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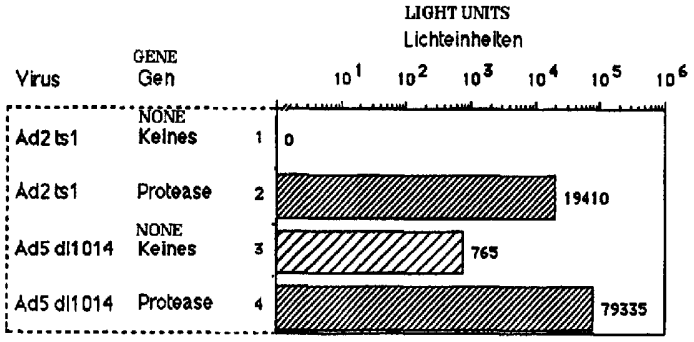

(57) Abstract

The invention concerns a process for preparing viruses and viral vectors by the transfection of mammal cells with complexes of viral DNA and a polycation, which is optionally conjugated with a ligand for the target cell, in the presence of an endosomolytic agent. The process affords particular advantages for the production of recombinant retroviruses, adenoviruses and adeno-associated viruses in the cell, permitting high virus titres in packing cell lines and in primary cells. The area of application is above all gene therapy.

(57) Zusammenfassung

Verfahren zur Herstellung von Viren und viralen Vektoren mittels Transfektion von Säugetierzellen mit Komplexen aus viraler DNA und einem Polykation, das gegebenenfalls mit einem Liganden für die Zielzelle konjugiert ist, in Gegenwart eines endosomolytischen Mittels. Das Verfahren bringt besondere Vorteile für die Produktion von rekombinanten Retroviren, Adenoviren und Adeno-assoziierten Viren in der Zelle, wobei es hohe Virustiter in Verpackungszellinien sowie in primären Zellen ermöglicht. Anwendungsgebiet ist vor allem die Gentherapie.

Abstract

Process for preparing viruses and viral vectors by transfection of mammalian cells with complexes of viral DNA and a polycation which is optionally conjugated with a ligand for the target cell, in the presence of an endosomolytic agent. The process confers particular advantages for the production of recombinant retroviruses, adenoviruses and adeno-associated viruses in the cell, making it possible to obtain high virus titres in packaging cell lines and in primary cells. The primary field of application is gene therapy.

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Process for preparing viruses and viral vectors

The invention relates to the preparation of viruses and viral vectors, particularly for use in gene therapy.

Genetic changes with respect to therapeutic activity require a viable gene transfer into primary cells. Proposed transporting means for the genes include recombinant viruses, such as retroviruses, adenoviruses, herpes viruses, which are currently used in a number of clinical studies (Miller, 1993). These vectors are recombinant viruses, the viral genes of which have partly or wholly been replaced by foreign genetic material. After infection of cells the recombinant virus genome is stably integrated into the genome of the host cell in certain cases, depending on the vector system used. The gene transfer by means of retroviral vectors are characterised particularly by high infective efficiency and stable integration of the provirus into the host cell genome. They thus permit stable gene transfer into the majority of dividing primary cells. Up till now, it has only been possible to introduce genetic material into haematopoietic parent cells in this way. One restriction of this gene transfer system is its limited packaging capacity; because of size restrictions (about 6 kb) retroviral vectors can transduce only cDNAs or small genes. A further disadvantage is that titres of infectious particles are generally low compared with DNA viruses, such as adenovirus. The lower titres are a general feature of retroviruses; thus, fibroblasts which have been infected with Moloney-Murine Leukemia Virus (M-MuLV) produce about 1 - 5 x 10^7 infectious particles per millilitre of culture medium (cfu/ml). The titres of recombinant retroviruses are usually substantially lower

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(1 - 10 x 10^5 cfu/ml) and can often only be achieved after laborious cloning of virus-producing cells. Relatively high titres may be obtained from small retroviral vectors which carry a single gene, e.g. N2 (Armentano et al., 1987), whilst the titres generally reduce as the vector size increases, e.g. if a second gene is transduced. It has been observed that the titres frequently correlate to the number of integrated virus copies.

With respect to the use of recombinant retroviruses in gene therapy the low titre is a major disadvantage, whereas the problem of the limited packaging capacity is generally of less significance, since in most cases cDNAs are expressed.

Some attempts have already been made to increase the retroviral titre. First of all, it was shown that the infection of packaging cells with a virus of a different host spectrum yields higher titres compared with the transfection of packaging cells (Miller et al., 1993). This increase in the titre can be explained by the observation that the expression of the retroviral genome is higher after infection than after transfection (Hwang and Gilboa, 1984). The co-cultivation of two packaging cell lines which produce viruses with different host spectra can lead to enormous increases in the efficiency of virus production as the consequence of a significant increase in the number of copies of proviruses (Bestwick et al., 1988; Bodine et al., 1990; Lynch and Miller, 1993). However, a serious disadvantage of this method is the formation of replication-competent helper viruses in large amounts (Lynch and Miller, 1993).

The packaging cell lines used hitherto are therefore obviously unsuitable for gene transfer *in vivo*. Moreover, this method is labour intensive and time

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consuming, since co-cultivation takes a long time and afterwards the cells still have to be cloned. Recently, a new packaging cell line was described which is derived from the Ad5-transformed human embryonic kidney cell line 293. This cell line makes it possible to form high titres of retroviral vectors after transient transfection (Pear et al., 1993).

Apart from retroviruses, the parvovirus AAV ("adeno-associated virus"), dependent on a helper virus, and herpes viruses and adenoviruses are used for applications in gene therapy (review article by Jolly, 1994; Trapnell and Gorziglia, 1994).

In the preparation of recombinant AAV-virus the first step is normally the transfection of vector DNA sequences. Since the AAV vectors are deficient in important replication functions and suitable packaging lines are not yet available, the preparation of AAV particles requires infection with helper virus in addition to the transfection: the methods currently used for preparing recombinant AAV require transfection in order to introduce into the cell a plasmid which has the Rep- and Cap-functions and thus acts as a packaging plasmid, as well as a plasmid which carries the gene of interest, flanked by the AAV "terminal repeats". Overlying this transfection is infection with adenovirus which prepares the helper functions required for replication of the AAV virus.

In view of its advantages for gene therapy, AAV is used more and more as a vector system (Carter et al., 1992; Samulski et al., 1982; Kotin, 1994). The most widely used method of producing recombinant AAV is based on a two plasmid system: the first plasmid codes for the Rep- and Cap-functions and a second plasmid codes for the gene concerned, which has a length of less than 5000 bp,

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flanked by the 145 nucleotide long ITRs (inverted terminal repeats). In the presence of adenovirus or herpes virus the AAV Rep-sequence causes amplification of the gene sequence flanked by the ITRs, controlled by cis-acting signals in the ITRs. Then the Cap-gene products encapsulate the single stranded genome, which is about 5000 nucleotides in size, again using signals coded by the ITRs. The virus particle descendants obtained by this method have no AAV-genes of any kind and can infect a wide spectrum of cell types and integrate into their genome. These properties as well as the theoretical simplicity of production of new recombinant viruses are the properties which have made AAV the preferred vector for gene transfer applications. However, for clinical applications, these vectors are subject to restrictions; the reason for this is the problems involved with preparing the virus in a quantity and purity as required for safety and quality testing. One restriction in manufacture was the impossibility, hitherto, of preparing stable cell lines which express the AAV Rep- and Cap-genes required for the packaging of the vectors. Hitherto, production was restricted to transient transfection systems by means of which both the vector skeleton and also the Cap- and Rep-functions could be introduced. The low performance of the transfection systems based on lipids or calcium phosphate precipitation, which was normally used for this purpose, restricted the amount of AAV-vector which can be produced per cell type. The fact that an adenovirus helper virus is necessary in these systems also means that the initial virus harvest is a mixture of adenovirus particles with the desired AAV-vector, the adenoviruses predominating in quantity. This imbalance (high adenovirus titre/low AAV-titre) also makes subsequent purification of the AAV-vector difficult.

There are several methods of preparing recombinant

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adenovirus vectors which are used in gene therapy (Ghosh-Choudhury et al., 1986; Bett et al., 1994; review by Berkner, 1992). These are based on the fact that, following the transfection of adenovirus DNA, infectious adenovirus can be formed (Graham and Van der Eb, 1973). One of these methods envisages co-transfection of two plasmids which can recombine with one another. One plasmid codes for the entire adenovirus genome with the exception of one of the early virus genes (e.g. E1) the sequence of which was removed to make space for the insertion of a foreign gene. A second plasmid carries sequences which are connected directly upstream and downstream to the point in the virus genome which was removed from the first plasmid. These regions are thus present in both plasmids and are able to recombine with one another. If a foreign gene with a corresponding promoter is inserted in the second plasmid between the flanking regions complementary to the adenoplasmid, a subsequent recombination of the two plasmids leads to the incorporation of the foreign gene in the viral genome. In order to avoid the production of particles which contain only the sequence of the first plasmid, the viral packaging signal Psi is removed from this plasmid and/or bacterial sequences are inserted which make the virus DNA too long for packaging and can only be removed by recombination with the second plasmid. The two plasmids are introduced into cells of a cell line which carries the adenovirus E1-sequence and thus has the function required for virus replication, which is absent from the two plasmids. During recombination of the two plasmids, a DNA sequence is formed which contains all the functions necessary for the replication and packaging of adenovirus, apart from the E1-functions which are supplied by the cell line ("helper cells" or "production cells"), and also the new gene which is coded by the first plasmid (vector plasmid).

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Up till now, the standard method of preparing this class of recombinant adenoviruses was based on calcium phosphate transfection of the two plasmids into 293 cells. After transfection the cells are covered with a layer of agar. After an incubation period of 10 to 14 days the adenovirus-infected cells are visible as plaques. During this time, recombination takes place between the two plasmids. The plaques are formed by the lysing of an originally infected cell and infection of the adjacent cells by the released virus. The uppermost agar layer focuses and localises the virus infection and makes it possible to isolate a virus which originates from individual infection or recombination events. This plaque formation is frequently the limiting step in the preparation of recombinant adenovirus on account of the low transfection efficiency which can be achieved by the calcium phosphate method and the low efficiency of plaque formation.

