(19) DANMARK

(10) DK/EP 2403867 T3

(12) Oversættelse af europæisk patentskrift

Patent- og Varemærkestyrelsen

(51) Int.Cl.: *C 07 K 14/015 (2006.01)* *A 61 K 39/23 (2006.01)* *C 12 N 7/04 (2006.01)* *C 12 N 15/35 (2006.01)*

(45) Oversættelsen bekendtgjort den: **2019-08-12**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2019-05-22**

(86) Europæisk ansøgning nr.: **10708716.5**

(86) Europæisk indleveringsdag: **2010-03-04**

(87) Den europæiske ansøgnings publiceringsdag: **2012-01-11**

(86) International ansøgning nr.: **EP2010001343**

(87) Internationalt publikationsnr.: **WO2010099960**

(30) Prioritet: **2009-03-04 US 157436 P** **2010-02-19 US 306205 P**

(84) Designerede stater: **AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK SM TR**

(73) Patenthaver: **Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Tyskland**
Medigene AG, Lochhamer Strasse 11, 82152 Planegg, Tyskland

(72) Opfinder: **KLEINSCHMIDT, Jürgen, Weihwiesenweg 5, 69245 Bammental, Tyskland**
HÖRER, Markus, Josef-Beyerl-Str. 10, 82152 Planegg, Tyskland
SONNTAG, Florian, Oberrother Strasse 31, 89299 Unterroth, Tyskland
LUX, Kerstin, Axel-von-Ambesser-Strasse 7, 81371 München, Tyskland

(74) Fuldmægtig i Danmark: **Plougmann Vingtoft A/S, Strandvejen 70, 2900 Hellerup, Danmark**

(54) Benævnelse: **ASSEMBLY ACTIVATING PROTEIN (AAP) OG DETS ANVENDELSE TIL FREMSTILLING AF PARVOVIRUSPARTIKLER HOVEDSAGELIGT BESTÅENDE AF VP3**

(56) Fremdragne publikationer:
WO-A2-02/053703
WO-A2-2004/027019
WO-A2-2008/145401
HOQUE M ET AL: "Nuclear transport of the major capsid protein is essential for adeno-associated virus capsid formation" 19990901; 19990900, vol. 73, no. 9, 1 September 1999 (1999-09-01), pages 7912-7915, XP002290874
RUFFING M ET AL: "MUTATIONS IN THE CARBOXY TERMINUS OF ADENO-ASSOCIATED VIRUS 2 CAPSID PROTEINS AFFECT VIRAL INFECTIVITY: LACK OF AN RGD INTEGRIN-BINDING MOTIF" JOURNAL OF GENERAL VIROLOGY, SOCIETY FOR GENERAL MICROBIOLOGY, SPENCERS WOOD, GB, vol. 75, no. 12, 1 January 1994 (1994-01-01), pages 3385-3392, XP008030694 ISSN: 0022-1317 & DATABASE EMBL [Online] 8 December 2008 (2008-12-08), Ruffing, M. et al.: "Adeno-associated virus-2" XP002592832 Database accession no. NC_001401
RABINOWITZ J E ET AL: "Insertional Mutagenesis of AAV2 Capsid and the Production of Recombinant Virus" VIROLOGY, ACADEMIC PRESS,ORLANDO, US LNKD- DOI:10.1006/VIRO.1999.0045, vol. 265, no. 2, 20

Fortsættes ...

December 1999 (1999-12-20), pages 274-285, XP004439668 ISSN: 0042-6822
WARRINGTON K H ET AL: "ADENO-ASSOCIATED VIRUS TYPE 2 VP2 CAPSID PROTEIN IS NONESSENTIAL AND CAN TOLERATE LARGE PEPTIDE INSERTIONS AT ITS N TERMINUS" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US LNKD- DOI:10.1128/JVI.78.12.6595-6609.2004, vol. 78, no. 12, 1 June 2004 (2004-06-01), pages 6595-6609, XP001194467 ISSN: 0022-538X
WU P ET AL: "MUTATIONAL ANALYSIS OF THE ADENO-ASSOCIATED VIRUS TYPE 2 (AAV2) CAPSID GENE AND CONSTRUCTION OF AAV2 VECTORS WITH ALTERED TROPISM" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US LNKD- DOI:10.1128/JVI.74.18.8635-8647.2000, vol. 74, no. 18, 1 September 2000 (2000-09-01), pages 8635-8647, XP001028219 ISSN: 0022-538X
STEINBACH S ET AL: "Assembly of adeno-associated virus type 2 capsids in vitro" JOURNAL OF GENERAL VIROLOGY, SOCIETY FOR GENERAL MICROBIOLOGY, SPENCERS WOOD, GB, vol. 78, no. 6, 1 January 1997 (1997-01-01), pages 1453-1462, XP002346582 ISSN: 0022-1317
RUFFING M ET AL: "ASSEMBLY OF VIRUSLIKE PARTICLES BY RECOMBINANT STRUCTURAL PROTEINS OF ADENO-ASSOCIATED VIRUS TYPE 2 IN INSECT CELLS" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 66, no. 12, 1 December 1992 (1992-12-01), pages 6922-6930, XP001032438 ISSN: 0022-538X
SONNTAG FLORIAN ET AL: "A viral assembly factor promotes AAV2 capsid formation in the nucleolus." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 1 JUN 2010 LNKD- PUBMED:20479244, vol. 107, no. 22, 1 June 2010 (2010-06-01) , pages 10220-10225, XP002591761 ISSN: 1091-6490
F. SONNTAG ET AL: "The Assembly-Activating Protein Promotes Capsid Assembly of Different Adeno-Associated Virus Serotypes", JOURNAL OF VIROLOGY, vol. 85, no. 23, 14 September 2011 (2011-09-14), pages 12686-12697, XP55187887, ISSN: 0022-538X, DOI: 10.1128/JVI.05359-11

DESCRIPTION

[0001] The present invention relates to nucleic acids encoding the novel parvoviral protein "assembly activating protein" (AAP), the encoded polypeptides, antibodies specific for AAP, the use of the nucleic acids for the preparation of the polypeptides, the use of the nucleic acids or the polypeptides for the preparation of the parvoviral particle and methods of producing parvoviral particles essentially consisting of VP3 by providing in addition to the coding sequence of the parvoviral structural protein VP3 a sequence fragment Z/a nucleic acid encoding AAP in the cell and expressing VP3 and fragment Z under control of a rep-independent promoter. Furthermore, the present invention relates to parvoviral particles essentially consisting of VP3 and/or obtainable by the above method. The present invention further relates to said parvoviral particle for use as a medicament, particularly a vaccine, or for use in the prevention or treatment of specific diseases.

[0002] Mutated parvovirus structural protein-based virus-like particles (VLPs) have been shown to be suitable vaccine candidates (WO 2008/145401). Based on such mutated parvovirus structural proteins, VLPs were generated for the presentation of tolerogens or small antigens or even individual epitopes. These VLPs proved especially beneficial, where B cell tolerance has to be broken to have a therapeutic effect for the patient.

[0003] For the clinical development of vaccines based on VLPs it is generally necessary to generate a product which ideally is based on a single active compound/protein and which is as pure as possible. With respect of VLPs this is a problem in general as viruses are often composed of more than one protein and are capable of packaging specifically viral DNA or unspecifically DNA from the host cell. Accordingly it is desirable to obtain "pure" VLPs that contain as few different proteins as possible and preferably no nucleic acid. In the literature, several attempts have been made to efficiently produce those particles.

[0004] Rabinowitz et al. (e.g. Rabinowitz et al., 1999) have altered the structural genes of AAV2 by linker insertional mutagenesis in order to define critical components of virion assembly and infectivity. They generated the mutant H2634 that contains the *rep* and c*ap* ORFs and an insertion at the HaeIII restriction site at position 2634. Importantly, due to the presence of the *rep* ORF this insertion mutant expressed the respective Rep protein. It assembled intact virions and the capsid appeared to be composed only of VP3. According to the authors the undetectable expression of VP1 and VP2 in either cell lysates or purified virions could have been a problem of detection limits.

[0005] Warrington et al. (2004) and WO 2004/027019 also addressed the question of the specific roles of the individual capsid proteins in capsid formation to define where full-length peptides can be inserted into the AAV capsid ORF without disruption of critical structural domains. Generating constructs containing the *rep* and c*ap* ORF with mutations in the start codons of VP1, VP2 and/or VP3 and thus expressing only a single or two capsid protein(s) in the presence of Rep, Warrington et al. showed that genome-containing particles were formed as long as the VP3 protein was present. Hence, mutants expressing VP1 and/or VP2 as single capsid proteins or together did not form particles. Rather they concluded from their results that VP1 is necessary for viral infection but not essential for capsid assembly and particle formation whereas VP2 appears to be nonessential for viral infectivity. Moreover, they observed that expression of VP3 alone from constructs with

mutated start codons for VP1 and VP2 is sufficient to form VLPs.

[0006] Just as well, Grieger et al. (2007) generated VP3-only particles using the AAV2 helper plasmid pXR2 (containing *rep* and c*ap* genes, Li et al. (2008)) via mutagenesis of the VP1 and VP2 start codon. Expression of VP3- as well as VP2/VP3-only constructs in the presence of Rep resulted in noninfectious viral particles as long as they lacked the VP1 subunit.

[0007] From their results on the formation of genome-containing AAV-like particles from mutants expressing VP3 as only capsid protein in the presence of Rep it seemed that these particles can readily be obtained.

[0008] All the expression constructs described above expressed Rep proteins which should be omitted to assemble VLPs that are composed preferably of one protein and no DNA. Rep does not only represent a further protein that is attached to VLPs but also is held responsible for packaging of virus genomes and unspecific DNA into preformed capsids (King et al., 2001). Packaging of DNA is to be avoided as VLPs potentially can enter cells of a patient and thereby transfect such contaminating DNA, which may cause all sorts of unwanted effects.

[0009] To be sure that only VP3 is expressed, Hoque et al. (1999a, 1999b) and Handa et al. (JP 2001169777) generated expression constructs comprising the coding sequence (cds) of VP3 alone under control of a heterologous promoter in the absence of any Rep cds. Surprisingly, they could not produce viral particles from these expression constructs. By analyzing a series of deletion mutants of VP2 that started expression at different sites 5' of the VP3 start codon, they identified a region necessary for nuclear transfer of VP3 and found that the efficiency of nuclear localization of the capsid proteins and the efficiency of VLP formation correlated well. They observed that viral particles were formed as long as a region between amino acid 29 and 34 in the cds of VP2 or in other words in the 5' extension of VP3, was present. From the amino acid motif of this region which is PARKRL they concluded that it functions as a nuclear localization signal (NLS) which is important for the translocation of VP3 into the nucleus.

[0010] Alternatively, capsids also could be obtained if the NLS of simian virus 40 (SV40) large T antigen was fused to the N-terminus of the VP3 protein (NLS_{SV40}-VP3). This fusion protein could form VLPs indicating that the VP2-specific region located on the N-terminal side of the protein is not structurally required. Due to this finding the authors reasoned that VP3 has sufficient information for VLP formation and that VP2 is necessary only for nuclear transfer of the capsid proteins, which again is a prerequisite for VLP formation.

[0011] Due to the method for mutant construction used by them, all constructs started with an ATG start codon directly at the 5' end of the coding sequence. Since in general the "position effect" (Kozak, 2002) will cause the first (most upstream) ATG start codon of a transcript to initiate translation, the main protein to be expressed and generating the particle will be N-terminally extended VP3. Only a minor part of translation will start at the further downstream ATG start codon of VP3.

[0012] In agreement with Hoque et al. (supra) and Handa et al. (supra) and using constructs described by them, we could not detect VLPs consisting of VP3 alone from expression constructs

comprising the cds of VP3 alone under control of a constitutive promoter in neither mammalian cells nor insect cells in quantitative amounts (meaning that $<10^{10}$, particularly $<10^{8}$ capsids/ml were present) using the AAV2 Titration ELISA (quantified according to the instructions of the manufacturer Progen, Heidelberg, Germany, Fig. 15B). Nor could we detect AAV-like particles expressing VP1 or VP2 alone from expression constructs comprising the respective cds alone starting with an ATG codon under control of a constitutive promoter. The efficiency of capsid production of all constructs alone or in different combinations of different ratios in the presence or absence of Rep expression and in the presence or absence of co-delivery of adenoviral helper genes was at the lower detection limit of the AAV2 Titration ELISA ($<10^{8}$ capsids/ml, see above).

[0013] We could confirm that VLPs can be generated from expression constructs comprising some sequence 5' of the VP3 start codon together with the sequence coding for VP3, but in contrast to the results of Hoque et al., we could not quantify capsid assembly in detectable amounts ($\geq 10^{8}$ capsids/ml, see example 8) using the NLS_{sv40}-VP3 fusion construct. Accordingly, the method of Hoque et al. is not suitable for the generation of large amount of pure VLPs suitable for vaccination purposes for the market.

[0014] Taken together, the prior art techniques either use expression systems in the presence of Rep inevitably leading to the packaging of Rep and DNA or in the absence of Rep yields of VP3 VLPs are too low in order to generate a commercially viable process or product.

[0015] Accordingly, it was an object of the present invention to provide particles useful as a vaccine based on VLPs and methods of producing the same avoiding one or more of the above disadvantages. Particularly, it is desirable that the VLPs essentially consist of only one type of viral protein, contain no or only very little amounts of DNA and/or that they may be produced in an economical manner, e.g. in high yields.

[0016] The problem is solved by providing parvoviral particles consisting essentially of VP3, with essentially no VP1, VP2 and Rep proteins. They may be produced by expressing in a cell VP3 from a VP3 coding sequence (cds) of the parvoviral structural protein VP3 (VP3 cds) under control of a rep-independent promoter. Additionally, in this method a DNA sequence fragment (fragment Z) (partially) encoding a newly identified polypeptide designated "assembly activating protein" (AAP) is expressed, which allows for high yields, e.g. approximately about 10^{5}, preferably about 10^{6}, and more preferably about 10^{7} virus particles to be formed per cell. The identification of this novel protein is a totally new concept with respect to the assembly of parvoviral capsids in general and especially for VP3 capsids, as no sequence motif within a VP2 protein such as the postulated "PARKRL" motif or a heterologous nuclear localization sequence for VP3 is required as postulated (Hoque et al., 1999a, 1999b).

[0017] In contrast to the state of the art these VLPs do not contain a heterologous NLS or a VP2 protein. Upon epitope insertion at one or several of the preferred sites in the VP3, particles could be successfully assembled that presented epitopes for vaccine development. With this method 10^{11}, preferably about 10^{12}, and more preferably about 10^{13} virus particles are formed per ml crude lysate and therefore yields are sufficient for a commercially viable product.

[0018] Surprisingly and in line with its function of encoding a polypeptide, the sequence fragment Z can be provided either *in cis* or *in trans* to assemble capsids consisting essentially of VP3. Further, fragment Z and VP3 can be derived from the same or different species of parvovirus families, mutually trans-complementing each other regarding VP3 particle assembly.

[0019] The following definitions explain how the defined terms are to be interpreted in the context of the products, methods and uses of the present invention.

[0020] "AA" is used as abbreviation for amino acid(s), "nt" is used as abbreviation for nucleotide(s).

[0021] As used herein, a "parvovirus" or "parvoviral" relates to a member of the family of Parvoviridae wherein the wildtype expresses VP1, VP2 and VP3 as capsid proteins. The family of Parvoviridae contains several genera divided between 2 subfamilies Parvovirinae (Parvovirus, Erythrovirus, Dependovirus, Amdovirus and Bocavirus) and Densovirinae (Densovirus, Iteravirus, Brevidensovirus, Pefudensovirus and Contravirus) (Fields: Virology, fourth edition 2001, Volume 2, chapters 69 and 70, Lippincott Williams Wilkins, Philadelphia; http://virus.stanford.edu/parvo/parvovirus. html http://www.ncbi.nlm.nih.gov/ICTVdb/ Ictv/fs_parvo.htm#SubFamily1). The wildtype capsid is assembled of the three structural proteins VP1, VP2 and VP3 that form the 60 subunits of the AAV capsid in a ratio of 1:1:8 (Kronenberg et al., 2001). Hence, the term "VP3" stands for virus protein 3. The naturally occurring parvoviral particle is composed of the icosahedral capsid that encloses the single stranded DNA genome. Preferred parvoviruses are the Dependoviruses, including AAV.

[0022] As used herein, the term "serotype" stands for the kind of virus of a group of closely related viruses distinguished by their characteristic set of antigens. Thus, the serotype is characterized by serologic typing (testing for recognizable antigens on the virus surface). Accordingly, the AAV can also be selected from a serotype evolved from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 to AAV12 and AAV13, in particular from AAV2.

[0023] Parvoviral particles consisting "essentially of VP3" or "essentially only VP3" means that the capsid is assembled to at least 98%, preferably at least 99%, more preferably at least 99,6% and essentially at least 99,8% of VP3. This means that only 1/50, preferably 1/100, more preferably 1/250 and essentially only 1/500 or less of the proteins assembling the capsid are N-terminally extended versions of VP3 or completely different proteins. The capsid may be assembled to at least 98%, preferably at least 99%, more preferably at least 99.6% and essentially at least 99.8% of VP3, meaning that only 1/50, preferably 1/100, more preferably 1/250 and essentially only 1/500 or less of the proteins assembling the capsid are N-terminally extended versions of VP3 or different parvoviral proteins. It is especially preferred that the parvoviral capsid consists only of one protein, which is VP3 in its wildtype sequence or a mutated form of it.

[0024] A "coding sequence" or "cds" means that portion of a gene which directly specifies the amino acid (AA) sequence of its product. Hence, the "VP3 coding sequence" or "VP3 cds" defines that part of the cap gene from which the genetic code is translated into the amino acid (AA) sequence of a VP3, which can be wildtype or mutated as further defined in this invention. The VP3 cds is located at the 3' end of the cap ORF and starts with an ATG nucleotide triplet coding for a methionine. Depending from the individual parvovirus chosen, the VP3 cds is translated into about

533 Aas. E.g. for AAV2 the cds of the major coat protein VP3 can be obtained from the NCBI entree (http://www.ncbi.nlm.nih.gov/) NC_001401 (nucleotides 2809-4410) according to Ruffing et al. (1994), the AA sequence from the corresponding NCBI entree YP_680428. Here, a VP3 cds encodes a VP3 protein which is capable of particle formation according to the methods of this invention. An N-terminally extended VP3 protein comprises one or more of the respective Aas of VP2. Accordingly, VP2 can be seen as an N-terminally extended VP3, in contrast to a VP3 which has an N-terminal insertion of a heterologous sequence thereto, such as a Tag or an epitope as further defined below.

[0025] The genetic code defines a mapping between tri-nucleotide sequences, called "codons", and Aas. A triplet codon in a nucleic acid sequence usually specifies a single AA.

[0026] A "reading frame" is a contiguous and non-overlapping set of tri-nucleotide codons in DNA or RNA. There are 3 possible reading frames in an mRNA strand and six in a double stranded DNA molecule due to the two strands from which transcription is possible. An "open reading frame" (ORF) is a reading frame that contains a start codon, the subsequent region which usually has a length which is a multiple of 3 nucleotides, and ends with a stop codon. An ORF could potentially encode a protein. Insertion of one or two nucleotides unambiguously results in a shift to a different reading frame (frameshift mutation). Usually, ATG is used as the start codon. However, as already known from VP2 of AAV non-canonical start codons are sometimes used.

[0027] "Mutations" are changes to the nucleotide sequence of the genetic material of an organism. Such mutations may lead to a change of the encoded protein and therefore may have varying effects depending on where they occur and whether they alter the structure and/or function of the encoded protein. Structurally, mutations can be classified as point mutations, insertions adding one or more extra nt into the DNA/AA into the protein or deletions removing one or more nt/AA. An "insertion" of nt/AA is generally speaking an insertion of at least one heterologous nt/AA into the sequence of - for this invention - a parvovirus protein. 'Heterologous' in this context means heterologous as compared to the virus, from which the parvovirus protein is derived. Exemplified for a parvovirus structural protein, the inserted Aas can simply be inserted between two given Aas of the parvovirus structural protein. An insertion of Aas can also go along with a deletion of given Aas of the parvovirus structural protein at the site of insertion, leading to a complete substitution (e.g. 10 given Aas are substituted by 10 or more inserted Aas) or partial substitution (e.g. 10 given Aas are substituted by 8 inserted Aas) of Aas of the parvovirus structural protein.

[0028] In addition to an open reading frame beginning with a start codon close to its 5' end some further sequence requirements in the local environment of the start codon have to be fulfilled to initiate protein synthesis. One of these is the "Kozak sequence". The amount of protein synthesized from a given mRNA is dependent on the strength of the Kozak sequence. For a 'strong' consensus, relative to the translation initiation codon that is referred to as number 1 the nucleotides at positions +4 (i.e. G in the consensus) and -3 (i.e. either A or G in the consensus) must both match the consensus (there is no number 0 position). An 'adequate' consensus has only 1 of these sites, while a 'weak' consensus has neither. The cc at -1 and -2 are not as conserved, but contribute to the overall strength. There is also evidence that a G in the -6 position is important in the initiation of translation.

[0029] The term "percent identity" with respect to two sequences, particular amino acid sequences, indicates how many amino acids or bases are identical in an alignment of two sequences. For normalization, either the length of longer sequence, of shorter sequence or of columns of alignment occupied in both sequences, may be used. Usually, sequence alignment software is used in order to determine percent identity of sequences. Common software tools used for general sequence alignment tasks include for example ClustalW and T-coffee for alignment, and BLAST and FASTA3x for database searching. The skilled person will be able to select a suitable method or software and appropriate settings when assessing percent identity.

[0030] "Nucleic acid molecule" may be in the form of RNA, such as mRNA or cRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA e.g. obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The DNA may be triple-stranded, double- stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

[0031] A "Rep-independent promoter" is a promoter which can be activated in the absence of the Rep protein, whereas in the context of this invention Rep stands for the non-structural protein(s) encoded by a parvovirus, particularly Rep40, Rep52, Rep68 and Rep78 as described by Muzyczka and Berns (2001). These promoters include for example heterologous constitutive promoters and inducible promoters.

[0032] "Gene expression" is the process by which inheritable information from a gene, such as the DNA sequence, is made into a functional gene product, such as protein or nucleic acid. Thus, gene expression always includes transcription, but not necessarily translation into protein. rRNA and tRNA genes are an example for non-protein coding genes that are expressed into rRNA and tRNA, respectively, and not translated into protein. In order for gene expression to take place a promoter preferably has to be present near the gene to provide (a) binding site(s) and recruit (an) enzyme(s) to start transcription.

[0033] "Shut off" of gene expression means that expression of a gene is blocked. It may be either through genetic modification (a change in the DNA sequence including mutation or deletion of the start codon, at least part of the cds or at least part of a sequence element necessary for its expression like e.g. the promoter), or by treatment with a reagent such as a short DNA or RNA oligonucleotide with a sequence complementary to either an mRNA transcript or a gene. Latter can preferably be used for transient shut off.

[0034] "Poly (A)" sites at the 3' end of the transcript signal the addition of a series of adenines during the RNA processing step before migration to the cytoplasm. These so-called poly(A) tails increase RNA stability.

[0035] The "sequence fragment Z" or "fragment Z" is a DNA fragment that comprises

1. (i) at least 44 nucleotides upstream of the VP3 start codon and
2. (ii) more than 242 nucleotides of the VP3 cds starting with the start codon,
   derived from
    1. (a) a parvovirus, or

2. (b) a nucleotide sequence that is at least 60%, preferably 80%, more preferably 90%, especially 99% and advantageously 100% identical to the nucleotide sequence of fragment Z derived from AAV2 (sequence 1, Fig. 2), or
3. (c) a nucleic acid sequence that hybridizes in 4x SSC, 0.1% SDS at 65°C to the complementary strand of the fragment Z DNA molecule of AAV2, or
4. (d) a nucleic acid sequence that can be used in trans-complementation assays to cause assembly of VP3 VLPs.

This means that the sequence of fragment Z at the same time represents part of the VP2 and VP3 cds, since the AAV capsid genes are encoded by overlapping sequences of the same ORF using alternative mRNA splicing and alternative translational start codons. Thus, the VP2 gene contains the whole VP3 gene sequence with a specific 5' region (schematic representation in Fig. 1).

[0036] A "functionally active variant" of the claimed polypeptide or a nucleic acid is a polypeptide or a nucleic acid that is defined as in the claims and is a variant obtained by one or more mutations as detailed herein, which is functionally active in that the variant maintains its biological function, e.g. its capability to promote assembly of VP3. The biological activity may be determined in trans-complementation assays, where the expression of such polypeptide from such nucleic acid is able to promote assembly of VP3 VLPs from a VP3 coding construct whose expression under suitable conditions is insufficient for VP3 capsid assembly. Suitable insufficient AAV2 VP3 coding constructs are pCMV-VP3/2809 or pCI-VP3. A suitable test is described in the Examples, e.g. in Example 3. Preferably, maintenance of biological function is defined as having at least 50%, preferably at least 60%, more preferably at least 70%, 80% or 90%, still more preferably 95% of the activity of the natural occurring AAP.
Complementation assays can be performed as described in example 3 and either be analyzed by ELISA (example 1.5) or by immunofluorescence (1.6). Both assays are based on the detection of virus particles by the binding of a monoclonal antibody to the viral capsid in an assembled state. For example the monoclonal antibody A20 (Progen, Heidelberg, Germany) binds to the viral capsid of AAV2 and some other AAV serotypes, for more distantly related serotypes specific antibodies are commercially available. If no specific antibody is available, viral capsids can be detected by electron microscopy (for example see Hoque et al. (1999b)), or sucrose density gradient analysis (example 1.3.2.)

[0037] "Extended versions of VP3" comprise in general N-terminal extensions by several Aas. These N-terminal extensions represent the 3' part of the sequence coding for VP2 but not for VP3, since the AAV capsid genes are encoded by overlapping sequences of the same ORF using different start codons (Fig. 1). Thus, N-terminally extended VP3 is identical to N-terminally truncated VP2 meaning that parts of VP2 can be present within the N-terminal extension of VP3 but no complete and intact wildtype VP2 protein is expressed as e.g. given by Ruffing et al. (1994) and accessible from NCBI (number of entree: NC_001401. According to this invention the particles consist essentially of VP3 (as defined) and therefore extended versions of VP3 are very rare, whereas naturally occurring particles comprise VP1 :VP2:VP3 in a ratio of 1:1:8 (Kronenberg et al., 2001).

[0038] To determine the composition of capsid proteins expressed in a given sample Western blot analysis can be used. The cell lysate or purified VLPs can be fractionated on a sucrose gradient

and fractions analyzed upon gel electrophoresis and transfer to a nitrocellulose membrane, where they can be probed using binders specific to the target protein. The monoclonal antibody B1 reacts with all three capsid proteins and can be used to detect VP3, whereas the monoclonal antibody A69 reacts only with VP1 and VP2 and can be used to detect truncated VP2.

[0039] In the context of this disclosure "efficient particle formation" means that a high titer of particles is formed of about 10^{11}, preferably of about 10^{12}, and more preferably of about 10^{13} particles/ml in crude lysate (corresponding to about 10^5, preferably about 10^6, and more preferably about 10^7 particles/ transfected cell).

[0040] The term "about" means according to the invention a general error range of ± 20%, especially ± 10%, in particular ± 5%.

[0041] Virus particle titers can be quantified from lysates of transfected cells (see above) in their undiluted form or in a dilution using a commercially available titration ELISA kit which is based on the binding of the monoclonal antibody A20 to the viral capsid in an assembled state to measure the virus concentration. As already described above, if the antibody A20 does not bind to the capsid of e.g. a different virus serotype, particle titers can be visualized by electron microscopy and quantified by counting (Grimm et al., 1999, Grimm and Kleinschmidt, 1999, Mittereder et al., 1996).

[0042] To analyze protein expression and estimate its amount cell lysates of identical portions of transfected cells can be processed for SDS-PAGE. Upon gel electrophoresis and transfer to a nitrocellulose membrane, proteins can be probed using binders specific to the target protein (e.g. monoclonal antibodies B1, A69, anti-GFP). The amount of protein translation can be estimated from the amount of binders that specifically bind to the protein. These complexes can be visualized and quantified by e.g. immunohistochemical staining, immunofluorescent staining or radioactive labeling.

[0043] The term "binder" refers to a molecule that specifically binds to its respective binding partner. Commonly used binders are antibodies, especially monoclonal antibodies, antibody derivatives such as single chain antibodies or antibody fragments. In principle all classes of antibodies can be used, preferred are IgG antibodies. Fragments or multimers of antibodies can equally be used. Commonly used fragments are single chain antibodies, Fab- or $(Fab)_2$-fragments. Examples of other suitable binders are protein scaffolds such as anticalins or lipocalins (Nygren and Skerra, 2004), receptors or parts thereof (e.g. soluble T-cell receptors), ankyrine, microbodies or aptamers.

[0044] The term "specifically binds" means that two molecules A and B, preferably proteins, bind to each other thereby generating complex AB with an affinity ($K_D=k_{off}/k_{on}$) of at least $K_D=1x10^{-5}$ mol/l, preferably at least $1x10^{-7}$ mol/l, more preferably at least $1x10^{-8}$ mol/l, especially at least $1x10^{-9}$ mol/l.

[0045] An "epitope" is the part of a macromolecule that is recognized by the immune system, specifically by antibodies, B-cells, or T-cells.

[0046] A "mimotope" is a non-linear structural epitope composed of several Aas derived from different regions of the linear sequence of the structural protein located in close neighborhood due to the overall tertiary structure of the capsid or from a non-peptide structure such as carbohydrate residues, nucleic acids or lipids, and such non-linear structural epitope is specifically bound by an antibody. Thus, by mimicking the structure of an epitope the mimotope causes an antibody response identical to the one elicited by the epitope. The mimotope in the context of the present invention might consist of (parts of) the inserted peptide sequence alone or might be composed of inserted peptide and parvovirus core particle AA residues.

[0047] As used herein the term "B-cell epitope" is meant to include also mimotopes. Therefore, the epitopes can be both linear and structural.

[0048] The term "antigen" in the context of the products, methods and uses of the present invention refers to any target antigen against which an immune reaction should be induced. Such target antigens are usually antigens that are susceptible to the humoral immune response. They are usually proteins that may be posttranslationally modified, as for example glycosylated proteins.

[0049] The term "Immunoglobulin" (abbr. Ig) refers to any of the glycoproteins naturally occurring in the blood serum that are induced in response to invasion by immunogenic antigens and that protect the host by eradicating pathogens. In total, there are five human antibody classes, known as IgM, IgG, IgA, IgD and IgE, which belong to this group of proteins.

[0050] In a first aspect, this invention relates to a nucleic acid encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, and SEQ ID NO: 22, or encoding a polypeptide comprising a functionally active variant of any of these amino acid sequences, wherein the functionally active variant is capable of promoting the assembly of a parvoviral capsid consisting essentially of VP3 and wherein the functionally active variant has an amino acid sequence that is at least 60% identical to any of the amino acid sequences of SEQ ID NO: 1 to 22, wherein the nucleic acid is incapable of expressing any of the functional Rep proteins, VP1, VP2 and VP3.

[0051] As described are functionally active variants, wherein the functionally active variant

1. (i) has an amino acid sequence that is at least 60% identical to any of the amino acid sequences of SEQ ID NO: 1 to 22, and/or
2. (ii) is encoded by a cDNA that hybridizes in 6x SSC, 5x Denhardt's solution, 0.5% SDS at 40°C for 2 to 12 hours to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, and SEQ ID NO: 44, or to a nucleic acid sequence complementary to any of the nucleic acid sequences of SEQ ID NO: 23 to SEQ ID NO: 44; and/or

3. (iii) is encoded by a part of a parvoviral genome comprising an open reading frame (ORF) not in frame with that encoding VP1, VP2 and VP3, that includes more than 378 nucleotides of the VP3 ORF,

wherein the nucleic acid is incapable of expressing any of the functional Rep proteins, particularly incapable of expressing Rep40, Rep52, Rep68, Rep78, VP1, VP2 and VP3.

[0052] It was demonstrated that co-expression of a so far unidentified product of the AAV2 cap gene efficiently promotes assembly of VP3 into an icosahedral capsid. This protein, designated assembly activating protein or AAP is encoded by ORF2 of the cap gene (wherein the first ORF encodes VP1, VP2 and VP3) and has a molecular weight of approximately 23 kDa. The molecular weight of AAP estimated from Western blots was higher (about 30 kDa) maybe due to posttranslational modification(s). Its cellular localization is in the nucleolus and it targets the VP proteins to the nucleolus where capsid assembly takes place. However, nucleolar localization of VP3 alone is not sufficient for capsid formation, indicating that AAP provides an additional chaperon-type, scaffold and/or nucleation function also within the full length AAV genome.

[0053] Homologous polypeptides can be identified for different parvoviruses. Such an alignment of predicted AAP protein sequences derived from ORF2 of the *cap* gene of different parvoviruses are shown in Figure 28. Accordingly, the nucleic acid according to the invention is preferably characterized in that it encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 1 (AAV2), SEQ ID NO: 2 (AAV1), or the amino acid sequence of SEQ ID NO: 5 (AAV5).

[0054] It is envisaged by this invention that naturally occurring AAP may be modified but remains functionally active. Such functionally active variants may be generated e.g. in order to increase expression, stability and/or activity, or in order to facilitate easier cloning of constructs. Accordingly, an functionally active variant may have an amino acid sequence that is at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95%, most preferably at least 99% and especially 100% identical to any of the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, and SEQ ID NO: 22.

[0055] Described are also functionally active variants that are encoded by a cDNA that hybridizes in 6x SSC, 5x Denhardt's solution, 0.5% SDS at 45°C, more preferably at 50°C, more preferably at 55°C, more preferably at 60°C, especially at 65°C and advantageously at 68°C to a nucleic acid sequence complementary to any of the nucleic acid sequences of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, and SEQ ID NO: 44, such as a functionally active variant encoded by a cDNA that hybridizes at the conditions specified above in 6x SSC, 5x Denhardt's solution, 0.5% SDS to the nucleic acid sequence of SEQ ID NO: 23, or a nucleic acid sequence complementary to the nucleic acid sequence of SEQ ID NO: 23.

[0056] In a preferred embodiment of the invention, the nucleic acid encodes a polypeptide consisting of an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, and SEQ ID NO: 22, or encodes a polypeptide consisting of a functionally active variant of any of these amino acid sequences, as defined in the claims. The functionally active variant is defined above. More preferably the nucleic acid encodes a polypeptide consisting of an amino acid sequence selected from the group consisting of SEQ ID NO: 1, to SEQ ID NO: 22.

[0057] Due to N- and C-terminal truncation experiments with AAP it has been found that with respect to the 3'-end of AAP of AAV2 378 nt overlapping with the VP3 ORF starting at ATG_{2809} are not able to support VP3 capsid assembly, whereas 445 nucleotides of the VP3 ORF are about equally efficient in yield of capsids as wt AAV. Accordingly, the nucleic acid of the invention is characterized in that it includes more than 378 nucleotides (such as more than 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399 nucleotides), preferable at least 400 nucleotides (such as at least 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424 nucleotides), more preferably at least 425 nucleotides (such as at least 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444 or 445 nucleotides), and especially at least 445 nucleotides (such as 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487 or 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500 or more nucleotides) of the VP3 ORF.

[0058] With respect to the 5'-end of AAP of AAV2 an N-terminally truncated AAP encoded by a nucleic acid with a 44 nucleotide extension upstream of the VP3 start codon is about equally efficient in yield of capsids as wt AAV, if translation is started by an ATG inserted in frame to ORF2, and with lower efficiency if no ATG start codon is inserted (data not shown). An N-terminally truncated AAP encoded by a nucleic acid starting with an ATG instead of the ACG at position 2858 did not lead to detectable capsid formation. For AAV4 and AAV9 it was shown that a VP3 cds expression construct starting at the respective VP3 start codon is sufficient for detectable capsid assembly, therefore still encoding functional AAP (variant) (data not shown).

[0059] Accordingly, the nucleic acid of the invention is characterized in that it includes at least 44 nucleotides (such as 44, 45, 46, 47, 48, 49, or 50 nucleotides), preferably at least 20 nucleotides (such as 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42 or 43 nucleotides), more preferably at least 5 nucleotides (such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 nucleotides) of the adjacent VP2-encoding nucleotides, which are located in direct succession of the 5' of the VP3 start codon.

[0060] The nucleic acid encoding AAP or variants thereof may even start 3' of the VP3 start codon, as can be seen from AAV4 and AAV9 (above). Therefore, in another preferred embodiment, the nucleic acid of the invention is characterized in that its start codon is an ATG at 4 nucleotides, preferably 24 nucleotides, and more preferably 44 nucleotides downstream of the VP3 start codon.

[0061] Therefore, in preferred aspect the nucleic acid of the disclosure comprises nucleotides starting at least at 44 nucleotides upstream and 445 nucleotides downstream of the VP3 start codon (counting includes the ATG), preferably at least 20 nucleotides (such as 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41,. 42, 43 or 44 nucleotides) upstream and 425 nucleotides (such as 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444 or 445 nucleotides) downstream of the VP3 start codon, and especially at least 5 nucleotides (such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 nucleotides) upstream and 400 nucleotides (such as 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423 or 424 nucleotides) downstream of the VP3 start codon. Accordingly, total length of the nucleic acid of the invention is at least 489 nt (such as 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500 or more nt), preferably at least 445 nt (such as 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487 or 488 nt), and especially at least 405 nt (such as 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443 or 444 nt).

[0062] The nucleic acid of the invention is capable of expressing a protein promoting capsid assembly of VP3. It may be characterized in that it is derived from AAV2 and its translation start codon (found in wildtype AAV2 sequences) is $C_{2729}TG$, $A_{2735}CG$, $A_{2717}TT$ or $T_{2720}TG$ or that it is derived from another parvovirus and its translation start codon is at the homologous site to the translation start codons of AAV2. Homologous start codons for other parvoviruses can easily be identified by the given alignment (see Fig. 27) and looking for amino acids encoded by potential non-canonical start codons. Such potential non-canonical start codons can easily be verified by mutational analysis as done for AAV2 $C_{2729}TG$ in example 14. For parvoviruses not shown in Fig. 27 such a sequence can easily be added to the given alignment.

[0063] In a preferred embodiment the AAP encoding ORF is mutated in a way in order to generate an ATG start codon allowing for improved translation of the open reading frame, whereas "improved" means higher expression of AAP or variants thereof compared to the respective wildtype sequence. Preferably one of the translation start codons of AAV2 or the homologous sites of other parvoviruses is mutated into an ATG start codon. Starting translation with the canonical start codon ATG generally leads to optimized expression of AAP or variants thereof and therefore, when AAP or variants thereof is suboptimal, leads to increased yield of capsid assembly. This becomes especially beneficial if expression systems are switched to cells that the respective virus is not adapted to. It can be assumed that expression of AAP or variants thereof in non-host cells will be suboptimal. For example, it is foreseen to manufacture capsids in insect cells or other cells suitable for infection by Baculovirus, in yeasts or bacteria, where optimized expression of AAP or variants thereof may be highly beneficial or crucial in order to get high capsid formation.

[0064] Whereas such mutation of the start codon of AAP into an ATG may reduce capsid formation in a *cis* situation (where AAP is encoded by an overlapping nucleic acid with ORF1 encoding VP3), such mutation is especially beneficial in a *trans* situation, where AAP is encoded independently from ORF1 encoding VP3 (example 14).

[0065] It is well known in the art and part of the invention that the nucleic acid is characterized in that the polypeptide coding sequence of the nucleic acid is followed by a poly(A) signal.

[0066] In one aspect of the invention the nucleic acid of the invention comprises a promoter driving transcription of the polypeptide-encoding sequence. In a preferred embodiment, a heterologous promoter, i.e. which is not present in the virus from which AAP-encoding nucleic acid is derived or preferably not present in any parvovirus wildtype genome, is used. The promoter which can be used in the method described herein is not limited to the examples described herein. It may be any known or subsequently discovered one. Constitutive promoters like e. g. the early cytomegalovirus (CMV) promoter (US 4,168,062), that are continuously transcribed, are as useful in the invention as inducible promoters such as an antibiotic-specific or a cell-specific promoter. For expression in mammalian cell systems use of the CMV promoter is especially preferred, e.g. for use in manufacturing processes using transfection methods, whereas in insect cells use of the Polyhedrin promoter (PolH) is preferred. Inducible heterologous promoters are especially preferred, as they can be used to establish stable production cells for VP3.

[0067] Due to the high conservation of genome organization amongst the parvoviruses, the invention can easily be transferred to other parvovirus members. Within the parvoviruses preferred viruses, from which the nucleic acid of the invention is derived from, are adeno-associated virus (AAV), Goose parvovirus, Duck parvovirus, and Snake parvovirus. Preferred AAVs are selected from the group consisting of bovine AAV (b-AAV), canine AAV (CAAV), mouse AAV1, caprine AAV, rat AAV, avian AAV (AAAV), AAV1, AAV2, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, and AAV13, especially AAV2.

[0068] In a further aspect the nucleic acid of the invention is comprised in an expression cassette, construct, vector or cell line. A construct, typically a plasmid, is generally a nucleic acid comprising the nucleic acid of the invention and additional sequences such as polycloning sites, origin of replication, selection marker genes etc. An expression cassette is generally a construct that, once it is inside a cell, is able to produce the protein encoded by the nucleic acid of the invention by the cellular transcription and translation machinery. The expression construct is engineered to contain regulatory sequences that act as enhancer or promoter regions and lead to efficient transcription of the nucleic acid of the invention. It further usually comprises a poly(A)-site that is later polyadenylated which is important for nuclear export, translation and stabilization of the mRNA. Vectors are constructs that are used to introduce the nucleic acid of the invention into cells. Dependent on the cells to be transfected they are constructed according to standard skills of the artisan. These can be plasmids for calcium phosphate transfection or liposomal transfection, or viral vectors, e.g. baculoviruses. Cell lines are laboratory cell lines suitable for the expression of AAP or variants thereof or the replication of AAP (variant) encoding plasmids.

[0069] A further aspect of the invention is a polypeptide encoded by a nucleic acid according to the claims. The underlying naturally occurring polypeptide is referred to as Assembly Activating Protein (AAP). Accordingly, variants of this polypeptide encoded by the nucleic acid of the present invention are referred to as AAP variants. For example, a variant comprising the AAP protein and one or more further peptides would be referred to as an AAP-comprising polypeptide. The protein AAP is expressed from ORF2 (with the start codon for VP3 defining ORF1), has a calculated molecular weight of approximately 23 kDa and is able to provide capsid assembly of VP3 in the nucleolus. It is

also essential for capsid formation within the whole AAV genome. It targets VP proteins to the nucleolus and exerts there an additional function in promoting the assembly reaction.

[0070] A further aspect of the invention is a method of producing the polypeptide of the invention, i.e. AAP or an AAP variant, by expressing a nucleic acid according to this invention in a host cell. Such production is suitable to promote capsid formation of parvoviruses in general and specifically of capsid comprising VP3, but no VP1 and VP2 and Rep proteins. Suitable host cells can be selected by the skilled person according to his needs and preferences. Preferred host cells selected from a list consisting of a mammalian cell line, especially a human cell line, a cell line used for baculovirus infection, a bacterial strain and a yeast strain.

[0071] A further aspect of the invention is an antibody or a binder in general that specifically binds AAP as defined in the claims. Particularly, the antibody specifically binds to any of the sequences of SEQ ID NO: 1 to 22. Such antibodies can be used to further investigate the function of AAP or, when used as a transacting factor in heterologous expression systems, in order to verify and optimize AAP expression levels for commercial production of parvoviruses DNAor virus like particles. A preferred antibody is characterized in that it specifically binds AAP of AAV2 (SEQ ID NO:1). Antibodies according to this invention may be polyclonal or monoclonal. Further encompassed by the invention are corresponding antibody fragments like single chain antibodies, scF_Vs, F_{ab} fragments, nanobodies or alike, or antibody multimers.

[0072] A further aspect of the invention is the use of nucleic acid of the invention for the preparation of a polypeptide of the invention, including AAP and AAP variants.

[0073] A further aspect of the invention is the use of the nucleic acid or the polypeptide of the invention for the preparation of a parvovirus. The identification of AAP leads to previously unknown possibilities to manufacture such viruses as expression constructs can be optimized individually in order to increase yield or in order to generate inducible production systems using stable transfected producer cell lines. Expression can be increased through the use of heterologous promoters. Specifically, particles can be prepared in the absence of functional Rep and VP1 and VP2 encoding sequences enabling the manufacture of parvoviral particles not comprising any of the functional proteins VP1, VP2, Rep40, Rep52, Rep68 and Rep78. All these factors are important in the context of generating a robust, fast and cheap production system for such viruses and particles.

[0074] One aspect of the invention is a method of producing parvoviral particles consisting essentially of VP3 as defined in the claims, the method comprising the steps of (i) providing a cell capable of expressing VP3 from a VP3-coding sequence (cds) from a parvovirus, wherein the VP3 is under control of a rep-independent promoter and expressing a protein encoded by the nucleic acid according to the invention, (ii) incubating the cell at conditions conducive to the expression of VP3 and the protein from the nucleic acid according to the invention, thereby producing the parvoviral particle, and (iii) optionally purifying parvoviral particles from the cell, wherein at least 10^5 virus particles are formed per cell and no functional VP1, VP2 and Rep proteins are expressed in said cell. This method is equally applicable using fragment Z instead of the nucleic acid according to the disclosure.

[0075] Particularly, a method of producing parvoviral particles essentially consisting of VP3 is provided, the method comprising the steps of

1. i. expressing VP3 from a VP3 coding sequence (cds) from a parvovirus under control of a rep-independent promoter in a cell,
2. ii. expressing a DNA sequence fragment (fragment Z) in the cell under control of a rep-independent promoter, that comprises
    1. (1) at least 44 nucleotides upstream of the VP3 start codon and
    2. (2) more than 242 nucleotides of the VP3 cds starting with the start codon derived from
        1. a) a parvovirus, or
        2. b) a nucleotide sequence that is at least 60%, preferably 80%, more preferably 90%, especially 99% and advantageously 100% identical to the nucleotide sequence of fragment Z derived from AAV2 (sequence 1, Fig. 2), or
        3. c) a nucleic acid sequence that hybridizes in 4x SSC, 0.1% SDS at 65°C to the complementary strand of the fragment Z DNA molecule of AAV2 (sequence 2), or
        4. d) a nucleic acid sequence that can be used in trans-complementation assays to cause assembly of VP3 VLPs.
3. iii. incubating the cell at conditions suitable for VP3 expression, and
4. iv. purifying parvoviral particles from the cell,

wherein approximately about 10^5, preferably about 10^6, and more preferably about 10^7 virus particles are formed per cell and essentially no VP1, VP2 and Rep proteins (particularly Rep40, Rep52, Rep68 and Rep78) are expressed.

[0076] These methods are based on the generation of particles from a virus of the family of Parvoviridae wherein the wildtype expresses VP1, VP2 and VP3 as capsid proteins. Parvoviral particles consisting essentially of VP3 may be generated by expressing the parvoviral VP3 cds essentially in the absence of expression of functional VP1, VP2 and Rep proteins, particularly Rep40, Rep52, Rep68 and Rep78. As a result, the purified parvoviral particle consists essentially of only one capsid protein. Rep-mediated DNA packaging is completely avoided due to the absence of Rep in the particle. The invention allows production of high titers of parvoviral particles consisting essentially of VP3 which are amongst others suitable for vaccine development.

[0077] It is well known in the art that VP3 alone is not able to assemble into capsids. In the context of this disclosure a nucleic acid encoding a novel polypeptide designated AAP respectively a sequence element Z (fragment Z) was identified that, if expressed in the cell, mediates assembly of VP3 particles and that VP3 does not need additional viral proteins for capsid assembly.

[0078] Several lines of evidence led to the conclusion that VP3 requires RNA derived from the cap gene for capsid assembly. This factor required for VP3 capsid assembly could be provided *in trans* in a fragment of the *cap* gene fused to gfp (VP2N-gfp). Protein expression from the first ORF of this cap gene fragment (ORF that encodes VP1, VP2 and VP3) was not necessary as several constructs containing stop codons in the relevant region of the cap gene also provided helper function. Expression of VP2N-gfp from read-through transcripts could not be detected by Western

blot analysis. Such protein expression, initiated at non-conventional translation start sites and followed by a stop codon is very unlikely and their amount would be very low. Such protein expression of VP2N-gfp is also not sufficient for stimulating capsid assembly of VP3. This has clearly been shown by expression of this protein using alternative codons which resulted in high VP2N-gfp protein levels but not in VP3 capsid assembly. Because such a change of the codons implicates a change of the nucleotide sequence it is clear that the correct nucleotide sequence is necessary for the assembly helper effect and not the expressed protein of the first ORF. Finally, providing the correct nucleotide sequence by a plasmid which could not be transcribed in the first ORF resulted also not in capsid assembly, arguing that transcription of the correct nucleotide sequence is necessary.

[0079] As shown in Fig. 2 fragment Z comprises at least 44 nucleotides upstream and more than 242 nucleotides downstream of the VP3 start codon. Preferably, fragment Z does not comprise a full-length VP3 cds. The sequence of fragment Z can be derived from one of a number of different parvoviruses as listed in Fig. 2 where some examples for the nucleotide sequence of the respective region for fragment Z of parvoviruses AAV1, AAV2, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8, AAV10, AAV11, and b-AAV are given. This listing is not limited to the parvoviruses shown here. A further sequence can easily be aligned through its position of the VP3 start codon and selected as fragment Z. A nucleotide sequence can also be selected as fragment Z by its identity to the nucleotide sequence of fragment Z derived from AAV2 (SEQ ID NO: 45, see below) which is at least 60%, preferably 80%, more preferably 90%, especially 99% and advantageously 100%. Moreover, a nucleotide sequence hybridizing in 4x SSC, 0.1% SDS at 65°C to the complementary strand of the fragment Z DNA molecule of AAV2 (SEQ ID NO: 46, see below) can also be used in trans-complementation assays as fragment Z to cause assembly of VP3 VLPs. It is especially preferred that fragment Z is derived from AAV2 and comprises SEQ ID NO: 45.

[0080] Nucleotide sequence of DNA sequence fragment Z derived from AAV2 (SEQ ID NO: 45, as also given in Fig. 2):

```
  1 tcggacagcc accagcagcc cctctggtc tgggaactaa tacgatggct
 51 acaggcagtg gcgcaccaat ggcagacaat aacgagggcg ccgacggagt
101 gggtaattcc tcgggaaatt ggcattgcga ttccacatgg atgggcgaca
151 gagtcatcac caccagcacc cgaacctggg ccctgcccac ctacaacaac
201 cacctctaca aacaaatttc cagccaatca ggagcctcga acgacaatca
251 ctactttggc tacagcaccc cttgggggta ttttgac
```

[0081] Reverse and complementary sequence of SEQ ID NO: 45, that can be used in hybridization experiments to identify an unknown DNA fragment as fragment Z (SEQ ID NO: 46):

```
  1 gtcaaaatac ccccaagggg tgctgtagcc aaagtagtga ttgtcgttcg
 51 aggctcctga ttggctggaa atttgtttgt agaggtggtt gttgtaggtg
101 ggcagggccc aggttcgggt gctggtggtg atgactctgt cgcccatcca
151 tgtggaatcg caatgccaat ttcccgagga attacccact ccgtcggcgc
201 cctcgttatt gtctgccatt ggtgcgccac tgcctgtagc catcgtatta
251 gttcccagac cagaggggcc tgctggtggc tgtccga
```

[0082] For initiation of transcription of the VP3 cds and the sequence of fragment Z or of the nucleic acid of the disclosure one or two "Rep-independent promoter(s)" is/are chosen. A rep-

independent promoter is used in order to express VP3 and fragment Z in absence of the parvoviral factor Rep which is to be avoided as Rep is held responsible for packaging of virus genomes and unspecific DNA into parvoviral particles. For the purposes of this disclosure packaging of viral or unspecific DNA is to be avoided as the parvoviral particles could then unintentionally act as gene therapy vectors. By using a "Rep-independent promoter" for VP3 expression and transcription of fragment Z or the nucleic acid of the disclosure , RNA polymerase can initiate transcription in the absence of expression of Rep proteins enabling manufacture of capsids in the absence of Rep proteins, particularly Rep40, Rep52, Rep68 and Rep78. Rep-independent promoters are for example heterologous constitutive or inducible promoters.

[0083] Accordingly the nucleic acid of the invention may comprise a promoter driving transcription of the polypeptide-encoding sequence. Preferably a heterologous promoter, which is not present in any parvovirus wildtype genome, is used. The promoter which can be used in the method described herein is not limited to the examples described herein. It may be any known or subsequently discovered one. Constitutive promoters like e. g. the early cytomegalovirus (CMV) promoter (US 4,168,062), that are continuously transcribed, are as useful as inducible promoters such as an antibiotic-specific or a cell-specific promoter. For expression in mammalian cell systems use of the CMV promoter is especially preferred, e.g. for use in manufacturing processes using transfection methods, whereas in insect cells use of the Polyhedrin promoter (PolH) is preferred. Inducible heterologous promoters are especially preferred, as they can be used to establish stable production cells for VP3.

[0084] Suitable conditions for VP expression are well known in the art and can in principle be transferred to the expression of VP3 only. To produce parvoviruses or specifically parvoviral particles the respective DNA sequences have to be transfected into cells. One protocol is described within the examples. However, different transfection methods, different cells or stably transfected cells may be used instead. Different production methods are described for example by Grimm et al. (2002) and Grieger and Samulski (2005).

[0085] The methods of this invention lead to high yields of parvovirus particles, wherein 10^5, preferably about 10^6, and more preferably about 10^7 virus particles are formed per transfected cell. These numbers correspond to about 10^{11}, preferably about 10^{12}, and more preferably about 10^{13} particles/ml of crude lysate. The commercial use of VP3 particles requires an efficient method of production providing high yields of particles.

[0086] The particles can be purified by methods disclosed herein and the prior art.

[0087] It is especially preferred that the sequence of fragment Z or the nucleic acid according to the invention and the VP3 cds are arranged and expressed in such a way that parvoviral particles consisting only of VP3 are produced. "Consisting only" in this context means that no other proteinaceous molecules can be detected as part of the particles by common methods such as Western blotting. Such particles may comprise other molecules or salts such as water and other constituents of buffers. Additionally, the particle may comprise molecules that are incapsulated by chance during assembly of the particle within the cell.

[0088] According to one aspect, the sequence of fragment Z or the nucleic acid according to the invention do not overlap with the VP3 cds leading to parvoviral particles consisting only of VP3. This avoids the expression of a substoichiometrical number of N-terminally extended VP3 proteins present in the particles (see example 4). Such a small number of N-terminally extended VP3 proteins most likely would not affect activity or yield of the particles. However, under regulatory aspects of medicaments it is advantageous to have a one-protein product.

