Title: Means and methods for the production of adenovirus vectors.

FIELD OF THE INVENTION

The invention relates to nucleic acid delivery vehicles and use thereof, more in particular the invention relates to recombinant adenoviral vectors and the use thereof.

BACKGROUND OF THE INVENTION

To date 51 human adenovirus serotypes have been identified that are subdivided into 6 subgroups (A, B, C, D, E and F) based on hemagglutination properties and sequence homology (Francki et al. 1991). The adenovirus infectious cycle is divided into an early and a late phase. In the early phase, the virus is uncoated and the genome is transported to the nucleus after which the early gene regions (E1, E2, E3 and E4) become transcriptionally active. The E1 region contains two transcription regions: E1A and E1B. E1A encodes proteins that are involved in modification of the host cell cycle and activation of the other viral transcription regions (reviewed by Russell. 2000). The E1B region encodes two major proteins: E1B-19K and E1B-55K, that prevent the induction of apoptosis resulting from the activity of the E1A proteins (Rao et al. 1992; Yew and Berk 1992; Shenk 1996). In addition, the E1B-55K protein is required in the late phase for selective viral mRNA transport and inhibition of host protein expression (Pilder et al. 1986). E2 is also divided in two subdomains: E2A and E2B, that together encode three proteins (a DNA binding protein, a viral polymerase and a pre-terminal protein) that are all involved in replication of

the viral genome (Van der Vliet 1995). E3 is not necessary for replication *in vitro* but encodes several proteins that subvert the host defence mechanism towards viral infection (Horwitz 2001). E4 harbours at least 6 open reading frames (orf's) that encode proteins involved in several distinct functions related to viral mRNA splicing and transport, host cell mRNA transport, viral and cellular transcription and transformation (reviewed by Leppard 1997). The late proteins, necessary for formation of the viral capsids and packaging of viral genomes, are all generated from the major late transcription unit (MLTU) that becomes fully active after the onset of replication. A complex process of differential splicing and poly-adenylation gives rise to more than 15 mRNA species that share a tripartite leader sequence. The E1B-55K, E4-orf3 and E4-orf6 proteins play a pivotal role in the regulation of late viral mRNA processing and transport from the nucleus. For this process E1B-55K interacts with E4-orf6 to form a functional complex that stimulates transport of viral mRNA's to the cytoplasm, while the complex is also involved in inhibition of the transport of cellular mRNA's from the nucleus to the cytoplasm (reviewed in Leppard 1997 and 1998).

Production of E1-deleted vectors based on subgroup C serotypes Ad5 or Ad2 is achieved in E1 complementing cell lines such as 293 (Graham et al. 1970), 911 (Fallaux et al. 1996) and PER.C6™ (Fallaux et al. 1998; ECACC deposit no. 96022940). As disclosed in WO 99/55132 and WO 01/05945, vectors and cell line can be matched to avoid generation of replication competent adenoviruses through homologous recombination between adenovirus sequences in the cell line and the vector. For efficient production of replication incompetent adenoviruses derived from group C, the cell line

PER.C6™ is preferably used. Using this cell line adenovirus vectors can be matched, thereby allowing for the production of group C adenoviral vectors in the absence of replication competent adenovirus (Fallaux et al. 1998; US patent no. 6,033,908). However, group C vectors may not always be the ideal vehicles for direct *in vivo* applications since the infection efficiency is seriously hampered by the presence of high titers of neutralizing activity in most humans and the absence of sufficient amounts of the cellular receptor (Coxsackie-adenovirus receptor, CAR) on specific primary target cells (e.g. endothelial cells, smooth muscle cells, synoviocytes, monocytes and dentritic cells). Administration of higher amounts of viruses to increase transduction may lead to increased toxicity and unpredictable clinical outcome due to the variation in neutralizing titers of subjects that are treated. These limitations can be overcome by the use of other serotypes of adenoviruses. For example, the receptor binding part of a fiber of subgroup B viruses (in particular of serotype 16) when expressed on an Ad5-based vector, mediated significantly increased infection of human endothelial cells and smooth muscle cells (WO 00/31285) and of human synoviocytes (WO 00/52186). The fiber of another subgroup B adenovirus, Ad35, is most efficient in mediating infection of human monocytes and dendritic cells (WO 00/03029). Furthermore, Ad35 has been identified as a virus to which the vast majority of the human population has no neutralizing activity (WO 00/70071).

There is a generally felt need in the art to develop technology that has broader serotype utility. A particular problem is the lack of suitable packaging cell lines for these other serotypes. Packaging cell lines for Ad5 vectors typically comprises E1 encoded proteins derived from

adenovirus serotype 5. Examples of such 'standard' packaging cell lines are 293, 911 and PER.C6™. Attempts to produce vectors derived from other serotypes on these standard packaging cell lines have proven arduous if not unsuccessful. Occasionally, some production is seen, depending on the particular serotype used. However, the yields of recombinant adenovirus vectors derived from adenovirus subgroups other than subgroup C, produced on cell lines transformed and immortalized by E1 from Ad5, is poor. In a paper by Abrahamsen et al. (1997), improved plaque purification of an E1A-deleted adenovirus serotype 7 vector (subgroup B) was observed on 293 cells comprising E4-orf6 derived from adenovirus serotype 5, as compared to 293 cells lacking the E4-orf6 sequence from Ad5. However, a problem was encountered with the stability of the vector as unexpected recombinations were observed in plaque-purified stocks. An additional problem was encountered with wild type adenovirus virus contamination during production. Moreover, for large-scale production of adenoviruses it is not useful to co-transfect E4-orf6 to obtain titers that are high enough for application. One option for growing such adenoviruses is to provide cells with the E4-orf6 gene stably integrated into the genome of the complementing/packaging cell line. Such cells have been described in the art (e.g. WO 96/22378). A disadvantage of that system is the fact that new stable cell lines have to be generated, and numerous selection rounds have to be performed before stable and proper cells have been generated. This process is laborious and time-consuming. In general, it can be stated that generation and propagation of adenoviruses from serotypes other than serotype 5 (subgroup C), such as subgroup B viruses, has proven to be difficult on Ad5 complementing cells. As has been disclosed by the

applicants in WO 00/70071, recombinant viruses based on subgroup B virus Ad35 can be made by co-transfection of an expression construct containing the Ad35 early region-1 sequences (Ad35-E1). Furthermore, Ad35-based viruses that are deleted only for E1A sequences and not for E1B were shown to replicate efficiently on PER.C6 cells, suggesting that the E1A proteins of Ad5 are able to complement the Ad35-E1A functions (applicant's international application PCT/NL01/00824, not yet published). Moreover, the experiments show that lack of Ad35-E1B results in poor yields on Ad5 complementing cells. WO 00/70071 also discloses cell lines for the production of E1-deleted non-group C adenoviral vectors by further modifying cell lines that are capable of complementing adenovirus serotype 5. WO 00/70071 further suggests that one should establish new cell lines harbouring Ad35-E1 sequences for the complementation of recombinant adenovirus serotype 35 vectors lacking the E1 region (see also applicant's international application PCT/NL01/00824). However, as also discussed above, if one desires to apply a specific serotype for a specific need, one would have to establish a new cell line for every specific serotype, or one would have to modify the available cell lines that can complement adenovirus serotype 5 for complementation of the serotype of interest. It would clearly be advantageous to use the established cell lines that are available in the art, and not to modify these and use them for the production of all other, non-Ad5 serotypes, applying the established and efficient methods known in the art. It is concluded that until the present invention, no flexible and proper 'production platform' is available in the art that enables one to produce useful yields of adenovirus serotypes that are different from the serotypes of subgroup C.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of plasmid pΔMT.Orf6.Hygro (ECACC deposit no. P02041226). For the sake of clarity, the capital D stands for delta (Δ).

Figure 2 is a schematic representation of plasmid pAd35.ΔMT.Orf6. For the sake of clarity, the capital D stands for delta (Δ).

Figure 3 is a schematic representation of pUC.35-5E4.

Figure 4 is a representation of the cloning steps leading to pUC.35-5E4.

Figure 5 is a schematic representation of pBr.Ad35.PRn.

Figure 6 is a schematic representation of pBr.Ad35.PR5E4 (ECACC deposit no. P02041229).

Figure 7 is a schematic representation of pWE.Ad35.pIX-rITR.5E4.

Figure 8 is a schematic representation of pCRscriptAmp.NFI-NcoIR.

Figure 9 is a schematic representation of pCRscriptAmp.NcoIF-NR2.

Figure 10 is a schematic representation of pCR.NF1-NR2.