The aim of the present invention was to provide an improved method of preparing viruses and viral vectors.

In recent times, gene transfer systems have been developed based on mechanisms which the cell uses for transporting macromolecules, e.g. the extremely effective route of receptor-mediated endocytosis (Wu and Wu, 1987; EP-A1 0 388 758; WO 91/17773, WO 92/17210 and WO 92/19281). Using this method, which makes use of bifunctional molecular conjugates which have a DNA-binding domain and a domain with specificity for a cell surface receptor, it has been possible to achieve high gene transfer rates.

Gene transfer by the physiological route, as represented by receptor-mediated endocytosis using nucleic acid complexes, has major advantages (non-toxic mechanism of passage through the cell membrane; possibility of

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administering biologically active nucleic acids on a repetitive or continuous basis; possibility of cell-specific targeting; possibility of preparing the conjugates in large amounts). An additional improvement to the system was achieved by a technique which uses the ability of certain viruses and virus components to break open endosomes. By adding these endosomolytic agents it has been possible to achieve a substantial increase in the expression rates of the genes imported into the cell. This method is essentially characterised by a transfer of large DNA fragments, transfer in a high copy number and a generally transient gene transfer.

Using this gene transfer system it is possible to introduce even large DNA fragments (>50 kb) into dividing or non-dividing cells, including primary cells which resist standard methods of transfection. Although the gene expression investigated hitherto was transient, albeit long-lasting, hitherto it has not been possible to achieve gene transfer into haematopoietic parent cells of mammals or into early precursor cells.

The solution to the above problem originated with the idea of combining the advantages of the two gene transfer systems, namely the system based on recombinant viruses and that based on receptor-mediated endocytosis, with the aim of making use of the high transfection efficiency of the system based on receptor-mediated endocytosis, which uses endosomolytic agents to increase heterologous gene expression, in order to prepare viruses and viral vectors.

The present invention thus relates to a process for preparing viruses or viral vectors, characterised in that one or more plasmids containing viral vector DNA and optionally one or more sequences coding for viral genes which increase the infectiousness of the virus or viral

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vector are complexed with a polycation, optionally conjugated with a ligand for the target cell, and mammalian cells are transfected with the complex in the presence of an endosomolytic agent.

The viral vector DNA is preferably recombinant, i.e. it contains, in addition to viral vector sequences, a DNA sequence, e.g. a therapeutically active gene, which is to be expressed in the target cell to which the viral vectors are applied, e.g. in the course of gene therapy. (If wild-type virus vectors are prepared, these are used particularly for the production of vaccines).

The method used in the course of the present process for introducing DNA into eukaryotic cells is known *per se* (Wagner et al., 1991a and 1991b; Cotten et al., 1992; Wagner et al., 1992a and 1992b; Zatloukal et al., 1992; Cotten et al., 1993a and 1993b; Curiel et al., 1991; WO 93/07283 and WO 93/07282).

A preferred embodiment of the process according to the invention consists in delivering the plasmid DNA into the cells in the form of a ternary complex with, on the one hand, a conjugate of a ligand for the target cell and a polycation, e.g. transferrin-polylysine, and on the other hand a conjugate of an endosomolytic agent and a polycation, e.g. adenovirus polylysine. In this case, the endosomolytic agent is an adenovirus which is present in the form of a polylysine conjugate capable of being complexed with the DNA and thus integrated in the complex. Preferably an adenovirus inactivated, for example, by physical-chemical methods is used, optionally in admixture with non-inactivated adenovirus, which is bound to polylysine via a biotin-streptavidin bridge. (In the literature this method, in which the endosomolytic agent is adenovirus and the ligand is transferrin, is referred to as "adenovirus-aided

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transferrinfection").

With regard to carrying out the process, in the choice of complex partners for the plasmid DNA (ligand, polycation) and the endosomolytic agent the process permits wide variations; with respect to this, reference is made to the disclosure of WO 93/07283.

The present invention is used particularly in gene therapy.

Examples of genes which may be used in somatic gene therapy which may occur, within the scope of the present invention, as components of recombinant virus vector DNA, are factor VIII (haemophilia A) (see for example Wood et al., 1984), factor IX (haemophilia B), (see for example Kurachi et al. 1982), adenosinedeaminase (SCID), (see for example Valerio et al., 1984), α-1 antitrypsin (lung emphysema), (see for example Ciliberto et al., 1985) or the "Cystic fibrosis transmembrane conductance regulator gene" (see for example Riordan et al., 1989). These examples do not constitute any kind of restriction to the invention.

In a preferred embodiment, the plasmid DNA codes a retrovirus vector (this DNA is hereinafter referred to as "retrovirus vector DNA").

Application of the process according to the invention to retrovirus vector DNA yielded surprisingly high titre levels. The retrovirus titre levels achievable by means of the process according to the invention in packaging and primary cells are exceptionally high: the transfected cells contain, surprisingly, five to ten or more intact integrated provirus copies, which results in the production of 30 to 100 times higher titres of infectious vector particles. Thus, 30 to 100 times

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higher titres of infectious vector particles are obtained compared with the calcium phosphate method or using retroviral infection.

The main advantages of the process according to the invention, applied to retrovirus DNA, over the established methods are as follows:

1) In established packaging cell lines, high virus titres are obtained even after transient transfection, and a further increase in the titre is achieved after selection for stable integration. Moreover, production of virus in genetically unaltered cells is made possible after co-transfection of retroviral vector constructs and packaging functions.

2) Another crucial advantage of the process according to the invention is the fact that the transfected cells are free from replication-competent helper virus or replicating adenovirus. The high titres were achieved not only for transiently transfected cells but also for stable clonal or pooled transfectants. Remarkably, the virus titres of pooled populations or transfected clones were not significantly lower than those of isolated clones, which is generally not the case with calcium phosphate-transfected clones. The increased virus titres were most probably due to multiple integrated copies of the transfected plasmids. The infection of target cells (for example NIH3T3) with virus obtained from cells which had been transfected with transferrin-polylysine/adenovirus was indistinguishable from infection with viruses originating from cells which had been transfected with calcium phosphate.

With regard to the retrovirus vectors which may be used, a large number of which are available, there are no restrictions; all retroviral vectors which have at least

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two LTRs and a packaging signal, i.e. the cis-functions, may be used. The trans-functions, which constitute the packaging functions, are possibly part of the retroviral sequence on a single plasmid or, in a preferred embodiment, particularly for therapeutic use, they are located on a separate plasmid, a so-called "packaging plasmid" (in this case, therefore, the retrovirus vector-DNA consists of two plasmids).

There is extensive literature on retrovirus vectors, packaging lines and their use for gene therapy; examples of suitable retrovirus vectors and the mechanisms of the transduction of retroviruses are provided, *inter alia*, in the review articles by Miller and Rosman, 1989, Morgenstern and Land, 1990, and Swain and Coffin, 1992.

Packaging plasmids contain sequences which code for retroviral proteins, but do not carry any retroviral regulating units, as a result of which the formation of replication-competent helper viruses is avoided. Examples of packaging plasmids suitable for use according to the present invention are described *inter alia* by Markowitz, 1988.

The process according to the invention is suitable for the rapid and simple production of stable cell lines which produce high titres of recombinant virus. Unlike the processes according to the prior art, it does not require labour-intensive transfection, subsequent infection and isolation of clones. Another major advantage of the process is the possibility of transiently producing recombinant viruses, which are poorly tolerated by the packaging cell lines.

In addition to the production of packaging cell lines with a high titre the process can preferably also be used to produce viral vectors, e.g. retroviral vectors,

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even in non-packaging cells. This requires the embodiment of the invention in which "packaging plasmids" are co-transfected with the vector DNA containing the other virus sequences. In this case, the virus vector DNA thus also consists of two plasmids, one of which contains the sequences coding for the packaging functions while the other contains the sequences coding for the other virus proteins as well as the DNA which is to be expressed in the cell.

For applications using several plasmids, particularly for the production of viral vectors, the particular advantage of the process according to the invention is that the plasmids can be transported together into the cell, after having been complexed as a mixture in a defined ratio, preferably 3:1 to 1:3, with the other transfection components, e.g. with polylysine-transferrin and with adenovirus-polylysine-conjugates. Thus, a vector plasmid, or a retrovirus vector plasmid, which carries the sequence to be expressed in the cell, more particularly a sequence which is effective in gene therapy, and a packaging plasmid can be introduced into the cell in coordinated manner, whilst the ratio of amounts can be varied and adjusted to the optimum level with regard to the desired efficiency of expression. The optimum ratio is determined by empirical tests.

One of the problems of using recombinant retroviruses in gene therapy is the fact that retroviruses are rapidly broken down in the body and thus often do not reach the target cells. Efficient gene transfer can therefore only be achieved with the retroviral vectors prepared by conventional methods if they are administered directly to the target organ or even by perfusion of the organ in question, e.g. the liver. This requirement can be overcome if supernatants with a high retrovirus titre are used for transfection which have been prepared by

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the process according to the invention *in vitro* (using packaging cell lines or, in the case of cells without a packaging function, using co-transfection of vectors containing packaging functions).

The application of the invention to viral, e.g. retroviral, vectors in which cells without a packaging function are used together with a packaging plasmid offers the advantage of application to autologous cells, which is primarily of interest for transferring therapeutically effective genes by means of retroviruses *in situ*. There are two particular alternative methods available for gene transfer *in situ*:

1) Cells, e.g. fibroblasts, haematopoietic cells, myoblasts, hepatocytes or bone marrow stroma cells are taken from the body and these primary cells are transfected *ex vivo* with recombinant retrovirus vector-DNA using the method according to the invention. The vector-producing cells thus obtained are then re-implanted in the body in order to produce a large amount of vector material over a lengthy period at the implantation site, leading to *in situ* transduction of proliferating cells in the surrounding area. The main advantage of such a use is that the continuous production of the vector *in situ* makes it possible to transduce cells with low division rates, such as parent cells and cells which contribute to the physiological cell turnover.

