EHV-4 GLYCOPROTEIN VACCINE
VACCIN A BASE DE GLYCOPROTEINE DE VHE-4 (VIRUS D’HERPES EQUIN-4)

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- WO-A-90/01546
- JOURNAL OF VIROLOGY, vol. 63, no. 1, October 1989, American Society for Microbiology; P. GUO et al., pp. 4189-4198
- JOURNAL OF GENERAL VIROLOGY, vol. 69, 1988, SGM (GB); A.A. CULLINANE et al., pp. 1575-1590

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Description

The present invention is concerned with a nucleic acid sequence encoding an Equine herpesvirus-4 polypeptide, a recombinant nucleic acid molecule comprising such a nucleic acid sequence, a vector virus or host cell containing said nucleic acid sequence, an EHV-4 polypeptide, antibodies immuno-reactive with said polypeptide, a vaccine against EHV-4 infection, as well as methods for the preparation of each of such a vaccine.

Equine herpesvirus-4 (EHV-4) is, like the related equine herpesvirus-1, an alphaherpesvirus responsible for significant economic losses within the equine industry. EHV-4 is primarily associated with respiratory disease though EHV-4 induced abortions are occasionally reported.

The genome of EHV-4 has been characterized as a double-stranded linear DNA molecule consisting of two covalently linked segments (L, 109 kbp; S, 35 kbp) the latter being flanked by inverted repeats. The glycoproteins of herpesviruses mediate essential viral functions such as cellular attachment, penetration into cells and pathogenicity. Furthermore, herpesvirus glycoproteins are critical components in the interaction of the virus with the host immune system.

A number of studies, predominantly with the well-characterized glycoproteins of herpes simplex virus (HSV), have demonstrated the importance of herpesvirus glycoproteins in both antibody and cellular immune responses. Although considerable diversity exists among the herpesvirus glycoproteins in structure and function, some similarities in DNA and protein sequence have been identified. This has lead to the classification of several herpesvirus proteins into different groups, each consisting of homologous proteins being related by the presence of specific conserved regions or sites. Groups of such homologues are for example: Herpes Simplex virus-1 (HSV-1) gB, Pseudorabies virus (PRV) gL, Bovine herpesvirus (BHV) gL, EHV-1gP, HSV-1gD, PRV gp50, BHV gL, HSV-1gE, PRV gL. The gH proteins of Herpes simplex virus type 1, Varicella-zoster virus and Pseudorabies virus (PRV) have been mapped and sequenced and shown to be involved in protection against the virus (Gompels, U and A. Minson (1986), Virology 153, 230). Keller, P.M. et al. (1987), Virology 157, 526; Patent application WO 89/10965).


However, none of these documents disclose the characterization or exact localization of the EHV-4 gH or gC homologue on the EHV-4 genome nor do they disclose or teach the use of said proteins or genes encoding said proteins for the preparations of a vaccine to prevent EHV-4 infection.

Herein, the EHV-4 gH-type protein and gC-type protein are termed EHV-4 gH and EHV-4 gC, respectively.

Control by vaccination of EHV-4 infection has been a long-sought goal. Current vaccines comprise chemically inactivated virus vaccines and modified live-virus vaccines. However, inactivated vaccines generally induce only a low level of immunity, requiring additional immunizations, disadvantageously require adjuvants and are expensive to produce. Further, some infectious virus particles may survive the inactivation process and causes disease after administration to the animal.

In general, attenuated live virus vaccines are preferred because they evoke a more long-lasting immune response (often both humoral and cellular) and are easier to produce. Up to now, only live attenuated EHV-4 vaccines are available which are based on live EHV-4 virus attenuated by serial passages of virulent strains in tissue culture. However, because of this treatment uncontrolled mutations are introduced into the viral genome, resulting in a population of virus particles heterogeneous in their virulence and immunizing properties.

In addition it is well known that such traditional attenuated live virus vaccines can revert to virulence resulting in disease of the inoculated animals and the possible spread of the pathogen to other animals.

Vaccines containing only the necessary and relevant EHV-4 immunogenic material which is capable of eliciting an immune response against the pathogen, or genetic information encoding said material, do not display above-mentioned disadvantages of the live or inactivated vaccines.

According to the present invention an EHV-4 gH or gC nucleic acid sequence encoding EHV-4 gH or gC polypeptide, or an antigenic fragment thereof characterized in that said sequence encodes a polypeptide having an amino acid sequence shown in SEQ ID No:1 or SEQ ID No:2 or derivatives of said polypeptide can be applied for the preparation of a vaccine for the immunization of horses against EHV-4 infection which does not display above-mentioned drawbacks of inactivated or live attenuated vaccines.

"Nucleic acid sequence" as used herein refers to a polymeric form of nucleotides of any length, but excludes the natural EHV-4 genome, both to ribonucleic acid sequences and to deoxy-ribonucleic acid sequences. In principle, this term refers to the primary structure of the molecule. Thus, this term includes double and single stranded DNA, as well as double and single stranded RNA, and modifications thereof.