[0089] Accordingly, it is especially preferred that the parvoviral particles according to this invention are assembled only of VP3. For this purpose expression of VP1, VP2 and Rep, particularly Rep40, Rep52, Rep68 and Rep78, is shut off in the cell by a method well known to the skilled person, as for example deletion or mutation of the respective start codon, deletion (in whole or in part) of the cds specific for the protein, or mutation of the cds specific for the protein, avoiding expression of a functional gene product (examples are described for example in Warrington et al. (2004)).

[0090] Selection of the translational initiation site in most eukaryotic mRNAs appears to occur via a scanning mechanism which predicts that proximity to the 5' end plays a dominant role in identifying the start codon. This "position effect" causes that the first (most upstream) ATG start codon of a transcript initiates translation (Kozak, 2002).

[0091] Referring to the expression of parvovirus/AAV capsid proteins this means, that the minor spliced transcript mainly accounts for the synthesis of VP1 from the first ATG whereas translation of VP3 is primarily initiated from its ATG start codon which is the most upstream ATG of a major spliced transcript. This major spliced RNA also encodes the unusual ACG start codon of VP2 upstream of the VP3 start site. Therefore, in addition to VP3 that is effectively synthesized from the major spliced transcript, to a certain extent VP2 is expressed (Becerra et al., 1988, Becerra et al., 1985).

[0092] In general, the position effect is evident also in cases where a mutation inactivates or removes the normal start site and translation shifts to a downstream start site. Thus, a silent internal ATG codon can be activated and translational efficiency is increased, a problem well known in some disease states (Kozak, 2002).

[0093] Taken this knowledge into account, the mutagenesis of VP1 and VP2 start codons to inactivate their expression can activate translation of truncated proteins starting at downstream sites that are silent in the wildtype (as described by Warrington et al. (2004), and observed in example 2.2.).

[0094] Therefore, in addition to the main start codons known for capsid proteins such alternative start codons are preferably deleted or mutated to ensure that VP3 is the sole capsid protein to be expressed. Expression of VP3 only and shut off of any other capsid proteins may be controlled via Western blotting as described.

[0095] In a further preferred embodiment coding sequences for VP1 and VP2, which do not encode VP3 sequences, are completely deleted from the expression cassette encoding VP3. In such case fragment Z or the nucleic acid of the invention is provided *in trans* in order to enable production of VP3 capsids.

[0096] In a preferred aspect of the disclosure, the DNA sequence of fragment Z or the nucleic acid according to the invention is followed by a poly(A) signal. The poly(A) signal is able to recruit the polyadenylation machinery to add a stretch of adenines (the poly(A) tail) onto the RNA molecule once transcription of a gene has finished. This processing step increases stability of the factor transcribed from fragment Z within the cell. Poly(A) signals such as the poly(A) from SV40 large T-antigen are well known in the art and are regularly used in all kinds of expression cassettes and constructs.

[0097] Our analyses of a series of deletion mutants that started expression at different sites 5' of the VP3 start codon showed that the mutant pCMV-VP3/2765 is still able to cause capsid assembly (example 2.). Therefore, as already described above, fragment Z has to comprise at least 44 nucleotides upstream of the VP3 start codon. Since efficiency of particle formation was increased by using a fragment Z 5' extended by some nucleotides it is preferred that fragment Z comprises at least 113 nucleotides or especially at least 198 nucleotides upstream of the VP3 start codon, respectively. In our experiments we have chosen a construct providing a fragment Z of AAV2 that starts at nucleotide 2696 (corresponding to 113 nucleotides upstream of the VP3 start codon). In Fig. 2 the sequences of the different serotypes are listed relative to the VP3 start codon which is underlined. The sequences easily can be extended in the 5' or 3' direction according to the nucleotide sequences given in the respective NCBI entrees (compare legend of Fig. 2).

[0098] If the 5' extended sequence of fragment Z comprises the translation start codon of VP1 and/or VP2 or any other ATG start codon in ORF1, ORF2 or ORF3 they have to be inactivated by mutation or deletion to express VP3 as sole capsid protein.

[0099] Further, fragment Z has to comprise more than 242 nucleotides downstream of the VP3 start codon. It is preferred that fragment Z comprises more than about 275 nucleotides, more than about 300 nucleotides, more than about 325 nucleotides, more than about 350 nucleotides, more than about 375 nucleotides, more than about 400 nucleotides, more than about 425 nucleotides, and most preferably more than about 445 nucleotides of the VP3 cds starting with the start codon. An especially preferred fragment Z stops at about nucleotide 3254 (corresponding to about 445 nucleotides downstream of the VP3 start codon).

[0100] The active molecule encoded by fragment Z is most likely a diffusible molecule, i.e. a protein designated AAP. Based on the degenerated genetic code we optimized the sequence of fragment Z to get potentially higher expression of a putative diffusible protein possibly encoded within Z in the first reading frame (ORF1) that also encodes the capsid proteins VP1, VP2 and VP3, thereby leaving the AA sequence of proteins encoded in ORF1 unchanged but disturbing the protein sequence encoded by other ORFs by modifying the DNA sequence. This codon-optimized fragment, however, could not mediate particle formation as no virus particles could be detected anymore (example 5, Fig. 6). On the other hand, insertion of stop codons into ORF1 within fragment Z, leading to shut off of protein synthesis from ORF1 but leading only to minor changes in the DNA sequence of ORF1 and not disrupting protein synthesis from ORF2, still enabled efficient particle formation (example 6, Fig. 8).

[0101] It is especially preferred that the mutation of fragment Z comprises at least one stop codon

for protein translation in the first or third reading frame. As a consequence no protein with a length of 18 AA or above can be translated from these reading frames of fragment Z in particular no VP2 protein or part of it can be generated. Therefore it is a main advantage that no VP2 or part of it is included in the particles.

[0102] As a preferred aspect of this disclosure the main translation start codon ATG (AA 203, numbering according to VP1 of AAV2, (Girod et al., 1999)) of VP3 within fragment Z or the nucleic acid of the disclosure is mutated. It is further preferred that also one or both of the alternative minor start codons of VP3 (AA 211 and AA 235, (Warrington et al., 2004)) are mutated. In a more preferred aspect all ATG codons that can be used for translational start of VP3 are mutated (a number of the possible ones are listed in Warrington *et al.,* 2004) to completely avoid translation of VP3 from the expression construct providing fragment Z.

[0103] Since the product of fragment Z and the encoded function of the nucleic acid of the invention was characterized to be a trans-acting element meaning that fragment Z and the nucleic acid of the invention code for a diffusible molecule, the sequence of fragment Z or the nucleic acid of the invention can be provided on the same or a different nucleic acid molecule to the cell as the cap gene or part thereof, e.g. a VP3 cds.

[0104] In a preferred embodiment the nucleic acid of the invention is provided *"in cis"* relative to the VP3 cds. If fragment Z/the nucleic acid of the invention is provided *"in cis"* relative to an expression cassette coding for VP3 means that expression of fragment Z/the nucleic acid of the invention and VP3 are driven by the *same one* promoter. The sequence of fragment Z/the nucleic acid of the invention can be located upstream or downstream of the VP3 cds. The sequences coding for fragment Z and VP3 can be directly linked or separated by a variable number of nucleotides (Fig. 3.1.).

[0105] In a more specific embodiment of this invention the nucleic acid of the invention is located directly upstream of the VP3 cds. Since fragment Z/the nucleic acid of the invention comprises more than 242 nucleotides downstream of the VP3 start codon and this sequence has not to be present in duplicate, the directly following VP3 cds has only to provide the remaining DNA sequence of the VP3 ORF (a schema is given in Fig. 3.1.a)). In this case substoichiometrical amounts of N-terminally extended VP3 are expressed and presented in the capsid (example 4). In order not to increase this part of N-terminally extended VP3 it is one important embodiment of this invention not to add new or delete existing start codon(s) respectively at the 5' end or upstream of fragment Z/the nucleic acid of the invention.

[0106] Moreover, only the VP3-specific cds that does not overlap with fragment Z/the nucleic acid of the invention can be mutated easily. Mutation of the VP3 cds that overlaps with fragment Z/the nucleic acid of the invention also possibly changes the sequence of the diffusible molecule coded by fragment Z/the nucleic acid of the invention. As a result, in some cases the diffusible molecule will not be active any more. Mutations in this context include silent mutations as well as e.g. insertion of epitopes. In order to increase possibilities to mutate the VP3 cds it is beneficial to minimize the overlap i.e. to separate fragment Z/the nucleic acid of the invention and the VP3 cds. This can be done in a *cis* situation where one promoter drives expression of a VP3 cds that does not contain the 44 nucleotides upstream of the VP3 start codon essential for fragment Z, and of a

separate fragment Z place before such VP3 cds or thereafter.

[0107] It is especially preferred that fragment Z/the nucleic acid of the invention is provided *"in trans"* relative to the VP3 cds. If fragment Z/the nucleic acid of the invention is provided *"in trans"* relative to an expression cassette coding for VP3 it means that expression of fragment Z/the nucleic acid of the invention and VP3 are driven by *separate* promoters (in opposite to *"in cis",* see above). The sequence of fragment Z/the nucleic acid of the invention can be located upstream or downstream of the VP3 cds on the same construct, or on a different expression construct than the VP3 cds (examples are listed in Fig. 3.2.).

[0108] In this case fragment Z comprises only the 5' end of the VP3 cds and hence it is a main advantage that no N-terminally extended VP3 (see below) can be expressed and incorporated into the capsid. Therefore, if the sequence coding for VP3 and fragment Z are provided *in trans,* it is assumed that a more pure particle composition, preferably consisting only of the structural protein VP3, can be obtained.

[0109] It is one advantage of the *in trans* configuration that the VP3 cds can easily be modified e.g. to optimize its codon usage for the expression cell line in order to further increase the yield without changing the sequence of fragment Z/the nucleic acid of the invention. Also other modifications such as mutations, insertions, tags etc. can be done without affecting fragment Z/the nucleic acid of the invention. It can be assumed that during previous attempts to identify insertion sites within VP3 in overlapping sequences of fragment Z and the VP3 cds, potentially useful insertion sites were not identified as an insertion also interfered with expression of fragment Z or with the function of AAP. Accordingly, functional separation of these sequences either in a *cis* setting with no overlap or preferably in a *trans* setting enables independent mutagenesis of the VP3 coding sequence. Such mutagenesis has multiple commercial applicabilities. In the context of generation of novel gene therapy vectors limitations of generating chimeric parvovirus Cap sequences can be overcome. Several groups have tried direct evolution of AAV involving generation of randomly mutagenized viral libraries on one serotype (Koerber et al., 2008), by using STEP and shuffling methods to create multiple randomly recombined capsid species using known AAV serotype capsid sequences (Ward and Walsh, 2009, Li et al., 2008). In a similar way, independent expression of AAP can be used to identify further insertion sites that tolerate ligands (to be used for targeting to other cells), B-cell epitopes (to be used for generating epitope specific vaccines) or deletions/substitutions (to be used for detargeting of the virus or to reduce antigenicity of the virus).

[0110] A further advantage of the *in trans* configuration of fragment Z/the nucleic acid of the invention and the VP3 cds is that one construct can be stably transfected into a producer cell line whereas the other construct can be transiently transfected/transduced. For example an expression cassette comprising fragment Z/the nucleic acid of the invention can be stably transfected into a cell line suitable for efficient VP3 expression generating a single production cell line. Such production cell line then can be transiently transfected/transduced (e.g. infected with a virus) with a specific VP3 cds leading to expression of such VP3 and respective particle formation. Accordingly, one production cell line can be used for the production of different particles. Given the time and cost that is needed for qualification of production cell lines that are used for the manufacture of medicaments, this constitutes a considerable regulatory advantage. For this reason, it is especially preferred that fragment Z/the nucleic acid of the invention and the VP3 cds are provided on

separate expression constructs.

[0111] This setup can additionally be used e.g. to generate AAV/parvovirus particles of a distinct serotype by providing an expression cassette coding for VP3 of a specific serotype *in trans* to the cells that have been stably transfected with fragment Z/the nucleic acid of the invention of one serotype. In general, a VP3 cds specific for an AAV serotype selected from the group consisting of AAV1, AAV2, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 and AAV13 can be used for transfection of cells that have been stably transfected with fragment Z/the nucleic acid of the invention of only one serotype e.g. AAV2. Thereby, AAV particles consisting of VP3 of a selected serotype e.g. AAV1 particles can easily be generated. For AAV1 and AAV2 we could confirm that expression of fragment Z/the nucleic acid of the invention mediates capsid assembly of VP3 not only expressed from constructs cloned from the same serotype but also expressed from constructs cloned from the other serotype, namely AAV2 and AAV1, respectively (example 21). Nevertheless, it can not be generally expected that every serotype complements each other, but a person skilled in the art can easily identify the respective pairs of AAP from one parvovirus/serotype and VP3 from a different one to get assembly of VP3 VLPs in trans-complementation assays as described herein. Cross-complementation can be used for example if a stable cell line expressing a specific AAP has been obtained that can be used for the production of VP3 VLPs from different parvoviruses or AAV serotypes. The respective combinations can be chosen for time- and cost-effective VLP production at high titer.

[0112] Just as well the other way around is possible and one specific VP3 cds can be transfected *in trans* with an expression cassettes providing fragment Z/the nucleic acid of the invention chosen from a number of different sequences coding for fragment Z e.g. from different AAV serotypes/parvoviruses.

[0113] In another preferred embodiment of the invention the parvoviral particle does not contain Rep protein, particularly any functional Rep40, Rep52, Rep68 and Rep78 proteins. For details on this embodiment are given herein throughout the description.

[0114] In another preferred embodiment of the invention only/at most 1/50 of the expressed structural protein, preferably at most 1/100, more preferably at most 1/250 and essentially only/at most 1/500 of the structural proteins are N-terminally extended versions of VP3. Especially preferred are parvovirus particles that do not contain any structural proteins with N-terminally extended versions of VP3 or, vice e versa, none of the structural proteins are N-terminally extended versions of VP3. If the sequences of fragment Z and VP3 cds overlap and are expressed under control of the same promoter (*cis* situation), the ATG start codon of the VP3 cds is mainly used as start for protein translation whereas only a very small proportion of protein translation starts upstream. The resulting small part of proteins contains in addition to the VP3 cds an N-terminal extension by several Aas corresponding to the 3' part of the sequence coding for VP2 but not for VP3. Taken together, expression of these 5' extended versions of VP3 was visible in Western blots (example 4) but accounted for only 1/50 of the structural proteins, preferably 1/100, more preferably 1/250 and essentially only 1/500 of the structural proteins of the parvovirus particle.

[0115] In another preferred aspect of the disclosure only/at most 1/50 of the expressed structural

protein, preferably at most 1/100, more preferably at most 1/250 and essentially only/at most 1/500 of the structural proteins is a polypeptide according to the invention, i.e. AAP or variants thereof. Whereas no specific influence of AAP has been shown on host cells that might have an impact on medical applications of parvovirus vectors or particles, it is in principle beneficial to have as little impurities as possible.

[0116] Viral Rep proteins bind to genomic and viral DNA and are discussed to play a role in DNA packaging. In order to use AAV as a vaccine and avoid unspecific reactions against the packaged DNA or an undesired gene transfer, parvoviral particles as free of DNA as possible are especially preferred. Therefore, parvoviral particles are produced in the absence of expression of Rep proteins in the cell. Preferably, only 1/100 of the particles, preferably 1/1,000 and more preferably only 1/10,000 of the particles contain DNA. Especially preferred is that none of the parvovirus particles contains DNA. Preferably at most 1/100, more preferably only/at most 1/1,000, even more preferably only/at most 1/10,000 of the particles contain any DNA. As a result, no inactivation step to destroy packaged DNA (e.g. gamma or UV-irradiation) is necessary prior to vaccination purposes.

[0117] The parvoviruses for use according to this invention are preferably selected from the group consisting of adeno-associated virus (AAV), bovine AAV (b-AAV), canine AAV (CAAV), and avian AAV (AAAV).

[0118] Especially preferred are AAVs selected from the group consisting of AAV-1, AAV-2, AAV-3b, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12 and AAV13, especially AAV-2. AAV1 to AAV12 specify defined serotypes of adeno-associated virus (AAV). As described herein in more detail, it is especially preferred that the VP3 cds further comprises at least one mutation. The mutation is in comparison to the respective wildtype parvoviral sequence, preferably selected from the group consisting of one or more deletion(s), one or more insertion(s), one or more substitution(s), and a combination of these mutations.

[0119] The VP3 cds may comprise one or more silent mutation(s). By introducing DNA mutations that do not result in a change to the AA sequence of the VP3 protein it is possible to optimize the codon usage of the cds of VP3 e.g. to enhance its expression. Due to the degeneracy of the genetic code one AA may be specified by more then one codon, for example the AA glutamic acid is specified by GAA and GAG codons. Accordingly, for each AA of the structural protein VP3 one would select those codons that are translated with higher efficiency and mutate the cds respectively. As already discussed these mutations do not change the AA sequence of the protein, that is why they are called silent, but of course change the nucleotide sequence including the diffusible molecule coded by fragment Z/the nucleic acid of the invention. For this reason it is an especially preferred that only the part of the VP3 cds is modified by insertion or optimization of codon usage e.g. to get higher expression of VP3 in the chosen setup that does not overlap with the sequence of fragment Z, or in a *trans* situation as described above anywhere within the VP3 cds.

[0120] The one or more mutation(s) of the VP3 cds may lead to one or more mutations located on the surface of a VP3 VLP. The surface-located regions of the structural protein can be determined by analyzing the crystal structure, which is known for AAV2 (Xie et al., 2002). If the crystal structure

is still not available for the chosen serotype the chosen VP3 sequence can be aligned to the VP3 sequence of at least one different serotype with an already known crystal structure to identify homologous regions of interest. The alignment can be done using a commercially available software like e.g. Multialign (Corpet, 1988) and standard parameters described there.

[0121] The one or more mutation(s) of the VP3 cds may lead to one or more mutation(s) located at the N-terminus of VP3. Preferably, the N-terminus is defined as the N-terminal 10, preferably N-terminal 5, especially N-terminal 2 amino acids of the respective VP3. Especially preferred is an insertion at or corresponding to an insertion directly N- or C-terminal, preferably directly C-terminal of AA 203 (I-203).

[0122] The insertion(s) may be inserted into one or more positions selected from the group consisting of I-261, I-266, I-381, I-447, I-448, I-453, I-459, I-471, I-534, I-570, I-573, I-584, I-587, I-588, I-591, I-657, I-664, I-713 and I-716, preferably I-261, I-453, I-534, I-570, I-573 and I-587, especially I-587.

[0123] The used nomenclature I-### refers to the insertion site with ### naming the AA number relative to the VP1 protein of AAV-2, however meaning that the insertion may be located directly N- or C-terminal, preferably directly C-terminal of one AA in the sequence of 5 Aas Nor C-terminal of the given AA, preferably 3, more preferably 2, especially 1 AA(s) N- or C-terminal of the given AA. For parvoviruses other than AAV-2 the corresponding insertion sites can be identified by performing an AA alignment or by comparison of the capsid structures, if available. Such alignment has been performed for the parvoviruses AAV-1, AAV-2, AAV-3b, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-10, AAV-11, b-AAV, GPV, B19, MVM, FPV and CPV (Figure 3 of WO 2008/145400).

[0124] The AA position after which the insertion was introduced and which named the site is underlined. It is also possible likewise to introduce an insertion into the five directly adjacent Aas located next to the underlined AA, because these are likewise located within a loop in the AAV2 capsid.

[0125] For example the insertion site I-587 corresponds to an insertion before and/or after one of the following Aas indicated by emphasis:

FQSSS TDPAT of AAV1,

LQRG$\underline{N}_{587}$ RQAAT of AAV2,

LQSS$\underline{N}$ TAPTT of AAV-3b,

LQSS$\underline{S}$ TDPAT of AAV-6,

LQAA$\underline{N}$ TAAQT of AAV-7,

LQQQ$\underline{N}$ TAPQI of AAV-8,

LQQA$\underline{N}$ TGPIV of AAV10,

NQNA$\underline{T}$ TAPIT of AAV11 and

NQSST TAPAT of AAV-5.

[0126] Further, the insertion site I-453 corresponds to an insertion directly N- or C-terminal of the following ten Aas each, preferably directly C-terminal of the AA indicated by emphasis
QNQSG SAQNK of AAV-1,
NTPSG$_{453}$ TTTQS of AAV-2,
GTTSG TTNQS of AAV-3b,
QNQSG SAQNK of AAV-6,
SNPGG TAGNR of AAV-7,
QTTGG TANTQ of AAV-8,
QSTGG TQGTQ of AAV-10,
SGETL NQGNA of AAV-11 and
FVSTN NTGGV of AAV-5.

[0127] Relating to the AAV2 sequence insertion sites for AAV and other parvoviruses encompassed by this invention are listed Table 1.

Table 1: Insertion sites for parvoviruses

insertion site	**corresponding AA/sequence of AAV2**		**references**
I-261	S_{261}	YKQIS_{261} SQSGA	(Girod et al., 1999)
I-266	A_{266}	SQSGA_{266} SNDNH	(Wu et al., 2000)
I-381	N_{381}	YLTLN_{381} NGSQA	(Girod et al., 1999)
I-447	R_{447}	YYLSR_{447} TNTPS	(Girod et al., 1999, Wu et al., 2000)
I-448	T_{448}	YLSRT_{448} NTPSG	(Grifman et al., 2001)
I-453	G_{453}	NTPSG_{453} TTTQS	WO 2008/145400
I-459	R_{459}	TTQSR_{459} LQFSQ	(Shi et al., 2001, Arnold et al., 2006)
I-471	R_{471}	ASDIR_{471} DQSRN	(Asokan and Samulski, 2006, Moskalenko et al., 2000)
I-534	F_{534}	EEKFF_{534} PQSGV	(Girod et al., 1999)
I-570	P_{570}	RTTNP_{570} VATEQ	
I-573	T_{573}	NPVAT_{573} EQYGS	(Girod et al., 1999)

insertion site	corresponding AA/sequence of AAV2		references
I-584	Q_{584}	STNLQ$_{584}$ RGNRQ	(Shi et al., 2001, Shi and Bartlett, 2003)
I-587	N_{587}	LQRGN$_{587}$ RQAAT	(Girod et al., 1999, Shi et al., 2001, Maheshri et al., 2006, Ried et al., 2002, Grifman et al., 2001, Nicklin et al., 2001, Arnold et al., 2006)
I-588	R_{588}	QRGNR$_{588}$ QAATA	(Shi and Bartlett, 2003)
I-591	A_{591}	NRQAA$_{591}$ TADVN	(Wu et al., 2000)
I-657	P_{657}	VPANP$_{657}$ STTFS	
I-664	A_{664}	TFSAA$_{664}$ KFASF	(Wu et al., 2000)
I-713	T_{713}	NVDFT$_{713}$ VDTNG	
I-716	T_{716}	FTVDT$_{716}$ NGVYS	(Maheshri et al., 2006)

[0128] I-570 is especially suitable as an insertion site that goes along with a deletion of given Aas of the parvovirus structural protein at the site of insertion, leading to a complete substitution. In this case the Aas RTTNPVATEQ can be substituted by an epi- or mimotope.

[0129] Insertions have been successfully made into AAV-serotypes other than AAV2.

Table 2: Insertions into AAV-serotypes other than AAV2

AAV serotype	sequence	insertion site/ AA relative to AAV2		references
AAV1	FQSSS$_{588}$ TDPAT	I-587	N_{587}	own data
AAV1	SSSTD$_{590}$ PATGD	I-589	Q_{589}	(Arnold et al., 2006, Stachler and Bartlett, 2006)
AAV-3	NNLQS$_{586}$-SNTAP	I-585	R_{585}	(Arnold et al., 2006)
AAV-4	GGDQS$_{584}$-NSNLP	I-585		(Arnold et al., 2006)
AAV-5	TNNQS$_{575}$-STTAP	I-585		(Arnold et al., 2006)

[0130] The most preferred insertion sites are:

1. i) I-587 as various insertions have been made in the AA stretch around N_{587} ($LQRGN_{587}$ RQAAT) of AAV2. Within this stretch insertions of various peptides were made C-terminal of Aas Q_{584}, N_{587}, R_{588} and A_{591} in AAV2 and C-terminal of Aas of other AAV-serotypes corresponding to R_{585} and Q_{589} of AAV2.
2. ii) I-453 as epitopes have been successfully inserted C-terminal of G_{453} in AAV2.
3. iii) $FQSSS_{588}$ TDPAT or $SSSTD_{590}$ PATGD of AAV1.
4. iv) I-261 as according to this invention epitopes have been successfully inserted C-terminal of S_{261} in AAV2.
5. v) I-534 as according to this invention epitopes have been successfully inserted C-terminal of F_{534} in AAV2.
6. vi) I-570 as according to this invention epitopes have been successfully inserted C-terminal of P_{570} in AAV2.
7. vii) I-573 as according to this invention epitopes have been successfully inserted C-terminal of T_{573} in AAV2.

[0131] Corresponding Aas for all insertion sites specified herein for parvoviruses disclosed herein can be retrieved from the alignment in Figure 3 of WO 2008/145400. For those parvoviruses not listed therein an alignment under standard parameters as used there can be performed with the provided AA sequence of such parvovirus and the corresponding AA can be retrieved from such alignment.

[0132] According to this invention two insertions may be preferred and are made into two positions selected from the group consisting of I-261, I-453, I-534, I-570, I-573 and I-587, preferably I-261 in combination with I-587, I-261 in combination with I-453 or I-453 in combination with I-587. With respect to triple insertions, preferred combinations are made into three positions of VP3, preferably an insertion in position 453 in combination with an insertion in position 587 and in combination with an additional mutation, more preferably in positions I-453, I-587 combined with one of the I-534, I-570 and I-573.

[0133] Particularly for vaccination applications AAV particles presenting the selected epitope have to be generated. Therefore, it is preferred that the VP3 cds comprises at least one epitope heterologous to the virus.

[0134] It is further preferred that the epitope of the VP3 protein is a B-cell epitope. Preferably the B-cell epitope is a part of an antigen. Preferred antigens are serum proteins, proteins that can be found at least under certain conditions (e.g. in a disease state) in the blood, membrane proteins, especially receptor proteins (e.g. CD20, acetylcholine receptors, IL13R, EGFR), and surface antigens of infectious agents, preferably not immuno-dominant epitopes of such surface antigens. Especially preferred antigens are IgE, tumor-antigens (e.g. Melan A, high molecular weight melanoma associated antigen (HMW MAA), CA125, IL13R, Her2/NEU, L1 cell adhesion molecule), VEGF, EGFR, CD20, IL1, IL4, IL5, IL6, IL9, IL13, IL17, IL18, IL33, TSLP (thymic stromal lymphopoietin), CETP (cholesterol ester transfer protein), TNF-family members (e.g. TNF-α), or β-amyloid.

[0135] The VP3 may comprise at least one B-cell epitope heterologous to the parvovirus, which is preferably not identical to a pathogen, particularly to a B-cell epitope of a pathogen, wherein the B-cell epitope is located on the surface of the virus. Preferably, the VP3 is capable of inducing an immunoglobulin capable of binding to the antigen the B-cell epitope is derived from.

[0136] Preferably, the B-cell epitope is inserted into I-453 and/or I-587, especially into I-453 and/or I-587 of AAV1, AAV2 or AAV4.

[0137] It is especially preferred that an identical B-cell epitope is inserted at two or more different insertion sites, if it is key to have a large number of identical peptides being optimally presented on the surface of a capsid, especially in case direct B-cell receptor crosslinking should be required for T-cell independent priming of B-cells and breaking of B cell tolerance against self-antigens. A higher density of B-cell epitopes increases the likelihood of optimal peptide-specific B-cell receptor crosslinking which requires a defined distance between B-cell receptors, and therefore, respective B-cell epitopes being presented on a parvovirus capsid.

[0138] Moreover, a larger number of inserted B-cell epitopes decreases the probability for undesired immune reactions against the parvovirus backbone due to i) masking of natural parvovirus B-cell epi-/mimotopes and/or ii) slight structural capsid changes rendering these natural B-cell epi-/mimotopes less immunogenic. Accordingly, parvovirus structural proteins comprising at least three insertions are especially preferred.

[0139] Taken together, preferred insertion sites for superficial presentation of epitopes are the positions following the amino acids that correspond to the AAV2 amino acids number I-261, I-266, I-381, I-447, I-448, I-453, I-459, I-471, I-534, I-570, I-573, I-584, I-587, I-588, I-591, I-657, I-664, I-713 and I-716, especially 1-261, I-453, I-534, I-570, I-573, I-587, and I-588, most preferably I-453 and I-587.

[0140] The insertions, whether terminal or internal, may be combined with the deletion of one or more amino acids, leading to a partial or 1:1 substitution of amino acids by different amino acids, wherein partial substitution means that e.g. 8 amino acids are substituted by 6 different amino acids, and a 1:1 substitution means that e.g. 8 amino acids are substituted by 8 different amino acids.

[0141] The VP3 may be comprised in a fusion protein, e.g. fused to a second protein or peptide. In an especially preferred example B-cell epitopes in particular epitopes larger than 20 amino acids are fused to the N-terminus of VP3.

[0142] The VP3 may comprise at least one tag useful for binding to a ligand. In an especially preferred embodiment said tag is introduced in the parvovirus mutated structural protein by a further mutation. Such tags are well known in the art, Table 3.

Table 3: Tags and corresponding ligands

Tag	Ligand
AU1	Anti AU1 monoclonal antibody

Tag	Ligand
HIS	Nickel
GST	Glutathione
Protein A	IgG
Biotin or Strep	Streptavidin
Calmodulin-binding peptide	Calmodulin
Fc-Peptide of IgG	Protein A
Flag	GLAG- or 3xFLAG peptide
HA (hem agglutinin)	HA peptide

[0143] The VP3 may comprise at least one further mutation. The mutation may be any suitable mutation, such as any of those defined above.

[0144] For example, one or several further mutation(s) of the VP3 might be adequate to e.g. i) introduce additional or even identical B-cell epitopes of the same target antigen, and/or ii) B-cell epitopes of one or more further target protein(s) (multi-target vaccine), T-cell epitope(s) to further promote the desired T-cell immune response, peptide sequence(s) to target and/or activate antigen-presenting cells, or to obtain capsid mutants with reduced immunogenicity of the core particle. The latter might be one possibility to setup an efficient prime/boost regimen.

[0145] Besides, a further mutation of the parvovirus mutated structural protein at a different position can be used to compose more complex mimotopes, to modify certain properties of the virion, e.g. it can be used to modify its natural antigenicity (e.g. Huttner et al., 2003, and WO 01/05990), to modify its chromatographic properties (e.g. WO 01/05991), to insert a second B-cell epitope, to insert a T-helper epitope, or to insert a CTL epitope. Such further mutation is selected from a point mutation, an internal or terminal deletion, an insertion and a substitution. Preferably, the further (second) insertion is internally e.g. by an N- or C-terminal fusion.

[0146] Another aspect of the disclosure is a parvoviral particle obtainable from any of the methods disclosed above. Based upon the above described methods we were able to produce parvoviral particles which essentially consist only of VP3 and do not comprise a heterologous nuclear localization signal (NLS). Such particles do not contain Rep protein, particularly Rep40, Rep52, Rep68 and Rep78. The described methods enable the production of sufficient quantities/yields for the manufacture of medicaments in a commercial scale.

[0147] Another aspect of the invention relates to a parvoviral particle consisting essentially of VP3, wherein the VP3 optionally comprises one or more mutation(s) as compared to the corresponding wildtype VP3, and wherein the VP3 does not contain a heterologous nuclear localization signal (NLS), and wherein the particle does not contain Rep proteins.

[0148] Especially preferred is a parvoviral particle wherein the capsid consists only of VP3.

[0149] With respect to the one or more mutations it is referred to the mutations as described

before.

[0150] The description provides an expression cassette A comprising a VP3 cds as defined before and a heterologous promoter thereto, wherein transcription of VP3 is driven by the heterologous promoter, and wherein the expression cassette is capable of expressing essentially only VP3. Especially preferred are expression cassettes that express only VP3. The construct is used to express mutated VP3. The sequence of VP3 can be mutated as described. As understood by the skilled person the expression cassette further comprises a poly(A) sequence.

[0151] The description also provides an expression cassette B comprising a fragment Z as defined before and a promoter heterologous thereto, wherein transcription of fragment Z is driven by the heterologous promoter. The construct is used to express fragment Z. The sequence of fragment Z can be mutated as described. Optionally, this expression cassette further comprises a poly(A) sequence.

[0152] The description further provides an expression cassette C comprising (i) a VP3 cds as defined before and fragment Z as defined before, and (ii) a promoter heterologous thereto, wherein the expression of VP3 and fragment Z is driven by this one heterologous promoter.

[0153] Moreover, the nucleic acid of the invention may be an expression cassette comprising a heterologous promoter, a VP3 cds as described above and the nucleic acid of the invention are combined, wherein the expression of VP3 and of the polypeptide encoded by the nucleic acid (AAP or AAP variant) is driven by this one heterologous promoter. Optionally, this expression cassette further comprises a poly(A) sequence.

[0154] At least one expression cassette A/VP3 expression cassette and at least one expression cassette B/expression cassette comprising the nucleic acid of the invention may be combined in a kit. By combining expression of parvoviral VP3 from the VP3 cds and expression of fragment Z/the nucleic acid of the invention it is possible to generate particles consisting essentially only of VP3 according to this invention.

[0155] The description relates to a kit comprising at least one VP3 expression cassette A and at least one nucleic acid of the invention or a kit comprising at least one expression cassette C for the combined and simultaneous expression of VP3 and fragment Z in the cell and generation of VP3 VLPs. Such kits preferably additionally contain a manual.

[0156] Still another aspect of the present invention relates to the parvovirus particle according to the invention for use as a medicament. Such medicaments have numerous advantages over the prior art. The immune system of a mammal is specialized to generate strong antibody responses against viral capsid proteins due to the co-evolution of mammals and their immune system on one hand and viruses on the other hand. Strong antibody responses means titers of 1,000 to >100,000 measured in a standard ELISA. Virus-like particles are highly immunogenic due to resemblance of a virus, the repetitive and highly structural pattern of antigens, and efficient uptake of such particles by antigen-presenting cells. The size of the virion, the density and symmetric order of B-cell epitopes and the optimal distance of about 50 to 100 Å between any two B-cell epitopes plays a major role regarding very strong T-cell independent B-cell responses mediated by direct cross-

linking of the respective B-cell receptor breaking even B-cell tolerance against self-antigens or tolerogens (Szomolanyi-Tsuda and Welsh, 1998, Szomolanyi-Tsuda et al., 1998, Szomolanyi-Tsuda et al., 2000, Szomolanyi-Tsuda et al., 2001, Zinkernagel, 2002, Bachmann et al., 1993).

[0157] Taken together, such medicaments are capable of inducing a polyclonal immune response against certain B-cell epitopes that leads to an active immune response resulting in high and long lasting antibody titers. The multimeric structure of the virion contains a large number of densely packed identical epitopes directly cross-linking the respective receptor on B-cells and, thereby, inducing a T-cell independent B-cell response. The particulate structure of the medicament further supports the immune response via efficient uptake by antigen-presenting cells which activate T-cells finally triggering IgG class switch and hypermutation of activated B-cells, leading to the persistent release of high-affinity IgG antibodies and differentiation of B-cells into memory cells.

[0158] Using the methods of the current invention such medicaments can easily be produced.

[0159] The parvovirus particle according to the invention for use as a medicament according to the present invention may further additionally comprise one or more excipients. The excipient is a pharmaceutically acceptable carrier and/or excipient. Excipients are conventional and may include buffers, stabilizers, diluents, preservatives, and solubilizers. Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the (parvo)viral particles herein disclosed. In general, the nature of the carrier or excipients will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e. g. powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

[0160] In a further embodiment the medicament is a vaccine. In general, a vaccine is a preparation consisting of antigens of a disease-causing agent which, when introduced into the body, stimulates the production of specific antibodies or altered cells. This produces generally an immune response as an active principle. Particularly, the parvovirus particles assembled of VP3 comprise at least one B-cell epitope heterologous to the parvovirus, preferably for preventing or treating an autoimmune disease (e.g. diabetes type 1), a tumor disease (examples are: melanoma: e.g. HMW MAA, glioblastome multiforme: e.g. CA125, anti-IL13R, colon cancer: e.g. CA125 or anti-EGFAND/OR, breast cancer: e.g. Her2/NEU, ovarian cancer: e.g. L1 adhesion molecule, B-cell lymphoma: e.g. CD20), an allergic disease (asthma, allergies such as allergic rhinitis, examples for targets are IgE, IL4, IL5, IL9, IL13, IL18, IL33, TSLP), a metabolic disease (e.g. high cholesterol, intervention into the cholesterol metabolism (target example: CETP), obesity, hypertension (target example angiotensin II), an inflammatory disease (e g. rheumatoid arthritis, Crohn's disease, target examples' IL6, IL17 and TNF-α), a neurological disease (e.g. Alzheimer's disease; target example: β-Amyloid) or to be used in ophthalmology (e.g. AMD, target example VEGF).

[0161] Also encompassed by the present inventions are the parvovirus particle according to the invention for use in methods for vaccination and/or for treating or preventing the diseases specified herein by administering to a patient an effective amount of parvovirus particles of the invention.

[0162] The vaccine may further comprise one or more adjuvants, particularly as an immunostimulatory substance. The adjuvant can be selected based on the method of administration and may include mineral oil-based adjuvants such as Freund's complete and incomplete adjuvant, Montanide incomplete Seppic adjuvant such as ISA, oil in water emulsion adjuvants such as the Ribi adjuvant system, syntax adjuvant formulation containing muramyl dipeptide, or aluminum salt adjuvants.

[0163] One embodiment of this invention is the parvovirus particle according to the invention for use in the prevention or treatment of an autoimmune disease, an infectious disease, a tumor disease, an allergic disease, a metabolic disease, a (chronic) inflammatory disease, a neurological disease, addiction or to be used in ophthalmology. Preferred autoimmune diseases and/or chronic inflammatory diseases are rheumatoid arthritis, psoriasis and Crohn's disease. A preferred tumor disease is a disease eligible for treatment with a monoclonal antibody, e.g. trastuzumab. Preferred allergic diseases are asthma and allergies, e.g. allergic rhinitis. Examples for preferred allergens are birch pollen, house dust mite and grass pollen. A preferred neurological disease is Alzheimer's disease. A preferred metabolic disease is atherosclerosis. A preferred ophthalmological disease is age-related macular degeneration. The parvovirus particle or the medicament may preferably be used in a method of breaking B-cell tolerance, meaning inducing antibodies against a self-antigen.

[0164] The disease treated by the medicament is preferably not an infectious disease.

[0165] Moreover, the parvovirus mutated structural protein of the medicament is not used as a vector in gene therapy.

[0166] The parvoviral particle of the invention may be used for gene therapy.

[0167] The following examples and figures are intended to explain the invention in detail without restricting it.

FIGURES

[0168]

FIG. 1: Schematic organization of the AAV capsid gene.
The coding DNA for the cap gene is shown in the first line, the Cap proteins VP1, VP2 and VP3 in the following ones. Nucleotide numbers correspond to the genome sequence of AAV-2 given by Ruffing et al. (1994) accessible from NCBI (number of entree: NC_001401). Numbering of amino acid (AA) sequences according to VP1 of AAV2 (Girod et al. 1999). EcoNI and BsiWI restriction sites are marked. Not to scale.

FIG. 2: Nucleotide sequences of fragment Z of different AAVs.

The nucleotide sequences of fragment Z of the parvoviruses AAV1 (NC_002077), AAV2 (AF043303), AAV3b (AF028705), AAV4 (U89790), AAV5 (NC_006152), AAV6 (AF028704), AAV7 (AF513851), AAV8 (AF513852), AAV10 (AY631965), AAV11 (AY631966), and b-AAV (NC_005889) are given (numbers of nucleotide entrees according to NCBI are given in brackets). +1 indicates the position of the first nucleotide coding for the ATG start codon of VP3. The 44 nucleotides upstream and 242 nucleotides downstream of the +1 position are shown. The ATG start codon of VP3 is underlined.

FIG. 3: Schematic representation of the different expression constructs suitable for assembly of VP3 particles.
Six possible expression constructs differing in the set-up of the fragment Z sequence and VP3 cds are shown by different boxes as indicated. In the c*is* situation they are expressed under the same one promoter whereas *in trans* two separate promoters drive their expression, as indicated by the circle. +1 indicates the position of the first nucleotide coding for the ATG start codon of VP3. The DNA of fragment Z comprising at least 44 nucleotides upstream and more than 242 nucleotides downstream of the +1 position are boxed (compare Fig. 2). +1602 marks the number of the last nucleotide of the TAA stop codon at the 3' end of the VP3 cds (as outlined in Fig. 1). An arbitrary number of nucleotides can separate the VP3 cds and fragment Z and is marked by //. Not to scale.

FIG. 4: Schematic organization of the *rep* and *cap* genes, as well as position of different restriction sites used for cloning of expression constructs.
Schematic representation of the rep and cap genes in the parvovirus genome. The position of the restriction sites R1 to R5 used for cloning of the different expression constructs, as well as the positions of the translation start codons of the three capsid proteins are marked. Not to scale

FIG. 5: Comparison of capsid assembly using different VP protein expression constructs.
A) Schematic representation of the c*ap* gene expression constructs used for analysis of VP protein expression and to study capsid assembly. Plasmids pCMV-VP3/1882 to pCMV-VP3/2809 are derived from plasmid pVP3. Numbers indicate nucleotide positions in the AAV2 genome according to Ruffing et al., 1994 (supra). Arrows represent translation start sites of the VP proteins, mutated translation start sites are labeled with a cross. The ability of the proteins expressed from these expression constructs to assemble capsids is given in the right column (corresponding to the quantification in C, ++ corresponds to peak titer of capsids, - means that no capsids could be detected, + means that capsid assembly is detectable. B) Western blot analysis of expressed VP proteins was performed using antibody B1 which detects all three capsid proteins or antibody A69 which detects only VP1 and VP2. In each lane a different expression construct is separated, name according to A. The position of the three capsid proteins is marked C) Capsid formation was quantified by an ELISA based on monoclonal antibody A20. Means +/- standard deviations of at least three independent experiments are shown; asterisk indicates constructs for which no capsids could be detected.

FIG. 6: Complementation of VP3 capsid assembly by VP2N-gfp.
A) Schematic representation of the fusion construct, pVP2N-gfp, as well as of its transcripts VP2N-gfp, VP3N-gfp and GFP as indicated. B) Western blot detection of VP3 (B1 antibody), VP3N-gfp fusion protein (anti-gfp antibody) and VP2N-gfp (A69 antibody) expression in HeLa cells after co-transfection of pVP3/2809 (1) and decreasing amounts of pVP2N-gfp (1, 1/5, 1/50, 1/500, del meaning 0) as indicated. C) Detection of capsid formation by indirect immunofluorescence using

antibody A20 in HeLa cells co-transfected with pVP3/2809 and pVP2N-gfp in different ratios as marked and shown in B. D) Quantification of capsid formation in HeLa cells co-transfected with pVP3/2809 and pVP2N-gfp in different ratios using the A20 based capsid ELISA. Again, the different plasmid ratios are marked and correspond to those shown in B and C. For each experiment the mean concentration of capsids +/- standard deviations of at least two independent experiments are shown; asterisk indicates samples for which no capsids could be detected.

FIG. 7: Substoichiometric incorporation of truncated VP2 within VP3 particles in the cis situation.

Western blot analysis of purified wt AAV and capsids derived from pVP3/2696 or pVP3/2809 trans-complemented with pVP2N-gfp. Detection of VP1 and VP2 occurred with antibody A69. Different amounts of capsids as indicated were loaded to the gel for a qualitative estimation of the ratio of different signals (VP2tru = truncated VP2).

FIG. 8: Characterization of helper plasmid pVP2Ncm-gfp with alternative codon usage.

1. A) Alignment of wt (VP2N, SEQ ID NO: 145) and codon modified VP2N (VP2Ncm, SEQ ID NO: 146) DNA sequences of the respective constructs pVP2N-gfp (details in Fig. 6A) and pVP2Ncm-gfp.
2. B) Western blot of 293-T cell extracts after transfection of the indicated plasmids with monoclonal antibody A69.
3. C) Fluorescence images of HeLa cells transfected with pVP2N-gfp: The upper and lower left panels represent total GFP fluorescence. The upper and lower right panels show indirect immunofluorescence of the VP2 part within VP2N-gfp visualized by the A69 antibody and the respective secondary Cy3-labeled goat anti-mouse antibody.
4. D) Quantification of capsid formation in 293-T cells co-transfected with pCMV-VP3/2809 and the indicated plasmids using the A20 based capsid ELISA. Means +/- standard deviations of at least three independent experiments are shown; asterisk indicates sample for which no capsids could be detected.

FIG. 9: Stop codon mutagenesis within the trans-complementation construct

A) Schematic representation of pVP2N-gfp constructs with translation stop codons in the VP2N reading frame at four different positions. Numbers of the substituted nucleotides refer to the nucleotide positions of the AAV2 genome. In pVP2N/stopA the cag-codon starting at nucleotide 2770 and coding for glutamine has been mutated into tag, in pVP2N/stopB the gga-codon starting at nucleotide 2797 and coding for glycine has been mutated into tga, in pVP2N/stopC the agt-codon starting at nucleotide 2821 and coding for serine has been mutated into tga, and in pVP2N/stopD the gga-codon starting at nucleotide 2878 and coding for glycine has been mutated into tga. B) Western blot of 293-T cell extracts after co-transfection of pCMV-VP3/2809 and the indicated plasmids with monoclonal antibodies B1 and A69. C) Quantification of capsid formation in 293-T cells co-transfected with pCMV-VP3/2809 and the indicated plasmids using the A20 based capsid ELISA. Means +/- standard deviations of at least three independent experiments are shown; asterisk indicates sample for which no capsids could be detected.

FIG. 10: Cellular localization of capsid proteins and capsids obtained by expression of different *cap* gene mutants.

Localization of capsid proteins expressed from different constructs in HeLa cells was visualized by double immunofluorescence using a polyclonal rabbit antiserum detecting total capsid proteins

(VPs) and monoclonal antibody A20 detecting assembled capsids. The transfected plasmids are indicated at the left margin.
Immunofluorescence staining of transfected HeLa cells with the A20 antibody showed that the VP protein of mutant pCMV-VP3/2696RKR168-170AAA was as efficient in capsid assembly as wt AAV. For the construct pCMV-VP3/2696RKR168-170AAA the postulated NLS was mutated by converting the RKR peptide (AA 168-170).

Fig. 11: Capsid assembly of VP3 modified by a NLS or an N terminal extension of human serum albumin.

1. A) Schematic representation of NLS-VP3 and HSA-VP3 used for analysis of capsid assembly.
2. B) Indirect double immunofluorescence of HeLa cells transfected with plasmids indicated above the images using a polyclonal VP antiserum (VPs) to localize total expressed capsid proteins (upper row) and antibody A20 to detect assembled capsids (lower row). VP2N-egfp is a synonym for pVP2N-gfp.
3. C) Immuno dot blot analysis of fractions obtained from COS-1 cell extracts separated on sucrose gradients. The cells were harvested 48 h post transfection of the plasmids indicated in the left margin. Note that reaction with the A20 antibody was performed under non-denaturing conditions to detect assembled capsids, whereas reaction with B1 antibody was performed after denaturation of the capsids to detect single capsid proteins. The sedimentation constant of the viral capsid is indicated (60 S).

Fig. 12: VP3 particle production in insect cells

1. A) Schematic representation of constructs used for AAV production in insect cells.
2. B) Western blot analysis of expressed VP proteins was performed using antibody SA7885 (1:10000 dilution) a polyclonal rabbit serum that detects all three capsid proteins and subsequent the secondary antibody anti rabbit IgG-HRP 1:2500 (Dianova, Hamburg, Germany).
3. C) Capsid formation was quantified by an ELISA based on monoclonal antibody A20. Means +/- standard deviations of 2 (VP2 construct) or 4 (VP3 and VP1_Mod4) independent experiments are shown.

Fig. 13: Western Blot analyses of different AAV1 constructs
Western blot analysis of expressed VP proteins in crude lysates of 293 cells transfected with different AAV1 constructs: pCI_VP2/2539_AAV1, pCI_VP3/2539_AAV1mutACG, pCI_VP3/2634_AAV1mutACG and pUCAV1. Detection of VP proteins was performed using the B1 antibody (dilution: 1:250) (Progen Heidelberg, Germany) and subsequent the secondary antibody anti mouse IgG-HRP 1:2500 (Dianova, Hamburg, Germany).
2E10 particles per construct were loaded according to AAV1 titration by an AAV1 capsid ELISA (Progen Heidelberg, Germany).
The Western Blot shows that construct pUCAV1 expresses the three capsid proteins VP1, VP2 and VP3 (lane 5) whereas pCI_VP2/2539_AAV1 leads to expression of VP2 and VP3 (lane 2) and within lysates of cells transfected with pCI_VP3/2539_AAV1mutACG and pCI_VP3/2634_AAV1mutACG only VP3 could be detected (lane 3 and 4).

Fig. 14: Trans-complementation of an AAV1 VP2 construct with pVP2N-gfp of AAV2
Western blot analysis of cell extracts transfected with VP3 expression construct of AAV2 pCMV-

VP3/2809 or of AAV1 pCMV-AAV1VP3/2828 (indicated in the figure as AAV2 or AAV1, respectively) with or without cotransfection of pVP2N-gfp. AAV1 and AAV2 VP3 was detected by the antibody B1 (Progen, Heidelberg, Germany) which recognizes an epitope completely conserved between AAV1 and AAV2. The VP2N-gfp protein was detected by antibody A69 (Progen, Heidelberg, Germany).

Fig. 15: Comparison of particle production efficiency using different pCMV-VP expression vectors

1. A) Schematic representation of constructs. pCI-VP, pCI-VP2 and pCI-VP3 were cloned by PCR amplification of the respective VP coding regions using primer with XhoI (5'-) and NotI (3'-) overhangs and subcloning of the XhoI-/NotI-digested PCR products into the XhoI-/NotI-digested vector pCI (PROMEGA). In case of pCI-VP2, the start codon for VP2 was changed from ACG to ATG at the same time.
   For cloning of the constructs pCI-VP2mutACG, pCMV-NLS-VP3, and pCMV-VP3/2696 please refer to elsewhere.
2. B) For transfection 5.0E+05 293-T cells were seeded into each well of a 6-well cell culture plate in a total volume of 3 ml medium (DMEM containing 10% FCS and ABAM). Cells were cultivated at 37°C and 5% CO_2 in a humidified atmosphere for 24h. Subsequently cells were transfected using the calcium phosphate transfection protocol as disclosed in US 2004/0053410. Briefly, for transfection of one well with 293-T cells 6 µg of the indicated plasmids (pCI-VP, pCI-VP2, pCI-VP3, pCI-VP2 and pCI-VP3 in a 1:10 molar ratio, pCMV-NLS-VP3, pCI-VP2mutACG, and pCMV-VP3/2696, respectively) were mixed in 150 µl 270 mM $CaCl_2$. 150 µl 2x BBS (50 mM BES (pH 6.95), 280 mM NaCl and 1.5 mM Na_2HPO_4) was added to the mixture and the resulting solution was carefully mixed by pipetting. The solution was incubated for 20 min at room temperature and then added drop-wise to the cells. Cells were incubated at 35°C, 3% CO_2 in a humidified atmosphere for 18h. After 18h at 35°C and 3% CO_2 cells were cultivated for an additional 3d at 37°C, 5% CO_2 in a humidified atmosphere. Subsequently, 293-T cells were lysed in the medium by three rounds of freeze (-80°C) and thaw (37°C) cycles. The lysate (3 ml total volume) was cleared by centrifugation and the VLP capsid titer was determined using a commercially available ELISA (AAV Titration ELISA, Progen). Average values of 4 to 6 independent transfections per construct are indicated with respective error bars.
   Notably, particle production efficacy with construct pCMC-NLS-VP3 was below the detection limit (about 1E+09 / ml) and, therefore, at least 3-4 logs lower compared to the best VP3 particle production vectors described in this invention (pCI-VP2mutACG and pCMV-VP3/2696).
3. C) For transfection 5.0E+05 293-T cells were seeded into each well of a 6-well cell culture plate in a total volume of 3 ml medium (DMEM containing 10% FCS and ABAM). Cells were cultivated at 37°C and 5% CO_2 in a humidified atmosphere for 24h. Subsequently cells were transfected using the calcium phosphate transfection protocol as disclosed in US 2004/0053410. Briefly, for transfection of one well with 293-T cells 6 µg of the indicated plasmids (pCI-VP, pCI-VP2, pCI-VP3, pCI-VP2 and pCI-VP3 in a 1:10 molar ratio, pCMV-NLS-VP3, pCI-VP2mutACG, and pCMV-VP3/2696, respectively) were mixed in 150 µl 270 mM $CaCl_2$. 150 µl 2x BBS (50 mM BES (pH 6.95), 280 mM NaCl and 1.5 mM Na_2HPO_4) was added to the mixture and the resulting solution was carefully mixed by pipetting. The solution was incubated for 20 min at room temperature and then added drop-wise to the cells. Cells

were incubated at 35°C, 3% CO_2 in a humidified atmosphere for 18h. After 18h at 35°C and 3% CO_2 cells were cultivated for an additional 3d at 37°C, 5% CO_2 in a humidified atmosphere.

Subsequently, supernatant of 293-T cells was removed, cells were rinsed with PBS and finally lysed in 300 µl RIPA buffer (25 mM Tris.Cl pH 7.4, 150 mM NaCl, 1% IGEPAL, 1% Na.DOC, 0.1% SDS). 100 µl 3xGeba sample buffer (Gene Bio-Application Ltd) and 25 mM DTT were added, and samples were heated at 95°C for 10 min. Samples were centrifuged and 30 µl cleared supernatant were subjected to SDS page (10% GeBa gels, Gene Bio-Application Ltd). Proteins were transferred to a nitrocellulose membrane (1h, 230 mA) which was blocked for 1 h at RT subsequently. VP proteins were detected with the antibody B1 (Progen) by overnight incubation at 4°C in blocking buffer (1:500 dilution), subsequent washing and incubation with secondary antibody (anti-mouse IgG-HRP; 1:2500 in blocking buffer). Finally, the membrane was rinsed again and incubated with super signal pico west substrate (Pierce) for 5 min at RT. AAV capsid proteins are expressed as expected from the different VP expression vectors.

FIG. 16: Schematic organization of the AAV capsid gene.

The position of ORF2 and the encoded protein AAP is shown in relation to the position of translation start codons of the Cap proteins VP1, VP2 and VP3, as well as the EcoNI and BsiWI restriction sites (as given and described in more detail in Fig. 1). The arrows mark the translation start site and indicate that VP1, VP2 and VP3 are translated from the same one reading frame (named first ORF, ORF1, herein) of the cap gene, whereas AAP is translated from a different reading frame (ORF2). For VP1, VP2 and VP3 the well-defined numbers of the translation start points are given.

FIG. 17: Nucleotide sequence of ORF2 and protein sequence of AAP of AAV2.

