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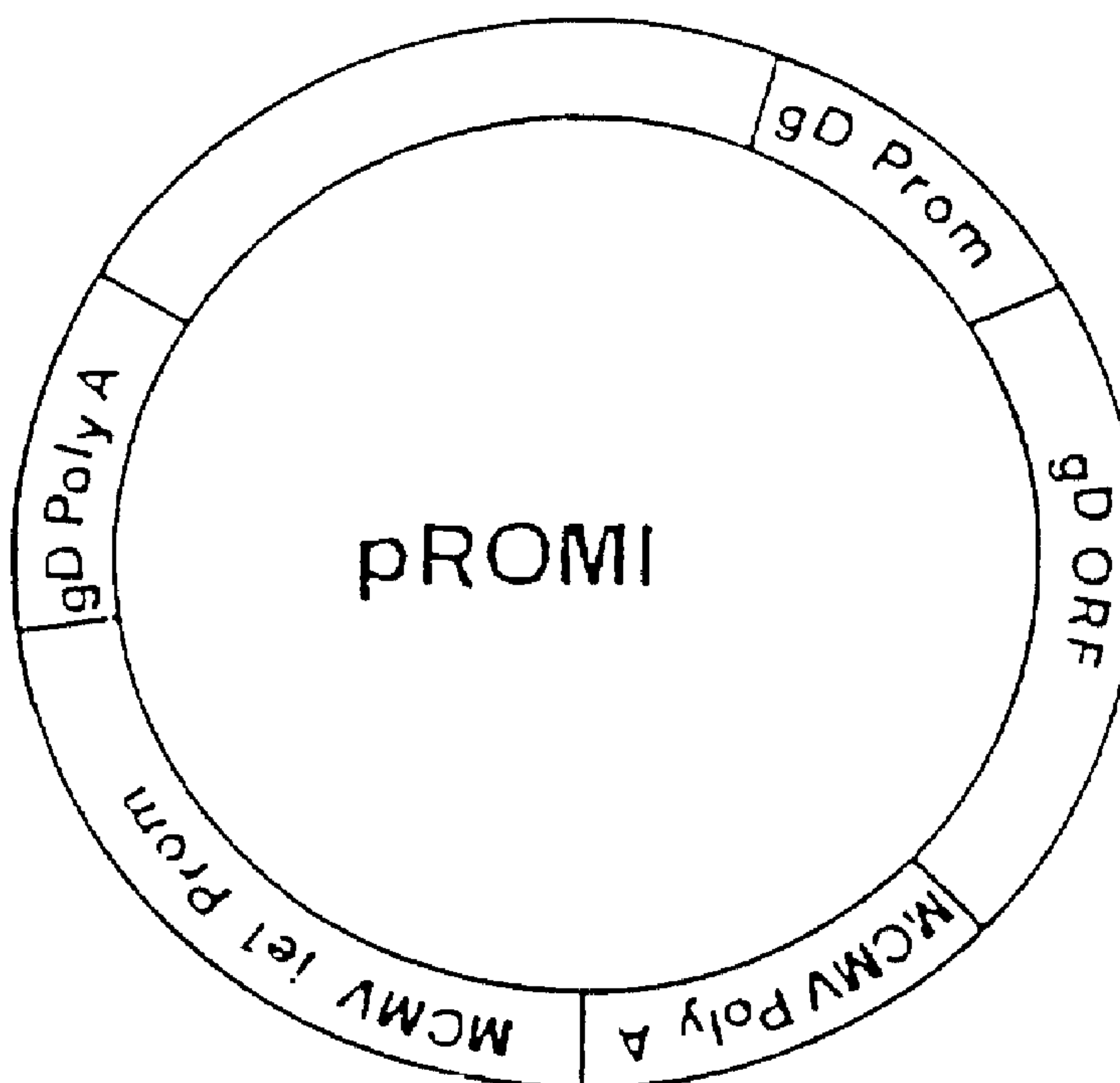
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(54) Title: SYNTHETIC GENE OF BOVINE VIRAL DIARRHOEA VIRUS



(57) Abrégé/Abstract:

The present invention refers to synthetic Bovine Viral Diarrhoea virus genes, live attenuated Bovine Herpesvirus recombinants carrying such genes, Bovine Herpesvirus recombinant virus particles carrying the proteins encoded by such genes, vaccines based on these recombinants, methods for the preparation of such recombinants and methods for the preparation of such vaccines and to diagnostic tools.



Abstract

The present invention refers to synthetic Bovine Viral Diarrhoea virus genes, live attenuated Bovine Herpesvirus recombinants carrying such genes, Bovine Herpesvirus recombinant virus particles carrying the proteins encoded by such genes, vaccines based on these recombinants, methods for the preparation of such recombinants and methods for the preparation of such vaccines and to diagnostic tools.

SYNTHETIC GENE OF BOVINE VIRAL DIARRHOEA VIRUS

The present invention refers to synthetic Bovine Viral Diarrhoea virus genes, live attenuated Bovine Herpesvirus recombinants, live attenuated and inactivated Bovine Herpesvirus recombinants carrying such genes, vaccines based on these live attenuated recombinants, methods for the preparation of such live attenuated recombinants and to methods for the preparation of such vaccines.

Bovine Viral Diarrhoea virus (BVDV), a member of the *Flaviviridae*, is a cause of congenital and enteric disease with a wide range of clinical manifestations. The disease was first described by P. Olafson et al. (Cornell Vet. 36 (1946) 205-213) as a transmissible disease of cattle with a high morbidity and low mortality. Affected cattle displayed elevated temperature, diarrhoea, and coughing. The condition was termed bovine viral diarrhoea.

Today, BVDV is considered to be a major pathogen of cattle with a world-wide economic impact (M.L. Doherty ML, Irish Veterinary Journal 40 (1986) 189-190; H.R. Frey et al., Dtsch. Tierärztl. Wschr. 94 (1987) 580-583; C.E. Ross et al., J. Am. Vet. Med. Assoc. 188 (1986) 618-619). The clinical pattern of BVDV infection within a herd and consequently its impact on production depend on the interaction of several factors, including the strain of the virus, age of the cattle, immunity in the herd, and interplay of stressors.

BVD is an acute postnatal infection in seronegative immunocompetent cattle. Acute BVDV infection enhances clinical diseases caused by other pathogens and is an important component of the bovine respiratory disease complex. Postnatal exposure of immunocompetent cattle to concytopathic biotypes of BVDV causes clinical BVD of varying severity. Naturally acquired BVD is usually characterised by high morbidity, low mortality, a normal host immune response, and minimal mucosal lesions. Pyrexia, nasal discharge, and transient leukopenia are frequently seen.

The failure to respond to BVDV infection until around day 125 of gestation can result in embryonic death, resorption, and stillbirths or, more frequently, in a whole array of non fatal teratogenic effects

(H.-J. Thiel et al., Fields Virology, Eds. B.N. Fields, D.M. Knipe, P.M. Howley et al., 3rd Edn., Raven Publishers Philadelphia 1996, p. 1059 ff.).

Since BVDV-infection leads to high economic losses, there is a substantial need for vaccines against BVDV.

To control the disease, inactivated vaccines are currently preferred. They induce, however, only a limited immune response which has been shown to be insufficient for protection against antigenic variants of BVDV (Bolin et al., Am. J. Vet. Res. 52 (1991) 1033-1037). Thus there is a clear need for improved, efficacious vaccines against BVDV.

However, vaccine development has been hampered because it is not known how a protective immune response can be induced by subunit or vector vaccines. First attempts to vaccinate calves using a baculovirus expressed glycoprotein E2 provided only limited protection (Bolin and Ridpath, Arch. Virol. 141 (1996) 1463-1477).

Since, as mentioned above, it is not known how a protective immune response can be induced, it is important for potential vaccines to at least mimic the natural infection as closely as possible. Therefore, in order to improve the immune response it is tempting to express the immunologically relevant BVDV E2 glycoprotein by a live recombinant vector virus.

These vector viruses mimic the natural infection in the sense that they do infect host cells and express, next to their own genetic material, the additional genetic information cloned into their genome.

An obvious vector for essaying the possible expression of the BVDV E2 glycoprotein under laboratory conditions is the vaccinia virus. This virus has successfully been used as an expression vector for a multitude of different genes for many years (see e.g. Paoletti, US 4,603,112 regarding the general principle for integration of foreign DNA into vaccinia virus to produce a modified virus capable of expressing foreign genes. For a general review about vaccinia virus vectors, see e.g. D.J. Jolly, Semin-Immunol. 2(5) (1990) 329-339).

For experimental purposes, the use of vaccinia virus as a live recombinant vector virus is attractive, because much is known about the virus and many tools for making vaccinia recombinants are available.

Vaccinia virus, however, is known to have an extremely broad host range. It is known to be infectious for all mammalian species tested so far, ranging from rabbits, mice, racoons, sheep, goats and camels to humans (D.J. Jolly, *Semin-Immunol.* 2(5) (1990) 329-339).

From an environmental point of view, this broad host range of live recombinant viruses in general is an unwanted situation, especially where only bovine animals are target animals for BVDV-vaccination. Vaccinia virus, although a bovine pathogen, would therefore certainly not be the vector of preference for use in animal vaccines for the protection of bovine animals against BVDV.

Promising live recombinant vector virus candidates however would be the live attenuated bovine herpesviruses (BHV), since such bovine herpesviruses, if expressing the BVDV E2-gene, would have the following advantages:

- ⇒ they are host-specific: they only infect bovine species;
- ⇒ they protect against two different diseases: Bovine herpesvirus infection and BVDV-infection.