In general, the term "polypeptide" refers to a molecular chain of amino acids, with a biological activity, does not refer to a specific length of the product but excludes the natural EHV-4 protein in its natural environment, and if required
can be modified in vivo or in vitro, for example by glycosylation, amidation, carboxylation or phosphorylation; thus inter alia peptides, oligopeptides and proteins are included.

Said gH or gC polypeptide are homologous with their gH or gC counter-parts of other herpesviruses and can be identified and characterized by the conserved regions and sites within the gH or gC polypeptide homologues.

The gene encoding EHV-4 gH polypeptide maps to the BamHI C fragment (figure 1) and encodes a protein of 855 amino acids in length with a predicted molecular weight of 94,100 D. From the amino acid sequence (SEQ ID No. 1) the following structural features characteristic of membrane glycoproteins can be derived:

- A signal peptide within the extreme N-terminal region of the primary translation product comprising a stretch of hydrophobic amino acid residues is identified. The cleavage site is at about Ala<sub>28</sub>, the predicted molecular weight of gH after cleavage of the signal peptide being about 92,130 D.
- Residues 20-616 constitute the hydrophilic external domain which possesses 11 N-linked glycosylation sites (N-X-S/T).
- The hydrophobic transmembrane domain of about 20 amino acid residues is located towards the C terminus.
- The cytoplasmic domain of EHV-4 gH stretches from about amino acid position 837-855.

A comparison of the amino sequence of the gH proteins of alpha, beta and gamma herpesviruses by Gompels et al. (J. Gen. Virol. 69, 2819, 1988) and Cranage et al. (J. Virol. 62, 1416, 1988) highlighted several features of the gH protein conserved throughout the herpesvirus family

- an unusually short cytoplasmic domain of 14 or 15 amino acids in alpha herperviruses and of 7 or 8 amino acids in beta and gammaherpesviruses
- four conserved cysteine residues at similar positions relative to the putative transmembrane domain and within conserved local sequence, and
- a conserved glycosylation site sequence NGTV 13-18 amino acids N-terminal to the transmembrane domain.

EHV-4 gH exhibits all above features: the proposed cytoplasmic domain is under 20 amino acids in length, the four conserved cysteines are present at positions 556, 591, 663 and 716, and the C-terminal glycosylation site is located within the sequence NGTV (amino acids 796-799) which is positioned 19 amino acids N-terminal to the putative EHV-4 transmembrane domain. The Cys residues at 737 and 740 in the EHV-4 gH occur at sites of cysteine conservation throughout most herpesvirus gHs, with the exception of HSV-1. The strong conservation of cysteine residues between the EHV-4 and HSV-1 gHs and, indeed, throughout the alpha, beta and gammaherpesvirus gHs investigated implies some degree of conservation of the secondary and tertiary structure of these proteins presumably involving disulphide bonding (Gompels et al., 1988, ibid).

The gene encoding the EHV-4 gC polypeptide maps to the BamHI G fragment (figure 2) and encodes a protein of 485 amino acids in length with a molecular weight of about 52,500 D. From the amino acid sequence (SEQ ID NO: 2) the following structural features characteristic of membrane glycoproteins can be derived:

- The signal peptide is identified at the N-terminus spanning about 32 amino acids with cleavage occurring between the Ala and Ser residues at positions 32 and 33 respectively
- The external domain of EHV-4 gC spans about residues 33 to 444 and possesses 11 N-linked glycosylation sites (N-X-S/T).
- An antigenic determinant of EHV-4 gC is located at about residue 409 (Asn) (Hopp and Woods (1981), PNAS 78, 3624).
- Amino acids 445-468 constitute the glycoprotein transmembrane domain.
- The C-terminal cytoplasmic domain spans residues 469 to 485, is hydrophilic and possesses a net positive charge of 2.

gC homologues comprise inter alia conserved amino acids in the C-terminal half positioned around six sites of cysteine conservation. A few of the N-linked glycosylation sites exist in similar positions but are not strictly conserved. A further common feature of gCs is that the C-terminal cytoplasmic domain is short and positively charged (Fitzpatrick, D.R. et al. (1989), Virology 173, 46; Allen, G.P. and Googol, L.D., ibid).

For the purpose of comparing the EHV-4 gC to other gCs in terms of the specifically conserved features an alignment of EHV-4 gC, BHV-1 gII, PRV gII, HSV-1 gC, and MDV A antigen is carried out. EHV-4 gC possesses cysteine residues at each of the six conserved positions, amino acids 256, 316, 357, 361, 390 and 416. Nine putative EHV-4 gC glycosylation sites are conserved in EHV-1 gp13 and three in PRV gII.