2) The transduction of the target cells with the retrovirus vector is carried out directly *in vivo*, by local application of transfection complexes according to the invention which contain recombinant retrovirus vector-DNA. Thus, terminally differentiated target cells such as blood cells, bronchial or intestinal epithelial cells, keratinocytes or mammary gland cells

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may be reached.

One example of a local *in vivo* application is the direct injection of the gene transfer complexes into the liver parenchyma or into the bile duct system with the aim of bringing the retroviral vectors and packaging plasmids into the bile duct epithelium in order to achieve *in situ* gene transfer into precursor cells. Another advantage of the use of the complexes according to the invention in gene therapy is the introduction of the human β-interferon gene into the breast, in which direct *in vivo* transduction into the breast epithelium can take place.

In another preferred embodiment of the process according to the invention the plasmid DNA contains AAV-vector DNA; the process according to the invention in this embodiment thus serves to prepare recombinant AAV-vectors.

The AAV-sequences are preferably present in the form of the two-plasmid-system described above, in which a first plasmid codes for the Rep- and Cap-functions and a second plasmid codes for the gene in question, flanked by the 145 nucleotide long ITRs ("inverted terminal repeats").

The preparation of AAV requires on the one hand transportation of the AAV-genes into a high percentage of the cells or into the total cell population, and on the other hand infection with a helper virus such as adenovirus or herpes virus. Since gene transfer according to WO 93/07283, in the embodiment in which the endosomolytic agent is an adenovirus, satisfies both the parameters required for meeting these two conditions, this system is outstandingly suitable for the preparation of recombinant AAV-vectors. Moreover, the

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system has a further peculiarity which makes it particularly suitable for this application, namely the possibility of using inactivated adenovirus without affecting the functions required for entry of the virus into the cell (Cotten et al., 1992; Cotten et al., 1994b). In view of this fact, a concentration of adenovirus particles may be used in a level sufficient for efficient gene transfer, without overloading the cell with replication-competent adenovirus which would compete for essential factors with AAV and thus reduce AAV production. The process according to the invention, in the embodiment in which the endosomolytically active adenovirus simultaneously constitutes the helper virus for AAV, offers the possibility of using a mixture of active and inactive adenovirus particles which makes it possible to adapt the transfection system in order to achieve optimum AAV production. The optimum ratio of inactivated and non-inactivated adenovirus can be determined empirically; basically, it is desirable to keep the proportion of non-inactivated virus as low as possible. Within the framework of the present invention, ratios of inactivated to non-inactivated adenovirus of 4:1 and 9:1 have proved equally suitable.

In another embodiment, the process according to the invention can be used to prepare AAV particles without helper virus infection occurring at the same time. Since only four gene functions of the adenovirus helper virus are necessary for AAV-replication, namely E1, E2a, E4 and VA (Richardson and Westphal, 1981), these helper functions may be provided in the form of plasmids, each of which carries the relevant adenovirus gene regions, instead of using complete active adenovirus as the helper virus. In addition, the helper functions, if they are not present on the plasmid, may be provided by the production cells (the helper cells). These plasmids are co-transfected with the plasmids carrying the AAV-

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sequences as components of the transfection complexes. Since the presence of adenovirus as a helper virus is not essential in this embodiment of the invention, an endosomolytically active agent may be used which is free from non-inactivated adenovirus.

In a variant of this embodiment, a single DNA molecule which contains the sequence coding for a non-packagable adenovirus genome may be used for co-transfection of the cells (in addition to the AAV-sequences). An example of such a plasmid is pBHG11 (Bett et al., 1994), which contains the complete adenovirus genome with the exception of the deletions of the E3- and E1-regions. These two functions are not essential in the plasmid, since the production of AAV can be carried out in cells which provide the necessary adenovirus E1-functions e.g. in 293 cells, and also because the E3-region is not essential either for the infectious growth of adenovirus nor of AAV in cell culture. The plasmid pBHG11 also has a deletion which removes the packaging signal Psi; moreover, the sequence coded thereby is too big for efficient adenovirus packaging. The essential advantage of this embodiment of the invention is the absence of the production of helper virus. If only the gene functions necessary for the helper function are provided, but not sufficient genes required for the formation of a complete adenovirus particle, as a result of infection only AAV-particles are obtained and no adenoviruses, which makes it easier to purify the AAV-particles.

In another preferred embodiment of the invention the plasmid DNA contains adenovirus vector DNA; in this embodiment the process serves to prepare adenovirus and adenovirus vectors.

In a special variant of this embodiment for preparing

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adenoviral vectors, two plasmids containing adenovirus DNA sequences which are to be imported into the cells are selected so that the sequences can recombine. Using the process according to the invention it is possible to introduce the two plasmids into the cell in such quantities that the formation of recombinant adenovirus is made easier. The first plasmid contains, for example, the ITR (Aleström et al., 1982) and the adenovirus packaging signals (base pairs 194 to 358; Gräble and Hearing, 1992) with a strong promoter which drives the foreign gene, plus an additional region of the adenovirus sequence from which the E1-sequences has been eliminated, it contains pIX-sequences plus further sequences downstream from E1 which make it possible for recombination to occur with the second plasmid. The E1 region has been eliminated from this vector system (plasmid) to make room for the insertion of foreign genes. The second plasmid carries the entire adenovirus genome from which the packaging signal has been eliminated and in which bacterial sequences have been inserted in the E1-region. The absence of the packaging signal and the increased size caused by the bacterial insert prevent this second adenovirus sequence from being packaged in virions. The two plasmids are introduced into cells of the line 293 (Graham et al., 1977), a human embryonic kidney cell line which carries the adenovirus E1-sequence and thus has the function necessary for virus replication which is missing from the two plasmids. When the two plasmids are recombined a DNA sequence is obtained which contains all the functions necessary for the growth of adenovirus and DNA packaging (apart from the E1-functions which are prepared by the cell line), and also the new gene which is coded by the first plasmid.

Alternatively, instead of a cell line such as 293 which provides the E1-function ("E1-cell line"), an E4-cell

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line may be used, e.g. the cell line W162, or an E1/E4-cell line which has both gene functions; in these cases, the E4-function or both the E1- and the E4-function are absent from the adenovirus vector-DNA plasmids.

In another preferred embodiment the process according to the invention is used to prepare recombinant adenoviruses, capable of penetrating into cells which carry a foreign DNA molecule of 35 kb but completely lack adenovirus genes. In order to obtain such adenoviruses, the following plasmid constructs may be used, for example: a first plasmid (vector plasmid) contains the ITRs of adenovirus and the packaging signal Psi which flanks the ca. 35 kb non-adenoviral DNA, including therapeutic and/or marker genes, and optionally filler DNA; the flanking ITRs are necessary for amplification of the ca. 35 kb sequence during the production cycle; the Psi sequence ensures that the amplified 35 kb sequence is packaged into virions. A second plasmid contains the complete adenovirus sequence, but it lacks the packaging function Psi, and an insert size of more than 35 kb prevents its packaging. Examples of plasmids which are suitable for the purposes of the present invention are the plasmid pBHG11 described by Bett et al., 1994, which is E1-negative, and structurally similar plasmids. (Alternatively, E1-positive plasmids may be used, in which case the use of a production or helper cell is not necessary; normal cells can be used for the transfection.) The second plasmid (packaging plasmid) provides all the gene functions which are necessary in order to form an infectious adenovirus particle which is built up and is charged with the sequence coded by the first plasmid. This system has the advantage of providing infectious adenovirus particles which do not have any adenovirus genes since the two plasmids do not recombine with each other.

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This embodiment of the present invention with which large amounts of both plasmids are conveyed into the packaging cell line by means of transient transfection gets round the problem of needing cell lines which carry stably large amounts of the adenovirus genome, which has proved difficult, presumably because of the toxic effects of some adenovirus products. Another advantage of this "blunted" adenovirus vector is the increased capacity for foreign genes. Whereas the recombinant adenovirus vectors according to the prior art can absorb about 8 kb of a foreign sequence, the vector obtained by the process according to the invention, which requires only the about 200 bp ITR-sequences plus a 180 bp packaging sequence, can absorb more than 34 kb of foreign genes including therapeutically effective genes.

A further advantage of this embodiment of the invention is its great flexibility, as the adenovirus genes can be distributed over different plasmids (vector plasmid, packaging plasmid and possibly other plasmids). Unlike the embodiment in which the vector plasmid carries only the packaging function psi apart from the foreign gene and the ITRs and the packaging plasmid carries all the other adenovirus sequences, some of these adenovirus sequences may be present on the vector plasmid itself, whilst the other functions are wholly or partly coded by the packaging plasmid. Apart from being on the packaging plasmid and the vector plasmid, these functions may also be provided by the cell and/or they may be on another plasmid.

It is also possible to prepare adenoviruses or adenoviral vectors which have changes or additions to their various gene functions in order to improve the infectiousness of the virus, using the process according to the invention. The principle for such manipulations is that the manipulated or supplementary genes are used

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in trans to prepare recombinant or non-recombinant adenoviruses (in accordance with the principle proposed, for example, for the E1 sequence by Goldsmith et al., 1994), but that these genes may also be present on the packaging or vector plasmid, if the multiplasmid system is used to produce adenoviral vectors.

One example of such a manipulation is the deliberate changing of the filaments of an adenovirus in order to achieve binding to specific types of target cells; the basis for this is the possibility of changing the filament genes between different adenovirus serotypes. The presence of a manipulated filament gene, e.g. on the packaging plasmid or on a separate plasmid, makes it possible to produce adenovirus particles which have new filament molecules and are thus available for alternative uses in connection with specific target cells.

