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(54) Title: LENTIVIRAL VECTOR

(57) Abstract: The present invention relates to a lentivirus based DNA virus comprising a bacterial selection marker, a bacterial origin of replication and recognition sequences for endonucleases suitable for receiving a transgene, wherein the recognition sequences for endonucleases are arranged between two lentivirus-derived LTR sequences, at least one of which carries a deletion of the U3 gene making it self-inactivating (SIN), characterized in that the vector comprises, arranged between two lentivirus-derived LTR sequences, a lentivirus packaging signal (⊗) and at least one homing endonuclease recognition site for receiving at least one expression cassette comprising a transgene.
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
**Lentiviral Vector**

The present invention relates to nucleic acids and cloning methods utilizing such nucleic acids useful for gene transfer into animal cells, especially into vertebrate and mammalian cells, e.g. human cells. The nucleic acid described herein can be employed to effectively transfer a gene, containing a structural and/or regulatory sequence into animal cells *in vitro*, e.g. cultivated cells, as well as *in vivo*. The transferred DNA containing the structural and/or regulatory sequence is subsequently termed the transgene, the cell or organism comprising the transgene is termed transgenic cell and organism, respectively.

In detail, the present invention relates to a vector and cloning system which uses a lentiviral vector to introduce an expression cassette into cells for stable integration into the cellular genome and subsequent expression of the transgene.

Lentivirus based vectors for transforming animal cells have the advantages of allowing a stable integration of introduced DNA sequences and their subsequent stable expression without considerable levels of gene silencing, and expression seems to be relatively

Although derived from HIV-1 or, alternatively, from SIV, these vectors display high safety levels, which are achieved by the following measures: 1. Only the genes essential for infection and certain regulatory functions are included in those sequences finally introduced into the target cell. 2. The LTRs have been partially deleted, including their promoter regions to create self-inactivating LTRs, also termed SIN. 3. The viral genes necessary for the steps of production of viral particles, packaging of the RNA derived from the virus based DNA vector, and infection are distributed on up to three independent plasmids. Thus, the probability for an accidental recombination of sequences to form a replication competent virus is minimized. Accordingly, only the nucleotide construct intended to be packaged contains a respective packaging signal. 4. Presence of a central polypurine tract (cPPT) containing sequence (Follenzi et al., 2000, Nat. Genet. 25, No. 2: 217) leads to a strong nuclear import, facilitating integration of the sequence into the cellular genome independent form the stage of the cell cycle. 5. The replacement of the HIV-env by vesicular stomatitis virus glycoprotein (VSV-G) mediates a receptor-independent entry into target cells, allowing the infection of a wide range of host cell types.

Despite the advantages of known lentiviral vectors for delivering a transgene for expression into an animal cell, the cloning of the lentiviral vector and the subsequent gene transfer is time-consuming and in most laboratories it is inappropriate for use as a standard procedure. Reasons are the labor-intensive production of viral particles and insufficient titers to be achieved and especially the sophisticated construction of vector constructs. In detail, especially the introduction of additional and/or alternative functional genetic elements, e.g. promoters, transgenes, enhancers or complete expression cassettes is difficult due to the following reasons: 1. A large size of the vector leads to a low cloning efficiency. 2. Lentiviral sequences result in high recombination rates during the in vitro cloning procedures. 3. The absence of comfortable multiple cloning sites in known virus based vectors requires complex
cloning strategies. However, introduction of a multiple cloning site directly into a virus based vector would probably be of limited use for cloning as most of its restriction sites are already present in the backbone of the lentiviral vector.

A known lentiviral cloning and expression system addressing these problems is available from Invitrogen Life Technologies, Karlsruhe, Germany (ViraPower Lentiviral Expression System), which employs a first bacterial vector for cloning an expression cassette including the desired transgene. The transfer of the expression cassette into the vector containing the viral sequences is achieved either by site specific recombination using lambda phage or via oriented ligation employing one blunt end and one partially single stranded sticky end for receiving an insert with respective blunt and sticky ends.