The nucleotide sequence of ORF2 of AAV2 (NCBI entrée number NC_001401) from position 2717 to 3343 (including the tga stop codon), as well as the respective protein sequence of AAP obtained upon translation of ORF2 starting with the first nucleotide of ORF2 is given. 2809 marks the nucleotide position of the ATG start codon of VP3 which is underlined and given in bold.. The predicted AAP translation initiation codon CTG coding for L (leucine) also is underlined and marked in bold.

Fig. 18: Sequence of ORF1cm and ORF2cm.

1. A) DNA sequence of the codon modified EcoNI-BsiWI restriction fragment ORF1cm.
2. B) DNA sequence of the codon modified EcoNI-BsiWI restriction fragment ORF2cm.
   Translation start codons of VP2 and VP3 are underlined. Start of ORF2 is marked (↓) and position of the predicted non-canonical AAP translation initiation codon CTG intact in ORF2cm is highlighted by a frame. And/orote that the translation start codon of AAP is mutated into CCG in ORF1cm.

Fig. 19: Trans-complementation of VP3 expressing plasmid with pVP2N-gfp.

1. A) Schematic representation of construct pVP2N-gfp, containing the *EcoNI-BsiWI* fragment derived of the AAV2 genome and a *gfp*-cassette,
2. B) pVP2N-gfp was co-transfected with pCMV-VP3/2809 in decreasing amounts into 293-T cells, starting with equimolar ratios, in order to complement VP3 expression of plasmid

pCMV-VP3/2809. For comparison empty vector pBS (commercially available Bluescript vector) or plasmid pCMV-VP3/2696 were transfected. Samples were analyzed by Western blot using monoclonal antibodies B1 for detection of VP3 and A69 for detection of VP2N-gfp and VP2tru (truncated VP2).

3. C) Capsid formation was quantified by an ELISA based on monoclonal antibody A20. Means +/- standard deviations of at least three independent experiments are shown; asterisks indicate samples for which no capsids could be detected.

Fig. 20: Trans-complementation of VP3 expressing plasmid with pVP2N/ ORF1cm and pVP2N/ORF2cm.

Same experimental setup as described in Fig. 19 with the difference that the constructs pVP2N/ORF1cm and pVP2N/ORF2cm have been used for trans-complementation. Codon modified DNA sequences (detailed sequences are given in Fig. 18) are represented as shaded boxes in A).

Fig. 21: Trans-complementation of VP3 expressing plasmid with pORF2/CTG-AU1, pORF2/ATG-AU1 and pORF2/TTG-AU1.

Same experimental setup as described in Fig. 19 with the difference that the constructs pORF2/CTG-AU1, pORF2/ATG-AU1 and pORF2/TTG-A have been used for trans-complementation. They comprise the entire ORF2 of the cap gene (as given in Fig. 17) fused to sequences coding for an AU1-tag. The predicted AAP translation initation codon (CTG) was additionally mutated to ATG and TTG

Monoclonal antibody anti-AU1 for detection of AAP-AU1 or polyclonal anti-AAP serum for detection of AAP-AU1 or C-terminally truncated AAP (AAPtru).

Fig. 22: Trans-complementation of VP3 expressing plasmid with pVP2N/ORF2stopA, pVP2N/ORF2stopB, and pVP2N/ORF2stopC.

Derivates of pVP2N-gfp harbouring stop codons in ORF2 of the cap gene fragment were co-transfected with VP3 expression plasmid pCMV-VP3/2809 into 293-T cells.

1. A) Schematic representation of the constructs pVP2N/ORF2stopA, pVP2N/ORF2stopB, and pVP2N/ORF2stopC, respectively, containing stop codons in ORF2 of the cap gene fragment at the indicated positions. In pVP2N/ORF2stopA the tgg-codon starting at nucleotide 2810 has been mutated into tag, in pVP2N/ORF2stopB the caa-codon starting at nucleotide 2831 has been mutated into taa, and in pVP2N/ORF2stopC the gaa-codon starting at nucleotide 2879 has been mutated into tga. All mutations do not disrupt ORF1.
2. B) Samples were analyzed by Western blot using monoclonal antibodies B1 for detection of VP3 and A69 for detection of VP2N-gfp.
3. C) Capsid formation was quantified by an ELISA based on monoclonal antibody A20. Means +/- standard deviations of at least three independent experiments are shown; asterisks indicate samples for which no capsids could be detected.

Fig. 23: Trans-complementation of full length AAV2 genome deficient in AAP expression with different constructs.

1. A) Schematic representation of plasmid pTAV2.0, harbouring the wildtype AAV2 genome and of plasmid pTAV/ORF1cm, containing the ORF1 codon modified EcoNI/BsiWI fragment of the c*ap* gene (shaded box).
2. B) Plasmids were co-transfected with the indicated constructs into 293-T cells. Western blot

analysis of VP protein expression was performed using monoclonal antibody B1. AAP and truncated AAP (AAPtru) were detected with polyclonal anti-AAP serum.

3. C) and D) Capsid formation upon co-transfection of plasmids as indicated in 293-T cells was quantified by an ELISA based on monoclonal antibody A20. Means +/- standard deviations of at least three independent experiments are shown; asterisks indicate samples for which no capsids could be detected.

Fig. 24: Trans-complementation of full length AAV2 genome containing a stop codon in ORF2 of the cap gene by wt genome.

1. A) Schematic representation of plasmid pTAV2.0, harbouring the wt AAV2 genome and of plasmid pTAV/ORF2stopB, containing a stop codon in ORF2 of the *cap* gene (equivalent position as in plasmid pVP2N/ORF2stopB, Fig. 22).
2. B) Plasmids were co-transfected with empty vector pBS or with pVP2N-gfp (as indicated) into 293-T cells. Western blot analysis of VP protein expression was performed using monoclonal antibody B1. AAP and AAPtru were detected with polyclonal anti-AAP serum.
3. C) Capsid formation upon co-transfection of plasmids as indicated in 293-T cells was quantified by an ELISA based on monoclonal antibody A20. Means +/- standard deviations of three independent experiments are shown; asterisk indicates sample for which no capsids could be detected.

Fig. 25: Immunofluorescence images for intracellular localization of VP3 and NoLS-VP3, as well as assembled capsids.

1. A) Schematic representation of the construct used for expression of VP3 fused to the nucleolar localization signal of HIV Rev (NoLS-VP3) in comparison to the construct expressing NLS-VP3 due to fusion of VP3 to the nuclear localization signal of the SV40 large T-antigen (as used in Fig. 11).
2. B) Indirect double immunofluorescence of HeLa cells transfected with plasmids indicated at the left using a polyclonal VP antiserum (VPs) to localize total expressed capsid proteins (left images) and antibody A20 to detect assembled capsids (right images).
3. C) Indirect double immunofluorescence of HeLa cells transfected with plasmids indicated at the left using a monoclonal antibody against the AU1-tag (anti-AU1) to localize expressed AAP (left image) and polyclonal Fibrillarin antibody (anti-Fibrillarin) as a marker for nucleoli localization (middle image). On the right the phase contrast image of the same sector is shown.

Fig. 26: Expression and capsid assembly activity of VP3, NLS-VP3 and NoLS-VP3.

1. A) Western blot analysis of extracts of 293-T cells expressing VP3 or VP3 fusion proteins as indicated was performed using monoclonal antibody B1.
2. B) Capsid formation in 293-T cells was quantified by an ELISA based on monoclonal antibody A20. Means +/- standard deviations of at least three independent experiments are shown; asterisks indicate samples for which no capsids could be detected.

Fig. 27: Comparison of parvovirus AAP sequences.

Alignment of predicted AAP protein sequences derived from ORF2 of the cap gene of different parvoviruses. Conserved amino acids that are 100% identical in at least 60% of aligned sequences

are represented as lines in the lower row. Position of the predicted AAV2 AAP translation start is highlighted by a frame. Non-translated sequences upstream of the potential translation initiation codons are included as well. NCBI entrée numbers of the corresponding DNA sequences are listed in table 8.

Fig. 28: EM analysis of AAV2 empty particle preparations
Virus-like particles assembled of VP1, VP2 and VP3 (VP1,2,3 VLP) or assembled only of VP3 (VP3 VLP) as indicated.

Fig. 29: Capsid assembly upon trans-complementation
Capsid formation in 293-T cells from constructs pCMV_VP3/2809 of AAV2 (AAV2-VP3), pCMV_AAV1VP3/2829 from AAV1 (AAV1-VP3) and a corresponding AAV5 VP3 construct (AAV5-VP3) co-transfected with pVP2N-gfp from AAV2, AAV1 and AAV5 as indicated was quantified by an ELISA based on monoclonal antibody A20. Bluescript vector (pBS) was used as negative control. Asterisks indicate samples for which no capsids could be detected.

AMINO ACID SEQUENCES

[0169]

SEQ ID NO: 1
ILVRLETQTQ YLTPSLSDSH QQPPLVWELI RWLQAVAHQW QTITRAPTEW VIPREIGIAI
PHGWATESSP PAPEPGPCPP TTTTSTNKFP ANQEPRTTIT TLATAPLGGI LTSTDSTATF
HHVTGKDSST TTGDSDPRDS TSSSLTFKSK RSRRMTVRRR LPITLPARFR CLLTRSTSSR
TSSARRIKDA SRRSQQTSSW CHSMDTSP

SEQ ID NO: 2
SSRHKSQTPP RASARQASSP LKRDSILVRL ATQSQSPIHN LSENLQQPPL LWDLLQWLQA
VAHQWQTITK APTEWVMPQE IGIAIPHGWA TESSPPAPAP GPCPPTITTS TSKSPVLQRG
PATTTTTSAT APPGGILIST DSTATFHHVT GSDSSTTIGD SGPRDSTSNS STSKSRRSRR
MMASQPSLIT LPARFKSSRT RSTSFRTSSA LRTRAASLRS RRTCS

SEQ ID NO: 3
ISVRLATQSQ SQTLNLSENH QQPPQVWDLI QWLQAVAHQW QTITRVPMEW VIPQEIGIAI
PNGWATESSP PAPEPGPCPL TTTISTSKSP ANQELQTTTT TLATAPLGGI LTLTDSTATS
HHVTGSDSLT TTGDSGPRNS ASSSSTSKLK RSRRTMARRL LPITLPARFK CLRTRSISSR
TCSGRRTKAV SRRFQRTSSW SLSMDTSP

SEQ ID NO: 4
LNPPSSPTPP RVSAKKASSR LKRSSFSKTK LEQATDPLRD QLPEPCLMTV RCVQQLAELQ
SRADKVPMEW VMPRVIGIAI PPGLRATSRP PAPEPGSCPP TTTTSTSDSE RACSPTPTTD
SPPPGDTLTS TASTATSHHV TGSDSSTTTG ACDPKPCGSK SSTSRSRRSR RRTARQRWLI
TLPARFRSLR TRRTNCRT

SEQ ID NO: 5
TTTFQKERRL GPKRTPSLPP RQTPKLDPAD PSSCKSQPNQ PQVWELIQCL REVAAHWATI
TKVPMEWAMP REIGIAIPRG WGTESSPSPP EPGCCPATTT TSTERSKAAP STEATPTPTL
DTAPPGGTLT LTASTATGAP ETGKDSSTTT GASDPGPSES KSSTFKSKRS RCRTPPPPSP
TTSPPPSKCL RTTTTSCPTS SATGPRDACR PSLRRSLRCR STVTRR

SEQ ID NO: 6
SSRHKSQTPP RALARQASSP LKRDSILVRL ATQSQSPTHN LSENLQQPPL LWDLLQWLQA
VAHQWQTITK APTEWVMPQE IGIAIPHGWA TESSPPAPEH GPCPPITTTS TSKSPVLQRG
PATTTTTSAT APPGGILIST DSTAISHHVT GSDSSTTIGD SGPRDSTSSS STSKSRRSRR

MMASRPSLIT LPARFKSSRT RSTSCRTSSA LRTRAASLRS RRTCS

SEQ ID NO: 7

SRHLSVPPTP PRASARKASS PPERDSISVR LATQSQSPTL NLSENLQQRP LVWDLVQWLQ
AVAHQWQTIT KVPTEWVMPQ EIGIAIPHGW ATESLPPAPE PGPCPPTTTT STSKSPVKLQ
VVPTTTPTSA TAPPGGILTL TDSTATSHHV TGSDSSTTTG DSGPRSCGSS SSTSRSRRSR
RMTALRPSLI TLPARFRYSR TRNTSCRTSS ALRTRAACLR SRRTSS

SEQ ID NO: 8

SHHPSVLQTP LRASARKANS PPEKDSILVR LATQSQFQTL NLSENLQQRP LVWDLIQWLQ
AVAHQWQTIT KAPTEWVVPR EIGIAIPHGW ATESSPPAPE PGPCPPTTTT STSKSPTGHR

EEPPTTTPTS ATAPPGGILT LTDSTATFHH VTGSDSSTTT GDSGPRDSAS SSSTSRSRRS
RRMKAPRPSP ITSPAPSRCL RTRSTSCRTF SALPTRAACL RSRRTCS

SEQ ID NO: 9

SSLLRNRTPP RVLANRVHSP LKRDSISVRL ATQSQSQTLN QSENLPQPPQ VWDLLQWLQV
VAHQWQTITK VPMEWVVPRE IGIAIPNGWG TESSPPAPEP GPCPPTTITS TSKSPTAHLE
DLQMTTPTSA TAPPGGILTS TDSTATSHHV TGSDSSTTTG DSGLSDSTSS SSTFRSKRLR
TTMESRPSPI TLPARSRSSR TQTISSRTCS GRLTRAASRR SQRTFS

SEQ ID NO: 10

TLGRLASQSQ SPTLNQSENH QQAPLVWDLV QWLQAVALQW QTITKAPTEW VVPQEIGIAI
PHGWATESSP PAPEPGPCPP TTTTSTSKSP TGHREEAPTT TPTSATAPPG GILTSTDSTA
TSHHVTGSDS STTTGDSGQK DSASSSSTSR SRRSRRMKAP RPSPITLPAR FRYLRTRNTS
CRTSSAPRTR AACLRSRRMS S

SEQ ID NO:11

SHHKSPTPPR ASAKKANNQP ERGSTLKRTL EPETDPLKDQ IPAPCLQTLK CVQHRAEMLS
MRDKVPMEWV MPRVIGIAIP PGLRARSQQP RPEPGSCPPT TTTCTCVSEQ HQAATPTTDS
PPPGDILTST DSTVTSHHVT GKDSSTTTGD YDQKPCALKS SISKLRRSQR RTARLRSLIT
LPARFRYLRT RRMSSRT

SEQ ID NO: 12

KRLQIGRPTR TLGRPRPRKS KKTANQPTLL EGHSTLKTLE QETDPLRDHL PEKCLMMLRC
VRRQAEMLSR RDKVPMEWVM PPVIGIAIPP GQRAESPPPA PEPGSYPRTT TTCTCESEQR
PTATPTTDSP PPGDTLTLTA STATFPHATG SDSSTTTGDS GRNRCVLKSS TYRSRRSRRQ
TARLRSLITL PARFRSLRIR RMNSHT

SEQ ID NO: 13

SRVLKSQTPR AELARKANSL PERDSTLTTN LEPETGLPQK DHLPELCLLR LKCVQQLAEM
VAMRDKVPRE WVMPPVIGIA IPLGQRATSP PPQPAPGSCR PTTTTCTCGS ARATPATPST
DSPPPGDTLT LTASTATSRQ ETGKGSSTTT GDCAPKACKS ASSTSKLRRS RRLTGRRPYP
TTSPARSRSL RTARTSSRT

SEQ ID NO: 14

VKPSSRPKRG FSNPLVWWKT QRRLRPETSG KAKTNLVCPT LLHRLPRKTR SLARKDLPAG
QKIRAKAPLP TLEQQHPPLV WDHLSWLKEV AAQWAMQARV PMEWAIPPEI GIAIPNGWKT
ESSLEPPEPG SCPATTTTCT NESKDPAEAT TTTNSLDSAP PGDTLTTIDS TATFPRETGN
DSSTTTGASV PKRCALDSLT SRLKRSRSKT STPPSATTSP VRSRSLRTRT TNCRTSSDRL
PKAPSRRSQR ISTRSRSTGT AR

SEQ ID NO: 15

ILVRLATQSQ SQTLNHSDNL PQPPLVWDLL QWLQAVAHQW QTITRVPMEW VIPQEIGIAI
PNGWATESSP PAPAPGPCPP TTITSTSKSP ANQEPPTTTT TLATAPPGGI LTSTDSTATF
HHVTGKDSST TTGDSDPRDS TSSSLTFKSK RSRRMTVRRR LPITLPARFR CLLTPSTSSR
TSSARRIRDA SRRSQQTSSW SHSMDTSP

SEQ ID NO: 16

TRRTVSSLPL QRRPKLEALP PPAIWDLVRW LEAVARQSTT ARMVPMEWAM PREIGIAIPH
GWTTVSSPEP LGPGICQPTT TTSTNDSTER PPETKATSDS APPGDTLTST ASTVISPLET
GKDSSTITGD SDQRAYGSKS LTFKLKKSRR KTQRRSSPIT LPARFRYLRT RSTSSRT

SEQ ID NO: 17

LNNPTTRPGP GRSVPNASTT FSRKRRRPRP SKAKPLLKRA KTPEKEPLPT LDQAPPLVWD
HLSWLKEVAV QWAMQAKVPT EWAIPREIGI AIPNGWTTES LPEPLEPGSC PATTTTCTSG
SKDREEPTPT INSLDSAPPG GTLTTTDSTA TSPPETGNDS STTTGASDPK RCALDSLTSR
LKKSLSKTPT PPSPTTSPAR SKSLRTRTTS CRTSSDRLQR APSRRSQRIS TRSRSMVTAR

SEQ ID NO: 18

TTTFQKERRL GPKRTPSLPP RQTPKLDPAD PSSCKSQHNQ PQVWELIQCL REVAAHWATI
TKVPMEWAMP REIGIAIPRG WGTESSPSPP APGCCPATTT TSTERSKAAP STEATPTPTL
DTAPPGGTLT LTASTATGAP ETGKDSSTTI GASDPGLSES KSSTSKSKRS RCRTPPPPSP
TTSPPPSKCL RTTTTNSRTS SATGPRDACR PSPRRSLRCR STATRR

SEQ ID NO: 19

ASRSRSWLLQ SSVHTRPRKP QRTRRVSRDR IPGRRPRRGS SSPISLDLQQ TYLHPHNSPS
LPQGFPVWFL VRCLQEEALQ WTMLNKVPTE WAMPREIGIA IPNGWATEFS PDPPGPGCCP
ATTTTCTSRS QTPPACTASP GADTLATAPP GGTSTSIAST ATSRPETGSA SSITTGASDP
RDCESNSSTS RSRRSRLLIR RPRSPTTSRA RSRSSQTTST SCRTSAATPP RDACRRSPRT
SSRCRSTATR R

SEQ ID NO: 20

KTEEPPRRAP NLWQHLKWQR EEAELWATLQ GVPMEWVMPR EIGIAIPNGW ETQSSQRPPE
PGSCQATTTT STKQLPVEPL KMQMSSMQDT VPPGGTLIST ASTATSPLET GRDLSTTIGE
SDPNLLNSRS SMSKSKKSQR RIKQRPLQTI SPQRFKSLRM MSINSRMSWA RLRKAPCRRS
RRMSMPCRST GTAQCTPTRM EHGSMTVVHS TA

SEQ ID NO: 21

KSLNYLKKTL LHPVIVEEKQ VQLPPKAPNL WQHLTWQREE AELWATLQGV PMEWVMPQEI
GIAIPNGWET QSLPRLQEPG SCQATTTTST KPSQAEQTQT QIPNMLDTAP PGGTLISTDS
TAISLQETGR DSSTTIGGLD RKHSNSRYSM CKLKKSRRKT RQRLLLTTLP LQSRYSRIMN
TSCPMFWARP RRGRCHRSPQ MCMPCPSTAT AQCTPTRVEL DSMTEVPSIA

SEQ ID NO: 22

TNTILKLKRP NKACRYQLHL KAEKKKLHRH NLEGAQQVPI LAAHLSWLQE EAVRWQTITR
APREWVIPQV IGIAIPSGWE TTSLQSQPEL GCSPLTGIIS TGLSTLTAPQ VRVLMQPMQD
TRLPGGTLTS IDSIATSPPE TGKDSSTTTQ ASGRKDSKSK SLTSKSKKLQ HKIQRKQLPT
ISPAPYRSLR TRTTTYHMY

SEQ ID NO: 143

LNNPTTRPGP GRSVPNASTT FSRKRRRPRP SKAKPLLKRA KTPEKEPLPT LDQAPPLVWD
HLSWLKEVAV QWAMQAKVPT EWAIPREIGI AIPNGWTTES LPEPLEPGSC PATTTTCTSG
SKDREEPTPT INSLDSAPPG GTLTTTDSTA TSPPETGNDS STTTGASDPK RCALDSLTSR
LKKSLSKTPT PPSPTTSPAR SKSLRTRTTS CRTSSDRLQR APSRRSQRIS TRSRSMVTAR

NUCLEIC ACID SEQUENCES

[0170]

SEQ ID NO: 23

ATTTTGGTCA GACTGGAGAC GCAGACTCAG TACCTGACCC CCAGCCTCTC GGACAGCCAC
CAGCAGCCCC CTCTGGTCTG GGAACTAATA CGATGGCTAC AGGCAGTGGC GCACCAATGG
CAGACAATAA CGAGGGCGCC GACGGAGTGG GTAATTCCTC GGGAAATTGG CATTGCGATT
CCACATGGAT GGGCGACAGA GTCATCACCA CCAGCACCCG AACCTGGGCC CTGCCCACCT
ACAACAACCA CCTCTACAAA CAAATTTCCA GCCAATCAGG AGCCTCGAAC GACAATCACT
ACTTTGGCTA CAGCACCCCT TGGGGGTATT TTGACTTCAA CAGATTCCAC TGCCACTTTT
CACCACGTGA CTGGCAAAGA CTCATCAACA ACAACTGGGG ATTCCGACCC AAGAGACTCA
ACTTCAAGCT CTTTAACATT CAAGTCAAAG AGGTCACGCA GAATGACGGT ACGACGACGA
TTGCCAATAA CCTTACCAGC ACGGTTCAGG TGTTTACTGA CTCGGAGTAC CAGCTCCCGT
ACGTCCTCGG CTCGGCGCAT CAAGGATGCC TCCCGCCGTT CCCAGCAGAC GTCTTCATGG
TGCCACAGTA TGGATACCTC ACCCTGA

SEQ ID NO: 24

AGCAGTCGCC ACAAGAGCCA GACTCCTCCT CGGGCATCGG CAAGACAGGC CAGCAGCCCG
CTAAAAAGAG ACTCAATTTT GGTCAGACTG GCGACTCAGA GTCAGTCCCC GATCCACAAC
CTCTCGGAGA ACCTCCAGCA ACCCCCGCTG CTGTGGGACC TACTACAATG GCTTCAGGCG
GTGGCGCACC AATGGCAGAC AATAACGAAG GCGCCGACGG AGTGGGTAAT GCCTCAGGAA
ATTGGCATTG CGATTCCACA TGGCTGGGCG ACAGAGTCAT CACCACCAGC ACCCGCACCT
GGGCCTTGCC CACCTACAAT AACCACCTCT ACAAGCAAAT CTCCAGTGCT TCAACGGGGG
CCAGCAACGA CAACCACTAC TTCGGCTACA GCACCCCCTG GGGGTATTTT GATTTCAACA
GATTCCACTG CCACTTTTCA CCACGTGACT GGCAGCGACT CATCAACAAC AATTGGGGAT
TCCGGCCCAA GAGACTCAAC TTCAAACTCT TCAACATCCA AGTCAAGGAG GTCACGACGA
ATGATGGCGT CACAACCATC GCTAATAACC TTACCAGCAC GGTTCAAGTC TTCTCGGACT
CGGAGTACCA GCTTCCGTAC GTCCTCGGCT CTGCGCACCA GGGCTGCCTC CCTCCGTTCC
CGGCGGACGT GTTCATGA

SEQ ID NO: 25

ATTTCGGTCA GACTGGCGAC TCAGAGTCAG TCCCAGACCC TCAACCTCTC GGAGAACCAC
CAGCAGCCCC CACAAGTTTG GGATCTAATA CAATGGCTTC AGGCGGTGGC GCACCAATGG
CAGACAATAA CGAGGGTGCC GATGGAGTGG GTAATTCCTC AGGAAATTGG CATTGCGATT
CCCAATGGCT GGGCGACAGA GTCATCACCA CCAGCACCAG AACCTGGGCC CTGCCCACTT
ACAACAACCA TCTCTACAAG CAAATCTCCA GCCAATCAGG AGCTTCAAAC GACAACCACT
ACTTTGGCTA CAGCACCCCT TGGGGGTATT TTGACTTTAA CAGATTCCAC TGCCACTTCT
CACCACGTGA CTGGCAGCGA CTCATTAACA ACAACTGGGG ATTCCGGCCC AAGAAACTCA
GCTTCAAGCT CTTCAACATC CAAGTTAAAG AGGTCACGCA GAACGATGGC ACGACGACTA
TTGCCAATAA CCTTACCAGC ACGGTTCAAG TGTTTACGGA CTCGGAGTAT CAGCTCCCGT
ACGTGCTCGG GTCGGCGCAC CAAGGCTGTC TCCCGCCGTT TCCAGCGGAC GTCTTCATGG
TCCCTCAGTA TGGATACCTC ACCCTGA

SEQ ID NO: 26

TTGAATCCCC CCAGCAGCCC GACTCCTCCA CGGGTATCGG CAAAAAAGGC AAGCAGCCGG
CTAAAAAGAA GCTCGTTTTC GAAGACGAAA CTGGAGCAGG CGACGGACCC CCTGAGGGAT
CAACTTCCGG AGCCATGTCT GATGACAGTG AGATGCGTGC AGCAGCTGGC GGAGCTGCAG
TCGAGGGCGG ACAAGGTGCC GATGGAGTGG GTAATGCCTC GGGTGATTGG CATTGCGATT
CCACCTGGTC TGAGGGCCAC GTCACGACCA CCAGCACCAG AACCTGGGTC TTGCCCACCT
ACAACAACCA CCTCTACAAG CGACTCGGAG AGAGCCTGCA GTCCAACACC TACAACGGAT
TCTCCACCCC CTGGGGATAC TTTGACTTCA ACCGCTTCCA CTGCCACTTC TCACCACGTG
ACTGGCAGCG ACTCATCAAC AACAACTGGG GCATGCGACC CAAAGCCATG CGGGTCAAAA
TCTTCAACAT CCAGGTCAAG GAGGTCACGA CGTCGAACGG CGAGACAACG GTGGCTAATA
ACCTTACCAG CACGGTTCAG ATCTTTGCGG ACTCGTCGTA CGAACTGCCG TACGTGA

SEQ ID NO: 27

ACGACCACTT TCCAAAAAGA AAGAAGGCTC GGACCGAAGA GGACTCCAAG CCTTCCACCT
CGTCAGACGC CGAAGCTGGA CCCAGCGGAT CCCAGCAGCT GCAAATCCCA GCCCAACCAG
CCTCAAGTTT GGGAGCTGAT ACAATGTCTG CGGGAGGTGG CGGCCCATTG GGCGACAATA
ACCAAGGTGC CGATGGAGTG GGCAATGCCT CGGGAGATTG GCATTGCGAT TCCACGTGGA
TGGGGGACAG AGTCGTCACC AAGTCCACCC GAACCTGGGT GCTGCCCAGC TACAACAACC
ACCAGTACCG AGAGATCAAA AGCGGCTCCG TCGACGGAAG CAACGCCAAC GCCTACTTTG
GATACAGCAC CCCCTGGGGG TACTTTGACT TTAACCGCTT CCACAGCCAC TGGAGCCCCC
GAGACTGGCA AAGACTCATC AACAACTACT GGGGCTTCAG ACCCCGGTCC CTCAGAGTCA
AAATCTTCAA CATTCAAGTC AAAGAGGTCA CGGTGCAGGA CTCCACCACC ACCATCGCCA
ACAACCTCAC CTCCACCGTC CAAGTGTTTA CGGACGACGA CTACCAGCTG CCCTACGTCG
TCGGCAACGG GACCGAGGGA TGCCTGCCGG CCTTCCCTCC GCAGGTCTTT ACGCTGCCGC
AGTACGGTTA CGCGACGCTG A

SEQ IDNO: 28

AGCAGTCGCC ACAAGAGCCA GACTCCTCCT CGGGCATTGG CAAGACAGGC CAGCAGCCCG
CTAAAAAGAG ACTCAATTTT GGTCAGACTG GCGACTCAGA GTCAGTCCCC GACCCACAAC
CTCTCGGAGA ACCTCCAGCA ACCCCCGCTG CTGTGGGACC TACTACAATG GCTTCAGGCG
GTGGCGCACC AATGGCAGAC AATAACGAAG GCGCCGACGG AGTGGGTAAT GCCTCAGGAA
ATTGGCATTG CGATTCCACA TGGCTGGGCG ACAGAGTCAT CACCACCAGC ACCCGAACAT
GGGCCTTGCC CACCTATAAC AACCACCTCT ACAAGCAAAT CTCCAGTGCT TCAACGGGGG
CCAGCAACGA CAACCACTAC TTCGGCTACA GCACCCCCTG GGGGTATTTT GATTTCAACA
GATTCCACTG CCATTTCTCA CCACGTGACT GGCAGCGACT CATCAACAAC AATTGGGGAT
TCCGGCCCAA GAGACTCAAC TTCAAGCTCT TCAACATCCA AGTCAAGGAG GTCACGACGA
ATGATGGCGT CACGACCATC GCTAATAACC TTACCAGCAC GGTTCAAGTC TTCTCGGACT
CGGAGTACCA GTTGCCGTAC GTCCTCGGCT CTGCGCACCA GGGCTGCCTC CCTCCGTTCC

CGGCGGACGT GTTCATGA

SEQ ID NO: 29

AGCCGTCACC TCAGCGTTCC CCCGACTCCT CCACGGGCAT CGGCAAGAAA GGCCAGCAGC
CCGCCAGAAA GAGACTCAAT TTCGGTCAGA CTGGCGACTC AGAGTCAGTC CCCGACCCTC
AACCTCTCGG AGAACCTCCA GCAGCGCCCT CTAGTGTGGG ATCTGGTACA GTGGCTGCAG
GCGGTGGCGC ACCAATGGCA GACAATAACG AAGGTGCCGA CGGAGTGGGT AATGCCTCAG
GAAATTGGCA TTGCGATTCC ACATGGCTGG GCGACAGAGT CATTACCACC AGCACCCGAA

CCTGGGCCCT GCCCACCTAC AACAACCACC TCTACAAGCA AATCTCCAGT GAAACTGCAG
GTAGTACCAA CGACAACACC TACTTCGGCT ACAGCACCCC CTGGGGGTAT TTTGACTTTA
ACAGATTCCA CTGCCACTTC TCACCACGTG ACTGGCAGCG ACTCATCAAC AACAACTGGG
GATTCCGGCC CAAGAAGCTG CGGTTCAAGC TCTTCAACAT CCAGGTCAAG GAGGTCACGA
CGAATGACGG CGTTACGACC ATCGCTAATA ACCTTACCAG CACGATTCAG GTATTCTCGG
ACTCGGAATA CCAGCTGCCG TACGTCCTCG GCTCTGCGCA CCAGGGCTGC CTGCCTCCGT
TCCCGGCGGA CGTCTTCATG A

SEQ ID NO: 30

AGCCATCACC CCAGCGTTCT CCAGACTCCT CTACGGGCAT CGGCAAGAAA GGCCAACAGC
CCGCCAGAAA AAGACTCAAT TTTGGTCAGA CTGGCGACTC AGAGTCAGTT CCAGACCCTC
AACCTCTCGG AGAACCTCCA GCAGCGCCCT CTGGTGTGGG ACCTAATACA ATGGCTGCAG
GCGGTGGCGC ACCAATGGCA GACAATAACG AAGGCGCCGA CGGAGTGGGT AGTTCCTCGG
GAAATTGGCA TTGCGATTCC ACATGGCTGG GCGACAGAGT CATCACCACC AGCACCCGAA
CCTGGGCCCT GCCCACCTAC AACAACCACC TCTACAAGCA AATCTCCAAC GGGACATCGG
GAGGAGCCAC CAACGACAAC ACCTACTTCG GCTACAGCAC CCCCTGGGGG TATTTTGACT
TTAACAGATT CCACTGCCAC TTTTCACCAC GTGACTGGCA GCGACTCATC AACAACAACT
GGGGATTCCG GCCCAAGAGA CTCAGCTTCA AGCTCTTCAA CATCCAGGTC AAGGAGGTCA
CGCAGAATGA AGGCACCAAG ACCATCGCCA ATAACCTCAC CAGCACCATC CAGGTGTTTA
CGGACTCGGA GTACCAGCTG CCGTACGTTC TCGGCTCTGC CCACCAGGGC TGCCTGCCTC
CGTTCCCGGC GGACGTGTTC ATGA

SEQ ID NO: 31

AGCAGTCTCC TCAGGAACCG GACTCCTCCG CGGGTATTGG CAAATCGGGT GCACAGCCCG
CTAAAAAGAG ACTCAATTTC GGTCAGACTG GCGACACAGA GTCAGTCCCA GACCCTCAAC
CAATCGGAGA ACCTCCCGCA GCCCCCTCAG GTGTGGGATC TCTTACAATG GCTTCAGGTG
GTGGCGCACC AGTGGCAGAC AATAACGAAG GTGCCGATGG AGTGGGTAGT TCCTCGGGAA
ATTGGCATTG CGATTCCCAA TGGCTGGGGG ACAGAGTCAT CACCACCAGC ACCCGAACCT
GGGCCCTGCC CACCTACAAC AATCACCTCT ACAAGCAAAT CTCCAACAGC ACATCTGGAG
GATCTTCAAA TGACAACGCC TACTTCGGCT ACAGCACCCC CTGGGGGTAT TTTGACTTCA
ACAGATTCCA CTGCCACTTC TCACCACGTG ACTGGCAGCG ACTCATCAAC AACAACTGGG
GATTCCGGCC TAAGCGACTC AACTTCAAGC TCTTCAACAT TCAGGTCAAA GAGGTTACGG
ACAACAATGG AGTCAAGACC ATCGCCAATA ACCTTACCAG CACGGTCCAG GTCTTCACGG
ACTCAGACTA TCAGCTCCCG TACGTGCTCG GGTCGGCTCA CGAGGGCTGC CTCCCGCCGT
TCCCAGCGGA CGTTTTCATG A

SEQ ID NO: 32

ACTTTGGGCA GACTGGCGAG TCAGAGTCAG TCCCCGACCC TCAACCAATC GGAGAACCAC
CAGCAGGCCC CTCTGGTCTG GGATCTGGTA CAATGGCTGC AGGCGGTGGC GCTCCAATGG
CAGACAATAA CGAAGGCGCC GACGGAGTGG GTAGTTCCTC AGGAAATTGG CATTGCGATT
CCACATGGCT GGGCGACAGA GTCATCACCA CCAGCACCCG AACCTGGGCC CTGCCCACCT
ACAACAACCA CCTCTACAAG CAAATCTCCA ACGGGACATC GGGAGGAAGC ACCAACGACA
ACACCTACTT CGGCTACAGC ACCCCCTGGG GGTATTTTGA CTTCAACAGA TTCCACTGCC
ACTTCTCACC ACGTGACTGG CAGCGACTCA TCAACAACAA CTGGGGATTC CGGCCAAAAA
GACTCAGCTT CAAGCTCTTC AACATCCAGG TCAAGGAGGT CACGCAGAAT GAAGGCACCA
AGACCATCGC CAATAACCTT ACCAGCACGA TTCAGGTATT TACGGACTCG GAATACCAGC
TGCCGTACGT CCTCGGCTCC GCGCACCAGG GCTGCCTGCC TCCGTTCCCG GCGGATGTCT
TCATGA

SEQ ID NO: 33

AGTCACCACA AGAGCCCGAC TCCTCCTCGG GCATCGGCAA AAAAGGCAAA CAACCAGCCA
GAAAGAGGCT CAACTTTGAA GAGGACACTG GAGCCGGAGA CGGACCCCCT GAAGGATCAG
ATACCAGCGC CATGTCTTCA GACATTGAAA TGCGTGCAGC ACCGGGCGGA AATGCTGTCG
ATGCGGGACA AGGTTCCGAT GGAGTGGGTA ATGCCTCGGG TGATTGGCAT TGCGATTCCA
CCTGGTCTGA GGGCAAGGTC ACAACAACCT CGACCAGAAC CTGGGTCTTG CCCACCTACA
ACAACCACTT GTACCTGCGT CTCGGAACAA CATCAAGCAG CAACACCTAC AACGGATTCT
CCACCCCCTG GGGATATTTT GACTTCAACA GATTCCACTG TCACTTCTCA CCACGTGACT

GGCAAAGACT CATCAACAAC AACTGGGGAC TACGACCAAA AGCCATGCGC GTTAAAATCT

TCAATATCCA AGTTAAGGAG GTCACAACGT CGAACGGCGA GACTACGGTC GCTAATAACC
TTACCAGCAC GGTTCAGATA TTTGCGGACT CGTCGTATGA GCTCCCGTAC GTGA

SEQ ID NO: 34

AAAAGACTCC AAATCGGCCG ACCAACCCGG ACTCTGGGAA GGCCCCGGCC AAGAAAAAGC
AAAAAGACGG CGAACCAGCC GACTCTGCTA GAAGGACACT CGACTTTGAA GACTCTGGAG
CAGGAGACGG ACCCCCTGAG GGATCATCTT CCGGAGAAAT GTCTCATGAT GCTGAGATGC
GTGCGGCGCC AGGCGGAAAT GCTGTCGAGG CGGGACAAGG TGCCGATGGA GTGGGTAATG
CCTCCGGTGA TTGGCATTGC GATTCCACCT GGTCAGAGGG CCGAGTCACC ACCACCAGCA
CCCGAACCTG GGTCCTACCC ACGTACAACA ACCACCTGTA CCTGCGAATC GGAACAACGG
CCAACAGCAA CACCTACAAC GGATTCTCCA CCCCCTGGGG ATACTTTGAC TTTAACCGCT
TCCACTGCCA CTTTTCCCCA CGCGACTGGC AGCGACTCAT CAACAACAAC TGGGGACTCA
GGCCGAAATC GATGCGTGTT AAAATCTTCA ACATACAGGT CAAGGAGGTC ACGACGTCAA
ACGGCGAGAC TACGGTCGCT AATAACCTTA CCAGCACGGT TCAGATCTTT GCGGATTCGA
CGTATGAACT CCCATACGTG A

SEQ ID NO: 35

AGCAGAGTCC TCAAGAGCCA GACTCCTCGA GCGGAGTTGG CAAGAAAGGC AAACAGCCTG
CCAGAAAGAG ACTCAACTTT GACGACGAAC CTGGAGCCGG AGACGGGCCT CCCCCAGAAG
GACCATCTTC CGGAGCTATG TCTACTGAGA CTGAAATGCG TGCAGCAGCT GGCGGAAATG
GTGGCGATGC GGGACAAGGT GCCGAGGGAG TGGGTAATGC CTCCGGTGAT TGGCATTGCG
ATTCCACTTG GTCAGAGAGC CACGTCACCA CCACCTCAAC CCGCACCTGG GTCCTGCCGA
CCTACAACAA CCACCTGTAC CTGCGGCTCG GCTCGAGCAA CGCCAGCGAC ACCTTCAACG
GATTCTCCAC CCCCTGGGGA TACTTTGACT TTAACCGCTT CCACTGCCAC TTCTCGCCAA
GAGACTGGCA AAGGCTCATC AACAACCACT GGGGACTGCG CCCCAAAAGC ATGCAAGTCC
GCATCTTCAA CATCCAAGTT AAGGAGGTCA CGACGTCTAA CGGGGAGACG ACCGTATCCA
ACAACCTCAC CAGCACGGTC CAGATCTTTG CGGACAGCAC GTACGAGCTC CCGTACGTGA

SEQ ID NO: 36

GTAAAGCCAT CTTCCAGGCC AAAAAGAGGG TTCTCGAACC CTTTGGTCTG GTGGAAGACT
CAAAGACGGC TCCGACCGGA GACAAGCGGA AAGGCGAAGA CGAACCTCGT TTGCCCGACA
CTTCTTCACA GACTCCCAAG AAAAACAAGA AGCCTCGCAA GGAAAGACCT TCCGGCGGGG
CAGAAGATCC GGGCGAAGGC ACCTCTTCCA ACGCTGGAGC AGCAGCACCC GCCTCTAGTG
TGGGATCATC TATCATGGCT GAAGGAGGTG GCGGCCCAGT GGGCGATGCA GGCCAGGGTG
CCGATGGAGT GGGCAATTCC TCCGGAAATT GGCATTGCGA TTCCCAATGG CTGGAAAACG
GAGTCGTCAC TCGAACCACC CGAACCTGGG TCTTGCCCAG CTACAACAAC CACCTGTACA
AACGAATCCA AGGACCCAGC GGAGGCGACA ACAACAACAA ATTCTTTGGA TTCAGCACCC
CCTGGGGATA CTTTGACTAC AATCGATTCC ACTGCCACTT TTCCCCGCGA GACTGGCAAC
GACTCATCAA CAACAACTGG GGCATCCGTC CCAAAGCGAT GCGCTTTAGA CTCTTTAACA
TCCAGGTTAA AGAGGTCACG GTCCAAGACT TCAACACCAC CATCGGCAAC AACCTCACCA
GTACGGTCCA GGTCTTTGCG GACAAGGACT ACCAACTGCC GTACGTCCTC GGATCGGCTA
CCGAAGGCAC CTTCCCGCCG TTCCCAGCGG ATATCTACAC GATCCCGCAG TACGGGTACT
GCACGCTAA

SEQ ID NO: 37

ATTTTGGTCA GACTGGCGAC ACAGAGTCAG TCCCAGACCC TCAACCACTC GGACAACCTC
CCGCAGCCCC CTCTGGTGTG GGATCTACTA CAATGGCTTC AGGCGGTGGC GCACCAATGG
CAGACAATAA CGAGGGTGCC GATGGAGTGG GTAATTCCTC AGGAAATTGG CATTGCGATT
CCCAATGGCT GGGCGACAGA GTCATCACCA CCAGCACCCG CACCTGGGCC CTGCCCACCT
ACAACAATCA CCTCTACAAG CAAATCTCCA GCCAATCAGG AGCCACCAAC GACAACCACT
ACTTTGGCTA CAGCACCCCC TGGGGGTATT TTGACTTCAA CAGATTCCAC TGCCACTTTT
CACCACGTGA CTGGCAAAGA CTCATCAACA ACAACTGGGG ATTCCGACCC AAGAGACTCA
ACTTCAAGCT CTTTAACATT CAAGTCAAAG AGGTCACGCA GAATGACGGT ACGACGACGA
TTGCCAATAA CCTTACCAGC ACGGTTCAGG TGTTTACTGA CTCCGAGTAC CAGCTCCCGT
ACGTCCTCGG CTCGGCGCAT CAGGGATGCC TCCCGCCGTT CCCAGCAGAC GTCTTCATGG
TCCCACAGTA TGGATACCTC ACCCTGA

SEQ ID NO: 38

ACGAGGAGGA CCGTGAGTTC GCTGCCGCTG CAGCGGAGAC CGAAACTGGA AGCGCTCCCC
CCACCGGCAA TTTGGGACCT GGTACGATGG CTGGAGGCGG TAGCGCGCCA ATCGACGACG
GCTCGTATGG TGCCGATGGA GTGGGCAATG CCTCGGGAGA TTGGCATTGC GATTCCACAT
GGCTGGACAA CTGTGTCATC ACCCGAACCA CTCGGACCTG GAATCTGCCA ACCTACAACA
ACCACATCTA CAAACGACTC AACGGAACGA CCTCCGGAGA CCAAAGCTAC TTCGGATTCA

GCACCCCCTG GGGATACTTT GACTTCAACC GCTTCCACTG TCATTTCTCC CCTCGAGACT
GGCAAAGACT CATCAACAAT AACTGGGGAC TCCGACCAAA GAGCCTACGG TTCAAAATCT
TTAACATTCA AGTTAAAGAA GTCACGACGC AAGACTCAAC GAAGATCATC TCCAATAACC
TTACCAGCAC GGTTCAGGTA TTTGCGGACA CGGAGTACCA GCTCCCGTAC GTGA

SEQ ID NO: 39

TTGAACAACC CGACAACACG GCCGGGACCG GGGAGAAGCG TCCCGAACGC GTCGACGACT
TTTTCCCGAA AAAGAAGAAG GCCAAGACCG AGCAAGGCAA AGCCCCTGCT CAAACGGGCG
AAGACCCCGG AGAAGGAACC TCTTCCAACG CTGGATCAAG CGCCCCCTCT AGTGTGGGAT
CATCTGTCAT GGCTGAAGGA GGTGGCGGTC CAATGGGCGA TGCAGGCCAA GGTGCCGACG
GAGTGGGCAA TTCCTCGGGA AATTGGCATT GCGATTCCCA ATGGCTGGAC AACGGAGTCG
TTACCCGAAC CACTCGAACC TGGGTCCTGC CCAGCTACAA CAACCACTTG TACAAGCGGA
TCCAAGGACC GGGAGGAACC GACCCCAACA ATAAATTCTT TGGATTCAGC ACCCCCTGGG
GGTACTTTGA CTACAACCGA TTCCACTGCC ACTTCTCCCC CCGAGACTGG CAACGACTCA
TCAACAACAA CTGGGGCATC CGACCCAAAG CGATGCGCTT TAGACTCTTT AACATCCAGG
TTAAAGAAGT CACTGTCCAA GACTCCAACA CCACCATCGC CAACAACCTC ACCAGCACGG
TCCAAGTCTT TGCGGACAAG GACTACCAGC TGCCGTACGT CCTCGGATCG GCTACAGAGG
GCACCTTCCC GCCGTTCCCA GCGGATATCT ACACGATCCC GCAGTATGGT TACTGCACGC
TAA

SEQ ID NO: 40

ACGACCACTT TCCAAAAAGA AAGAAGGCTC GGACCGAAGA GGACTCCAAG CCTTCCACCT
CGTCAGACGC CGAAGCTGGA CCCAGCGGAT CCCAGCAGCT GCAAATCCCA GCACAACCAG
CCTCAAGTTT GGGAGCTGAT ACAATGTCTG CGGGAGGTGG CGGCCCATTG GGCGACAATA
ACCAAGGTGC CGATGGAGTG GGCAATGCCT CGGGAGATTG GCATTGCGAT TCCACGTGGA
TGGGGGACAG AGTCGTCACC AAGTCCACCC GCACCTGGGT GCTGCCCAGC TACAACAACC
ACCAGTACCG AGAGATCAAA AGCGGCTCCG TCGACGGAAG CAACGCCAAC GCCTACTTTG
GATACAGCAC CCCCTGGGGG TACTTTGACT TTAACCGCTT CCACAGCCAC TGGAGCCCCC
GAGACTGGCA AAGACTCATC AACAACTATT GGGGCTTCAG ACCCCGGTCT CTCAGAGTCA
AAATCTTCAA CATCCAAGTC AAAGAGGTCA CGGTGCAGGA CTCCACCACC ACCATCGCCA
ACAACCTCAC CTCCACCGTC CAAGTGTTTA CGGACGACGA C

SEQ ID NO: 41

GCGTCGAGGA GCCGGAGCTG GCTCCTCCAG TCAAGCGTCC ACACTCGCCC GAGAAAACCC
CAGAGAACCA GAAGGGTCAG CCGCGACCGG ATCCCCGGAC GCCGGCCAAG AAGAGGCTCG
AGTTCTCCGA TCAGCCTGGA TCTTCAGCAG ACTTACCTGC ATCCTCACAA CAGTCCCAGC
CTCCCGCAGG GGTTCCCGGT GTGGTTCCTG GTACGATGTC TGCAGGAGGA GGCGCTCCAG
TGGACGATGC TCAACAAGGT GCCGACGGAG TGGGCAATGC CTCGGGAGAT TGGCATTGCG
ATTCCAAATG GCTGGGCAAC CGAGTTCTCA CCCGATCCAC CCGGACCTGG GTGCTGCCCA
GCTACAACAA CCACCTGTAC AAGCAGATCT CAGACGCCTC CGGCGTGCAC AGCCTCCCCG
GGAGCCGATA CTTTGGCTAC AGCACCCCCT GGGGGTACTT CGACTTCAAT CGCTTCCACT
GCCACTTCTC GCCCAGAGAC TGGCAGCGCC TCGTCAATAA CCACTGGGGC TTCCGACCCA
AGAGACTGCG AGTCAAACTC TTCAACATCC AGGTCAAGGA GGTCACGACT ACTGATTCGA
CGACCACGGT CTCCAACAAC CTCACGAGCA CGGTCCAGGT CTTCACAGAC GACGAGTACC
AGCTGCCGTA CGTCTGCGGC AACGCCACCG AGGGATGCCT GCCGCCGTTC CCCCCGGACG
TCTTCACGCT GCCGCAGTAC GGCTACGCGA CGCTGA

SEQ ID NO: 42

AAGACGGAGG AGCCACCGCG GAGGGCACCG AACCTGTGGC AGCATCTGAA ATGGCAGAGG
GAGGAGGCGG AGCTATGGGC GACTCTTCAG GGGGTGCCGA TGGAGTGGGT AATGCCTCGG
GAAATTGGCA TTGCGATTCC CAATGGATGG GAAACACAGT CATCACAAAG ACCACCAGAA
CCTGGGTCCT GCCAAGCTAC AACAACCACA TCTACAAAGC AATTACCAGT GGAACCTCTC
AAGATGCAAA TGTCCAGTAT GCAGGATACA GTACCCCCTG GGGGTACTTT GATTTCAACC
GCTTCCACTG CCACTTCTCC CCTAGAGACT GGCAGAGACT TATCAACAAC CATTGGGGAA

TCCGACCCAA ATCTCTTAAA TTCAAGATCT TCAATGTCCA AGTCAAAGAA GTCACAACGC
AGGATCAAAC AAAGACCATT GCAAACAATC TCACCTCAAC GATTCAAGTC TTTACGGATG
ATGAGCATCA ACTCCCGTAT GTCCTGGGCT CGGCTACGGA AGGCACCATG CCGCCGTTCC
CGTCGGATGT CTATGCCCTG CCGCAGTACG GGTACTGCAC AATGCACACC AACCAGAATG
GAGCACGGTT CAATGACCGT AGTGCATTCT ACTGCTTAG

SEQ ID NO: 43

AAAAGCCTAA ATTATCTGAA GAAAACTCTC CTTCACCCAG TAATAGTGGA GGAGAAGCAA
GTGCAGCTGC CACCGAAGGC TCCGAACCTG TGGCAGCACC TAACATGGCA GAGGGAGGAA
GCGGAGCTAT GGGCGACTCT GCAGGGGGTG CCGATGGAGT GGGTAATGCC TCAGGAAATT
GGCATTGCGA TTCCCAATGG CTGGGAGACA CAGTCATTAC CAAGACTACA AGAACCTGGG

TCCTGCCAAG CTACAACAAC CACATCTACA AAGCCATCAC AAGCGGAACA AACCCAGACA
CAAATACCCA ATATGCTGGA TACAGCACCC CCTGGGGGTA CTTTGATTTC AACAGATTCC
ACTGCCATTT CTCTCCAAGA GACTGGCAGA GACTCATCAA CAACCATTGG GGGATTAGAC
CGAAAGCACT CAAATTCAAG ATATTCAATG TGCAAGTTAA AGAAGTCACG ACGCAAGACC
AGACAAAGAC TATTGCTAAC AACCTTACCT CTACAATCCA GATATTCACG GATAATGAAC
ACCAGCTGCC CTATGTTCTG GGCTCGGCCA CGGAGGGGAC GATGCCACCG TTCCCCTCAG
ATGTGTATGC CTTGCCCCAG TACGGCTACT GCACAATGCA CACCAACCAG AGTGGAGCTA
GATTCAATGA CAGAAGTGCC TTCTATTGCT TAG

SEQ ID NO: 44

ACGAATACTA TCCTAAAGCT AAAAAGGCCA AACAAGGCTT GCAGATACCA GCTCCACCTA
AAGGCGGAGA AGAAGAAGCT ACATCGTCAC AATCTGGAGG GAGCCCAGCA GGTTCCGATA
CTAGCGGCAC ATCTGTCATG GCTACAGGAG GAGGCGGTCC GATGGCAGAC GATAACCAGG
GCGCCGAGGG AGTGGGTAAT TCCTCAGGTG ATTGGCATTG CGATACCAAG TGGATGGGAG
ACCACGTCAT TACAAAGTCA ACCAGAACTT GGGTGCTCCC CACTTACGGG AATCATCTCT
ACGGGCCTAT CAACTTTGAC GGCACCACAG GTTCGGGTGC TAATGCAGCC TATGCAGGAT
ACAAGACTCC CTGGGGGTAC TTTGACTTCA ATCGATTCCA TTGCCACTTC TCCCCCCGAG
ACTGGCAAAG ACTCATCAAC AACCACACAG GCATCAGGCC GAAAGGACTC AAAATCAAAG
TCTTTAACGT CCAAGTCAAA GAAGTTACAA CACAAGATTC AACGAAAACA ATTGCCAACA
ATCTCACCAG CACCGTACAG ATCTTTGCGG ACGAGAACTA CGACTTACCA TATGTATTAG

SEQ ID NO: 142

TTGAACAACC CGACAACACG GCCGGGACCG GGGAGAAGCG TCCCGAACGC GTCGACGACT
TTTTCCCGAA AAAGAAGAAG GCCAAGACCG AGCAAGGCAA AGCCCCTGCT CAAACGGGCG
AAGACCCCGG AGAAGGAACC TCTTCCAACG CTGGATCAAG CGCCCCCTCT AGTGTGGGAT
CATCTGTCAT GGCTGAAGGA GGTGGCGGTC CAATGGGCGA TGCAGGCCAA GGTGCCGACG
GAGTGGGCAA TTCCTCGGGA AATTGGCATT GCGATTCCCA ATGGCTGGAC AACGGAGTCG
TTACCCGAAC CACTCGAACC TGGGTCCTGC CCAGCTACAA CAACCACTTG TACAAGCGGA
TCCAAGGACC GGGAGGAACC GACCCCAACA ATAAATTCTT TGGATTCAGC ACCCCCTGGG
GGTACTTTGA CTACAACCGA TTCCACTGCC ACTTCTCCCC CCGAGACTGG CAACGACTCA
TCAACAACAA CTGGGGCATC CGACCCAAAG CGATGCGCTT TAGACTCTTT AACATCCAGG
TTAAAGAAGT CACTGTCCAA GACTCCAACA CCACCATCGC CAACAACCTC ACCAGCACGG
TCCAAGTCTT TGCGGACAAG GACTACCAGC TGCCGTACGT CCTCGGATCG GCTACAGAGG
GCACCTTCCC GCCGTTCCCA GCGGATATCT ACACGATCCC GCAGTATGGT TACTGCACGC
TAA

EXAMPLES

[0171] The following examples exemplify the invention for AAV, especially for AAV2. Due to the general similarities within the structures of the adeno-associated viruses and other parvoviruses the invention can be easily transferred to other parvoviruses encoding 3 viral capsid proteins.