The adenovirus or adenovirus vector can also be modified in order to bring about the overexpression of adenovirus protease; in this case, therefore, it is the viral gene which increases infectiousness, the protease gene. The adenovirus protease is necessary for processing six of the capsid proteins in the construction of the virus; the specific cleaving of the viral capsid proteins causes activation of the adenovirus particle for the interaction with the membrane. In the infectious particle, the proteases necessary for the entry of DNA into the nucleus, both of adenovirus DNA and of DNA which is coupled to the surface of adenovirus, in the case of adenovirus-aided transferrinfection. With the aid of this embodiment of the present invention it is possible to provide larger quantities of protease during the productive adenovirus infection. The presence of additional protease during the production of the virus has two advantages: the increased amount ensures that

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all the virions obtained are fully processed and have full activity for breaking down the membranes. The larger quantity of protease may also cause the additional protease to be encapsulated in larger quantities in the virions obtained. As a result, these virions may be more efficient in the reactions which take place during the transfer of DNA into the nucleus of the infected cells.

There are two possibilities for preparing larger quantities of protease: in one embodiment, the optionally recombinant adenovirus is used in order to form complexes with polylysine, and optionally a conjugate of a ligand and polylysine, and DNA which contains the sequence coding for the protease. The complexes obtained trigger a productive virus infection; the protease gene contained on the plasmid ensures an excess of protease in the virion (under normal conditions there are about 10 protease copies per virion), in relation to the quantity achieved by the genomic copy of the gene. The advantage of this embodiment is that the virus genome occurs separately from the modified protease gene (the modification consists in the presence of a strong constitutive promoter). Inactivation of non-recombinant adenovirus obtained by this process, e.g. by means of psoralen/UV, yields adenovirus particles which differ from a normal infection only in their increased protease content and the increased processing of the virion. Such adenoviruses may be used, for example, to produce tumour vaccines by the process described in WO94/21808, in order to introduce a cytokine gene into tumour cells.

Alternatively, with this embodiment of the process according to the invention, it is possible to prepare a recombinant virus which carries the protease gene under the control of a strong constitutive promoter. In this

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case, the two-plasmid or multi-plasmid system is used, whilst the protease gene may be contained on the vector plasmid, possibly in addition to the foreign gene, e.g. a therapeutically active gene such as a cytokine gene. (Alternatively, the protease gene may be present on the packaging plasmid or on a third plasmid). The advantage of this approach is, in particular, the simplicity of use of the recombinant adenovirus thus obtained. In this alternative embodiment it should be borne in mine that the presence of a protease gene in the resulting virus takes up genome capacity, which limits the space available for the therapeutic gene. One possible way of getting round this restriction, e.g. in the case of large therapeutic genes, is to modify the promoter of the protease gene present so as to increase its activity and achieve increased protease expression without taking up space in the genome.

When using an adenovirus which produces larger quantities of protease, it should be remembered that the protease plays a part in the inflammatory response to the entry of adenovirus; the increased protease content of adenovirions can raise the inflammatory potential of the virus. Whereas this may be a disadvantage for certain applications in the production of long-term modified cells, this property is an advantage in the production of tumour vaccines with which the body's immune response is to be strengthened.

The changes in adenovirus (vectors) may also be carried out on the basis of the YAC ("Yeast Artificial Chromosome") system, developed by Ketner et al., 1994, for the manipulation of the adenovirus genome. This system makes use of the efficient homologous recombination reactions in yeast in order to introduce the desired modifications into the adenovirus genome. After the changes have been put in position the

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adenovirus sequence is excised from the YAC and introduced into a cell line which is suitable for the growth of adenovirus. However, this system has the disadvantage that the transfection of the large adenovirus sequence (35 kb) using the calcium phosphate transfection method is inefficient and therefore transfection is the limiting step in this process. This disadvantage can be overcome by using the process according to the invention in order to transfect the adenovirus vector systems manipulated with the YAC-system, which allows rapid triggering of the adenovirus infection.

For all applications in which adenovirus or AAV-vectors are prepared, instead of using "helper cells" which help to control the helper functions missing from the AAV or adenovirus vector plasmids, these helper functions such as E1 and/or E4 may also be contained on another plasmid which is transfected together with the other vector DNA contained on plasmids. In this case, instead of the helper cells, the target cells in which the foreign DNA, e.g. the therapeutically active DNA, is finally brought to expression, are transfected; in these cases, therefore, production of the vector occurs not in the helper cell but directly in the target cell.

The present invention relates, according to another aspect, to transfection complexes containing DNA in the form of one or more plasmids containing viral vector DNA and optionally one or more sequences coding for viral genes which increase the infectiousness of the virus or viral vector, which is complexed with a polycation optionally conjugated with a ligand for the target cell, as well as an endosomolytically acting agent.

In a preferred embodiment the plasmid DNA is retroviral vector-DNA.

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In another preferred embodiment the plasmid DNA is AAV-vector-DNA. In one variant of this embodiment the endosomolytically active agent consists of a mixture of inactivated and non-inactivated adenovirus, the latter simultaneously acting as a helper virus for the AAV.

In another preferred embodiment the plasmid DNA is adenovirus-vector-DNA.

In another embodiment the plasmid DNA codes for adenovirus protease; in this case the endosomolytically active agent is optionally recombinant adenovirus.

Summary of Figures

Fig. 1: Throughflow cytometric analysis of the HERc-expression

Fig. 2: Analysis of genomic DNA for determining the integration of retrovirus-DNA

Fig. 3: Western blot for detecting AAV-virus

Fig. 4: Preparation of adenovirus with increased protease expression.

In Examples 1 - 5 which follow, unless otherwise specified, the following materials and methods were used:

a) Cells used

Mouse hepatocytes BNL CL.2 (ATCC No. TIB 73); cells of the packaging cell line GP+E86 described by Markowitz et al., 1988, NIH3T3 (TK-)-cells.

b) Preparation of recombinant retroviruses

The retroviral vectors which express the normal human

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EGF-receptor (HERc), c-kit or tsp53, were derived from the vector LXSN described by Miller and Rosman, 1989. The plasmid LXSN-HERc was prepared by inserting a 3.9 kb XhoI-fragment obtained from the plasmid NTK-HERc (von Rüden and Wagner, 1988), into the XhoI site of pLXSN. The LXSN-tsp53 virus was obtained by inserting the cDNA coding for the p53val135 mutant (Gottlieb et al., 1993) into the EcoRI site of pLXSN. The vector pLXSN kit was described by Alexander et al., 1991; the plasmid pLXSN-tsb53 was described by Gottlieb et al., 1993. The plasmid pMOV9.2 was described by Harbers et al., 1981. The titres of the recombinant viruses were determined by infecting NIH3T3 (TK-)-cells (10^5 cells per 6 cm dish for 2 hours) and then selecting with G418 (Gibco, 1 mg/ml), using as medium IMDM (Iscoves modified Dulbeccos Medium (Gibco) and 10% FCS).

c) Transfection of cells

The transfection with transferrin-polylysine/adenovirus-DNA-complexes (hereinafter referred to as "Adenovirus-Transferrinfection") was carried out essentially as described in WO 93/07283. The procedure used was as follows: the transfection complexes were prepared in a three-step process. In the first step 8 x 10^9 particles of biotinylated, psoralen/UV-inactivated adenovirus dl312 were modified with streptavidin-modified polylysine 290, by incubating for 30 minutes at ambient temperature in a total volume of 200 µl of HBS (20 mM HEPES, 150 mM NaCl, pH 7.4). Then 6 µg of DNA in 150 µl of HBS were added, mixed thoroughly and left to bind to the polylysine-modified adenovirus for an incubation period of 30 minutes. Finally, 5.4 µg of polylysine-modified transferrin in 150 µl of HBS were added to the mixture in order to bind to the DNA as well. After 30 minutes the complexes were diluted with 1.5 ml of culture medium (for GP+E86-cells: IMDM, containing 5%

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FCS, 50 μM β-mercaptoethanol and antibiotics; for the mouse hepatocytes BNL CL.2: high glucose DMEM containing FCS and antibiotics) and left to act on 3 x 10^5 cells per 6 cm culture dish for 4 hours at 37°C. The medium was then replaced by fresh culture medium. 48 hours after transfection the supernatants were examined for the formation of recombinant retroviruses. The efficiency of transfection was monitored by means of the β-galactosidase expression in cells which had been transfected in parallel with a β-galactosidase reporter gene, using the procedure as described by Zatloukal et al., 1992. The transfection of GP+E86-cells yielded 39% of β-galactosidase-expressing cells; of the transfected BNL CL.2-cells, 10% of the cells were positive.

The transfection of GP+E86-cells using calcium phosphate was carried out using standard procedures.

d) Throughflow cytometric analysis

The cells were washed twice with phosphate buffered saline solution (PBS) supplemented with FCS and 0.05% sodium azide (washing buffer) before being incubated for 30 minutes with the monoclonal antibody R1 (Waterfield et al., 1982) which recognises the extracellular domain of the human EGF-receptor. As secondary antibody, fluorescein-labelled F(ab')2-fragments of goat anti-mouse-IgG (Dianova) were used. The living cells were analysed using a Becton-Dickinson FACScan Analyser.

e) Determining the virus titre

NIH3T3 (TK-) fibroblasts were infected using standard methods and selected for the expression of the transduced Neo^R gene: 1 x 10^5 NIH3T3 were incubated with 1 ml of supernatant from cultures producing cells for 1 to 2 hours. The supernatants were previously diluted in

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culture medium (IMDM + 10% FCS). After the infection the virus-containing medium was exchanged for virus-free medium and the cells were then cultivated for 24 to 30 hours. In order to select the infected cells G418 (1 mg/ml) was then added to the medium. After about 10 days the number of G418 resistant colonies was counted and the virus titre was calculated (number of colonies multiplied by the dilution factor gives the number of infectious particles per ml of culture supernatant cfu/ml).

f) DNA-analysis

Genomic DNA was prepared from 3 x 10^6 cells (Sambrook et al., 1989) and 10 μg were digested with the suitable endonucleases. The cut DNA was separated on agarose gels and then applied to nylon membranes. The blots were hybridised with ^{32}P-labelled cDNA probes. In order to determine the intact integration of provirus, the DNA was cleaved with Asp718 which cuts once inside the proviral LTRs, liberating a diagnostic DNA fragment. Digestion with HindIII, which cuts once in the provirus genome, yields restriction fragments which are characteristic for each integration site. The blots were analysed using a neo-specific probe.