Live attenuated recombinant BHV carrier viruses are known to be good potential expression vectors and vaccine viruses at the same time (R.S. Schrijver et al., *Vaccine* 15 (1997) 1908-1916; S. Chowdhury, *Vet. Microbiol.* 52 (1996) 13-23; F. Fehler et al., *J. Virol.* 66 (1992) 831-839; C. Schröder et al., *J. Virol.* 70 (1996) 25-33; J. Schmitt et al., *J. Virol.* 70 (1996) 1091-1099; G.M. Keil et al., *J. Virol.* 70 (1996) 3032-3038; E. Hanon, *J. Virol.* 70 (1996) 4116-4120; J.C. Whitbeck et al., *J. Virol.* 70 (1996) 7878-7884; A. Miethke et al., *J. Gen. Virol.* 76 (1996) 1623-1635).

BHV-1 has e.g. been used for the expression of LacZ (J. Schmitt et al., *J. Virol.* 70 (1996) 1091-1099). BHV-4 has been used e.g. for the expression of the LacZ-gene (Vanderplasschen et al., *Virology* 213 (1995) 328-340), and the herpes simplex virus I thymidine kinase gene (Keil, G.M., VIIIth International Congress of Virology, Berlin, 26-31 August, 1990).

Until now, however, expression of BVDV genes cloned in BHV as a live attenuated recombinant carrier virus, let alone showing in vivo protection by such a BHV-recombinant, has not been reported in the art. This is due to the fact that BHV is replicating/expressing in the nucleus, whereas BVDV is replicating/expressing in the cytoplasm.

Two main problems are known to exist when genes of a cytoplasmatically replicating virus are expressed in the nucleus, which is an evident consequence of cloning into the genome of a virus that replicates in the nucleus, e.g. BHV:

- a) For genes from cytoplasmatically replicating viruses, e.g. the E2 gene of the closely related classical swine fever virus - which is also a member of the *Flaviviridae* - it has been shown that the presence of cryptic splice signals caused unwanted splicing of E2 mRNA in the nucleus of mammalian cells. This is explained in more detail below. Consequently no expression of the E2 glycoprotein occurs (J.S. Shiu et al., J. Virol. Meth. 69 (1997) 223-230). The problem of the existence of cryptic splice sites is a general problem when expressing genes originating from cytoplasmatically replicating viruses in the nucleus.
- b) Moreover, cytoplasmatically replicating viruses have a low G/C content. For genes of such viruses to be expressed successfully in the nucleus, the low G/C content of the genes to be expressed must necessarily be brought up to the high G/C level of the genes of the vector virus. This necessity was shown e.g. for the related Bovine Respiratory Syncytial Virus (BRSV; R.S. Schrijver et al., Vaccine 15 (1997) 1908-1916; G. Kühnle et al., J. Virol. 72 (1998) 3804-3811). Expression of the G glycoprotein of BRSV by recombinant BHV was only possible after increasing the G+C content of the open reading frame (ORF) from 42 % to 62 % and by simultaneous removal of all potential splice donor signals. The same has been shown for the BRSV-F-gene.

Comparison of the GC-content of BVDV-genes and BHV-genes showed the following: the average GC-content of the BVDV-E2-gene is about 46 %, whereas the G/C content of e.g. the BHV-gD-gene is about 72 %. Therefore, on the basis of the prior art, it was to be expected that the GC-content of the synthetic BVDV-gene had to be raised dramatically by replacing the nucleotides A and T by G and C, as far as possible within the framework of what is allowed by the degeneracy of the genetic code. This is an arduous and labour-intensive task since first of all for each G/C-enhancing change in each codon, it must be checked if the changed codon still encodes the native

amino acid. Secondly, after this has been done, the whole G/C-enriched E2 gene has to be synthetically prepared.

Surprisingly it was found now, that if the original native BVDV E2-gene is replaced by a synthetic BVDV-gene having a synthetic sequence from which only splice-sites were removed, and still encoding the naturally occurring amino acid sequence, the gene is successfully expressed in the nucleus. This is fully unexpected in view of the extreme difference between the BVDV GC-content (46 %) and the average GC-content of BHV-genes, which is above 70 %.

Therefore, one embodiment of the invention relates to synthetic BVDV-genes having a not naturally occurring nucleotide sequence, from which only splice-sites were removed but still encoding a naturally occurring amino acid sequence.

Since the E2-protein is an important immunogenic protein of BVDV, a preferred form of the invention relates to the synthetic BVDV-gene encoding the BVDV E2 protein.

The use of BHV (replicating in the nucleus) for the expression of BVDV genes, normally replicating in the cytoplasm, may lead to the formation of unstable (BVDV-)RNA due to the presence of obvious and/or cryptic splice-donor and/or -acceptor sites, that are recognised by the nuclear RNA-splicing mechanism. Splice-sites have small consensus sequences for both splice-donor sites (GTRAGT as a consensus sequence where R is A or G; splice donor sites can also be GTATGT and GTAGAT) and splice acceptor sites (Y_nNYAG where Y is C or T, and N can be any nucleotide). In e.g. the nucleotide sequence of the wild-type E2-gene at least four potential splice-donor sites at positions 152-157, 419-424, 614-619, and 1061-1066 matching the AGGU consensus sequence were found, which are not present in the synthetic E2-gene.

Consensus sequences of both the splice-donor- and -acceptor as known in the art, have been mentioned above. Splice-sites can be removed by replacing one or more nucleotides of the potential splice-site within the framework of what is allowed by the degeneracy of the genetic code. This can be done by e.g. site-directed mutagenesis, or replacement of small or longer fragments by fragments with an alternative sequence. Alternatively, the whole gene can be replaced by synthetic DNA-fragments. Techniques for site-directed mutagenesis, and for DNA-synthesis are known in the art.

It may not be necessary to remove all potential splice sites, since in many cases, the removal of only a splice-donor, or splice-acceptor site will suffice to suppress splicing. Therefore, a more preferred form of this embodiment relates to the BVDV E2-gene modified in such a way that at least one possible splice-donor or acceptor site found in the naturally occurring nucleic acid sequence is removed in the synthetic BVDV-gene.

Comparison of the nucleotide sequence of the synthetic E2-gene according to the invention and the original E2-gene shows, that the GC-content of the synthetic gene is only slightly higher than the GC-content of the native E2-gene. Due to the removal of splice donor sequences the G+C content of a synthetic E2 ORF inherently increased from 46 % to approximately 48%. Nevertheless the synthetic gene of the present invention can, in spite of its very low G/C content, surprisingly efficient be expressed in BHV-recombinant viruses under the control of eukaryotic promoters, and the so obtained BHV/BVDV-recombinant viruses can be propagated in a stable manner.

Therefore, in an even more preferred form, the synthetic BVDV E2-gene according to the invention has a GC content between 46 and 60 %, or even between 48 and 50 %.

Although it is in principle possible to fully adapt the GC-content to the high GC-content of BHV, a very efficient expression was already obtained when the GC-content was 48%. Thus in a still even more preferred form, the synthetic BVDV-gene has a GC-content of approximately 48 %.

A naturally occurring nucleic acid sequence is understood to be a nucleotide sequence as it is found in naturally occurring viruses, such as the viruses isolated from the field.

The synthetic gene according to the present invention has a nucleotide sequence that, albeit different from the original nucleotide sequence (i.e. the nucleotide sequence found in virus isolated from the field), still encodes exactly the same amino acid sequence, and thus encodes the native protein. This is possible due to the degeneracy of the genetic code: all amino acids (with the exception of Met and Trp) are coded for by at least two different triplets. In the case of Leu, there are even 6 different triplets encoding this amino acid. Thus, there is a large number of possibilities to modify the nucleic acid sequence encoding a certain gene, without at the same time modifying the amino acid sequence. This principle is applicable to each and every gene and therefore also to the BVDV E2-gene.

The degeneracy of the genetic code also allowed e.g. the introduction of a number of restriction enzyme cleavage sites in the nucleic acid sequence encoding the E2-protein gene, without modifying the amino acid sequence of the E2-protein.

As an example may serve the modification of the BVDV E2-gene made from position 599 onwards, where the sequence GGCTCT was replaced by GGATCC, thus providing the BamHI restriction site at position 600 without changing the amino acids glycine and serine encoded by these two triplets.

New unique restriction enzyme cleavage sites for PstI, HindIII, EagI, BamHI, SacII, and NotI were introduced in the gene encoding the BVDV-E2 protein.

Synthetic DNA is defined as a DNA that is made by synthesis, instead of being isolated from a natural source.

This does not necessarily mean that each and every nucleotide of the DNA is synthesised. It is also possible to modify an existing DNA by replacing part thereof by a part with another nucleic acid sequence using recombinant DNA technology. The result of this modification is also considered to be a synthetic DNA.

The synthetic DNA can be made in a number of different ways, all known in the art. One useful method for modifying a nucleotide sequence, is e.g. site-directed mutagenesis. With this generally known method, modifications are made deliberately at predetermined sites. It is also possible to replace small or longer fragments by fragments with an alternative sequence. Many different techniques for DNA-manipulation are currently available. One possible method of replacing (parts of) a naturally found sequence with a synthetically made sequence, is e.g. to cut the nucleic acid sequence with restriction enzymes to remove the sequence to be replaced, followed by ligation of a synthetically made fragment with the same restriction sites.

Alternatively, the whole gene can be replaced by synthetic DNA-fragments.

The synthetic BRSV E2 gene from which a splice site has been removed, and that shows a minor raise in GC-content as depicted in the overview of replacements in the sequence of SEQ ID NO: 14 was shown to be very efficiently expressed. Therefore, in the most preferred form of this

embodiment, the BVDV gene has the nucleotide sequence presented in the overview of replacements in the sequence of SEQ ID NO: 14.

It is clear, that the BVDV-gene, in order to be expressed, must be placed under the control of a promoter.

A large number of suitable promoters is known in the art, that are recognised for their very efficient level of expression. Such promoters are e.g. the Pseudorabies gX-promoter, the Pseudorabies TK-promoter, the Adenovirus Major Late promoter, the Retroviral Long Terminal Repeat, the SV40 Early and Late promoters, the Mouse Cytomegalovirus immediate early (MCMVie1) promoter, the Mouse Cytomegalovirus early (MCMVe1) promoter, the Human Cytomegalovirus immediate early (HCMVie1) promoter, and the BHV-gE promoter. All these promoters have been described and are known in the art for many years.