Figure 11 shows the alignment between the amino acid sequence of E4-orf6 of Adenovirus serotype 5 (SEQ ID NO:21; upper sequence) with the amino acid sequence of the E4-orf6 protein from Adenovirus serotype 5 cloned into the Ad35 backbone (SEQ ID NO:21; middle sequence; NB. identical to E4-orf6 of Ad5, upper sequence) as obtained by determination of the nucleotide order, and the amino acid sequence of the E4-orf6 protein of Adenovirus serotype 35 (SEQ ID NO:22; lower sequence), showing that the entire fragment encoding the E4-orf6 protein in the Adenovirus serotype 35 backbone has been replaced by the fragment encoding the E4-orf6 protein of Adenovirus serotpe 5.

Figure 12 shows the alignment between the amino acid sequence of the E4-orf6/7 protein of Adenovirus serotype 5 (SEQ ID NO:23; upper sequence) with the amino acid sequence of the E4-orf6/7 fusion protein encoded partly by the Adenovirus serotype 5 E4-orf6/7 fragment replacing the corresponding part of the adenovirus serotype 35 E4-orf6/7 fragment in the Adenovirus serotype 35 backbone (SEQ ID NO:24; middle sequence) and the amino acid sequence of the E4-orf6/7 protein of Adenovirus serotype 35 (SEQ ID NO:25; lower sequence), showing that the orf6/7 sequence is partly chimeric, with the fusion approximately at the lysine (K) residue at position 138. For the sake of clarity, the notation orf6+7 should be read as the open reading frame orf6/7, which is a separate open reading frame within the E4 region of adenoviruses besides orf6 and orf7, being a notation well known to persons skilled in the art.

Figure 13 is a schematic representation of pBr.Ad35.PR.5Orf6 (ECACC deposit no. P02041227).

Figure 14 is a schematic representation of pWE.Ad35.pIX-rITR.5Orf6.

Figure 15 is a schematic representation of pBr.Ad35.PRnΔE3. For the sake of clarity, the capital D stands for delta (Δ).

Figure 16 is a schematic representation of pBr.Ad35.ΔE3.PR5E4. For the sake of clarity, the capital D stands for delta (Δ).

Figure 17 is a schematic representation of pBr.Ad35.ΔE3.PR5Orf6. For the sake of clarity, the capital D stands for delta (Δ).

Figure 18 shows the system for producing recombinant adenoviral particles in cells such as PER.C6 through a double homologous recombination event.

Figure 19 is a schematic representation of pWE.Ad35.pIX-EcoRV.

SUMMARY OF THE INVENTION

The present invention provides recombinant adenovirus vectors comprising structural and non-structural elements of an adenovirus of a first serotype, wherein said vector further comprises a sequence encoding an E4-orf6 protein, wherein said sequence is selected from the group consisting of: a) an E4-orf6 encoding sequence derived from an adenovirus of a second serotype different from said first serotype; b) an E4-orf6 encoding sequence derived from an adenovirus of said first serotype by way of a deletion, mutation, addition and/or substitution in one or more codons; and c) an E4-orf6 encoding sequence comprising a fusion between a part of an E4-orf6 encoding sequence derived from a second serotype different from said first serotype and a part of an E4-orf6 encoding sequence derived from a third serotype, wherein said third serotype may be identical to-, or different from said first serotype.

The invention further provides methods for the production of such recombinant adenovirus vectors comprising structural and non-structural elements of an adenovirus of a first serotype, said method comprising the steps of: a) providing a complementing cell harbouring an E1B-55K encoding sequence, derived from an adenovirus of a second serotype in expressible form, with the necessary elements of an adenovirus such as to allow assembly of said recombinant adenovirus vector by said complementing cell, wherein said elements comprise at least some structural and non-structural elements from an adenovirus of said first serotype different from said second serotype and a sequence encoding a functional E4-orf6 protein, or a functional part, derivative and/or analogue thereof, which is compatible with said

expressible E1B-55K protein in said complementing cell; b) culturing said complementing cell in a medium under conditions allowing for production and assembly of the adenovirus vector to take place; and c) harvesting the recombinant adenovirus vector so produced from the medium and/or the complementing cell, wherein the sequence encoding the compatible E4-orf6 protein is present in the recombinant adenovirus vector so produced.

The invention further relates to pharmaceutical compositions comprising adenoviral vectors according to the invention and the treatment of individuals using the adenoviral vectors provided by the present invention. The invention also relates to a kit of parts comprising cell lines and adenoviral vectors provided by the invention for executing the methods provided by the invention.

DETAILED DESCRIPTION

The present invention discloses methods and means that solve certain difficulties related to diminished complementation of non-group C adenoviral vectors in Ad5 packaging/complementing cells. Although in the Ad5 complementing cell lines functional Ad5 E1B-55K expression is present, it was found that only very low titers of adenoviral vectors could be produced when the adenoviral backbone was of a non-group C adenoviral origin; this finding implies a serotype-specificity in the interaction of E1B-55K with another (viral) protein. It is disclosed here that this serotype-dependency can be circumvented by providing E4-orf6 protein compatible with the E1B-55K protein provided by the complementing cell line. As discussed herein, E1B-55K and E4-orf6 form a complex that is involved in inhibiting transport of cellular mRNA's from the nucleus to the cytoplasm, while the complex is also involved in stimulation of transport of viral mRNA's from the nucleus to the cytoplasm. It has been observed by the present inventors that proper complementation of viral vectors in packaging cells requires the presence of E1B-55K and E4-orf6 gene products that are compatible. Packaging cells are also referred to as complementing cells, if the cells comprise certain sequences encoding proteins that complement functions not provided by the vector that should be packaged. 'Compatible' as used herein therefore means that a complex between the available E1B-55K gene product is able to form a functional complex with the available E4-orf6 gene product in a sense that this protein complex supports viral replication, propagation and/or packaging to a level that is comparable to the wild-type situation or that is comparable to the situation found when a

recombinant adenovirus serotype 5 vector is produced on a Ad5 complementing cell line such as 293 or PER.C6. Vector replication in packaging cells is efficient if, during production period in which the virus is formed, the cell comprises at least E1B-55K protein and E4-orf6 protein that are compatible. Preferably, the E1B-55K and E4-orf6 sequences are from adenoviruses within the same adenovirus subgroup (such as A, B, C, D, E or F). More preferably, the E1B-55K and E4-orf6 sequences are from the same serotype. Since established cell lines are available in the art that are capable of supporting the growth of adenoviruses of subgroup C, such as serotype 5, it is even more preferred that the E1B-55K and E4-orf6 genes are derived from adenovirus serotype 5. As will be understood by the skilled person compatibility may be determined in complementation tests or assays as such in the realm of those skilled in the art of adenoviral vector production. The person skilled in the art will also understand that the present invention can also be used for the production of any adenovirus serotype on any complementing cell line as long as the E1B-55K and E4-orf6 proteins are compatible.

It has further been observed that the E4-orf6 gene product, 'matching' with the E1B in the complementing cell line, can be provided by the adenoviral vector, by replacing the E4-orf6 in the adenoviral vector of choice with an E4-orf6 encoding sequence that is compatible with the E1B gene present within the packaging cell line. This modification was surprisingly found not to have a severe effect on the stability, replication, packaging, assembly and production of the vector.

It is a specific aspect of the invention that one is now able to efficiently produce adenovirus serotypes different

than those from subgroup C on cell lines normally applied for the production of adenovirus serotype 5 or other serotype from subgroup C, such as serotype 1, 2 and 6. The present invention provides methods for the production of non-group C adenoviruses without the necessity of separately providing the complementing (packaging) cell with E4-orf6 because the E4-orf6 sequence, that is compatible with the complementing E1B-55K sequence, is incorporated in the adenoviral backbone.

The present invention provides a recombinant adenovirus vector comprising structural and non-structural elements of an adenovirus of a first serotype, wherein said vector further comprises a sequence encoding a functional E4-orf6 protein, or a functional part, derivative and/or analogue thereof, wherein said sequence is selected from the group consisting of: a) an E4-orf6 encoding sequence derived from an adenovirus of a second serotype different from said first serotype; b) an E4-orf6 encoding sequence derived from an adenovirus of said first serotype comprising a deletion, mutation, addition and/or substitution in one or more codons; and c) an E4-orf6 encoding sequence comprising a fusion between a part of an E4-orf6 encoding sequence derived from a second serotype different from said first serotype and a part of an E4-orf6 encoding sequence derived from a third serotype, wherein said third serotype may be identical to-, or different from said first serotype. In one embodiment, the present invention provides a recombinant adenovirus vector according to the invention, wherein said first serotype and said second serotype are from different adenovirus subgroups. In a preferred embodiment, a recombinant adenovirus vector according to the invention is provided, wherein said first serotype is from a subgroup other than subgroup C and wherein said E4-orf6 encoding sequence is derived from an adenovirus

serotype of subgroup C. More preferred is a recombinant adenovirus according to the invention, wherein said first serotype is from subgroup B and said second serotype is from subgroup C. Even more preferably, said E4-orf6 encoding sequence is derived from adenovirus serotype 5.

