructed, utilizing the homology vector 523-78.72 (see MATERIALS AND METHODS) and virus S-IBR-044 in the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. The transfection stock would be screened by the SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES for a white plaque recombinant virus (lacZ substrate). This virus will be useful as a vaccine to protect cattle from infection with IBR. The combination of deletions will provide the appropriate attenuation which is required for a superior vaccine. This virus will also provides two negative serological markers which may be used to distinguish vaccinated from infected animals. The availability of two negative markers permits one marker to be used as a confirmatory test, greatly increasing the reliability of such a diagnostic determination.
Example 21
S-IBR-046

S-IBR-046, a recombinant IBR virus with deletions in the Tk, US2, gpG and gpE genes and the bovine viral diarrhea virus gp53 gene inserted in place of the gpE gene, may be constructed in the following manner. S-IBR-046 would be derived from S-IBR-044 (see example 20). It would be constructed utilizing the homology vector 523-78.72, into which the bovine viral diarrhea virus gp53 gene has been inserted, and virus S-IBR-044 in the HOLOMOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. Note that the bovine diarrhea virus gene would be cloned using techniques described in the methods section. The gp53 gene would be placed under the control of the HCMV immediate early promoter. The transfection stock would be screened by the SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES for a white plaque recombinant virus (lacZ substrate). This virus will be useful as a vaccine to protect cattle from infection with IBR virus and bovine viral diarrhea virus.
Example 22

S-IBR-047

S-IBR-047, a recombinant IBR virus with deletions in the Tk, US2, gpG and gpE genes and the parainfluenza type 3 genes for hemagglutinin and fusion protein inserted in place of the gpE gene may be constructed in the following manner. S-IBR-047 would be derived from S-IBR-044 (see example 20). It would be constructed utilizing the homology vector 523-78.72, into which the parainfluenza type 3 virus hemagglutinin and fusion genes has been inserted, and virus S-IBR-044 in the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. Note that the parainfluenza type 3 virus genes would be cloned using techniques described in the methods section. The transfection stock would be screened by the SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES for a white plaque recombinant virus (lacz substrate). This virus will be useful as a vaccine to protect cattle from infection with IBR virus and parainfluenza type 3 virus.
Example 23

S-IBR-049

S-IBR-049, a recombinant IBR virus with deletions in the Tk, US2, gpG and gpE genes and the bovine respiratory syncytial virus genes for the attachment, nucleocapsid and fusion proteins inserted in place of the gpE gene may be constructed in the following manner. S-IBR-049 would be derived from S-IBR-044 (see example 20). It would be constructed utilizing the homology vector 523-78.72, into which the bovine respiratory syncytial virus attachment nucleocapsid and fusion genes had been inserted and virus S-IBR-044 in the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. Note that the bovine respiratory syncytial virus genes would be cloned using techniques described in the methods section. The attachment protein gene would be placed under the control of the HCMV immediate early promoter and the fusion and nucleocapsid protein genes would be placed under the PRV gpX promoter. The transfection stock would be screened by the SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES for a white plaque recombinant virus (lacZ substrate). This virus will be useful as a vaccine to protect cattle from infection with IBR virus and bovine respiratory syncytial.
Example 24

S-IBR-051

S-IBR-051, a recombinant IBR virus with deletions in the Tk, US2, gpG and gpE genes and the Pasteurella haemolytica genes for the leukotoxin and iron regulated outer membrane proteins inserted in place of the gpE gene, may be constructed in the following manner. S-IBR-051 would be derived from S-IBR-044 (see example 20). It would be constructed utilizing the homology vector 523-78.72, into which the Pasteurella haemolytica leukotoxin and iron regulated outer membrane protein genes had been inserted, and virus S-IBR-044 in the HOMOLOGOUS RECOMBINATION PROCEDURE FOR GENERATING RECOMBINANT HERPESVIRUS. Note that the Pasteurella haemolytica genes would be cloned using the techniques described in the methods section. The leukotoxin gene would be placed under the control of the HCMV immediate early promoter and the iron regulated outer membrane protein genes would be placed under the PRV gpX promoter. The transfection stock would be screened by the SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES for a white plaque recombinant virus (lacZ substrate). This virus will be useful as a vaccine to protect cattle from infection with IBR virus and Pasteurella haemolytica.
Example 25

Shipping Fever Vaccine

Shipping fever or bovine respiratory disease (BRD) complex is manifested as a result of a combination of infectious diseases of cattle and additional stress related factors (70). Respiratory virus infections, augmented by pathophysiological effects of stress, alter the susceptibility of cattle to Pasteurella organisms that are normally present in the upper respiratory tract by a number of mechanisms. Control of the viral infections that initiate BRD as well as control of the terminal bacterial pneumonia is essential to preventing the disease syndrome (71).

The major infectious diseases that contribute to BRD are: infectious bovine rhinotracheitis virus, parainfluenza type 3 virus, bovine viral diarrhea virus, bovine respiratory syncytial virus, and Pasteurella haemolytica (71). The applicants' examples 1 through 24 describe vaccine inventions that individually immunize against the various components of BRD. An extension of the applicants' approach is to combine vaccines in a manner so as to control the array of disease pathogens with a single immunization. To this end, at least two approaches can be taken: first, mixing of the various IBR vectored antigens (BRSV, PI-3, BVDV and P. Haemolytica) in a single vaccine dose, and secondly, the individual antigens (BRSV, BVDV, PI-3 and P. haemolytica) can be simultaneously cloned into the same IBR backbone virus. Note that a combination of antigens could be included in one or more IBR backbone viruses so as to limit the number of IBR viruses required for BRD protection. Also, conventionally derived vaccines (killed virus, inactivated bacterins and modified live viruses) could be included as part of the BRD vaccine
formulation should such vaccine components prove to be more effective.
References


30. B. Roizman et al., Cold Spring Harbor Conference on New Approaches to Viral Vaccines (September, 1983).


What is claimed is:

1. A recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpG glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpG glycoprotein.

2. A recombinant IBR virus of claim 1, wherein DNA encoding gpG glycoprotein has been deleted.

3. A recombinant IBR virus of claim 2, wherein foreign DNA has been inserted in place of the deleted DNA encoding gpG glycoprotein.

4. A recombinant IBR virus of claim 1, wherein foreign DNA has been inserted into the DNA encoding gpG glycoprotein.

5. A recombinant IBR virus of claim 1, further characterized in that DNA encoding the gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpE glycoprotein.

6. A recombinant IBR virus of claims 2, 3, or 4, further characterized in that DNA encoding the gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpE glycoprotein.

7. A recombinant IBR virus of claim 5, wherein DNA encoding gpE glycoprotein has been deleted.

8. A recombinant IBR virus of claim 7, wherein foreign DNA is inserted in place of the deleted DNA encoding gpE glycoprotein.
9. A recombinant IBR virus of claim 5, wherein foreign DNA has been inserted into the DNA encoding gpE glycoprotein.