From these, the MCMVie1 promoter, the MCMVe1 promoter, the HCMVie1 promoter, and the BHV-gE promoter are preferred promoters.

Therefore, preferably the synthetic BVDV-gene according to the invention is placed under the control of one of the promoters of the group of promoters consisting of the MCMVie1 promoter, the MCMVe1 promoter, the HCMVie1 promoter, and the BHV-gE promoter.

Another embodiment of the invention relates to live attenuated carrier BHV viruses carrying a synthetic BVDV-gene according to the present invention.

As motivated above, BHV-viruses are the carriers of choice for BVDV-genes.

In a preferred form, as a recombinant BHV carrier, the BHV-1 virus is used. This virus is a very commonly found pathogen in cattle, also (as is the case with BVDV) causing high economical losses.

The most common manifestation of Bovine Herpes Virus-I infection is bovine rhinotracheitis which varies from a mild respiratory disease to a severe infection of the entire respiratory tract.

From an economical point of view, Infectious Bovine Rhinotracheitis (IBR) is also the most dramatic manifestation of BHV-I infection. Morbidity rate in IBR is usually close to 100%.

Therefore, using BHV-1 as the live attenuated recombinant carrier virus for carrying and expressing the BVDV-E2-gene is very efficient: vaccines based on such a live attenuated recombinant virus protect against both BVDV and BHV-1. Animals so vaccinated are protected against the two most frequently found causes of respiratory disease in cattle.

A large number of potential insertion sites can be used for the insertion of the synthetic BVDV-gene in the BHV carrier virus. The most suitable technique for such an insertion is homologous recombination, known in the art and frequently used. Preferably, the BVDV-gene is cloned in a non-essential BHV-gene, since in that case, a viable BHV-BVDV recombinant is obtained that is not disturbed in essential functions.

Several non-essential genes are known for BHV. The genes coding for the (glyco)proteins gE, TK, gI, gG, and US2 (=PrV 28k) are e.g. very suitable as integration sites.

Attenuated BHV-strains having deletions or insertions in such genes have been described i.a. in U.S.Patent No. 4,824,667, U.S.Patent No. 4,703,011, By Kit et al., in the Veterinary Record 127: 363-364 (1990), in European patent Publication EP 0.326.127 and in European Patent EP 0.587.805.

In a more preferred form, the BVDV-gene to be expressed is integrated in the gE-gene of BHV.

In an equally more preferred form, the BVDV-gene to be expressed is integrated in the gI-gene of BHV-1.

A live attenuated recombinant BHV carrier virus according to the invention may, next to one or more BVDV-genes, comprise other genes encoding antigens from micro-organisms or viruses that are pathogenic for cattle.

Therefore, in a preferred form, the present invention provides live attenuated recombinant BHV carrier viruses comprising, next to a BVDV-gene, a gene encoding an antigen from micro-organisms or viruses that are pathogenic for cattle. Such genes are further referred to as "additional genes".

In a more preferred form of this embodiment, the additional gene is chosen from the group of cattle pathogens, consisting of Bovine Rotavirus, Bovine Respiratory Syncytial Virus, Parainfluenza type 3 virus, Bovine Paramyxovirus, Foot and Mouth Disease virus, and *Pasteurella haemolytica*.

Also, an additional gene may be introduced into the live attenuated recombinant BHV carrier virus according to the invention, that encodes a cytokine. Several cytokines, e.g. interferons are known to play an important role as immune modulators. Thus it may be advantageous to include genetic information for this kind of molecules into said section.

Still another embodiment of the present invention refers to vaccines for the protection of cattle against virus infection, based upon live attenuated recombinant BHV carrier virus expressing a BVDV-gene, according to the invention.

Vaccines based thereon have the advantage that they mimic the natural infection of not only BHV, but to a large extend also of BVDV, as motivated above.

Therefore, they provide protection against more than one pathogen, in this case against at least BHV-infection and BVDV-infection.

Vaccination is in many cases at least a two-step process: a first immunisation with an antigen triggers the immune response, and a second immunisation, the booster, actually enhances both the speed and the strength of the immune response. After a first vaccination, immunity against both the carrier itself and the antigens encoded by the heterologous gene carried by the carrier is triggered. This is the result of the fact that the recombinant carrier infects a cell and during viral replication both the viral proteins and the heterologous protein of the carried gene are expressed and become presented at the cell membrane. There they are detected by the immune system. A known problem however, when using viruses as live recombinant carriers is the following: antibodies against the recombinant carrier raised during first immunisation prevent a successful second round of infection, a booster, with the recombinant carrier. Thus no expression of proteins after boosting takes place, and therefore the immune system will not see the encoded proteins a second time. For the carrier virus this problem can be circumvented by just giving a higher dose of virus particles, since the viral proteins are per se present on the virus particle. In this approach the virus particles act as an inactivated vaccine and as such stimulates the immune system. Infection is however necessary for the heterologous gene to be expressed. Therefore, since no second round of infection occurs, the

heterologous gene carried by the carrier is not expressed a second time. Thus no booster immunisation against the heterologous gene product will be generated.

It was now surprisingly found, that the BVDV E2 protein is incorporated into the envelope of the BHV-1 virus particles during the maturation of the virus. This is indeed surprising since BVDV-E2 lacks any herpesspecific targeting signals and therefore could not be expected to become incorporated into the envelope of the BHV-1 particle. The unexpected incorporation of BVDV-E2 in the envelope of BHV means that the problem mentioned above can be circumvented: a booster immunisation with a high dose of BHV virus particles carrying the BVDV E2 protein on their envelope causes a second immune response to be triggered against both the BHV envelope proteins and the BVDV protein on the envelope, without infection being necessary.

Therefore, the vaccine according to the invention may also comprise a recombinant BHV carrier virus particle according to the invention as such, regardless if the particle is still viable or not.

This embodiment of the invention thus relates also to inactivated Recombinant BHV carrier virus particles carrying the BVDV E2 glycoprotein on their surface.

Thus the invention relates to vaccines suitable for booster vaccination that comprise inactivated BHV carrier viruses according to the invention. Moreover, the invention relates to the use of inactivated BHV carrier viruses according to the invention for the manufacture of vaccines for booster vaccination against BVDV-infection.

The vaccine according to the present invention may comprise a pharmaceutically acceptable carrier. One possible carrier is a physiological salt-solution.

Other pharmaceutically acceptable carriers are for instance the solutions in which adjuvants are provided.

The invention also relates to methods for the preparation of vaccines according to the invention. Such methods comprise i.a. the admixing of the live attenuated BHV-recombinant and/or the BHV-recombinant virus particle and a pharmaceutically acceptable carrier.

If desired, an adjuvant and possibly one or more emulsifiers such as Tween[®] and Span[®] are also incorporated in the live or inactivated vaccine according to the invention. Suitable adjuvants are for example vitamin-E acetate solubilisate, aluminum hydroxide, -phosphate or -oxide, (mineral) oil

emulsions such as Bayol[®] and Marcol52[®] and saponins. Incorporation of the antigens in Iscoms is also a possible way of adjuvation.

It is advantageous to add a stabiliser to live or inactivated viruses, particularly if a dry composition of live viruses is prepared by lyophilisation. Suitable stabilisers are, for example, SPGA (Bovarnik et al., J. Bacteriology 59 (1950) 509), carbohydrates (such as sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose), proteins (such as albumin or casein), or degradation products thereof, and buffers (such as alkali metal phosphates). If desired, one or more compounds with adjuvant activity as described above can also be added.

A vaccine according to the invention may be administered by intramuscular or subcutaneous injection or via intranasal, intratracheal, oral, cutane, percutane or intracutane administration.

The natural route of infection is through the respiratory tract. Therefore, in a preferred form, the vaccine is formulated for intranasal administration. For the preparation of such a vaccine, a physiological salt solution is very attractive as a carrier, since it is isotonic with mucosal fluid

Alternatively or in addition, the DNA isolated from the live attenuated BHV-recombinant according to the present invention can be directly administered. Vaccination methods using naked DNA instead of viruses are sufficiently known in the art, and have been described i.a. by Cohen (Science 259: 1691-1692 (1993)), by Donnelly et al., The Immunologist 2: 20-26 (1993), by Pardoll (Immunity 3: 165-169 (1995)) and by Montgomery (Current Biology 5: 505-510 (1994)).

The useful effective amount to be administered in a vaccine will vary depending on the age, weight, and mode of administration. A suitable vaccine dosage can for example comprise about 10^3 - 10^{10} pfu of the live recombinant per animal.

Another embodiment of the present invention relates to methods for the preparation of vaccines according to the invention. Such methods comprise admixing a live attenuated BHV-recombinant or an inactivated recombinant according to the present invention and a pharmaceutically acceptable carrier.

For the preparation of a live vaccine the BHV-I mutant according to the present invention can be grown on susceptible cells. Such susceptible cells are e.g. Madin Darby Bovine Kidney cells (MDBK-cells) or bovine embryonic cells.

The viruses thus grown can be harvested by collecting the tissue cell culture fluids and/or cells. The live vaccine may be prepared in the form of a suspension or may be lyophilised.

Still another embodiment of the present invention refers to methods for the preparation of live attenuated BHV-recombinants according to the invention.

Such methods comprise bringing together in a suitable host cell BHV-DNA and a vector comprising the BVDV-gene to be expressed, placed under the control of a suitable promoter and flanked by 3' and 5' flanking regions that share homology with BHV-sequences, in order to facilitate homologous recombination.

This can be done by first transfecting the suitable host cell with the vector, followed by infection with the BHV-virus. Alternatively, both the vector and the isolated viral DNA may be transfected together into a suitable host cell.

A generally acknowledged problem in the field of vaccination is the following: the presence of antibodies against a certain pathogen in the serum of a host mammal indicates that the host has been infected with the pathogen, either in a virulent form (e.g. through field infection) or an attenuated form. When an animal becomes infected with BVDV this means that antibodies against all immunogenic BVDV-proteins are induced. It is however impossible to discriminate between field-infected mammals and mammals vaccinated with a vaccine strain, regardless if this vaccine strain is live or inactivated, since antibodies against the viral proteins as such will be abundantly present.