It has been recognized by those skilled in the art that different levels of neutralizing antibodies circulate in humans that are directed against different serotypes. It has been found that certain adenoviral serotypes encountered high titers of such neutralizing antibodies and that many individuals in different populations carried neutralizing antibodies against such serotypes. It was also found that certain serotypes were only neutralized in a minority of the samples (WO 00/70071). Apparently, certain serotypes from subgroup B were only neutralized in a small group of samples. Therefore, in a preferred embodiment of the present invention, recombinant adenovirus vectors according to the invention are provided, wherein said first serotype is selected from the group consisting of adenovirus serotypes 11, 14, 16, 21, 34, 35 and 50. Highly preferred are recombinant adenoviral vectors, wherein said first serotype is serotype 11 or 35, while these encountered neutralizing antibodies only in a very small percentage of the tested samples.

The vectors of the present invention can be used in different settings such as gene therapy, functional genomics, tumour vaccination and/or anti-viral vaccination. For this, it is necessary that the adenoviral vector functions as a gene delivery vehicle, wherein a non-native gene is incorporated into the adenoviral genome. The adenoviral particle can subsequently be targeted specifically to target cells of interest; the adenovirus binds to that specific cell

either through capsid-receptor binding or through other means, and delivers the transgene. Targeting of adenoviruses can be performed in many different ways. Persons skilled in the art of adenoviral vector targeting will be aware of all the different possibilities that are applied to deliver the adenoviral vectors to the cells of interest. Such possibilities include but are not limited to capsid alterations (fiber, hexon and/or penton modifications, such as deletions, swaps between fibers of different serotypes, and additions of peptides and/or other binding moieties), wherein chimeric fibers are produced that recognize a receptor present on the cell of interest, or wherein the binding of the penton-base is utilized. Other possibilities are linking targeting moieties to the capsid proteins, wherein for instance binding peptides, known and strong binding proteins, or antibodies or parts thereof are linked to the capsid proteins to achieve specific targeting. Such vectors can all be produced using the methods and means provided by the present invention. Therefore, the present invention also discloses recombinant adenovirus vectors according to the invention, further comprising a sequence encoding a non-adenoviral protein, polypeptide or peptide. Such sequences can be present on different locations within the adenoviral backbone, but preferably it is located in the E1 region, which is lacking in the recombinant adenoviral vectors of the invention. The E1 region is complemented by (the complementation) elements present in the complementing cells. The direction of the promoter, transgene and other regulatory sequences can be directed towards the left-, as well as to the right inverted terminal repeat.

The present invention can also be used for the production of viral vectors based on adenovirus and/or on

other viruses such as the Adeno-Associated Virus (AAV), wherein the combination, such as an Ad-AAV chimeric virus, can integrate into the host cell genome. Several methods are known in the art for generating integrating adenoviruses. Generally, the invention is also useful for the production of adenovirus forms that (specifically, or non-specifically) can integrate.

As mentioned, several non-adenoviral transgenes can be cloned into the recombinant adenoviral vectors of the present invention. These do not only include regulatory nucleic acid sequences such as enhancers, promoters (e.g. strong non-adenoviral promoters such as the cytomegalovirus promoter, the SV40 promoter and the RSV promoter) and polyadenylation signals, but also heterologous genes for therapeutic purposes. Therefore, in one aspect of the invention, recombinant adenovirus vectors according to the invention are provided, wherein said non-adenoviral protein, polypeptide or peptide is selected from the group consisting of: a cell-death inducing polypeptide, an antigenic determinant of a pathogenic organism, a tumor specific antigen, a viral protein, a hormone and a cytokine. Examples of pathogenic organisms are, but are not limited to bacteria, viruses and fungi. Non-limiting examples of non-adenoviral factors, proteins, polypeptides and petides are transcription factors, intracellular signalling proteins, phosphatases, kinases, apoptosis inhibiting factors, receptor antagonists, soluble forms of membrane-bound receptors, RNA inhibitors, anti-sense RNA's, decoy factors, ribozymes, and more specifically, thymidine kinase, erythropoietin, novel-erythropoiesis stimulating protein (NESP), IL3, ceNOS, gamma-interferon and gp100. Non-adenoviral viral proteins can be cloned into the recombinant adenoviral vectors provided by the methods and

means of the present invention for vaccination purposes. Such viral proteins include, but are not limited to, gag, pol, env, nef, etc. for HIV vaccines, E6 and E7 proteins for Human Papilloma Virus vaccines, circumsporozoite proteins from *Plasmodium* protozoa for malaria vaccines, rotavirus components for rotavirus vaccines, ebola proteins for ebola vaccines, the F and G gene products from Respiratory syncytial virus (RSV) for RSV vaccines, HA and NA for influenza vaccines, etc.

The recombinant adenoviruses of the present invention comprise structural and non-structural elements. Examples of structural elements are the genes encoding the capsid proteins, such as fiber, hexon and penton proteins, as well as the gene products itself. Examples of non-structural elements are the early genes that are expressed upon infection into a cell and that are downregulated when the infection cycle proceeds. Other examples of non-structural elements are the genes encoding the proteins active during replication, such as pol and pTP. It is to be understood that the recombinant adenoviral vectors may comprise structural and non-structural elements derived from different serotypes. Examples of such vectors are for instance the adenoviral particles carrying chimeric fibers (see applicant's patent application WO 00/03029). Molecular biology techniques have made it possible to construct endless combinations of nucleic acid sequences. It is clear to the person skilled in the art of molecular biology that combining different sequences can be performed using different molecular techniques, such as polymerase chain reaction (PCR) as well as direct subcloning. Many of the sequences used in the present invention as well as sequences and chimeric constructs known in the art are derived from different adenovirus serotypes. 'Derived' as

used herein therefore means that such sequence combinations can be obtained through direct cloning from wild-type sequences obtained from wild-type viruses, while they can for instance also be obtained through PCR by using different pieces of DNA as a template. This means also that such sequences may be in the wild-type form as well as in altered form. Another option for reaching the same result is through combining synthetic DNA. It is to be understood that 'derived' does not exclusively mean a direct cloning of the wild type DNA. A person skilled in the art will also be aware of the possibilities of molecular biology to obtain mutant forms of a certain piece of nucleic acid. These mutations may render a different functionality, but they may also be silent in a way that certain mutations do not alter the functionality of that particular piece of DNA and its encoded protein. Therefore, the terms 'functional part, derivative and/or analogue thereof' are to be understood as equivalents of the nucleic acid they are related to. A person skilled in the art will appreciate the fact that certain deletions, swaps, (point)mutations, additions, etc. may still result in a nucleic acid that has a similar function as the original nucleic acid. It is therefore to be understood that such alterations that do not significantly alter the functionality of the proteins such as the E4-orf6 and E1B-55K gene product are within the scope of the present invention.

Some alterations in the nucleic acid, such as a deletion, a mutation, addition and/or substitution in one or more codons may also significantly change the structure and/or functionality of the encoded gene product. The present invention therefore also relates to E4-orf6 encoded sequences that are derived from the same adenovirus serotype as the backbone harbouring the genes for instance the structural and

non-structural elements, but wherein the E4-orf6 encoding sequence has been mutated such that it has become compatible with the E1 proteins (such as the E1B-55K gene product) present in the complementing cell in which the adenoviral vector is to be produced. The codon may be altered completely to change the encoded amino acid, but it may also be mutated partly to change the encoded amino acid. Deletions of nucleic acids may result in loss of one or more encoded amino acids; while it may also result in frame-shifts. The present invention also relates to E4-orf6 sequences present in the adenoviral nucleic acid that comprise different parts derived from different serotypes, wherein the domains that render the protein functional in compatibility may be used from one serotype, while the remainder of the E4-orf6 sequence or a part thereof is derived from another (un)related serotype (for instance from the same subgroup, from different subgroups or from different species, or combinations thereof). It is therefore also within the scope of the present invention to apply E4-orf6 fusion proteins that are compatible. Such fusion protein may be the product of several pieces of nucleic acid.