10. A recombinant IBR virus of claim 1, further characterized in that (1) DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and (2) DNA encoding the gpE glycoprotein has been altered or deleted.

11. A recombinant IBR virus of claims 2, 3, or 4, further characterized in that (1) DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and (2) DNA encoding the gpE glycoprotein has been altered or deleted.

12. A recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which (1) DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted, and (2) DNA encoding gpG glycoprotein has been altered or deleted.

13. A recombinant IBR virus of claim 12, wherein DNA encoding the gpG glycoprotein has been deleted.


15. A recombinant IBR virus of claim 12, wherein foreign DNA is inserted in place of the deleted DNA encoding gpG glycoprotein.

16. A recombinant IBR virus of claim 12, wherein foreign DNA is inserted in place of the deleted DNA corresponding to the US2 region of the naturally-occurring IBR virus.
17. A recombinant IBR virus of claim 15, wherein the foreign DNA is a sequence which encodes *Escherichia coli* S-galactosidase.

18. A recombinant IBR virus of claim 17 designated S-IBR-035.

19. A recombinant IBR virus of claim 17 designated S-IBR-036.

20. A recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which DNA encoding gpE glycoprotein has been altered or deleted so that upon replication the recombinant IBR virus produces no gpE glycoprotein.

21. A recombinant IBR virus of claim 20, wherein DNA encoding gpE glycoprotein has been deleted.

22. A recombinant IBR virus of claim 21, wherein foreign DNA is inserted in place of the deleted DNA encoding gpE glycoprotein.

23. A recombinant IBR virus of claim 20, wherein foreign DNA has been inserted in DNA encoding gpE glycoprotein.

24. A recombinant IBR virus of claim 20, further characterized in that DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted.

25. A recombinant IBR virus of claims 21, 22, or 23, further characterized in that DNA corresponding to the US2 region of the naturally-occurring IBR virus has been deleted.
26. A recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which DNA in the unique short region of the naturally-occurring IBR virus has been deleted.

27. A recombinant IBR virus of claim 26, wherein foreign DNA has been inserted into the DNA of the recombinant IBR virus.

28. A recombinant IBR virus of claim 27, wherein foreign DNA has been inserted into the XbaI site in the long unique region.

29. A recombinant IBR virus of claim 27, wherein the foreign DNA is a sequence which encodes bovine rotavirus glycoprotein 38.

30. A recombinant IBR virus of claim 29, wherein the sequence which encodes bovine rotavirus glycoprotein 38 has been inserted into the XbaI site in the long unique region.

31. A recombinant IBR virus of claim 30 designated S-IBR-008.

32. A recombinant IBR virus of claim 26, wherein at least a portion of both repeat sequences has been deleted.

33. A recombinant IBR virus of claim 32 designated S-IBR-027.

34. A recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which at least a portion of both repeat sequences has been deleted.
35. A recombinant IBR virus of claim 34, wherein DNA encoding one or more EcoRV restriction sites has been deleted.

36. A recombinant IBR virus of claim 35 designated S-IBR-002.

37. A recombinant IBR virus of claim 34, wherein foreign DNA has been inserted into the DNA of the recombinant IBR virus.

38. A recombinant IBR virus of claim 37, wherein the foreign DNA is a sequence which encodes the Tn5 NEO gene.

39. A recombinant IBR virus of claim 38 designated S-IBR-020.

40. A recombinant IBR virus of claim 38, wherein at least a portion of the thymidine kinase gene has been deleted.

41. A recombinant IBR virus of claim 40, wherein the Tn5 NEO gene is under the control of an inserted, upstream, HSV-1 alpha-4 promoter.

42. A recombinant IBR virus of claim 41 designated S-IBR-028.

43. A recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which a foreign DNA sequence which encodes the Tn5 NEO gene has been inserted into the viral DNA.

44. A recombinant IBR virus of claim 43, wherein the Tn5 NEO gene is under the control of an inserted, upstream, pseudorabies virus glycoprotein X promoter.
45. A recombinant IBR virus of claim 44 designated S-IBR-004.

46. A recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which a foreign DNA sequence which encodes the *Escherichia coli* β-galactosidase and Tn5 NEO genes, and the parainfluenza type 3 virus hemagglutinin gene, HN, has been inserted into the viral DNA.

47. A recombinant IBR virus of claim 46 designated S-IBR-018.

48. A recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus in which a foreign DNA sequence which encodes the *Escherichia coli* β-galactosidase and Tn5 NEO genes, and the parainfluenza type 3 virus fusion gene, F, has been inserted into the viral DNA.

49. A recombinant IBR virus of claim 48 designated S-IBR-019.

50. A vaccine which comprises a suitable carrier and an effective immunizing amount of the recombinant virus of claim 26, 34, 43, 46, or 48.

51. A method of immunizing an animal against infectious bovine rhinotracheitis virus which comprises administering to the animal an effective immunizing dose of the vaccine of claim 50.

52. A method of claim 51, wherein the animal is a bovine.

53. A method for distinguishing an animal vaccinated with a vaccine which comprises an effective
immunizing amount of a recombinant virus of claims 1, 5, 10, or 12 from an animal infected with a naturally-occurring IBR virus which comprises analyzing a sample of a body fluid from the animal for the presence of gpG glycoprotein of IBR virus and at least one other antigen normally expressed in an animal infected by a naturally-occurring IBR virus, identifying antigens which are present in the body fluid and determining whether gpG glycoprotein is present in the body fluid, the presence of antigens which are normally expressed in an animal by a naturally-occurring IBR virus and the absence of gpG glycoprotein in the body fluid being indicative of an animal vaccinated with the vaccine and not infected with a naturally-occurring IBR virus.

54. The method of claim 53, wherein the presence of antigens and gpG glycoprotein in the body fluid is determined by detecting in the body fluid antibodies specific for the antigens and gpG glycoprotein.

55. A method for distinguishing an animal vaccinated with a vaccine which comprises an effective immunizing amount of a recombinant virus of claims 5, 10, 20, or 24 from an animal infected with a naturally-occurring IBR virus which comprises analyzing a sample of a body fluid from the animal for the presence of gpE glycoprotein of IBR virus and at least one other antigen normally expressed in an animal infected by a naturally-occurring IBR virus, identifying antigens which are present in the body fluid and determining whether gpE glycoprotein is present in the body fluid, the presence of antigens which are normally expressed in an animal by a naturally-occurring IBR virus and the absence of gpE glycoprotein in the body fluid being
indicative of an animal vaccinated with the vaccine and not infected with a naturally-occurring IBR virus.