Also included within the present invention are nucleic acid sequences encoding an antigenic fragment of the EHV-4 gH or gC polypeptide, i.e. a fragment of said gH or gC polypeptide comprising a molecular configuration capable of
eliciting any type of immune response, humoral and/or cellular, against said gH or gC polypeptide in a susceptible animal, when presented in a suitable form. Furthermore, said fragment is characteristic for an EHV-4 gH or gC polypeptide.

Particularly, a nucleic acid sequence according to the invention can be used that encodes an EHV-4 polypeptide having an amino acid sequence depicted in SEQ ID NO: 1 or SEQ ID NO: 2, or a derivative of said polypeptide.

The gene encoding the EHV-4 gH and gC polypeptide have been localized on the EHV-4 genome and the nucleotide sequences thereof are depicted in SEQ ID NO: 1 and SEQ ID NO: 2, respectively. This information can be used to genetically manipulate said genes or derivatives thereof, for example to clone the genes by recombinant DNA techniques generally known in the art and to express the polypeptides encoded thereby in vitro or in vivo. Nucleic acid sequences having above-mentioned nucleotide sequences or derivatives thereof are preferably used for the expression of the EHV-4 gH or gC polypeptides.

It will be understood that for the particular EHV-4 gH or gC polypeptide embraced herein, natural variations can exist between individual EHV-4 viruses or strains. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. All such derivatives are included within the scope of this invention. Moreover, the potential exists to use recombinant DNA technology for the preparation of nucleic acid sequences encoding these various derivatives.

As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in an other codon but still coding for the same amino acid, e.g. the codon for the amino acid glutamic acid is both GAT and GAA. Consequently, it is clear that for the expression of a polypeptide with the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2, an antigenic fragment thereof use can be made of a derivate nucleic acid sequence with such an alternative codon composition different from the nucleic acid sequence shown in said SEQ ID's.

Furthermore, also fragments derived from the EHV-4 gH or gC polypeptide or from the amino acid sequences depicted in SEQ ID NO: 1 or SEQ ID NO: 2 which still display EHV-4 gH or gC antigenic properties, or fragments derived from the nucleotide sequences encoding the EHV-4 gH or gC polypeptide or derived from the nucleotide sequences depicted in said SEQ ID's encoding antigenic fragments of said gH or gC polypeptides are also included in the present invention.

All such modifications mentioned above resulting in such derivatives of the EHV-4 gH or gC polypeptide or gene are covered by the present invention so long as the characteristic EHV-4 gH or gC features remain unaffected in essence.

A nucleic acid sequence according to the present invention can be ligated to various expression effecting DNA sequences, optionally containing portions of DNA encoding fusion protein sequences such as β-galactosidase, resulting in a so called recombinant nucleic acid molecule which can be used for the transformation of a suitable host. Such hybrid DNA molecules, are preferably derived from, for example plasmids, or from nucleic acid sequences present in bacteriophages or viruses.

Specific vectors which can be used to clone nucleic acid sequences according to the invention are known in the art (e.g. Rodriguez, R.L. and D.T. Denhardt, ed., Vectors: A survey of molecular cloning vectors and their uses, Buttenworths, 1988).

The methods to be used for the construction of a recombinant nucleic acid molecule according to the invention are known to those of ordinary skill in the art and are inter alia set forth in Maniatis, T. et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

"Transformation", as used herein, refers to the introduction of a heterologous nucleic acid sequence into a host cell, irrespective of the method used, for example direct uptake or transduction. The heterologous nucleic acid acid may be maintained through autonomous replication or alternatively, may be integrated into the host genome. The recombinant DNA molecules preferably are provided with appropriate control sequences compatible with the designated host which can regulate the expression of the inserted nucleic acid sequence.

A suitable host cell is a cell which can be transformed by a nucleic acid sequence encoding a polypeptide or by a recombinant nucleic acid molecule comprising such a nucleic acid sequence and which can be used to express said polypeptide coded by said nucleic acid sequence. The host cell can be of procaryotic origin, e.g. bacteria such as E. coli, B. subtilis and Pseudomonas species; or of eucaryotic origin such as yeasts, e.g Saccharomyces cerevisiae or higher eucaryotic cells such as insect, plant or mammalian cells, including HeLa cells and Chinese hamster ovary (CHO) cells. Insect cells include the Sf9 cell line of Spodoptera frugiperda. Information with respect to the cloning and expression of the nucleic acid sequences of the present invention in eucaryotic cloning systems can be found in Esser, K. et al. (Plasmids of Eukaryotes, Springer-Verlag, 1986).

The nucleic acid sequences of the present invention are preferably operably linked to expression control sequences.

Such control sequences may comprise promoters, operators, inducers, ribosome binding sites etc.