A person skilled in the art will be aware of the fact that besides all human adenoviruses numerous non-human adenoviruses have been identified in the art. Obviously, also non-human adenoviruses can be applied to reach the same results as disclosed by the present invention. It will be clear to the skilled person that compatibility between E1B-55K and E4-orf6 may not be limited to human adenoviruses but that also elements from adenoviruses specific for different species can be compatible. Thus, it also another aspect of the invention that non-human adenoviruses can be produced to high titers on known packaging cell lines available in the

art as long as the E1B-55K and E4-orf6 gene products are compatible. Non-limiting examples of non-human adenoviruses that can be produced using the methods and means of the present invention are canine-, bovine-, ovine-, frog-, porcine, equine-, monkey- and avian adenoviruses. Serotypes as used herein therefore goes beyond species-restricted serotypes. If for instance a monkey adenovirus E4-orf6 gene product is compatible with the E1B-55K provided by the packaging cell, then this combination is within the scope of the present invention. Also, when fusions are applied between different serotypes, or between E4-orf6 sequences derived from for instance a human and an avian adenovirus that is compatible with the E1B gene of the packaging cell, then that particular combination is also within the scope of the present invention.

The present invention provides a method for producing a recombinant adenovirus vector comprising structural and non-structural elements of an adenovirus of a first serotype, said method comprising the steps of: a) providing a complementing cell harbouring an E1B-55K encoding sequence, or a functional part, derivative and/or analogue thereof, derived from an adenovirus of a second serotype in expressible form, with the necessary elements of an adenovirus such as to allow assembly of said recombinant adenovirus vector by said complementing cell, wherein said elements comprise at least some structural and non-structural elements from an adenovirus of said first serotype different from said second serotype and a sequence encoding a functional E4-orf6 protein, or a functional part, derivative and/or analogue thereof, which is compatible with said expressible E1B-55K protein in said complementing cell; b) culturing said complementing cell in a medium under

conditions allowing for production and assembly of the adenovirus vector to take place; and c) harvesting the recombinant adenovirus vector so produced from the medium and/or the complementing cell, wherein the sequence encoding the compatible E4-orf6 protein is present in the recombinant adenovirus vector so produced.

In one aspect of the invention, a method according to the invention is provided, wherein said E4-orf6 encoding sequence is selected from the group consisting of: i) an E4-orf6 encoding sequence derived from an adenovirus of said second serotype; ii) an E4-orf6 encoding sequence derived from an adenovirus of a third serotype different from said first and second serotype; iii) an E4-orf6 encoding sequence derived from an adenovirus of said first serotype comprising a deletion, mutation, addition and/or substitution in one or more codons; and iv) an E4-orf6 encoding sequence comprising a fusion between a part of an E4-orf6 encoding sequence derived from a third serotype and a part of an E4-orf6 encoding sequence derived from an adenovirus of said second serotype, wherein said third serotype may be identical to-, or different from said first serotype. In a preferred embodiment, said first and said second serotypes are from different subgroups. In a more preferred embodiment, said second serotype is an adenovirus serotype of subgroup C. In an even more preferred embodiment, said second serotype is adenovirus serotype 5. In other particular aspect of the invention, said first serotype is an adenovirus serotype of subgroup B. Preferably, said first serotype is selected from the group consisting of adenovirus serotypes 11, 14, 16, 21, 34, 35 and 50.

There are several packaging cells known in the art that are used for complementing recombinant adenoviral vectors and

to produce, assemble and package the adenoviral particles. Non-limiting examples of such cell lines are HEK-293, 911 and PER.C6™ cells. It is preferred to use cell lines that have already proven to deliver high titers of adenoviral stocks. Such cell lines express E1 proteins in a stable manner, it is therefore a preferred aspect of the invention to use cell lines and methods, wherein said E1B-55K encoding sequence is integrated into the genome of said complementing cell. More preferred are complementing cells that are derived from a primary, diploid human cell, or a progenitor cell thereof. Even more preferred, said complementing cell is derived from a primary human retinoblast cell, a primary human embryonic kidney cell, a primary human neuronal cell or a primary human amniocyte. Highly preferred is the use of a complementing cell in the methods provided by the present invention, wherein said complementing cell is a PER.C6 cell or a derivative thereof. PER.C6 cells are well known in the art for not giving rise to replication competent adenovirus when adenoviral DNA is used that has no overlap with the nucleic acid provided by the cells. Many of the adenoviral vectors used in the art lack the E1 region, therefore in one aspect of the invention, said complementing cell comprises, integrated into its genome, a nucleic acid encoding at least one adenovirus E1A protein. Preferably, said nucleic acid encoding at least one adenovirus E1A protein is derived from an adenovirus serotype of a subgroup different than subgroup B. More preferably, said nucleic acid encoding at least one adenovirus E1A protein is derived from an adenovirus serotype of subgroup C. Highly preferred are embodiments wherein said nucleic acid encoding at least one adenovirus E1A protein is derived from an adenovirus serotype 5. In another embodiment of the invention, the invention provides a method, wherein

said E4-orf6 encoding sequence and said E1B-55K encoding sequence are derived from different adenovirus serotypes and wherein said different adenovirus serotypes are members of the same adenovirus subgroup. Preferably, said E4-orf6 encoding sequence and said E1B-55K encoding sequence are derived from different adenovirus serotypes and wherein said different adenovirus serotypes are both members of subgroup C. More preferably, said E4-orf6 encoding sequence and said E1B-55K encoding sequence are derived from the same adenovirus serotype. Highly preferred are methods, wherein said E4-orf6 encoding sequence and said E1B-55K encoding sequence are derived from adenovirus serotype 5.

The present invention also relates to a pharmaceutical composition comprising a recombinant adenoviral vector according to the invention or obtainable by a method provided by the invention. The pharmaceutical composition further comprises an acceptable pharmaceutical carrier, generally applied by persons skilled in the art of preparation of pharmaceuticals. Furthermore, the present invention relates to a method of treating a human body comprising administering to a human body a recombinant adenoviral vector according to to the invention, or a pharmaceutical composition provided by the invention. The invention also relates to methods in which adenoviral vectors can be produced using the proper complementing/packaging cells and the adenoviral vector of interest. For an efficient production process it is useful to apply the correct cells with the proper adenoviral vector. Therefore, the invention also relates to a kit of parts comprising: a) a complementing cell for producing a recombinant adenovirus vector comprising structural and non-structural elements of an adenovirus of a first serotype, said cell harbouring an E1B-55K encoding sequence, or a

functional part, derivative and/or analogue thereof, derived from an adenovirus of a second serotype in expressible form; and b) on one or more replicable nucleic acid vectors all necessary adenoviral elements such as to allow assembly of said recombinant adenovirus vector by said complementing cell, wherein said elements comprise at least some structural and non-structural elements from an adenovirus of said first serotype different from said second serotype and a sequence encoding a functional E4-orf6 protein, or a functional part, derivative and/or analogue thereof, which is compatible with said expressible E1B-55K protein in said complementing cell. Preferably, a kit of parts is used, wherein said E4-orf6 encoding sequence is selected from the group consisting of: a) an E4-orf6 encoding sequence derived from an adenovirus of said second serotype; b) an E4-orf6 encoding sequence derived from an adenovirus of a third serotype different from said first and second serotype; c) an E4-orf6 encoding sequence derived from an adenovirus of said first serotype comprising a deletion, mutation, addition and/or substitution of one or more codons; and d) an E4-orf6 encoding sequence comprising a fusion between a part of an E4-orf6 encoding sequence derived from a third serotype and a part of an E4-orf6 encoding sequence derived from an adenovirus of said second serotype, wherein said third serotype may be identical to-, or different from said first serotype.

The invention is particularly useful for the replication of E1 deleted chimeric adenoviruses that are derived almost entirely from a serotype other than adenovirus 5. Such vectors need only to be provided with a nucleic acid encoding adenovirus 5 E4orf6 or a functional part, derivative and/or analogue thereof. Once provided therewith the vector can be efficiently replicated on normal adenovirus 5 E1-

complementing packaging cell lines. Stability of the vectors is improved, and vectors may be complemented for deletions in both E1A and E1B. By providing such vectors with a nucleic acid encoding adenovirus E4orf6, it is possible to enable efficient plaque purification and good yields in the absence of an additional wild type contamination problem, when grown on 293 or 911 cells. In PER.C6, of course, wild type adenovirus contamination can also be prevented in other ways.

An additional advantage of a recombinant vector of the invention is that there is no need to generate special cell lines adenovirus E4-orf6 from a nucleic acid integrated into the genome. Although such cell lines exist, they are not easily maintained in good order. This is at least in part due to the fact that with more and more foreign genes inserted into the genome of cell line it is difficult to maintain stability of all foreign sequences (or the expression thereof). In the present invention it was found that at least some of the problems associated with low yields of non-adenovirus serotype 5 based vectors and stability of adenovirus serotype vectors from subgroup B, such as adenovirus serotype 7, 11 and 35 on adenovirus serotype 5 packaging cell lines can be overcome with a recombinant adenovirus vector of the invention.