When using the phage-mediated transfer of the expression cassette into the virus based vector, the known lentiviral expression system suffers from the fact that a procaryotic selection marker along with its bacterial promoter sequence is necessarily present in that vector to select for recombinant viral vectors including the transduced expression cassette because the recombination using a phage is rather inefficient. The additional selection sequences enlarge the total sequence of the virus based vector, leading to a reduced capacity for desired sequences to be integrated between the ITRs without improving the performance of the vector. When using the alternative cloning procedure ligating PCR-generated fragments in an oriented fashion into one sticky end having topoisomerase as a ligase linked to it and one blunt end, the limitation of PCR to fragments of at most 4 kb is easily encountered when amplifying entire expression cassettes. In view of the introduction of larger PCR amplificates, a further difficulty is that the recognition site for the sticky end to be ligated needs to be added to one end of the amplification fragment, usually by adding the required additional nucleotides to one primer, which is a strong hinderance for using proof-reading DNA polymerase for the amplification. This is because proof-reading polymerase eliminates non-pairing nucleotides from the amplification product, which applies to the added nucleotides of the restriction/ligation site. Accordingly, the introduction of amplificates generated by long-distance PCR using a mixture of Taq-DNA polymerase without proof-reading activity and proof-reading DNA polymerase suffers from the difficulty to combine the exactness required from proofreading polymerase with the necessity to add non-pairing nucleotides to the amplificate for cloning purposes.
A further viral vector system is based on the human adenovirus, a double-stranded DNA virus causing mild infections. This cloning system is available from Clontech Laboratories Inc. (Becton Dickinson Biosciences) and uses a two-step procedure for cloning the desired transgene between flanking viral sequences. In a first step, the desired transgene is cloned downstream of a promoter element and upstream of a poly-adenylation site contained within a bacterial vector. In a second step, the resulting expression cassette including the promoter and poly-adenylation sites is excised via adjacent recognition sites for homing endonucleases I-Ceu I and PI-Sce I for ligation into an equally digested adenovirus based vector. Within the adenovirus based vector, the expression cassette is arranged between the homing endonuclease recognition sites, which are flanked by inverted terminal repeat sequences (ITRs).

One aspect discouraging a person skilled in the art to merely transfer functional sites from an Adenovirus derived vector, which is a DNA virus, to a lentiviral vector are the inherent differences between the virus types, especially the dependency of the lentivirus on reverse transcription and different regulatory systems as well as different life cycles.

The life cycle, functional elements and nucleic acid sequences of Adenoviruses, which are double-stranded DNA viruses, widely differ from lentiviruses, which are single-stranded RNA viruses using an intermediate state in the form of DNA. As the highly organized viral genomes are the result of long term evolution it is frequently observed that minor mutations, including even single base exchanges or other modifications of the underlying natural nucleic acid sequence result in major deficiencies of the overall function of the virus or of viral vectors, respectively. For example, the modification of genetic elements frequently negatively interferes with the packaging process and the introduction of additional sequences can introduce regulatory regions that inadvertently negatively regulate expression of the desired transgene. It has also been found by the inventor that in respect of lentiviral vectors, the introduction of a eucaryotic poly-adenylation signal, which is necessarily included in expression cassettes of adenoviral vectors, leads to a dramatic decrease in titers.

It is therefore not obvious that nucleic acid sequences, which are functional in an adenoviral vector will equally well function in a completely different type of virus. In this respect, one needs to take into account not only the possible effects of the sequence of the expression cassette but also the effects of flanking nucleotides originating from the cloning process.
These additional sequences, which are not intentionally included for a desired purpose, can lead to unpredicted effects, such as forming a binding site for cellular repressor proteins.

In view of the known lentiviral cloning systems aiming at the provision of a DNA construct that can be used to introduce a transgene into animal cells, the present invention seeks to overcome the drawbacks of the state of art, especially by providing a virus based cloning vector useful for introducing a transgene into animal cells with high efficiency. Preferably, introduction of the transgene leads to stable integration thereof into the genome of the cell transfected in vitro or in vivo, the latter being applicable for pharmaceutical or medical use or for animal breeding, e.g. animal models for research use. It is a further object of the present invention to provide a virus based vector which can be packaged in viral particles using permissive cells to obtain high titers of recombinant viral particles. In addition, the present invention aims at providing a method of cloning and a respective cloning system useful for cloning and transferring a transgene to be transfected into animal cells in an easy to approach way, avoiding recombination events during the in vitro and in vivo cloning steps or cumbersome cloning methods.

In order to achieve the above objects, the invention provides a cloning system comprising two vectors, one exclusively bacterial vector and one lentivirus based vector. The system provides for a method avoiding the drawbacks of state of art methods for cloning a transgene into a virus based vector which can be packaged into viral particles. According to the invention, the cloning is achieved in a two-step procedure. In a first step, the desired transgene, which in the case of a structural gene is preferably combined with regulatory sequences, is integrated into a conventional high copy number bacterial plasmid by ligation into its multiple cloning site. Within the cloning site of this merely bacterial plasmid, an expression cassette of the desired transgene can be constructed by conventional cloning methods using digestion by restriction enzymes and subsequent ligation to generate the appropriate arrangement of regulatory and/or structural sequences.

Within the disclosure and claims of this invention, the term “expression cassette” can both refer to a transgene containing regulatory sequences comprising an adjacent structural gene, or alternatively it can refer a transgene comprising regulatory sequences only. The transgene to be introduced by means of the inventive lentivirus based vector may comprise at least one structural and/or regulatory gene, preferably of animal origin, more preferably of vertebrate or
mammalian origin, or of human origin. Alternatively or additionally, the transgene may comprise nucleotide sequences encoding antisense RNA, a ribozyme, or siRNA (inhibitory RNA).