When the host cells are bacteria, illustrative useful expression control sequences include the trp promoter and operator (Goeddel, et al., Nucl. Acids Res. 8, 4057, 1980); the lac promoter and operator (Chang, et al., Nature 275, 1046, 1978).
615, 1978); the outer membrane protein promoter (EMBO J. 1, 771-775, 1982); the bacteriophage λ promoters and operators (Nucl. Acids Res. 11, 4677-4686, 1983); the α-amylase (B. subtilis) promoter and operator, termination sequences and other expression enhancement and control sequences compatible with the selected host cell. When the host cell is yeast, illustrative useful expression control sequences include, e.g., α-mating factor. For insect cells the polyhedrin promoter of baculoviruses can be used (Mol. Cell. Biol. 3, 2156-65, 1983). When the host cell is of insect or mammalian origin illustrative useful expression control sequences include, e.g., the SV-40 promoter (Science 222, 524-527, 1983) or, e.g., the metallothionein promoter (Nature 296, 39-42, 1982) or a heat shock promoter (Voellmy et al., Proc. Natl. Acad. Sci. USA 82, 4949-53, 1985).

Alternatively, also expression control sequence present in EHV-4, in particular those regulating the expression of gH or gC may be applied. The present invention also comprises an EHV-4 gH or gC polypeptide or an antigenic fragment thereof, essentially free from the whole virus or other protein with which it is ordinarily associated.

In particular, a polypeptide comprising at least part of the amino acid sequence depicted in SEQ ID NO: 1 or SEQ ID NO: 2 or derivatives thereof is included in the present invention.

In an other embodiment of the invention a polypeptide having an amino acid sequence encoded by a nucleic acid sequence mentioned above is used. Immunization of horses against EHV-4 infection can, for example be achieved by administering to the horse a polypeptide according to the invention as a so-called subunit vaccine. The subunit vaccine according to the invention may comprise a polypeptide in a pure form, optionally in the presence of a pharmaceutically acceptable carrier. The polypeptide can optionally be covalently bonded to a non-related protein, which, for example can be of advantage in the purification of the fusion product. Examples are β-galactosidase, protein A, prochymosine, blood clotting factor Xa, etc.

In some cases the ability to raise neutralizing antibodies against these polypeptides per se may be low. Small fragments are preferably conjugated to carrier molecules in order to raise their immunogenicity. Suitable carriers for this purpose are macromolecules, such as natural polymers (proteins, like key hole limpet hemocyanin, albumin, toxins), synthetic polymers like polyamino acids (polylysine, polyalanine), or micelles of amphiphilic compounds like saponins. Alternatively these fragments may be provided as polymers thereof, preferably linear polymers.

Polypeptides to be used in such subunit vaccines can be prepared by methods known in the art, e.g. by isolation of said polypeptides from EHV-4, by recombinant DNA techniques or by chemical synthesis.

If required the polypeptides according to the invention to be used in a vaccine can be modified in vitro or in vivo, for example by glycosylation, amidation, carboxylation or phosphorylation.

An alternative to subunit vaccines are live vector vaccines. A nucleic acid sequence according to the invention is introduced by recombinant DNA techniques into a micro-organism (e.g. a bacterium or virus) in such a way that the recombinant micro-organism is still able to replicate thereby expressing a polypeptide coded by the inserted nucleic acid sequence. Next, this recombinant micro-organism can be administered to the horse for immunization whereafter it maintains itself for some time, or even replicates, in the body of the inoculated horse, expressing in vivo a polypeptide coded for by the inserted nucleic acid sequence according to the invention resulting in the stimulation of the immune system of the inoculated horse. Suitable vectors for the incorporation of a nucleic acid sequence according to the invention are derived from, for example viruses such as EHV-1, adenovirus, vaccinia virus or other pox viruses, papilloma virus or bacteria such as E. coli or specific Salmonella species. With recombinant micro-organisms of this type, the polypeptide synthesized in the host cell can be exposed as a surface antigen. In this context fusion of the said polypeptide with OMP proteins or pilus proteins of Escherichia coli or synthetic provision of signal and anchor sequences which are recognized by the organism are conceivable. It is also possible that the said immunogenic polypeptide, if desired as part of a larger whole, is released inside the animal to be immunized. In all of these cases it is also possible that one or more immunogenic products will find expression which generate protection against various pathogens and/or against various antigens of a given pathogen.

A vaccine according to the invention can be prepared by culturing a host cell comprising a nucleic acid sequence according to the invention, whereafter the cells and/or vector viruses grown in the cells can be collected, optionally in a pure form, and formed to a vaccine optionally in a lyophilized form.

Above mentioned host cells comprising a nucleic acid sequence according to the invention can also be cultured under conditions which are favourable for the expression of a polypeptide coded by said nucleic acid sequence. Vaccines may be prepared using samples of the crude culture, host cell lysates or host cell extracts, although in another embodiment more purified polypeptides according to the invention are formed to a vaccine, depending on its intended use. In order to purify the polypeptides produced, host cells containing a nucleic acid sequence according to the invention are cultured in an adequate volume and the polypeptides produced are isolated from such cells or from the medium if the protein is excreted. Polypeptides excreted into the medium can be isolated and purified by standard techniques, e.g. salt fractionation, chromatography, centrifugation, whereas intracellular polypeptides can be isolated by first collecting said cells, lysing the cells followed by separation of the polypeptides from the other intracellular
components and forming the polypeptides to a vaccine.