56. The method of claim 55, wherein the presence of antigens and gpE glycoprotein in the body fluid is determined by detecting in the body fluid antibodies specific for the antigens and gpE glycoprotein.

57. Isolated DNA encoding the gpG glycoprotein of IBR virus.

58. Purified recombinant gpG glycoprotein encoded by the DNA of claim 57.

59. A recombinant cloning vector which comprises the DNA of claim 57.

60. A recombinant expression vector which comprises the DNA of claim 57.

61. A host cell which comprises the recombinant expression vector of claim 60.

62. A method of producing a polypeptide which comprises growing the host cell of claim 61 under conditions such that the recombinant expression vector expresses gpG glycoprotein and recovering the gpG glycoprotein so expressed.

63. An antibody directed to an epitope of the purified gpG glycoprotein of IBR virus of claim 58.

64. A monoclonal antibody of claim 63.

65. A method of detecting the presence or absence of gpG glycoprotein of IBR virus in a sample which
comprises contacting the sample with the antibody of claim 63 under conditions such that the antibody forms a complex with any gpE glycoprotein present in the sample and detecting the presence or absence of such complex.

66. A method of claim 65, wherein the sample is bovine-derived.

67. Isolated DNA encoding the gpE glycoprotein of IBR virus.

68. Purified recombinant gpE glycoprotein encoded by the DNA of claim 67.

70. A recombinant cloning vector which comprises the DNA of claim 67.

71. A recombinant expression vector which comprises the DNA of claim 70.

72. A method of producing a polypeptide which comprises growing the host cell of claim 70 under conditions such that the recombinant expression vector expresses gpE glycoprotein and recovering the gpE glycoprotein so expressed.

73. An antibody directed to an epitope of the purified gpE glycoprotein of IBR virus of claim 68.

74. A monoclonal antibody of claim 73.

75. A method of detecting the presence or absence of gpE glycoprotein of IBR virus in a sample which comprises contacting the sample with the antibody of claim 73 under conditions such that the antibody forms a complex with any gpE glycoprotein present in
the sample and detecting the presence or absence of such complex.

76. A method of claim 75, wherein the sample is bovine-derived.

77. A method of producing a fetal-safe, live recombinant IBR virus which comprises treating viral DNA from a naturally-occurring live IBR virus so as to delete from the virus DNA corresponding to the US2 region of the naturally-occurring IBR virus.

78. A recombinant pseudorabies virus designated S-PRV-160.

79. An antibody which recognizes the recombinant pseudorabies virus of claim 78.

80. Isolated DNA encoding the US2 gene of an IBR virus.

81. A homology vector for producing a recombinant IBR virus by inserting foreign DNA into the genomic DNA of an IBR virus which comprises a double-stranded DNA molecule consisting essentially of:

(a) double-stranded foreign DNA encoding RNA which does not naturally occur in an animal into which the recombinant IBR is introduced;

(b) at one end of the foreign DNA, double-stranded IBR viral DNA homologous to genomic DNA located at one side of a site on the genomic DNA which is not essential for replication of the IBR virus; and

(c) at the other end of the foreign DNA, double-stranded IBR viral DNA homologous to genomic
DNA located at the other side of the same site on the genomic DNA.

82. A homology vector of claim 81, wherein double-stranded foreign DNA further comprises a promoter.

83. A homology vector of claim 82, wherein the promoter is from HSV-1 α 4 immediate early gene, Human cytomegalovirus immediate early gene or pseudorabies virus glycoprotein X gene.

84. A homology vector of claim 81, wherein double-stranded foreign DNA further comprises a polyadenylation signal.

85. A homology vector of claim 84, wherein the polyadenylation signal is from HSV-1 thymidine kinase gene or pseudorabies virus glycoprotein X gene.

86. A homology vector of claim 81, wherein the RNA encodes a polypeptide.

87. A homology vector of claim 86, wherein the polypeptide is a detectable marker.

88. A homology vector of claim 87, wherein the polypeptide is Escherichia coli β-galactosidase or bacterial transposon neomycin resistance protein.

89. A homology vector of claim 88, wherein DNA which encodes the polypeptide is flanked on each side by restriction sites permitting said DNA to be cut out with a restriction endonuclease which cuts at a limited number of sites on the genome.
90. A homology vector of claim 81, wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 860 bp NcoI to BamHI subfragment of the HindIII A fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 1741 bp BglII to StuI subfragment of the HindIII A fragment of IBR virus.

91. A homology vector of claim 90, wherein upstream double-stranded foreign DNA which comprises a promoter and downstream double-stranded foreign DNA which comprises a polyadenylation signal flank on each side double-stranded foreign DNA which encodes a detectable marker.

92. A homology vector of claim 91, wherein the upstream promoter is homologous to genomic DNA present within the approximately 490 bp PvuII to BamHI subfragment of the BamHI N fragment of HSV-1 and the downstream polyadenylation signal is homologous to genomic DNA present within the approximately 784 bp SmaI to SmaI subfragment of the BamHI Q fragment of HSV-1.

93. A homology vector of claim 91, wherein the DNA which encodes a detectable marker is homologous to the approximately 1541 bp BglIII to BamHI fragment of Tn5.

94. A homology vector of claim 81, wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 3593 bp HindIII to XhoI subfragment of the HindIII K fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 785 bp XhoI to NdeI subfragment of the HindIII K fragment of IBR virus.
95. A homology vector of claim 94, wherein upstream double-stranded foreign DNA which comprises a promoter and downstream double-stranded foreign DNA which comprises a polyadenylation signal flank on each side double-stranded foreign DNA which encodes a detectable marker.

96. A homology vector of claim 95, wherein the upstream promoter is homologous to genomic DNA present within the approximately 1191 bp AvaII to PstI subfragment of the XbaI B fragment of HCMV and the downstream polyadenylation sequence is homologous to genomic DNA present within the approximately 753 bp SalI to NdeI subfragment of the BamHI #7 fragment of PRV.

97. A homology vector of claim 95, wherein the DNA which encodes a detectable marker is homologous to the approximately 3347 bp BstII to BamHI fragment of pJF751.

98. A homology vector of claim 81, wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 888 bp MluI to SmaI subfragment of the HindIII K fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 785 bp XhoI to NdeI subfragment of the HindIII K fragment of IBR virus.

99. A homology vector of claim 98, wherein upstream double-stranded foreign DNA which comprises a promoter and double-stranded foreign DNA which comprises a polyadenylation signal flank on each side double-stranded foreign DNA which encodes a detectable marker.
100. A homology vector of claim 99, wherein the upstream promoter is homologous to genomic DNA present within the approximately 1191 bp AvaII to PstI subfragment of the XbaI B fragment of HCMV and the downstream polyadenylation signal is homologous to genomic DNA present within the approximately 753 bp SalI to NdeI subfragment of the BamHI #7 fragment of PRV.