1. General Methods

1.1. Production of AAV (like particles) in insect cells

[0172] For production of AAV particles in Sf9 cells (cultivated in Graces (JHR Bioscience, USA)/ 10 % FCS) cells were transfected with the vector plasmid pVL_VP1_MOD4, pVL_VP2 or pVL_VP3, derivates of the pVL1393 Polyhedrin Promoter-Based Baculovirus Transfer Vector (BD Bioscience, San Jose, CA, USA) harboring a modified AAV VP1 open reading frame. (Cloning of pVL_VP1_MOD4, pVL_VP2 and pVL_VP3 is described in example 9)

[0173] Transfection was performed using the BaculoGold™ Transfection Kit according to manufacturer's manual (BD Bioscience, San Jose, CA, USA). Following transfection cells were incubated at 27°C. 5 days after transfection the supernatant was used for single clone separation via an end point dilution assay (EPDA). For that purpose Sf9 cells were cultivated in 96 well plates ($2x10^4$ cells/well) and infected with serial dilutions of the transfection supernatant. 7 days after incubation at 27°C the supernatant was transferred into a new 96 well plate (master plate) and stored at 2-8°C. The cells of the EPDA are lysed with sodium hydroxide, neutralized with sodium acetate and treated with Proteinase K. Following an Immune detection with the DIG-DNA wash and Block Buffer Kit (Roche, Mannheim, Germany) single clones could be detected.

[0174] To amplify single clones the according well from the master plate was used to infect Sf9 cells. Amplification of the recombinant Baculovirus was performed through several passages. Each passage was incubated for 3 days at 27°C prior of use of the supernatant to infect cells for the next passage. In the first passage $1.2x10^5$ Sf9 cells (12 well plates) were infected with 50µl of the supernatant out of the according well of the master plate. Supernatant was used to infect $2x10^6$ Sf9 (T25 Flask) (passage 1B). For passage $2,2x10^7$ Sf9 (T175 Flasks) were infected with 1 ml supernatant from passage 1B.

[0175] The virus titer of supernatant of passage 2 (P2) was analyzed via an end point dilution assay. To produce AAV $1x10^6$/well Sf9 (6 well plates) were infected with supernatant of P2 with a multiplicity of infection (MOI) of 1. Cultures were incubated at 27°C for 2-3 days. Cells were harvested and disrupted by a freeze and thaw process and analyzed for AAV production. AAV2 titer was analyzed using a commercially available AAV2 titration ELISA kit (Progen, Heidelberg, Germany) according to the manufacturer's manual.

1.2. Production of AAV (like particles) in mammalian cells

1.2.1. Plasmids

• Ad helper plasmid

[0176] An Ad helper plasmid encoding adenoviral proteins E2, E4 and VAI-VAII was used for AAV manufacturing in 293 or 293-T cells. The helper plasmid pUCAdE2/E4-VAI-VAII was constructed by subcloning the BamHI restriction fragment encoding the adenovirus (Ad) E2 and E4-ORF6 from pAdEasy-1 (Stratagene, La Jolla, USA) into the BamHI site of pUC19 (Fermentas, St. Leon-Rot, Germany). The resulting plasmid is referred to as pUCAdE2/E4. The VAI-VAII fragment from pAdVAntage™ (Promega, Mannheim, Germany) was amplified by PCR using the primers

XbaI-VAI-780-3': 5'-TCT AGA GGG CAC TCT TCC GTG GTC TGG TGG-3' (SEQ ID NO: 59), and

XbaI-VAII-1200-5': 5'-TCT AGA GCA AAA AAG GGG CTC GTC CCT GTT TCC-3' (SEQ ID NO: 60)

cloned into pTOPO (Invitrogen, Carlsbad, USA) and then subcloned into the XbaI site of pUCAdE2/E4. This plasmid was named pUCAdV.

• AAV encoding plasmids

[0177] The construction of pUCAV2 is described in detail in US 6,846,665. Plasmid pTAV2.0 is described in (Heilbronn et al., 1990), pVP3 is described in (Warrington et al., 2004). Further AAV viral protein encoding plasmids are described within the respective examples.

1.2.2. Transfection for large scale virus production

[0178] 293-T cells (ATCC, Manassas, USA) (7.5 x 10^6/dish) were seeded in 15 cm dishes (i.e. dish with a diameter of 15 cm) 24 h prior to transfection (cultivated in DMEM/10% FCS). Cells were transfected by calcium phosphate precipitation as described in US 2004/0053410.

[0179] In case of AAV promoter p40 dependent transcription a co-transfection with an adenoviral helper plasmid was performed. For co-transfection of the AAV encoding plasmid and pUCAdV a molar ratio of the plasmids of 1:1 was chosen. For transfection of one culture plate with 293-T cells the calcium phosphate transfection protocol was used as described above, 12 µg AAV Cap encoding plasmid (pUCAV2, pTAV2.0, and pVP3, respectively) and 24 µg pUCAdV were used. In case of p40 independent transcription cells were transfected with the respective AAV VP1, VP2 and/or VP3 encoding plasmid. For transfection of one culture plate of 293-T cells the calcium phosphate transfection protocol was used as disclosed in US 2004/0053410, 36 µg total DNA were mixed in 875 µl 270 mM $CaCl_2$. In brief, 875 µl 2x BBS (50 mM BES (N,N-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid) (pH 6.95), 280 mM NaCl and 1.5 mM Na_2HPO_4) was added to the mixture and the resulting solution was carefully mixed by pipetting. The solution was incubated for 20 min at room temperature (RT) and then added drop-wise to the cell culture plate. After 18 h incubation of cells in a humidified atmosphere at 35°C and 3% CO_2, medium was changed into a serum free DMEM (Invitrogen Carlsbad, USA) and cells were cultivated for an additional 3 d at 37°C, 5% CO_2 in a humidified atmosphere.

[0180] 293-T cells were harvested with a cell lifter, transferred into 50 ml plastic tubes (Falcon) and centrifuged at 3000g, 4°C for 10 min. The cell pellet was resuspended in 0.5 ml lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.5) per 15 cm dish and objected to three rounds of freeze and thaw cycles (liquid nitrogen/37°C). The cell lysate was cleared by two centrifugation steps (3700g, 4°C, 20 min) and the AAV-containing supernatant was used for further purification. Alternatively the whole dishes were objected to freeze and thaw cycles (-50°C/RT). The remaining supernatant was collected and further purified as described in 1.3.

1.2.3. Small scale transfection and preparation of virus supernatants

[0181] Cells (5 x 10^5/dish) were seeded in 6 cm dishes 24 h prior to transfection. 293-T cells were transfected by calcium phosphate precipitation as described in US 2004/0053410. For HeLa and COS-1 cells transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's manual. In case of promoter p40 dependent transcription of the c*ap* gene (pTAV2.0, derivates thereof, and pVP3) cells were infected with adenovirus type 5 (Ad5) (MOI=10). After additional incubation for 24-48 h, cells were harvested in the medium and lysed by three freeze-thaw cycles (-80°C and 37°C). Lysates were incubated at 56°C for 30 min to inactivate Ad5. Cell debris was removed by centrifugation at 10000g for 5 min.

1.2.4. Cell culture

[0182] HeLa and 293-T cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.

1.3. Purification

1.3.1 Tangential Cross Flow Filtration (TFF) and Benzonase treatment

[0183] After harvest the cleared cell culture medium was further concentrated using a Tangential Cross Flow Filtration Unit (Sartoflow Slice 200 Benchtop Crossflow System, Sartorius Biotech GmbH, Göttingen, Germany) using a 100 kDa cut off membrane (SARTOCON Slice 200). The resulting TFF concentrate was pooled with the supernatant (obtained as described in 1.2) and immediately treated with 100 U/ml benzonase (Merck, Darmstadt, Germany) at 37°C for 2 h. After benzonase treatment the cell lysate was cleared by centrifugation at 3700g, 4°C for 20 min. Cleared supernatant was purified using size exclusion chromatography (ÄKTA explorer system, GE Healthcare, Munich, Germany).

1.3.2 Size exclusion chromatography (SEC)

[0184] Cleared supernatant was separated through a Superdex 200 (prep grade) packed XK 50 chromatography column (250 mm in height and 50 mm in diameter; GE Healthcare, Munich, Germany). SEC fractions (5 ml each) were collected and the capsid titer was determined using the AAV2 capsid-specific A20 ELISA (Progen, Heidelberg, Germany, Cat. No: PRATV). SEC fractions containing AAV2 particles were pooled and further purified using iodixanol- or sucrose-density ultracentrifugation.

(i) Purification of AAV particles by density gradient centrifugation using iodixanol

[0185] The virus-containing SEC pool was transferred to Qickseal ultracentrifugation tubes (26x77 mm, Beckman Coulter, Marseille, France). Iodixanol solutions (purchased from Sigma, Deisenhofen, Germany) of different concentrations were layered beneath the virus containing lysate. By this an Iodixanol gradient was created composed of 6 ml 60% on the bottom, 5 ml 40%, 6 ml 25% and 9 ml 15% Iodixanol with the virus solution on top. The gradient was spun in an ultracentrifuge at 416000g for 1 h at 18°C. The 40% phase containing the AAV particles was then extracted with a canula by puncturing the tube underneath the 40% phase and allowing the solution to drip into a collecting tube until the 25% phase was reached.

(ii) Sucrose density gradient analysis

[0186] 1.5 x 10^6 cells were seeded in 10 cm dishes 24 h prior to transfection. They were harvested 48 h post transfection and lysed in 300 µl PBS-MK (phosphate-buffered saline: 18.4 mM Na_2HPO_4, 10.9 mM KH_2P0_4, 125 mM NaCl supplemented with 1 mM $MgCl_2$, 2.5 mM KCl) by five freeze-thaw cycles (-80°C and 37°C). After treatment with 50 U/ml Benzonase (Sigma, Deisenhofen, Germany) for 30 min at 37°C and centrifugation at 3700g for 20 min the supernatant was loaded onto a 11 ml 5-30% or 10-30% sucrose gradient (sucrose in PBS-MK, 10 mM EDTA, containing one tablet of complete mini EDTA free protease inhibitor (Roche, Mannheim, Germany)) in polyallomer centrifuge tubes (14 by 89 mm; Beckman Coulter, Marseille, France). After centrifugation at 160000g for 2 h at 4°C (SW41 rotor; Beckman), 500 µl fractions were collected from the bottom of the tubes. As reference empty AAV2 capsids (60 S) were analyzed in a separate gradient. For immuno dot blot assay 50 µl of heat denatured (99°C for 10 min) or non denatured aliquots of the fractions were transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using a vacuum blotter. Membranes were blocked for 1 h in PBS containing 10% skim milk powder and then incubated for 1 h with monoclonal antibodies B1 (Progen, Heidelberg, Germany, Cat. No: 65158) to detect denatured capsid proteins or A20 to detect non denatured capsids. Antibodies B1 and A20 were applied in 1:10 dilutions. Membranes were washed several times with PBS and incubated for 1 h with a peroxidase-coupled goat anti-mouse antibody (1:5000 dilution) (Dianova, Hamburg, Germany). Then, membranes were washed again and the antibody reaction was visualized using an enhanced chemiluminescence detection kit (Amersham, Braunschweig, Germany). For Western blot analysis 15 µl per fraction were processed for SDS-PAGE and then probed with monoclonal antibodies A69 (Progen, Heidelberg, Germany, Cat. No: 65157) or B1.

(iii) Purification of AAV particles by chromatography

Purification of empty wtVP3# and modified AAVLPs*

[0187] Indices # and * refer to slight differences in the purification protocol between wtAAV# and modified AAVLPs*. Buffer ingredients are marked correspondingly.

Cation exchange chromatography (AKTA explorer system)

[0188] Total lysate containing empty wtVP3# and modified AAVLPs* was obtained by performing three freeze thaw cycles (-54°C/37°C). Total lysate was cleared by centrifugation at 4100 rpm, 4°C, 20 min (MULTIFUGE L-R; Heraeus, Hanau, Germany). The pH of the resulting cleared supernatant was adjusted to 6. In addition, the conductivity of salt was reduced to approximately 10 mS/cm by adding sterile water.

[0189] A Fractogel EMD SO_3^- (M) chromatography column (100 mm in height; 15 mm in diameter, XK16, GE Healthcare, München, Germany) was packed and equilibrated using 5 CV running buffer consisting of 80 mM NaCl, 2% sucrose, 50 mM HEPES (pH 6.0), 2.5 mM $MgCl_2$.

[0190] After equilibration, cleared supernatant was separated through the Fractogel EMD $S0_3^-$ (M) packed chromatography column (flow rate 10 ml/min). After separation, column was washed using 5 CV running buffer mentioned above. Bound particles (wtVP3 or modified AAVLPs) were effectively eluted at a sodium chloride concentration of 350 mM (peak 1≈45ml).

Buffer exchange (AKTA explorer system)

[0191] To adjust the pH and the salt concentration of the eluted proteins (peak 1) for successive anion exchange chromatography, buffer exchange was performed using a Sephadex G25 packed chromatography column (500 mm in height; 15 mm in diameter, XK26, GE Healthcare, München, Germany) (flow rate 10 ml/min). After column equilibration using 3 CV SOURCE 15Q running buffer consisting of 25 mM Tris (pH 8.2), 150 mM NaCl#/100 mM NaCl*, 2.5 mM $MgCl_2$ peak 1 was separated through the column. Protein fraction (≈120ml) was collected.

Anion exchange chromatography (AKTA explorer system)

[0192] A SOURCE 15Q chromatography column (80 mm in height; 15 mm in diameter, XK16, GE Healthcare, München, Germany) was equilibrated using 5 CV SOURCE 15Q running buffer consisting of 25 mM Tris (pH 8.2), 150 mM NaCl#/100 mM NaCl*, 2.5 mM $MgCl_2$. After equilibration, the protein fraction obtained after buffer exchange (appr. 120 ml) was loaded and separated through the chromatography column (flow rate 10 ml/min). Flow-through containing 90% of the particles (appr. 120 ml) was collected.

Particle concentration using centrifugal filter devices

[0193] Flow-through containing wtVP3# or modified AAVLPs* was concentrated using Centricon

Plus-70 (cut off 100 kDa) centrifugal filter devices (Millipore). Concentration was carried out using a swinging-bucket rotor (MULIFUGE L-R; Heraeus, Hanau, Germany) at 3500g, 20°C for 15 min. Resulting concentrate (appr. 45 ml) was immediately separated through a size exclusion chromatography.

Size exclusion chromatography (ÄKTA explorer system)

[0194] A Superdex 200 (prep grade) chromatography column (500 mm in height; 50 mm in diameter, XK50, GE Healthcare, München, Germany) was packed and equilibrated using 2 CV running buffer consisting of 200 mM NaCl, 2% sucrose, 50 mM HEPES (pH 6.0), 2.5 mM $MgCl_2$. The concentrate mentioned above (appr. 45 ml) was separated through the column (fow-rate 10 ml/min). Particles eluted first (SEC fraction no. 1-13; each 5 ml). SEC fractions with a particle purity of greater than 95% were pooled, sterile filtered (0.2 µm) (Minisart; Sartoriusstedim) and stored at -84°C.

1.4. Analysis of protein expression by Western blot

[0195] Identical portions of harvested cells or identical amounts of purified particles were processed for SDS-PAGE. Protein expression was analyzed by Western blot assay using monoclonal antibodies A69, B1 (Progen, Heidelberg, Germany), anti-AU1 (Covance, Emeryville, USA), anti-GFP (clone B-2; Santa Cruz Biotechnology, Santa Cruz, USA) or polyclonal antibody anti-AAP (see 1.7.) as described previously (Wistuba et al., 1995). Variations of the protocols are indicated within the description of the respective examples.

1.5. Titer analysis

[0196] Capsid titers were determined using a commercially available AAV2 titration ELISA kit (Progen, Heidelberg, Germany Cat. No: PRATV) or the respective AAV1 titration ELISA kit (Progen, Heidelberg, Germany Cat. No: PRAAV1) according to the manufacturer's manual.

1.6. Immunofluorescence analysis

[0197] For immunofluorescence analysis HeLa cells were cultivated for 24 h on coverslips, transfected and in case of promoter p40 dependent transcription of the cap gene (pTAV2.0 and pVP3) infected with Ad5 (MOI=4). After 20-48 h cells were fixed with 100% methanol (10 min, -20°C) and washed with PBS (phosphate-buffered saline: 18.4 mM Na_2HPO_4, 10.9 mM KH_2PO_4, 125 mM NaCl). Incubation with primary antibodies was performed for 1 h at RT or over night at 4°C. As primary antibodies hybridoma supernatants A20 or A69 were used to detect assembled capsids or VP2 respectively. A20 and A69 were used undiluted (Progen, Heidelberg, Germany). For detection of unassembled capsids a rabbit polyclonal serum was used in a 1:500 dilution to

label all three free VP proteins. Coverslips were washed three times with PBS and thereafter incubated with appropriate secondary antibodies (Cy 3 labeled goat anti mouse in 1:400 dilution or FITC labeled goat anti rabbit 1:150 purchased from Dianova, Hamburg, Germany or Molecular Probes, Leiden, The Netherlands) for 1 h at RT. Coverslips were washed again, dipped into 100% ethanol and embedded in Permafluor mounting medium (Beckman Coulter, Marseille, France). Confocal images (0.3 µm sections) were obtained with a Leica TCS SP2 laser scanning microscope and further processed using Adobe Photoshop CS software. Variations of the protocols are indicated within the description of the respective examples.

[0198] To visualize GFP expression, cells were fixed with 2% paraformaldehyde for 15 min, quenched twice with 50 mM NH_4Cl for 5 min, and permeabilized with 0.2% Triton X-100 for 10 min.

1.7. Preparation of polyclonal antibody

[0199] The polyclonal AAP antiserum (anti-AAP) was generated by immunization of a guinea pig with a peptide comprising the sequence GKDSSTTTGDSDPRDSTS (SEQ ID NO: 61) conjugated to KLH (Keyhole Limpet Hemocyanin) following standard procedures.

1.8. Negative staining of virus particles for electron microscopy

[0200] For electron microscopy according to (Grimm et al., 1999, Grimm and Kleinschmidt, 1999, Mittereder et al., 1996), negative staining of virus particles was performed as described in detail below.

[0201] Five µl of sample (about $5x10^{10}$ virus particles) were applied onto the freshly air-glow discharged carbon coated side of a grid and incubated for 2 min. Excess solution was removed by blotting the edge of the grid onto Whatman filter paper. To avoid salt precipitates, the grid was washed with 3 drops of water followed by four drops of 2% (w/v) uranyl acetate solution. The last droplet of staining solution was allowed to sit on the grid for 5 min before blotting and air drying. Electron micrographs were taken with a Morgagni 268D FEI microscope at 100 kV.

2. Analysis of VLP formation by N-terminal deletion analysis of VP2

[0202] Our as well as previous studies (compare above) reported a lack of capsid assembly when VP3 is expressed from constructs comprising the cds of VP3 alone. Since expression of VP3 is not sufficient for VLP formation, we tried to identify further sequences which could overcome this defect. In this experiment we checked whether a sequence upstream of the VP3 cds was necessary for VLP formation. If yes, the sequence should be characterized.

2.1. Cloning of deletion mutants

[0203] Plasmids pTAV2.0 (Heilbronn et al., 1990), pVP3 (Warrington et al., 2004), pCMV-VP (Wistuba et al., 1997) and pKEX-VP3 (Ruffing et al., 1992) have been described previously. The deletion mutants pCMV-VP3/1882, pCMV-VP3/2193, pCMV-VP3/2596, pCMV-VP3/2611, pCMV-VP3/2696, pCMV-VP3/2765 and pCMV-VP3/2809 were cloned from plasmid pVP3. Numbers behind the name of the pCMV-VP3 plasmid indicate the nucleotide position in the AAV2 genome according to Ruffing et al. (1994). Constructs are schematically shown in Fig. 5A.

[0204] For cloning of deletion mutants, the HindIII/BsiWI fragment of pVP3 (with mutated VP1 and VP2 translation start codons) was subcloned into the HindIII/BsiVVI backbone of pCMV-VP resulting in the construct pCMV-VP3/1882 (Fig. 5). Constructs pCMV-VP3/2193 and pCMV-VP3/2596 were generated by subcloning of the DraI/BsiVVI or the EcoNI (blunted)/BsiVVI fragment from pVP3 into the HindIII (blunted)/BsiVVI backbone of pCMV-VP (EcoNI and HindIII sites were blunted by digestion of the single stranded overhang) (the position of the different restriction sites used for cloning relative to the genomic sequence is shown in Fig. 4). For further deletions pVP3 was used as a template for site-directed mutagenesis reactions. Mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's manual. For each mutation, two complementary PCR primers were designed to generate a new HindIII restriction site at the designated area. Primer sequences:

5'-CCTCTGGTCTGGGAACTAAGCTTATGGCTACAGGCAGTGGCG-3' (SEQ ID NO: 62)

5'-CGCCACTGCCTGTAGCCATAAGCTTAGTTCCCAGACCAGAGG-3' (SEQ ID NO: 63)

HindIII/BsiVVI fragments from mutated plasmids were then subcloned into the HindIII/BsiVVI backbone of pCMV-VP resulting in constructs pCMV-VP3/2611, pCMV-VP3/2696, pCMV-VP3/2765 and pCMV-VP3/2809 (Fig. 5A).

2.2. Analyses of constructs by Western blot and ELISA

[0205] For analysis of protein expression identical portions of harvested cells were processed for SDS-PAGE.

[0206] As shown in Fig. 5B transfection of 293-T cells with all constructs listed in Fig. 5A except pTAV2.0 (wt AAV) and pCMV-VP resulted in expression of only VP3 when analyzed by Western blotting using antibody B1 which reacts with all three capsid proteins. In contrast cells transfected with pTAV2.0 (wt AAV) or pCMV-VP, a plasmid in which the corresponding translation start sites were not mutated, VP1 and VP2 were well detected in addition to VP3. Antibody B1 reacted with two polypeptide bands migrating slower than VP3 e.g. for mutated plasmids pKEX-VP3, pCMV-VP3/2765 and pCMV-VP3/2809. At least for plasmids pKEX-VP3 and pCMV-VP3/2809 the corresponding polypeptides can not contain VP1 or VP2 amino acid sequences since the nucleotide sequences coding for VP1 or VP2 were completely deleted. Moreover, VP1 and VP2 could not be detected upon expression of all three mutant plasmids, using the antibody A69. Hence, the presence of VP1 and VP2 in these samples could clearly be excluded. We concluded that the two polypeptide bands migrating slower than VP3 were a consequence of higher VP3 levels, which were not completely denatured.

[0207] When, however, extracts of cells transfected with pVP3 were probed with antibody A69 which detects only VP1 and VP2, thus omitting the reaction with the abundant VP3, one could detect faint bands in the region of VP1 and VP2 which were absent in extracts of cells transfected with pKEX-VP3. This result suggests that transfection of the pVP3 construct leads to the expression of small amounts of VP1 and VP2 or VP1- and VP2-like proteins. They are possibly translated from alternative translation initiation codons or by unscheduled initiation at the mutated VP1 and VP2 translation initiation sites.

[0208] Antibody A69 revealed in all deletion mutants of pVP3 up to pCMV-VP3/2696 one or several polypeptide band(s), only Western blots with extracts of cells transfected with pCMV-VP3/2765 and pCMV-VP3/2809 showed no reaction with A69 because the antibody epitope was already deleted in these proteins.

[0209] Capsid assembly was confirmed by an antibody A20 based capsid ELISA (Fig. 5C). In contrast, expression of VP3 by pKEX-VP3 did not yield detectable amounts of capsids (Fig. 5C), although the amount of expressed VP3 protein was even higher compared to pVP3 (Fig. 5B).

[0210] In agreement with our previous results, expression of VP3 alone by transfecting pCMV-VP3/2809 - which is equivalent to pKEX-VP3 - did not lead to detectable capsid formation (Fig. 5C). The formation of capsids which might not react with the A20 ELISA was excluded by analysis of cell extracts on sucrose gradients followed by Western blotting with the B1 antibody (data not shown). Interestingly, analyzing the capsid assembly efficiency of the different deletion mutants it was detected that the capsid assembly efficiency increased from one deletion mutant to the next, before decreasing upon a certain extent of deletion. Peak efficiencies in capsid assembly were seen for mutants pCMV-VP3/2596 and pCMV-VP3/2611 (Fig. 5A and C).

2.3 Conclusion

[0211] This result shows a clear correlation between the presence of N-terminally extended VP3 sequence (due to the presence of DNA sequence upstream of the VP3 start codon) and capsid assembly. We identified a DNA sequence of about 44 nucleotides upstream of the VP3 cds that has to be present in addition to the VP3 cds for VP3 VLP formation. This 44 nt confers to construct pCMV-VP3/2765 which still is able to cause capsid assembly.

[0212] The presence of some more DNA sequence upstream of the 2765' site increases efficiency of capsid assembly which is in line with ORF2 starting at nucleotide position 2717 and the putative start of the full-length AAP possibly located between nucleotide 2717 and 2765.

3. Sequence fragment of the *cap* gene is able to induce capsid assembly *in trans*

[0213] In example 2, we identified some sequence upstream of the VP3 start codon (comprised by fragment Z) that has to be present in addition to the VP3 cds for particle formation. To prove the hypothesis that the product of fragment Z functions transiently and *in trans,* we tested whether a

capsid sequence fragment comprising the EcoNI/BsiVVI restriction fragment fused to the cds of GFP can rescue the capsid assembly deficiency of VP3.

3.1. Cloning of pVP2N-gfp for trans-complementation

[0214] For generation of construct pVP2N-gfp, EcoNI and BsiVVI restriction sites were introduced into the multiple cloning site of the vector pEGFP-N1 (BD Biosciences, Erembodegem, Belgium). Afterwards the EcoNI/BsiVVI fragment from pTAV2.0 (position of restriction sites is given in Fig. 4) was inserted downstream of a CMV promoter and upstream of the GFP cds and its poly(A) signal. Expression of this fusion construct pVP2N-gfp results in three transcripts VP2N-gfp, VP3N-gfp and GFP, depending on the initiation of transcription at one of the three existing start codons for VP2, VP3 or GFP as schematically shown in Fig. 6A.

[0215] A number of derivates containing e.g. codon modifications or stop codons originated from pVP2N-gfp as schematically indicated in the respective figures. They always include the GFP cds and were named accordingly (with the addition -gfp). To simplify matters this appendix (-gfp) is missing to names of the respective constructs in some figures (e.g. Figs. 20, 22, 23).

3.2. Analysis of functional substitution *in trans*

[0216] The following experiments were performed in HeLa cells. Plasmids pCMV-VP3/2809 and pVP2N-gfp were co-transfected in different molar ratios and analyzed for gene expression and capsid assembly (Fig. 6). While Western blot analysis confirmed that the amount of VP3 was the same in cell extracts transfected in each molar ratio of the two plasmids (detection with antibody B1, Fig. 6B upper part), VP2N-gfp (detection with antibody A69, Fig. 6B lower part) could only be detected after transfection in a 1:1 or 1:1/5 ratio, respectively. In the 1:1 or 1:1/5 ratio, antibody anti-gfp (Fig. 6B, middle) additionally detects all three transcripts resulting from expression of the fusion construct pVP2N-gfp as schematically shown in Fig. 6A, namely VP2N-gfp, VP3N-gfp and GFP. Due to the strong start codon of VP3 and corresponding to the *in vivo* situation the transcript of VP3N-gfp dominates. Surprisingly, capsid assembly could be observed by immunofluorescence up to a pCMV-VP3/2809 to GFP-fusion-plasmid ratio of 1:1/50 (Fig. 6C). Quantification of capsid formation using the antibody A20 based capsid ELISA showed that capsid formation of mutant pCMV-VP3/2809 supplemented with pVP2N-gfp was similarly efficient as mutant pCMV-VP3/2696 where the N terminally extended VP3 was co-expressed (Fig. 6D).

3.3. Conclusion

[0217] This result shows that presence of an EcoNI-BsiVVI restriction fragment of the c*ap* gene *in trans* rescues capsid assembly of constructs expressing VP3 as only capsid protein. Since assembly could be detected even at a 50-fold reduced amount of pVP2N-gfp plasmid co-transfected, a substoichiometric action of the helper factor for VP3 capsid assembly can be assumed.

4. C-terminally truncated VP2 proteins are expressed in substoichometric amounts and become incorporated into capsids

[0218] Here it was investigated if the generated AAV like particles consist of VP3 only. Empty particles were produced from plasmid pCMV-VP3/2696 or in a trans-complementation assay of cotransfection of pCMV-VP/2809 and pVP2N-GFP Particles were purified via sucrose cushion according to Steinbach et al. (1997) with modification described by Kronenberg et al. (2001) and with the modification that the 293 cells were transfected without adenoviral infection and cells were harvested after 48h. Incorporation of truncated VP2 protein was analyzed by Western blot (Fig.7).

[0219] pVP2N-GFP could not be detected within maximal loading of $5x10^{11}$ particles. But transfecting pCMV-VP3/2696 an A69 signal was detected which shows that a truncated VP2 is incorporated into the capsids substoichiometrically.

4.1. Result

[0220] In conclusion VP3 only particles are generated within the *trans* situation. In contrast in the c*is* situation a truncated VP2 is incorporated substochiometrically. From Western blot the signal intensity of VP1 from $2x10^9$ wt AAV particles is about the same as the signal from $1x10^{11}$ particles generated from pCMV-VP3/2696. This means the amount of truncated VP2 is about 50 fold lower than the amount of VP1. Assuming a stoichiometric ratio of VP1:VP2:VP3 of 1:1:10 within a wt capsid there is approximately 500-fold less truncated VP2 than VP3. Since one capsid is composed of 60 VP subunits also capsids must exist that are composed of VP3 only.

4.2. Conclusion

[0221] This result strengthens the conclusion that the truncated VP2 protein itself is not required for the capsid itself.

5. Codon modification of the construct used for trans-complementation can inhibit the trans-complementation process

[0222] To investigate the nature of the trans-complementing agent of the fragment Z, the VP2N part (part between restriction sites EcoNI and BsiWI) within pVP2N-gfp was codon modified. That means the DNA sequence was altered without changing the amino acid sequence of the first ORF. Codon modification was performed by GENEART (Regensburg, Germany). Codons were modified for codons preferentially used in mammalian cells. Sequence is shown in Figure 8A. Identity of the DNA sequence of pVP2N-gfp versus pVP2N-gfp codon modified (cm, pVP2Ncm-gfp) is 71% while protein identity is 100%.

[0223] Protein expression of pVP2Ncm-gfp was compared in Western blot analysis (Fig. 8B) and by immunoflourescence within transfected 293 cells (Fig. 8C). The ability to rescue capsid formation of pCMV-VP3/2809 was tested in trans-complementation assays as described in example 3 (Fig. 8D). Plasmids were cotransfected in a molar ratio of 1:1.

Result and conclusion

[0224] Western blot showed that the protein expression from the codon modified construct (pVP2Ncm-gfp) was even higher than protein expression from the non-modified construct (pVP2N-gfp), not unexpected since the codon modification was optimized for mammalian cells (Fig. 8B). Also the localization within the cells of the codon modified protein did not differ from the non-modified protein (Fig. 8C). Surprisingly the pVP2Ncm-gfp lost its ability to rescue capsid formation of pCMV-VP3/2809 (Fig. 8D).

[0225] To exclude a negative effect of the large amounts of pVP2Ncm-gfp protein on capsid assembly, we co-transfected the codon modified pVP2Ncm-gfp with pCMV-VP3/2696. In this combination capsid assembly was normal, showing that the assembly activity was not suppressed by the high amount of pVP2Ncm-gfp (data not shown). Also expression of lower amounts of pVP2Ncm-gfp by transfection of reduced amounts of plasmid pVP2Ncm-gfp together with pCMV-VP3/2809 did not rescue capsid assembly (data not shown).

[0226] This result strengthens the hypothesis that no protein translated from the first ORF is responsible for the trans-complementing activity.

6. Insertion of stop codons into the construct used for trans-complementation does not inhibit the trans-complementation process

[0227] To further analyze the nature of the trans-complementing agent stop codons were inserted within the EcoNI-BsiWI restriction fragment. To insert Stop codons point mutations were performed.

6.1. Insertion of Stop Codons into pVP2N-gfp

[0228] For construction of pVP2N/stopA-gfp (also named pVP2N/ORF1stopA-gfp), pVP2N/stopB-gfp (identical to pVP2N/ORF1stopB-gfp), pVP2N/stopC-gfp (also named pVP2N/ORF1stopC-gfp) and pVP2N/stopD-gfp (identical to pVP2N/ORF1stopD-gfp) site-directed mutagenesis reactions (QuickChange site-directed mutagenesis kit, Stratagene) were performed using template pVP2N-gfp and two complementary PCR primers which included the desired substitutions. In each case the EcoNI/BsiVVI fragment was then cloned into the EcoNI/BsiVVI backbone of pVP2N-gfp.

[0229] For generation of StopA cytosine at nucleotide position 2770 were substituted to thymine resulting in a tag stop codon. For generation of StopB adenine at nucleotide position 2797 was substituted to thymine resulting in a tga stop codon. Stop C was generated by substituting adenine

at nucleotide position 2821 to thymine and thymine at position 2823 to adenine, resulting in a tga stop codon. Stop D was created by substituting guanine at nucleotide position 2878 to thymine resulting in a tga stop codon. Positions are according to Ruffing et al. (1994).

[0230] Primer pairs used for insertion of Stop codons at four different sites within the pVP2N-gfp

StopA 5'-CCA GCC TCT CGG ATA GCC ACC AGC AGC C-3' (SEQ ID NO: 64)

i-StopA 5'-GGC TGC TGG TGG CTA TCC GAG AGG CTG G-3' (SEQ ID NO: 65)

StopB 5'-GCC CCC TCT GGT CTG TGA ACT AAT ACG ATG GC-3' (SEQ ID NO: 66)

i-StopB 5'-GCC ATC GTA TTA GTT CAC AGA CCA GAG GGG GC-3' (SEQ ID NO: 67)

StopC 5'-CGA TGG CTA CAG GCT GAG GCG CAC CAA TGG C-3' (SEQ ID NO: 68)

i-StopC 5'-GCC ATT GGT GCG CCT CAG CCT GTA GCC ATC G-3' (SEQ ID NO: 69)

StopD 5'-GGA GTG GGT AAT TCC TCG TGA AAT TGG CAT TGC G-3' (SEQ ID NO: 70)

i-StopD 5'-CGC AAT GCC AAT TTC ACG AGG AAT TAC CCA CTC C-3' (SEQ ID NO: 71)

[0231] Schematic presentation of the inserted stop codons is depicted in Fig. 9A. In pVP2N/stopA-gfp nucleotide C_{2770} has been mutated into t, therefore the cag-codon encoding glutamine is changed into tag (silent mutation in ORF2), in pVP2N/stopB-gfp nucleotide g_{2797} has been mutated into t, hence the gga-codon encoding glycine is changed into tga (Trp → Cys mutation in ORF2), in pVP2N/stopC-gfp nucleotide a_{2821} has been mutated into t (silent in ORF2) and nucleotide t_{2823} has been mutated into a, therefore the agt-codon encoding serine is changed into tga (Val → Glu mutation in ORF2), and in pVP2N/stopD-gfp nucleotide g_{2878} has been mutated into t, hence the gga-codon encoding glycine is changed into tga (silent in ORF2). Positions are according to Ruffing et al. (1994). All substitutions do not disrupt ORF2. The resulting pVP2N-gfp stop constructs were used for trans-complementation of the construct pCMV-VP3/2809 as described in example 3. Plasmids pCMV-VP3/2809 and the respective pVP2N/stop-gfp construct were cotransfected in a molar ratio of 1:1.

[0232] Further protein expression of the Stop constructs was tested by Western blot analysis using the A69 antibody.

6.2. Result and conclusion

[0233] Western blot analysis confirmed that VP3 is expressed in all samples (detected by monoclonal antibody B1 in Fig. 9B). As expected Bluescript vector (pBS) did not cause capsid assembly in the trans-complementation assay and therefore served as a negative control (Fig. 9C). Interestingly, although no protein expression was detected for the pVP2N/stop-gfp constructs in contrast to the pVP2N-gfp construct (Fig. 9B), the insertion of stop codons did not influence the

trans-complementing activity of the EcoNI-BsiVVI restriction fragment of the cap gene. VP3 particles could easily be assembled (Fig. 9C). The reduction in capsid titers obtained with mutants pVP2N/stopB-gfp and pVP2N/stopC-gfp could be due to the nucleotide changes introduced by generating the respective mutations (stopB in ORF1 led to a Trp→Cys mutation in ORF2, stopC in ORF1 led to a Val→Glu mutation in ORF2). These experiments together show that the nucleic acid sequence of the EcoNI-BsiVVI fragment is the basis for the capsid assembly helper activity and not an expressed protein from the first ORF, since all mutants contain stop codons in the first ORF. Although the substitutions resulting in stop codons in ORF 1 did not stop amino acid synthesis of AAP from ORF2, differences in capsid titers indicated that the functionality of AAP was influenced.

7. The postulated NLS is not necessary for VLP formation

[0234] While mutant pCMV-VP3/2696 formed high capsid levels, the slightly shorter mutant pCMV-VP3/2765 assembled to clearly reduced amounts of capsids (Fig. 5C). This shorter mutant had lost a group of AA which had been suggested to function as a NLS for AAV VP2 proteins (Hoque et al., 1999a) and showed reduced nuclear transport of the VP protein (Fig. 10) To test whether the postulated NLS is responsible for this difference, we substituted the respective sequence element by converting the RKR peptide (AA 168-170) into AAA in the construct pCMV-VP3/2696 in order to destroy the proposed NLS activity by site directed mutagenesis according to standard procedures using two complementary PCR primers which included the desired substitutions. Primers used for substitution of RKR by AAA:

BC3^{-}ala forward:	5'-GGC GGG CCA GCA GCC TGC AGC AGC AGC ATT GAA TTT TGG TCA GAC TGG-3' (SEQ ID NO: 72)
BC3^{-}ala reverse:	5'-CCA GTC TGA CCA AAA TTC AAT GCT GCT GCT GCA GGC TGC TGG CCC GCC-3' (SEQ ID NO: 73)

[0235] Immunofluorescence of transfected HeLa cells with the A20 antibody (Fig. 10) and the capsid ELISA (data not shown) showed that the VP protein of mutant pCMV-VP3/2696RKR168-170AAA was as active in capsid assembly as wt AAV.

[0236] This supports the interpretation that the sequence element comprising RKR168-170 does not act as a NLS in this context and might play a different role in capsid assembly.

8. Nuclear localization (and N terminal extension) of VP proteins is not sufficient for capsid assembly

[0237] It has been reported that fusion of an NLS derived from the SV40 large T antigen to VP3 translocates VP3 into the nucleus and leads to capsid assembly (Hoque et al., 1999a). We

repeated this experiment and observed efficient nuclear accumulation of VP3 protein, however, there was no capsid assembly detectable with antibody A20 (Figs. 11A, 11B and 15B).

[0238] Further, a heterologous N terminal extension upstream of VP3 (HSA) was tested to restore assembly competence to VP3.

[0239] Further several constructs were transfected in 293 cells to compare protein expression and assembly efficiency.

8.1. Cloning of constructs

[0240] pCI-VP, pCI-VP2 and pCI-VP3 were cloned by PCR amplification of the respective VP coding regions using primer with XhoI (5'-) and NotI (3'-) overhangs and subcloning of the XhoI-/NotI-digested PCR products into the XhoI-/NotI-digested vector pCI (PROMEGA). In case of pCI-VP2, the start codon for VP2 was changed from ACG to ATG at the same time

[0241] Cloning of the construct pCMV-NLS-VP3 was carried out by site-directed mutagenesis reaction using the construct pCMV-VP3/2809 as template and the complementary PCR primers
5'-GGAAT TCGAT ATCAA GCTTG CCATG GCACC ACCAA AGAAG AAGCG AAAGG TTATG GCTAC AGGCA GTGG-3' (SEQ ID NO: 74) and 5'-CCACT GCCTG TAGCC ATAAC CTTTC GCTTC TTCTT TGGTG GTGCC ATGGC AAGCT TGATA TCGAA TTCC-3' (SEQ ID NO: 75).

[0242] Then the HindIII/BsiWI fragment was subcloned from the amplicon into the HindIII/BsiWI backbone of pCMV-VP3/2809. The cap gene product NLS-VP3 contains the amino acid sequence of SV40 NLS MAPPKKKRKV at the N-terminus of VP3.

[0243] The construct pCMV-HSA-VP3 is also based on pCMV-VP3/2809 and contains a nucleic acid sequence coding for amino acids 25-58 of human serum albumin (HSA) directly upstream of the VP3 cds. Fragment
5'-GGTAC CAAGC TTACG GACGC CCACA AGAGC GAGGT GGCCC ACCGG TTCAA GGACC TGGGC GAGGA AAACT TCAAG GCCCT GGTGC TGATC GCCTT CGCCC AGTAC CTGCA GCAGT GCAAG CTTGA GCTC-3' (SEQ ID NO: 76)
(with a HindIII restriction site at both ends) was obtained via gene synthesis (Geneart, Regensburg, Germany). After HindIII digestion of the corresponding vector the resulting 111 bp fragment was subcloned into the HindIII linearized pCMV-VP3/2809 backbone. Translation of VP3 is initiated at a standard ATG start codon whereas translation of HSA-VP3 (with 37 Aas elongation at VP3 N-terminus) is initiated at an ACG start codon.

8.2. Analyses of constructs by immunofluorescence and sucrose gradient

[0244] We transfected HeLa cells with the different constructs: pCMV-NLS-VP3 or pCMV-VP3/2809 either alone or in a co-transfection with pVP2N-GFP. Further pCMV-HSA-VP3 was transfected. Expression of capsid proteins and formation of capsids was analyzed by immunofluorescence as

described above using a polyclonal VP antiserum or the monoclonal A20 antibody. Further capsid formation was analyzed within following a sucrose gradient.

Results

[0245] Just as Hoque et al. (1999a) and comparable to the wildtype (wt) and the proteins expressed from the N-terminally truncated construct pCMV/2696, we could express VP3 from the construct pCMV-NLS-VP3 and observed efficient nuclear accumulation of VP3 protein. However, in contrast to the wt and the N-terminally truncated construct pCMV/2696 we could not detect capsid assembly using the antibody A20 (Fig. 11B).

[0246] As expected, expression of the VP3 protein with a prolonged N-terminus consisting of 36 AA of human serum albumin (HSA-VP3), equivalent in length to the VP3 N-terminal extension of mutant pCMV-VP3/2696 could be detected by antibody staining (Fig. 11B). In comparison to the expression product of pCMV-NLS-VP3 those of the mutant pCMV-HSA-VP3 showed a much higher fraction of cytoplasmic staining. Again, we could not detect capsid assembly using the antibody A20 (Fig. 11B).

[0247] Co-transfection of pVP2N-gfp induced capsid assembly, readily detectable by antibody A20 (Fig. 11B).

[0248] Analysis of possible assembly products - not reacting with the A20 antibody - by sucrose density gradient sedimentation showed very low amounts of VP protein containing material (sedimenting over the whole range of the gradient) which reacted with antibody B1 (Fig. 11C). This indicates the formation of incorrectly assembled or aggregated VP protein in rather low, hardly detectable quantities.

8.3. Analyses of constructs by Western blot and ELISA

[0249] A set of different constructs was analyzed for gene expression in Western Blot and in ELISA for capsid assembly (Fig. 15A):

- pCI-VP2: The VP2 sequence of AAV2 was cloned into the multiple cloning site of pCI (Promega, Mannheim, Germany). The VP2 start codon ACG was changed into an ATG.
- pCI-VP3: The wildtype VP3 sequence was cloned into pCI.
- pCI-VP: The complete cap ORF was cloned into pCI. The start codons of VP2 and VP3 were not mutated.
- pCMV-NLS-VP3: (described in example 8 and by Hoque et al. (1999a))
- pCI-VP2mutACG: This is a modification of the pCI VP2: the VP2 start-codon is destroyed and replaced by a GAG codon
- pCMV-VP3/2696 (described in example 2)

Results

[0250] Western Blot analysis showed similar capsid protein expression of the different constructs with the expected size of the VP proteins (Fig. 15C). The efficiency in capsid assembly however was quite different (Fig. 15B). Particle titer obtained with the construct cloned analogue to Hoque et al (pCMV-NLS-VP3) was below detection limit. That also means that the favorised constructs pCI-VP2mutACG or pCMV-VP3/2696 are more than 3 log more efficient in VP3 particle formation efficiency when compared to the Hoque construct pCMV-NLS-VP3. The construct pCI-VP2 corresponds to pCMV-VP3/2611 except for a mutation of the minor ACG start codon to an ATG in pCI-VP2 whereas the ACG codon is completely deleted in pCMV-VP3/2611. Capsid formation efficiency of the pCI-VP2 construct is strongly reduced (Fig. 15B). We did not analyze whether the particles obtained from pCI-VP2 are mainly composed of VP2, VP3 or a mixture of both proteins. Fig. 15C shows that VP3 is still expressed from this construct even though with significantly (about 10 fold) lower efficiency compared to VP2. We hypothesize that the particles obtained mainly consist of VP3. The low titer is explained by i) 10-fold reduced amounts of VP3 from pCI-VP2 compared to pCMV-VP3/2611. Furthermore, we speculate that the ATG start codon in pCI-VP2 interferes with AAP expression as the ATG probably dominates the non-canonical start codon of AAP. pCI-VP3 showed only low capsid formation efficiency as expected. Efficiency of particle assembly could partially be rescued by co-transfection of pCI-VP3 with pCI-VP2 (Fig. 15B) in a ratio of 10:1. However, the overall particle formation is still reduced by 1-2 log compared to pCI-VP2mutACG or pCMV-VP3/2696 supporting our hypothesis that the ATG start codon in the VP2 coding region of pCI-VP2 interferes with AAP expression. Particle formation from pCI-VP is much lower when compared to pCMV-VP (Fig. 5). This is explained as follows: pCI-VP differs from pCMV-VP by lack of the splice donor site. Therefore, only one messenger RNA is transcribed from pCI-VP expressing mainly VP1, whereas two messenger RNAs are transcribed from pCMV-VP. The minor transcript mainly expresses VP1, whereas the major transcript encodes VP2 and VP3 in a ration of 1:8. Therefore, pCMV-VP expresses VP1:VP2:VP3 in the expected ratio of 1:1:8, whereas VP2 and VP3 can hardly if at all be detected with construct pCI-VP.

Conclusion

[0251] The results show that nuclear accumulation of VP3 alone is not sufficient for capsid assembly and that a heterologous N-terminal extension upstream of VP3 is not able to bring about assembly competence to VP3.

[0252] Further our favored constructs pCI-VP2mutACG or pCMV-VP3/2696 lead to more than 3 log higher VP3 particle titers when compared to the NLS-VP3 fusion construct described by Hoque et al. (1999a). These experiments also demonstrate that VP3 N-terminal fusion constructs can assemble into VLPs. Therefore I-203 is a suitable insertion site for foreign peptide sequences.

9. VP3 capsid assembly can be achieved in insect cells

9.1. Cloning of the VP1 mutant "Modification 4"

[0253] The construct pVL_VP1_MOD4 was generated to produce viral particles consisting essentially of the capsid protein VP3 in the absence of any Rep expression.

[0254] In detail, pUC19AV2 (described in detail in US 6,846,665) was used as template to amplify VP1 according to standard PCR conditions in the presence of the following primers:

Insect_mod_4_s:	5'-CAC CCG CGG GGA TCC **GCC** GCT GCC GAC GGT TAT CTA CCC GAT TGG CTC-3' (SEQ ID NO: 77), and
E_VP2_rev:	5'-CGC GAA TTC CTA **TTA** CAG ATT ACG AGT CAG G-3' (SEQ ID NO: 78)

[0255] Thereby, the wildtype translation start codon ATG (coding for Methionin) of VP1 was changed into GCC (Alanin) and inactivated. The resulting EcoRI/BamHI fragment was cloned into pBSIIKS (Stratagene, La Jolla, CA, USA). This vector was used to inactivate the translation start codon of VP2 by site directed mutagenesis according to the instructions of the QuickChange II Site directed mutagenesis kit (Stratagene) using the following primers:

Insect-muta_4_s:	5'-ACC TGT TAA G**AC A**GC TCC GGG AAA AAA G-3' (SEQ ID NO: 79)
Insect-muta_4_as:	5'-CTT TTT TCC CGG AGC **TGT** CTT AAC AGG T-3' (SEQ ID NO: 80)

[0256] Thereby, the wildtype translation start codon ACG of VP2 was changed into ACA (both coding for Threonin). The resulting construct was digested with restriction enzymes BamHI and EcoRI and cloned into the baculo transfer vector pVL1393. As a result, the construct contained the complete AAV cap gene with mutations of the VP1 and VP2 start codons but no rep cds. (Fig. 12)

9.2. cloning of pVL_VP2

[0257] AAV2 VP2 was amplified using the primers E_VP2_for and E_VP2_rev listed below. Thereby, the wildtype VP2 translation start codon ACG (coding for Threonine) was changed into ATG (Methionine). Primers:

E_VP2_for:	5'-CAC CCG CGG GGA TCC ACT **ATG**GCT CCG GGA AAA AAG AGG-3' (SEQ ID NO: 81)
E_VP2_rev:	5'-CGC GAA TTC CTA **TTA** CAG ATT ACG AGT CAG G-3' (SEQ ID NO: 82)

[0258] The resulting construct was cloned into the baculo transfer vector pVL1393.

9.3. cloning of pVL_VP3

[0259] AAV2 VP3 was amplified using the primers E_VP3_for and E_VP3_rev listed below. Primers:

E_VP3_for:	5'-CAC CCG CGG GGA TCC ACT **ATG** GCT ACA GGC AGT GGC GCA C-3' (SEQ ID NO: 83)E_VP2_rev:5'-CGC GAA TTC CTA **TTA** CAG ATT ACG AGT CAG G-3' (SEQ ID NO: 84)

[0260] The resulting construct was cloned into the baculo transfer vector pVL1393.

9.4. Analysis of particle production

[0261] AAV particles were produced as described in 1.1. Cell lysates were investigated by Western blot analysis for protein expression. pVL_VP1_MOD4 showed only VP3 expression, pVL_VP2 VP2 expression, while pVL_VP3 showed in addition to VP3 smaller degradation signals (Fig. 12 B). Titers were obtained by an A20 ELISA. A titer of $1x10^{12}$ particles/ml was observed for the modification 4 construct while VP2 pVL_VP2 showed a titer of $9x10^{8}$ particles/ml and pVL_VP3 only a titer of $1x10^{8}$ particles/ml (Fig. 12 C).

Conclusion

[0262] This result shows that AAV VLPs can be produced in insect cells as efficiently as in mammalian cells. The data show that in insect cells the N-terminal sequence of VP3 also seems to be required and sufficient for efficient VP3 capsid assembly. Further a change of the VP2 start codon from ACG into ATG comes along with loss of efficiency in capsid assembly (Fig. 12 C). We speculate that particle assembly from pVL_VP2 goes along with minor VP3 expression initiated from a VP3 ATG which was left intact in the construct.

10. Capsids composed essentially of VP3 tolerate insertions of polypeptides

10.1. Generation of virus-like particles (VLP) containing epitopes at position I-587

[0263] For cloning of expression vectors encoding VLPs composed of VP3 capsid proteins containing a particular epitope sequence at position I-587, the epitope sequence was first cloned into the VP coding sequence of pUCAV2 at the site corresponding to I-587 (amino acid number relative to the VP1 protein of AAV-2) and was subsequently sub-cloned into the vector pCIVP2mutACG.

[0264] Generation of vector pUCAV2 is described in detail in US 6,846,665. Basically, this vector contains the complete AAV2 genome (BgIII fragment) derived from pAV2 (Laughlin et al., 1983) cloned into the BamHI restriction site of pUC19. pUCAV2 was further modified by introduction of a NotI and AscI restriction site allowing the insertion of epitope sequences at position I-587 of the AAV2 capsid (PCT/EP2008/004366). In addition, an FseI restriction site located between position 453 (amino acid number relative to the VP1 protein of AAV-2) and I-587 587 was introduced in-frame into the VP coding sequence of the vector by site directed mutagenesis.

[0265] For cloning of epitope sequences into modified pUCAV2 sense- and anti-sense oligonucleotides were designed that encode the respective epitope with an alanine or glycine adaptor sequence and contain a 5'-site extension. The 5'-site extension of the oligonucleotides was designed so that annealing of the sense and anti-sense oligonucleotides results in a dsDNA with 5'-site and 3'-site overhangs compatible with overhangs generated by NotI and AscI restriction of the modified pUCAV2. The sequences of the oligonucleotides and the respective epitope sequences are summarized in Table 4. Each of the inserted epitopes is flanked by an adaptor according to the following scheme (X_n represents the epitope sequence):

Type I adaptor: $(Ala)_2$-$(Gly)_3$-X_n-$(Gly)_4$-Ala

Type II adaptor: $(Ala)_2$-$(Gly)_4$-X_n-$(Gly)_4$-Ala

Type III adaptor: $(Ala)_3$-$(Gly)_5$-X_n-$(Gly)_5$-$(Ala)_2$

Type IV adaptor: $(Ala)_5$-X_n-$(Ala)_5$

[0266] To anneal the oligonucleotides 50.0 µg of the sense oligonucleotide and 50.0 µg of the anti-sense oligonucleotide were mixed in a total volume of 200 µl 1x PCR-Buffer (Qiagen) and incubated for 3 min at 95°C in a thermomixer. After 3 min at 95°C the thermomixer was switched off and the tubes were left in the incubator for an additional 2h to allow annealing of the oligonucleotides during the cooling down of the incubator. To clone the annealed oligonucleotides into pUCAV2 at I-587 the vector was linearized by restriction with NotI and AscI and the cloning reaction was performed using the Rapid DNA Ligation Kit (Roche). Briefly, the annealed oligonucleotides were diluted 10-fold in 1x DNA Dilution Buffer and incubated for 5 min at 50°C. 100 ng of the annealed oligonucleotides and 50 ng of the Not*I* /AscI linearized vector pUCAV2 were used in the ligation reaction, which was performed according to the instructions of the manufacturer of the Rapid DNA Ligation Kit (Roche). *E. co*l*i* XL1 blue or DH5a were transformed with an aliquot of the ligation reaction and plated on LB-Amp agar plates. Plasmids were prepared according to standard procedures and were analyzed by sequencing.