Surprisingly the recombinant BHV-virus carrying the BVDV-E2 gene according to the present invention offers a solution to this problem as follows:

The E2 gene is expressed in vivo in all BVDV field strains and induces anti-E2 antibodies in infected animals. In addition, BVDV field strains induce significant amounts of antibodies against all other immunogenic BVDV-proteins. This implicates, that serum obtained from mammals infected with wild-type BVDV will always have antibodies against the E2-protein as well as

antibodies against all other immunogenic BVDV-proteins. The BHV/BVDV-recombinant according to the present invention however can only induce antibodies against the BVDV E2 protein. Therefore mammals vaccinated with a BHV/BVDV-recombinant according to the invention, regardless the live or inactivated character, can only have antibodies against the BVDV E2 protein in their serum. Thus sera obtained from animals vaccinated with the BHV/BVDV-recombinant according to the invention will only react with the BVDV E2 protein, whereas sera obtained from animals infected with a BVDV field strain will contain antibodies against all immunogenic BVDV proteins.

Diagnostic tests based upon E2 protein and another immunogenic BVDV protein can therefore easily be used to discriminate between field-infection and vaccination with the vaccine according to the present invention.

The BHV/BVDV-recombinant according to the present invention thus is a very suitable marker vaccine, i.e. a vaccine strain that can be serologically discriminated from a field strain.

A diagnostic test for the discrimination between vaccine strains and field strains can be e.g. a simple ELISA-test, or the incubation of a Western blot comprising the BVDV proteins with serum of mammals to be tested.

Thus, in still another embodiment, the invention relates to diagnostic tests for the discrimination between sera from mammals infected with BVDV field strains and from mammals vaccinated with a vaccine according to the invention.

The invention is further illustrated by way of the following Examples.

EXAMPLES

Example 1.

Construction of BVDV E2syn open reading frame (ORF) from synthetic oligonucleotides.

The cDNA sequence of the E2-gene of the BVDV strain 68 was determined (see SEQ ID NO: 14). This sequence was used as a guiding sequence to compose a synthetic gene called BVDV E2syn ORF that has many convenient restriction enzyme cleavage sites which also will facilitate the construction of a whole range of smaller and larger deletion mutants. The E2syn ORF does not contain any splice donor consensus sequences but still codes for an E2 protein with an amino acid sequence identical to the BVDV strain 68 encoded E2 protein.

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To reach this goal a total of 13 fragments as given in SEQ ID NO: 1-13 were obtained by mixing equimolar amounts of complementary oligonucleotides in 10 mM tris-HCl, pH 7.5, boiled for 5 minutes and then slowly cooled down to room temperature.

Fragments with SEQ ID NO: 1-7 were cloned adjacently in pSP73 yielding plasmid pNH7. This was done by cleaving pSP73 with BglII and XhoI. Fragment 1 was ligated into this DNA via its cohesive ends resulting in Plasmid pNH1 which was cleaved with StuI and XhoI to receive fragment 2. The resulting plasmid pNH2 was cleaved with PstI and XhoI and fragment 3 was integrated to obtain pNH3 which was cleaved with HindIII and XhoI to construct pNH4 by integration of fragment 4. pNH4 was cleaved with SfuI and XhoI and received fragment 5 resulting in pNH5 that was cleaved with Bsu36I and XhoI for integration of fragment 6. The resulting plasmid pNH6 was cleaved with EagI and XhoI received fragment 7 to give plasmid pNH7.

Fragments A-F (SEQ ID NO: 8-13) were integrated in that order in pSP73 (Promega) to yield pSPCF. This was done by cleaving pSP73 with BglII and XhoI. Fragment A was ligated into this DNA via its cohesive ends resulting in Plasmid pCA which was cleaved with BglII and BstXI to receive fragment B. The resulting plasmid pCB was cleaved with BglII and SacII and fragment C was integrated to obtain pCC which was cleaved with BglII and ApaI to construct pCD by integration of fragment D. pCD was cleaved with BglII and BsrGI and received fragment E resulting in pCE that was cleaved with BglII and AccI for integration of fragment F to obtain plasmid pCF.

Finally plasmid pNH7 was cleaved with BamHI and XhoI and received the purified BamHI-XhoI insert from plasmid pCF to yield plasmid pSPE2syn containing the reconstructed BVDV E2 ORF.

Example 2

Construction and analysis of BHV-1 BVDV-E2-recombinants

Cloning of the MCMV promoters ie1 and e1 in pAT-gD PLUS.

In order to test the expression of the E2 gene under various conditions, it was decided to clone the gene under the control of various alternative promoters.

First of all, a multicloning site was inserted in pAT-gIV (Vector pAT-gIV has been described extensively in EP 0 663 403. It should be mentioned here that the vector pAT-gIV is also referred to as pAT-gD, due to the new nomenclature of the herpesvirus (glycoprotein-)genes) between the MCMVie2-polyA fragment and the gD-polyA fragment, and this new plasmid was named pAT-gD PLUS.

Starting with pAT-gD PLUS, two constructs were made:

Construction of pROMI: Plasmid pAMB33 (Dorsch-Häsler et al., Proc. Natl. Acad. Sci. 82 (1985) 8325-8329) was digested with HpaI and XbaI. Fragments were filled with Klenow-polymerase and isolated from agarose gels. A 1.4 kB fragment comprising the MCMV ie1 promoter was obtained.

The plasmid pAT-gD PLUS was cleaved in its polylinker site with EcoRV. In this site, the isolated MCMV ie1 fragment was cloned, to yield pROMI (see Fig. 1).

Construction of pROME: Plasmid MM354, comprising the MCMV e1 promoter (sequence of the Murine Cytomegalovirus early 1 gene is present in the EMBL Gene Bank at Heidelberg, Germany) was digested with SmaI and HindIII. Fragments were filled with Klenow-polymerase and isolated from agarose gels. A 1.1 kB fragment comprising the MCMV e1 promoter was obtained. The plasmid pAT-gD PLUS was cleaved in its polylinker site with EcoRV. In this site, the isolated MCMV e1 fragment was cloned, to yield pROME (See Fig. 2).

Cloning of pROMEsig.

To provide a signal peptide for correct transport and processing of BVDV E2, the pestivirus signal sequence of CSFV strain Alfort (See SEQ ID NO: 16) was ligated into pROME (see above) after cleavage with BamHI and KpnI to give plasmid pROMEsig.

Cloning of the BVDV E2syn ORF into pROMEsig. Plasmid pROMEsig was cleaved with KpnI and NotI and used for the integration of the BVDV E2syn ORF cleaved out of plasmid pSPE2syn with KpnI and NotI. The resulting plasmid was named pROME2syn.

Integration of pROME2syn into BHV-1. MDBK cells were transfected with 5 mg pROME2syn and 2.5 mg of purified DNA of gD negative virus BHV-1/80-221 (Fehler et al., J.Virol. 66 (1992) 831-

839). Transfected cells were shocked with glycerol 4 hours after addition of the DNA. After development of CPE the cell culture supernatant was titrated on MDBK cells. Under these conditions only genotypically gD positive virions can grow productively and form plaques on monolayers. At 3 days p.i. virions from single plaques were isolated and DNA from MDBK cells was purified, cleaved with HindIII, size separated by agarose gel electrophoresis and transferred to nitrocellulose filters which were hybridised to ³²P-labeled probes either from the gD ORF, the lacZ ORF, the E2syn ORF or sequences downstream the E2syn ORF presented for homologous recombination. The results demonstrated that the isolated virions lack the β -galactosidase sequences and that the BVDV E2syn ORF was integrated. The resulting virus BHV-1/E2syn was plaque purified twice and used for further characterization.

Identification and characterization of BHV-1 expressed BVDV E2.

To demonstrate expression by recombinant BHV-1 and to identify the BVDV E2syn ORF encoded gene product proteins from wild type BHV-1 strain Schönböken (Fig. 3, lanes 1 and 3) and recombinant BHV-1/E2syn (Fig. 3, lanes 2 and 4), infected MDBK cells were analysed by immunoblotting using an immune serum from a BVDV strain 68 infected calf (panel a) or a rabbit serum directed against BHV-1 glycoprotein D. The anti-BVDV serum did not specifically react with proteins from BHV-1 strain Schönböken infected cells but bound to a 53 kDa apparent molecular mass protein among BHV-1/E2syn infected cell proteins. This apparent molecular mass corresponds very well to the 53 kDa apparent molecular mass reported for the E2 glycoprotein expressed in BVDV infected cells. Binding of the anti-gD serum to the 72 and 68 kDa apparent molecular mass forms of BHV-1 gD (panel b) demonstrates comparable infection with BHV-1 strain Schönböken and BHV-1/E2syn, respectively.

Fig. 4 demonstrates incorporation of the BHV-1/E2syn expressed E2 glycoprotein into recombinant virions. For this purpose MDBK cells were infected with BHV-1/E2syn in the presence of ³⁵S-methionine. Proteins from lysed cells (lane 1) and purified virions (lane 2) were immunoprecipitated using the anti BVDV serum and bound proteins were visualised by fluorography after size separation in SDS-polyacrylamide gels. The result shows that the E2 glycoprotein is associated with BHV-1/E2syn virions.

LEGENDS TO THE FIGURES

Fig. 1 shows a map of pROMI

Fig. 2 shows a map of pROME

Fig. 3 demonstrates expression by recombinant BHV-1 and identifies the BVDV E2syn ORF encoded gene product.

MDBK cells were infected with wild type BHV-1 strain Schönböken (Fig. 3, lanes 1 and 3) and recombinant BHV-1/E2syn (Fig. 3, lanes 2 and 4), and their protein content analysed by immunoblotting using an immune serum from a BVDV strain 68 infected calf (panel a) or a rabbit serum directed against BHV-1 glycoprotein D (panel b).