101. A homology vector of claim 99, wherein the DNA which encodes a detectable marker is homologous to the approximately 3347 bp BalI to BamHI fragment of pJF571.

102. A homology vector of claim 81, wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 1704 bp SmaI to SmaI subfragment of the HindIII K fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 742 bp NheI to BglII subfragment of the SmaI 2.5KB fragment of IBR virus.

103. A homology vector of claim 102, wherein upstream double-stranded foreign DNA which comprises a promoter and downstream double-stranded foreign DNA which comprises a polyadenylation signal flank on each side double-stranded foreign DNA which encodes a detectable marker.

104. A homology vector of claim 103, wherein the upstream promoter is homologous to genomic DNA present within the approximately 413 bp SalI to BamHI subfragment of the BamHI #10 fragment of PRV and the downstream polyadenylation signal is homologous to genomic DNA present within the approximately 754 bp NdeI to SalI subfragment of the BamHI #7 fragment of PRV.
105. A homology vector of claim 103, wherein the detectable marker is homologous to the approximately 3010 bp BamHI to PvuII fragment of pJF751.

106. A homology vector for producing a recombinant IBR virus by deleting DNA which encodes a detectable marker which had been inserted into the genomic DNA of an IBR virus comprising a double-stranded DNA molecule consisting essentially of double-stranded IBR viral DNA homologous to the genomic DNA which flank on each side the DNA to be deleted.

107. A homology vector of claim 106, wherein the upstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 888 bp MluI to SmaI subfragment of the HindIII K fragment of IBR virus and the downstream double-stranded IBR viral DNA is homologous to genomic DNA present within the approximately 785 bp XhoI to NdeI subfragment of the HindIII K fragment of IBR virus.

108. A vaccine which comprises a suitable carrier and an effective immunizing amount of the recombinant virus of claim 1, 5, 10, 12, 20 or 24.

109. A method of immunizing an animal against infectious bovine rhinotracheitis virus which comprises administering to the animal an effective immunizing dose of the vaccine of claim 108.

110. A method of claim 109, wherein the animal is a bovine.

111. A recombinant IBR virus of claim 37, wherein the foreign DNA is a sequence which encodes Bovine viral diarrhea virus glycoprotein gp53.
112. A recombinant IBR virus of claim 111, designated S-IBR-032.

113. A recombinant IBR virus comprising viral DNA from a naturally-occurring IBR virus from which DNA from the US2 gene, the gpE glycoprotein gene and the gpG glycoprotein gene has been deleted so that upon replication, the recombinant IBR virus produces no gpE glycoprotein and no gpG glycoprotein.

114. A recombinant IBR virus of claim 113, wherein a foreign DNA sequence is inserted in place of the DNA which encodes gpE glycoprotein.

115. A recombinant IBR virus of claim 114, wherein the foreign DNA sequence encodes *Escherichia coli* β-galactosidase.

116. A recombinant IBR virus of claim 115, designated S-IBR-039.

117. A recombinant IBR virus of claim 113, wherein DNA from the thymidine kinase gene has been deleted so that upon replication, the recombinant IBR virus produces no thymidine kinase.

118. A recombinant IBR virus of claim 117, designated S-IBR-045.

119. A recombinant IBR virus of claim 117, wherein a foreign DNA sequence is inserted in place of the DNA encoding gpE glycoprotein.

120. A recombinant IBR virus of claim 119, wherein the foreign DNA sequence encodes *Escherichia coli* β-galactosidase.
121. A recombinant IBR virus of claim 120 designated S-IBR-044.

122. A recombinant IBR virus of claim 119, wherein the foreign DNA sequence encodes Bovine viral diarrhea virus gp53 glycoprotein.

123. A recombinant IBR virus of claim 122 designated S-IBR-046.

124. A recombinant IBR virus of claim 119, wherein a foreign DNA sequence is inserted in place of the DNA encoding thymidine kinase.

125. A recombinant IBR virus of claim 124, wherein the foreign DNA sequence inserted in place of the DNA encoding thymidine kinase encodes *Escherichia coli* β-glucuronidase.

126. A recombinant IBR virus of claim 125, wherein the foreign DNA sequence inserted in place of the DNA encoding gpE glycoprotein encodes *Escherichia coli* β-galactosidase.

127. A recombinant IBR virus of claim 126, designated S-IBR-043.

128. A recombinant IBR virus of claim 119, wherein the foreign DNA sequence encodes Parainfluenza virus type 3 fusion protein and Parainfluenza virus type 3 hemagglutinin protein.

129. A recombinant IBR virus of claim 128, designated S-IBR-047.

130. A recombinant IBR virus of claim 119, wherein the foreign DNA sequence encodes Bovine respiratory
syncytial virus fusion protein, Bovine respiratory syncytial virus attachment protein and Bovine respiratory syncytial virus nucleocapsid protein.

131. A recombinant IBR virus of claim 130, designated S-IBR-049.

132. A recombinant IBR virus of claim 119, wherein the foreign DNA sequence encodes Pasteurella haemolytica leukotoxin and Pasteurella haemolytica iron regulated outer membrane proteins.

133. A recombinant IBR virus of claim 132, designated S-IBR-051.

134. A vaccine which comprises an effective immunizing amount of the recombinant IBR virus of claim 118 and a suitable carrier.

135. A vaccine which comprises an effective immunizing amount of the recombinant IBR virus of claim 123 and a suitable carrier.

136. A vaccine which comprises an effective immunizing amount of the recombinant IBR virus of claim 129 and a suitable carrier.

137. A vaccine which comprises an effective immunizing amount of the recombinant IBR virus of claim 131 and a suitable carrier.

138. A vaccine which comprises an effective immunizing amount of the recombinant IBR virus of claim 133 and a suitable carrier.

139. A vaccine which comprises an effective immunizing amount of a recombinant virus protective against
Bovine respiratory disease complex and a suitable carrier.

140. A vaccine of claim 139, wherein the recombinant virus is a recombinant IBR virus.

141. A vaccine of claim 140, wherein the recombinant virus consists essentially of any or all of the recombinant IBR viruses of claims 118, 123, 129, 131 and 133.

142. A vaccine of claim 139 further comprising non-recombinant virus.

143. A vaccine of claim 140 further comprising non-recombinant virus.

144. A vaccine of claim 143, wherein the recombinant IBR virus consists essentially of any or all of the recombinant viruses of claims 117, 122, 134, 136 and 138.

145. A method of immunizing an animal against infectious bovine rhinotracheitis which comprises administering to the animal an immunizing dose of the vaccine of claim 134, 135, 136, 137 or 138.

146. A method of immunizing an animal against bovine viral diarrhea which comprises administering to the animal an immunizing dose of the vaccine of claim 135.