[0267] For generation of empty VLPs composed of VP3 proteins containing an epitope sequence at I-587 the BsiVVI / XcmI restriction fragment of pUCAV2 containing the epitope at I-587 was sub-cloned into the vector pCIVP2mutACG according to standard procedures. The vector pCIVP2mutACG contains the overlapping AAV2 VP2 and VP3 coding sequences cloned into the XhoI / NotI site of pCI (Promega). In pCIVP2mutACG the ACG start-codon of VP2 is destroyed and

replaced by a GAG codon. Substitution was performed by PCR amplification of the AAV2 VP2 and VP3 coding sequences using VP2 specific primers and the plasmid pCIVP2 as template (the vector pCIVP2 contains the wildtype VP2 and VP3 coding sequence cloned into the polylinker of pCI). The forward primer used for PCR anneals to the 5' site of the VP2 coding sequence and contains the substitution of the VP2 ACG start codon by a GAG codon. In addition, the forward primer contains an XhoI recognition sequence at the 5'-site. The reverse primer annealed to the 3' end of the VP2 / VP3 coding sequence and contained a NotI recognition sequence at its 5'-site. The resulting PCR product was cloned into the XhoI / NotI site of pCI.

[0268] The resulting vectors were used for production of VLPs by transfection of 293-T cells. Cells (5×10^5/dish) were seeded in 6 cm dishes 24 h prior to transfection. 293-T cells were transfected by calcium phosphate precipitation as described in US 2004/0053410. Subsequently, 293-T cells were lysed in the medium by three rounds of freeze (-80°C) and thaw (37°C) cycles. The lysate (3 ml total volume) was cleared by centrifugation and the VLP capsid titer was determined using a commercially available ELISA (AAV Titration ELISA; Progen, Heidelberg, Germany). VLP titers ranged between 2.1 E+12 and 9.8 E+12 capsids/ml (Table 5). The VLP TP18 clone was directly used for large scale packaging (as described in example 1). It contained 1.2E+13 capsids/ml within the crude lysate (Table 5).

10.2. Generation of virus-like particles (VLP) containing epitopes at position I-587 and I-453 of the capsid

[0269] For cloning of expression vectors encoding VLPs composed of VP3 capsid proteins containing epitope sequences at position I-453 and I-587 (amino acid number relative to the VP1 protein of AAV-2), the first epitope sequence was cloned into pCIVP2mutACG at the site corresponding to I-587 as described above.

[0270] The second epitope sequence was initially cloned into the NotI / AscI restriction site of the vector pCIVP2-I453-NotI-AscI (described in: WO 2008/145400). Briefly, the vector pCI-VP2-I453-Not-AscI was created by PCR amplification of the AAV2 VP2 gene and cloning of the respective PCR product into the XhoI / NotI site of vector pCI (Promega). The resulting vector pCIVP2 was modified by destruction of the NotI restriction site of the cloning site by site-directed mutagenesis. The vector was further modified by introduction of a novel singular NotI and AscI restriction site allowing the insertion of epitope sequences at position I-453 of the AAV2 capsid. In addition, an FseI site located between I-453 and I-587 was introduced in-frame into the VP coding sequence of pCIVP2-I453-NotI-AscI by site directed mutagenesis. For cloning of epitope sequences into the NotI / AscI site of the vector sense- and anti-sense oligonucleotides were designed that encode the respective epitope with a alanine / glycine adaptor sequence and contain a 5'-site extension. The 5'-site extension of the oligonucleotides was designed so that annealing of the sense and anti-sense oligonucleotides results in a dsDNA with 5'-site and 3'-site overhangs compatible with overhangs generated by NotI and AscI restriction of pCIVP2-I453-Not-AscI. Cloning of the annealed oligonucleotides was performed as described above.

[0271] The sequences of the oligonucleotides and the respective epitope sequences are

summarized in Table 6. Each of the inserted epitopes is flanked by an adaptor according to the following scheme (X_n represents the epitope sequence):

$(Ala)_2$-$(Gly)_3$-X_n-$(Gly)_4$-Ala

[0272] For generation of bivalent VLPs displaying epitopes (murine TNFα or IL-17 epitope) at I-453 and I-587 the BsiWI / FseI fragment of pCIVP2-I453-NotII-AscI containing a given epitope inserted at I-453 was subcloned into the vector pCIVP2mutACG containing a particular epitope inserted into I-587 (described above). The resulting vector was used for production of bivalent VLPs by transfection of 293-T cells as described above (example 1.2) (6-well plate scale). Particle production was analyzed by ELISA (AAV2 Titration ELISA; Progen). Results are shown in Table 7. These data demonstrate that VLPs composed of VP3 proteins with epitope insertions at I-453 and I-587 can be produced with high capsid titers.

Table 4: Oligonucleotides used for cloning of epitope sequences into I-587

Name / Peptide Seq.	Type	sense Oligonucleotide	anti-sense Oligonucleotide	Adaptor
CETP TP18 DISVTGAPVIT ATYL	Rabbit CETP epitope	5'GGCCGGCGGAGGTGACAT CAGCGTGACCGGTGCACCCG TGATCACCGCCACCTACCTG GGGGGTGGCGGTG 3' (SEQ ID NO: 85)	5'CGCGCACCGCCACCCCC CAGGTAGGTGGCGGTGATC ACGGGTGCACCGGTCACGC TGATGTCACCTCCGCC 3' (SEQ ID NO: 86)	Type I
3Depi-3 DSNPRGVSAY LSR	Human IgE epitope	5'GGCCGGCGGAGGTGGTGA CAGCAACCCTAGAGGCGTGA GCGCCTACCTGAGCAGAGGG GGTGGCGGTG 3' (SEQ ID NO: 87)	5'CGCGCACCGCCACCCCC TCTGCTCAGGTAGGCGCTC ACGCCTCTAGGGTTGCTGT CACCACCTCCGCC 3' (SEQ ID NO: 88)	Type II
Kricek VNLTWSRASG	Human IgE epitope	5'GGCCGCAGCGGCGGTGAA CCTGACCTGGAGCAGAGCCT CCGGCGCGGCGGCGGCGG 3' (SEQ ID NO: 89)	5'CGCGCCGCCGCCGCCGC GCCGGAGGCTCTGCTCCAG GTCAGGTTCACCGCCGCTG C3' (SEQ ID NO: 90)	Type IV
TNFα-V1 SSQNSSDKPV AHVVANHQVE	Murine TNFα epitope	5'GGCCGGCGGAGGTAGCAG CCAGAACAGCAGCGACAAGC CCGTGGCCCACGTGGTGGCT AACCACCAGGTGGAGGGGGG TGGCGGTG 3' (SEQ ID NO: 91)	5'CGCGCACCGCCACCCCC CTCCACCTGGTGGTTAGCC ACCACGTGGGCCACGGGCT TGTCGCTGCTGTTCTGGCT GCTACCTCCGCC 3' (SEQ ID NO: 92)	Type I
IL-17-V1 NAEGKLDHH MNSVL	Murine IL-17 epitope	5'GGCCGGCGGAGGTAACGC CGAGGGCAAGCTTGACCACC ACATGAACAGCGTGCTGGGG GGTGGCGGTG 3' (SEQ ID NO: 93)	5'CGCGCACCGCCACCCCC CAGCACGCTGTTCATGTGG TGGTCAAGCTTGCCCTCGG CGTTACCTCCGCC 3' (SEQ ID NO: 94)	Type I
		5'GGCCGGCGGAGGTCTGGA	5'CGCGCACCGCCACCCCC	

Name / Peptide Seq.	Type	sense Oligonucleotide	anti-sense Oligonucleotide	Adaptor
IL-6-V2 LEEFLKVTLRS	Murine IL-6 epitope	GGAATTCCTGAAGGTGACCC TGAGAAGCGGGGGTGGCGGT G 3' (SEQ ID NO: 95)	GCTTCTCAGGGTCACCTTC AGGAATTCCTCCAGACCTC CGCC 3' (SEQ ID NO: 96)	Type I
Aβ(1-9) DAEFRHDSG	Human amyloid-β epitope	5'GGCCGCAGGCGGAGGGGG AGGCGACGCCGAGTTCAGAC ACGACAGCGGCGGCGGAGGG GGAGGCGCGG 3' (SEQ ID NO: 97)	5'CGCGCCGCGCCTCCCCC TCCGCCGCCGCTGTCGTGT CTGAACTCGGCGTCGCCTC CCCCTCCGCCTGC 3' (SEQ ID NO: 98)	Type III

Table 5: Small scale production of different VLPs

Name	Epitope at I-587	Titer (capsids/ml)
VLP-TP18	**CETP TP18** DISVTGAPVITATYL (SEQ ID NO: 99)	1.2 E+13(*)
VLP-3Depi3	**3Depi-3** DSNPRGVSAYLSR (SEQ ID NO: 100)	2.1 E+12
VLP-Kricek	**Kricek** VNLTWSRASG (SEQ ID NO: 101)	2.6 E+12
VLP-TNFα	**TNFα-V1** SSQNSSDKPVAHWANHQVE (SEQ ID NO: 102)	9.8 E+12
VLP-IL-17	**IL-17-V1** NAEGKLDHHMNSVL (SEQ ID NO: 103)	5.6 E+12
VLP-IL-6	**IL-6-V2** LEEFLKVTLRS (SEQ ID NO: 104)	5.6 E+12
VLP-Aβ	**Aβ(1-9)** DAEFRHDSG (SEQ ID NO: 105)	6.2 E+12
(*) Large-scale packaging		

Table 6: Oligonucleotides used for cloning of epitope sequences into I-453

Name / Peptide Seq.	Type	sense Oligonucleotide	anti-sense Oligonucleotide
TNFα-V1 SSQNSSDKPVA HWANHQVE	**Murine TNFα epitope**	5'GGCCGCCGGTGGAGGCAG CAGCCAGAACAGCAGCGACA AGCCCGTGGCCCACGTGGTG GCTAACCACCAGGTGGAGGG CGGTGGAGGG 3' (SEQ ID NO: 106)	5'CGCGCCCTCCACCGCCCTCCAC CTGGTGGTTAGCCACCACGTGGGC CACGGGCTTGTCGCTGCTGTTCTG GCTGCTGCCTCCACCGGC 3' (SEQ ID NO: 107)
		5'GGCCGCCGGTGGAGGCAA	5'CGCGCCCTCCACCGCCCAGCAC

Name / Peptide Seq.	Type	sense Oligonucleotide	anti-sense Oligonucleotide
IL-17-V1 NAEGKLDHHMN SVL	**Murine IL-17 epitope**	CGCCGAGGGCAAGCTTGACC ACCACATGAACAGCGTGCTG GGCGGTGGAGGG 3' (SEQ ID NO: 108)	GCTGTTCATGTGGTGGTCAAGCTT GCCCTCGGCGTTGCCTCCACCGGC 3' (SEQ ID NO: 109)
IL-6-V2 LEEFLKVTLRS	**Murine IL-6 epitope**	5'GGCCGCCGGTGGAGGCCT GGAGGAATTCCTGAAGGTGA CCCTGAGAAGCGGCGGTGGA GGG 3' (SEQ ID NO: 110)	5'CGCGCCCTCCACCGCCGCTTCT CAGGGTCACCTTCAGGAATTCCTC CAGGCCTCCACCGGC 3' (SEQ ID NO: 111)

Table 7: Production of VLPs carrying epitopes at I-453 and I-587

combination	Epitope at I-453	Epitope at I-587	Titer (capsids/ml)
TNF-α / IL-17	**TNFα-V1**	**IL-17-V1**	**7.9E+12**
	SSQNSSDKPVAHWANHQVE (SEQ ID NO: 112)	NAEGKLDHHMNSVL (SEQ ID NO: 113)	
TNF-α / IL-6	**TNF α-V1**	**IL-6-V2**	**8.5E+12**
	SSQNSSDKPVAHWANHQVE (SEQ ID NO: 114)	LEEFLKVTLRS (SEQ ID NO: 115)	
IL-17 / TNF-α	**IL-17-V1**	**TNFα-V1**	**1.0E+13**
	NAEGKLDHHMNSVL (SEQ ID NO: 116)	SSQNSSDKPVAHWANHQVE (SEQ ID NO: 117)	
IL-6 / TNF-α	**IL-6-V2**	**TNFα-V1**	**1.0E+13**
	LEEFLKVTLRS (SEQ ID NO: 118)	SSQNSSDKPVAHWANHQVE (SEQ ID NO: 119)	
IL-17 / IL-6	**IL-17-V1**	**IL-6-V2**	**3.9E+12**
	NAEGKLDHHMNSVL (SEQ ID NO: 120)	LEEFLKVTLRS (SEQ ID NO: 121)	
IL-6 / IL-17	**IL-6-V2**	**IL-17-V1**	**8.9E+12**
	LEEFLKVTLRS (SEQ ID NO: 122)	NAEGKLDHHMNSVL (SEQ ID NO: 123)	

10.3. Conclusion

[0273] VP3 particles tolerate insertions and can therefore be used as a medicament such as a vaccine for example by insertion of B-Cell epitopes.

11. VP3 capsid assembly of different AAV serotypes

11.1. AAV1 deletion constructs

[0274] To analyze whether these findings can be conferred to other serotypes an analogue setting of constructs for AAV1 were tested.

[0275] Following constructs were cloned:

- pCI_VP2/2539_AAV1: The complete AAV1 VP2 plus 95 bp of VP1 were cloned into pCI (Promega, Mannheim, Germany). The VP2 ACG start codon was not mutated.
- pCI_VP3/2539_AAV1mutACG: The complete AAV1 VP2 plus 95bp of VP1 were cloned into pCI. The VP2 ACG start codon was mutated to ACC.
- pCLVP3/2634_AAV1mutACG: The VP1 part was deleted completely and the VP2 ACG start codon was mutated into an ACC.

Cloning

[0276] Cloning of all constructs was performed by site directed mutagenesis standard procedures using modified primers (primers used for site directed mutagenesis are listed below).

[0277] pCI_VP2/2539_AAV1 was generated by inserting a NheI site 95 bp upstream of the VP2 ACG start codon and a XmaI site downstream of the VP3 stop codon. Mutations were generated within pUCrep/fs/cap_AAV1_I588 (described within PCT/EP2008/004366). The resulting plasmid was digested with NheI and XmaI. The generated fragment was cloned into the pCI-VP2 Vector (described in PCT/EP2008/004366). Primers:

AAV1 NheI VP2plus95bp:	5'-GAG CGT CTG **CTA** GCA GAT ACC TCT TTT GGG G-3' (SEQ ID NO: 124)
AAV1 VP3 Xma rev:	5'-GAA ACG AAT CAC CCG GGT TAT TGA TTA AC-3' (SEQ ID NO: 125)

[0278] pCI_VP3/2539_AAV1mutACG was generated by mutating the ACG start codon to ACC within pCIVP2/2539_AAV1. Primer:

AAV1 VP2ko for:	5'-GGC GCT AAG ACC GCT CCT GGA AAG-3' (SEQ ID NO: 126)
AAV1 VP2ko rev:	5'-CTT TCC AGG AGC GGT CTT AGC GCC-3' (SEQ ID NO: 127)

[0279] pCI_VP3/2634_AAV1mutACG was generated by deleting the 95 bp directly upstream of the VP2 ACG start codon and mutating by the same step the ACG start codon to ACC within pCIVP2_AAV1. Primer:

AAV1 VP2ko VP1del for:	5'-ACG ACT CAC TAT AGG CTA GCA GGC GCT AAG ACC GCT CCT GGA AAG-3' (SEQ ID NO: 128)
AAV1 VP2ko VP1del rev:	5'-CTT TCC AGG AGC GGT CTT AGC GCC TGC TAG CCT ATA GTG AGT CGT-3' (SEQ ID NO: 129)

[0280] Assembly of AAV1 capsids was controlled within crude lysates after transfection of 293 cells with the respective plasmid. The capsid titer was determined by an AAV1 titration ELISA (Progen, Heidelberg, Germany) according to manufacturer's manual. The assembly efficiency of the three AAV1 constructs was comparable. The construct pCI_VP3/2634_AAV1mutACG gave a titer of 10^{13} particles/ml, confirming the fact that capsid generation of AAV1 particles is generally more efficient than of AAV2 particles. In Western blot analyses VP2 and VP3 proteins were detectable for construct pCI- VP2/2539_AAV1 and only VP3 was detectable for pCI_VP3/2539 _AAV1 mutACG and pCI_VP3/2634_AAV1 mutACG respectively (Fig.13).

[0281] As a control for capsid protein expression, pUCAV1 was transfected. pUCAV1 contains the complete AAV1 Cap open reading frame encoding VP1, VP2 and VP3 of AAV1. pUCAV1 is described in detail in the PCT submission PCT/EP2008/004366 (there referred to as "pUCAV1_AgeI").

11.2. Trans-complementation of pCMV driven AAV1 VP3 constructs

[0282] To see whether trans-complementation experiments described in example 5 can be conferred to other serotypes analogue constructs of pCMV-VP3/2809 (AAV2) were cloned for AAV1.

11.2.1. Cloning

[0283] pCMV_AAV1VP3/2829 was cloned as following: By mutagenesis a HindIII restriction site was introduced directly before the VP3 ATG start codon of plasmid pUCrep/fs/cap_AAV1 (described within PCT/EP2008/004366) using the primers indicated below. The resulting plasmid was digested with AgeI. The Age I site was blunt ended with Klenow polymerase and the construct was subsequently digested with HindIII. The generated fragment was cloned into the HindIII/HincII-digested pBSCMV backbone. pBSCMV was generated by insertion of a 650bp BamHI CMV promoter fragment into the BamHI site of BlueskriptII SK+ vector (Stratagene, Amsterdam, Netherlands) described by Wistuba et al, 1997. Primer Hind III mutagenesis:

Forward:	5'-CGC TGC TGT GGG ACC T**AA GCT T**AT GGC TTC AGG CGG TGG CG-3' (**SEQ ID NO:** 130)
Reverse:	5'-CGC CAC CGC CTG AAG CCA T**AA GCT T**AG GTC CCA CAG CAG

	CG-3' (**SEQ ID NO:** 131)

11.2.2. Trans-complementation assay

[0284] Trans-complementation was performed with the pVP2N-gfp construct from AAV2 as described in example 3. Cells were transfected with plasmid pCMV-VP3 of either AAV2 pCMV_VP3/2809) or AAV1 (pCMV_AAV1VP3/2829) with or without cotransfection of pVP2N-gfp (Fig.14). Same molar ratios of VP3 construct and pVP2N-gfp were transfected. Protein expression was analyzed by Western blot and particle formation efficiency was measured by ELISA.

11.2.3. Result and conclusion

[0285] Particle assembly of AAV1 analyzed by an AAV1 ELISA (Progen, Heidelberg) was rescued by trans-complementation with pVP2N-gfp derived from AAV2. Rescue efficiency cannot be indicated as we did not compare cotransfection of pCMV_AAV1VP3/2829 and pVP2N-gfp with transfection of pCIVP3/2634_AAV1mutACG (see chapter 11.1 above). Also, we did not yet clone and test an AAV1 trans-complementation plasmid pVP2N-Gfp
Particle titer measured for trans-complemented AAV2 VP3 was 2.1E11. For AAV1 VP3 the titer obtained was 3.4E10 (a direct comparison of AAV1 and AAV2 titers is not possible due to the use of different ELISAs).

[0286] The results indicate that AAV1 makes use of the same mechanism for capsid assembly as AAV2 and that fragment Z and VP3 are interchangeable with different AAV serotypes.

11.3. Insertion of polypeptides within AAV1 I588 is tolerated

[0287] Here it was investigated whether empty AAV1 essentially VP3 particles tolerate insertions within amino-acid position 588.

[0288] For cloning of epitope sequences into pUCAV1-AgeI-1588 (described in PCT/EP2008/004366), sense- and anti-sense oligonucleotides were designed that encode the respective epitope with a glycine adaptor sequence. Upon hybridization of both oligonucleotides, 5'- and 3'-overhangs are generated that are compatible with overhangs generated by NotI and AscI restriction of the pUCAV1-AgeI-1588. The sequences of the oligonucleotides and the respective epitope sequences investigated are summarized in Table 4. Each of the inserted epitopes is flanked by an adaptor according to the following scheme (X_n represents the epitope sequence):
Ser(588)-$(Ala)_2$-$(Gly)_5$-X_n-$(Gly)_5$-Thr(589)
Oligo nucleotides for cloning the human IgE epitope "Kricek"

Amino acid sequence:	VNLTWSRASG

Sense oligo:	5'-g gcc gca gcc gca gtg aac ctg acc tgg agc aga gcc tcc ggc gcg gca gct gca gct-3' (SEQ ID NO: 132)
antisense oligo:	5'-c gcg agc tgc agc tgc cgc gcc gga ggc tct gct cca ggt cag gtt cac tgc ggc tgc-3' (SEQ ID NO: 133)

Oligo nucleotides for cloning the human IgE epitope "3Depi-3"

Amino acid sequence:	DSNPRGVSAYLSR
Sense oligo:	5'-GGCC GGC GGT GGA GGC GGT GAC AGC AAC CCT AGA GGC GTG AGC GCC TAC CTG AGC AGA GGA GGC GGT GGA GGG-3' (SEQ ID NO: 134)
antisense oligo:	5'-CGCG CCC TCC ACC GCC TCC TCT GCT CAG GTA GGC GCT CAC GCC TCT AGG GTT GCT GTC ACC GCC TCC ACC GCC-3' (SEQ ID NO: 135)

[0289] The precise cloning procedure used corresponds to the protocol used for insertion of epitopes into AAV2 I587 described in example 10.

[0290] For generation of empty AAV1 VLPs composed of essentially VP3 proteins containing an epitope sequence at I-588 the BsiVVI / SphI restriction fragment of pUCAV1-AgeI-I588 carrying the epitope at I-588 was sub-cloned into the vector pCIVP3/2634_AAV1 mutACG (described in example 11.1) according to standard procedures.

[0291] The resulting vectors were used for production of AAV1 VLPs by transfection of 293-T cells as described above (example 1.2.)
Titers were determined by a commercial AAV1 ELISA (Progen, Heidelberg, Germany). High titers of 3.6E13/ml (Kricek) and 9.2E13/ml (3Depi-3) were obtained, indicating that insertions within AAV1 588 (being homologous to AAV2 587) are well tolerated and that AAV1 VP3 particles can be used as vaccine carrier.

12. ORF2 comprises fragment Z and encodes AAP.

[0292] Detailed sequence analysis revealed that fragment Z encodes a significant part of the new "assembly activating protein" (AAP). Fig. 16 gives an overview and Fig. 17 shows in more detail the position of ORF2 and the encoded protein AAP in relation to the cap gene and the position of the translation start codons of the Cap proteins VP1, VP2 and VP3, as well as the location of fragment Z and EcoNI and BsiWI restriction sites. The three Cap proteins VP1, VP2 and VP3 are translated

from the same one ORF of the cap gene (also named the first ORF, ORF1), whereas AAP is translated from a different reading frame (named the second ORF, ORF2). For VP1, VP2 and VP3 numbers of the well-defined translation start points are given, whereas for AAP it is not definitely known.

[0293] In Fig. 17 the sequence of ORF2 (627 nucleotides, SEQ ID NO: 23) and the respective AAP protein sequence (208 amino acids, SEQ ID NO: 1) is given for AAV2 as extracted from NCBI entrée number NC_001401.

[0294] The sequences of the respective open reading frames and proteins of some other parvoviruses were extracted from the capsid gene sequences available in the NCBI database and given in detail in SEQ ID Nos 2-44 as listed in table 8.

Table 8: NCBI entrée numbers and numbers of corresponding SEQ IDs of AAP encoding nucleotide and protein sequences from different parvoviruses.

parvovirus	No. of nt entrée at NCBI	respective ORF2	Length of ORF2 / nt	encoded protein AAP	Length of AAP / AA
AAV2	NC_001401	SEQ ID NO: 23	627	SEQ ID NO: 1	208
AAV1	NC_002077	SEQ ID NO: 24	678	SEQ ID NO: 2	225
AAV3b	AF028705	SEQ ID NO: 25	627	SEQ ID NO: 3	208
AAV4	NC_001829	SEQ ID NO: 26	597	SEQ ID NO: 4	198
AAV5	NC_006152	SEQ ID NO: 27	681	SEQ ID NO: 5	226
AAV6	AF028704	SEQ ID NO: 28	678	SEQ ID NO: 6	225
AAV7	NC_006260	SEQ ID NO: 29	681	SEQ ID NO: 7	226
AAV8	NC_006261	SEQ ID NO: 30	684	SEQ ID NO: 8	227
AAV9	AY530579	SEQ ID NO: 31	681	SEQ ID NO: 9	226
AAV10	AY631965	SEQ ID NO: 32	606	SEQ ID NO: 10	201
AAV11	AY631966	SEQ ID NO: 33	594	SEQ ID NO: 11	197
AAV12	DQ813647	SEQ ID NO: 34	621	SEQ ID NO: 12	206
b-AAV (bovine)	NC_005889	SEQ ID NO: 35	600	SEQ ID NO: 13	199
Avian AAV ATCC VR-865	AY186198	SEQ ID NO: 36	789	SEQ ID NO: 14	262

parvovirus	No. of nt entrée at NCBI	respective ORF2	Length of ORF2 / nt	encoded protein AAP	Length of AAP / AA
Avian AAV strain DA-1	AY629583	SEQ ID NO: 142	723	SEQ ID NO: 143	240
AAV13	EU285562	SEQ ID NO: 37	627	SEQ ID NO: 15	208
Mouse AAV1	DQ100362	SEQ ID NO: 38	534	SEQ ID NO: 16	177
Avian AAV strain DA-1	AY629583	SEQ ID NO: 39	723	SEQ ID NO: 17	240
Caprine AAV1 isolate AAV-Go.1	AY724675	SEQ ID NO: 40	581	SEQ ID NO: 18	226
Rat AAV1	DQ100363	SEQ ID NO: 41	756	SEQ ID NO: 19	251
Goose parvovirus strain DB3	EU088102	SEQ ID NO: 42	639	SEQ ID NO: 20	212
Duck parvovirus strain 90-0219	AY382892	SEQ ID NO: 43	693	SEQ ID NO: 21	230
Snake parvovirus 1	AY349010	SEQ ID NO: 44	600	SEQ ID NO: 22	199

[0295] For sequence comparison an alignment of the predicted AAP protein sequences derived from ORF2 of the cap gene of some parvoviruses is given in Fig. 27.

[0296] In construct pVP2N-gfp the EcoNI/BsiVVI fragment from pTAV2.0 was inserted downstream of a CMV promoter and upstream of the GFP cds of vector pEGFP-N1 (example 3.1/Fig. 6A and example 13/Fig. 19A). Since the BsiVVI site is located about 90 nucleotides upstream of the 3' end of ORF2, the vector pVP2N-gfp encodes C-terminally truncated AAP (named AAPtru) that is as active in trans-complementation as AAP expressed from full-length ORF2 (see e.g. Fig. 21).

13. Codon modification confirms that expression of functional protein from ORF2 is necessary for trans-complementation

[0297] To investigate the nature of the trans-complementing activity of ORF2, the sequence between the EcoNI/BsiVVI restriction fragment was codon modified (cm).

[0298] The first mutant DNA sequence was named ORF1cm. The DNA sequence of the mutant was altered in such a way that the first reading frame coding for the capsid protein remained intact whereas the second reading frame coding for AAP was changed. As a result the sequence encodes wildtype capsid protein but no functionally active AAP any more. Identity of the DNA sequence of pVP2N-gfp versus pVP2N/ORF1cm-gfp is 71% while protein identity in the first reading frame is 100%.

[0299] The second mutant DNA sequence was named ORF2cm and altered in the first reading frame meaning that it did not code for a functionally active capsid protein any more but functionally intact AAP could be expressed. Identity of the DNA sequence of pVP2N-gfp versus pVP2N/ORF2cm-gfp is 79% while protein identity in the second reading frame is 100%.

[0300] The sequences of ORF1cm and ORF2cm are given in Figs. 18A and 18B, respectively. As already described in example 5, codon modification was performed by GENEART (Regensburg, Germany). Codons were modified for codons preferentially used in mammalian cells.

[0301] As described in example 3.1, pVP2N-gfp was generated by inserting the EcoNI/BsiVVI restriction fragment of pTAV2.0 into the multiple cloning site of pEGFP-N1. Constructs pVP2N/ORF1cm-gfp and pVP2N/ORF2cm-gfp were generated in the same way with the difference that the codon modified EcoNI/BsiVVI fragments were inserted into the corresponding vector backbone.

[0302] Protein expression of pVP2N/ORF1cm-gfp and pVP2N/ORF2cm-gfp (Fig. 20A) was compared with that of unmodified pVP2N-gfp (Fig. 20B) in Western blot analysis. The ability to rescue capsid formation of pCMV-VP3/2809 was tested in trans-complementation assays as described in example 3. Plasmids were cotransfected in a molar ratio of 1:1 (Fig. 20C).

Result and conclusion

[0303] As already described in example 3 and shown in Fig. 6, Western blot analysis using monoclonal antibody A69 confirmed expression of a capsid protein comprising the VP2 N-terminus (VP2N-gfp, Fig. 19B) in the GFP fusion construct pVP2N-gfp (Fig. 19A). Complementation of plasmid pCMV-VP3/2809 with different molar ratios of pVP2N-gfp in 293-T cells corresponding to decreasing amounts of co-transfected pVP2N-gfp showed decreasing capsid assembly upon its quantification (Fig. 19C). Determination of the number of assembled capsids also revealed that deletion mutant pCMV-VP3/2809 co-transfected with pVP2N-gfp was nearly as efficient in capsid assembly as mutant pCMV-VP3/2696, the deletion mutant that showed normal capsid formation (Fig. 5). Assembly could be detected even at a 500-fold reduced amount of co-transfected pVP2N-gfp plasmid.

[0304] Hence it was clear, that the assembly promoting activity associated with the constructs containing c*ap* sequences upstream of the VP3 translation start site can be provided *in trans*.

[0305] As already described for example 5, Fig. 8B codon-modified construct pVP2N/ORF1cm protein expression from codon-modified constructs was even higher than protein expression from the non-modified construct pVP2N-gfp, since the codon modification was optimized for mammalian cells. VP3 levels from co-expressed pCMV-VP3/2809 were normal. However, capsid assembly was not detected when using the helper construct pVP2N/ORF1cm (Fig. 20C). Also reduced expression of the respective protein by transfecting lower amounts of pVP2N/ORF1cm did not support capsid formation of VP3 (data not shown).

[0306] In contrast, assembled capsid could be detected using the helper construct pVP2N/ORF2cm (Fig. 20C). As described above, only ORF2cm expresses functionally intact AAP, whereas in pVP2N/ORF1cm the sequence of AAP is non-functional and this codon-modified construct encodes solely capsid protein. Accordingly, only pVP2N/ORF2cm rescued capsid assembly in trans-complementation.

[0307] This result clearly indicates, that the trans-complementing activity of fragment Z is mediated by its encoded protein AAP in ORF2. Codon modification experiments confirmed that expression of functional capsid protein in ORF1 is not necessary for trans-complementation but expression of functional AAP in ORF2.

14. Mutation of the predicted translation start codon of AAP

[0308] The sequence of ORF2 as given in Fig. 17 was analyzed in detail to further characterize AAP mediating capsid assembly. ORF2 does not contain an ATG prior to the VP3 start codon. It has to be assumed that a non-canonical start codon is utilized which is upstream of the defined minimal 5'-end of fragment Z at nt 2765. Taken into account the sequence requirements in the local environment of a start codon i.a. as defined by Kozak (2002) we predict the fifth codon at position 2729-2731, which is CTG and encodes a leucine (underlined in Fig. 17), to be the non-canonical start codon for translation of AAP. To observe its influence on expression efficiency, the site was mutated into ATG and TTG.

[0309] Protein expression of AU1 tagged versions of ORF2, namely pORF2/CTG-AU1, pORF2/ATG-AU1 and pORF2/TTG-AU1 (Fig. 21A), was compared with that of unmodified pVP2N-gfp in Western blot analysis (Fig. 21B). The ability to rescue capsid formation of pCMV-VP3/2809 was tested in trans-complementation assays as described in example 3. Plasmids were cotransfected in a molar ratio of 1:1 (Fig. 21C).

[0310] Constructs pORF2/CTG-AU1, pORF2/ATG-AU1 and pORF2/TTG-AU1 comprise the entire ORF2 of the cap gene (AAV2 nt2717-3340) fused to sequences coding for an AU1-tag (Fig. 21A).

[0311] For generation of constructs pORF2/CTG-AU1, pORF2/ATG-AU1 and pORF2/TTG-AU1 PCRs were performed with template pTAV2.0 and forward primer

5'-GGATCGCAAGCTTATTTTGGTCAGACTGGAGACGCAGACTCAGTACCTGACCC-3' (SEQ ID NO: 136),

5'-GGATCGCAAGCTTATTTTGGTCAGAATGGAGACGCAGACTCAG-3' (SEQ ID NO: 137),
or

5'-GGATCGCAAGCTTATTTTGGTCAGATTGGAGACGCAGACTCAG-3' (SEQ ID NO: 138)
and reverse primer
5`-GCGGTGTCTCGAGTTATATATAGCGATAGGTGTCGGGTGAGGTATCC

ATACTGTGGCACCATGAAGAC-3` (SEQ ID NO: 139).

[0312] The HindIII/XhoI digested amplification products were inserted into the HindIII/XhoI backbone of pBS-CMVsense, which was generated by insertion of a 560 bp BamHI human cytomegalovirus (CMV) promoter fragment from pHCMV-Luci (kindly provided by K. Butz, Germen Cancer Research Center, Heidelberg, Germany) into the BamHI site of plasmid Bluescript II SK+ (pBS, Stratagene, La Jolla, CA, USA).

Results and conclusion

[0313] The expression of the postulated proteins could be demonstrated using a monoclonal antibody against the AU1-tag (anti-AU1) for the constructs pORF2/CTG-AU1 and pORF2/ATG-AU1 (Fig. 21B), whereas expression from construct pORF2/TTG was below the detection level. Co-transfection of the ORF2 containing plasmids pORF2/CTG-AU1, pORF2/ATG-AU1 and pORF2/TTG-AU1 with the VP3 expression plasmid pCMV-VP3/2809 yielded capsid formation (Fig. 21C) wherein the number of assembled capsids measured per volume correlated with the amount of expressed protein estimated from the Western blot. Capsid titers obtained after transfection of pORF2/ATG-AU1 with pCMV-VP3/2809 were comparable to those obtained after co-transfection of pVP2N-gfp with pCMV-VP3/2809. In contrast, the TTG start codon encoding plasmid stimulated capsid assembly by a factor of approximately 10^3 fold less compared to the pVP2N-gfp plasmid. A polyclonal antiserum directed against a peptide of ORF2 clearly indicated expression of AAP and detected in addition to the AU1-tagged full length AAP also the C-terminally truncated AAP (AAPtru) expressed from pVP2N-gfp (Fig. 21B).

[0314] Taken together, mutation of the putative non-canonical CTG start codon into a strong ATG start codon enhanced protein synthesis and capsid assembly whereas mutation into a codon which normally is not preferred as initiation codon for protein synthesis significantly reduces protein levels and the number of assembled capsids. This result not only corroborates our conclusion that the protein product of ORF2 promotes the capsid assembly process. The results further indicate that the non-canonical CTG start codon is likely used as a start for translation, as its mutation into TTG leads to a significant reduction of AAP expression.

15. Insertion of stop codons in ORF2 confirm that expression of functional AAP is necessary for trans-complementation

[0315] Additionally, mutations were performed in the AAP encoding reading frame by introduction of stop codons into ORF2 in order to confirm that expression of functional AAP is necessary for trans-complementation.

[0316] Plasmids pVP2N/ORF2stopA-gfp, pVP2N/ORF2stopB-gfp, and pVP2N/ORF2stopC-gfp were created by site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene) of template pVP2N-gfp using two complementary PCR primers which included the desired substitutions. In pVP2N/ORF2stopA-gfp codon tgg_{2811} has been mutated into tag, in pVP2N/ORF2stopB-gfp codon $c_{2831}aa$ has been mutated into taa, and in pVP2N/ORF2stopC-gfp

codon g_{2879}aa has been mutated into tga (Fig. 22A). Positions are according to Ruffing et al. (1994). All mutations do not disrupt ORF1. In each case the EcoNI/BsiVVI fragment was then cloned into the EcoNI/BsiWI backbone of pVP2N-gfp.

Results and conclusion

[0317] Western blot analysis confirmed that VP3 is expressed in all samples (detected by monoclonal antibody B1 in Fig. 22B). Again, Bluescript vector (pBS) did not cause capsid assembly in the trans-complementation assay (Fig. 22C). Introduction of stop codons into ORF2 of the cap gene at the three different sites (as indicated in Fig. 22A) did not influence expression of VP2N-gfp (Fig. 22B), whereas all mutants harboring stop codons in ORF2 did not show any activity in capsid assembly (Fig. 22C).

[0318] Accordingly, Cap expression from pVP2n-gfp is not sufficient for capsid assembly in the trans-complementation assay. This result clearly supports the existence of AAP expressed from a different reading frame (ORF2) overlapping with the cap gene, which provides the capsid assembly helper function.

16. Expression of functional AAP rescues capsid assembly in the context of the AAV genome

[0319] Next we wanted to analyze whether expression of the newly discovered "assembly activating protein" AAP is necessary for capsid assembly in the context of the whole AAV genome. Therefore, construct pTAV/ORF1cm was created by cloning the EcoNI/BsiWI fragment of pVP2N/ORF1cm-gfp (example 13) into the EcoNI/BsiWI backbone of pTAV2.0 (example 1.2.1.). Hence, plasmid pTAV/ORF1cm (schematically shown in Fig. 23A) encodes the known AAV2 capsid and Rep proteins but should be deficient in the synthesis of AAP, because the codons of the cap gene were modified in the second reading frame (ORF2) without changing the first one encoding the Cap proteins (ORF1).

Results and conclusion

[0320] Indeed, the four Rep proteins (Rep40, Rep52, Rep68, and Rep78) were correctly expressed (data not shown). Western blot analysis showed that the expression pattern of the three VP proteins was slightly altered. Expression of endogenous AAP from wildtype plasmid pTAV2.0 but not from the codon modified one pTAV/ORF1cm was directly proven using polyclonal anti-AAP serum (Fig. 23B). As expected, truncated AAP is detectable upon co-expression of pVP2N-gfp.

[0321] Capsid assembly of the two constructs was compared after co-transfection of wildtype plasmid pTAV2.0 and codon modified plasmid pTAV/ORF1cm with empty Bluescript vector (pBS) or with pVP2N-gfp. As expected, transfection of pTAV/ORF1cm with pBS showed no detectable capsid formation, since pTAV/ORF1cm expresses all three capsid proteins but neither pTAV/ORF1cm nor

pBS express functionally active AAP. In contrast, transfection of pTAV/ORF1cm with pVP2N-gfp restored capsid assembly at least partially (Fig. 23C), since C-terminally truncated but active AAP is expressed from pVP2N-gfp.

[0322] Complementation of pTAV/ORF1cm that is deficient in expression of functional active AAP with mutant plasmids like pVP2N/ORF1cm-gfp (as described in example 13) and pVP2N/ORF2stopA-gfp (see example 15) which both were unable to express the AAP protein (due to codon modification or introduction of a stop codon, respectively) also did not lead to capsid formation. In contrast, in addition to pVP2N-gfp functionally active AAP can be expressed from plasmids pVP2N/ORF2cm-gfp (described in example 13), pORF2/CTG-AU1 and pORF2/ATG-AU1 (see example 14) and rescued capsid assembly in trans-complementation (Fig. 23D).

[0323] Taken together, capsid formation in the context of the complete viral genome is dependent on the expression of endogenous or complemented AAP.

17. Expression of functional AAP is necessary for capsid assembly in the context of the AAV genome

[0324] To further prove that AAP is necessary for capsid assembly in the context of the whole AAV genome, a stop codon was introduced in ORF2 disrupting AAP amino acid sequence.

[0325] Therefore, construct pTAV/ORF2stopB was created by cloning the EcoNI/BsiVVI fragment of pVP2N/ORF2stopB-gfp (for details see example 15) into the EcoNI/BsiVVI backbone of pTAV2.0. (example 1.2.1). In pVP2N/ORF2stopB-gfp the caa codon starting at nucleotide 2831 was mutated into a taa stop codon. Hence, plasmid pTAV/ORF2stopB (schematically shown in Fig. 24A) encodes the known AAV2 capsid and Rep proteins but should be deficient in the synthesis of AAP, because of the inserted stop codon.

Results and conclusion

[0326] Again, correct expression of the four Rep proteins could be detected in Western blot analysis (data not shown), as well as a slightly altered expression pattern of the three VP proteins. Expression of endogenous AAP from wildtype plasmid pTAV2.0 but not from the one containing the stop codon was directly proven using polyclonal anti-AAP serum (Fig. 24B). Capsid assembly of the two constructs was compared after co-transfection of wildtype plasmid pTAV2.0 and mutant plasmid pTAV/ORF2stopB with empty Bluescript vector (pBS) or with pVP2N-gfp. As expected, transfection of pTAV/ORF2stopB with pBS showed no detectable capsid formation, since pTAV/ORF2stopB expresses all three capsid proteins but neither pTAV/ORF2stopB nor pBS express functionally active AAP. In contrast, transfection of pTAV/ORF2stopB with pVP2N-gfp restored capsid assembly at least partially (Fig. 24C), since C-terminally truncated but active AAP is expressed from pVP2N-gfp.

[0327] This result further confirmed that capsid formation in the context of the complete viral

genome is dependent on the expression of functional AAP.

18. The "assembly activating protein" AAP targets VP proteins to the nucleolus.

[0328] In addition to example 8, several constructs were transfected in 293-T cells to compare the location of expressed proteins within the transfected cell and assembly efficiency.

18.1. Cloning of constructs

[0329] Cloning of construct pCMV-NLS-VP3 is described in example 8.1. The approach for generation of pCMV-NoLS-VP3 was concordant to that of pCMV-NLS-VP3 with the difference that the complementary primer pair

5`-GGAAT TCGAT ATCAA GCTTG CCATG GCACG GCAGG CCCGG CGGAA TAGAC GGAGA

CGGTG GCGGG AACGG CAGCG GATGG CTACA GGCAG TGG-3` (SEQ ID NO: 140),

and

5`-CCACT GCCTG TAGCC ATCCG CTGCC GTTCC CGCCA CCGTC TCCGT CTATT CCGCC

GGGCC TGCCG TGCCA TGGCA AGCTT GATAT CGAAT TCC-3` (SEQ ID NO: 141)

was used. Accordingly, the c*ap* gene product NoLS-VP3 contains the amino acid sequence of the nucleolar localization signal of HIV Rev MARQARRNRRRRWRERQR at the N terminus of VP3. Both constructs are schematically shown in Fig. 25A.

18.2. Analyses of constructs by immunofluorescence

[0330] Analogous to the experimental setup described in example 8, HeLa cells were transfected with the different constructs as indicated. Expression of capsid proteins and formation of capsids was analyzed by immunofluorescence as described above using a polyclonal VP antiserum or the monoclonal A20 antibody.

18.3. Results and conclusion

[0331] From literature analyzing productive AAV infection (e.g. Wistuba et al., 1997) it is known that capsid assembly can first be detected in the nucleoli of infected cells. Capsid protein VP3 expressed from pCMV-VP3/2809 in HeLa cells was distributed throughout the cell nucleus and the cytoplasm and excluded from nucleoli (as shown in Fig. 11B) and no capsids were detectable in these cells upon staining with capsid specific monoclonal antibody A20. But if AAP is co-expressed by co-transfecting pVP2N-gfp, translocation of a significant part of the VP3 protein to nucleoli and the formation of capsids could be detected.

[0332] As described in example 8, we expressed the construct pCMV-NLS-VP3 and observed strong nuclear accumulation of VP3 fused to the nuclear localization signal (NLS) of SV40, which however was excluded from nucleoli and did not cause capsid assembly (Fig. 11B). Co-expression

of AAP from plasmid pVP2N-gfp however again targeted a portion of NLS-VP3 proteins to the nucleoli where capsid formation was detectable.

[0333] Interestingly, AAP protein expressed from pORF2/ATG-AU1 (described in example 14) and stained with anti-AU1 antibody co-located with Fibrillarin to the nucleoli (Fig. 25C, the phase contrast image on the right confirms location of nucleoli at the site of staining).

[0334] This result suggested that AAP co-transports VP proteins to the nucleoli, which is a prerequisite for subsequent capsid assembly.

[0335] When expressing the construct pCMV-NoLS-VP3 we observed at least partially nucleolar localization of VP3 fused to the nucleolar localization signal derived from HIV REV, but surprisingly no capsid assembly could be detected (Fig. 25B). Therefore it seemed that the transfer of VP proteins to nucleoli is not sufficient for capsid formation. Again, co-expression of AAP from pVP2N-gfp promoted capsid formation, substantiating that AAP not only targets VP proteins to the nucleoli but plays an additional positive role in the assembly reaction. This example also shows that VP3 N-terminal insertions (I-203) are tolerated even if a highly positively charged 17mer NoLS-sequence seems to partially interfere with VLP titers.

19. Expression of functional AAP is necessary for capsid assembly.

[0336] In addition to the immunofluorescence images seen in example 18 we analyzed protein expression of the respective mutant constructs pCMV-NLS-VP3 and pCMV-NoLS-VP3 on Western blots. Moreover, we quantified capsid assembly activity of the respective constructs by monoclonal antibody A20 capsid ELISA.

Results and conclusion

[0337] Western blot analysis confirmed expression of VP3 from pCMV-VP3/2809 and the slightly longer proteins NLS-VP3 and NoLS-VP3 from pCMV-NLS-VP3 and pCMV-NoLS-VP3, respectively (Fig. 26A).

[0338] As already observed in example 18, neither NLS-VP3 nor NoLS-VP3 rescue capsid formation upon cotransfection with Bluescript vector (pBS), whereas in the presence of AAP expression (from pVP2N-gfp) capsid formation was detectable (Fig. 26B).

[0339] This result confirms that AAP not only targets VP proteins to the nucleoli (which is also accomplished by the NoLS-VP3 fusion construct not leading to capsid assembly) but also plays an essential role in the assembly reaction itself.

20. Assembly of wildtype and VP3 VLPs

[0340] To compare the morphology of virus-like particles assembled of VP1, VP2 and VP3 (VP1,2,3 VLP) with that of VLPs assembled only of VP3 (VP3 VLP) the respective samples have been investigated by electron microscopy after negative staining using 2% uranylacetate as described above.

[0341] Virus-like particles assembled of VP1, VP2 and VP3 corresponding to the wildtype capsid were produced in 293-T cells by expression of the complete cap gene. VLPs assembled only of VP3 were produced by co-transfection of pCMV-VP3/2809 and pVP2N-gfp (VP3 VLP).

Results and conclusion

[0342] Electron microscopic images confirmed that the morphology of virus-like particles assembled of VP1, VP2 and VP3 (VP1,2,3 VLP) is comparable to that of VLPs assembled only of VP3 (VP3 VLP, Fig. 28). In both images, no staining of the interior is visible, therefore clearly confirming that all particles are empty. An image of full (DNA-containing) particles in comparison to empty particles is shown e.g. in Xie et al.(2004).

21. Trans-complementation of AAP and VP3 cloned from different serotypes

[0343] To confirm that expression of AAP from one parvovirus is capable of mediating capsid assembly of VP3 from another parvovirus, we used the respective sequences of AAV1, AAV2 and AAV5 in trans-complementation assays.

[0344] Cloning of pVP2N-gfp of AAV1 and AAV5 was performed analogous to that of AAV2 (compare 3.1) with the difference that primer pairs were selected to amplify the respective sequences for AAV1 and AAV5 as given in SEQ ID NO: 24 and SEQ ID NO: 27 respectively. For trans-complementation cells were transfected with plasmid pCMV-VP3 of either AAV2 (pCMV_VP3/2809), AAV1 (pCMV_AAV1VPS/2829) as described above or a corresponding AAV5 VP3 expression construct with or without cotransfection of pVP2N-gfp of the respective AAV serotype (Fig. 29). Same molar ratios of VP3 construct and pVP2N-gfp were transfected. Particle formation efficiency was measured by ELISA

Results and conclusion

[0345] Capsid assembly of VP3 cloned from AAV1, AAV2 and AAV5, respectively, was compared after co-transfection of pVP2N-gfp cloned from AAV2 and AAV1, respectively, or Bluescript vector (pBS) (see Fig. 29). As expected, expression of VP3 in the absence of any other viral protein (pBS control) showed no detectable capsid formation, irrespective of its origin. In contrast, expression of AAP (expressed from the respective pVP2N-gfp construct) from serotype AAV1 completely restored AAV2 VP3 assembly (compared to assembly mediated by AAP from AAV2). Also vice e versa, AAP from AAV2 completely restored AAV1 VP3 assembly (compared to assembly mediated by AAP from AAV1). AAP from AAV5 was only partially able to complement AAV2 VP3 assembly and failed to

complement AAV1 VP3 assembly. Further, AAV2 and AAV1 AAP failed to complement AAV5 VP3 assembly. The failure of trans-complementation with respect to AAV5 constructs may be due to the fact that AAPs in these experiments were fused to GFP leading to a short C-terminal deletion of AAP which might interfere with the complementation of more distant parvoviruses while activity is sufficient for closely related serotypes. A further likely explanation is that more distant AAV serotypes are only partially able to complement each other with respect to VP3 assembly. Whereas AAP from AAV1 and AAV2 have a 71.5% identity and 81.0% similarity (Smith-Waterman Alignment), AAV2 and AAV5 only have a 56.2% identity and 60.8% similarity. These numbers are even lower with respect to AAV1 compared to AAV5 (53.8% identity and 58.1% similarity). Accordingly, the skilled artisan will be able to select functionally active AAPs from different serotypes and/or other functionally active variants by looking at identities / similarities of AAP.

[0346] Still, in addition to example 11 these result confirm that parvoviruses other than AAV2 encode functional AAP and make use of the same mechanism for capsid assembly. Further, AAP and VP3 are in principal interchangeable between different parvoviruses, especially between closely related viruses.

LITERATURE

[0347]

ARNOLD, G. S., SASSER, A. K., STACHLER, M. D. & BARTLETT, J. S. (2006) Metabolic biotinylation provides a unique platform for the purification and targeting of multiple AAV vector serotypes. Mol Ther, 14, 97-106.

ASOKAN, A. & SAMULSKI, R. J. (2006) AAV does the shuffle. Nat Biotechnol, 24, 158-60.

BACHMANN, M. F., ROHRER, U. H., KUNDIG, T. M., BURKI, K., HENGARTNER, H. & ZINKERNAGEL, R. M. (1993) The influence of antigen organization on B cell responsiveness. Science, 262, 1448-51.

BECERRA, S. P., KOCZOT, F., FABISCH, P. & ROSE, J. A. (1988) Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiations from a single transcript. J Virol, 62, 2745-54.

BECERRA, S. P., ROSE, J. A., HARDY, M., BAROUDY, B. M. & ANDERSON, C. W. (1985) Direct mapping of adeno-associated virus capsid proteins B and C: a possible ACG initiation codon. Proc Natl Acad Sci USA, 82, 7919-23.

CORPET, F. (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res, 16, 10881-90.

GIROD, A., RIED, M., WOBUS, C., LAHM, H., LEIKE, K., KLEINSCHMIDT, J., DELEAGE, G. & HALLEK, M. (1999) Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. Nat Med, 5, 1438.

GRIEGER, J. C., JOHNSON, J. S., GURDA-WHITAKER, B., AGBANDJE-MCKENNA, M. &

SAMULSKI, R. J. (2007) Surface-exposed adeno-associated virus Vp1-NLS capsid fusion protein rescues infectivity of noninfectious wild-type Vp2/Vp3 and Vp3-only capsids but not that of fivefold pore mutant virions. J Virol, 81, 7833-43.

GRIEGER, J. C. & SAMULSKI, R. J. (2005) Adeno-associated virus as a gene therapy vector: vector development, production and clinical applications. Adv Biochem Eng Biotechnol, 99, 119-45.

GRIFMAN, M., TREPEL, M., SPEECE, P., GILBERT, L. B., ARAP, W., PASQUALINI, R. & WEITZMAN, M. D. (2001) Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsids. Mol Ther, 3, 964-75.

GRIMM, D. (2002) Production methods for gene transfer vectors based on adeno-associated virus serotypes. Methods, 28, 146-57.

GRIMM, D., KERN, A., PAWLITA, M., FERRARI, F., SAMULSKI, R. & KLEINSCHMIDT, J. (1999) Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. Gene Ther, 6, 1322-30.

GRIMM, D. & KLEINSCHMIDT, J. A. (1999) Progress in adeno-associated virus type 2 vector production: promises and prospects for clinical use. Hum Gene Ther, 10, 2445-50.

HEILBRONN, R., BURKLE, A., STEPHAN, S. & ZUR HAUSEN, H. (1990) The adeno-associated virus rep gene suppresses herpes simplex virus-induced DNA amplification. J Virol, 64, 3012-8.

HOQUE, M., ISHIZU, K., MATSUMOTO, A., HAN, S. I., ARISAKA, F., TAKAYAMA, M., SUZUKI, K., KATO, K., KANDA, T., WATANABE, H. & HANDA, H. (1999a) Nuclear transport of the major capsid protein is essential for adeno-associated virus capsid formation. J Virol, 73, 7912-5.

HOQUE, M., SHIMIZU, N., ISHIZU, K., YAJIMA, H., ARISAKA, F., SUZUKI, K., WATANABE, H. & HANDA, H. (1999b) Chimeric virus-like particle formation of adeno-associated virus. Biochem Biophys Res Commun, 266, 371-6.

HUTTNER, N. A., GIROD, A., PERABO, L., EDBAUER, D., KLEINSCHMIDT, J. A., BUNING, H. & HALLEK, M. (2003) Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodies. Gene Ther, 10, 2139-47.

KING, J. A., DUBIELZIG, R., GRIMM, D. & KLEINSCHMIDT, J. A. (2001) DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. Embo J, 20, 3282-91.

KOERBER, J. T., JANG, J. H. & SCHAFFER, D. V. (2008) DNA shuffling of adeno-associated virus yields functionally diverse viral progeny. Mol Ther, 16, 1703-9.

KOZAK, M. (2002) Pushing the limits of the scanning mechanism for initiation of translation. Gene, 299, 1-34.

KRONENBERG, S., KLEINSCHMIDT, J. A. & BOTTCHER, B. (2001) Electron cryo-microscopy and image reconstruction of adeno-associated virus type 2 empty capsids. EMBO Rep, 2, 997-1002.

LAUGHLIN, C. A., TRATSCHIN, J. D., COON, H. & CARTER, B. J. (1983) Cloning of infectious adeno-associated virus genomes in bacterial plasmids. Gene, 23, 65-73.

LI, W., ASOKAN, A., WU, Z., VAN DYKE, T., DIPRIMIO, N., JOHNSON, J. S., GOVINDASWAMY, L., AGBANDJE-MCKENNA, M., LEICHTLE, S., REDMOND, D. E., JR., MCCOWN, T. J., PETERMANN, K. B., SHARPLESS, N. E. & SAMULSKI, R. J. (2008) Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticles. Mol Ther, 16, 1252-60.