Fig. 4 demonstrates incorporation of the BHV-1/E2syn expressed E2 glycoprotein into recombinant virions. MDBK cells were infected with BHV-1/E2syn in the presence of ³⁵S-methionine. Proteins from lysed cells (lane 1) and purified virions (lane 2) were immunoprecipitated using the anti BVDV serum and bound proteins were visualised by fluorography after size separation in SDS-polyacrylamide gels. The result shows that the E2 glycoprotein is associated with BHV-1/E2syn virions.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: AKZO NOBEL N.V.
- (ii) TITLE OF INVENTION: SYNTHETIC GENE OF BOVINE VIRAL DIARRHOEA VIRUS
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: FETHERSTONHAUGH & CO.
 - (B) STREET: P.O. BOX 2999, STATION D
 - (C) CITY: OTTAWA
 - (D) STATE: ONT
 - (E) COUNTRY: CANADA
 - (F) ZIP: K1P 5Y6
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: ASCII (text)
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: CA 2,304,991
 - (B) FILING DATE: 26-APR-2000
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: FETHERSTONHAUGH & CO.
 - (B) REGISTRATION NUMBER:
 - (C) REFERENCE/DOCKET NUMBER: 23804-572
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (613)-235-4373
 - (B) TELEFAX: (613)-232-8440

(2) INFORMATION FOR SEQ ID NO.: 1

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 92
- (ii) MOLECULAR TYPE: DNA
 - (A) ORGANISM: bovine viral diarrhoea virus
- (ix) FEATURE:
 - (A) NAME/KEY: misc_recomb
 - (B) LOCATION: (1)..(4)
 - (D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(ix) FEATURE:

- (A) NAME/KEY: misc_recomb
- (B) LOCATION: (5)..(57)
- (D) OTHER INFORMATION: "double stranded part of the oligonucleotide"

(ix) FEATURE:

- (A) NAME/KEY: misc_recomb
- (B) LOCATION: (58)..(61)
- (D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; nucleotides TCGA are not present in this fragment and only indicate that the complementary strand contains nucleotides AGCT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 1

```
GATCTGGTAC CACTTAGACT GCAAACCTGA ATACTACTAT GCCATAGCCA AGAATGATAG 60
AGTCGGCCTA CTAGGAGCTG AAGGCCTCTC GA                               92
```

(2) INFORMATION FOR SEQ ID NO.: 2

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 101

(ii) MOLECULAR TYPE: DNA

- (A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

- (A) NAME/KEY: misc_recomb
- (B) LOCATION: (1)..(97)
- (D) OTHER INFORMATION: "double stranded part of the nucleotide"

(ix) FEATURE:

- (A) NAME/KEY: misc_recomb
- (B) LOCATION: (98)..(101)
- (D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; nucleotides TCGA are not present in this fragment and only indicate that the complementary strand contains nucleotides AGCT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 2

```
CCTTACCACT GTTTGGAAGG AATACTCACC TGAAATGACG CTGGAAGACA CAATGGTCAT 60
AGCCTCCTGC AGAGAAGGCA AATTACATA CCGCTCCTCG A                               101
```

(2) INFORMATION FOR SEQ ID NO.: 3

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 114

(ii) MOLECULAR TYPE: DNA

- (A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

- (A) NAME/KEY: misc_recomb
- (B) LOCATION: (1)..(4)

(D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; the nucleotides TCGA are not present in this fragment and only indicate that the complementary strand contains nucleotides AGCT"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
 (B) LOCATION: (5)..(110)
 (D) OTHER INFORMATION: "double stranded part of the oligonucleotide"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
 (B) LOCATION: (111)..(114)
 (D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; nucleotides TCGA are not present in this fragment and only indicate that the complementary strand contains nucleotides AGCT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 3

TGCAGAGAAG GCAAATTTAC ATACCGCTCA AGATGCACAA GGGAAGCTAG ATATCTTGCA 60
 ATTTTGCATT CAAGAGCCTT ACCGACCAGC GTGGTATTTG AGAAGCTTAC TCGA 114

(2) INFORMATION FOR SEQ ID NO.: 4

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 62

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
 (B) LOCATION: (1)..(4)
 (D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
 (B) LOCATION: (5)..(58)
 (D) OTHER INFORMATION: "double stranded part of the oligonucleotide"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
 (B) LOCATION: (59)..(62)
 (D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; nucleotides TCGA are not present in this fragment and only indicate that the complementary strand contains nucleotides AGCT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 4

AGCTTTTCGA GGGCCAAAAG CAAGAGGACA CGGTTGAGAT GGATGACAAC TTCGAATCTC 60
GA 62

(2) INFORMATION FOR SEQ ID NO.: 5

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 108

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (1)..(2)

(D) OTHER INFORMATION: "5' sticky end: the indicated sequence
is single stranded; no nucleotides present
on the complementary strand"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (3)..(104)

(D) OTHER INFORMATION: "double stranded part of the
oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 5

CGAATTTGGA CTCTGCCCCAT GCGACGCCAA GCCCGTAGTA AAGGGGACTT TCAATACAAC 60
ACTGCTAAAT GGACCGGCTT TCCAGATGGT ATGCCCCTTA GGGCTCGA 108

(2) INFORMATION FOR SEQ ID NO.: 6

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 96

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_difference

(B) LOCATION: (1)..(3)

(D) OTHER INFORMATION: "5' sticky end: the indicated sequence
is single stranded; no nucleotides present
on the complementary strand"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (4)..(92)

(D) OTHER INFORMATION: "double stranded part of the
oligonucleotide"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (93)..(96)

(D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; nucleotides TCGA are not present in this fragment and only indicate that the complementary strain contain nucleotides AGCT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 6

```
TTAGGGTGGG CAGGGACCGT GAGCTGCATG TTAGCTAATA GGGATACCCT AGATACAGCA 60
GTAGTGCGGA CGTATAGGAG ATATCGGCCG TCTCGA                               96
```

(2) INFORMATION FOR SEQ ID NO.: 7

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 131

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (1)..(4)

(D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (5)..(127)

(D) OTHER INFORMATION: "double stranded part of the oligonucleotide"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (128)..(131)

(D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; nucleotides TCGA are not present in this fragment and only indicate that the complementary strand contains nucleotides AGCT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 7

```
GGCCGTTCCC TTACAGGCAA GACTGCATCA CCCAGAAGGT TCTGGGAGAG GATCTCTATA 60
ACTGCATTCT TGGAGGAAAC TGGACCTGCA TAACTGGAGA CCAACTACAA TACTCAGGAG 120
GATCCACTCG A                                                         131
```

(2) INFORMATION FOR SEQ ID NO.: 8

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 122

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (1)..(4)

(D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; no nucleotides present in the complementary strand"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
(B) LOCATION: (5)..(120)
(D) OTHER INFORMATION: "double stranded part of the oligonucleotide"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
(B) LOCATION: (121)..(122)
(D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; nucleotides AG are not present in this fragment and only indicate that the complementary strand contains nucleotides TC"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 8

```
GATCTACGGA TCCATTGAAT CCTGCAAATG GTGCGGCTTT AAATTCCAAA GAAGCGAGGG 60
GTTACCACAC TACCCCATTTG GCAAGTGCAG GCTGAAGAAT GAGACTGGCT ACAGATTAGT 120
AG                                                    122
```

(2) INFORMATION FOR SEQ ID NO.: 9

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 91

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
(B) LOCATION: (1)..(4)
(D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
(B) LOCATION: (5)..(87)
(D) OTHER INFORMATION: "double stranded part of the oligonucleotide"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb
(B) LOCATION: (88)..(91)
(D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; nucleotides GTAC are not present in this fragment and only indicate that the complementary strand contains nucleotides CATG"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 9

```
GATCTGGTAG ACGACACCTC TTGCAATATA GGAGGCGTGG CGATAGTACC ACAGGGGATG 60
GTAAAATGCA AGATAGGAGA CACAATTGTA C                               91
```

25

(2) INFORMATION FOR SEQ ID NO.: 10

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 84

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (1)..(4)

(D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (5)..(80)

(D) OTHER INFORMATION: "double stranded part of the oligonucleotide"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (81)..(84)

(D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 10

GATCTCTGTA CAGGTCATAG CTCTTGACAC CAAACTTGGG CCTATGCCCT GCAAGCCATA 60
 TGAGATCATT TCAAGCGAGG GGCC 84

(2) INFORMATION FOR SEQ ID NO.: 11

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 76

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (1)..(4)

(D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (5)..(74)

(D) OTHER INFORMATION: "double stranded part of the oligonucleotide"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (75)..(76)

26

(D) OTHER INFORMATION: "3' sticky end; the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 11

GATCTCGGGC CCGTAGAAAA GACGGCATGC ACCTTCAACT ACACGAGGAC ATTAAAGAAC 60
AAGTACTTTG AGCCGC 76

(2) INFORMATION FOR SEQ ID NO.: 12

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 114

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (1)..(4)

(D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: (5)..(110)

(D) OTHER INFORMATION: "double stranded part of the oligonucleotide"

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (111)..(114)

(D) OTHER INFORMATION: "3' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 12

GATCTACCGC GGGACAGCTA CTTCCAGCAA TATATGCTAA AAGGAGAATA TCAATACTGG 60
TTTGACCTGG AGGTAACCGA CCATCACCGG GATTACTTGG CCGAATCCAT ATTA 114

(2) INFORMATION FOR SEQ ID NO.: 13:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 115

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:

(A) NAME/KEY: misc_recomb

(B) LOCATION: (1)..(4)

(D) OTHER INFORMATION: "5' sticky end: the indicated sequence is single stranded; no nucleotides present on the complementary strand"

(ix) FEATURE:
 (A) NAME/KEY: misc_recomb
 (B) LOCATION: (5)..(111)
 (D) OTHER INFORMATION: "double stranded part of the
 oligonucleotide"