EXAMPLES

Example 1. Generation of E1-deleted Ad35 viruses expressing Ad5 E4-Orf6 on an Ad5 complementing cell line.

The sequencing of the Adenovirus serotype 35 genome as well as the construction of a plasmid-based vector system and generation of recombinant Ad35-based viruses have been described in detail in WO 00/70071.

The cloning of the Ad5-E4-orf6 coding sequence into pAdApt35IP1 (ECACC deposit no. P02041228, for cloning details of this plasmid see WO 00/70071) was performed as follows. The plasmid was digested with NheI and AvrII and dephosphorylated with Calf Intestine Phosphatase (New England Biolabs). Digested DNA was isolated from gel using the GeneClean kit. Plasmid pΔMT.Orf6.Hygro (Figure 1, ECACC deposit no. P02041226) was digested with NheI and subsequently partially digested with XbaI. After separation of the resulting bands on gel, the 1350 bp fragment corresponding to the ΔMT promoter linked to the E4-orf6 sequence was purified from gel. Next, both isolated fragments were ligated and transformed into electro-competent DH10B cells Invitrogen/LifeTechnologies) after which a colony with the insert in the correct orientation with respect to the SV40 poly(A) signal was selected for large scale DNA preparation. This resulted in construct pAd35.ΔMT.Orf6 (Figure 2), which contains the Ad5 E4-orf6 coding sequence functionally linked to a mutated metallothionein promoter (ΔMT). The ΔMT promoter has been described by Hagmeyer et al. (1996). The Ad5 E4-orf6 sequence corresponds to nucleotide 33193 to nucleotide 34077 in the Ad5 sequence (Genbank accession number M73260). To test whether the expression of Ad5 E4-orf6 proteins would make production of fully E1-

deleted Ad35 vectors possible on Ad5 complementing cells, pAd35.ΔMT.Orf6 was co-transfected with the Ad35 backbone construct pWE.Ad35.pIX-rITR onto PER.C6 cells. Hereto, pAd35.ΔMT.Orf6 was digested with PI-Psp-1 and pWE.Ad35.pIX-rITR was digested with NotI to liberate the adenoviral inserts from the backbone. 2 µg of digested pAd35.ΔMT.Orf6 and 6 µg of digested pWE.Ad35.pIX-rITR were transfected using LipofectAmine. The transfection mixture was added to PER.C6 cells that were seeded the day before at a density of $3.5x10^6$ cells per T25 flask. The next day the medium was changed for PER.C6 culture medium (DMEM with 10% FBS and 10 mM $MgCl_2$) and cells were further incubated at 37°C/10% CO_2. Control transfections were performed with pAdApt35.Luc co-transfected with pWE.Ad35.pIX-rITR, and pWE.Ad35.pIX-rITR alone. Two days after transfection, cells were passed from T25 to T80 flasks and incubated as described. Again three days later the culture transfected with pAd35.ΔMT.Orf6 together with the Ad35 backbone showed cytopathogenic effect (CPE) indicative of virus replication, and was harvested (including cells and medium) after a further incubation of 2 days. The cell suspension was subjected to 2 rounds of freeze/thaw cycles and the resulting material (crude lysate) was kept at -20°C until further use. The other flasks did not show CPE and were passed 1:3 in T80 flasks 6 days after transfer to T80. Again 5 days later the pAdApt35.Luc + pWE.Ad35.pIX-rITR transfected flask showed a few CPE-like events but this did not progress further. 0.2 and 0.5 ml of the crude lysate resulting from the pAd35.ΔMT.Orf6 transfection was used to re-infect PER.C6 cells at approximately 85% confluency in T80 flasks. This resulted in full CPE after one day of incubation indicating that infectious virus was present in the crude lysates. These

cultures were also harvested by two freeze/thaw cycles. Additional control transfections with construct pAd35.ΔMT.Orf6 alone onto PER.C6 were performed to confirm that orf6 expression by itself did not result in cell toxicity and CPE-like cell death. In conclusion, only the transfections with pAd35.ΔMT.Orf6 together with pWE.Ad35.pIX-rITR did result in CPE and virus replication.

PCR analysis was performed to confirm the presence of Ad35-based viral genomes with Ad5-E4-orf6 replacing the former E1 region. Hereto, viral DNA was isolated from the crude lysate samples as follows. 275 µl of crude lysate material was incubated with 10 µl DNaseI (10 mg/ml) at 37°C for 30 min. Subsequently, 6.0 µl 0.5 M EDTA (pH 8.0), 7.5 µl 20% SDS and 1.5 µl 20 mg/ml Proteinase K was added and mixed by vortexing. The mixture was then incubated at 50°C for 1 h. Finally, the viral DNA was isolated using the GeneClean Spin Kit (Bio 101, Inc.). 2 µl of the isolated DNA was then PCR amplified using primers 35psi-For and 35R4 (Table 1). The program was set at 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 5 min, and ended by an incubation at 72°C for 10 min. The primers are specific for Ad35 sequences and generate a fragment of 2.9 kb ranging from the packaging sequence to nt 4669 (numbering as in wt Ad35 sequence) thus including the Ad5 orf6 transgene cassette. Electrophoresis of the obtained PCR fragments showed that the fragments had the expected length matching with the control PCR fragments generated on the adapter plasmid pAd35.ΔMT.Orf6. Thus, fully E1-deleted Ad35-based vectors can be made on Ad5 complementing cells if the virus also expresses Ad5-E4orf6.

Example 2. Construction of pWE.Ad35.pIX-rITR5E4

A first PCR fragment was amplified using primers DF35-1 and 35FR (Table 1). Amplification was done with pWE.Ad35.pIX-rITR (see WO 00/70071) as template DNA using Pwo DNA polymerase (Roche) with additional DMSO (Sigma, final concentration 3%). The program was as follows: 94°C for 2 min followed by 30 cycles of (94°C for 30 sec, 52°C for 30 sec, 72°C for 3 min) and a final step of 72°C for 8 min to ensure complete fragments. Amplification resulted in a 1.6 kb fragment corresponding to nt 30224 to 31805 of the Ad35 sequence. A BamHI site was introduced at the 3' end. The amplified DNA was purified from gel using the GeneClean kit and ligated to the pCRScript/Amp cloning vector kit (Stratagene). Following transformation into electro-competent DH10B cells white colonies were selected for further analysis. This resulted in construct pCR-fiber35. Due to the blunt cloning the PCR fragment could be inserted in 2 orientations. A clone that had the insert with the BamHI site in the polylinker of the pCRScript/Amp vector at the 5' end was selected. Digestion with BamHI thus results in a 1.6 kb fragment. Sequencing confirmed correct amplification of the PCR fragment. A second PCR fragment was amplified using primers: 5E4F and 5E4R (Table 1). Amplification was done with pWE.Ad5.AflII-rITRsp, which is a cosmid vector containing an extra PacI site in pWE.Ad5.AflII-rITR (ECACC deposit no. P97082116 described in applicant's international patent application PCT/NL01/00824). pWE.Ad5.AflII-rITRsp served as a template using Pwo DNA polymerase as described above, although pWE.Ad5.AflII-rITR could also be used for the same purpose. After purification from gel, the DNA was digested with SstI and BamHI (both sites introduced during the PCR) and the 3 kb fragment was purified from agarose gel using the

GeneClean kit. The Ad5 E4 region that is amplified corresponds to bp 32794 to bp 35828 of the Ad5 sequence. A third PCR fragment was generated on pWE.Ad35.pIX-rITR using primers: 355ITR and 353ITR (Table 1). PCR amplification was performed as described above. The resulting 160 bp fragment is flanked by an SstI site (5' end) and an EcoRI site (3' end). After purification from gel as above, the DNA was digested with SstI and EcoRI. The 160 bp fragment corresponding to the right ITR of Ad35 was then separated from digested ends on a low melting point agarose gel and collected in gel. Next, pUC119 was digested with BamHI and EcoRI and the 3.1 kb fragment was purified from gel using the GeneClean kit. The above treated second and third PCR fragments were then ligated with BamHI/EcoRI digested pUC119 resulting in pUC.Ad5E4-35ITR. The cloned PCR derived inserts were sequenced to verify correct amplification. Next, the 1.6 kb insert in pCR-fiber35 was excised with BamHI and the fragment was purified from gel as above. pUC.Ad5E4-35ITR was also digested with BamHI and the linear fragment was purified from gel. Ligation of both fragments and selection of the clones that had the correct orientation relative to each other resulted in pUC.35-5E4 (Figure 3). The steps leading to the construction of pUC.35-5E4 are schematically represented in Figure 4. The adenovirus insert in pUC.35-5E4 was sub-cloned into pBr.Ad35.PRn (Figure 5; see WO 00/70071), a construct with Ad35 3' sequences. Hereto, construct pUC.35-5E4 is digested with MluI and NotI and the 4.7 kb fragment is purified from gel using the GeneClean kit. This fragment is then ligated with the vector fragment resulting from MluI and NotI digestion of construct pBr.Ad35.PRn. This 16.3 kb fragment was purified from gel using agarase enzyme (Roche). Ligations were then transformed into competent DH10B cells.