The expression cassette can include one or more, e.g. two, three or more structural genes, preferably each one adjacent its regulatory sequences, e.g. tissue specific promoters, enhancer sequences, internal ribosomal entry sites (IRES-sequences) and additional selection markers.

In a second step, the expression cassette is specifically excised from the bacterial plasmid used in the first step and ligated into the lentivirus based vector. It is only after completing the construction of the expression cassette of the desired transgene that the lentivirus based vector needs to be employed in the cloning method according to the invention. The lentivirus based vector contains sequences necessary for integrating the expression cassette into the genome of animal cells, either by transfection with vector DNA or by infection with viral particles containing the vector DNA.

According to the invention, the transfer of expression cassette from the bacterial vector into the lentivirus based is achieved using at least one homing endonuclease, also referred to as mega endonuclease having rather long recognition sequences which is both present on the bacterial plasmid surrounding the expression cassette, and on the lentivirus based vector arranged between LTRs. In a preferred embodiment, recognition sites for two different homing endonucleases surround the multiple cloning site and the expression cassette, respectively, on the bacterial plasmid as well as they are contained adjacent each other between the LTRs on the lentiviral vector, serving as the transfer vector during transfection.

In addition to the packaging signal sequence and the at least one homing endonuclease recognition site, optional elements present on the lentivirus based vector between the LTRs include splice donor (SD) and splice acceptor (SA) sequences, a Rev responsive element (RRE), an internal ribosomal entry site (IRES) sequence, and a poly purine tract (cPPT). Preferably, the LTR sequences are of the SIN type, LTR variants which are self inactivating upon integration into the host cell’s genome.
It has been found in preliminary experiments that the presence of a poly-adenylation site within the expression cassette leads to the generation of non-functional, i.e. incomplete viral transcripts. Accordingly, the inventive vectors do not contain a poly-adenylation site.

It is generally known that the effects of genetic elements constituting a viral vector on the titer generated when packaging the viral sequences into viral particles as well as the expression levels achieved in transduced cells cannot be predicted. Therefore it could not be expected that the lentivirus based vector according to the invention is capable of producing titers of viral particles and expression levels of the transgene in transduced cells in at least the same order as state of the art vectors while allowing for stable integration into a cellular genome and expressing the transgene. Surprisingly, this can be achieved while using the simplified cloning method of the invention employing a bacterial plasmid for construction of the desired expression cassette and subsequent transfer thereof into the lentiviral vector making use of at least one homing endonuclease restriction site on both vectors.

As an example for the unpredictability of the effects of sequence alterations within a lentiviral vector on its functional properties it was found that the inversion of the expression cassette can result in a dramatic reduction of functional viral particles, although subsequent computer analysis did not reveal that the inversion had created any functional elements able to abort transcription or translation. The expression cassette whose inversion was investigated consisted of the CMV promoter (CMV) and the humanized Renilla reniformis derived green fluorescent protein (hrGFP).

The simplified cloning properties of the inventive lentiviral vector in combination with the bacterial shuttle vector suitable for cloning an expression cassette have the advantages of allowing an easy and simplified construction of a larger number of variations of constructs in comparison to state of art cloning systems. This is especially important when investigating the expression of structural and/or regulatory sequences in different cell types, because the titer obtained for viral particles and the infection efficiency as well as the activity of the transgene within the infected cell depends *inter alia* on the various sequences and their arrangement contained in the lentiviral vector. The inventive lentiviral vector is demonstrated to have suitable properties for accepting large transgene sequences and obtaining high titers of viral particles when produced in permissive cells and high activity levels of the transgene after infection. In addition, the simplified access to cloning constructs according to the invention
allows to efficiently create and test a large number of variations of the expression cassette, which is helpful as also the transgene may influence infectivity and activity thereof once introduced into cells.

Furthermore, the lentiviral vector of the invention allows the integration of large transgene sequences, firstly within the vector and secondly within the cellular genome of infected cells. According to the invention, these large transgene sequences, e.g. expression cassettes, are conveniently assembled in the bacterial vector and then transferred into the lentiviral vector.

The invention is now described in detail with reference to the accompanying figures, wherein

Figure 1 depicts a schematic graphical representation of the bacterial shuttle vector pBSHuttle, including the Ampicillin resistance, which is exchangeable for another selection marker, like the resistance against Kanamycin or another antibiotic,

Figure 2 depicts a schematic graphical representation of the virus based vector pLentiShuttle,

Figure 3 shows the titers obtained for different lentiviral constructs,

Figure 4 is a graph of the results of the flow cytometrical determination of expression levels determined in human HEK293T cells infected with virus based vector according to the invention, and

Figure 5 shows the flow-cytometrical analysis of expression of fluorescent reporter genes in transduced rat cells.