It goes without saying that horses already infected by EHV-4 can be treated with antibodies directed against said EHV-4. Antiserum or antibodies characteristic for a polypeptide according to the invention can be used for the therapeutic treatment of EHV-4 infection. Said characteristic antiserum or antibodies may be obtained by immunizing animals with an effective amount of EHV-4 gH or gC polypeptide in order to elicit an appropriate immune response. Thereafter the animals are bled and antiserum can be prepared.

Monoclonal antibodies directed against a polypeptide according to the invention can also be used for the therapy of horses infected with EHV-4. Said monoclonal antibodies can be produced by methods known in the art for this purpose, e.g. by immunizing mice with said polypeptide, immortalizing mouse spleen cells and selecting hybridomas producing useful antibodies. Immortal antibody-producing cell lines can also be created by direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus.

Monoclonal antibodies, in particular, may be used to raise anti-idiotypic antibodies by methods known in the art. These anti-idiotypic antibodies may also be useful for prevention of EHV-4 infection in horses.

Above mentioned antiserum and monoclonal antibodies can also be used for the immunological diagnosis of horses infected with EHV-4.

The vaccine according to the invention can be administered in a conventional active immunization scheme: single or repeated administration in a manner compatible with the dosage formulation and in such amount as will be prophylactically and/or therapeutically effective and immunogenic. The administration of the vaccine can be done, e.g. intra-dermally, subcutaneously, intramuscularly, intra-venously or intranasally.

Additionally the vaccine may also contain an aqueous medium or a water containing suspension, often mixed with other constituents, e.g. in order to increase the activity and/or shelf life. Those constituents may be salts, pH buffers, stabilizers (such as skimmed milk or casein hydrolysate), emulsifiers, adjuvants to improve the immune response (e.g. oils, muramyldipeptide, aluminium hydroxide, saponin, polyions and amphipathic substances) and preservatives.

It is clear that a vaccine according to the invention may also contain immunogens related to other pathogens of horses or may contain nucleic acid sequences encoding these immunogens, like antigens of EHV-1, equine influenza virus, -retrovirus, -infectious anemia virus, -arteritis virus, -encephalitis virus, Borna disease virus of horses, Berue virus of horses, E.coli or Streptococcus equi to produce a multivalent vaccine.

Example 1

Isolation and characterization of gH gene

1. Culturing of EHV-4 virus

Roller bottles of slightly sub-confluent monolayers of equine dermal cells (NBL-6) grown in Earle's Minimum Essential Medium (Flow) supplemented with 0.2% sodium bicarbonate, 1% non-essential amino acids, 1% glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 10% foetal calf serum were infected with virus of the EHV-4 strain 1942 at a m.o.i. of 0.003 and allowed to adsorb for 60 min at 37 °C. They were incubated at 31 °C until extensive c.p.e. was evident and the majority of cells had detached from the bottle surface (2-6 days). The infected cell medium was centrifuged at 5,000 r.p.m. for 5 min to pellet the cells, and the supernatant was centrifuged at 12,000 r.p.m. for 2 hours in a Sorvall GSA 6 X 200 ml rotor. The pellet was resuspended in 5 ml PBS, sonicated and centrifuged at 11,000 r.p.m. in a Sorvall SS34 rotor for 5 min to spin down cellular debris. Virus was then pelleted by centrifugation at 18,000 r.p.m. in a Sorvall SS34 rotor for 1 hour. Ratios of virus particles to plaque-forming units were approximately 1,000 to 5,000.

2. Preparation of EHV-4 DNA

The pelleted virus was resuspended in 10 ml NTE (NaCl/Tris/EDTA) and briefly sonicated. Contaminating cellular DNA was degraded by adding DNase at 10 µg/ml and incubating at 37 °C for 1 hour. SDS was added to a final concentration of 2%, and the preparation was extracted approximately 3 times with NTE equilibrated phenol until a clear interphase was obtained. A chloroform extraction was followed by ethanol precipitation of the DNA as described above. The DNA was pelleted, washed with 70% ethanol, resuspended in 10 ml of 100 mM NaCl and 10 µg/ml RNase and left overnight at room temperature. Further purification was achieved by treatment with 1 mg/ml proteinase K for 2 hours at 31 °C. The DNA was extracted once with phenol/chloroform (1:1 vol/vol), once with chloroform, ethanol precipitated, drained well and resuspended in 0.1 X SSC.
3. Cloning of EHV-4 DNA

EHV-4 BamHI DNA fragments were ligated into the vector pUC9, a plasmid which includes the ampicillin-resistance gene from pBR322 and the polylinker region from M13mp9 (Vieira, J. and Messing, J. (1982), Gene 19, 259). 5 μg of EHV-4 DNA and 5 μg pUC9 DNA were separately digested with BamHI.