(ix) FEATURE:
 (A) NAME/KEY: misc_recomb
 (B) LOCATION: (112)..(115)
 (D) OTHER INFORMATION: "3' sticky end: the indicated sequence is
 single stranded; nucleotides TCGA are not
 present in this fragment and only indicate
 that the complementary strand contains
 nucleotide AGCT"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 13

```
GATCTCCATA TTAGTGGTCG TCGTCGCTCT CCTGGGCGGC AGATACGTCC TCTGGCTACT 60
GGTCACATAC ATGGTTCTAT CAGAACAAAA GGTCTTAGGG TGAGCGGCCG CTCGA      115
```

(2) INFORMATION FOR SEQ ID NO.: 14

(i) SEQUENCE CHARACTERISTICS
 (A) LENGTH: 1122

(ii) MOLECULAR TYPE: DNA
 (A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: (1)..(1122)
 (D) OTHER INFORMATION: "cDNA sequence of the BVDV strain 68"

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: (1)..(1122)
 (D) OTHER INFORMATION:

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (107)..(108)
 (D) OTHER INFORMATION: replace by "AG"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (140)..(141)
 (D) OTHER INFORMATION: replace by "CC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (152)..(153)
 (D) OTHER INFORMATION: replace by "GC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (155)..(156)
 (D) OTHER INFORMATION: replace by "AA"

- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (173)..(174)
 (D) OTHER INFORMATION: replace by "GA"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (230)..(231)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (242)..(243)
 (D) OTHER INFORMATION: replace by "AG"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (245)..(246)
 (D) OTHER INFORMATION: replace by "AG"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (251)..(252)
 (D) OTHER INFORMATION: replace by "TC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (257)..(258)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (278)..(279)
 (D) OTHER INFORMATION: replace by "TT"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (419)..(420)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (473)..(474)
 (D) OTHER INFORMATION: replace by "GA"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (477)..(478)
 (D) OTHER INFORMATION: replace by "TC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (479)..(480)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (482)..(483)
 (D) OTHER INFORMATION: replace by "CG"

- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (512)..(513)
 (D) OTHER INFORMATION: replace by "AG"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (515)..(516)
 (D) OTHER INFORMATION: replace by "AG"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (524)..(525)
 (D) OTHER INFORMATION: replace by "GA"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (542)..(543)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (566)..(567)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (575)..(576)
 (D) OTHER INFORMATION: replace by "GA"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (599)..(600)
 (D) OTHER INFORMATION: replace by "GA"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (602)..(603)
 (D) OTHER INFORMATION: replace by "CC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (614)..(615)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (617)..(618)
 (D) OTHER INFORMATION: replace by "AA"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (623)..(624)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (626)..(627)
 (D) OTHER INFORMATION: replace by "GC"

- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (635)..(636)
 (D) OTHER INFORMATION: replace by "TT"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (644)..(645)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (677)..(678)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (637)..(638)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (767)..(768)
 (D) OTHER INFORMATION: replace by "AA"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (770)..(771)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (884)..(885)
 (D) OTHER INFORMATION: replace by "GC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (914)..(915)
 (D) OTHER INFORMATION: replace by "AC"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (917)..(918)
 (D) OTHER INFORMATION: replace by "AG"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (929)..(930)
 (D) OTHER INFORMATION: replace by "CG"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (931)..(932)
 (D) OTHER INFORMATION: replace by "CG"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (933)..(934)
 (D) OTHER INFORMATION: replace by "GG"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (938)..(939)
 (D) OTHER INFORMATION: replace by "GC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (998)..(999)
 (D) OTHER INFORMATION: replace by "CC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (1025)..(1026)
 (D) OTHER INFORMATION: replace by "AA"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (1039)..(1040)
 (D) OTHER INFORMATION: replace by "GC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (1042)..(1043)
 (D) OTHER INFORMATION: replace by "GC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (1046)..(1047)
 (D) OTHER INFORMATION: replace by "TC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (1058)..(1059)
 (D) OTHER INFORMATION: replace by "GC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (1061)..(1062)
 (D) OTHER INFORMATION: replace by "GC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (1070)..(1071)
 (D) OTHER INFORMATION: replace by "TC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (1077)..(1078)
 (D) OTHER INFORMATION: replace by "GC"

(ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: (1112)..(1113)
 (D) OTHER INFORMATION: replace by "AG"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 14:

CAC	TTA	GAC	TGC	AAA	CCT	GAA	TAC	TAC	TAT	GCC	ATA	GCC	AAG	AAT	GAT	48
His	Leu	Asp	Cys	Lys	Pro	Glu	Tyr	Tyr	Tyr	Ala	Ile	Ala	Lys	Asn	Asp	
1				5					10					15		

AGA	GTC	GGC	CTA	CTA	GGA	GCT	GAA	GGC	CTT	ACC	ACT	GTT	TGG	AAG	GAA	96
Arg	Val	Gly	Leu	Leu	Gly	Ala	Glu	Gly	Leu	Thr	Thr	Val	Trp	Lys	Glu	
			20					25					30			
TAC	TCA	CCT	GAA	ATG	ACG	CTG	GAA	GAC	ACA	ATG	GTC	ATA	GCC	TCG	TGC	144
Tyr	Ser	Pro	Glu	Met	Thr	Leu	Glu	Asp	Thr	Met	Val	Ile	Ala	Ser	Cys	
		35					40					45				
AGA	GAA	GGT	AAG	TTT	ACA	TAC	CGC	TCA	AGG	TGC	ACA	AGG	GAA	GCT	AGA	192
Arg	Glu	Gly	Lys	Phe	Thr	Tyr	Arg	Ser	Arg	Cys	Thr	Arg	Glu	Ala	Arg	
	50					55				60						
TAT	CTT	GCA	ATT	TTG	CAT	TCA	AGA	GCC	TTA	CCG	ACC	AGT	GTG	GTA	TTT	240
Tyr	Leu	Ala	Ile	Leu	His	Ser	Arg	Ala	Leu	Pro	Thr	Ser	Val	Val	Phe	
	65				70				75						80	
GAA	AAA	CTT	TTT	GAG	GGG	CAA	AAG	CAA	GAG	GAC	ACG	GTC	GAG	ATG	GAT	288
Glu	Lys	Leu	Phe	Glu	Gly	Gln	Lys	Gln	Glu	Asp	Thr	Val	Glu	Met	Asp	
				85				90						95		
GAC	AAC	TTC	GAA	TTT	GGA	CTC	TGC	CCA	TGC	GAC	GCC	AAG	CCC	GTA	GTA	336
Asp	Asn	Phe	Glu	Phe	Gly	Leu	Cys	Pro	Cys	Asp	Ala	Lys	Pro	Val	Val	
			100					105					110			
AAG	GGG	ACT	TTC	AAT	ACA	ACA	CTG	CTA	AAT	GGA	CCG	GCT	TTC	CAG	ATG	384
Lys	Gly	Thr	Phe	Asn	Thr	Thr	Leu	Leu	Asn	Gly	Pro	Ala	Phe	Gln	Met	
		115					120					125				
GTA	TGC	CCC	TTA	GGG	TGG	ACA	GGG	ACC	GTG	AGC	TGT	ATG	TTA	GCT	AAT	432
Val	Cys	Pro	Leu	Gly	Trp	Thr	Gly	Thr	Val	Ser	Cys	Met	Leu	Ala	Asn	
	130					135					140					
AGG	GAT	ACC	CTA	GAT	ACA	GCA	GTA	GTG	CGG	ACG	TAT	AGG	AGG	TAT	AGA	480
Arg	Asp	Thr	Leu	Asp	Thr	Ala	Val	Val	Arg	Thr	Tyr	Arg	Arg	Tyr	Arg	
	145				150				155						160	
CCA	TTC	CCT	TAC	AGG	CAA	GAC	TGC	ATC	ACC	CAA	AAA	GTT	CTG	GGG	GAG	528
Pro	Phe	Pro	Tyr	Arg	Gln	Asp	Cys	Ile	Thr	Gln	Lys	Val	Leu	Gly	Glu	
				165					170					175		
GAT	CTC	TAT	AAC	TGT	ATT	CTT	GGA	GGA	AAC	TGG	ACC	TGT	ATA	ACT	GGG	576
Asp	Leu	Tyr	Asn	Cys	Ile	Leu	Gly	Gly	Asn	Trp	Thr	Cys	Ile	Thr	Gly	
			180					185					190			
GAC	CAA	CTA	CAA	TAC	TCA	GGA	GGC	TCT	ATT	GAA	TCC	TGT	AAG	TGG	TGT	624
Asp	Gln	Leu	Gln	Tyr	Ser	Gly	Gly	Ser	Ile	Glu	Ser	Cys	Lys	Trp	Cys	
		195					200					205				
GGT	TTT	AAA	TTC	CAA	AGA	AGT	GAG	GGG	TTA	CCA	CAC	TAC	CCC	ATT	GGC	672
Gly	Phe	Lys	Phe	Gln	Arg	Ser	Glu	Gly	Leu	Pro	His	Tyr	Pro	Ile	Gly	
	210					215					220					
AAG	TGT	AGG	CTG	AAG	AAT	GAG	ACT	GGC	TAC	AGA	TTA	GTA	GAC	GAC	ACC	720
Lys	Cys	Arg	Leu	Lys	Asn	Glu	Thr	Gly	Tyr	Arg	Leu	Val	Asp	Asp	Thr	
	225				230					235					240	
TCT	TGC	AAT	ATA	GGA	GGT	GTG	GCG	ATA	GTA	CCA	CAG	GGG	ATG	GTA	AAG	768
Ser	Cys	Asn	Ile	Gly	Gly	Val	Ala	Ile	Val	Pro	Gln	Gly	Met	Val	Lys	
				245					250					255		