147. A method of immunizing an animal against Parainfluenza type 3 which comprises administering to the animal an immunizing dose of the vaccine of claim 136.
148. A method of immunizing an animal against Bovine respiratory syncytial virus disease which comprises administering to the animal an immunizing dose of the vaccine of claim 137.

149. A method of immunizing an animal against Pneumonic pasteurellosis which comprises administering to the animal an immunizing dose of the vaccine of claim 138.

150. A method of immunizing an animal against bovine respiratory disease complex which comprises administering to an animal an immunizing dose of the vaccine of claim 141.

151. A method of immunizing an animal against bovine respiratory disease complex which comprises administering to an animal an immunizing dose of the vaccine of claim 144.

152. The method of claim 145, wherein the animal is a bovine.

153. The method of claims 146-151, wherein the animal is a bovine.

154. A method for distinguishing an animal vaccinated with a vaccine which comprises an effective immunizing amount of a recombinant virus of claim 118 from an animal infected with a naturally-occurring IBR virus which comprises analyzing a sample of a body fluid from the animal for the presence of gpE glycoprotein of IBR virus and at least one other antigen normally expressed in an animal infected by a naturally-occurring IBR virus, identifying antigens which are present in the body fluid and determining whether gpE glycoprotein is
present in the body fluid, the presence of antigens which are normally expressed in an animal by a naturally-occurring IBR virus and the absence of gpE glycoprotein in the body fluid being indicative of an animal vaccinated with the vaccine and not infected with a naturally-occurring IBR virus.
Figure 3

TTAACGTTGCCGTCGGCTGCCATGTTGACTATAGTCACGTTGGCCGGATAGGCGC
MetValThrIle

TCGGCTTCCCAAGAACCGCCGCACTGACGCGCCGCGGCAGTTGCCTTTCTGCCGACAG
AGCCGGGCGCTGACGCAAGCGCCTGGGAGACGATCGCTTGGCTTCGATACGCCTGA
GTCATACCGTCAGATCGAACGCGGCCGTCGGGTCGTCCTGACCGGGTCGCTGGCC
TTGTCGAGAGGGCTCCCTGGCCGGCCTGGCGGCGGGCTTAGCGCCACCGGACGCGG
AGTTGATCGCTGCGACGGTCAACAATTTGCTGAACACTCGGCGCCGCGAGGGCTGGCC
CTGCCACATGTGGGTGTTGTCGGCGCGCGATTTTGTACGCCCTATTTTTCGCACATTGC
GGACACGACCGGTGGTTTACGCAGCAGCTGAACTGTACGTACGGGTACGAGCGGCTGGCC
CGTCCCGCGCCGCGGGCCGCCATCGGTACGCGGTCGCGCCGCCCTACGATACCCGCACACTC
CCGGTACCGGCTGCTGCCTGCTCCTTTCCGGCTGGTCTCTACGAAACTGCTAGACCGGCGG
CGGCCCGCCCGCCCGCGGGACACCGGAGCGCCCGCGGGCGCGGGCGCGGGCCTAGCTG
GCTCTATACCTTTAAGGCCGACCCGACCGCCGCGGCTTGGTCTATTTGCTTTTGTCGACC
CGGCGAGGCCACATGTTCCGGAGGGCAACCCCAACCAGGGTGGGTGATCAGACAGTGCG
CTTGGACAGAGGGCGACCCCGACCCCGCACCCCGCCAGGCTCCCGGATCGGAGGGGGG
GGCGGGCTGCTGGGCTGGGCCACTTTAACGTCGGACACGAAAGACGCGGGTTGCTAGC
TCGTCGCCCGGACATTTTTCGCGTTGCTCCCTCGTCCAGCCGCTGCTGGAAGTTCC
..........ProSerProCysTrp---

CCGCTCCCGCGCGAGTGCCCAGACCGAATGGGGGCGCAGTTACCTTGGATGTGTTCCCG
CGCCCGGCGCCGACCGGCTGCTGGTCTTTCTGCAGGTTCTCATTGGTAAAGCTT
Figure 4(a)

IBR US2 (309)

HSV-1 US2 (291)
Figure 4(b)

H-MWGFADLYAPFAN
H-LWVGADLCVFIFYA
H-LWILGAADLQQVLLA
H-LWVGADLCVFIFEYA
HSLWVGADICRIALECI

115 124 148 123 132

IBR US2
HSV-1 US2
PRV US2
HSV-2 US2
MDV US2

SUBSTITUTE SHEET
Figure 5-2

IBR Cooper HindIII O
TGAGCGCGCCGCTGCATGCTGGTGCGAACTC

IBR Nasalgen HindIII K
CTAGTAAAAAGGCGAAGGGCTGGTGCGAACTC

IBR Cooper HindIII K
CTAGTAAAAAGGCGAAGGGCTGCAACGTGACG

LVKTAKGCTST

US2 (58)

ACGCGAGCGCGCTGCAGCAAGCTT

ACGCGAGCGCGCTGCAGCAAGCTT

TCAACGTCAGCGACGCGCGGCTGG

S T S S Q R R G W
Figure 7-2

TK (156-357)  
TK (1-62)  
NEO (1-264)  
IBR  
HSV-1  
Tn5  
PA  
Frag. 1  
Frag. 2  
Frag. 3  
Frag. 4  
Frag. 5  
800 BP

SUBSTITUTE SHEET
Figure 7-4

Junction B

TTA AGT GGG ATC CCG-GCG CGC AGG CGC GCA-CGT CGG TCG CGG TCG-CGC
Leu Ser Gly Ile

TK (62)

HindIII A

Ncol BamHI XbaI

GCC ATG GGG GAT-CCT CTA GAG CTT GGG-CTG CAG GTC CTG ATT-GAT ACA CTG

[ ] [PvuII]

HSV-1 BamHI N
Junction D

GGA CCT TGC ACA GAT-AGC GTG GTC CGG CCA-GGA CGA CGA GGC

BamHI

TTG-CAG GAT CCT CTA GAG-TCG GGA GAT GGG GGA-GGC TAA CTG AAA CAC-GGA AGG AGA Gly Asp Gly Gly Gly

Tn5

[Smal]

[Smal] → TK (350)

[Smal] → HSV-1 BamHI Q

Figure 7-6
Figure 7-7

Junction E

GTG TTG CTG CGT TCC CGA CCT GCA GCC CAA GCT CTA GAG TCG ACC TGC AGC

HSV-1 BamHI Q

BglII

CCA AGC TCA GAT CTG CTC ATG CTC GCG GCC GCC ATG CCC CCG GAA GCG

Asp Leu Leu MET Leu Ala Ala Ala MET Pro Pro Glu Ala

| → TK (156) |
| → HindIII A |
19/83

Figure 9(a)

IBR gpG (4412)

PRV gpG (498)
Figure 9(b)