MAHESHRI, N., KOERBER, J. T., KASPAR, B. K. & SCHAFFER, D. V. (2006) Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. Nat Biotechnol, 24, 198-204.

MITTEREDER, N., MARCH, K. L. & TRAPNELL, B. C. (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J Virol, 70, 7498-509.

MOSKALENKO, M., CHEN, L., VAN ROEY, M., DONAHUE, B. A., SNYDER, R. O., MCARTHUR, J. G. & PATEL, S. D. (2000) Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure. J Virol, 74, 1761-6.

MUZYCZKA, N. & BERNS, K. I. (2001) Parvoviridae: the viruses and their replication. IN KNIPE, T. M. & HOWLEY, P. M. (Eds.) Fields Virology. Fourth Edition ed. Philadelphia, Lippincott-Raven.

NICKLIN, S. A., BUENING, H., DISHART, K. L., DE ALWIS, M., GIROD, A., HACKER, U., THRASHER, A. J., ALI, R. R., HALLEK, M. & BAKER, A. H. (2001) Efficient and selective AAV2-mediated gene transfer directed to human vascular endothelial cells. Mol Ther, 4, 174-81.

NYGREN, P. A. & SKERRA, A. (2004) Binding proteins from alternative scaffolds. J Immunol Methods, 290, 3-28.

RABINOWITZ, J. E., XIAO, W. & SAMULSKI, R. J. (1999) Insertional mutagenesis of AAV2 capsid and the production of recombinant virus. Virology, 265, 274-85.

RIED, M. U., GIROD, A., LEIKE, K., BUNING, H. & HALLEK, M. (2002) Adeno-associated virus capsids displaying immunoglobulin-binding domains permit antibody-mediated vector retargeting to specific cell surface receptors. J Virol, 76, 4559-66.

RUFFING, M., HEID, H. & KLEINSCHMIDT, J. A. (1994) Mutations in the carboxy terminus of adeno-associated virus 2 capsid proteins affect viral infectivity: lack of an RGD integrin-binding motif. J Gen Virol, 75 (Pt 12), 3385-92.

RUFFING, M., ZENTGRAF, H. & KLEINSCHMIDT, J. A. (1992) Assembly of viruslike particles by recombinant structural proteins of adeno-associated virus type 2 in insect cells. J Virol, 66, 6922-30.

SHI, W., ARNOLD, G. S. & BARTLETT, J. S. (2001) Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptors. Hum Gene Ther, 12, 1697-711.

SHI, W. & BARTLETT, J. S. (2003) RGD inclusion in VP3 provides adeno-associated virus type 2 (AAV2)-based vectors with a heparan sulfate-independent cell entry mechanism. Mol Ther, 7, 515-25.

STACHLER, M. D. & BARTLETT, J. S. (2006) Mosaic vectors comprised of modified AAV1 capsid proteins for efficient vector purification and targeting to vascular endothelial cells. Gene Ther, 13,

926-31.

STEINBACH, S., WISTUBA, A., BOCK, T. & KLEINSCHMIDT, J. A. (1997) Assembly of adeno-associated virus type 2 capsids in vitro. J Gen Virol, 78 (Pt 6), 1453-62.

SZOMOLANYI-TSUDA, E., BRIEN, J. D., DORGAN, J. E., GARCEA, R. L., WOODLAND, R. T. & WELSH, R. M. (2001) Antiviral T-cell-independent type 2 antibody responses induced in vivo in the absence of T and NK cells. Virology, 280, 160-8.

SZOMOLANYI-TSUDA, E., BRIEN, J. D., DORGAN, J. E., WELSH, R. M. & GARCEA, R. L. (2000) The role of CD40-CD154 interaction in antiviral T cell-independent IgG responses. J Immunol, 164, 5877-82.

SZOMOLANYI-TSUDA, E., LE, Q. P., GARCEA, R. L. & WELSH, R. M. (1998) T-Cell-independent immunoglobulin G responses in vivo are elicited by live-virus infection but not by immunization with viral proteins or virus-like particles. J Virol, 72, 6665-70.

SZOMOLANYI-TSUDA, E. & WELSH, R. M. (1998) T-cell-independent antiviral antibody responses. Curr Opin Immunol, 10, 431-5.

WARD, P. & WALSH, C. E. (2009) Chimeric AAV Cap sequences alter gene transduction. Virology, 386, 237-48.

WARRINGTON, K. H., JR., GORBATYUK, O. S., HARRISON, J. K., OPIE, S. R., ZOLOTUKHIN, S. & MUZYCZKA, N. (2004) Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminus. J Virol, 78, 6595-609.

WISTUBA, A., KERN, A., WEGER, S., GRIMM, D. & KLEINSCHMIDT, J. A. (1997) Subcellular compartmentalization of adeno-associated virus type 2 assembly. J Virol, 71, 1341-52.

WISTUBA, A., WEGER, S., KERN, A. & KLEINSCHMIDT, J. A. (1995) Intermediates of adeno-associated virus type 2 assembly: identification of soluble complexes containing Rep and Cap proteins. J Virol, 69, 5311-9.

WU, P., XIAO, W., CONLON, T., HUGHES, J., AGBANDJE-MCKENNA, M., FERKOL, T., FLOTTE, T. & MUZYCZKA, N. (2000) Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. J Virol, 74, 8635-47.

XIE, Q., BU, W., BHATIA, S., HARE, J., SOMASUNDARAM, T., AZZI, A. & CHAPMAN, M. S. (2002) The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. Proc Natl Acad Sci U S A, 99, 10405-10.

XIE, Q., HARE, J., TURNIGAN, J. & CHAPMAN, M. S. (2004) Large-scale production, purification and crystallization of wild-type adeno-associated virus-2. J Virol Methods, 122, 17-27.

ZINKERNAGEL, R. M. (2002) Uncertainties - discrepancies in immunology. Immunological Reviews, 185, 103-125.

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO2008145401A [0002]
- WO2004027019A [0005]
- JP2001169777B [0009]
- US4168062A [0066] [0083]
- WO2008145400A [0123] [0127] [0131] [0270]
- WO0105990A [0145]
- WO0105991A [0145]
- US20040053410A [0168] [0168] [0178] [0179] [0181] [0268]
- US6846665B [0177] [0254] [0264]
- EP2008004366W [0264] [0277] [0277] [0281] [0283] [0288]

Non-patent literature cited in the description

- **FIELDS**VirologyLippincott Williams Wilkins20010000vol. 2, [0021]
- **E. W. MARTIN**Remington's Pharmaceutical SciencesMack Publishing Co.19750000 [0159]
- **ARNOLD, G. S.SASSER, A. K.STACHLER, M. D.BARTLETT, J. S.**Metabolic biotinylation provides a unique platform for the purification and targeting of multiple AAV vector serotypesMol Ther, 2006, vol. 14, 97-106 [0347]
- **ASOKAN, A.SAMULSKI, R. J.**AAV does the shuffleNat Biotechnol, 2006, vol. 24, 158-60 [0347]
- **BACHMANN, M. F.ROHRER, U. H.KUNDIG, T. M.BURKI, K.HENGARTNER, H.ZINKERNAGEL, R. M.**The influence of antigen organization on B cell responsivenessScience, 1993, vol. 262, 1448-51 [0347]
- **BECERRA, S. P.KOCZOT, F.FABISCH, P.ROSE, J. A.**Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiations from a single transcriptJ Virol, 1988, vol. 62, 2745-54 [0347]
- **BECERRA, S. P.ROSE, J. A.HARDY, M.BAROUDY, B. M.ANDERSON, C. W.**Direct mapping of adeno-associated virus capsid proteins B and C: a possible ACG initiation codonProc Natl Acad Sci USA, 1985, vol. 82, 7919-23 [0347]
- **CORPET, F.**Multiple sequence alignment with hierarchical clusteringNucleic Acids Res, 1988,

vol. 16, 10881-90 [0347]

- **GIROD, A.RIED, M.WOBUS, C.LAHM, H.LEIKE, K.KLEINSCHMIDT, J.DELEAGE, G.HALLEK, M.**Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2Nat Med, 1999, vol. 5, 1438- [0347]
- **GRIEGER, J. C.JOHNSON, J. S.GURDA-WHITAKER, B.AGBANDJE-MCKENNA, M.SAMULSKI, R. J.**Surface-exposed adeno-associated virus Vp1-NLS capsid fusion protein rescues infectivity of noninfectious wild-type Vp2/Vp3 and Vp3-only capsids but not that of fivefold pore mutant virionsJ Virol, 2007, vol. 81, 7833-43 [0347]
- **GRIEGER, J. C.SAMULSKI, R. J.**Adeno-associated virus as a gene therapy vector: vector development, production and clinical applicationsAdv Biochem Eng Biotechnol, 2005, vol. 99, 119-45 [0347]
- **GRIFMAN, M.TREPEL, M.SPEECE, P.GILBERT, L. B.ARAP, W.PASQUALINI, R.WEITZMAN, M. D.**Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsidsMol **T**her, 2001, vol. 3, 964-75 [0347]
- **GRIMM, D.**Production methods for gene transfer vectors based on adeno-associated virus serotypesMethods, 2002, vol. 28, 146-57 [0347]
- **GRIMM, D.KERN, A.PAWLITA, M.FERRARI, F.SAMULSKI, R.KLEINSCHMIDT, J.T**itration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2Gene **T**her, 1999, vol. 6, 1322-30 [0347]
- **GRIMM, D.KLEINSCHMIDT, J. A.**Progress in adeno-associated virus type 2 vector production: promises and prospects for clinical useHum Gene **T**her, 1999, vol. 10, 2445-50 [0347]
- **HEILBRONN, R.BURKLE, A.STEPHAN, S.ZUR HAUSEN, H.T**he adeno-associated virus rep gene suppresses herpes simplex virus-induced DNA amplificationJ Virol, 1990, vol. 64, 3012-8 [0347]
- **HOQUE, M.ISHIZU, K.MATSUMOTO, A.HAN, S. I.ARISAKA, F.TAKAYAMA, M.SUZUKI, K.KATO, K.KANDA, T.WATANABE, H.**Nuclear transport of the major capsid protein is essential for adeno-associated virus capsid formationJ Virol, 1999, vol. 73, 7912-5 [0347]
- **HOQUE, M.SHIMIZU, N.ISHIZU, K.YAJIMA, H.ARISAKA, F.SUZUKI, K.WATANABE, H.HANDA, H.**Chimeric virus-like particle formation of adeno-associated virusBiochem Biophys Res Commun, 1999, vol. 266, 371-6 [0347]
- **HUTTNER, N. A.GIROD, A.PERABO, L.EDBAUER, D.KLEINSCHMIDT, J. A.BUNING, H.HALLEK, M.**Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodiesGene **T**her, 2003, vol. 10, 2139-47 [0347]
- **KING, J. A.DUBIELZIG, R.GRIMM, D.KLEINSCHMIDT, J. A.**DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsidsEmbo J, 2001, vol. 20, 3282-91 [0347]
- **KOERBER, J. T.JANG, J. H.SCHAFFER, D. V.**DNA shuffling of adeno-associated virus yields functionally diverse viral progenyMol **T**her, 2008, vol. 16, 1703-9 [0347]
- **KOZAK, M.**Pushing the limits of the scanning mechanism for initiation of translationGene, 2002, vol. 299, 1-34 [0347]
- **KRONENBERG, S.KLEINSCHMIDT, J. A.BOTTCHER, B.**Electron cryo-microscopy and image reconstruction of adeno-associated virus type 2 empty capsidsEMBO Rep, 2001, vol. 2, 997-1002 [0347]
- **LAUGHLIN, C. A.TRATSCHIN, J. D.COON, H.CARTER, B. J.**Cloning of infectious adeno-

associated virus genomes in bacterial plasmidsGene, 1983, vol. 23, 65-73 [0347]

- **LI, W.ASOKAN, A.WU, Z.VAN DYKE, T.DIPRIMIO, N.JOHNSON, J. S.GOVINDASWAMY, L.AGBANDJE-MCKENNA, M.LEICHTLE, S.REDMOND, D. E., JR.**Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticlesMol Ther, 2008, vol. 16, 1252-60 [0347]
- **MAHESHRI, N.KOERBER, J. T.KASPAR, B. K.SCHAFFER, D. V.**Directed evolution of adeno-associated virus yields enhanced gene delivery vectorsNat Biotechnol, 2006, vol. 24, 198-204 [0347]
- **MITTEREDER, N.MARCH, K. L.TRAPNELL, B. C.**Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapyJ Virol, 1996, vol. 70, 7498-509 [0347]
- **MOSKALENKO, M.CHEN, L.VAN ROEY, M.DONAHUE, B. A.SNYDER, R. O.MCARTHUR, J. G.PATEL, S. D.**Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structureJ Virol, 2000, vol. 74, 1761-6 [0347]
- Parvoviridae: the viruses and their replication**MUZYCZKA, N.BERNS, K. I.**Fields VirologyLippincott-Raven20010000 [0347]
- **NICKLIN, S. A.BUENING, H.DISHART, K. L.DE ALWIS, M.GIROD, A.HACKER, U.THRASHER, A. J.ALI, R. R.HALLEK, M.BAKER, A. H.**Efficient and selective AAV2-mediated gene transfer directed to human vascular endothelial cellsMol Ther, 2001, vol. 4, 174-81 [0347]
- **NYGREN, P. A.SKERRA, A.**Binding proteins from alternative scaffoldsJ Immunol Methods, 2004, vol. 290, 3-28 [0347]
- **RABINOWITZ, J. E.XIAO, W.SAMULSKI, R. J.**Insertional mutagenesis of AAV2 capsid and the production of recombinant virusVirology, 1999, vol. 265, 274-85 [0347]
- **RIED, M. U.GIROD, A.LEIKE, K.BUNING, H.HALLEK, M.**Adeno-associated virus capsids displaying immunoglobulin-binding domains permit antibody-mediated vector retargeting to specific cell surface receptorsJ Virol, 2002, vol. 76, 4559-66 [0347]
- **RUFFING, M.HEID, H.KLEINSCHMIDT, J. A.**Mutations in the carboxy terminus of adeno-associated virus 2 capsid proteins affect viral infectivity: lack of an RGD integrin-binding motifJ Gen Virol, 1994, vol. 75, 3385-92 [0347]
- **RUFFING, M.ZENTGRAF, H.KLEINSCHMIDT, J. A.**Assembly of viruslike particles by recombinant structural proteins of adeno-associated virus type 2 in insect cellsJ Virol, 1992, vol. 66, 6922-30 [0347]
- **SHI, W.ARNOLD, G. S.BARTLETT, J. S.**Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptorsHum Gene Ther, 2001, vol. 12, 1697-711 [0347]
- **SHI, W.BARTLETT, J. S.**RGD inclusion in VP3 provides adeno-associated virus type 2 (AAV2)-based vectors with a heparan sulfate-independent cell entry mechanismMol Ther, 2003, vol. 7, 515-25 [0347]
- **STACHLER, M. D.BARTLETT, J. S.**Mosaic vectors comprised of modified AAV1 capsid proteins for efficient vector purification and targeting to vascular endothelial cellsGene Ther, 2006, vol. 13, 926-31 [0347]
- **STEINBACH, S.WISTUBA, A.BOCK, T.KLEINSCHMIDT, J. A.**Assembly of adeno-associated virus type 2 capsids in vitroJ Gen Virol, 1997, vol. 78, 1453-62 [0347]
- **SZOMOLANYI-TSUDA, E.BRIEN, J. D.DORGAN, J. E.GARCEA, R. L.WOODLAND, R. T.WELSH, R. M.**Antiviral T-cell-independent type 2 antibody responses induced in vivo in the

absence of **T** and NK cellsVirology, 2001, vol. 280, 160-8 [0347]

- **SZOMOLANYI-TSUDA, E.BRIEN, J. D.DORGAN, J. E.WELSH, R. M.GARCEA, R. L.T**he role of CD40-CD154 interaction in antiviral **T** cell-independent IgG responsesJ Immunol, 2000, vol. 164, 5877-82 [0347]
- **SZOMOLANYI-TSUDA, E.LE, Q. P.GARCEA, R. L.WELSH, R. M.T**-Cell-independent immunoglobulin G responses in vivo are elicited by live-virus infection but not by immunization with viral proteins or virus-like particlesJ Virol, 1998, vol. 72, 6665-70 [0347]
- **SZOMOLANYI-TSUDA, E.WELSH, R. M.T**-cell-independent antiviral antibody responsesCurr Opin Immunol, 1998, vol. 10, 431-5 [0347]
- **WARD, P.WALSH, C. E.**Chimeric AAV Cap sequences alter gene transductionVirology, 2009, vol. 386, 237-48 [0347]
- **WARRINGTON, K. H., JR.GORBATYUK, O. S.HARRISON, J. K.OPIE, S. R.ZOLOTUKHIN, S.MUZYCZKA, N.**Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminusJ Virol, 2004, vol. 78, 6595-609 [0347]
- **WISTUBA, A.KERN, A.WEGER, S.GRIMM, D.KLEINSCHMIDT, J. A**Subcellular compartmentalization of adeno-associated virus type 2 assemblyJ Virol, 1997, vol. 71, 1341-52 [0347]
- **WISTUBA, A.WEGER, S.KERN, A.KLEINSCHMIDT, J. A.**Intermediates of adeno-associated virus type 2 assembly: identification of soluble complexes containing Rep and Cap proteinsJ Virol, 1995, vol. 69, 5311-9 [0347]
- **WU, P.XIAO, W.CONLON, T.HUGHES, J.AGBANDJE-MCKENNA, M.FERKOL, T.FLOTTE, T.MUZYCZKA, N.**Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropismJ Virol, 2000, vol. 74, 8635-47 [0347]
- **XIE, Q.BU, W.BHATIA, S.HARE, J.SOMASUNDARAM, T.AZZI, A.CHAPMAN, M. S.T**he atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapyProc Natl Acad Sci U S A, 2002, vol. 99, 10405-10 [0347]
- **XIE, Q.HARE, J.TURNIGAN, J.CHAPMAN, M. S.**Large-scale production, purification and crystallization of wild-type adeno-associated virus-2J Virol Methods, 2004, vol. 122, 17-27 [0347]
- **ZINKERNAGEL, R. M.**Uncertainties - discrepancies in immunologyImmunological Reviews, 2002, vol. 185, 103-125 [0347]

Patentkrav

1. Nukleinsyre, der koder for et polypeptid betegnet som Assembly Activating Protein (AAP), idet polypeptidet omfatter en aminosyresekvens valgt fra gruppen bestående af SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21 og SEQ ID NO: 22, eller der koder for et polypeptid omfattende en funktionelt aktiv variant af en hvilken som helst af disse aminosyresekvenser, hvor den funktionelt aktive variant er i stand til at fremme samlingen af et parvoviral kapsid bestående hovedsageligt af VP3, og hvor den funktionelt aktive variant har en aminosyresekvens, der er mindst 60% identisk med en hvilken som helst af aminosyresekvenserne af SEQ ID NO: 1 til 22, hvor nukleinsyren ikke er i stand til at udtrykke et hvilket som helst af de funktionelle Rep-proteiner, VP1, VP2 og VP3.

2. Nukleinsyre ifølge krav 1,

a) **kendetegnet ved at** den inkluderer mere end 378 nukleotider af VP3 ORF;

b) **kendetegnet ved at** den inkluderer mindst 5 nukleotider af de tilstødende VP2-kodende nukleotider, som er placeret direkte opstrøms af det 5' af VP3-startkodonet, eller **kendetegnet ved at** startkodonet er et ATG ved 4 nukleotider, eller 24 nukleotider, eller 44 nukleotider nedstrøms af VP3-startkodonet;

c) **kendetegnet ved at** den er afledt fra AAV2 og dens translationsstartkodon er $C_{2729}TG$, $A_{2735}CG$, $A_{2717}TT$ eller $T_{2720}TG$, eller at den er afledt fra en anden parvovirus, og dens translationsstartkodon er ved det homologe sted til translationsstartkodonerne af AAV2;

d) omfattende en mutation, der genererer et ATG-startkodon, der muliggør forbedret translation af den åbne læseramme;

e) **kendetegnet ved at** den polypeptidkodende sekvens af nukleinsyren efterfølges af et poly(A)-signal;

f) omfattet i en ekspressions-kassette, -konstrukt, -vektor eller -cellelinje; og/eller

g) hvor nukleinsyren ikke er i stand til at udtrykke Rep40, Rep52, Rep68, Rep78, VP1, VP2 og VP3.

3. Polypeptid, kodet af en nukleinsyre ifølge krav 1 eller 2.

4. Polypeptid ifølge krav 3, hvor polypeptidet er Assembly Activating Protein (AAP).

5. Antistof, der specifikt binder et polypeptid ifølge krav 3.

6. Antistof ifølge krav 5, hvor det specifikt binder AAP af AAV2 (SEQ ID NO. 1).

7. Anvendelse af nukleinsyren ifølge krav 1 eller 2 til fremstilling af et polypeptid, som defineret i krav 3 eller 4.

8. Anvendelse af nukleinsyren ifølge krav 1 eller 2, eller polypeptidet ifølge krav 3 eller 4 til fremstilling af en parvoviral partikel.

9. Anvendelse af nukleinsyren eller polypeptidet som defineret i krav 8, til fremstilling af en parvoviral partikel, der ikke omfatter nogen af de funktionelle proteiner VP1, VP2 og Rep.

10. Fremgangsmåde til at producere parvovirale partikler bestående hovedsageligt af VP3, hvor kun 1/50 eller mindre af proteinerne, der samler kapsidet er N-terminalt forlængede versioner af VP3 eller fuldkommen anderledes proteiner, idet fremgangsmåden omfatter trinnene at

(i) tilvejebringe en celle, der er i stand til at udtrykke VP3 fra en VP3-kodende sekvens (cds) fra en parvovirus, hvor VP3 er under kontrol af en rep-uafhængig promoter og udtrykker et protein kodet af nukleinsyren ifølge krav 1 eller 2,

(ii) inkubere cellen ved betingelser, der bidrager til udtrykket af VP3 og proteinet fra nukleinsyren ifølge krav 1 eller 2, hvorved den parvovirale partikel produceres, og

(iii) eventuelt at oprense parvovirale partikler fra cellen,

hvor mindst 10^5 virus partikler dannes per celle
og ingen funktionelle VP1-, VP2- og Rep-proteiner udtrykkes i nævnte celle.

11. Fremgangsmåde ifølge krav 10,

a) hvor nukleinsyren ifølge krav 1 eller 2 tilvejebringes *in cis* i forhold til VP3-cds;

b) hvor nukleinsyren ifølge krav 1 eller 2 tilvejebringes *in trans* i forhold til VP3-cds;

c) hvor den parvovirale partikel ikke indeholder nogen af de funktionelle Rep-proteiner;

d) hvor den parvovirale partikel ikke indeholder Rep40, Rep52, Rep68 og Rep78; og/eller

e) hvor højest 1/100 af partiklerne indeholder DNA.

12. Fremgangsmåde ifølge et hvilket som helst af kravene 10 eller 11, hvor VP3-cds omfatter

a) mutation(er) valgt fra gruppen bestående af en deletion, en indsættelse, en substitution og en kombination deraf;

b) en eller flere stille mutation(er);

c) en eller flere mutation(er), der fører til en eller flere mutationer placeret på overfladen af en VP3 VLP;

d) en eller flere mutation(er), der fører til en eller flere indsættelser i en eller flere positioner valgt fra gruppen bestående af 1-261, 1-266, 1-381, 1-447, I-448, I-453, I-459, I-471, I-534, I-570, I-573, I-584, I-587, I-588, I-591, I-657, I-664, I-713 og I-716 eller til to indsættelser i to positioner valgt fra gruppen bestående af I-261, I-453, I-534, I-570, I-573 og I-587; eller til tre indsættelser i tre positioner af VP3; og/eller

e) en eller flere mutation(er), der fører til to indsættelser i position 1-453 i kombination med 1-587.

13. Parvoviral partikel bestående hovedsageligt af VP3, hvor kun 1/50 eller mindre af proteinerne, der samler kapsidet er N-terminalt forlængede versioner af VP3 eller fuldkommen forskellige proteiner,

(i) hvor VP3 eventuelt omfatter en eller flere mutation(er), og

(ii) hvor VP3 ikke indeholder et heterologt kernelokaliseringssignal, og

(iii) hvor partiklen ikke indeholder nogen af de funktionelle Rep-proteiner.

14. Parvoviral partikel ifølge krav 13, hvor kapsidet kun består af VP3, og/eller hvor mutation(erne) af VP3 er en mutation som beskrevet i krav 12.

15. Parvoviruspartikel ifølge krav 13 eller 14 til anvendelse som et medikament.

16. Parvoviruspartikel til anvendelse ifølge krav 15, hvor medikamentet yderligere omfatter en eller flere excipienser;

17. Parvoviruspartikel til anvendelse ifølge krav 15,

a) hvor medikamentet er en vaccine; og/eller

b) til anvendelse i forebyggelse eller behandling af en autoimmun sygdom, en infektionssygdom, en tumor sygdom, en allergisygdom, en stofskiftesygdom, en (kronisk) inflammatorisk sygdom, en neurologisk sygdom, afhængighed eller til anvendelse i oftalmologi.

DRAWINGS

Fig. 1

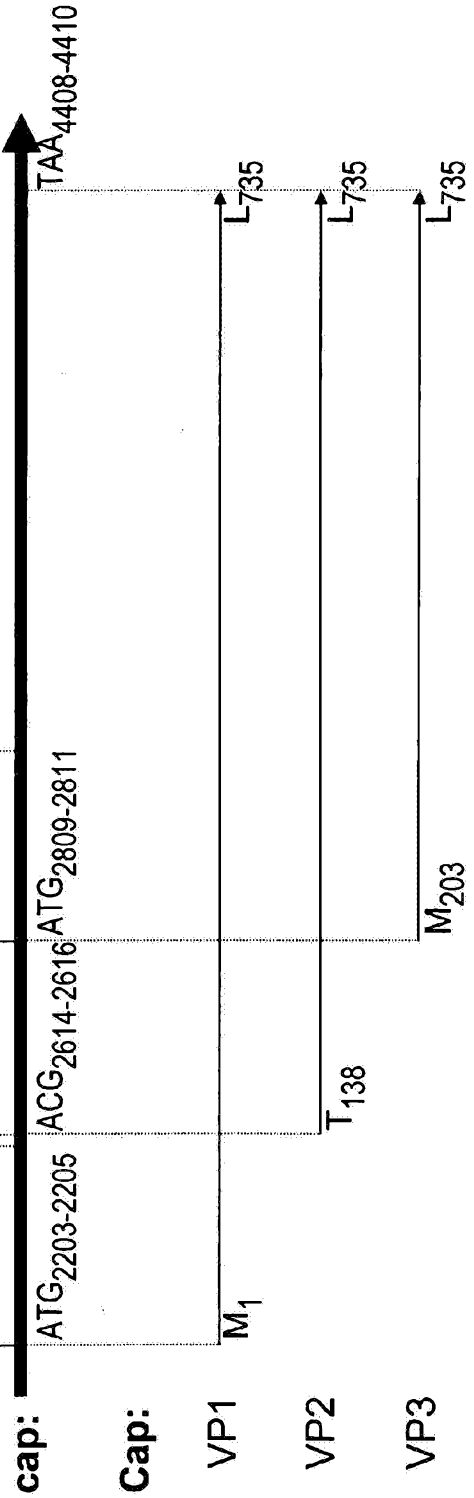
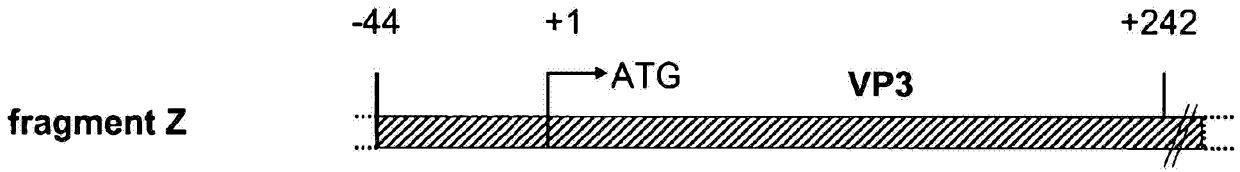


Fig. 2-1




```
AAV2 1 tcggacagcc accagcagcc ccctctggtc tgggaactaa tacgatggct acaggcagtg
    61 gcgcaccaat ggcagacaat aacgagggcg ccgacggagt gggtaattcc tcgggaaatt
   121 ggcattgcga ttccacatgg atgggcgaca gagtcatcac caccagcacc cgaacctggg
   181 ccctgcccac ctacaacaac cacctctaca aacaaatttc cagccaatca ggagcctcga
   241 acgacaatca ctactttggc tacagcaccc cttgggggta ttttga (SEQ ID NO: 144)

AAV1 1 tcggagaacc tccagcaacc cccgctgctg tgggacctac tacaatggct tcaggcggtg
    61 gcgcaccaat ggcagacaat aacgaaggcg ccgacggagt gggtaatgcc tcaggaaatt
   121 ggcattgcga ttccacatgg ctgggcgaca gagtcatcac caccagcacc cgcacctggg
   181 ccttgcccac ctacaataac cacctctaca agcaaatctc cagtgcttca acgggggcca
   241 gcaacgacaa ccactacttc ggctacagca ccccctgggg gtattt (SEQ ID NO: 47)
```

Fig. 2-2

```
AAV3b  1 tcggagaacc accagcagcc cccacaagtt tgggatctaa tacaatggct tcaggcggtg
       61 gcgcaccaat ggcagacaat aacgagggtg ccgatggagt gggtaattcc tcaggaaatt
      121 ggcattgcga ttcccaatgg ctgggcgaca gagtcatcac caccagcacc agaacctggg
      181 ccctgcccac ttacaacaac catctctaca agcaaatctc cagccaatca ggagcttcaa
      241 acgacaacca ctactttggc tacagcaccc cttgggggta ttttga (SEQ ID NO: 48)

AAV4   1 cccctgaggg atcaacttcc ggagccatgt ctgatgacag tgagatgcgt gcagcagctg
       61 gcggagctgc agtcgagggc ggacaaggtg ccgatggagt gggtaatgcc tcgggtgatt
      121 ggcattgcga ttccacctgg tctgagggcc acgtcacgac caccagcacc agaacctggg
      181 tcttgcccac ctacaacaac cacctctaca agcgactcgg agagagcctg cagtccaaca
      241 cctacaacgg attctccacc ccctggggat actttgactt caaccg (SEQ ID NO: 49)

AAV5   1 tgcaaatccc agcccaacca gcctcaagtt tgggagctga tacaatgtct gcgggaggtg
       61 gcggcccatt gggcgacaat aaccaaggtg ccgatggagt gggcaatgcc tcgggagatt
      121 ggcattgcga ttccacgtgg atgggggaca gagtcgtcac caagtccacc cgaacctggg
      181 tgctgcccag ctacaacaac caccagtacc gagagatcaa aagcggctcc gtcgacggaa
      241 gcaacgccaa cgcctacttt ggatacagca ccccctgggg gtactt (SEQ ID NO: 50)

AAV6   1 tcggagaacc tccagcaacc cccgctgctg tgggacctac tacaatggct tcaggcggtg
       61 gcgcaccaat ggcagacaat aacgaaggcg ccgacggagt gggtaatgcc tcaggaaatt
      121 ggcattgcga ttccacatgg ctgggcgaca gagtcatcac caccagcacc cgaacatggg
      181 ccttgcccac ctataacaac cacctctaca agcaaatctc cagtgcttca acgggggcca
      241 gcaacgacaa ccactacttc ggctacagca ccccctgggg gtattt (SEQ ID NO: 51)
```

Fig. 2-3

```
AAV7   1 ctagtgtggg atctggtaca gtggctgcag gcggtggcgc accaatggca gacaataacg
      61 aaggtgccga cggagtgggt aatgcctcag gaaattggca ttgcgattcc acatggctgg
     121 gcgacagagt cattaccacc agcacccgaa cctgggccct gcccacctac aacaaccacc
     181 tctacaagca aatctccagt gaaactgcag gtagtaccaa cgacaacacc tacttcggct
     241 acagcacccc ctgggggtat tttgacttta acagattcca ctgcca (SEQ ID NO: 52)

AAV8   1 tcggagaacc tccagcagcg ccctctggtg tgggacctaa tacaatggct gcaggcggtg
      61 gcgcaccaat ggcagacaat aacgaaggcg ccgacggagt gggtagttcc tcgggaaatt
     121 ggcattgcga ttccacatgg ctgggcgaca gagtcatcac caccagcacc cgaacctggg
     181 ccctgcccac ctacaacaac cacctctaca agcaaatctc caacgggaca tcgggaggag
     241 ccaccaacga caacacctac ttcggctaca gcacccccctg ggggta (SEQ ID NO: 53)

AAV10  1 tcggagaacc accagcaggc ccctctggtc tgggatctgg tacaatggct gcaggcggtg
      61 gcgctccaat ggcagacaat aacgaaggcg ccgacggagt gggtagttcc tcaggaaatt
     121 ggcattgcga ttccacatgg ctgggcgaca gagtcatcac caccagcacc cgaacctggg
     181 ccctgcccac ctacaacaac cacctctaca agcaaatctc caacgggaca tcgggaggaa
     241 gcaccaacga caacacctac ttcggctaca gcacccccctg ggggta (SEQ ID NO: 54)

AAV11  1 cccctgaagg atcagatacc agcgccatgt cttcagacat tgaaatgcgt gcagcaccgg
      61 gcggaaatgc tgtcgatgcg ggacaaggtt ccgatggagt gggtaatgcc tcgggtgatt
     121 ggcattgcga ttccacctgg tctgagggca aggtcacaac aacctcgacc agaacctggg
     181 tcttgcccac ctacaacaac cacttgtacc tgcgtctcgg aacaacatca agcagcaaca
     241 cctacaacgg attctccacc ccctggggat attttgactt caacag (SEQ ID NO: 55)
```

Fig. 2-4

```
b-AAV 1 ccccagaagg accatcttcc ggagctatgt ctactgagac tgaaatgcgt gcagcagctg
     61 gcggaaatgg tggcgatgcg ggacaaggtg ccgagggagt gggtaatgcc tccggtgatt
    121 ggcattgcga ttccacttgg tcagagagcc acgtcaccac cacctcaacc cgcacctggg
    181 tcctgccgac ctacaacaac cacctgtacc tgcggctcgg ctcgagcaac gccagcgaca
    241 ccttcaacgg attctccacc ccctggggat actttgactt taaccg (SEQ ID NO: 56)
```

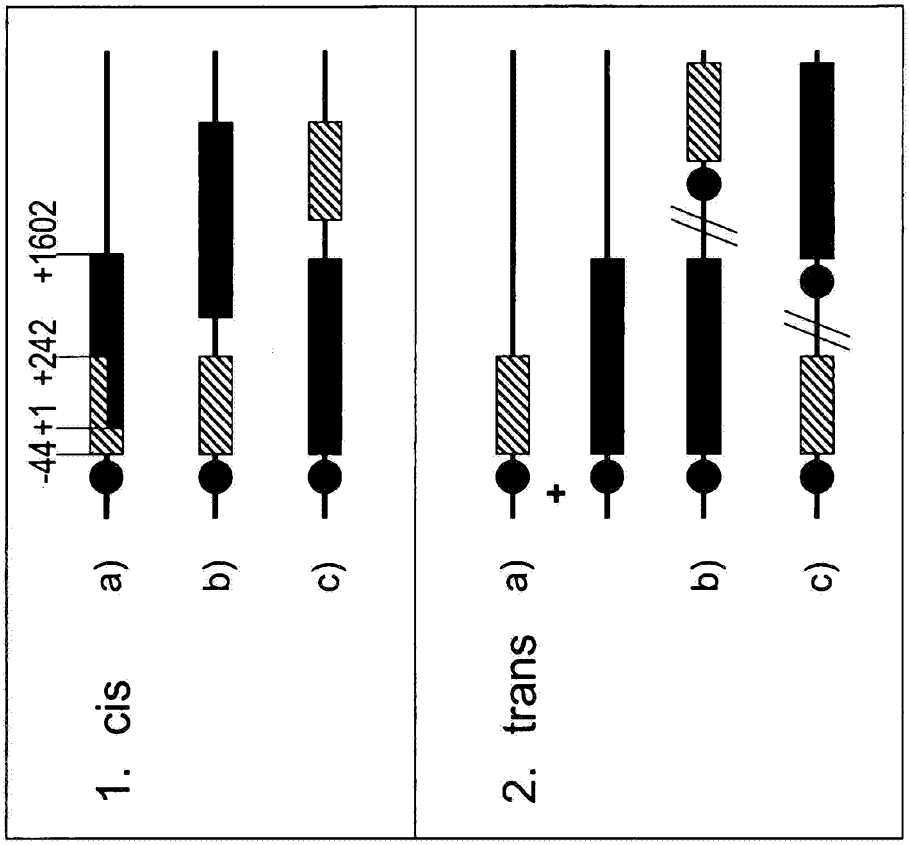
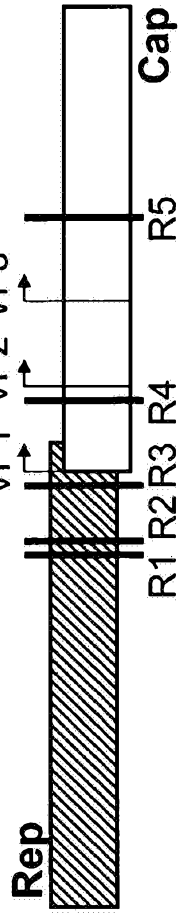


Fig. 3



Restriction site 1 (R1): FspI bp 1858

Restriction site 2 (R2): HindIII bp 1882

Restriction site 3 (R3): DraI bp 2193

Restriction site 4 (R4): EcoNI bp 2596

Restriction site 5 (R5): BsiWI bp 3254

Fig. 4

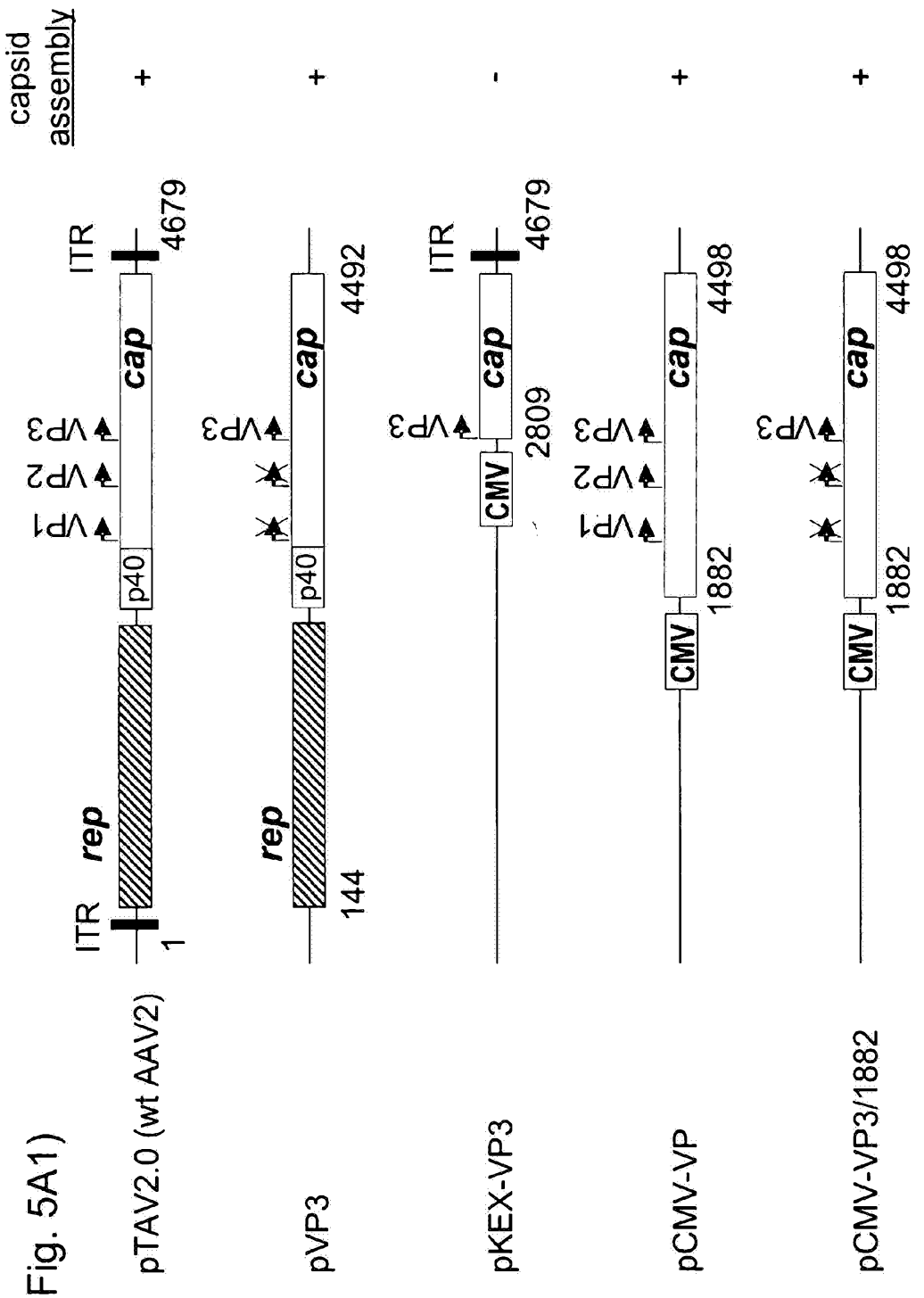

capsid assembly
+
+
-
+
+
ITR
rep
cap
p40
VP1
VP2
VP3
CMV
1
144
2809
1882
4492
4498
4679
pTAV2.0 (wt AAV2)
pVP3
pKEX-VP3
pCMV-VP
pCMV-VP3/1882

Fig. 5A1)

Fig. 5A2)

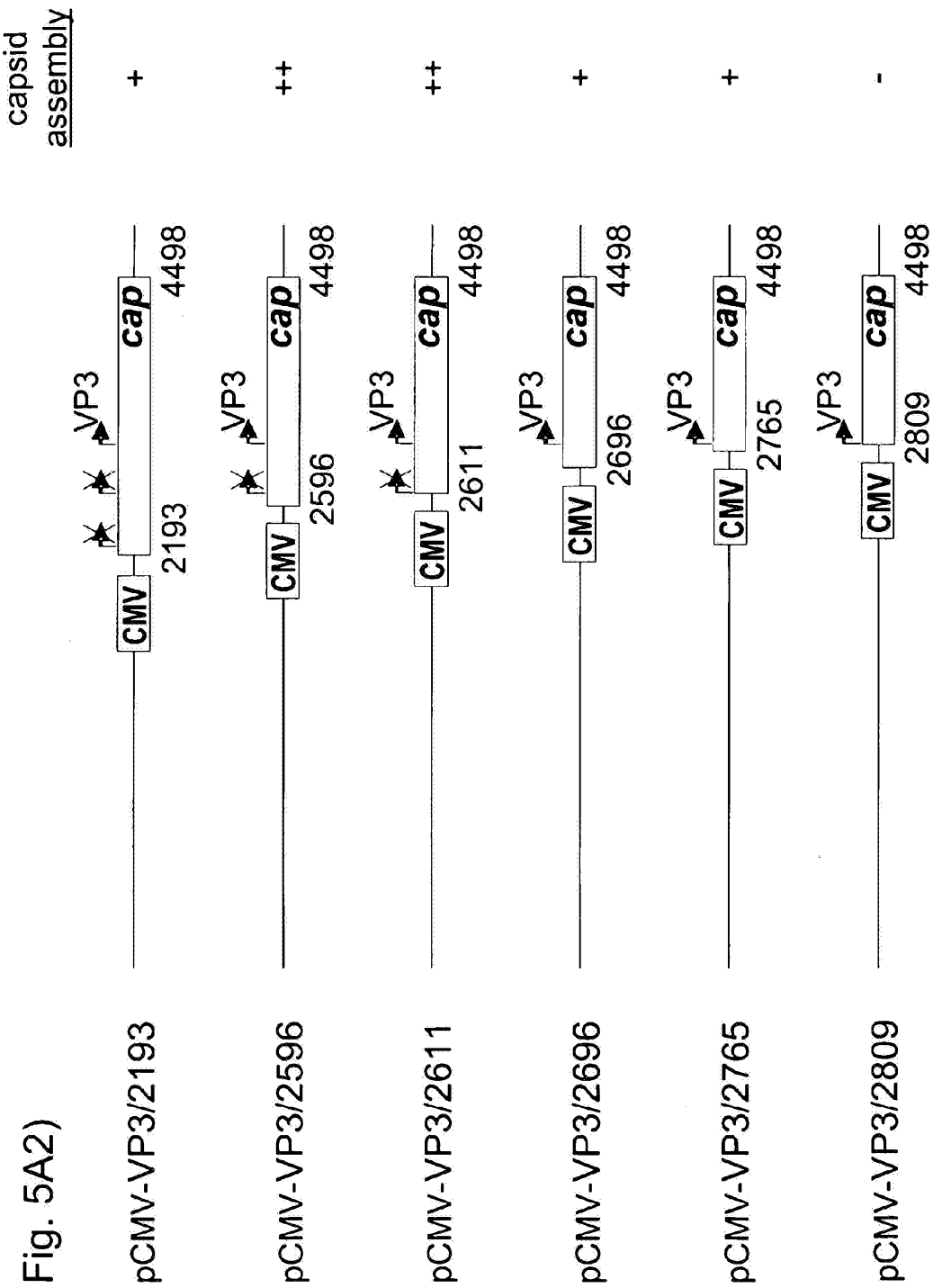

capsid assembly
+
++
++
+
+
-
pCMV-VP3/2193
pCMV-VP3/2596
pCMV-VP3/2611
pCMV-VP3/2696
pCMV-VP3/2765
pCMV-VP3/2809
CMV
VP3
cap
2193
2596
2611
2696
2765
2809
4498

Fig. 5

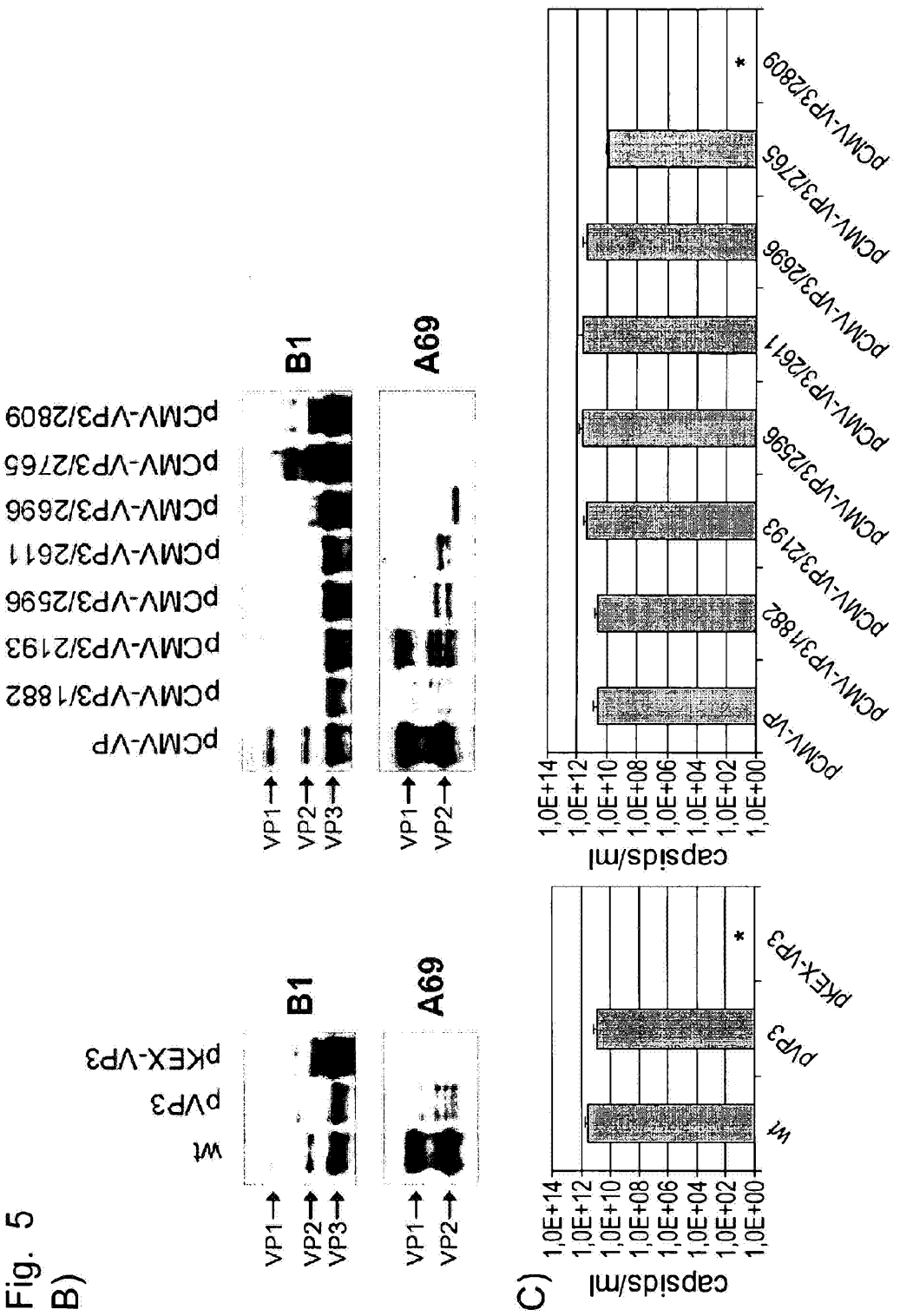
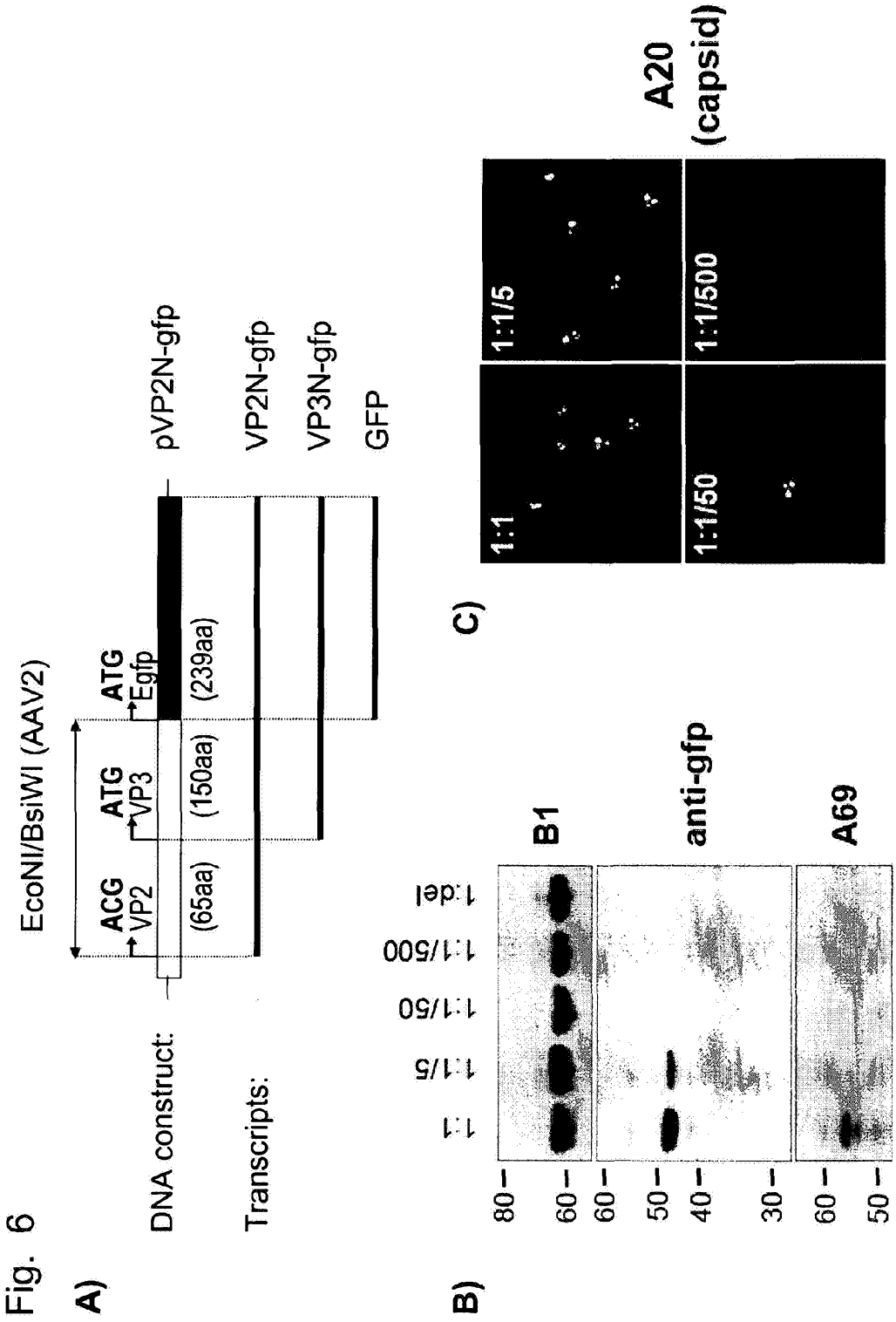

B)
wt
pVP3
pKEX-VP3
B1
A69
VP1→
VP2→
VP3→
pCMV-VP
pCMV-VP3/1882
pCMV-VP3/2193
pCMV-VP3/2596
pCMV-VP3/2611
pCMV-VP3/2696
pCMV-VP3/2765
pCMV-VP3/2809
C)
capsids/ml
1,0E+14
1,0E+12
1,0E+10
1,0E+08
1,0E+06
1,0E+04
1,0E+02
1,0E+00
*

Fig. 6


A)
EcoNI/BsiWI (AAV2)
ACG
VP2
(65aa)
ATG
VP3
(150aa)
ATG
Egfp
(239aa)
DNA construct:
Transcripts:
pVP2N-gfp
VP2N-gfp
VP3N-gfp
GFP
B)
B1
anti-gfp
A69
1:1
1:1/5
1:1/50
1:1/500
1:del
80
60
60
50
40
30
60
50
C)
A20
(capsid)
1:1
1:1/5
1:1/50
1:1/500