TGT	AAG	ATA	GGA	GAC	ACA	ATT	GTA	CAG	GTC	ATA	GCT	CTT	GAC	ACC	AAA	816
Cys	Lys	Ile	Gly	Asp	Thr	Ile	Val	Gln	Val	Ile	Ala	Leu	Asp	Thr	Lys	
			260					265					270			
CTT	GGG	CCT	ATG	CCC	TGC	AAG	CCA	TAT	GAG	ATC	ATT	TCA	AGT	GAG	GGG	864
Leu	Gly	Pro	Met	Pro	Cys	Lys	Pro	Tyr	Glu	Ile	Ile	Ser	Ser	Glu	Gly	
		275					280					285				
CCC	GTA	GAA	AAG	ACG	GCA	TGT	ACC	TTC	AAC	TAC	ACG	AGG	ACA	TTA	AAG	912
Pro	Val	Glu	Lys	Thr	Ala	Cys	Thr	Phe	Asn	Tyr	Thr	Arg	Thr	Leu	Lys	
	290					295					300					
AAT	AAA	TAC	TTT	GAG	CCC	AGA	GAC	AGT	TAC	TTC	CAG	CAA	TAT	ATG	CTA	960
Asn	Lys	Tyr	Phe	Glu	Pro	Arg	Asp	Ser	Tyr	Phe	Gln	Gln	Tyr	Met	Leu	
305					310					315					320	
AAA	GGA	GAG	TAT	CAA	TAC	TGG	TTT	GAC	CTG	GAG	GTA	ACT	GAC	CAT	CAC	1008
Lys	Gly	Glu	Tyr	Gln	Tyr	Trp	Phe	Asp	Leu	Glu	Val	Thr	Asp	His	His	
				325					330					335		
CGG	GAT	TAC	TTG	GCC	GAG	TCC	ATA	TTA	GTG	GTG	GTG	GTA	GCT	CTC	CTG	1056
Arg	Asp	Tyr	Leu	Ala	Glu	Ser	Ile	Leu	Val	Val	Val	Val	Ala	Leu	Leu	
			340					345					350			
GGT	GGT	AGA	TAC	GTG	CTC	TGG	TTA	CTG	GTC	ACA	TAC	ATG	GTT	CTA	TCA	1104
Gly	Gly	Arg	Tyr	Val	Leu	Trp	Leu	Leu	Val	Thr	Tyr	Met	Val	Leu	Ser	
		355				360						365				
GAA	CAA	AAA	GTC	TTA	GGG											1122
Glu	Gln	Lys	Val	Leu	Gly											
		370														

(2) INFORMATION FOR SEQ ID NO.: 15

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 374

(ii) MOLECULAR TYPE: POLYPEPTIDE

(A) ORGANISM: bovine viral diarrhoea virus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 15

His	Leu	Asp	Cys	Lys	Pro	Glu	Tyr	Tyr	Tyr	Ala	Ile	Ala	Lys	Asn	Asp
1				5				10						15	
Arg	Val	Gly	Leu	Leu	Gly	Ala	Glu	Gly	Leu	Thr	Thr	Val	Trp	Lys	Glu
			20					25					30		
Tyr	Ser	Pro	Glu	Met	Thr	Leu	Glu	Asp	Thr	Met	Val	Ile	Ala	Ser	Cys
		35					40					45			
Arg	Glu	Gly	Lys	Phe	Thr	Tyr	Arg	Ser	Arg	Cys	Thr	Arg	Glu	Ala	Arg
	50					55					60				
Tyr	Leu	Ala	Ile	Leu	His	Ser	Arg	Ala	Leu	Pro	Thr	Ser	Val	Val	Phe
65					70				75						80
Glu	Lys	Leu	Phe	Glu	Gly	Gln	Lys	Gln	Glu	Asp	Thr	Val	Glu	Met	Asp
				85				90						95	

(2) INFORMATION FOR SEQ ID NO.: 16

(A) LENGTH: 70

(ii) MOLECULAR TYPE: DNA
(A) ORGANISM: bovine viral diarrhoea virus

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: (10)..(69)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: (1)..(70)
(D) OTHER INFORMATION: "Pestivirus signal sequence of CSFV strain
Alfort

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 16

GGATCCACC ATG GCC CTG TTG GCT TGG GCG GTG ATA ACA ATC TTG CTG TAC 51
Met Ala Leu Leu Ala Trp Ala Val Ile Thr Ile Leu Leu Tyr
1 5 10

CAG CCT GTA GCA GGG TAC C 70
Gln Pro Val Ala Gly Tyr
15 20

Claims

1. Synthetic BVDV-gene, characterised in that said BVDV-gene has a not naturally occurring nucleotide sequence encoding a naturally occurring amino acid sequence.
2. Synthetic BVDV-gene according to claim 1, characterised in that said BVDV-gene encodes the BVDV E2 protein.
3. Synthetic BVDV-gene according to claim 1 or 2, characterised in that in said BVDV-gene at least one naturally occurring putative splice-donor- or -acceptor site is removed.
4. Synthetic BVDV-gene according to claim 2 or 3, characterised in that said BVDV gene has a GC-content between 48 and 60 %, preferably between 48 and 50% %.
5. Synthetic BVDV-gene according to claim 4, characterised in that said BVDV gene has a GC-content of approximately 48 %.
6. Synthetic BVDV-gene according to claim 2, characterised in that said BVDV gene has the nucleic acid sequence presented in the overview of replacements in the sequence of SEQ ID NO: 14.
7. Synthetic BVDV-gene according to claims 1 to 6, characterised in that said BVDV gene is under the control of the MCMVie1-promoter, the MCMVe1-promoter, the HCMVie1-promoter or the BHV gE-promoter.
8. Live attenuated recombinant BHV carrier virus, characterised in that it carries a synthetic BVDV-gene according to claims 1 to 7.
9. Live attenuated recombinant BHV carrier virus according to claim 8, characterised in that said BHV-recombinant is a BHV-1 recombinant.

10. Live attenuated recombinant BHV carrier virus according to claim 9, characterised in that the synthetic BVDV-gene is inserted in the gE-gene of said BHV recombinant.
11. Live attenuated recombinant BHV carrier virus according to claim 9, characterised in that the synthetic BVDV-gene is inserted in the gI-gene of said BHV recombinant.
12. Live attenuated recombinant BHV carrier virus according to claims 8 to 11, characterised in that it comprises an additional gene encoding an antigen from a micro-organism or virus that is pathogenic for cattle.
13. Live attenuated recombinant BHV carrier virus according to claim 12, characterised in that the gene is chosen from the group of cattle pathogens, consisting of Bovine Rotavirus, Bovine Viral Diarrhoea virus, Parainfluenza type 3 virus, Bovine Paramyxovirus, Foot and Mouth Disease virus, Bovine Respiratory Syncytium virus and *Pasteurella haemolytica*.
14. Inactivated Recombinant BHV carrier virus particle, characterised in that it carries the BVDV E2 glycoprotein on its surface.
15. Vaccine for the protection of cattle against virus infection, characterised in that said vaccine comprises a live attenuated recombinant BHV carrier virus according to claims 8 to 13 and/or the DNA thereof and/or a BHV-recombinant virus particle according to claim 14 and a pharmaceutically acceptable carrier.
16. Vaccine according to claim 15, characterised in that it is formulated for intranasal administration.
17. Method for the preparation of a vaccine according to claim 15 or 16, characterised in that said method comprises the admixing of a live attenuated BHV-recombinant according to claims 8 to 13 and/or the DNA thereof and/or a BHV-recombinant virus particle according to claim 14 and a pharmaceutically acceptable carrier.
18. Method for the preparation of a live attenuated BHV-recombinant according to claims 8 to 13, said method comprising bringing together in a suitable host cell isolated BHV-DNA and a

vector comprising the synthetic BVDV-gene, placed under the control of a suitable promoter and flanked by 3' and 5' flanking regions that share homology with BHV-sequences.

19. Method for the preparation of a live attenuated BHV-recombinant according to claims 8 to 13, said method comprising bringing into a suitable host cell a vector comprising the synthetic BVDV-gene, placed under the control of a suitable promoter and flanked by 3' and 5' flanking regions that share homology with BHV-sequences, followed by infection of said suitable host cell with BHV.
20. Diagnostic test for the discrimination between sera from cattle infected with BVDV field strains and cattle vaccinated with a vaccine according to claim 15 or 16, said test being characterised in that the test comprises purified BVDV E2 protein.
21. Use of inactivated BHV carrier viruses according to the invention for the manufacture of vaccines for booster vaccination against BVDV-infection.