The resulting construct was named pBr.Ad35.PR5E4 (Figure 6, ECACC deposit no. P02041229). The last step entails cloning of the modified 3' end of the Ad35 sequence into the viral cosmid clone pWE.Ad35.pIX-rITR. Hereto, two fragments are combined in a lambda phage packaging reaction (Stratagene) according to manufacturer's instructions. The first is the 16.8 kb modified Ad35 insert from pBr.Ad35.PR5E4 obtained by digestion with PacI and SwaI and the second is a 22.8 kb fragment obtained by digestion of pWE.Ad35.pIX-rITR with PacI and SwaI. The correct combination of the two fragments yields pWE.Ad35.pIX-rITR.5E4 (Figure 7). Thus, in this construct the E4 region in the Ad35 backbone is replaced with the corresponding region derived from Ad5.

Example 3. Construction of pWE.Ad35.pIX-rITR5Orf6.

To obtain an adenoviral backbone construct that contains the Ad35 sequences from the pIX gene (nt 3401 in the Ad35 sequence) to the end of the right ITR but with the sequences for E4-orf6 and -orf6/7 exchanged for the corresponding sequences of Ad5, Ad35 and Ad5 sequences were PCR amplified and combined as described below. PCR fragments were generated with Pwo DNA polymerase with addition of DMSO up to 3%. The first PCR was done with pBr.Ad35.PRn (Figure 5; see WO 00/70071) as template and the primers E4-F1 and E4-R2 (Table 1). The program was set as follows: 94°C for 2 min, 5 cycles of (94°C for 30 sec, 50°C for 30 sec and 72°C for 1 min) followed by 30 cycles of (94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min) and ended with a final step at 68°C for 8 min. The resulting 1.8 kb fragment was purified using the GeneClean kit. The second PCR was done with pWE.Ad5.AflII-rITRsp, which is a cosmid vector containing a PacI site in pWE.Ad5.AflII-rITR (ECACC deposit no. P97082116, described in

applicant's international patent application PCT/NL01/00824, not yet published), as template and the primers E4-F3 and E4-R4 (Table 1). The program was set as follows: 94°C for 2 min followed by 30 cycles of (94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min) and ended with a final step at 68°C for 8 min. The 1.1 kb fragment was purified as above. The third PCR was done with pBr.Ad35.PRn as template and the primers E4-F5 and E4-R6 (Table 1). The program was set as follows: 94°C for 2 min, 5 cycles of (94°C for 30 sec, 48°C for 30 sec and 72°C for 45 sec) followed by 30 cycles of (94°C for 30 sec, 56°C for 30 sec and 72°C for 45 sec) and ended with a final step at 68°C for 8 min. The 366 bp fragment was purified as above. Samples of the purified fragments were loaded on a gel to estimate the concentration and then the fragments were mixed together to contain 700 ng PCR-1, 650 ng PCR-2 and 430 ng PCR-3 in a total of 30 µl. To this mixture 3 µl EcoPol buffer (New England Biolabs), 3 µl 2 mM dNTP solution and 3 µl milliQ H_2O was added. The resulting mixture was incubated at 94°C for 3 min and then cooled down to 65°C in a PCR machine at a rate of 0.5°C/sec. Following incubation at 65°C for 10 min, the mixture was further cooled down to 20°C at a rate of 0.05°C per sec and incubated for 10 min at 20°C. Then 1 µl (5 units) Klenow enzyme (New England Biolabs) was added followed by an incubation of 60 min at 37°C. 5 µl of this Klenow mixture was used as a template to separately amplify two fragments as follows. Primer set 1: NF-1 and NcoI-R (Table 1) was used in a reaction using Pwo DNA polymerase (Roche) with addition of DMSO to a final concentration of 3% and using the following settings of the PCR machine: 94°C for 2 min followed by 30 cycles of (94°C for 30 sec, 66°C for 30 sec and 72°C for 3 min) followed by a final incubation at 68°C for 8 min. Primer set 2: NcoI-F and NR-2 (Table 1) was used

in a reaction using Pwo DNA polymerase (Roche) with addition of DMSO to a final concentration of 3% and using the following settings of the PCR machine: 94°C for 2 min followed by 30 cycles of (94°C for 30 sec, 62°C for 30 sec and 72°C for 90 sec) followed by a final incubation at 68°C for 8 min. The resulting fragments of 2.7 kb (primer set 1) and 1.1 kb (primer set 2) were purified from gel using the GeneClean kit and each was ligated to the pCRscriptAmp vector (Stratagene) and transformed into DH10B electrocompetent cells. This resulted in construct pCRscriptAmp.NFI-NcoIR (Figure 8) and construct pCRscriptAmp.NcoIF-NR2 (Figure 9). Since the inserts contained blunt ends two orientations were obtained of each cloning. Using KpnI digestions the constructs with the orientation needed for further cloning were selected (see Figures 8 and 9). The inserts were then sequenced to verify correct amplification. Next, part of the insert from pCRscriptAmp-NcoIF-NR2 was excised using BamHI and NcoI and purified from gel as above. pCRscriptAmp-NFI-NcoIR was digested with the same enzymes and the vector containing fragment was also purified from gel. Ligation of these fragments resulted in pCR.NF1-NR2 (Figure 10). pCR.NF1-NR2 contains Ad35 sequences between nt 30162 and 33234 of the Ad35 sequence with E4-orf6 and E4-orf6/7 sequences between nt 31879 and 32974 replaced for Ad5 derived sequences located between 32968 and 34077 from the published Ad5 sequence in Genbank (Accession Number M73260). Thus, as can be seen in the amino acid alignments presented in Figure 11 and 12, the amino acid sequence of the cloned E4-orf6 protein is identical to the E4-orf6 sequence found in Ad5 and the E4-orf6/7 amino acid sequence is for the greater part identical to the E4-orf6/7 sequence present in Ad5. Obviously, different hybrid Ad35-Ad5 E4 constructs can be designed using

the general method outlined above without departing from the invention. This chimeric insert from pCR.NF1-NR2 was then cloned into pWE.Ad35.pIX-rITR: pCR.NF1-NR-2 was digested with MluI and NdeI and the resulting 2.8 kb fragment was purified from gel using the GeneClean kit. Construct pBr.Ad35.PRn was also digested with MluI and NdeI and the 18 kb vector fragment was isolated from gel using agarase enzym (Roche). Ligation of both fragments resulted in construct pBr.Ad35.PR.5Orf6 (Figure 13, ECACC deposit no. P02041227). The Ad35 sequences between PacI and SwaI containing the chimeric E4 region in this construct are then cloned into construct pWE.Ad35.pIX-rITR using lambda-phage packaging as described above. The resulting pWE.Ad35pIX-rITR.5Orf6 (Figure 14) is then used to generate recombinant Ad35-based viruses by co-transfection on PER.C6 packaging cells with an Ad35 adapter plasmid.

Example 4. Construction of pWE.Ad35.pIX-rITRΔE3, pWE.Ad35.pIX-rITRΔE3.5E4 and pWE.Ad35.pIX-rITRΔE35Orf6.

The Ad35 backbone was further modified by a deletion of E3 sequences. E3 proteins are known to modulate the host immune response to adenovirus infection and are therefore not necessary for *in vitro* propagation of recombinant viruses. Furthermore, the deletion of E3 sequences allows for insertion of larger heterologous sequences in the vectors without compromising the packaging efficiency. Also, for the application of adenoviral vectors as vaccination vehicles, expression of immunomodulatory genes encoded by the E3 region is not preferred. Methods for deleting E3 sequences in the pBr.Ad35.PRn plasmid (Figure 5) are described below.