Complete digestion was verified by gel electrophoresis of aliquots of the reactions and then the DNA was extracted twice with an equal volume of phenol:chloroform (1:1) and ethanol-precipitated. Ligation was performed essentially by the method of Tanaka and Weissblum (J. Bact. 121, 354, 1975). Approximately 0.1 μg of BamHI digested pUC9 and 1 μg of BamHI-digested EHV-4 DNA were mixed in 50 mM Tris-HCl pH 7.5, 8 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP in a final volume of 40 μl. 2 units of T4 DNA ligase (0.5 μl) were then added. The reaction was incubated at 4 °C for 16 hours.

Calcium-shocked E.coli DH1 cells (Hanahan, D. (1983), J Mol Biol. 166, 557) were transformed with the recombinant plasmids essentially described by Cohen et al. (Proc Natl Acad Sci. USA 69, 2110, 1972).

Additional clones were derived by restriction digestion of recombinant plasmid pUC9 containing BamHI C fragment (fig. 1), followed by recovering of the specific EHV-4 restriction fragments and sub-cloning thereof (Maniatis, T. et al., ibid) within the multi-cloning site of the Bluescript M13⁺ plasmid vector (Stratagene) for sequence analysis.

The nucleotide sequence of a region of BamHI C fragment spanning the gH gene was determined by using single stranded plasmid DNA as template and Bluescript-derived and custom-made oligonucleotides as primers in a Sanger dideoxy sequencing strategy (Sanger et al., Proc. Natl. Acad. Sci U.S.A. 74, 5463,1977) (fig. 1). The exact localisation, nucleic acid sequence and corresponding amino acid sequence of the gH gene is shown in the SEQ ID NO: 1.

Example 2
Isolation and characterization of gc gene

Culturing of EHV-4 virus, preparation of EHV-4 DNA and construction of a BamHI library in pUC9 was carried out as outlined above.

Recombinant plasmid pUC9:EHV-4 BamHI G was a restriction enzyme digested to generate subfragments of EHV-4 BamHI G which were then isolated from 0.7% agarose gels and cloned into a Bluescript M13⁺ plasmid vector (Stratagene) by standard techniques (Maniatis, T. et al., ibid.).

Recombinant plasmids were propagated in E. coli strain JM83 in 1-broth supplemented with ampicillin (100 μg/ml). Plasmid DNA was extracted from 50 ml bacterial cultures by the alkaline lysis method and purified by banding on CsCl gradients.

DNA sequencing was carried out by the Sanger dideoxy technique (Sanger et al., ibid.) using denatured recombinant plasmid DNA as template and M13⁺-specific or custom oligonucleotides as primers.

The nucleotide sequence of a region of the BamHI G fragment spanning the gc gene was determined by analysis of overlapping sequences according to the strategy detailed in figure 2. The exact localisation, nucleotide sequence and corresponding amino acid sequence of the gc gene is shown in the SEQ ID NO: 2.

Legend

Figure 1
(a) BamHI restriction map of the EHV-4 genome (Cullinan, A.A. et al., J. Gen. Virol. 69, 1575, 1988).
(b) Sequencing strategy and localisation of the EHV-4 gH gene.

Figure 2
(a) Idem figure 1.
(b) Restriction map of BamHI G indicating sites of cleavage of Sall, EcoRI, BglI and BgnI.
(c) Sequencing strategy and limits of open reading frames within BamHI G fragment.

Sequence Listing

SEQ ID NO: 1
Sequence type: nucleotide with corresponding protein
Sequence length: 2730 base pairs; 855 amino acids
Strandness: single
Topology: linear
Molecule type: genomic DNA

Original source
Organism: Equine herpesvirus-4
Immediate experimental source: genomic BamHI library
Properties: EHV-4 gH gene.

CAGCGCGCGC GAGATACCG AGGATCCAG TGGTTGATA TTGGGAATAA ATACTGCTGC

GATT ATG TCA CAA CGG TAT CTA AAA ATA GCT ATC TTA GTG GCC GCT ACT
Met Ser Gin Pro Tyr Leu Lys Ile Ala Ile Leu Val Ala Ala Thr

ATT GTG TCT GCC ATT CCC GTG ACA ACA CGG GTT TCA ACT TCA CCA
Ile Val Ser Ala Ile Pro Val Trp Thr Thr Pro Val Ser Thr Ser Pro

CCC CAA CAA ACA AAA TGG CAC TAT GTG GGA AAT GGT ACC TGG GTA GAC
Pro Gin Gin Thr Lys Leu His Tyr Val Gly Asn Gly Thr Thr Val His