IBR gpG 95

PRV gpX 89

HSV-2 gpG 111

V
C
P
R
Y
C
G
P
C
**Figure 11-1**

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<td>HCMV</td>
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Size:
- ~2999 BP
- ~182 BP
- ~2121 BP
- ~121 BP
- ~760 BP
Figure 11-3

Junction A

**HindIII**

CAC ATA CGA TTT AGG-TGA CAC TAT AGA ATA-CAG GCT TGG GCT GCA-G

**PstI**

SalI XbaI SalI PstI

GT CGA CTC TAG AGT-CGA CCT GCA GTG AAT-AAT AAA ATG TGT GTT-TGT CCG AAA TAC

pSP64 ←| HCMV XbaI B (Fragment 1) →
Figure 11-4

Fragment 1

GCG=TTC GAG ATT TCT GTC=CCG ACT AAA TTC ATG=TCG CGC GAT AGT

GGT=GTT TAT CGC CGA TAG=AGA TGG CGA TAT TGG=AAA AAT CGA TAT TTG=AAA ATA TGG
Figure 11-6

Junction C

ATG TCT CTC GAG GGG CTC GAG GCC GCT CTC GAG GCC
Met Ser Leu Gln Glu Pro Ala Arg Leu Glu Gly Leu Pro Ser Gln Leu Pro Val

XhoI

IBR HindIII K gpg (362)

PRV BamHI #2 gpIII (421)

TTC GAG GAC CAG CGC CTC GAC GCC TCC CCC GCG TCC
Phe Glu Asp Thr Gln Arg Tyr Asp Ala Ser Val Asp Ala Ser Val Ser Trp

(Fragment 3)
Figure 11-7

Junction D  CCC GTG AGC AGC ATG ATC GTC GTC ATC GCC GCC ATC GGG ATC CTG GCC
             Pro Val Ser Ser MET Ile Val Val Ile Ala Gly Ile Gly Ile Leu Ala

(Fragment 3)  PRV BamHI #2

NdeI
  -ATC GTG CTG GTC ATC CAT ATG GCG ATC ATC AGG GCC CGG GCC CGG AAC GAC GCC
  Ile Val Leu Val Ile His MET Ala Ile Ile Arg Ala Arg Ala Arg Asn Asp Gly

gpIII (467)  gpX (480)
            ➔ PRV BamHI #7
Figure 11-8

Junction E

GGG CCA GTA CCG GCG=CTT GGT GTC CGT CGA=CTC TAG AGT CGA CCT

PRV BamHI #7  \[\text{DNA sequence} \]

\|--|\|--|

pSP65

PstI HindIII

=GCA GCC CAA GCT TTG GCG TAA TCA TGG TCA
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<td>PRV BamHI #7</td>
<td>SalI - NdeI*</td>
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<td>~785 BP</td>
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* resected with ExoIII/S1
Figure 12-4

Junction B
GTC GAA GTG CTC GAA-ATT CGA GCT CGC CCG-GGG ATC CTC TAG
Val Glu Val Leu

gpG (262) ←
IBR HindIII K ←

SalI PstI SalI XbaI [SalI]
AGT-CGA CCT GCA GGT CGA-CTC TAG AGG ATC TCG=ACG GAC ACC AGG CGC=CGG TAC

--- PRV BamHI #7
Figure 12-6

Junction D  TCC CAG TCA CGA CGT=TGT AAA ACG ACG GGA=TCC ATG GTC CCG

BamHI  NcoI  AvaiI

lacZ (10) ←
pJF751 ←

Met<--

GTG=TCT TCT ATG GAG

→ HCMV IE promoter
→ HCMV XbaI B
Figure 12-7

Junction E

\[\text{PstI} \quad \text{SalI} \quad \text{XbaI} \quad \text{BamHI}\]

\[\text{ATT CAC TGC AGG TCG\text{-ACT} CTA GAG GAT CCC\text{-CGG}}\]

\[\text{HCMV IE promoter} \leftarrow\]
\[\text{HCMV XbaI B} \leftarrow\]

\[\text{SacI} \quad \text{[EcoRI]} \quad \text{[XhoI]}\]

\[\text{GCG AGC TCG AAT\text{-TTC} GAG CGC CGC} \quad \text{Glu Arg Arg}\]

\[\text{\rightarrow gpG (362)}\]
\[\text{\rightarrow IBR HindIII K}\]
Figure 12-8

Junction F

GCG CGC GCG TAC AAC-GCC ACG GTC ATA GGG-CGA GCT CGA ATT CGT
Ala Arg Ala Tyr Asn Ala Thr Val Ile

[NdeI][SmaI]  SacI  EcoRI

gpIV (106) <-
IBR HindIII K <-> pSP64

=AAT CAT GGT CAT
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* resected with ExoIII/S1
Figure 13-4

Junction B

IBR HindIII K <---

SalI  PstI  SalI  XbaI  [SalI]
GTC=GAC  CTG  CAG  GTC  GAC=TCT  AGA  GGA  TCT  CGA=CGG  ACA  CCA  GGC  GCC=GGT  ACT  GGC  CCT

--- PRV BamHI #7
Figure 13-5

Junction C

GGG CGG GGC CGG GTC-AGC CGG ATC TAG AGT-CGC AGG ACC CAA CGC-

XbaI [Ball] XbaI

gpX (term) ← PRV BamHI #7 ←

TGC CGG AGT TTG

---------→ lacY (71)

---------→ pJF751
Figure 13-7

```
PstI  SalI  XbaI  BamHI
     /     /     /     /
Junction E  ATT  CAC  TGC  AGG  TCG=ACT  CTA  GAG  GAT  CCC=CGG
       /                             /
HCMV IE promoter <-
HCMV XbaI B <-

Sacl  [EcoRI]  [XhoI]
     /     /     /
GCG  AGC  TCG  AAT=TTC  GAG  CGC  CGC  Glu  Arg  Arg
       /                             /
|-> gpg (362)
|-> IBR HindIII K
```
Figure 13-8

Junction F

[NdeI] [SmaI] SacI EcoRI

GCG CGC GCG TAC AAC = GCC ACG GTG ATA GGG = CGA GCT CGA ATT CGT

Ala Arg Ala Tyr Asn Ala Thr Val Ile

gplV (106) ← IBR HindIII K ← → pSP64

=AAT CAT GGT CAT
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Figure 14-1
Figure 14-4

Junction B

[SmaI][EcoRI] SacI

CGG GGT AGC CCC AAT TCG AGC TCG CCC GGG GAT CCT CTA GAG GAT CCC

IBR HindIII K ←

SacI [EcoRI] [XhoI]

CGG GCG AGC TCG AAT TTC GAG CGC CGC CCC GAT GCC

Glu Arg Arg Pro Asp Ala

gpG (362)