First a PCR product was generated with primers 35E3for and 35E3rev (Table 1) using Pwo DNA polymerase (Roche)

according to manufacturers instructions and pBr.Ad35.PRn as template DNA. The program was set at 94°C for 2 min and 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72 °C for 1 min, followed by 68°C for 8 min. The amplified fragment contains Ad35 sequences from nt 26814 to 27647 (see WO 00/70071) and is flanked at the 3' end by an MluI site. The resulting 833 bp fragment was purified using the Qiaquick PCR purification kit (Qiagen) and digested with MluI and StuI. The digested PCR fragment was then purified from an LMP agarose gel using the Qiaquick gel extraction kit (Qiagen). Construct pBr.Ad35.PRn was also digested with MluI and StuI and the 17.3 kb vector containing fragment was isolated from agarose gel using agarase enzym (Roche) using methods known to persons skilled in the art. Both isolated DNAs were ligated and transformed into Max-efficiency DH5α competent bacteria (Invitrogen/LTI) to give pBr.Ad35.PRnΔE3 (Figure 15). The deleted sequences encompass nt 27648 to 30320 of the Ad35 sequence resulting in a 2673 bp deletion. The E3 deletion was then introduced in construct pWE.Ad35.pIX-rITR using lambda-phage packaging extracts (Stratagene). Hereto, both pWE.Ad35.pIX-rITR and pBr.Ad35.PRnΔE3 were digested with PacI and SwaI and the respective 22.8 kb and 14 kb fragments were isolated from low melting point agarose gels using agarase enzym (Roche). After ligation and packaging using STBL-2 cells (Invitrogen/LTI), construct pWE.Ad35.pIX-rITRΔE3 was obtained.

To construct the E3-deleted versions of the E4-modified backbone constructs described above, the E4 modifications were introduced into the pBr.Ad35.PRnΔE3 construct as follows. Construct pUC.35-5E4 (Figure 3) was digested with MluI and NotI and the 4.7 kb fragment was isolated from gel using the GeneClean II kit. Construct pBr.Ad35.PRnΔE3 was

also digested with MluI and NotI and the 13.6 kb vector fragment was isolated from gel using the GeneClean spin kit. Ligation of these fragments resulted in construct pBr.Ad35.ΔE3.PR5E4 (Figure 16). Construct pCR.NF1-NR2 (Figure 10) was digested with MluI, NdeI and BglI (the latter to digest the vector fragment into smaller fragments), and the 2.8 kb fragment was isolated from gel using the GeneClean spin kit. Construct pBr.Ad35.PRnΔE3 was digested with MluI and NdeI, dephosphorylated using CIP enzym (New England Biolabs) and the 15.2 kb vector fragment was also isolated using the GeneClean spin kit. Ligation of these fragments gave construct pBr.Ad35.ΔE3.PR5Orf6 (Figure 17). pBr.Ad35.ΔE3.PR5E4 and pBr.Ad35.ΔE3.PR5Orf6 are then used to swap the 3' PacI-SwaI fragment in pWE.Ad35.pIX-rITR for the corresponding regions from pBr.Ad35.ΔE3.PR5E4 and pBr.Ad35.ΔE3.PR5Orf6 as described intra. This leads to constructs pWE.Ad35.pIX-rITRΔE3.5E4 and pWE.Ad35.pIX-rITRΔE3.5Orf6. An alternative method to generate these large cosmids is to use three fragments in the ligation reaction for packaging: a 14.7 kb NotI-PacI fragment from pWE.Ad35.pIX-rITR, the PacI-NotI insert from pBr.Ad35.ΔE3.PR5E4 or pBr.Ad35.ΔE3.PR5Orf6 and the NotI digested pWE15 cosmid vector fragment (Stratagene). This latter fragment can also be isolated from the NotI/PacI digestion of pWE.Ad35.pIX-rITR.

Example 5. Generation of E1- and E1/E3-deleted Ad35-based vectors on PER.C6 cells.

To enable generation of recombinant Ad35 viruses on the complementing cell line PER.C6 using the pBr.Ad35.PRn-based constructs, we first made a new construct containing Ad35 sequences from bp 3401 to bp 24650 of the Ad35 sequence (WO

00/70071) and thus overlaps with both the adapter plasmids and the pBr.Ad35.PRn-based constructs. Transfection of these three plasmids into PER.C6 cells and a double homologous recombination event leads to a complete viral genome and replication of recombinant viruses as outlined in Figure 18. The required plasmid was made by deletion of a large part of the Ad35 sequences in pWE.Ad35.pIX-rITR. Hereto, pWE.Ad35.pIX-rITR was digested with EcoRV and the 29 kb vector containing fragment was purified from a low melting point gel using the Geneclean spin kit. The purified DNA was self-ligated and used to transform DH10B electro-competent bacteria (Invitrogen/LTI) resulting in pWE.Ad35.pIX-EcoRV (Figure 19).

All DNAs used for transfection were digested as indicated in Table 2, heat-inactivated at 65°C for 15 min and used without further treatment in the transfection. PER.C6 cells were seeded the day prior to transfection in T25 flasks at a density of $3x10^6$ cells/flask and transfected as indicated in Table 2 using LipofectAmine (Invitrogen/LTI) according to manufacturers instructions, except that the transfection mixture in serum-free DMEM medium (Gibco/BRL) was replaced for PER.C6 culture medium (DMEM, 10% FBS and 10 mM $MgCl_2$) after 5 h. The day after, transfection efficiency was estimated at 50% by fluorescence microscopy. Two days later, cells were trypsinized and reseeded in T80 flasks and further incubated at 37°C/10%CO_2. Six days following transfection all cultures showed full cytopathogenic effect (CPE, indicative for virus propagation) except for the PER.C6 culture transfected with Ad35.AdApt.eGFP + pWE.Ad35.pIX-rITR. One day later, cells and medium in the flasks with CPE were harvested and subjected to two freeze/thaw cycles, clarified from cell debris by centrifugation (10 min at 1500 rpm) and

100 µl of these crude lysates were used to re-infect fresh PER.C6 cells at 85% confluency in T80 flasks. The transfection of Ad35.AdApt.eGFP + pWE.Ad35.pIX-rITR that did not show signs of CPE was harvested by trypsinisation and also treated as above. Two days following infection of fresh PER.C6 cells all flasks showed full CPE except for the one that showed no signs of CPE at the time of initial harvesting. This clearly shows that fully E1-deleted Ad35-based viruses can be made on PER.C6 cells when the Ad5 E4-orf6 gene product is expressed from the Ad35 backbone.

Table 1. Primer sequences.

35FR	5′-CGGGATCCACTTTATTTTAGTTGTCGTCTTC-3′	(SEQ ID NO:1)
35R4	5′-CGGAATTCTTAATTAAGGGAAATGCAAATCTGTGAGG-3′	(SEQ ID NO:2)
35psi-For	5′-GTGGTATTTATGGCAGGGTG-3′	(SEQ ID NO:3)
DF35-1	5′-CACTCACCACCTCCAATTCC-3′	(SEQ ID NO:4)
5E4F	5′-CGGGATCCGTTTGTGTTATGTTTCAACGTG-3′	(SEQ ID NO:5)
5E4R	5′-GCTGGCGAGCTCGGCGGAGTAACTTGTATGTG-3′	(SEQ ID NO:6)
355ITR	5′-GATCCGGAGCTCACAACGTCATTTTCCCACG-3′	(SEQ ID NO:7)
353ITR	5′-AGGAATTCGCGGCCGCATTTAAATC-3′	(SEQID NO:8)
E4-F1	5′-AGAGGAACACATTCCCCC-3′	(SEQ ID NO:9)
E4-R2	5′-GGGGAGAAAGGACTGTGTATTCTGTCAAATGG-3′	(SEQ ID NO:10)
E4-F3	5′-TTTGACAGAATACACAGTCCTTTCTCCCCGGCTGG- 3′	(SEQ ID NO:11)
E4-R4	5′-ACAAAATACGAGAATGACTACGTCCGGCGTTCC- 3′	(SEQ ID NO:12)
E4-F5	5′-GGACGTAGTCATTCTCGTATTTTGTATAGC-3′	(SEQ ID NO:13)
E4-R6	5′-TCACCAACACAGTGGGGG-3′	(SEQ ID NO:14)
NF-1	5′-CCACAACCCCCACTACTCCC-3′	(SEQ ID NO:15)
NR-2	5′-CGTCTCTTCCCTCTCCTCTCC-3′	(SEQ ID NO:16)
NcoI-R	5′-AGGATCATCCGCTGCTGCCC-3′	(SEQ ID NO:17)
NcoI-F	5′-CATCAGGATAGGGCGGTGG-3′	(SEQ ID NO:18)
35E3for	5′-AATGACTAATGCAGGTGCGC-3′	(SEQ ID NO:19)
35E3rev	5′-CGACGCGTTGTAGTCGTTGAGCTTCTAG-3′	(SEQ ID NO:20)

Table 2. List of constructs used to generation E1-deleted Ad35 based viruses on PER.C6 cells as described in the examples. Adapter constructs were digested with PacI, pWE.Ad35.pIX-EcoRV was digested with NotI and EcoRV, E4-modified pBr-based constructs were digested with PacI and NotI.