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Patent Agent

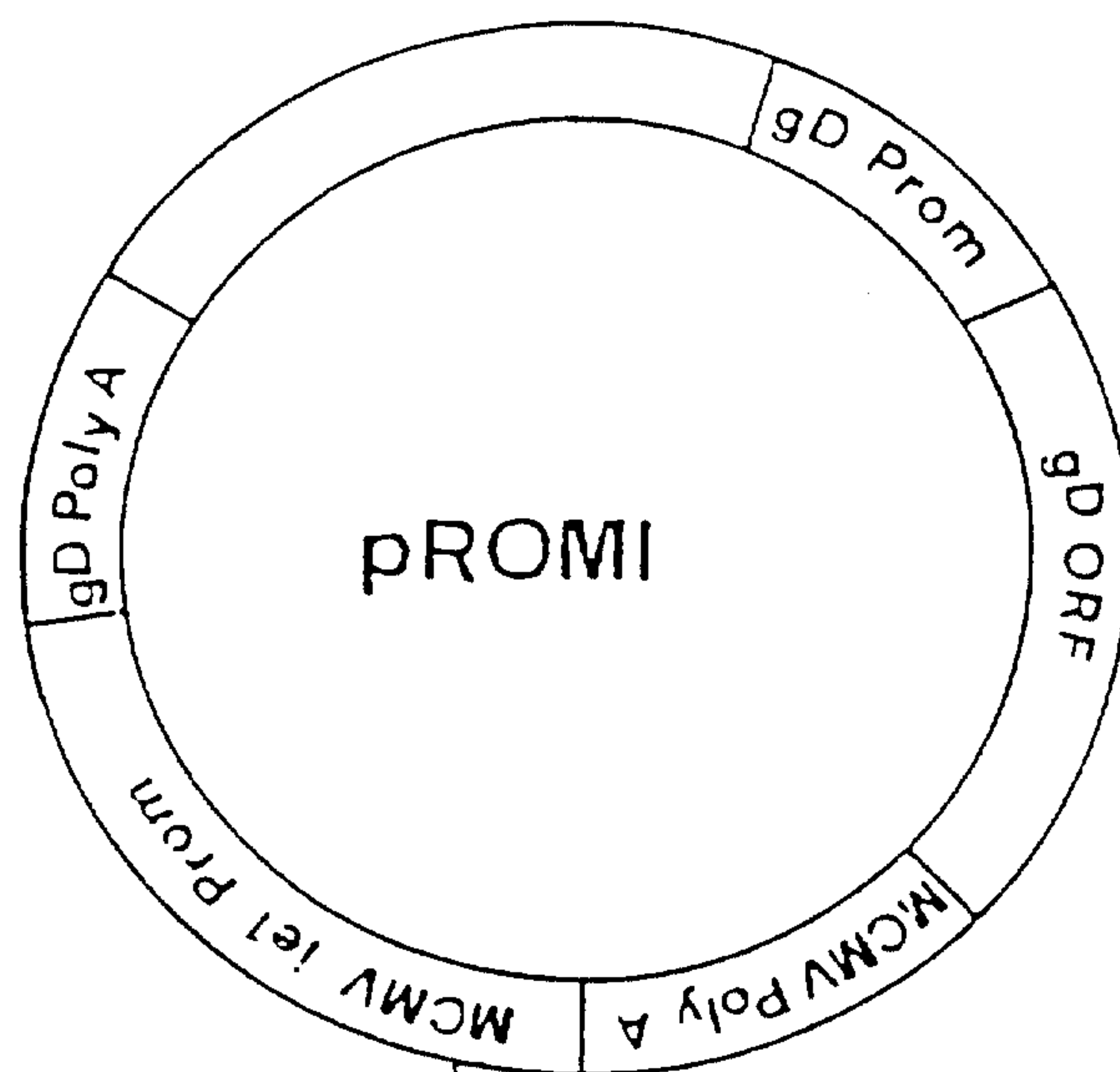
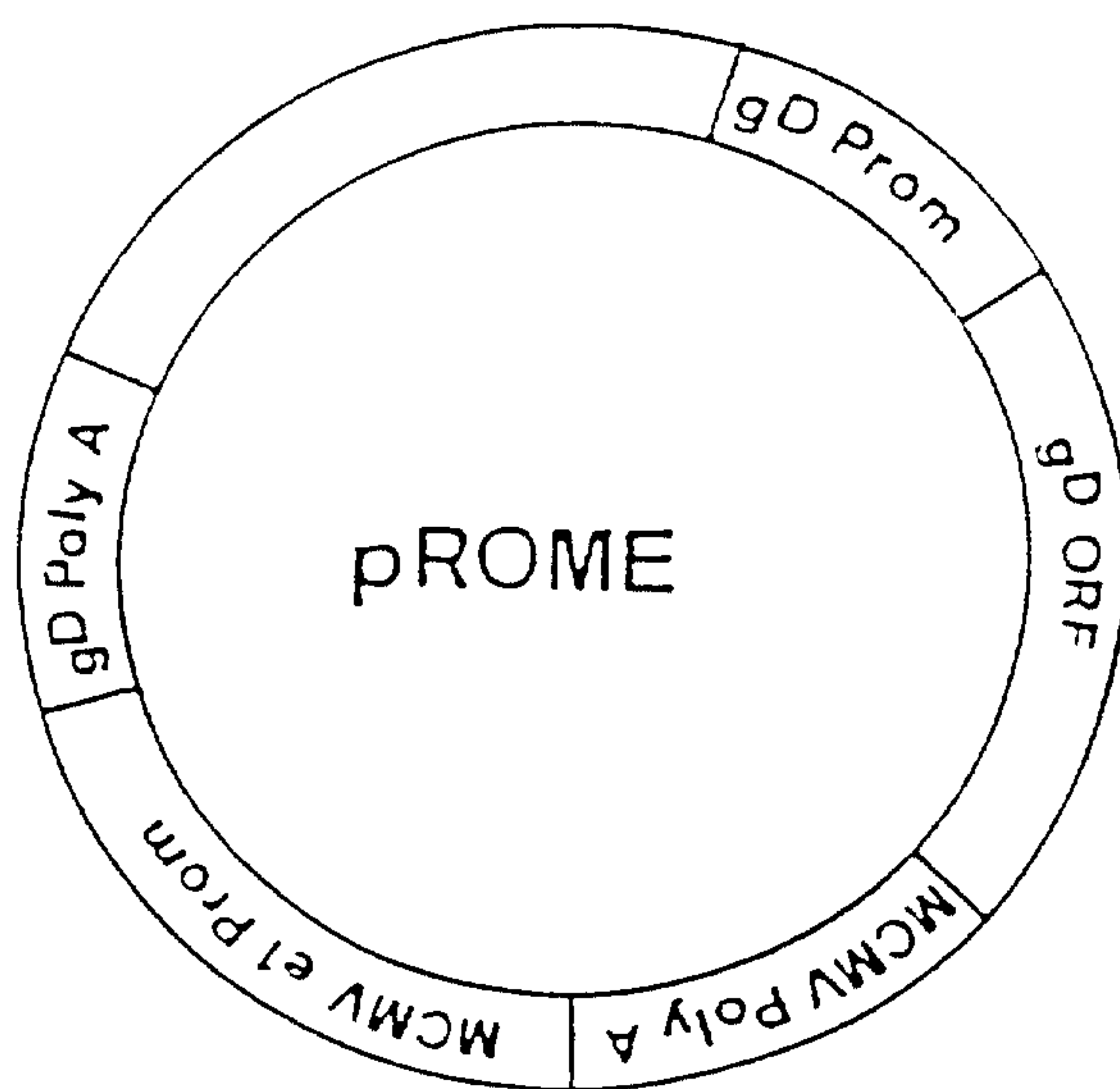
FIGURE 1FIGURE 2

FIGURE 3

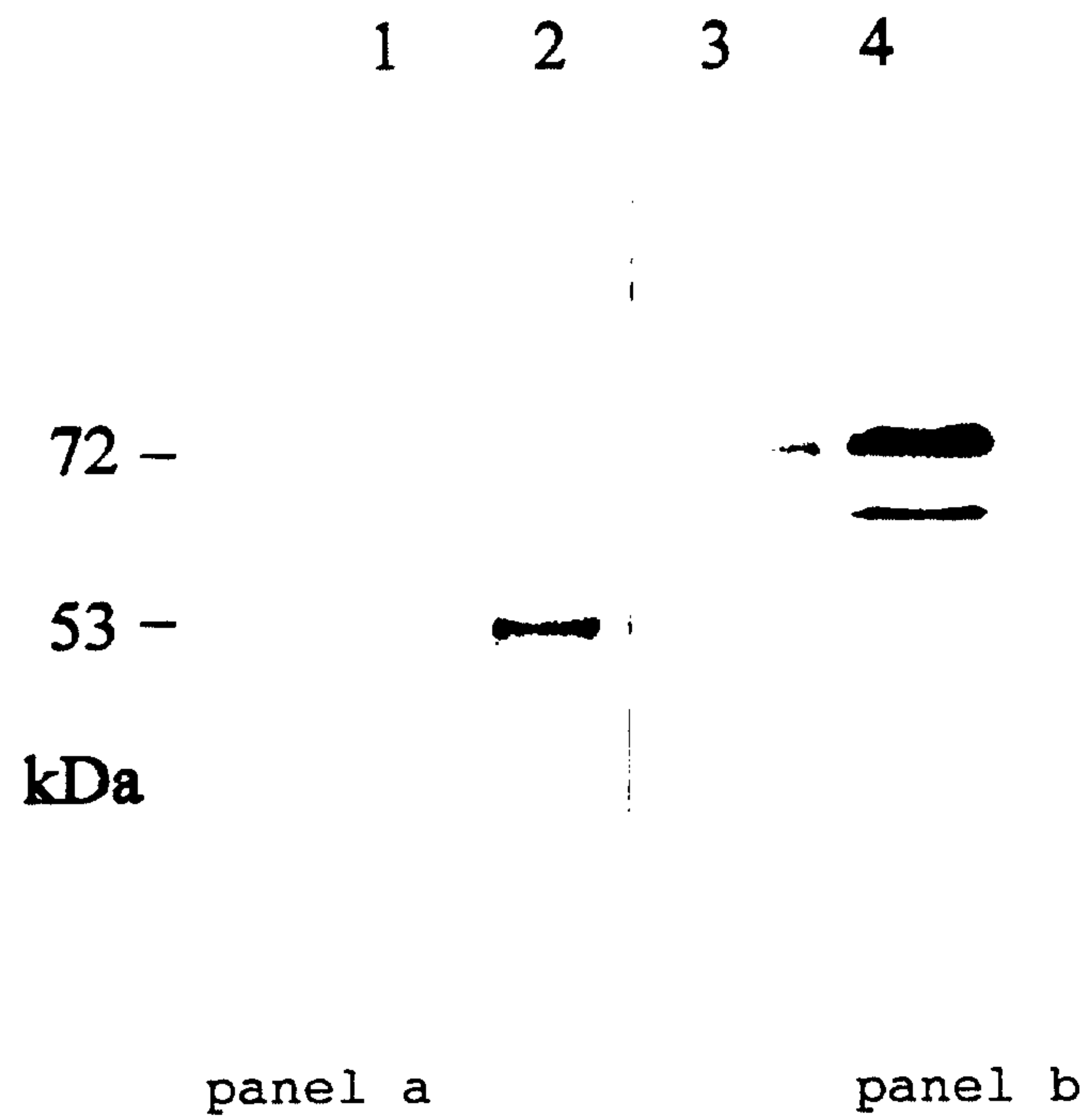


FIGURE 4