In Examples 6 to 8 the following methods were used:

a) Adenovirus preparations

Adenovirus 5 dl1014 (Bridge and Ketner, 1989) was cultured in W162-cells (Weinberg and Ketner, 1983). Ad5 wt300 (wild-type adenovirus Type 5) and Ad5 dl312 (adenovirus with a deletion in the E1a-region; Jones and Shenk, 1979) were cultured in 293-cells (Graham et al., 1977). The viruses were purified, where specified, biotinylated and, where specified, inactivated with

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psoralen/UV, as described by Cotten et al., 1994b. The virus preparations obtained were quantitatively defined by means of the protein content (1 mg protein = 3.4×10^{12} adenovirus particles) and stored at -70°C in 150 mM NaCl, 20 mM HEPES pH 7.4 and 40% glycerol.

b) Transfection reagents

All the plasmid-DNA was purified using Qiagen chromatography (Diagen GmbH) followed by Triton X-114 extraction in order to remove LPS (Cotten et al., 1994a). Streptavidin-polylysine was prepared using the method described by Wagner et al., 1992 and transferrin-polylysine was prepared using the method described by Wagner et al., 1991; these methods are also described in WO 93/07283. The transfection complexes were obtained as followed: a sample of biotinylated adenovirus with 1×10^{10} particles was diluted in 150 µl of HBS and mixed at ambient temperature for 30 minutes with 1 µg of streptavidin-polylysine in 150 µl of HBS. Then a sample of 6 µg of DNA in 100 µl of HBS was added, followed by 30 minutes incubation at ambient temperature. Finally, 100 µl of HBS containing 5 µg of transferrin-polylysine were added. After 30 minutes at ambient temperature, aliquots of the transfection complexes were applied to the particular target cells in 2% equine serum/DMEM.

For the preparation of AAV, 250 µl of transfection complex containing 6 µg of DNA were used per 180 cm^2 flask (2×10^7 cells).

c) AAV-plasmids

The preliminary tests were carried out using the wild-type AAV-2-vector pAV-1 (Laughlin et al., 1983; obtainable from ATCC). The subsequent attempts to prepare recombinant AAV-vectors were carried out using

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the AAV-vector pAB-11 (pAAVLACZ; Kaplitt et al., 1994), which contains the sequence coding for β-galactosidase and the plasmid pAd8 (Kaplitt et al., 1994). The original pAB-11-preparations were recloned; a clone which had received the second terminal repeat was isolated and expanded for further use.

d) Adenovirus plasmids

The adenovirus 5 plasmid which lacks the E1-sequence, the plasmid pΔE1sp1B (left-hand end) and the packaging plasmid pBGH11 (right-hand end) were obtained from Microbix.

e) Purification of AAV

Infected cell pellets were suspended in 20 mM HEPES, pH 7.4, at 2 ml per 2 x 10^7 cells. The material was frozen, thawed three times and then adjusted to 5 mM $MgCl_2$, treated with DNase 1 (0.1 mg/ml) for 20 minutes at 37°C and then with trypsin (0.25%) for 20 minutes at 37°C. The material was then mixed with an equal volume of Freon (FLUKA), treated in a vortex for 2 x 30 seconds and then centrifuged for 10 minutes at 3,000 rpm in a Heraeus-2705-Rotor. The supernatant was then transferred into a vertical centrifugal tube (either vTi 50 for volumes of 10 to 15 ml or vTi 65 for volumes of 2 to 5 ml), with an equal volume of 1.33 g/cm^3 CsCl, 20 mM HEPES, pH 4, followed by half a volume of 1.64 g/cm^3 CsCl, 20 mM HEPES, pH 4. The material was then centrifuged for 2 hours at 49,000 rpm (vTi 50) or 63,000 rpm (vTi 65) at 20°C.

The gradient fractions were harvested as follows: the clearly visible opalescent adenovirus band through the centre of the 1.33 g/cm^3 CsCl step was harvested, followed by a second fraction which comprised the region

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of the adenovirus up to the 1.64 g/cm^3 CsCl step. This second fraction containing the AAV was mixed with 1.40 g/cm^3 CsCl, 20 mM HEPES, pH 7.4, and centrifuged overnight in a vTi 65 rotor.

f) Measurement of luciferase

The luciferase activity was determined using standard methods as described, for example, in WO 93/07283.

g) β-Galactosidase measurement

The β-galactosidase content was determined using the method described by Jain and Magrath, 1991.

h) Detection of ECL

The ECL (Enhanced Chemoluminescence) of the Western-blot samples was detected in accordance with the manufacturer's instructions (Amersham).

Example 1

a) Preparation of high titres of retroviral LXSN-HERc-vectors after receptor-mediated transfection intensified by adenovirus

The DNA coding for the retroviral vector pLXSN-HERc was transfected into cells of the packaging cell line GP+E86, on the one hand using the calcium phosphate co-precipitation method and on the other hand using adenovirus transferrinfection. The release of infectious LXSN-HERc was determined 24 and 28 hours after transfection or after one week's selection for G418. The results are shown in Table I: as expected, only small virus titres (in the region of 10^3 infectious

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units per ml (cfu/ml)) could be detected in the cells which had been transiently transfected with calcium phosphate. In the pooled population of G418 resistant clones the titres rose significantly to about 10^5 cfu/ml. Greatly increased titres were obtained from the cells which had been transfected by means of adenovirus transferrinfection: 24 and 48 hours after transfection, titres of 2 x 10^5 cfu/ml were obtained (Table I) and after G418-selection the titres increased by 20 to 50 times up to 1 x 10^7 cfu/ml (Tables I, III and IV). Next the efficiency of the virus production of clonal packaging cells produced either by means of adenovirus transferrinfection, calcium phosphate or retroviral gene transfer was compared. (The cells were transfected as specified in the Table, or infected after treatment with tunicamycin with recombinant virus obtained from a pool of G418-resistant colonies (Table I)). The results of these experiments showed an even more marked difference (30-fold) in populations of pooled colonies (Table II). No replication-competent helper viruses could be detected in any of these clones.

b) Expression of the HERc-gene

In conformity with the increased virus titre in the cells transfected by adenovirus transferrinfection, the surface expression of HERc was also dramatically increased. Throughflow cytometric analysis showed a significant increase in fluorescence compared with calcium phosphate-transfected cells, but, as expected, no difference in HERc-expression could be detected in cells which had been infected with virus obtained from the packaging cells. The results of these experiments are shown in Fig. 1. Fig. 1A shows the expression of human EGF-R (HERc) on the surface of transfected clonal GP+E86 cells after G418 selection. Fig. 1B shows the expression of HERc on infected NIH3T3 TK-cells. All the

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cells which had been treated with the monoclonal mouse antibody R1 (Waterfield et al., 1982), which specifically recognises the extracellular part of the EGF-R-protein, and with fluorescein-labelled $F(ab')_2$-fragments of goat-anti-mouse-IgG, were stained. Viable cells were analysed using a Becton-Dickinson FACScan-Analyser.

Example 2

Co-transfection of vector and packaging functions

In order to test whether the packaging functions provided by the GP+E86-cells are synthesised in amounts which permit maximum virus production, a plasmid which expresses additional packaging functions was co-transfected. For this the plasmid pMOV9.2 was used which contains a complete Molony-mouse-leukaemia virus (M-MuLV). On average, only a slight increase (3-fold) in the virus titres was observed, although the frequency of clones with a high titre (>10^7 cfu/ml) increased significantly (Table III).

Example 3

Preparation of high titres of other retroviral vectors

In order to rule out the possibility that the increase in virus titre observed is restricted to a single retroviral vector construct, two other retroviral vectors derived from the vector LSXN were tested. The vector LXSN-Kit expresses the receptor coded by c-kit, the second construct LXSN-tsp53 expresses a temperature-sensitive mutant of the tumour suppressor gene p53. As can be seen from Table IV, the virus titres of the

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adenovirus-transferrinfection-transfected cells were uniformly 40 to 100 times higher than those in cells which had been transfected with calcium phosphate.

Example 4

Retrovirus production in cells other than packaging cells

In order to test the possibility of obtaining retroviral vectors of cells other than packaging cells, the plasmid pLXSN-HERc together with the plasmid pMOV9.2 (3 μg of each) was co-transfected into BML CL.2 hepatocytes. As shown in Table V about 50% of the isolated clones yielded a virus titre which was almost as high as that of the GP+E86 producer cells, and showed high expression values for HERc on the cell surface. Since a full length M-MuLV-virus was co-transfected in this experiment, these cells also freed replication-competent helper virus.

Example 5

Stable integration of multiple retrovirus-vector copies

In this Example a genomic DNA analysis was carried out (Sambrook) in order to determine whether the transfected plasmid had been integrated in the host cell genome and whether the numbers of integrated plasmid copies correlated with the increase in the titre. The Southern blot analysis of the DNA from cells which had been transfected by adenovirus transferrinfection showed at least two and up to more than ten integration sites, whereas the clones transfected with calcium phosphate generally contained only one or two copies (Fig. 2A).

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The number of integrated copies correlated well with the virus titres (cf. Table IV).

In the transfected BNL CL.2 cells (Fig. 2B) the number of integrated plasmids was generally lower by comparison with the GP+E86-cells, but it did correlate with the virus titres. Restriction digestion of the genomic DNA with an enzyme which cuts once in the LTRs confirmed the integrity of the integrated retroviral construct. Only a BNL CL.2 clone (No. 6) which contained more than ten integrated copies had two wrongly arranged (rearranged) vectors (Fig. 2B). Finally, the blots were re-hybridised with adenovirus-specific probes in order to establish whether adenovirus sequences had integrated into the genome of the cells which had been transfected by adenovirus transferrinfection. No adenovirus sequences could be detected, which was confirmed separately by PCR-analysis.