IBR HindIII K
Figure 14-5

Junction C

GCG CGC CGC TAC AAC=GCC ACG GTC ATA GGG=CGA GCT CGA ATT CGT
Ala Arg Ala Tyr Asn Ala Thr Val Ile

[NdeI][SmaI] SacI EcoRI

gLpIV (106) ←
IBR HindIII K ←

→ pSP64

=AAT CAT GGT CAT
Figure 15-1

GCGGGCAGCGCGCACAGAGACCGGGGAGGACGGGCAGCGCAGCGTTGAGCG
GCCGGACCGCGCGGCGGGTGTATGCGGTTACCACACCCCGACG
MetGlyLeuAlaArg

GGAGAACCAGCGCGACGCTTCGCTGCTGCTGCTGCCGGAGCTGCGTTCCGGGGAGGACGGCG
CGCGCGAGAGGTTTCGAAAAGGGCATTTGCGAATGCAACCCACCCGGCAGGGCAGCCCG
GGTGGCGCGCTGCTGCTGCGCAGCTTATTTGCTTTTCCGGGTGATGGCCAGGCCAGCAGCC
GCGAACGAAACCCCGGCTGGTGGCTGACAGGCTTCCTACGGCGCGCGCTGCGCGC
CCCGTCTTTCTCCAGGGCGCGCGCGCGCGACGTGCGCGCCGCTGCGGCTGGAGC
GTCTCGCGCGCCGCTGCCGCTGCGCAGCTGCGGAGGCTCTCAGCTGACGCAGCCGAG
TGCTTTCACCAGACGTGGCCCTGGACGGCGCGCGTGCTGCTGCGGAAACCCGGGGCGCGCTG
GCCATCCGCGGAAGCTCGCGAGCGCGCCGGCCGAACGACGCGACAGACCAGGGCCCGAAGGATTTGGTCTCGCC
GCCCGACGCTTCGCGCGACGATGCGGACCCCAGCGGGGTGCTGCTACGC CGCCCGCA
GCGAGGAGAGCCACGCACGCGCTTACCTTCGTCTGACGGCTCATCGCGCACGGCCGGCGAC
GAGGAGACGGAGTTTCGCGAGCGCTTACAGTCGCGACCGCGCGCGCGCGCGGACGGGCAGGGCAGGCGCG
CGGGAAGGAGGACGAGGCAGGGCCGCCAACGACGGGCGCGCCGCCGCCACCGACG
ACGACACGGCGGCGCCCCGCGCGCGACGCGCGAGCGGCGCGCTTCCGCGGTGCTGCCTCNTACGCTGG
CAGTTACGACCCCGGCGGATTCCCTTCTCTGTGCTATCGGTGCTGCTGCAGTCTGCTGGTTTTC
GACGAGCTCCCTTCCTGCGACGATCGACTGTCTCTTCTGCAGGGACGCCGCGGCACTGC
GCCCTATCCGCGATACAGAGACGTGCATCTTCACCACCCCGAGGCCACCGCCCTGCTGAC
CCCGCCGACCGCAACTGCGAGCTCTCGGCTGCGCGTACGCTCCGAGACCGTGACCTACGAGCCG
CTGATGCGAGACGGCGCCGCGACCTGCGTCGCGGCGACCGAAGCCGAGTGCTGCGAGGGCAGCC
GCCCTACCGGGCGCCGCTGGCGAATCCCGTGCCTCCGCGCAATAACAGCGTAGACCTGCTTTT
GACGACGCGGCGCCGCTCGGCGGCTCCGCGGGCTTTACGTCTTTGCTGCGTACGTAACGGCCAC
Figure 16(a)

IBR gpE (617)

PRV gI (577)
**Figure 17-1**

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<td>~832 BP</td>
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</table>
Figure 17-2

Substitute Sheet
Figure 17-3

Junction A

PstI  SalI  XbaI  BamHI  [SmaI]  [NdeI]

TTG  GGC  TGC  AGG  TCG-ACF  CTA  GAG  GAT  CCC=C TA  TGG

Trp

pSP64

TAC  AAG  ATC=GAG  AGC  GGG  TGC  GCC=C GG  CCG  CTG  TAC  TAC=ATG  GAG  TAC

Tyr  Lys  Ile  Glu  Ser  Gly  Cys  Ala  Arg  Pro  Leu  Tyr  Tyr  MET  Glu  Tyr

→ gIV (107)
→ IBR HindIII K
Junction B

TCC GGG CTT TAC GTC TTT GTG CTG CAG TAC AAC GGC CAC GTG
Ser Gly Leu Tyr Val Phe Val Leu Gln Tyr Asn Gly His Val

HindIII

GAA GCT GGG GAC TAC AGC CTA GTC GTT ACT TCG GAC CGT TTG
Glu Ala Trp Asp Tyr Ser Leu Val Val Thr Ser Asp Arg Leu

IBR HindIII K ←——

→ IBR SmaI 2.5 KB
Figure 17-5

Junction C  CCT TCA CCG CCG CCG-GAA GGC TCC ATC GTG-TCC ATC CCC ATC

IBR SmaI 2.5 KB  

\[\text{SaCl} \quad \text{[SmaI]} \quad \text{BamHI} \quad \text{XbaI} \quad \text{SalI} \quad \text{PstI}\]
\[\text{CTC-GAG CTC GAA TTG GGG-ATC CTC TAG AGT CGA-CCT GCA GCC}\]

\[\rightarrow \text{pSP65}\]
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**Figure 18-1**
Figure 18-3

Junction A

CTA TAG AAT ACA CGG=AAT TCG AGC TCG CCC=GGG TGA

Pro Gly ---

pSP65 <-

GCG GCC TAG=GCC CTC CCC CGA CCG

--> gIV (416)

--> IBR HindIII K
Figure 18-4

Junction B

ATG GCC GAG GCC AAG=CCC GCG ACC GAA ACC=CAG GGG ATC C
MET Ala Glu Ala Lys Pro Ala Thr Glu Thr Pro
gpe (76)
IBR HindIII K

XbaI SalI

TAG=AGT CGA CGT CTG GGG=CAGC GGG GGT GGT GCT=CTT CGA GAC GCT GCC

→ PRV BamHI #10
Junction C

ACC TTT GCG CAT CTC=CAC AGC TCA ACA ATG=AAG TGG GCA ACG TGG=ATC

MET Lys Trp Ala Thr Trp Ile

gpX (7) [---]

PRV BamHI #10 [---]

---

GAT CCC GTC GTT=TTA CAA CGT CGT GAC=TGG GAA AAC CCT GGC
Asp Pro Val Val Leu Gln Arg Arg Asp Trp Glu Asn Pro Gly