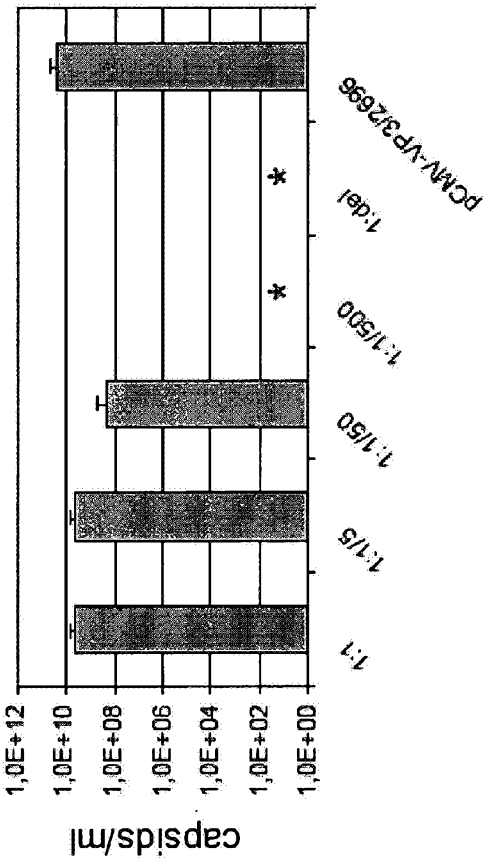

capsids/ml
1,0E+12
1,0E+10
1,0E+08
1,0E+06
1,0E+04
1,0E+02
1,0E+00
1:1
1:1/5
1:1/50
1:1/500
1:del
pCMV-VP3/2696
*
*

Fig. 6D)

1 = wt AAV2
2 = pCMV-VP3/2696
3 = pCMV-VP3/2808
+ pVP2N-gfp
A69
1
2
3
1 x 10¹¹
1 x 10¹⁰
4 x 10⁹
2 x 10⁹
1 x 10⁹
5 x 10⁸
2,5 x 10⁸
1 x 10¹¹
1 x 10¹¹
5 x 10¹¹
VP1
VP2
VP2 tru
VP2N-gfp

Fig. 7

Fig. 8A-1

```
========================================================================
 Aligned_sequences:
 1: pVP2N-gfp
 2: pVP2Ncm-gfp
 (from VP2 translation initation codon to BsiWI restriction site)
Identity DNA Sequence: 459/646 (71.1%)
Identity Codon Usage: 60/215 (27.9%)
Identity Protein Sequence: 215/215 (100%)
========================================================================
                10        20        30        40        50
VP2N     ACGGCTCCGGGAAAAAAGAGGCCGGTAGAGCACTCTCCTGTGGAGCCAGA
         ::::: :: :: :: ::  :::: :: ::::::   :: :::::::: ::
VP2Ncm   ACGGCCCCTGGCAAGAAACGGCCCGTGGAGCACAGCCCCGTGGAGCCCGA
                10        20        30        40        50
           T   A   P   G   K   K   R   P   V   E   H   S   P   V   E   P
                60        70        80        90       100
VP2N     CTCCTCCTCGGGAACCGGAAAGGCGGGCCAGCAGCCTGCAAGAAAAAGAT
         :   :   :   :: ::::: ::::: :: :::::::: :: :::::  :
VP2Ncm   CAGCAGCAGCGGCACCGGCAAGGCCGGACAGCAGCCCGCCAGAAAGCGGC
                60        70        80        90       100
         D   S   S   S   G   T   G   K   A   G   Q   Q   P   A   R   K   R
               110       120       130       140       150
VP2N     TGAATTTTGGTCAGACTGGAGACGCAGACTCAGTACCTGACCCCCAGCCT
         :::: :: :: ::::: :: ::::: ::    :: :: ::::: :::::
VP2Ncm   TGAACTTCGGCCAGACCGGCGACGCTGATAGCGTGCCCGACCCTCAGCCC
               110       120       130       140       150
          L   N   F   G   Q   T   G   D   A   D   S   V   P   D   P   Q   P
```

Fig. 8A-2

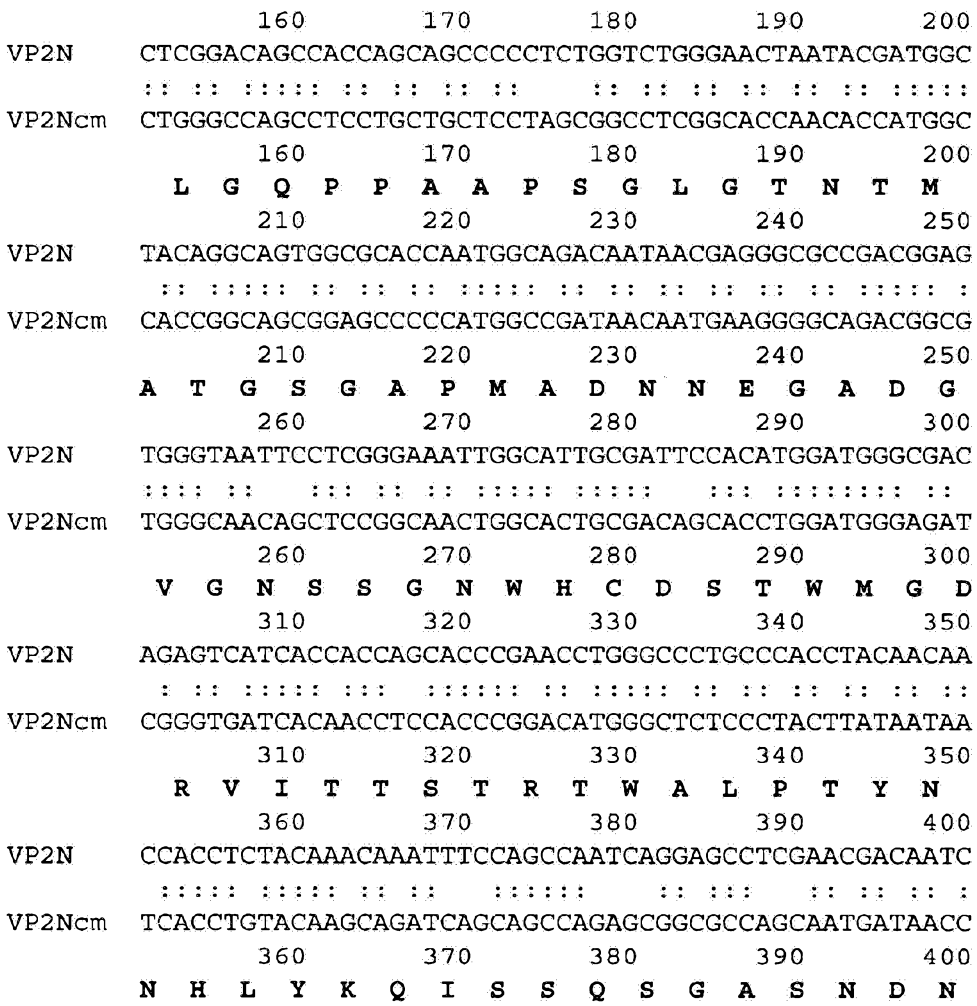

160 170 180 190 200
VP2N CTCGGACAGCCACCAGCAGCCCCCTCTGGTCTGGGAACTAATACGATGGC
VP2Ncm CTGGGCCAGCCTCCTGCTGCTCCTAGCGGCCTCGGCACCAACACCATGGC
160 170 180 190 200
L G Q P P A A P S G L G T N T M
210 220 230 240 250
VP2N TACAGGCAGTGGCGCACCAATGGCAGACAATAACGAGGGCGCCGACGGAG
VP2Ncm CACCGGCAGCGGAGCCCCCATGGCCGATAACAATGAAGGGGCAGACGGCG
210 220 230 240 250
A T G S G A P M A D N N E G A D G
260 270 280 290 300
VP2N TGGGTAATTCCTCGGGAAATTGGCATTGCGATTCCACATGGATGGGCGAC
VP2Ncm TGGGCAACAGCTCCGGCAACTGGCACTGCGACAGCACCTGGATGGGAGAT
260 270 280 290 300
V G N S S G N W H C D S T W M G D
310 320 330 340 350
VP2N AGAGTCATCACCACCAGCACCCGAACCTGGGCCCTGCCCACCTACAACAA
VP2Ncm CGGGTGATCACAACCTCCACCCGGACATGGGCTCTCCCTACTTATAATAA
310 320 330 340 350
R V I T T S T R T W A L P T Y N
360 370 380 390 400
VP2N CCACCTCTACAAACAAATTTCCAGCCAATCAGGAGCCTCGAACGACAATC
VP2Ncm TCACCTGTACAAGCAGATCAGCAGCCAGAGCGGCGCCAGCAATGATAACC
360 370 380 390 400
N H L Y K Q I S S Q S G A S N D N

Fig. 8A-3

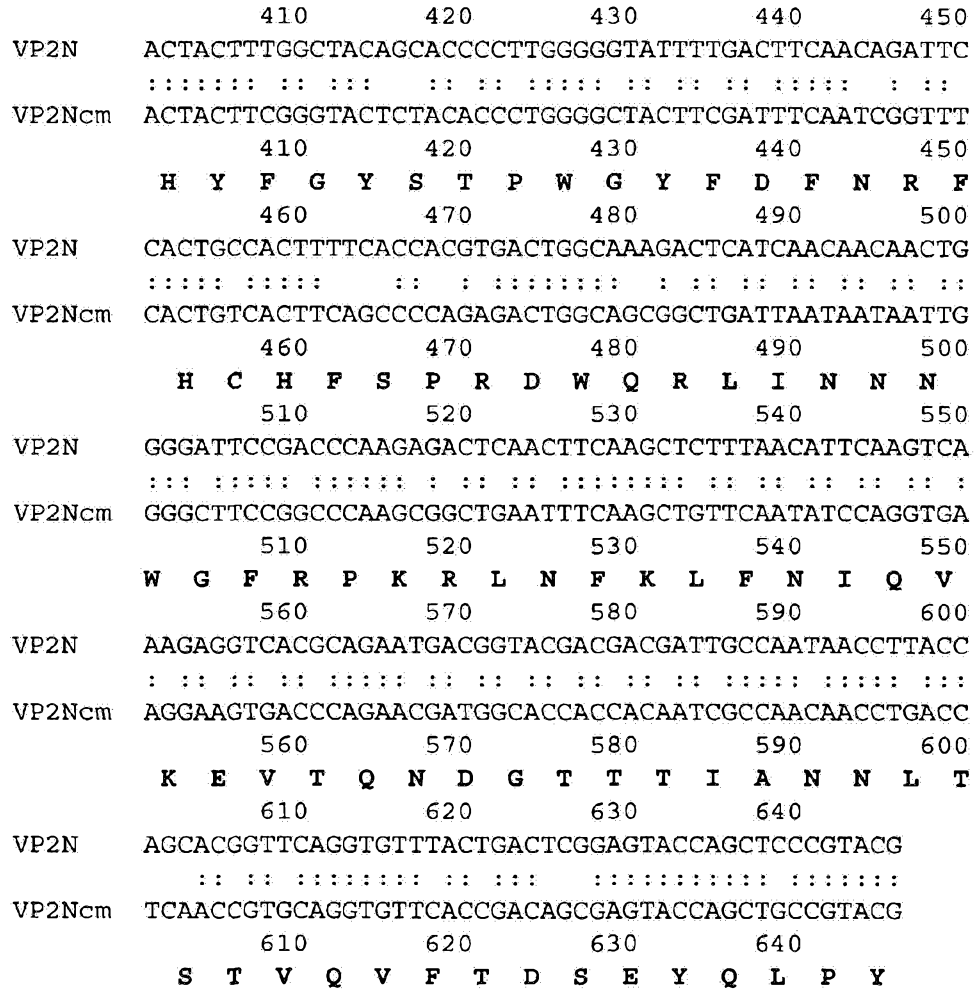

410 420 430 440 450
VP2N ACTACTTTGGCTACAGCACCCCTTGGGGGTATTTTGACTTCAACAGATTC
VP2Ncm ACTACTTCGGGTACTCTACACCCTGGGGCTACTTCGATTTCAATCGGTTT
410 420 430 440 450
H Y F G Y S T P W G Y F D F N R F
460 470 480 490 500
VP2N CACTGCCACTTTTCACCACGTGACTGGCAAAGACTCATCAACAACAACTG
VP2Ncm CACTGTCACTTCAGCCCCAGAGACTGGCAGCGGCTGATTAATAATAATTG
460 470 480 490 500
H C H F S P R D W Q R L I N N N
510 520 530 540 550
VP2N GGGATTCCGACCCAAGAGACTCAACTTCAAGCTCTTTAACATTCAAGTCA
VP2Ncm GGGCTTCCGGCCCAAGCGGCTGAATTTCAAGCTGTTCAATATCCAGGTGA
510 520 530 540 550
W G F R P K R L N F K L F N I Q V
560 570 580 590 600
VP2N AAGAGGTCACGCAGAATGACGGTACGACGACGATTGCCAATAACCTTACC
VP2Ncm AGGAAGTGACCCAGAACGATGGCACCACCACAATCGCCAACAACCTGACC
560 570 580 590 600
K E V T Q N D G T T T I A N N L T
610 620 630 640
VP2N AGCACGGTTCAGGTGTTTACTGACTCGGAGTACCAGCTCCCGTACG
VP2Ncm TCAACCGTGCAGGTGTTCACCGACAGCGAGTACCAGCTGCCGTACG
610 620 630 640
S T V Q V F T D S E Y Q L P Y

Fig. 8

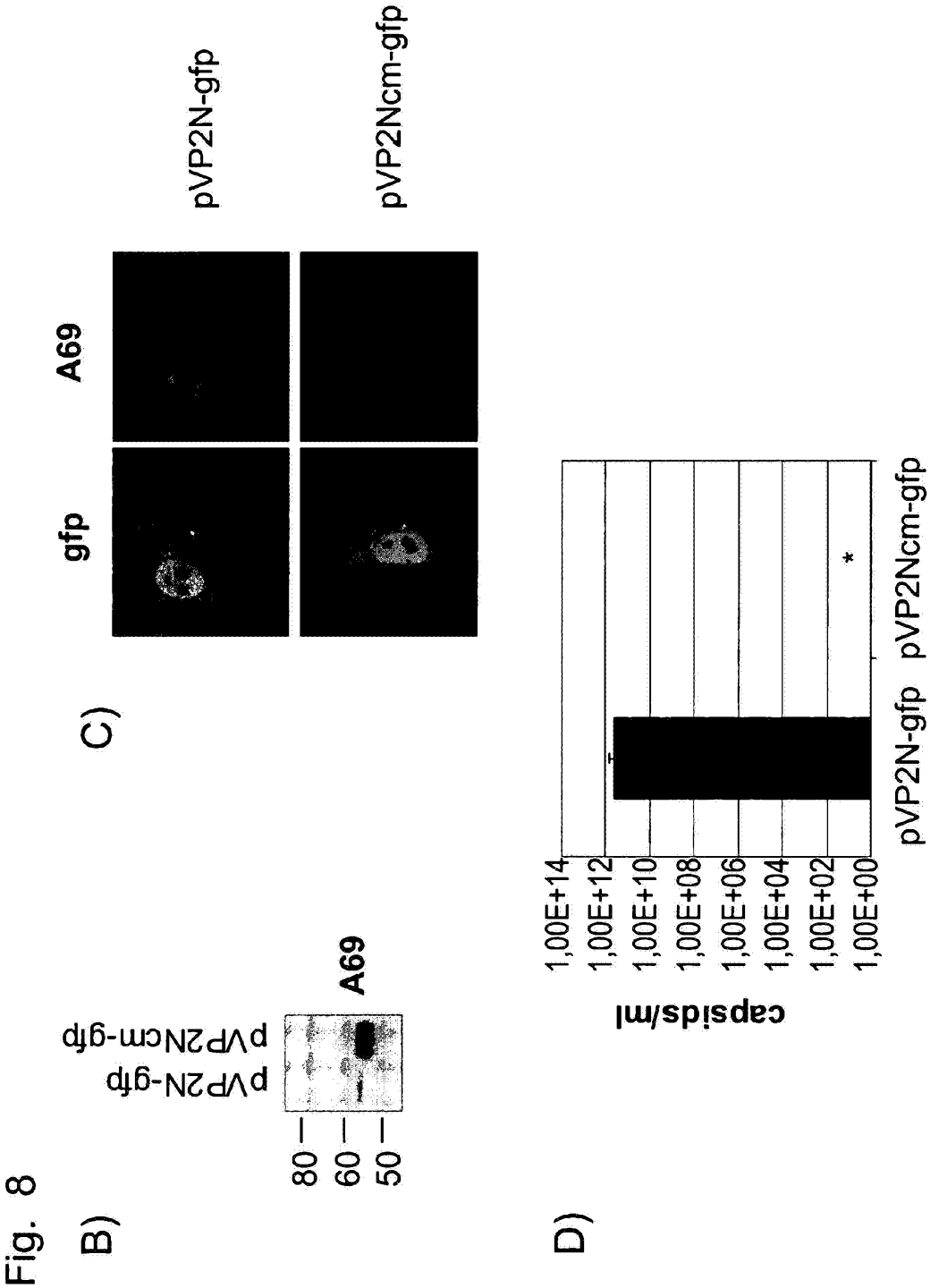

B)
A69
pVP2N-gfp
pVP2Ncm-gfp
80
60
50
C)
A69
gfp
pVP2N-gfp
pVP2Ncm-gfp
D)
capsids/ml
1,00E+14
1,00E+12
1,00E+10
1,00E+08
1,00E+06
1,00E+04
1,00E+02
1,00E+00
*
pVP2N-gfp
pVP2Ncm-gfp

Fig. 9

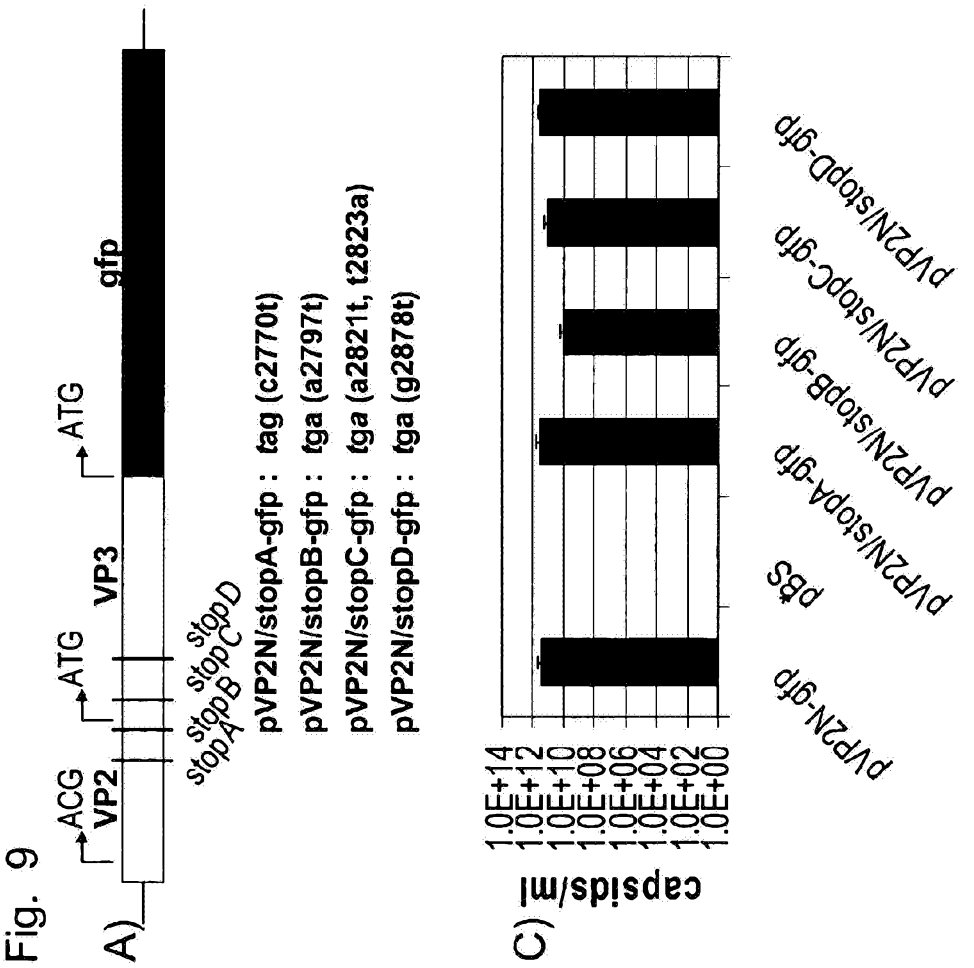

A)
ACG
VP2
ATG
VP3
ATG
gfp
stopA stopB stopC stopD
pVP2N/stopA-gfp : tag (c2770t)
pVP2N/stopB-gfp : tga (a2797t)
pVP2N/stopC-gfp : tga (a2821t, t2823a)
pVP2N/stopD-gfp : tga (g2878t)
B)
B1
A69
pVP2N- gfp
pBS
pVP2N/stopA- gfp
pVP2N/stopB- gfp
pVP2N/stopC- gfp
pVP2N/stopD- gfp
80
60
60
50
40
30
20
15
C)
capsids/ml
1.0E+14
1.0E+12
1.0E+10
1.0E+08
1.0E+06
1.0E+04
1.0E+02
1.0E+00
pVP2N-gfp
pBS
pVP2N/stopA-gfp
pVP2N/stopB-gfp
pVP2N/stopC-gfp
pVP2N/stopD-gfp

Fig. 10

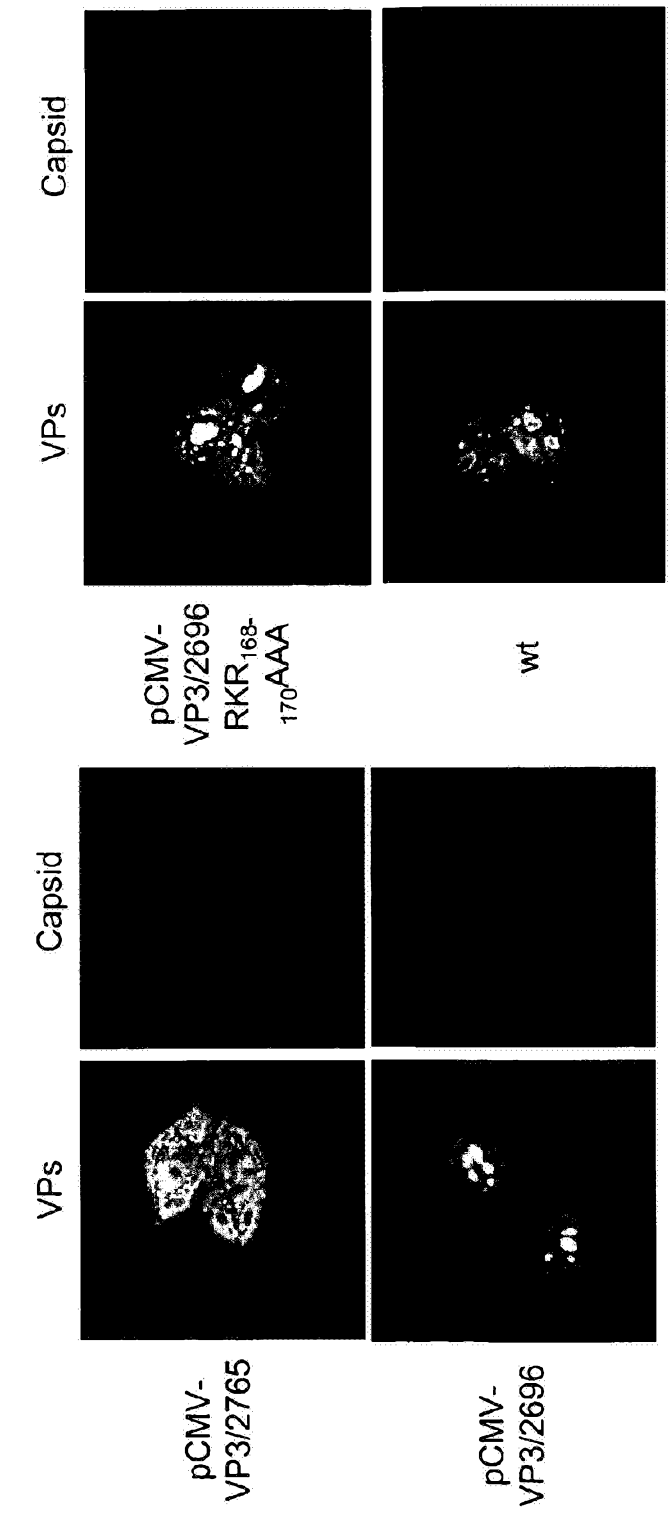

VPs
Capsid
pCMV-VP3/2765
pCMV-VP3/2696
pCMV-VP3/2696 RKR168-170AAA
wt

Fig. 11

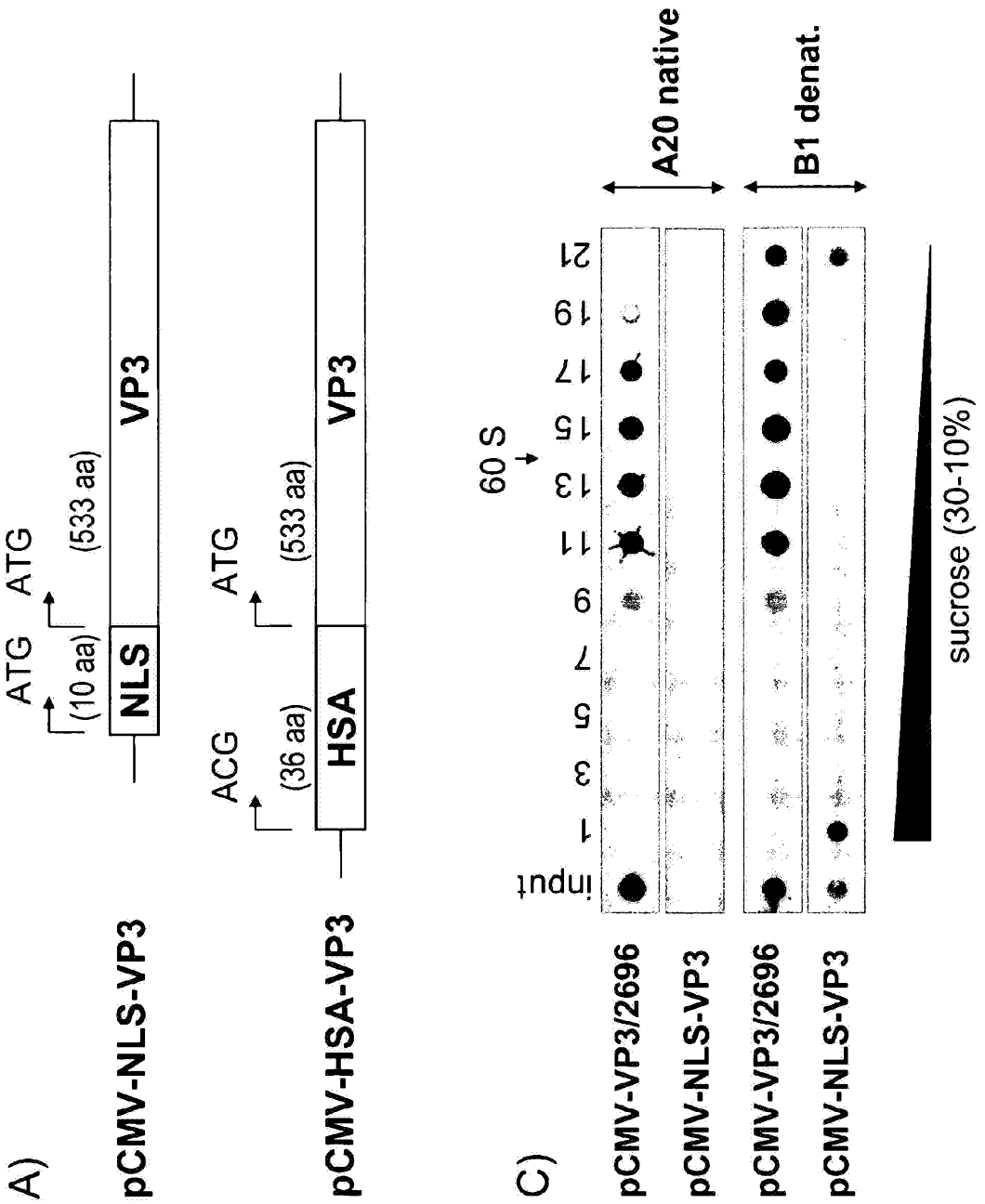
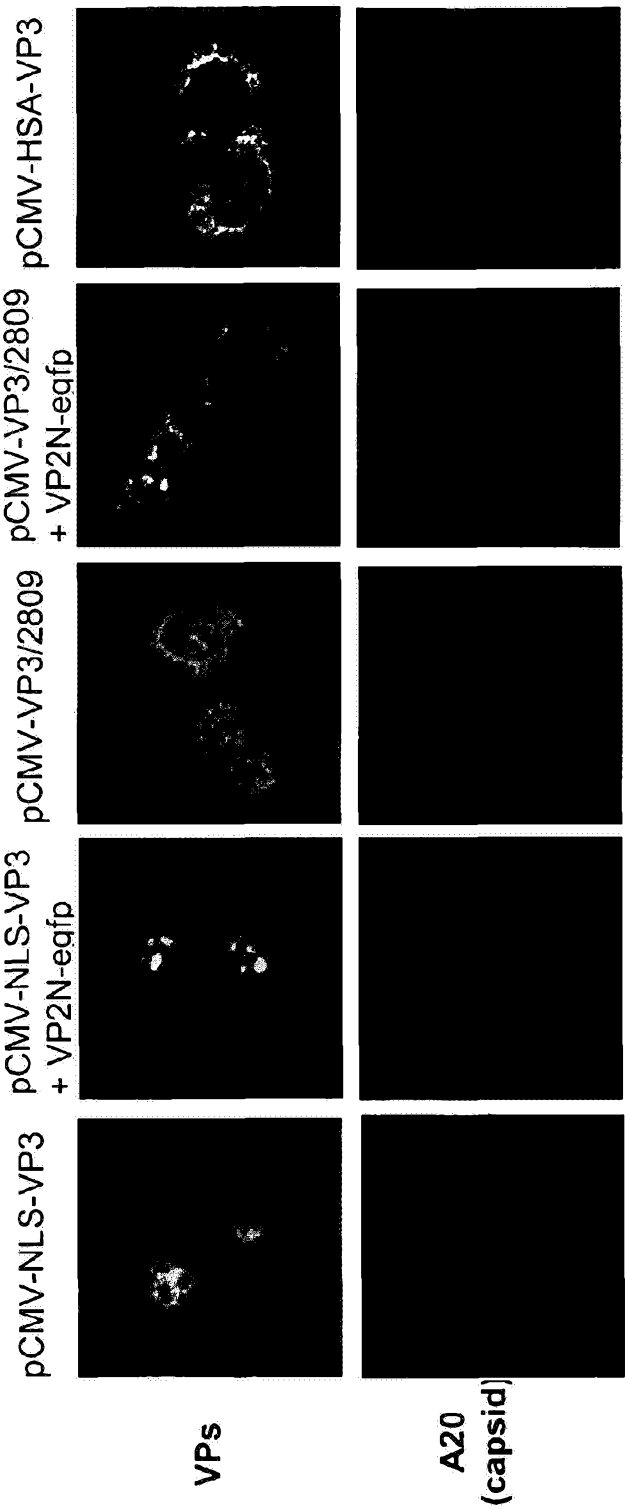

A)
pCMV-NLS-VP3
ATG
ATG
(10 aa)
NLS
(533 aa)
VP3
pCMV-HSA-VP3
ACG
ATG
(36 aa)
HSA
(533 aa)
VP3
C)
60 S
input
1
3
5
7
9
11
13
15
17
19
21
pCMV-VP3/2696
pCMV-NLS-VP3
pCMV-VP3/2696
pCMV-NLS-VP3
A20 native
B1 denat.
sucrose (30-10%)

Fig. 11B)


pCMV-NLS-VP3
pCMV-NLS-VP3 + VP2N-eqfp
pCMV-VP3/2809
pCMV-VP3/2809 + VP2N-eqfp
pCMV-HSA-VP3
VPs
A20 (capsid)

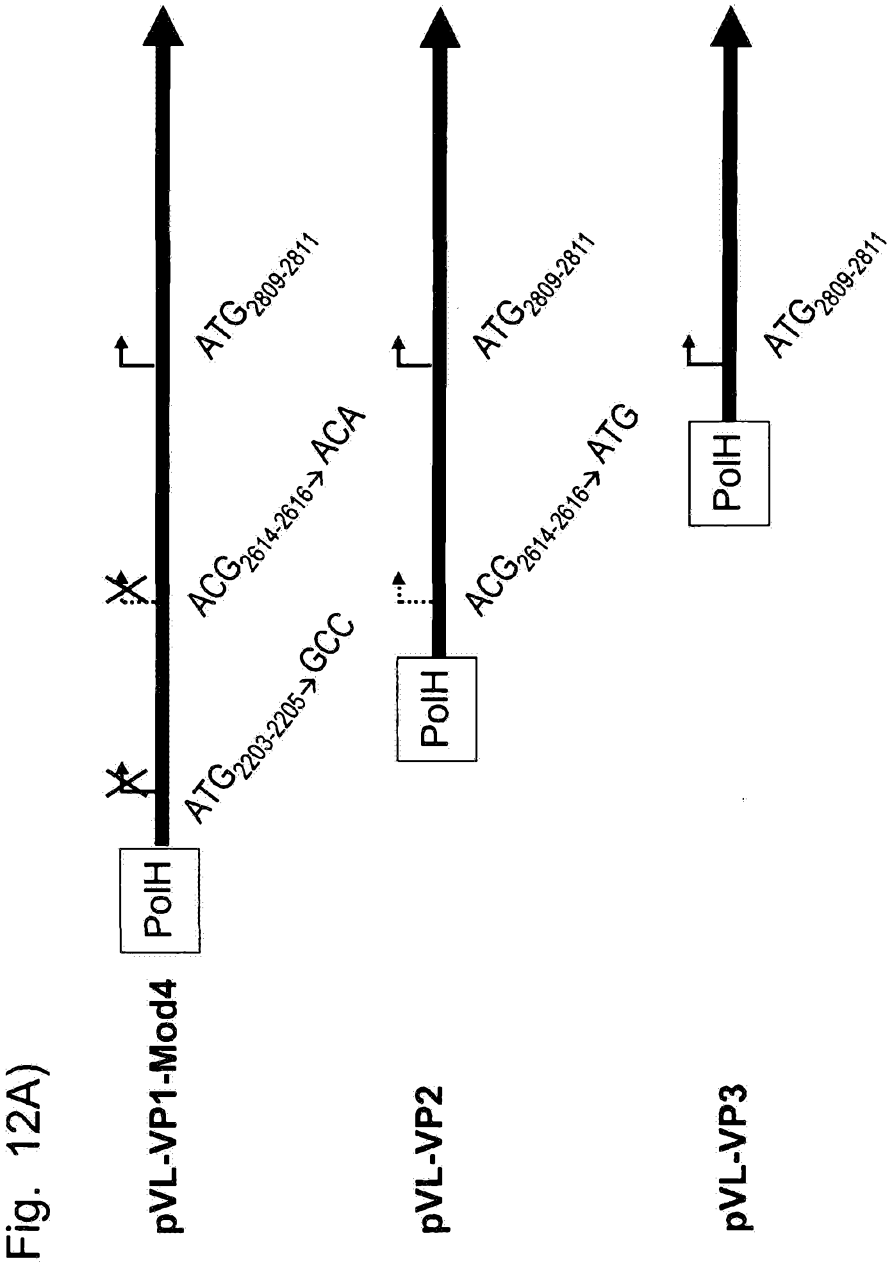

pVL-VP1-Mod4
PolH
ATG2203-2205→GCC
ACG2614-2616→ACA
ATG2809-2811
pVL-VP2
PolH
ACG2614-2616→ATG
ATG2809-2811
pVL-VP3
PolH
ATG2809-2811

Fig. 12A)


pVL_VP3
VP1
VP2
VP3
250 kD
148 kD
98 kD
64 kD
50 kD
36 kD
22 kD
16 kD
pVL_VP2
VP1
VP2
VP3
250 kD
148 kD
98 kD
64 kD
50 kD
36 kD
22 kD
16 kD
pVL_VP1_MOD4
250 kD
148 kD
98 kD
64 kD
50 kD
36 kD
22 kD
16 kD
VP1
VP2
VP3

Fig. 12B)

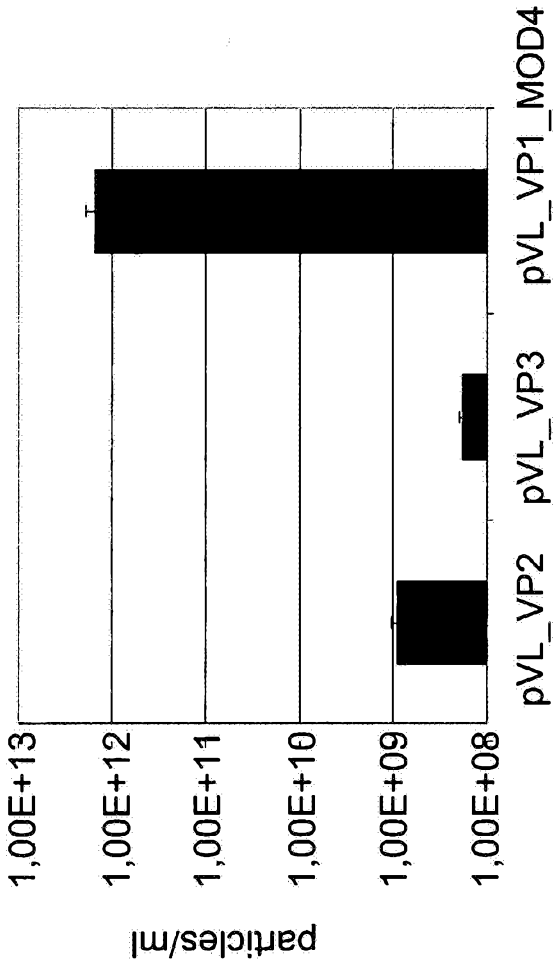
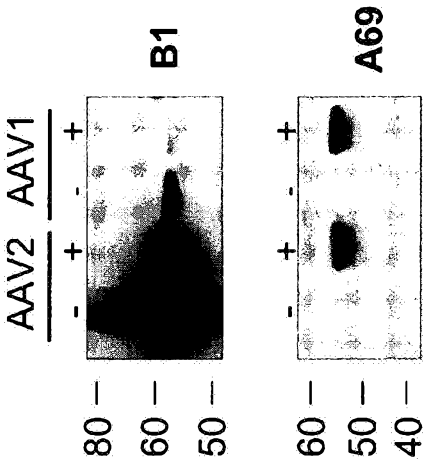

1,00E+13
1,00E+12
1,00E+11
1,00E+10
1,00E+09
1,00E+08
particles/ml
pVL_VP2
pVL_VP3
pVL_VP1_MOD4

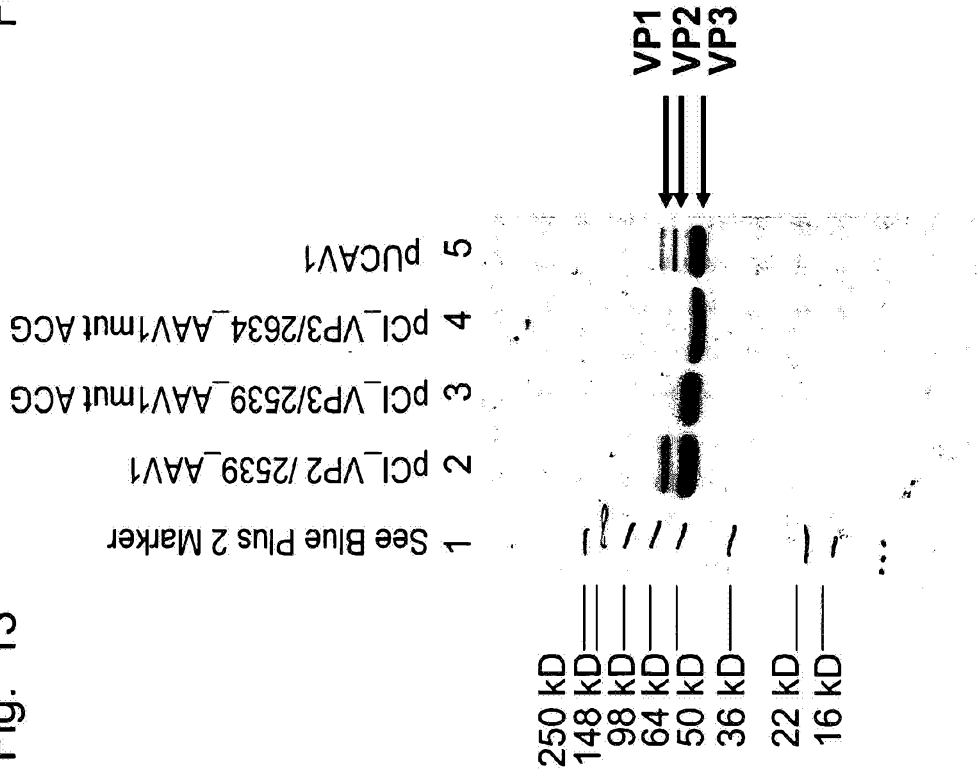
Fig. 12C)

Fig. 14



pVP2N-gfp

Fig. 13



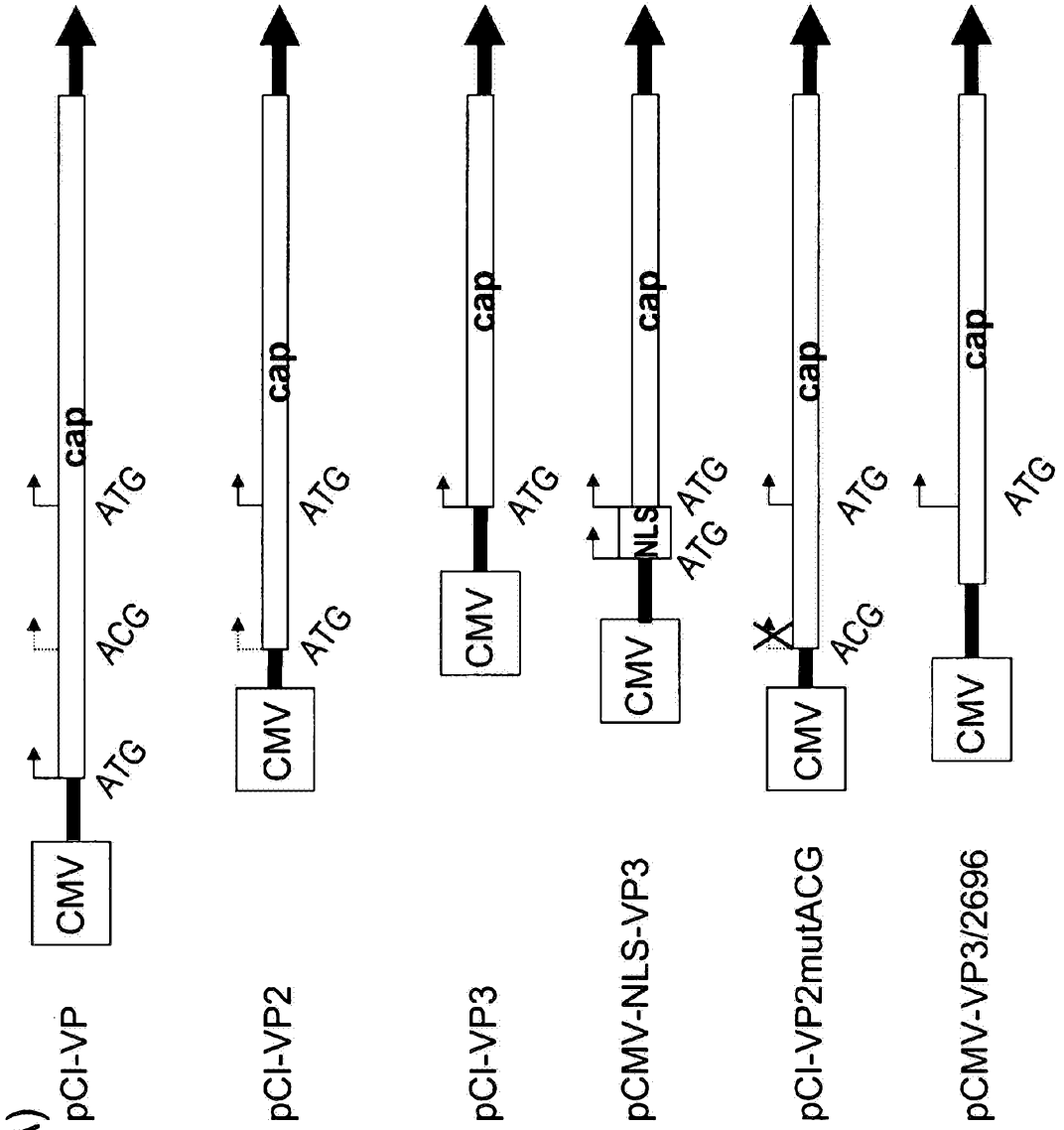

CMV
cap
ATG
ACG
ATG
pCI-VP
CMV
cap
ATG
ATG
pCI-VP2
CMV
cap
ATG
pCI-VP3
CMV
NLS
cap
ATG
ATG
pCMV-NLS-VP3
CMV
cap
ATG
ACG
pCI-VP2mutACG
CMV
cap
ATG
pCMV-VP3/2696

Fig. 15A)

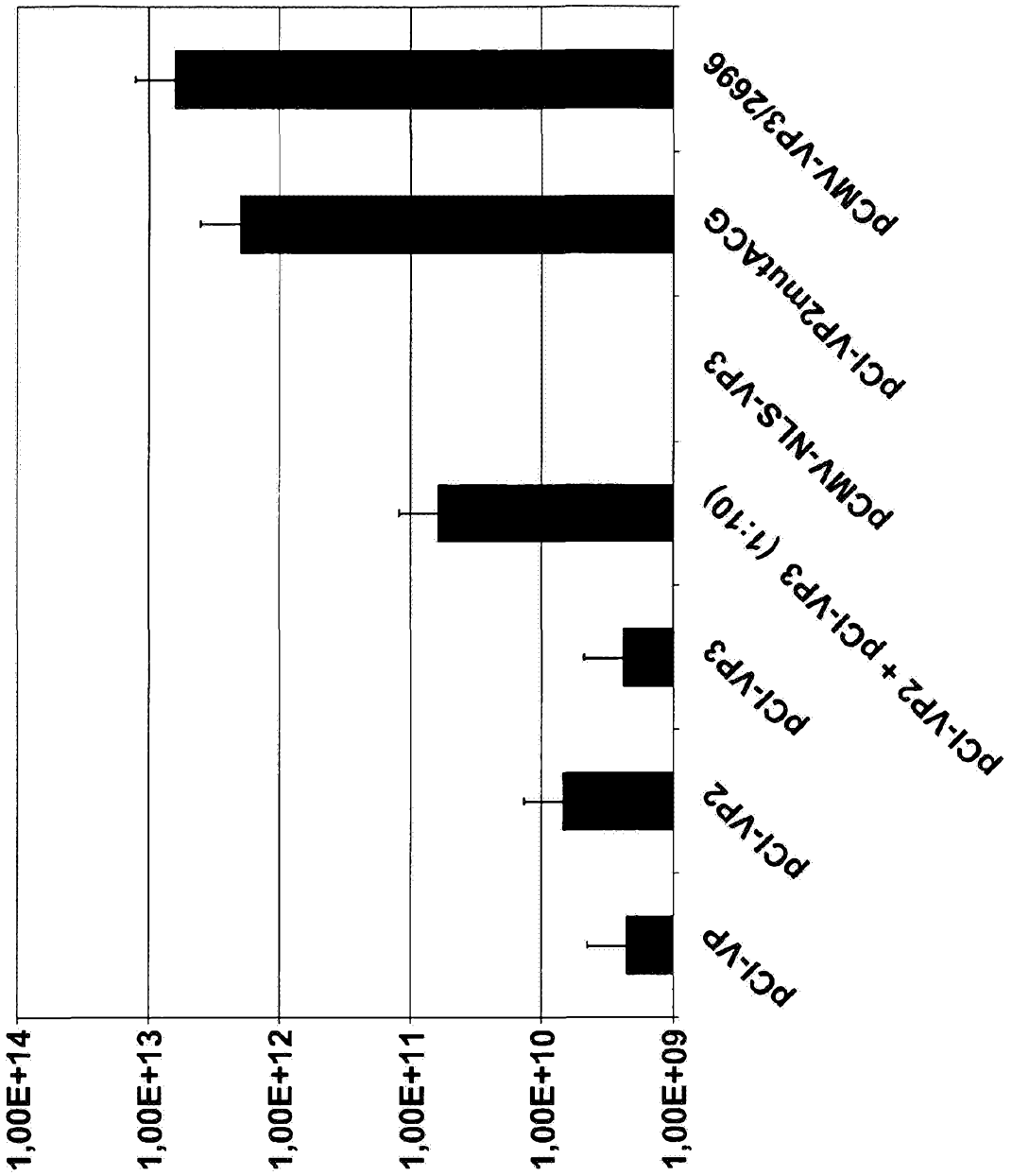

1,00E+14
1,00E+13
1,00E+12
1,00E+11
1,00E+10
1,00E+09
pCI-VP
pCI-VP2
pCI-VP3
pCI-VP2 + pCI-VP3 (1:10)
pCMV-NLS-VP3
pCI-VP2muACG
pCMV-VP3/2696

Fig. 15B)

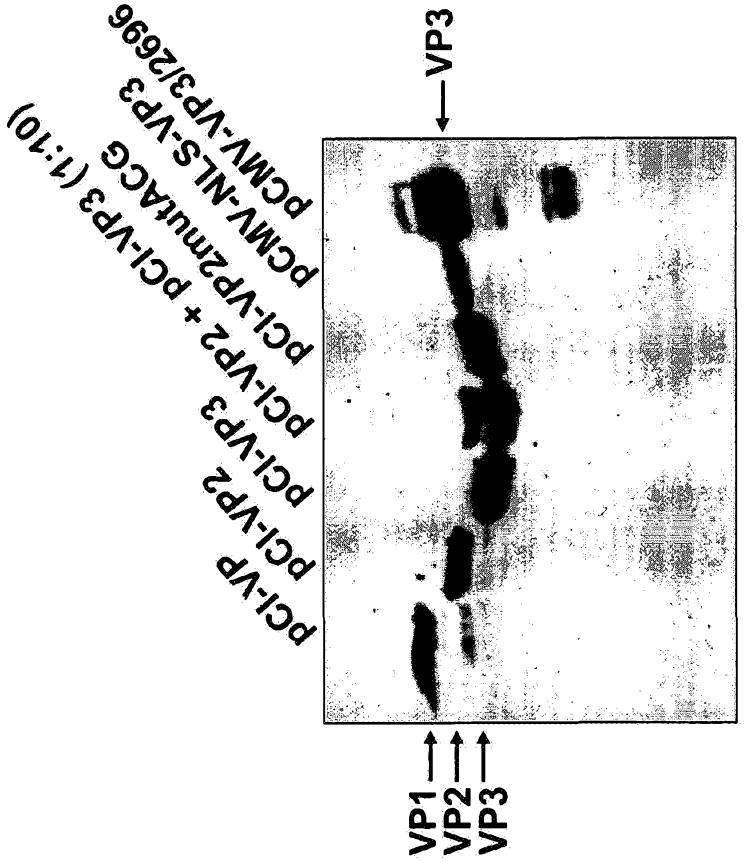

VP3
pCMV-VP3/2696
pCMV-NLS-VP3
pCI-VP2mutACG
pCI-VP2 + pCI-VP3 (1:10)
pCI-VP3
pCI-VP2
pCI-VP
VP1
VP2
VP3

Fig. 15C)

Fig. 16

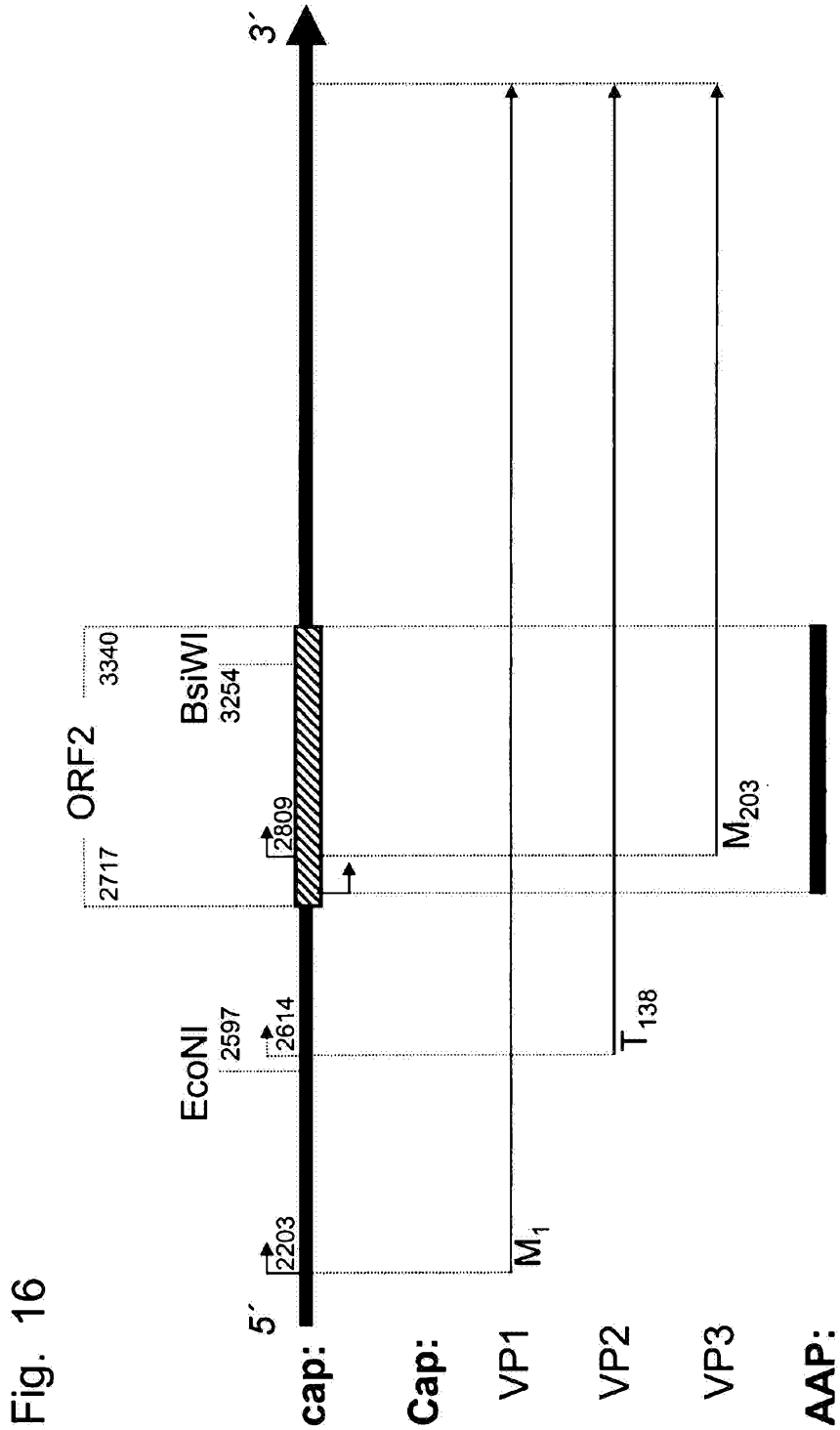
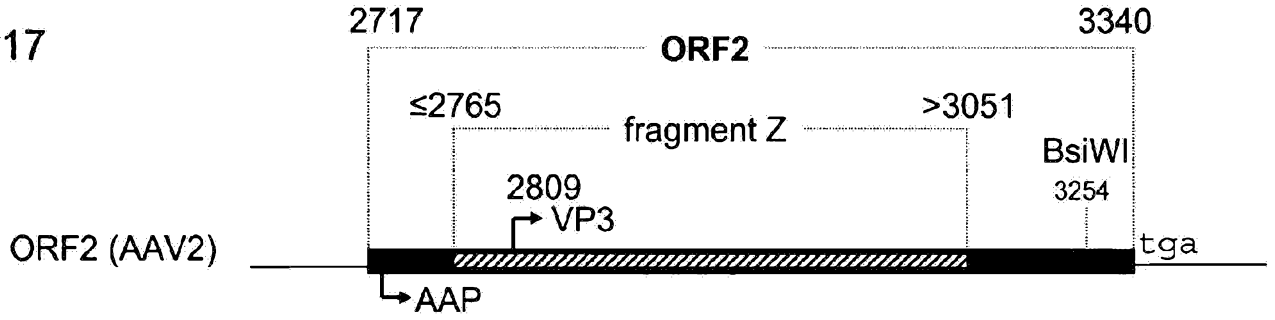