Fig. 2A shows the Southern analysis of the genomic DNA from cells which had been transfected with the plasmids given in the Figure. HindIII was used as restriction enzyme, which cuts the proviral DNA once and thus yields fragments which are characteristic of a single integration site (Fig. 2C). Fig. 2B shows the analysis of transfected BNL CL.2 cells, cut either with HindIII or with Asp718, which cuts once in each proviral LTR and thus yields fragments which are an indication of the intactness of proviral integration (Fig. 2C). In all the experiments, vector-specific DNA was detected by hybridisation with a ^{32}P-labelled neo-probe. Moreover, a CPE-assay showed that replicating adenovirus which might have developed from non-integrated adenovirus DNA could not be detected in retroviral supernatants.

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Example 6

Preparation of AAV

a) Preparation of wild-type-AAV

The plasmid pAV1 encoding the entire human wild-type AAV Type 2 (Laughlin et al., 1983), was transfected into W162 cells using transfection complexes containing a mixture of biotinylated Ad5 dl1014 and biotinylated psoralen/UV inactivated Ad5 dl1014 (1:9 or 1:4; transfections of the two parallel experiments in both proportions gave the same positive results). 3 days after transfection (as soon as virtually all the cells had become rounded and detached from the surface) the cells were harvested, suspended in 20 mM HEPES, pH 7.4, lysed by three freezing/thawing cycles and extracted with an equal volume of freon. The lysate was then fractionated on a CsCl step gradient by coating 15 ml of lysate with 15 ml of 1.33 g/cm^3 CsCl, 20 mM HEPES, pH 7.4 and then with 7 ml of 1.64 g/cm^3 CsCl, 20 mM HEPES, pH 7.4, in a vTi 50 tube. The sample was then centrifuged for 2 hours at 49,000 rpm. A fraction was harvested which contained the clear opalescent band in the centre through the 1.33 g/cm^3 stage plus the material of higher density. The sample was adjusted to 1.40 g/cm^3 using CsCl/20 mM HEPES, pH 7.4, and centrifuged overnight for 18 hours in a vTi 65-rotor at 63,000 rpm. A single sample with a density of 1.35 g/cm^3 was isolated. The presence of AAV was determined by resolving the fractions by SDS-PAGE, transferring to nitrocellulose and probing with an antibody which recognises the AAV-capsid proteins VP-1, VP-2 and VP-3 (Ruffing et al., 1992; ProGen, Heidelberg). It was established that the expected AAV-fractions contained proteins of the expected size. The Western blot is shown in Fig. 3; traces 1 to 3 show the reaction of the AAV-proteins VP1, VP2 and VP3 with the antibody; the molecular weights are

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shown on the left of the Figure.

b) Preparation of recombinant AAV

The plasmids pAB-11 and the plasmid pAD8 were used analogously to a) by transfecting them together into W162-cells in a ratio of 1:1 in order to obtain recombinant AAV-vectors. Using the recombinant AAV-virus obtained, HeLa cells were infected and the β-galactosidase-production in the cells was confirmed.

Example 7

Preparation of recombinant adenovirus

a) Use of transferrinfection for initiating adenovirus infection of DNA

Since it has been found that gene transfer using receptor-mediated endocytosis can transport large (50 kb) DNA-molecules into cells with comparable efficiency to small plasmids, this method was used in a preliminary trial in order to initiate an adenovirus infection of the ca. 35 kb adenovirus genome. Initially, 293-cells were transfected with the plasmid pFG140 (Graham, 1984; Graham et al., 1989), which codes for the entire adenovirus 5 genome and contains a bacterial origin of replication and an ampicillin-resistance gene instead of the E1-region. This DNA molecule codes for an E1-defective adenovirus which can replicate in the cell line 293 expressing the E1-region. 2 wells of a 6-well plate, each of which contain 40,000 293-cells, were treated with 15 µl of transfection complex, 500 µl of complex containing 6 µg of the plasmid pFG140; the complexes also contained transferrin-polylysine/streptavidin-polylysine and

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biotinylated psoralen/UV-inactivated Ad5 dl1014. 4 days after transfection a complete cytopathic effect (CPE) was observed, leading one to conclude that virus infection had been successfully initiated. The cells were harvested, lysed and the clarified lysate was used for the infection of 293-cells in T-75 flasks (5 x 10^6 cells/flask). Complete CPE had set in after 2 days and the cells were then harvested again, lysed and the lysate was subjected to 2 rounds of CsCl-density gradient centrifugation. This yielded two types of virus: a small amount of material which constituted a band at a density of 1.31 g/ml (the density of immature virus particles), and a large significant band at 1.34 g/ml (density of mature adenovirus). Both bands contained particles 110 nm in size (the expected size of adenovirus particles), measured by "Dynamic Light Scattering" analysis. The yield of mature virus was 6 x 10^{10} particles per 5 x 10^6 infected cells; this corresponds to 10,000 virus particles per cell, which is comparable with a yield such as is obtained with an authentic adenovirus preparation.

b) Use of transferrinfection for preparing recombinant adenovirus which contains the luciferase sequence

In order to test the suitability of receptor-mediated gene transfer for the preparation of recombinant adenovirus, the reporter gene uses luciferase under the control of the CMV-promoter to prepare an E1-deficient adenovirus. First of all a plasmid was prepared which contained the left ITR, the packaging function Psi, the sequence coding for luciferase under the control of the CMV-promoter (including cleavage site and polyA-tail), by removing the sequence coding for luciferase (De Wet et al., 1987) and the rabbit-globin-intron sequence from the plasmid pCLuc (Plank et al., 1992) and cloning them into the BamHI-site of the plasmid pΔE1sp1B (Bett et

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al., 1994). A clone with the sequence coding for luciferase in the same orientation as the natural E1a-sequence was identified and used further. The plasmid obtained was then co-transfected with a second plasmid pBHG11 (Bett et al., 1994) which codes for the complete Ad5-genome, with the exception of a deletion in the E3-region, a deletion in the E1-region, a deletion of the packaging signal Psi, and which contains a bacterial plasmid insert in the E1-region which makes the complete sequence for packaging into a virion too big. 6 wells each containing 40,000 293-cells were each treated with 15 μl of transfection complex. This corresponds to 0.09 μg of pE1Luc plus 0.09 μg of pBHG11 per well, complexed with transferrin-polylysine; streptavidin-polylysine and biotinylated psoralen/UV-inactivated Ad5 dl1014. After 5 days the full cytopathic effect was observed, indicating successful initiation of the virus infection. The cells from 2 wells were harvested, lysed in 10 ml of 2% equine serum/DMEM, and aliquots of the clarified lysate were used in order to infect 293-cells in two T-75 flasks (2 ml of lysate/flask, 5 x 10^6 cells/flask). When total CPE was observed after 2 days, the cells were harvested and subjected to density gradient centrifugation as described under a). Mature and immature adenovirus particles were obtained as in a); the lower band contained particles 107 nm in size, which was within the expected size range of adenovirus particles. Other passages of the virus were carried out, by infecting 293 cells with the CsCl-purified virus (about 100 particles/cell). The yield of virus as a function of the passage, the particle size and the luciferase activity after infection of HeLa cells, following infection thereof with the virus is shown in Table VI. (*1) Passage 0 is the first transfection into the 6 wells, passages 1-4 are the subsequent cultures of virus on the 2 large tissue culture flasks, followed by purification on CsCl-gradient. *2) Yield of purified

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virus of the CsCl-band at 1.34 g/ml; the numbers of virus particles were determined by protein measurement. *3) Size of the virus determined by "Light Scattering": virus samples (10 µl in 40% glycerol/HBS) were diluted in 1.5 ml of HBS. Scattering was determined at a laser setting of 0.5 W, 17 A, using a Brookhaven 9000AT correlator. *4) The luciferase activity was determined after infection of HeLa cells with a titration of virus particles per cell, the luciferase activity being measured 24 hours after infection. The values given represent the average of 3 measurements at the optimum gene transfer and correspond to equal amounts of cellular protein.) The results shown in Table VI show that the transfection system is suitable for producing recombinant virus which is capable of transmitting luciferase activity.

Example 8

a) Preparation of an adenovirus protease expression plasmid

A DNA fragment of adenovirus type 5 (position 21717 to 22426) coding for the viral protease (Yeh-Kai et al., 1983; Webster and Kemp, 1993) was isolated by PCR, treated with Klenow and T4-polymerase and ligated into the SmaI site of the vector pX (Superti-Furga et al., 1991). By restriction digestion and DNA sequencing, a plasmid clone was identified which carries a non-mutated gene fragment in the correct orientation (this clone was designated pPCRSpalt#9).

b) Preparation of virus, purification, biotinylation

Adenovirus 5 dl1014 was cultured as described in the method part. Adenovirus Ad2ts1 (temperature-sensitive

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for the adenovirus protease; Weber, 1976; Yeh-Kai et al., 1983) was cultivated at 32°C on W162 cells. At this temperature the virus produces particles which exhibit the capsids and the infectiousness of the wild type (Weber, 1976; Yeh-Kai et al., 1983). The infected cells were lysed by three freeze-thaw cycles, extracted with Freon and purified by means of a CsCl gradient (first a step gradient purification, followed by an equilibrium centrifugation in CsCl), as described by Cotten et al., 1994. The purified virus (0.3 to 1 mg/ml of protein, corresponding to 1 - 3.4 x 10^{12} virus particles per ml) were biotinylated with NHS-LC biotin (Wagner et al., 1992) and exhaustively dialysed against HBS/40% glycerol.

c) Transfections

Transfection or infection complexes of either Ad5 dl1014 (wild type protease gene) or Ad2 ts1 (temperature sensitive protease) were prepared as described in the previous Examples. In order to do this, virus samples (10^{10} virus particles) were diluted in 150 µl of HBS and mixed with 1 µg of streptavidin-polylysine in 150 mM HBS. After 30 minutes at ambient temperature, 6 µg of DNA (either pSP65 (Boehringer Mannheim) or pPCRSpalt#9) were added to 100 µl of HBS and after a further 30 minutes 4 µg of transferrin-polylysine in 100 µl of HBS were added. After 30 minutes incubation, 500 µl aliquots of the transfection complexes were added to the W162 cells (8 x 10^6 cells in a 180 cm^2 flask) in 10 ml of 2% equine serum/DMEM. After 2 hours at 37°C the medium was replaced by 10% FCS/DMEM and the cells were exposed to a temperature of 39°C. After 3 days, when the complete cytopathic effect had been observed, the cells were harvested, the adenovirus obtained was purified and the virus was subjected to quantitative measurement by means of the protein content.