No.	Constructs			CPE
1	pAdApt35.eGFP	pWE.Ad35.pIX-EcoRV	pBr.Ad35.PR5E4	Yes
2	pAdApt35.eGFP	pWE.Ad35.pIX-EcoRV	pBr.Ad35.PR5Orf6	Yes
3	pAdApt35.eGFP	pWE.Ad35.pIX-EcoRV	pBr.Ad35.ΔE3PR5E4	Yes
4	pAdApt35.eGFP	pWE.Ad35.pIX-EcoRV	pBr.Ad35.ΔE3.PR5Orf6	Yes
5	pAdApt35.eGFP	pWE.Ad35.pIX-rITRxNotI		No
6	pAdApt5.eGFP	pWE.Ad5.AflII-rITRxPacI		Yes

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Claims

1. A recombinant adenovirus vector comprising structural and non-structural elements of an adenovirus of a first serotype, wherein said vector further comprises a sequence encoding a functional E4-orf6 protein, or a functional part, derivative and/or analogue thereof, wherein said sequence is selected from the group consisting of:

1. an E4-orf6 encoding sequence derived from an adenovirus of a second serotype different from said first serotype;
2. an E4-orf6 encoding sequence derived from an adenovirus of said first serotype comprising a deletion, mutation, addition and/or substitution in one or more codons; and
3. an E4-orf6 encoding sequence comprising a fusion between a part of an E4-orf6 encoding sequence derived from a second serotype different from said first serotype and a part of an E4-orf6 encoding sequence derived from a third serotype, wherein said third serotype may be identical to-, or different from said first serotype.

2. A recombinant adenovirus vector according to claim 1, wherein said first serotype and said second serotype are from different adenovirus subgroups.

3. A recombinant adenovirus vector according to claim 1, wherein said first serotype is from a subgroup other than subgroup C and wherein said E4-orf6 encoding sequence is

derived from an adenovirus serotype of subgroup C.

4. A recombinant adenovirus according to claim 1, wherein said first serotype is from subgroup B and said second serotype is from subgroup C.

5. A recombinant adenovirus vector according to claim 1, wherein said E4-orf6 encoding sequence is derived from adenovirus serotype 5.

6. A recombinant adenovirus vector according to any one of claims 1 to 5, wherein said first serotype is selected from the group consisting of adenovirus serotypes 11, 14, 16, 21, 34, 35 and 50.

7. A recombinant adenovirus vector according to any one of claims 1 to 6, further comprising a sequence encoding a non-adenoviral protein, polypeptide or peptide.

8. A recombinant adenovirus vector according to claim 7, wherein said non-adenoviral protein, polypeptide or peptide is selected from the group consisting of: a cell-death inducing polypeptide, an antigenic determinant of a pathogenic organism, a tumor specific antigen, a viral protein, a hormone and a cytokine.

9. A method for producing a recombinant adenovirus vector comprising structural and non-structural elements of an adenovirus of a first serotype, said method comprising the steps of:

a. providing a complementing cell harbouring an E1B-55K encoding sequence, or a functional part, derivative

and/or analogue thereof, derived from an adenovirus of a second serotype in expressible form, with the necessary elements of an adenovirus such as to allow assembly of said recombinant adenovirus vector by said complementing cell, wherein said elements comprise at least some structural and non-structural elements from an adenovirus of said first serotype different from said second serotype and a sequence encoding a functional E4-orf6 protein, or a functional part, derivative and/or analogue thereof, which is compatible with said expressible E1B-55K protein in said complementing cell;

b. culturing said complementing cell in a medium under conditions allowing for production and assembly of the adenovirus vector to take place; and

c. harvesting the recombinant adenovirus vector so produced from the medium and/or the complementing cell,

wherein the sequence encoding the compatible E4-orf6 protein is present in the recombinant adenovirus vector so produced.

10. A method according to claim 9, wherein said E4-orf6 encoding sequence is selected from the group consisting of:

1. an E4-orf6 encoding sequence derived from an adenovirus of said second serotype;

2. an E4-orf6 encoding sequence derived from an adenovirus of a third serotype different from said first and second serotype;

3. an E4-orf6 encoding sequence derived from an adenovirus of said first serotype comprising a deletion, mutation, addition and/or substitution in one or more codons; and

4. an E4-orf6 encoding sequence comprising a fusion between a part of an E4-orf6 encoding sequence derived from a third serotype and a part of an E4-orf6 encoding sequence derived from an adenovirus of said second serotype, wherein said third serotype may be identical to-, or different from said first serotype.

11. A method according to any one of claims 9 or 10, wherein said first and said second serotype are from different subgroups.

12. A method according to any one of claims 9 to 11, wherein said second serotype is an adenovirus serotype of subgroup C.

13. A method according to claim 12, wherein said second serotype is adenovirus serotype 5.

14. A method according to any one of claims 9 to 13, wherein said first serotype is an adenovirus serotype of subgroup B.

15. A method according to claim 14, wherein said first serotype is selected from the group consisting of adenovirus serotypes 11, 14, 16, 21, 34, 35 and 50.

16. A method according to any one of claims 9 to 15, wherein said E1B-55K encoding sequence is integrated into the genome of said complementing cell.

17. A method according to any one of claims 9 to 16, wherein said complementing cell is derived from a primary, diploid human cell, or a progenitor cell thereof.

18. A method according to any one of claims 9 to 17, wherein said complementing cell is derived from a primary human retinoblast cell, a primary human embryonic kidney cell, a primary human neuronal cell or a primary human amniocyte.

19. A method according to any one of claims 9 to 18, wherein said complementing cell comprises, integrated into its genome, a nucleic acid encoding at least one adenovirus E1A protein.

20. A method according to claim 19, wherein said nucleic acid encoding at least one adenovirus E1A protein is derived from an adenovirus serotype of a subgroup different than subgroup B.

21. A method according to claim 19, wherein said nucleic acid encoding at least one adenovirus E1A protein is derived from an adenovirus serotype of subgroup C.

22. A method according to claim 20, wherein said nucleic acid encoding at least one adenovirus E1A protein is derived from an adenovirus serotype 5.

23. A method according to any one of claims 9 to 22, wherein said E4-orf6 encoding sequence and said E1B-55K encoding sequence are derived from different adenovirus serotypes and wherein said different adenovirus serotypes are members of the same adenovirus subgroup.

24. A method according to any one of claims 9 to 22, wherein said E4-orf6 encoding sequence and said E1B-55K encoding

sequence are derived from different adenovirus serotypes and wherein said different adenovirus serotypes are both members of subgroup C.

25. A method according to any one of claims 9 to 22, wherein said E4-orf6 encoding sequence and said E1B-55K encoding sequence are derived from the same adenovirus serotype.

26. A method according to claim 25, wherein said E4-orf6 encoding sequence and said E1B-55K encoding sequence are derived from adenovirus serotype 5.

27. A method according to claim 9, wherein said complementing cell is a PER.C6 cell or a derivative thereof.

28. A pharmaceutical composition comprising a recombinant adenoviral vector according to any one of claims 1 to 8 or obtainable by a method according to any one of claims 9 to 27.

29. A method of treating a human body comprising administering to a human body a recombinant adenoviral vector according to any one of claims 1 to 8, or a pharmaceutical composition according to claim 28.

30. A kit of parts comprising:

a) a complementing cell for producing a recombinant adenovirus vector comprising structural and non-structural elements of an adenovirus of a first serotype, said cell harbouring an E1B-55K encoding sequence, or a functional part, derivative and/or

analogue thereof, derived from an adenovirus of a second serotype in expressible form; and

b) on one or more replicable nucleic acid vectors all necessary adenoviral elements such as to allow assembly of said recombinant adenovirus vector by said complementing cell, wherein said elements comprise at least some structural and non-structural elements from an adenovirus of said first serotype different from said second serotype and a sequence encoding a functional E4-orf6 protein, or a functional part, derivative and/or analogue thereof, which is compatible with said expressible E1B-55K protein in said complementing cell.

31. A kit of parts according to claim 30, wherein said E4-orf6 encoding sequence is selected from the group consisting of:

1. an E4-orf6 encoding sequence derived from an adenovirus of said second serotype;
2. an E4-orf6 encoding sequence derived from an adenovirus of a third serotype different from said first and second serotype;
3. an E4-orf6 encoding sequence derived from an adenovirus of said first serotype comprising a deletion, mutation, addition and/or substitution in one or more codons; and
4. an E4-orf6 encoding sequence comprising a fusion between a part of an E4-orf6 encoding sequence derived from a third serotype and a part of an E4-orf6 encoding sequence derived from an adenovirus of said second serotype, wherein said third serotype may be identical to-, or different from said first serotype.