3´
5´
cap:
Cap:
VP1
VP2
VP3
AAP:
2203
EcoNI
2597
2614
2809
BsiWI
3254
ORF2
2717
3340
M1
T138
M203

Fig. 17




```
DNA 2717                    attt tggtcagact ggagacgcag actcagtacc tgacccccag
    2761 cctctcggac agccaccagc agccccctct ggtctgggaa ctaatacgat ggctacaggc
    2821 agtggcgcac caatggcaga caataacgag ggcgccgacg gagtgggtaa ttcctcggga
    2881 aattggcatt gcgattccac atggatgggc gacagagtca tcaccaccag cacccgaacc
    2941 tgggccctgc ccacctacaa caaccacctc tacaaacaaa tttccagcca atcaggagcc
    3001 tcgaacgaca atcactactt tggctacagc acccctgggg ggtattttga cttcaacaga
    3061 ttccactgcc acttttcacc acgtgactgg caaagactca tcaacaacaa ctggggattc
    3121 cgacccaaga gactcaactt caagctcttt aacattcaag tcaaagaggt cacgcagaat
    3181 gacggtacga cgacgattgc caataacctt accagcacgg ttcaggtgtt tactgactcg
    3241 gagtaccagc tcccgtacgt cctcggctcg gcgcatcaag gatgcctccc gccgttccca
    3301 gcagacgtct tcatggtgcc acagtatgga tacctcaccc tga (SEQ ID NO: 23)

AAP    1 ILVRLETQTQ YLTPSLSDSH QQPPLVWELI RWLQAVAHQW QTITRAPTEW
      51 VIPREIGIAI PHGWATESSP PAPEPGPCPP TTTTSTNKFP ANQEPRTTIT
     101 TLATAPLGGI LTSTDSTATF HHVTGKDSST TTGDSDPRDS TSSSLTFKSK
     151 RSRRMTVRRR LPITLPARFR CLLTRSTSSR TSSARRIKDA SRRSQQTSSW
     201 CHSMDTSP (SEQ ID NO: 1)
```

Fig. 18A)

```
ORF1cm     1  TTGAGGAACC TGTTAAGACG GCCCCTGGCA AGAAACGGCC CGTGGAGCAC
          51  AGCCCCGTGG AGCCCGACAG CAGCAGCGGC ACCGGCAAGG CCGGACAGCA
         101  GCCCGCCAGA AAGCGGCTGA↓ACTTCGGCCA GACCGGCGAC GCTGATAGCG
         151  TGCCCGACCC TCAGCCCCTG GGCCAGCCTC CTGCTGCTCC TAGCGGCCTC
         201  GGCACCAACA CCATGGCCAC CGGCAGCGGA GCCCCCATGG CCGATAACAA
         251  TGAAGGGGCA GACGGCGTGG GCAACAGCTC CGGCAACTGG CACTGCGACA
         301  GCACCTGGAT GGGAGATCGG GTGATCACAA CCTCCACCCG GACATGGGCT
         351  CTCCCTACTT ATAATAATCA CCTGTACAAG CAGATCAGCA GCCAGAGCGG
         401  CGCCAGCAAT GATAACCACT ACTTCGGGTA CTCTACACCC TGGGGCTACT
         451  TCGATTTCAA TCGGTTTCAC TGTCACTTCA GCCCCAGAGA CTGGCAGCGG
         501  CTGATTAATA ATAATTGGGG CTTCCGGCCC AAGCGGCTGA ATTTCAAGCT
         551  GTTCAATATC CAGGTGAAGG AAGTGACCCA GAACGATGGC ACCACCACAA
         601  TCGCCAACAA CCTGACCTCA ACCGTGCAGG TGTTCACCGA CAGCGAGTAC
         651  CAGCTGCCGT AC (SEQ ID NO: 57)
```

Fig. 18B)

```
ORF2cm     1  TTGAGGAACC TGTTAAGACG GCTCCGGGAA AAAAGAGGCC GGTAGAGCAC
           51  TCTCCTGTGG AGCCAGACTC CTCCTCGGGA ACCGGAAAGG CGGGCCAGCA
          101  GCCTGCAAGA AAAAGATTGA↓ATTTTGGTCA GACTGGAGAC GCAGACTCAG
          151  TACCTGACCC CCAGCCTCTC GGACAGCCAC CAGCAGCCCC CTCTGGTCTG
          201  GGAACTAATA CGATGGCTGC AGGCCGTGGC CCACCAGTGG CAGACCATCA
          251  CCAGAGCCCC CACCGAGTGG GTGATCCCTC GGGAGATCGG CATTGCCATC
          301  CCTCACGGCT GGGCCACAGA GTCTAGCCCT CCAGCCCCTG AGCCTGGCCC
          351  TTGTCCCCCT ACCACCACCA CCTCCACCAA CAAGTTCCCC GCCAACCAGG
          401  AACCCCGGAC CACCATCACC ACACTGGCCA CAGCCCCTCT GGGCGGCATC
          451  CTGACCAGCA CCGACAGCAC CGCCACCTTT CACCACGTGA CCGGCAAGGA
          501  CAGCAGCACC ACCACCGGCG ACAGCGACCC CAGAGACAGC ACCAGCTCCA
          551  GCCTGACCTT CAAGAGCAAG CGGAGCAGAC GGATGACCGT GCGGCGGAGA
          601  CTGCCTATCA CCCTGCCCGC CAGATTCCGG TGCCTGCTGA CCAGAAGCAC
          651  CAGCAGCCGT AC (SEQ ID NO: 58)
```

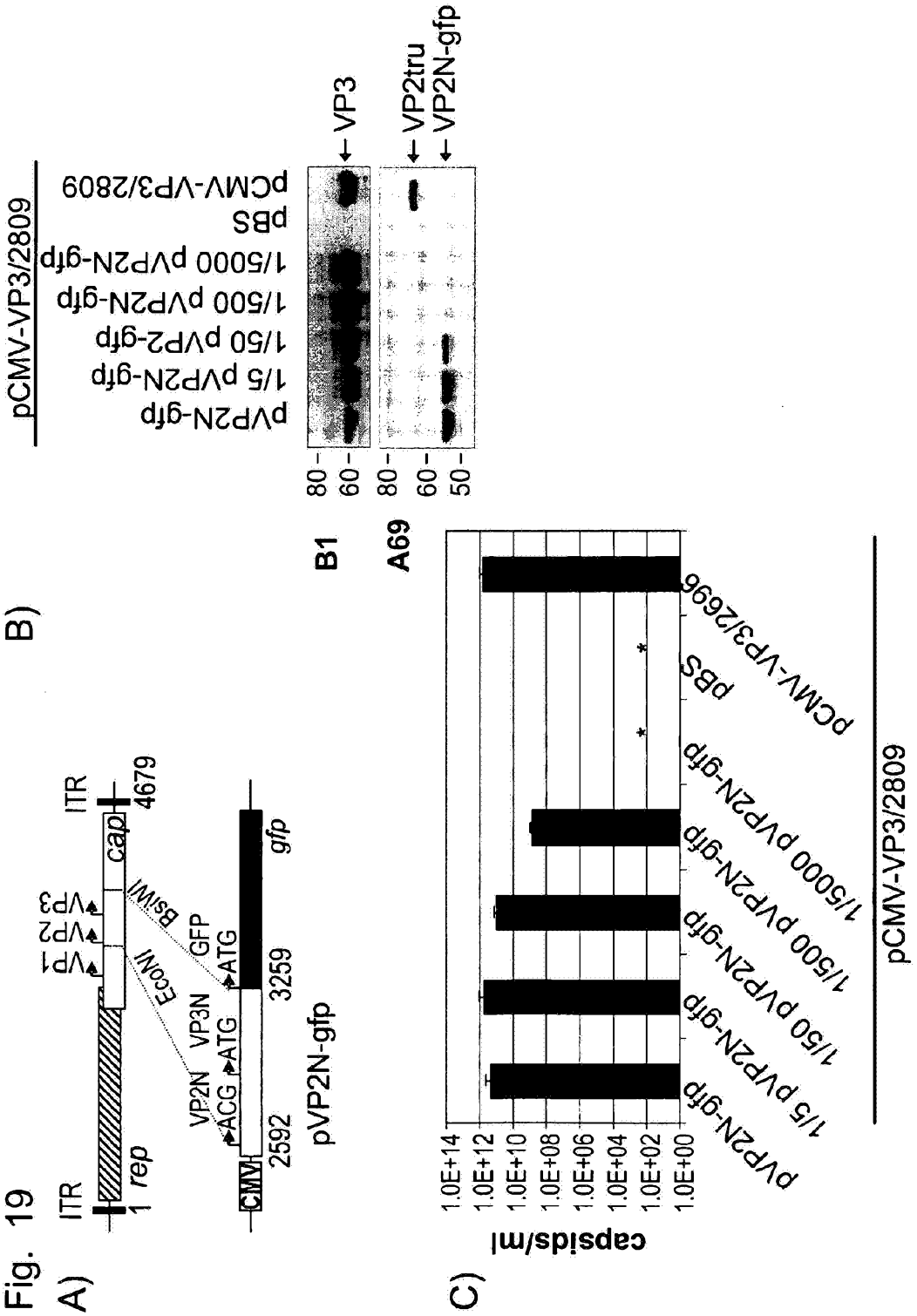


Fig. 19
A)
ITR
1 rep
cap
4679
VP3
VP2
VP1
BsiWI
EcoNI
GFP
ATG
VP3N
ATG
VP2N
ACG
ATG
CMV
2592
3259
gfp
pVP2N-gfp
B)
pCMV-VP3/2809
pVP2N-gfp
1/5 pVP2N-gfp
1/50 pVP2-gfp
1/500 pVP2N-gfp
1/5000 pVP2N-gfp
pBS
pCMV-VP3/2809
B1
A69
VP3
VP2tru
VP2N-gfp
80
60
80
60
50
C)
capsids/ml
1.0E+14
1.0E+12
1.0E+10
1.0E+08
1.0E+06
1.0E+04
1.0E+02
1.0E+00
pVP2N-gfp
1/5 pVP2N-gfp
1/50 pVP2N-gfp
1/500 pVP2N-gfp
1/5000 pVP2N-gfp
pBS
pCMV-VP3/2696
pCMV-VP3/2809

Fig. 20

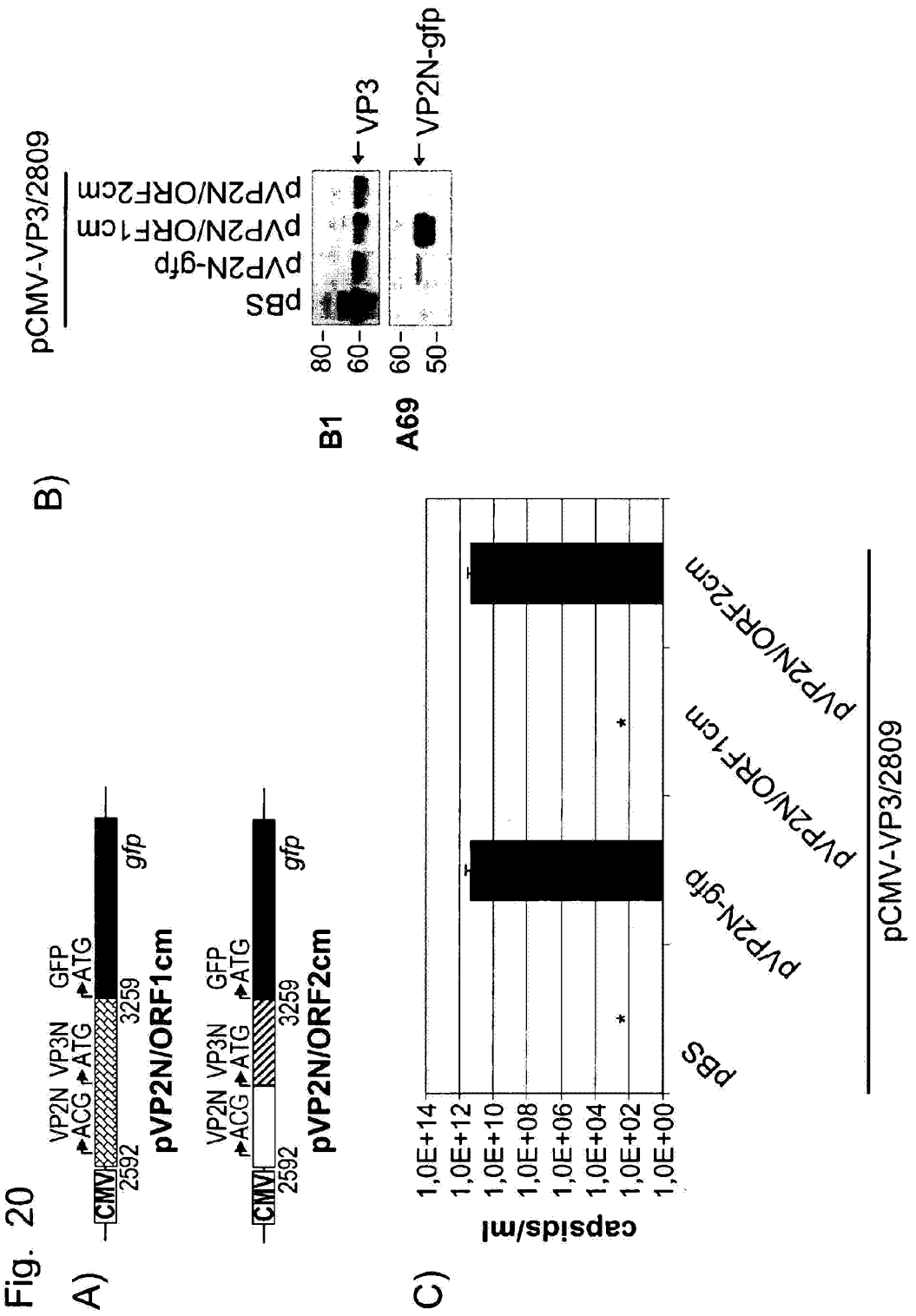
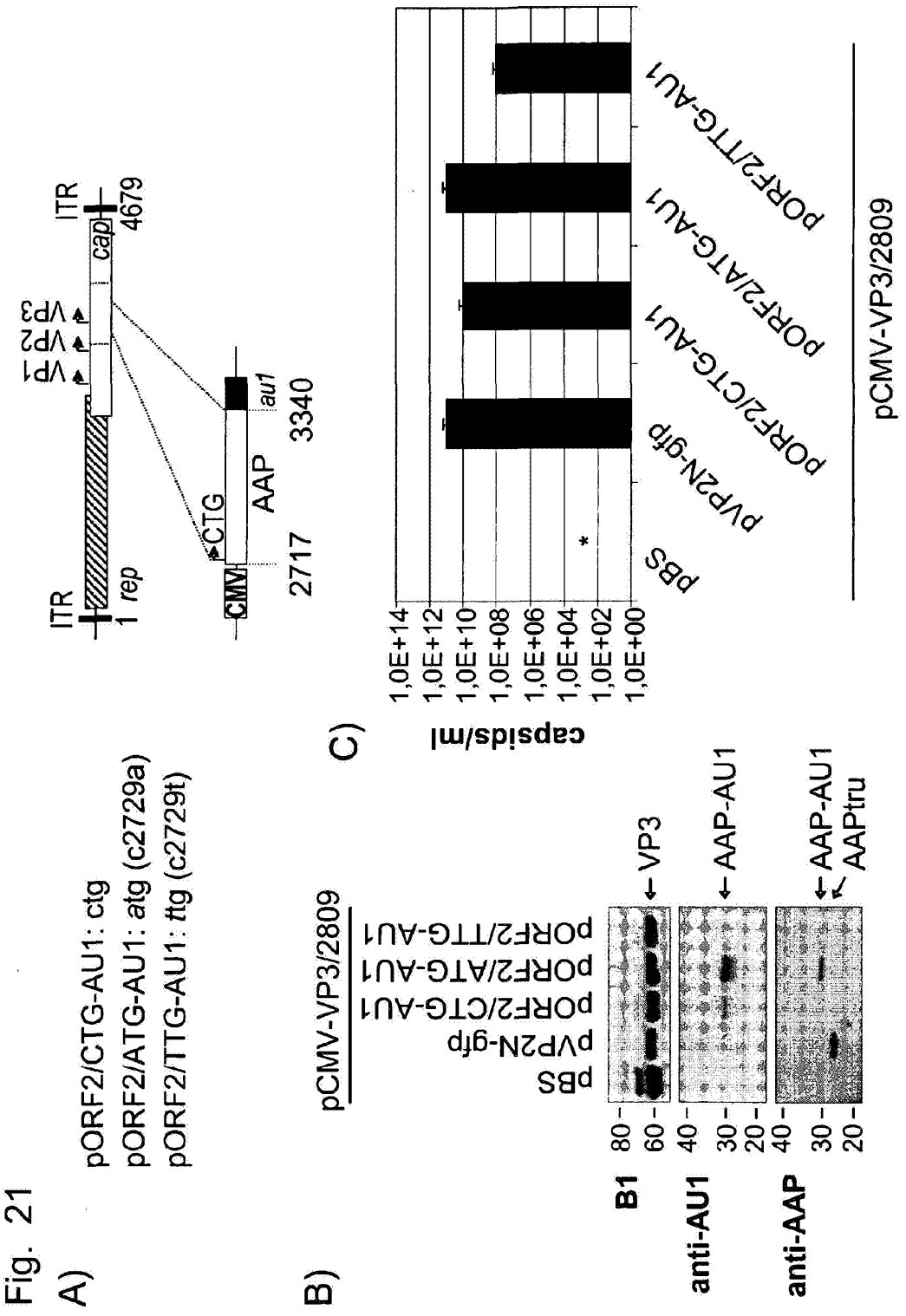

A)
VP2N VP3N GFP
ACG ATG ATG
CMV
2592
3259
gfp
pVP2N/ORF1cm
pVP2N/ORF2cm
B)
pCMV-VP3/2809
pBS
pVP2N-gfp
pVP2N/ORF1cm
pVP2N/ORF2cm
80-
60-
60-
50-
B1
A69
VP3
VP2N-gfp
C)
capsids/ml
1,0E+14
1,0E+12
1,0E+10
1,0E+08
1,0E+06
1,0E+04
1,0E+02
1,0E+00
pBS
pVP2N-gfp
pVP2N/ORF1cm
pVP2N/ORF2cm
pCMV-VP3/2809

Fig. 21


A)
pORF2/CTG-AU1: ctg
pORF2/ATG-AU1: atg (c2729a)
pORF2/TTG-AU1: ttg (c2729t)
ITR
cap
4679
VP3
VP2
VP1
ITR
1
rep
CTG
AAP
au1
3340
2717
CMV
B)
pCMV-VP3/2809
pBS
pVP2N-gfp
pORF2/CTG-AU1
pORF2/ATG-AU1
pORF2/TTG-AU1
B1
anti-AU1
anti-AAP
VP3
AAP-AU1
AAP-AU1
AAPtru
80
60
40
30
20
40
30
20
C)
capsids/ml
1,0E+14
1,0E+12
1,0E+10
1,0E+08
1,0E+06
1,0E+04
1,0E+02
1,0E+00
*
pBS
pVP2N-gfp
pORF2/CTG-AU1
pORF2/ATG-AU1
pORF2/TTG-AU1
pCMV-VP3/2809

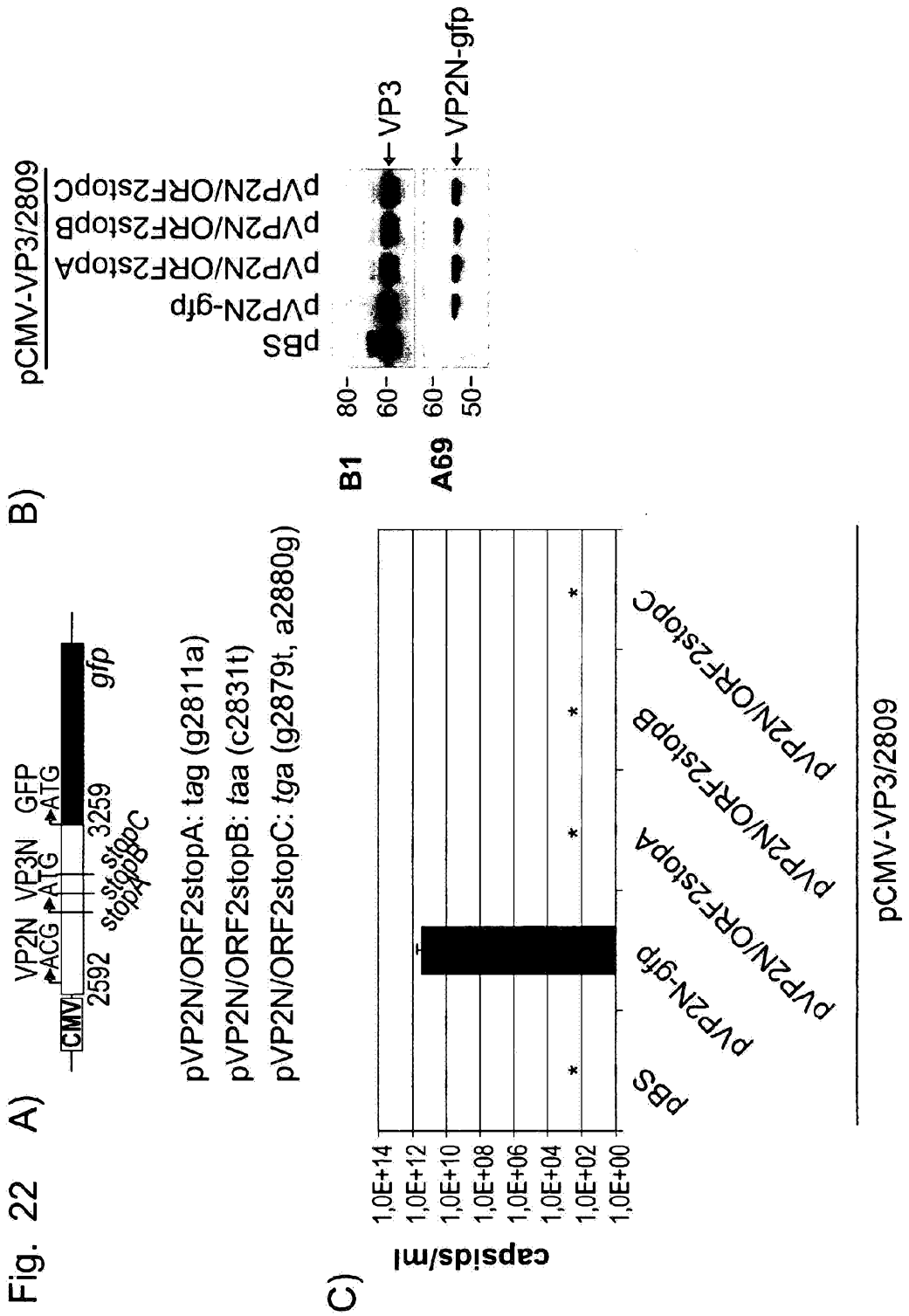
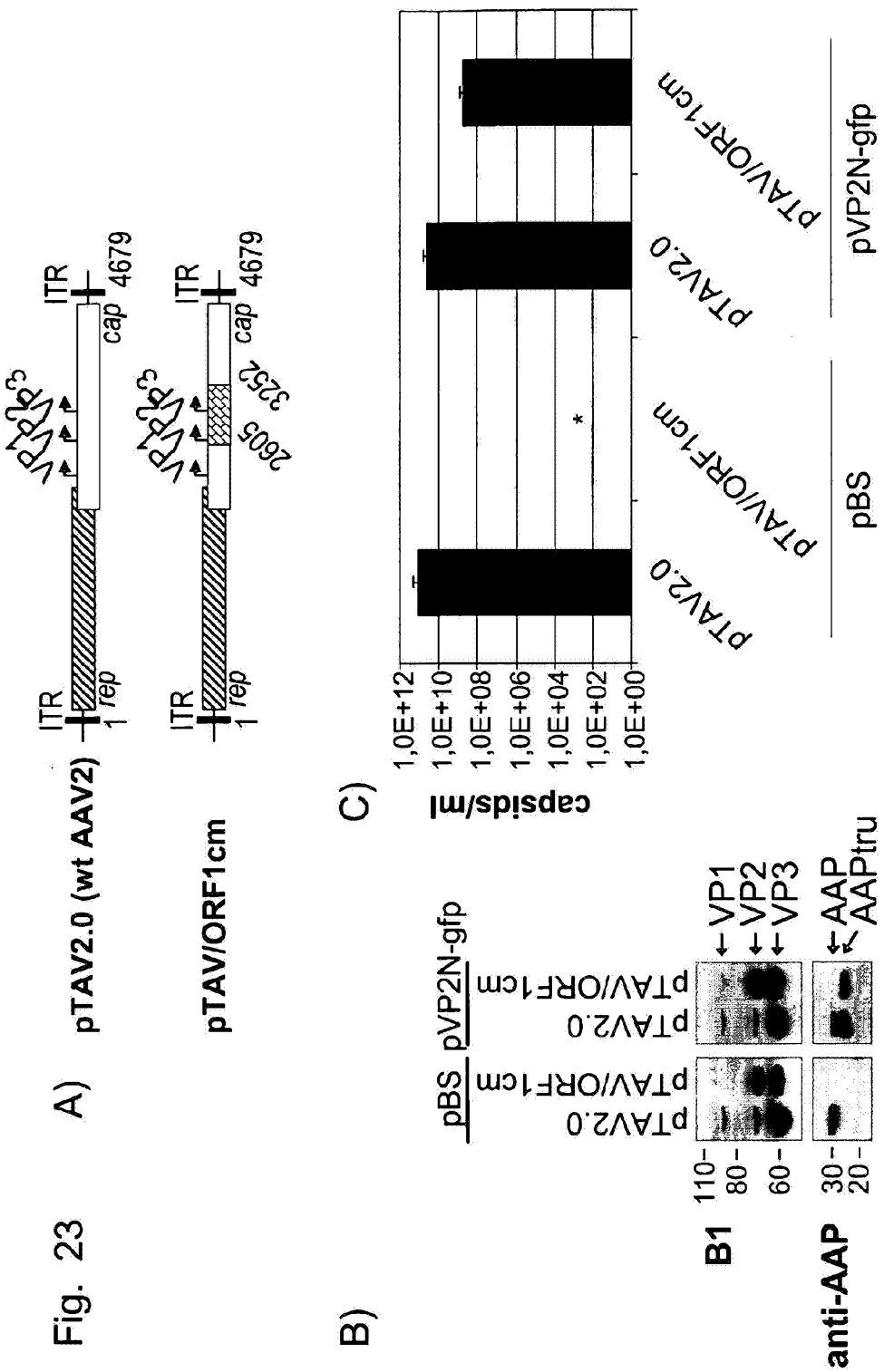


Fig. 22
A)
VP2N
ACG
VP3N
ATG
GFP
ATG
CMV
2592
3259
gfp
pVP2N/ORF2stopA: tag (g2811a)
pVP2N/ORF2stopB: taa (c2831t)
pVP2N/ORF2stopC: tga (g2879t, a2880g)
B)
pCMV-VP3/2809
pBS
pVP2N-gfp
pVP2N/ORF2stopA
pVP2N/ORF2stopB
pVP2N/ORF2stopC
B1
A69
80-
60-
60-
50-
VP3
VP2N-gfp
C)
capsids/ml
1,0E+14
1,0E+12
1,0E+10
1,0E+08
1,0E+06
1,0E+04
1,0E+02
1,0E+00
pBS
pVP2N-gfp
pVP2N/ORF2stopA
pVP2N/ORF2stopB
pVP2N/ORF2stopC
pCMV-VP3/2809

Fig. 23


A)
pTAV2.0 (wt AAV2)
ITR
rep
cap
4679
pTAV/ORF1cm
2605
3252
B)
pBS
pVP2N-gfp
pTAV2.0
pTAV/ORF1cm
VP1
VP2
VP3
AAP
AAPtru
110-
80-
60-
30-
20-
B1
anti-AAP
C)
capsids/ml
1,0E+12
1,0E+10
1,0E+08
1,0E+06
1,0E+04
1,0E+02
1,0E+00

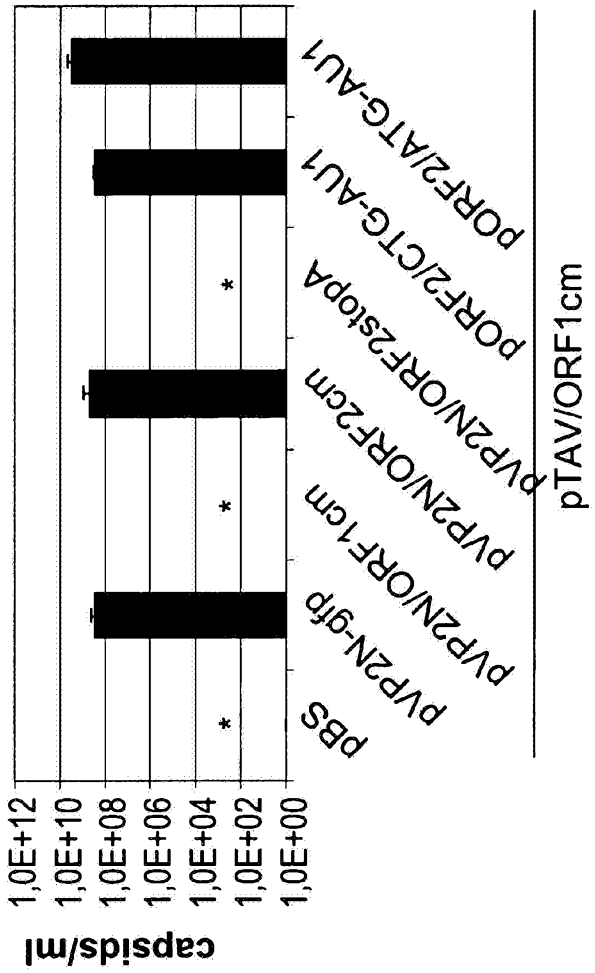
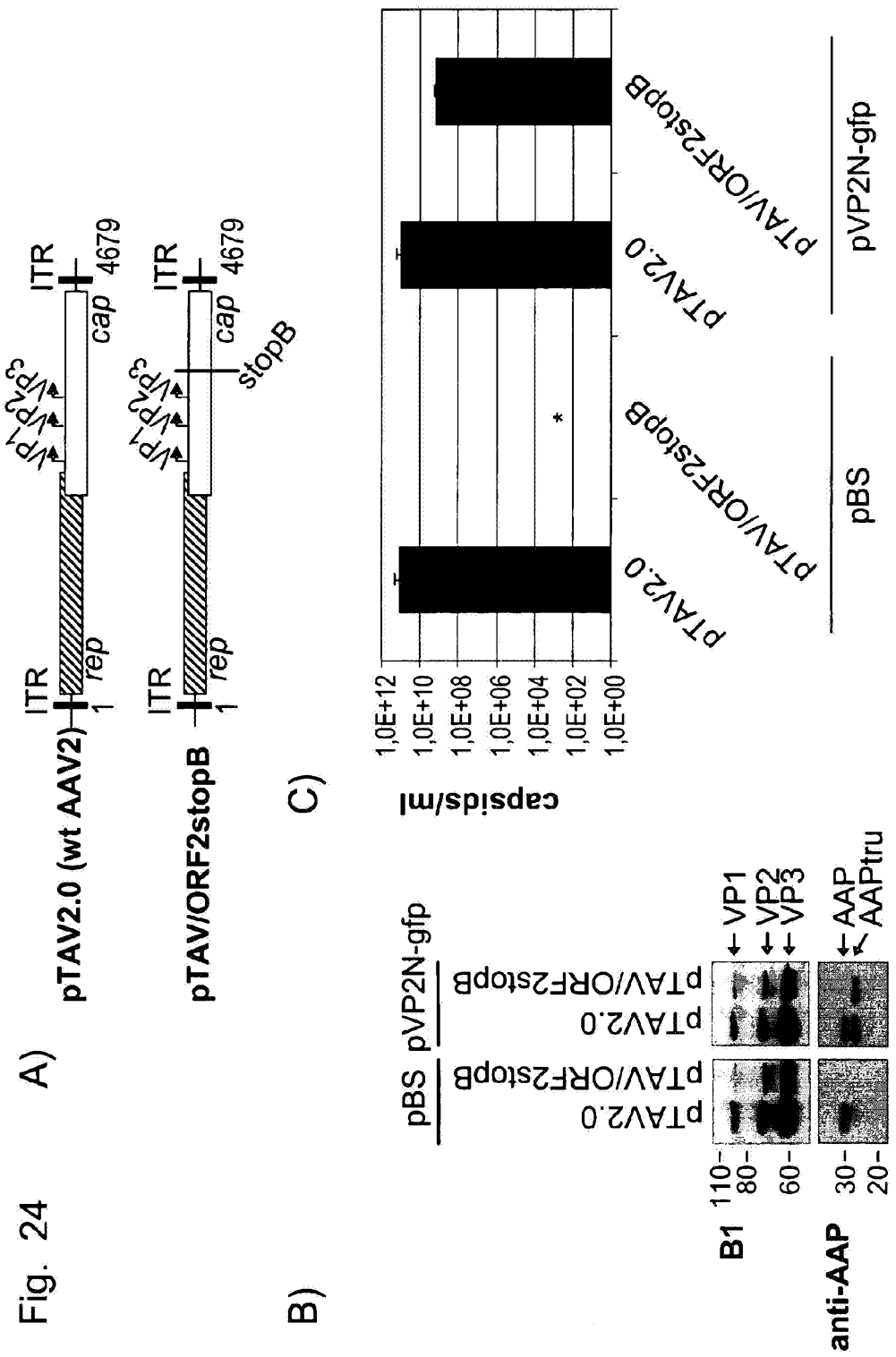

capsids/ml
1,0E+12
1,0E+10
1,0E+08
1,0E+06
1,0E+04
1,0E+02
1,0E+00
pBS
pVP2N-gfp
pVP2N/ORF1cm
pVP2N/ORF2cm
pVP2N/ORF2stopA
pORF2/CTG-AU1
pORF2/ATG-AU1
pTAV/ORF1cm
*
*
*

Fig. 23D)

Fig. 24


A)
pTAV2.0 (wt AAV2)
ITR
rep
cap
4679
VP1
VP2
VP3
pTAV/ORF2stopB
stopB
B)
pBS
pVP2N-gfp
pTAV2.0
pTAV/ORF2stopB
VP1
VP2
VP3
AAP
AAPtru
B1
anti-AAP
110-
80-
60-
30-
20-
C)
capsids/ml
1,0E+12
1,0E+10
1,0E+08
1,0E+06
1,0E+04
1,0E+02
1,0E+00
*

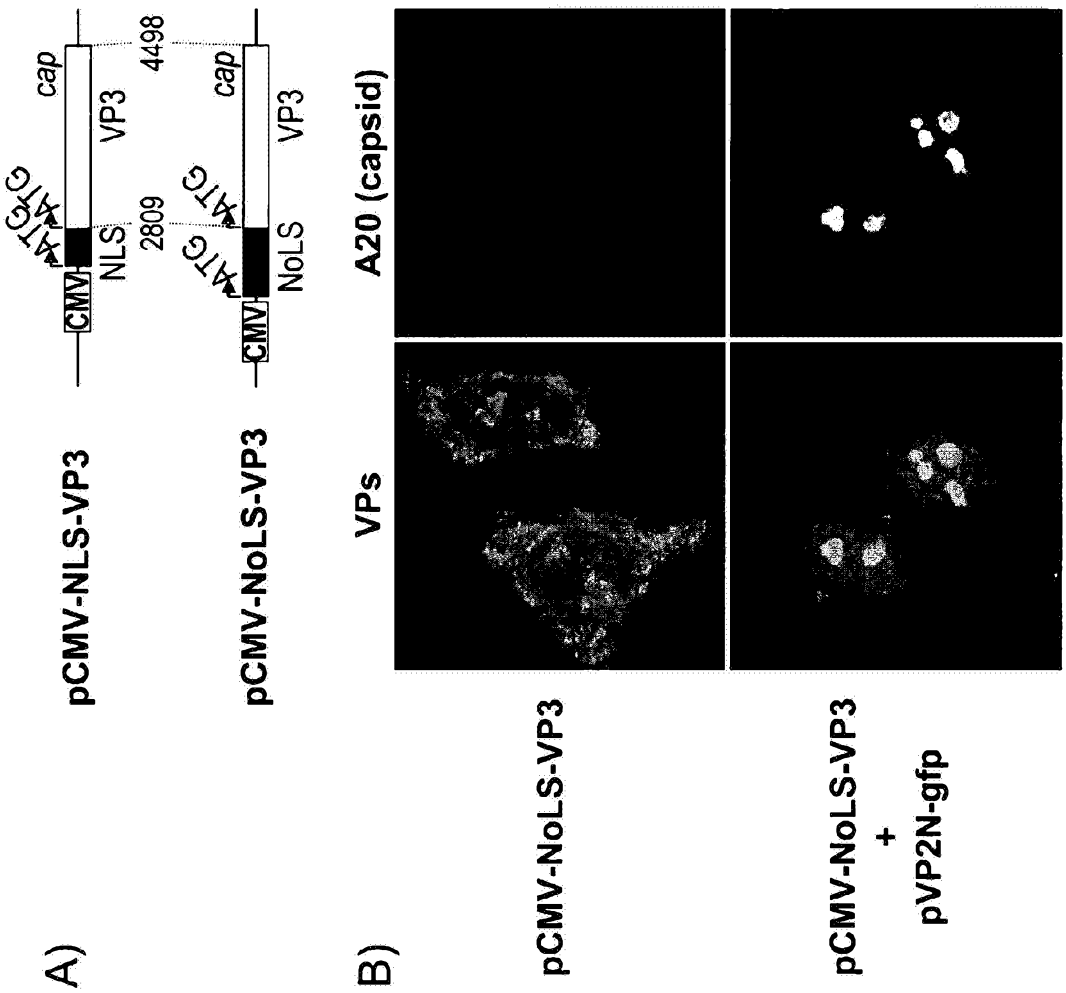

A)
pCMV-NLS-VP3
cap
VP3
4498
ATG
ATG
CMV
NLS
2809
pCMV-NoLS-VP3
cap
ATG
ATG
CMV
NoLS
VP3
B)
VPs
A20 (capsid)
pCMV-NoLS-VP3
pCMV-NoLS-VP3
+
pVP2N-gfp

Fig. 25

Fig. 25 C)

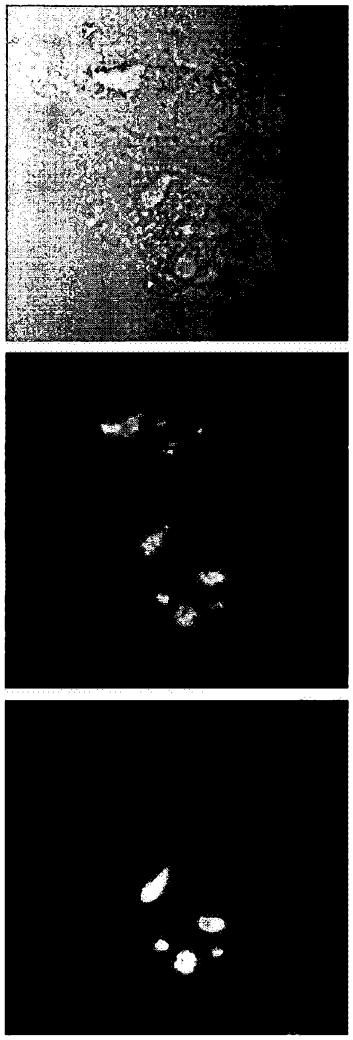


Fig. 26

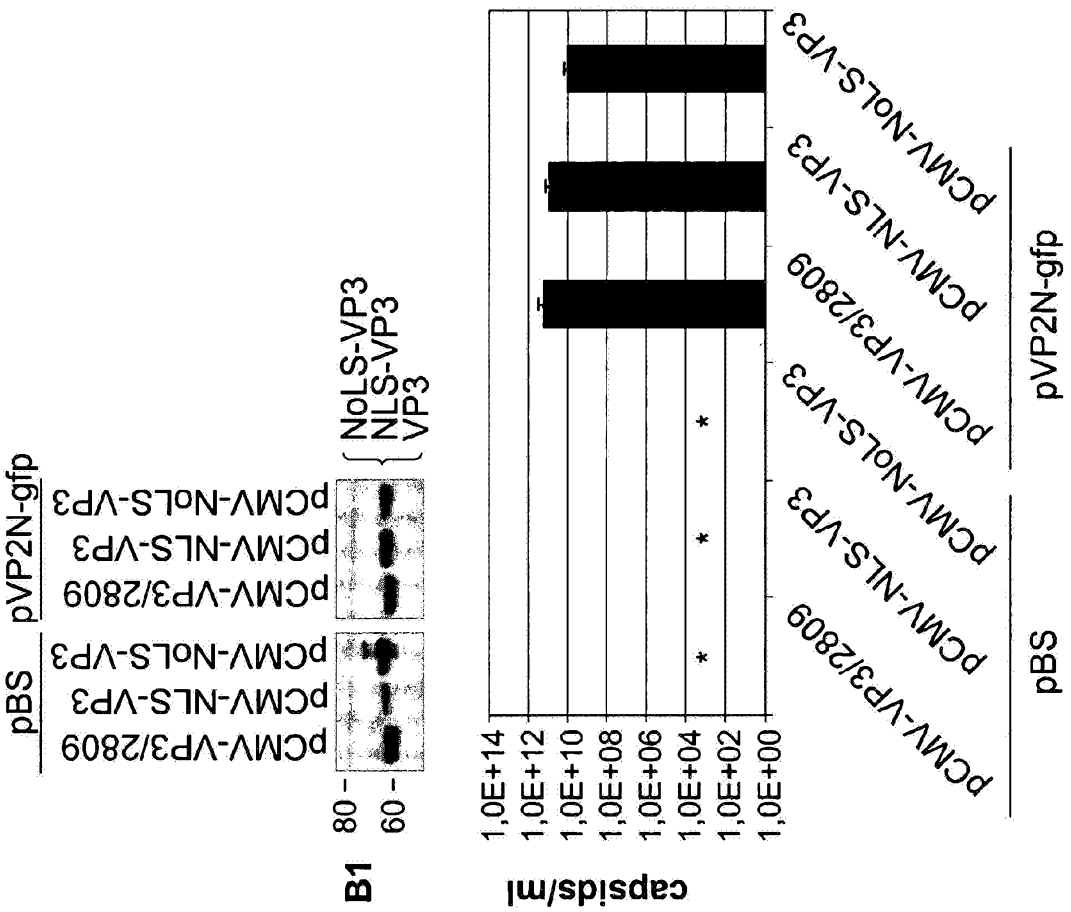

A)
pBS
pVP2N-gfp
pCMV-VP3/2809
pCMV-NLS-VP3
pCMV-NoLS-VP3
pCMV-VP3/2809
pCMV-NLS-VP3
pCMV-NoLS-VP3
NoLS-VP3
NLS-VP3
VP3
80 –
60 –
B1
B)
capsids/ml
1,0E+14
1,0E+12
1,0E+10
1,0E+08
1,0E+06
1,0E+04
1,0E+02
1,0E+00
*
*
*
pCMV-VP3/2809
pCMV-NLS-VP3
pCMV-NoLS-VP3
pCMV-VP3/2809
pCMV-NLS-VP3
pCMV-NoLS-VP3
pBS
pVP2N-gfp

Fig. 27-1

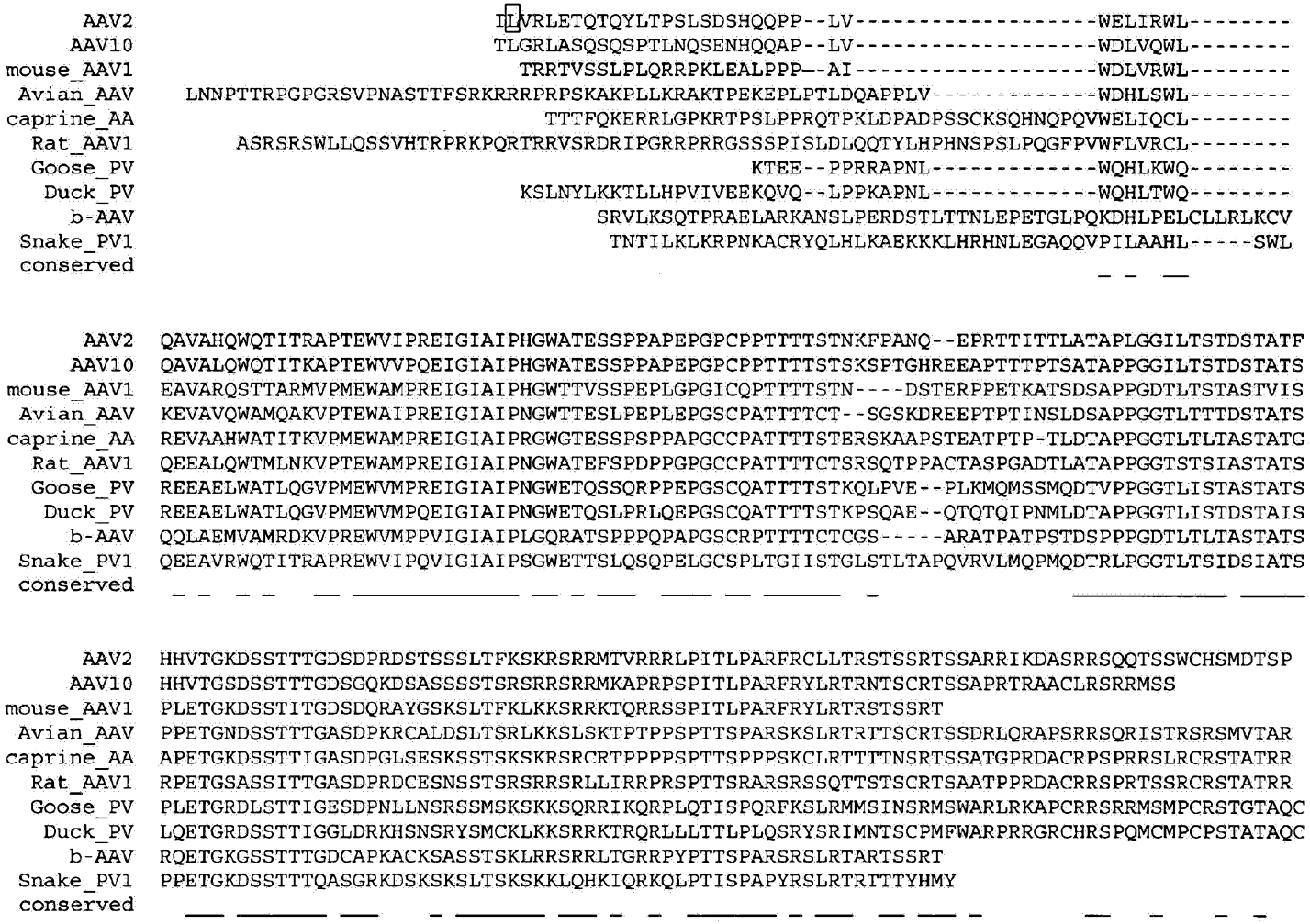

AAV2
AAV10
mouse_AAV1
Avian_AAV
caprine_AA
Rat_AAV1
Goose_PV
Duck_PV
b-AAV
Snake_PV1
conserved

Fig. 27-2

AAV2		(SEQ ID NO: 1)
AAV10		(SEQ ID NO: 10)
mouse_AAV1		(SEQ ID NO: 16)
Avian_AAV		(SEQ ID NO: 143)
caprine_AA		(SEQ ID NO: 18)
Rat_AAV1		(SEQ ID NO: 19)
Goose_PV	TPTRMEHGSMTVVHSTA	(SEQ ID NO: 20)
Duck_PV	TPTRVELDSMTEVPSIA	(SEQ ID NO: 21)
b-AAV		(SEQ ID NO: 13)
Snake_PV1		(SEQ ID NO: 22)
conserved		

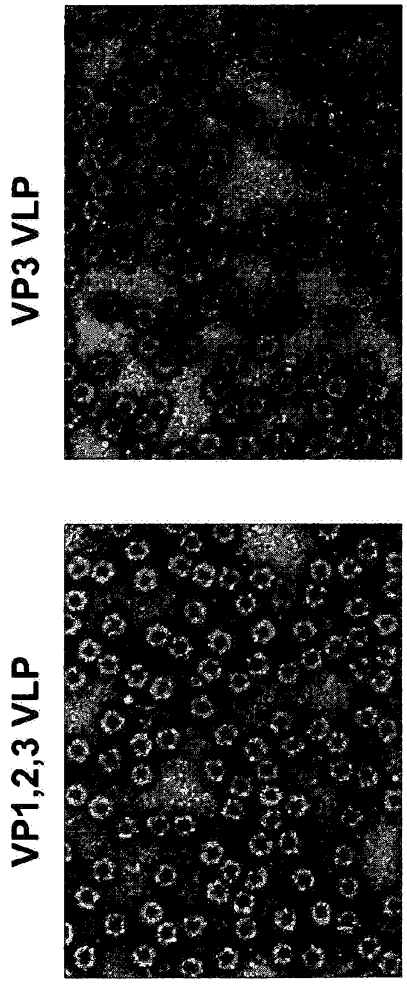
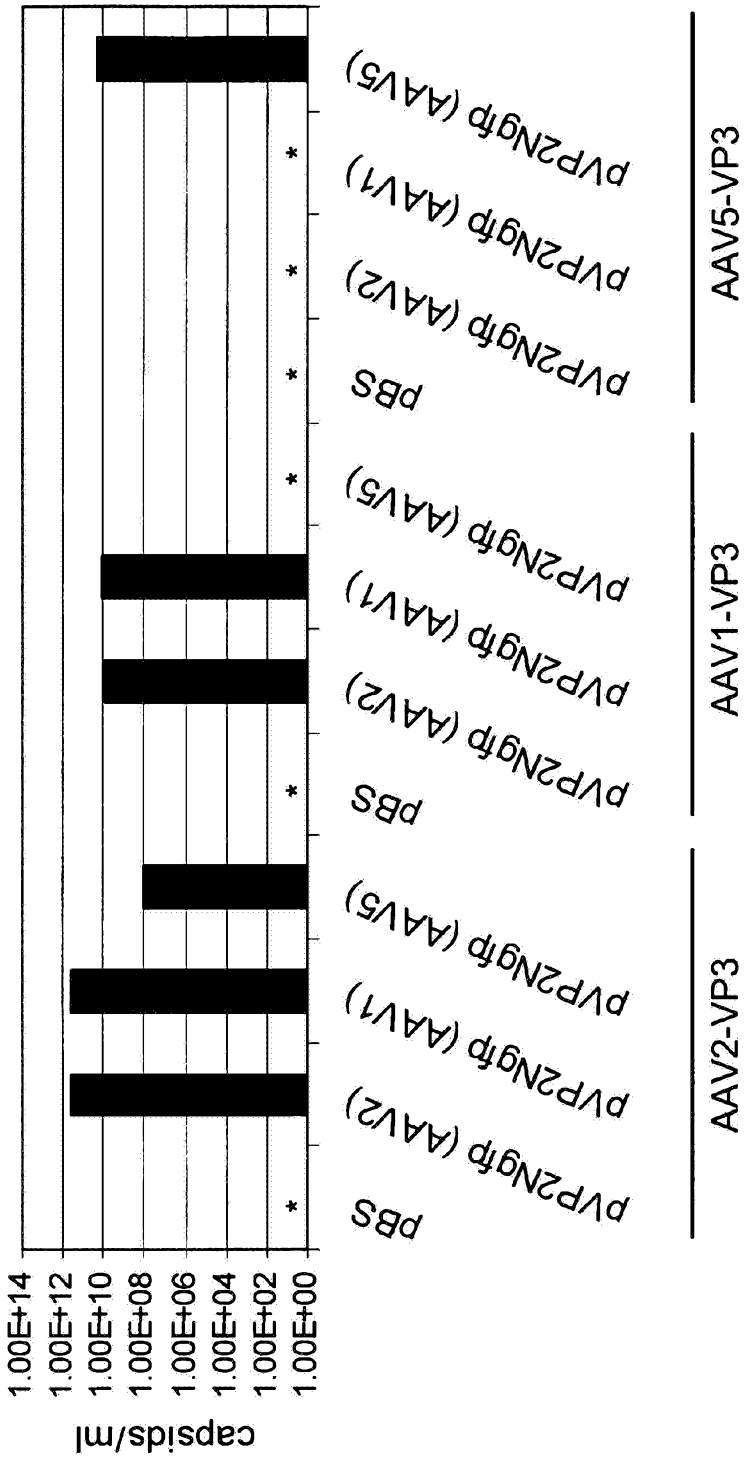


Fig. 28

Fig. 29


capsids/ml
1.00E+14
1.00E+12
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
*
*
*
*
*
*
*
*
*
PBS
pVP2N9fp (AAV2)
pVP2N9fp (AAV1)
pVP2N9fp (AAV5)
PBS
pVP2N9fp (AAV2)
pVP2N9fp (AAV1)
pVP2N9fp (AAV5)
PBS
pVP2N9fp (AAV2)
pVP2N9fp (AAV1)
pVP2N9fp (AAV5)
AAV2-VP3
AAV1-VP3
AAV5-VP3