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d) Determining the DNA transfer activity

In order to test the adenoviruses obtained in c) for their gene transfer activity, first of all TfpL/DNA complexes containing 6 µg of pCLuc/6 µg of TfpL were prepared in 500 µl of HBS. Aliquots of 25 µl were placed on HeLa cells (40,000 cells per well of a 24-well plate in 500 µl of equine serum/DMEM), and after that 10,000 adenovirus particles which originated either from the infection with Ad5 dl1014/pSP65, with Ad5 dl1014/pPCRSpalt#9, with Ad2 ts1/pSP65 or with Ad2 ts1/pPCRSpalt#9. After 2 hours the medium was changed for 10% FCS/DMEM and 24 hours after transfection the cells were analysed for luciferase expression. The results are shown in Fig. 4. It became apparent that Ad2 ts1 particles, when coupled with an empty DNA plasmid, produce virus at 39°C which is unable to aid the transfer of luciferase DNA (Fig. 4, column 1). By contrast, the coupling of the plasmid pPCRSpalt#9 which carries the wild type protease gene brought about an increase in the luciferase activity; this shows that the wild type protease gene which is transported into the cell with a plasmid coupled to the virus is able to replace the defective gene in the virus genome. The virus which was obtained when the protease expression plasmid pPCRSpalt#9 was coupled to Ad5 dl1014 (wild type for the protease) yielded adenovirus particles which were about 100 times better than when the empty plasmid was coupled to the virus (cf. Fig. 4, columns 3 and 4). This shows that the protease activity in normal infection may constitute a limiting factor in the production of virus particles with respect to gene transfer.

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Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

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Table I

Transfection	Titre ($G418^R$ cfu/ml)		
	24 h	48 h	stable
TF	2×10^5	2×10^5	26×10^5
$CaPO_4$	0.08×10^5	0.01×10^5	1×10^5

Table II

Clone	Titre ($G418^R$ cfu/ml) TF	$CaPO_4$	Infection
1	10×10^5	6×10^5	3×10^5
2	120×10^5	5×10^5	0.2×10^4
3	6×10^5	0.1×10^5	1×10^5
4	8×10^5	3×10^5	3.6×10^5
5	30×10^5	0.5×10^5	1.4×10^5
6	35×10^5	0.2×10^5	1.8×10^5
Average	35×10^5	2.5×10^5	1.8×10^5
Pool	28×10^5	1×10^5	0.8×10^5

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Table III

Clone	Titre ($G418^R$ cfu/ml) L-HERc	L-HERc+MOV
1	1×10^6	20×10^6
2	12×10^6	20×10^6
3	0.6×10^6	16×10^6
4	0.8×10^6	0.4×10^6
5	3×10^6	4×10^6
6	3×10^6	20×10^6
Average	3.4×10^6	13.4×10^6
Pool	3.2×10^6	10×10^6

Table IV

Vector	Titre ($G418^R$ cfu/ml) $CaPO_4$	TF
LXSN-HERc	1×10^5	1×10^7
LXSN-Kit	1×10^5	0.8×10^7
LXSN-tsp53	5×10^5	2×10^7

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Table V

Clone	Titre ($G418^R$ cfu/ml)
1	0.3×10^5
2	0.1×10^5
3	0.06×10^5
4	10×10^5
5	6×10^5
6	10×10^5
Pool	4×10^5

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Table VI

Virus (Passage) *1)	Virus yield (Particle/ infected cell) *2)	Size (nm) *3)	Light units/ virus particles *4)
Ad5Luc1 (Passage 1)	2.2×10^{11} Virus particles/ 1×10^{7} cells	107	8.5×10^{6}
Ad5Luc1 (Passage 2)	2.5×10^{12} Particles/ 5×10^{7} cells	102	3.4×10^{4}
Ad5Luc1 (Passage 3)	2.2×10^{11} Particles/ 5×10^{7} cells	105	1.0×10^{5}
Ad5Luc1 (Passage 4)	1.2×10^{11} Particles/ 5×10^{7} cells	106	3.9×10^{4}

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for preparing viruses or viral vectors comprising:

(a) complexing one or more plasmids with a polycation, thereby forming a complex, wherein said one or more of said plasmids comprise viral vector DNA;
and
(b) transfecting a mammalian cell with said complex in the presence of an endosomolytic agent;

wherein said transfection of the mammalian cell results in the production of viruses or viral vectors.

2. A process for preparing viruses or viral vectors as claimed in claim 1, wherein said DNA contains one or more sequences coding for viral genes which increase the infectivity of the virus or the viral vector.

3. Process according to claim 1 or claim 2, characterised in that the viral vector-DNA contains a DNA which is to be expressed in a target cell.

4. Process according to any one of claims 1 to 3, characterised in that the viral vector is a retrovirus-vector.

5. Process according to claim 4, characterised in that the retrovirus-vector-DNA consists of two plasmids, the first plasmid containing the sequences coding for the packaging functions and the second containing the sequences coding for the other virus proteins as well as the DNA which is to be expressed in the target cell.

6. Process according to claim 5, characterised in that the cells are primary cells.

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7. Process according to to any one of claims 1 to 3, characterised in that the viral vector is an AAV-virus vector.

8. Process according to claim 7, characterised in that the AAV-vector-DNA consists of two plasmids, the first containing the sections coding for the AAV-Rep- and -Cap-function and the second containing the foreign gene flanked by the inverted terminal repeats.

9. Process according to claim 8, characterised in that the cells are transfected with another plasmid which contains at least part of the helper genes required for the replication of AAV.

10. Process according to claim 8 or 9, characterised in that the endosomolytic agent consists at least partially of non-inactivated adenovirus.

11. Process according to one of claims 8 to 10, in which the plasmid DNA is used to transfect mammalian cells which contain the helper genes necessary for the replication of AAV, which are optionally not present on the plasmids and/or in the adenovirus.

12. Process according to any one of claims 1 to 3, characterised in that the virus is adenovirus or the viral vector is an adenovirus vector.

13. Process according to claim 12 for preparing adenovirus vectors, characterised in that mammalian cells which express one or more helper genes necessary for adenovirus replication are transfected with vector-DNA, the vector-DNA being present on two plasmids recombination of which yields a DNA molecule which has all the adenovirus gene functions with the exception of the helper genes.

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14. Process according to claim 13, characterised in that the first plasmid, which lacks the E1-region, contains the left terminal repeat, the packaging function and the DNA to be expressed in the target cell under the control of a promoter, and the second plasmid contains the entire adenovirus genome without a packaging signal and in the E1-region an insert of a size which prevents packaging of the adenovirus sequence encoded by the second plasmid, and in that the plasmids are used to transfect mammalian cells which express the E1-gene.

15. Process according to claim 12 for preparing adenovirus vectors, characterised in that the vector-DNA is present on two plasmids which cannot recombine with each other, the first plasmid containing the packaging function and the DNA to be expressed in the target cell under the control of a promoter, flanked by the two inverted terminal repeats, and the second plasmid having the other adenovirus sequences apart from the packaging function.

16. Process according to claim 15, characterised in that the second plasmid also contains the helper genes.

17. Process according to claim 15, characterised in that the second plasmid does not contain the helper genes.

18. Process according to claim 17, characterised in that the helper genes are contained on a third plasmid.

19. Process according to claim 17, characterised in that mammalian cells which express the helper genes are transfected.

20. Process according to one of claims 12 to 19,

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characterised in that, additionally, a plasmid which contains the adenovirus protease gene as a gene which increases the infectiousness of adenovirus is introduced into the cell.

21. Process according to claim 20, characterised in that the adenovirus protease gene is contained on the first, second or third plasmid.

22. Process according to claim 20 for preparing adenoviruses, characterised in that cells are brought into contact with a complex which contains optionally recombinant adenovirus, conjugated with a polycation, and possibly a conjugate of a ligand and a polycation, and a plasmid which codes for adenovirus protease.

23. Process according to any one of the preceding claims, characterised in that the plasmid DNA is complexed on the one hand with a conjugate of a ligand for the target cell and a polycation and on the other hand with a conjugate of an endosomolytic agent and a polycation.

24. Process according to claim 23, characterised in that the plasmid DNA is complexed with a transferrin-polylysine conjugate and with an adenovirus polylysine conjugate.

25. Transfection complex containing DNA which is to be expressed in a eukaryotic cell, which is complexed with a polycation optionally conjugated with a ligand for the target cell, and an endosomolytically active agent, characterised in that the DNA codes for a viral vector.

26. A transfection complex as claimed in claim 25 wherein said DNA contains one or more sequences coding for viral genes which increase the infectivity of the

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virus or the viral vector.

27. Transfection complex according to claim 25 or claim 26, characterised in that the viral vector-DNA contains DNA to be expressed in a target cell.

28. Transfection complex according to any one of claims 25 to 27, characterised in that the viral vector DNA including optionally helper genes required for replication thereof is present on at least two plasmids.

29. Transfection complex according to any one of claims 25 to 28, characterised in that the plasmid DNA is complexed with a transferrin-polylysine conjugate and with an adenovirus polylysine conjugate.

30. Transfection complex according to any one of claims 25 to 29, characterised in that the viral vector is a retrovirus vector.