---

lacZ (10) [---]

pJF751 [---]
Figure 18-6

Junction D

TGG AGC CCG TCA GTA=TGG GCG GAA ATC CAG=CTG AGC GCC GGT
Trp Ser Pro Ser Val Ser Ala Glu Ile Gln Leu Ser Ala Gly

pJF751 <---------------------->
BglII, XbaI

CGC=TAC CAT TAC CAG TTG=GTC TGG TGT CAA AAA=GAT CTA GAA TAA GCT=AGA
Arg Tyr His Tyr Gln Leu Val Trp Cys Gln Lys Asp Leu Glu --
lacZ (1024) <---------------------->

[NdeI]

GGA TCG ATC CCC=TAT GGC GAT CAT CAG=GGC CCG ATC CCC
TAT=GGC GAT CAT CAG
Me tAl aIl eIl eAr
|-> gpX(480)
|-> PRV BamHI #7

GGC=CCG GGC CCG GAA CGA=CGG CTA CCG CCA CGT=GGC CTC CGC CTG ACC=CCG CCC
gAl aAr gAl aAr gAs nAs pGl yTy rAr gHi sVa tAl aSe rAl a--

CGC CCG ACT=CCC CCG
Figure 18-7

Junction E

Ggc Gcc Tgg TGT Ccg=TCG Act Cta Gag Tcg=acc Tgc Agc Cca

PRV BamHI #7

[NheI]

AGC=TCT AGC AAC CCC CCT=CGG GAT GCC TAC GAC=CTC GCC GGC GCC CCA
Ser Asn Pro Pro Ala Asp Ala Tyr Asp Leu Ala Gly Ala Pro

→ gpE (548)

→ IBR SmaI 2.5KB
67/83
FIGURE 19
- PLASMID  ■ IBR  □ gpX promoter  □ Neo  □ PolyA

10kb

HindIII  IBR HindIII K
Sacl Ndel Xho Xho SaLI MLul SaLI
pSY524 pSP65

1. cut HindIII + Xhol (4.5kb)
2. ligate to HindIII + SaLI, cut psp65

HindIII  [Xhol/SaLI]
Sacl Ndel EcoRI
pSY846

1. cut Ndel+EcoRI
2. klenow fill in

HindIII  [Ndel/EcoRI]
Sacl
pSY862

1. cut Sacl
pSY845
1. cut HindIII

HindIII  [BamHI]
P → polyA
Xbal
+ IBR COOPER DNA
S-IBR-004

[Sacl]

10kb

Xbal  Xbal
P → → polyA
Neo

SUBSTITUTE SHEET
AGGAACAAAGTTGTCTCAACACACAGCAGCGAAGCAAGACCCAAAAGGCAGCGCAGGGCGACACCCGAACCC
AAATGGAATATTTGAAACACACAAAACAGCACAACAAAACACCAACATGAAAACGAACAAACACAGAGGG

METGluTyrTrpLys.............

ACACAGTAGCAAGGTTACAACATACATATAATGACACCTTCTGGACAATAAACATCAAAATATTATTAGTC
ATTTTTATAATGATATGGCAAAAATCTAAATTCAAGAGAAAACATATAAAATATATTGTCAGGAATAA
GAAAAAGAATTTCGCGGGCAATAGACACCAAGGATTCAGAGGAGCACCTCGGATGACATTTGGAACCTCAATACAGTC
AGGAATAAATACACAGACTTCCTCACATACTAGATGCTGTCACAAAACCTATATCCCACTATACACTACAACAC
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CCCATCTCTAAACAAGTAGCTCTAAGATGTTAATACACAGGGCAAGTTTATATAGCAACATCCTACTACA
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TCACCCAGGCTGTCAAAAATATAGGGAATCTTACACAAAGTAGGATAATTACATATAAATTC
GGACCTGATACTGATGACACATGAACTCATAATTTAATATTTGATGATAATAGGAATCTTGGC
TCTCTGGCAACTATTTGAAATACAGATGTTTATCATTTATGCTCAACACCAAAAGTGGATGAGAGATCCGATT
ATGCAATCAACAGGTATTTGAGATATTGTACTTGCACTAAATAATGGATTAATTTAACAACAG
Figure 23-1

A.

B

H X

UL

S

IR

B

1 KB

SUBSTITUTE SHEET

H

H

α4

PI-3 F

gX β-GAL gX NEO

73/83

Fig. 23-1

74/83

Fig. 23-2
Figure 24-2

A → CCT TAT GTA TCA TAC ACA TAC GAT TTA GGT GAC ACT ATA GAA

HindIII  PstI  SalI

TAC  AAG  CTT  GGG  CTG  CAG  GTC  GAC

pSP64  IBR HindIII A
Figure 24-3

B → GTC CGC GCT GCT GCG CCG CCT GGG CCG GCG GAA GAT CTG CTC ATG CTC
Val Arg Ala Ala Ala Pro Pro Gly Pro Ala Glu Asp Leu Leu MET Leu

TK (121) ←——-
IBR HindIII A

TK (156) ←——-
IBR HindIII A

GCG GCC GCC ATG•CCC CCG
Ala Ala Ala MET Pro Pro
Figure 24-4

C → GGA CAC CAT GTT TTT CCT GCC GCG CGC GGC CGT CGA CTC TAG A

IBR HindIII A

Sall  XbaI

→ pSP64

BamHI SmaI  SacI  EcoRI

GG ATC CCC GGG CGA GCT CGA ATT
Figure 25(a)-3

B→CAC ACC TTT GCG CAT CTC CAC AGC TCA ACA ATG AAT TCC
MET Asn Ser

EcoRI

gpX (1)
BamHI #10

ATG TTA CGT CCT GTA GAA ACC CCA ACC
MET Leu Arg Pro Val Glu Thr Pro Thr

uidA (1)
pRAJ260
Figure 25(b)-1

C → CAG GGA GGC AAA CAA TGA ATC AAC AAC TCT CCC GGG AGA TGG GGG AGG
Gln Gly Gly Lys Gln ---

uidA (602) ← pRAJ260 → BamHI Q

CTA ACT GAA ACA CGG AAG
Figure 25(b)-2

D→GTC GCC CCC CTT AAG•GGT CTC TTG CAC AAT•CCA GCC GCC TCC

SmaI  BamHI

GTG•TTG CTG CGT TCC CGG•GGA TCC

BamHI Q    ←|
A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C07K 7/10; C12N 7/01, 15/38, 15/63
US CL :435/235.1, 320.1; 424/93; 536/27; 530/387, 395
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/235.1, 320.1; 424/93; 536/27; 530/387, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CHEMICAL ABSTRACTS

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C. □ See patent family annex.

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Date of the actual completion of the international search: 11 October 1992
Date of mailing of the international search report: 22 OCT 1992

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Facsimile No. NOT APPLICABLE

Authorized officer
JAMES KETTER
Telephone No. (703) 308-0196

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