AAC AAT ACA TTC ACG GTA ACC AGG TAT GAC AGG ATA ACC ATG GAA CCA
Asn Asn Thr Phe Asn Val Thr Arg Tyr Asp Arg Ile Thr Met Glu Pro

GTT TAT AAT AAC AAT TTA TCC TCT ACT ACC TTT TTT GCT ATA TCG
Val Tyr Asn Asn Leu Ser Ser Thr Thr Pro Val Phe Val Ala Ile Ser

GAG AGA AAT TTT CCG AGC GTT AAG GCT ACA TTA GGA GCG TCC GTA TTT
Glu Arg Asn Phe Arg Thr Val Asn Thr Pro Leu Gly Ala Ser Val Phe

TGG ATT TTA AAA AGC GCT CTT AAT CAC CCC AAA CAC AAA CCC TGT ATA
Trp Ile Leu Lys Ser Ala Leu Asn Pro Pro Lys His Gln Pro Cys Ile

GCT AAT GTG CCA GAA CCC GTG GAC CCA CCC GGA CCG TGC GTC AAC TCA
Ala Asn Val Pro Glu Pro Gly Asp Pro Arg Gly Pro Cys Val Asn Ser

ACT GTG AGT CTA TTT TTT AAT GAC AAT TTT GAG CCC TTT TTA ATG ACA
Thr Val Ser Leu Phe Asn Asp Asn Leu Glu Pro Phe Leu Met Thr
AAA AAT CTT TTG GAG TTT GAA GTA TTG CCC GAC AAC TAC ATA ACC GGA
Lys Asn Leu Leu Glu Phe Glu Val Leu Pro Asp Asn Tyr Ile Thr Gly

TGG AGC TTT GAG CGG TCT AAA ACT GTG GCT AGC AAA GGC AAC CGG GTT
Trp Thr Phe Glu Arg Ser Lys Thr Val Ala Thr Lys Gly Asn Pro Val

GGA GTG GTT CTC TCC CCT CCC GGA ACA AGT CCG GAT GTA AAT AAC ACC
Gly Val Val Leu Ser Pro Arg Thr Ser Pro Asp Val Asn Thr

ATA AGA GAT GAT GGC ACC CCT AAA CAG CAC TTT AGC ATT ATA GAC GAA
Ile Arg Asp Asp Gly Thr Pro Lys Gin His Leu Ser Ile Ile Asp Glu

CAT ACT ACG TTT GTG CTC GAC GTC CAA AAT TTT ACA AAA ACT TTA ACT
His Thr Thr Phe Val Leu Asp Leu Gin Phe Thr Lys Thr Leu Thr

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Claims

1. EHV-4 gH or gC nucleic acid sequence encoding EHV-4 gH or gC polypeptide, or an antigenic fragment thereof characterised in that said sequence encodes a polypeptide having an amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2 or derivatives of said polypeptide.

2. Nucleic acid sequence according to claim 1, characterised in that said sequence comprises the deoxynucleic acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2 or derivatives of said deoxynucleic acid sequence.

3. Recombinant nucleic acid molecule comprising a nucleic acid sequence according to claims 1-2, operably linked to an expression control system.

4. Vector virus containing a recombinant nucleic acid molecule according to claim 3.

5. Host cell containing a nucleic acid sequence according to claims 1-2 or a recombinant nucleic acid molecule according to claim 3 or a vector virus according to claim 4.

6. EHV-4 gH or gC polypeptide or an antigenic fragment thereof comprising at least part of the amino acid sequence shown in SEQ ID No: 1 or SEQ ID No: 2 or derivatives of said polypeptide.

7. EHV-4 polypeptide encoded by a nucleic acid sequence according to claims 1-2.

8. Antibody or antiserum immuno-reactive with a polypeptide according to claims 6-7.

9. Vaccine for the protection of horses against EHV-4 infection, characterised in that it comprises a nucleic acid sequence according to claims 1-2, a recombinant nucleic acid molecule according to claim 3, a vector virus according to claim 4, a host cell according to claim 5 or a polypeptide according to claims 6-7.

10. Method for preparation of an EHV-4 vaccine, characterised in that a host cell according to claim 5 is cultured, whereafter EHV-4 containing material is collected and processed to a pharmaceutical preparation with immunizing activity.

11. Method for the preparation of an EHV-4 vaccine, characterised in that a polypeptide according to claims 6-7 is processed to a pharmaceutical preparation with immunizing activity.

12. Use of an effective amount of a vaccine according to claim 9 in the preparation of a medicament for the prophylactic and/or therapeutic treatment of EHV-4 infection.
Patentansprüche

1. EHV-4 gH- oder gC-Nucleinsäuresequenz, die ein EHV-4 gH- oder gC-Polypeptid kodiert, oder ein Antigenfragment hiervon, dadurch gekennzeichnet, daß die Sequenz ein Polypeptid kodiert, das eine Aminosäuresequenz aufweist, die in SEQ ID NO: 1 oder SEQ ID NO: 2 oder Derivaten des genannten Polypeptids dargestellt ist.