31. Transfection complex according to claim 30, characterised in that the retrovirus vector DNA consists of two plasmids, the first containing the sequences coding for the packaging functions and the second containing the sequences coding for the other virus proteins, as well as the DNA to be expressed in the cell.

32. Transfection complex according to one of claims 25 to 29, characterised in that the viral vector is an AAV-vector.

33. Transfection complex according to claim 32, characterised in that the AAV-vector DNA consists of two plasmids, the first containing the sections coding for the AAV-Rep- and Cap-function and the second containing the foreign gene flanked by the inverted terminal

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repeats.

34. Transfection complex according to claim 33, characterised in that it contains another plasmid which contains at least part of the adenovirus helper genes necessary for the replication of AAV.

35. Transfection complex according to any one of claims 32 to 34, characterised in that the endosomolytic agent contains non-inactivated adenovirus.

36. Transfection complex according to one of claims 25 to 29, characterised in that the plasmid DNA is adenovirus vector DNA and/or contains one or more adenovirus genes which increase the infectivity of the virus.

37. Transfection complex according to claim 36, characterised in that the adenovirus vector-DNA is present on two plasmids, recombination of which yields a DNA molecule which has all the adenovirus gene functions with the exception of the helper genes necessary for replication.

38. Transfection complex according to claim 37, characterised in that the first plasmid, which lacks the E1-region, contains the left terminal repeat, the packaging function and the DNA to be expressed in the target cell, under the control of a promoter, and the second plasmid contains the entire adenovirus genome without a packaging signal and in the E1-region has an insert of a size which prevents packaging of the adenovirus sequence coded by the second plasmid.

39. Transfection complex according to claim 36, characterised in that the adenovirus vector DNA is present on two plasmids which cannot recombine with each

other, the first plasmid having the packaging function and the DNA to be expressed in the target cell under the control of a promoter, flanked by the two inverted terminal repeats, and the second plasmid has the other adenovirus sequences apart from the packaging function.

40. Transfection complex according to claim 39, characterised in that the second plasmid also contains the helper genes.

41. Transfection complex according to claim 40, characterised in that the helper genes are contained on another plasmid.

42. Transfection complex according to claim 36, characterised in that the plasmid DNA contains the sequence coding for adenovirus protease.

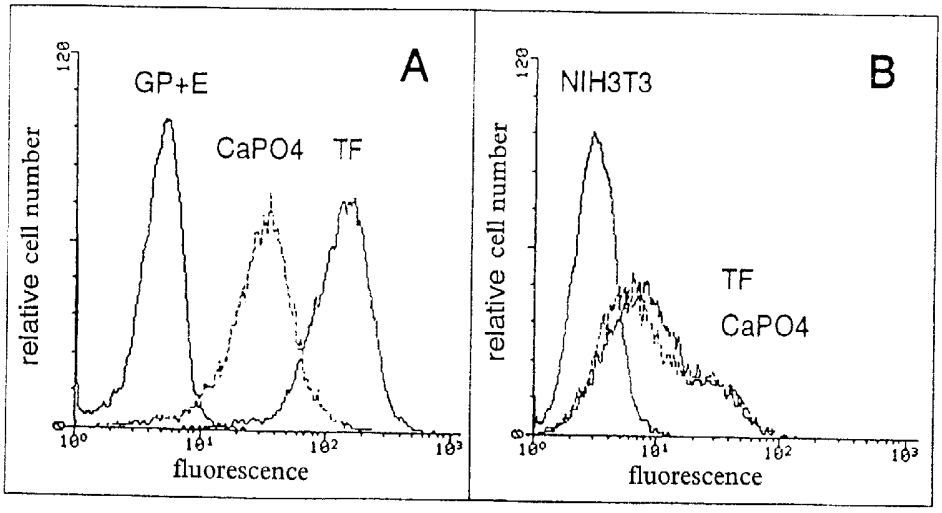
43. Transfection complex according to one of claims 27 to 42, characterised in that the DNA to be expressed in the target cell is a DNA which is effective in gene therapy.

44. A process as claimed in any one of claims 1 to 24 substantially as hereinbefore described.

45. A transfection complex as claimed in any one of claims 25 to 43 substantially as hereinbefore described.

Dated this 10th day of August, 1999
Boehringer Ingelheim International GmbH
by Davies Collison Cave
Patent Attorneys for the Applicants

Fig. 1


GP+E
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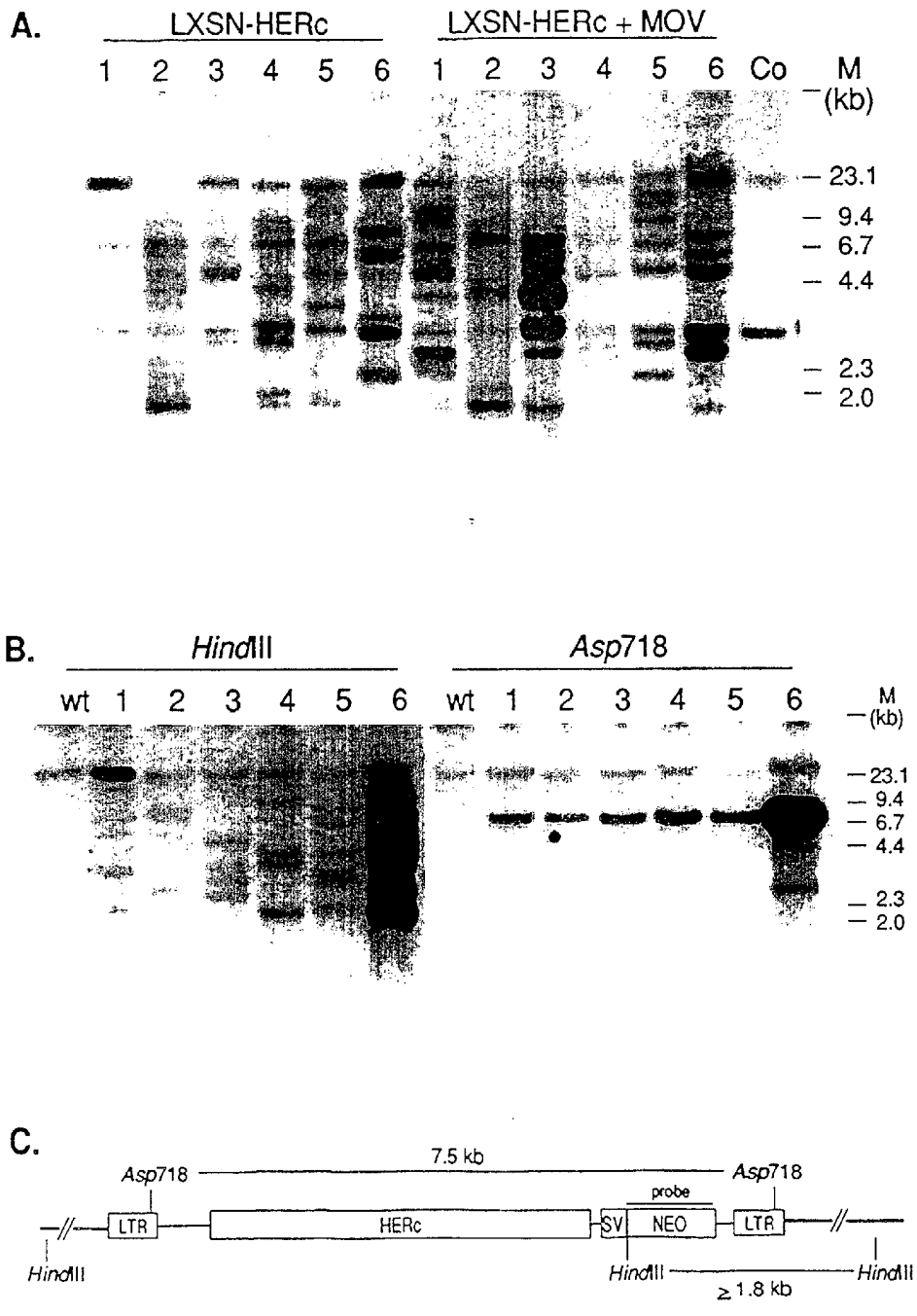
CaPO4
TF
relative cell number
fluorescence
NIH3T3
B
TF
CaPO4
relative cell number
fluorescence

Fig. 2


A.
LXSN-HERc
LXSN-HERc + MOV
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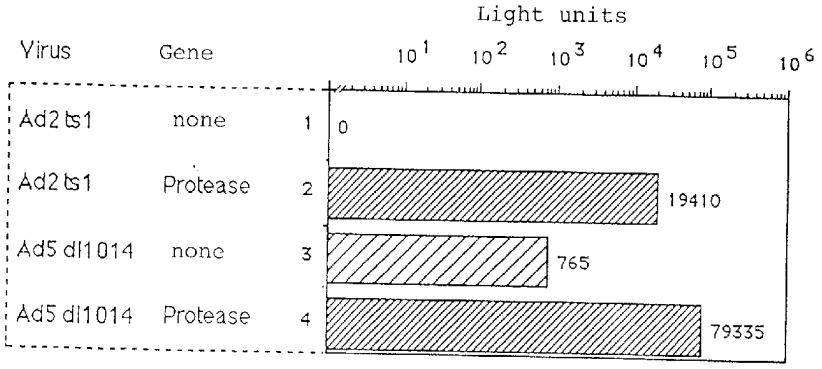
(kb)
23.1
9.4
6.7
4.4
2.3
2.0
B.
HindIII
Asp718
wt 1 2 3 4 5 6 wt 1 2 3 4 5 6 M
(kb)
23.1
9.4
6.7
4.4
2.3
2.0
C.
7.5 kb
Asp718
Asp718
probe
LTR
HERc
SV
NEO
LTR
HindIII
HindIII
HindIII
≥ 1.8 kb

Fig. 3

Fig. 4


Light units
Virus
Gene
10^1
10^2
10^3
10^4
10^5
10^6
Ad2 ts1
none
1
0
Ad2 ts1
Protease
2
19410
Ad5 dl1014
none
3
765
Ad5 dl1014
Protease
4
79335