2. Nucleinsäuresequenz nach Anspruch 1, dadurch gekennzeichnet, daß die Sequenz die Desoxynucleinsäuresequenz aufweist, die in SEQ ID NO: 1 oder SEQ ID NO: 2 oder Derivaten dieser Desoxynucleinsäuresequenz dargestellt ist.

3. Molekül einer rekombinanten Nucleinsäure, enthaltend eine Nucleinsäuresequenz nach Anspruch 1 oder 2, die mit einem Expressionssteuersystem funktionierbar verknüpft ist.


6. EHV-4 gH- oder gC-Polypeptid oder ein Antigenfragment hiervon, enthaltend mindestens einen Teil der Aminosäuresequenz, die in SEQ ID NO: 1 oder SEQ ID NO: 2 oder Derivaten des genannten Polypeptids dargestellt ist.

7. EHV-4-Polypeptid, das durch eine Nucleinsäuresequenz nach Anspruch 1 oder 2 kodiert ist.

8. Antikörper oder Antiserum, der bzw. das mit einem Polypeptid nach den Ansprüchen 6 oder 7 immunoreaktiv ist.


10. Verfahren zum Herstellen eines EHV-4-Impfstoffes, dadurch gekennzeichnet, daß eine Wirtszelle nach Anspruch 5 gezüchtet wird und anschließend EHV-4-enhaltendes Material gesammelt sowie zu einem pharmazeutischen Präparat mit immunisierender Wirkung verarbeitet wird.

11. Verfahren zum Herstellen eines EHV-4-Impfstoffes, dadurch gekennzeichnet, daß ein Polypeptid nach den Ansprüchen 6 oder 7 zu einem pharmazeutischen Präparat mit immunisierender Wirkung verarbeitet wird.

12. Verwendung einer wirksamen Menge eines Impfstoffes nach Anspruch 9 bei der Herstellung eines Arzneimittels für die prophylaktische und/oder therapeutische Behandlung einer EHV-4-Infektion.

Revendications

1. Séquence d’acides nucléiques gH ou gC de VHE-4 encodant le polypeptide gH ou gC de VHE-4, ou un fragment antigénique de celui-ci, caractérisée en ce que ladite séquence encodé un polypeptide ayant une séquence d’acides aminés présentée dans SEQ ID NO:1, ou SEQ ID NO:2, ou dans des dérivés dudit polypeptide.

2. Séquence d’acides nucléiques selon la revendication 1, caractérisée en ce que ladite séquence contient la séquence d’acides désoxy nucléiques présentée dans SEQ ID NO:1, ou SEQ ID NO:2, ou dans des dérivés de ladite séquence d’acides désoxy nucléiques.

3. Molécule d’acide nucléique recombinant comprenant une séquence d’acides nucléique selon les revendications 1 et 2, liée de façon opérationnelle à un système de contrôle d'expression.

4. Virus vecteur contenant une molécule d’acide nucléique selon la revendication 3.

5. Cellule hôte contenant une séquence d’acides nucléiques selon les revendications 1 et 2, ou une molécule d’acide nucléique recombinant selon la revendication 3, ou un virus vecteur selon la revendication 4.
6. Polypeptide gH ou gC de VHE-4, ou fragment antigénique de celui-ci, comprenant au moins une partie de la séquence d'acides aminés présentée dans SEQ ID NO:1, ou SEQ ID NO:2, ou dans des dérivés dudit polypeptide.

7. Polypeptide de VHE-4 encodé par une séquence d'acides nucléiques selon les revendications 1 et 2.

8. Anticorps ou antisérum immuno-réactif vis-à-vis d'un polypeptide selon les revendications 6 et 7.

9. Vaccin pour la protection des chevaux contre l'infection par le VHE-4, caractérisé en ce qu'il comprend une séquence d'acides nucléiques selon les revendications 1 et 2, une molécule d'acide nucléique recombinant selon la revendication 3, un virus vecteur selon la revendication 4, une cellule hôte selon la revendication 5, ou un polypeptide selon les revendications 6 et 7.

10. Méthode de préparation d'un vaccin à base de VHE-4, caractérisée en ce qu'une cellule hôte selon la revendication 5 est cultivée, après quoi le matériau contenant le VHE-4 est recueilli, et transformé en une préparation pharmaceutique présentant une activité immunsante.

11. Méthode de préparation d'un vaccin à base de VHE-4, caractérisée en ce qu'un polypeptide selon les revendications 6 et 7, est transformé en une préparation pharmaceutique présentant une activité immunsante.

12. Utilisation d'une quantité efficace d'un vaccin selon la revendication 9, dans la préparation d'un médicament pour le traitement prophylactique et/ou thérapeutique de l'infection par le VHE-4.
FIG. 1