**The bacterial shuttle vector**

The bacterial plasmid is subsequently termed pBSHuttle, a schematic map of its functional regions is given as Figure 1.

The multiple cloning site, which can be represented by any combination of cloning sites, is flanked by two identical, preferably by different homing endonuclease recognition sites, for example by recognition sites for I-Ceu I and PI-Sce I, one on each side of the multiple cloning site. The region of the multiple cloning site embraced by two homing endonuclease recognition sites can be used to construct the complete expression cassette necessary for later
activity of and/or synthesis from the transgene sequence. The expression cassette can for example be put together from promoter sequences, at least one desired structural gene and further regulatory elements like a internal ribosomal entry site sequence (IRES), enhancer sequences and/or selection markers.

Plasmid pBSHuttle is based on high copy number plasmid pBluescript II SK (-) (Stratagene, LaJolla, USA), into which the multiple cloning site flanked by recognition sites for I-Ceu I and PI-Sce I was transferred from pShuttle (BD Biosciences, Heidelberg, Germany). In detail, pBSHuttle is obtainable by deleting the fragment EcoRI/MfeI from pShuttle and amplifying a 310 bp fragment via PCR using forward-primer 5’-TAGGTACCGCCGCCCACCTGACGTTAA-3’ (the KpnI-site is underlined) (Seq ID No. 1) and reverse-primer 5’-GCGGAGCTCCGCGCGTTGGCCGTTACTC-3’ (the SacI-site is underlined) (Seq ID No 2). The resulting fragment was digested with SacI and KpnI and inserted between respective sites in pBluescript II SK (-) according to standard protocols. Then, the complete digestion fragment I-Ceu I / PI-Sce I isolated from pShuttle containing a human immediate early cytomegalovirus promoter (CMV promoter), a multiple cloning site, and bovine growth hormone polyadenylation signal (BGH poly A) was ligated into the newly introduced restriction sites Kpn I and Sac I of pBluescript II SK (-), yielding pBSHuttle CMV + poly A. Finally, the plasmid was transformed into dam<sup>ns8</sup> E.coli strain GM33, isolated therefrom, digested with Bcl I, followed by partial digestion with Bgl II and self-ligated, resulting in the elimination of a 269 bp fragment containing the BGH poly A signal to construct pBSHuttle CMV. Elimination of the CMV promoter sequences was achieved by digestion with Mfe I and Nhe I, followed by a fill-in reaction with the large Klenow fragment (NewEngland Biolabs) and self-ligation, leading to pBSHuttle.

It is an advantage of the cloning system and method according to the present invention that the first step of cloning the transgene is performed in a conventional high copy number bacterial vector devoid of viral sequences, especially devoid of viral long terminal repeat sequences (LTR) as these are a cause of recombination events during the cloning procedure. As a further advantage, the reduced size of this inventive vector in comparison to known vectors allows its more efficient amplification in bacteria. Furthermore, the lack of viral sequences allows the use of restriction sites within the multiple cloning site without contemporaneously digesting the vector at a further site, which is generally undesired when cloning.
In the alternative, cloning and constructing of the transgene expression cassette including the transgene within pBShuttle can be replaced by in vitro cloning techniques using the polymerase chain reaction (PCR) and site directed mutagenesis including hybrid primers to synthesize the expression cassette and for adding one of the homing endonuclease recognition sites to each terminus of the expression cassette. Hybrid primers for adding the homing endonuclease recognition sites to the expression cassette can for example both contain terminal parts of the expression cassette sequence and the sequence of the adjacent homing endonuclease recognition site for adding the latter to the respective terminus of the expression cassette. Following the addition of the homing endonuclease recognition sites, the expression cassette can be ligated into a conventional bacterial plasmid for further amplification in bacteria.

In a second step, following amplification of pBShuttle containing the transgene in E. coli, the transgene is provided with sticky ends at its homing endonuclease recognition sites via restriction with the at least one, preferably two different homing endonucleases, digesting the homing endonuclease recognition sites which flank the transgene expression cassette. In this way, the transgene can be excised from a bacterial vector like pBShuttle. In the alternative, the homing endonucleases may digest the in vitro amplification product of the PCR, in each case yielding the expression cassette including the desired transgene with both of its ends provided with homing endonuclease recognition sites in their sticky, i.e. digested state.

The lentivirus based vector

For integration into the virus based vector according to the invention, termed pLentiShuttle, the expression cassette constructed in the bacterial shuttle vector, now having sticky homing endonuclease recognition sites at both of its termini is ligated into the vector pLentiShuttle, equally digested with the same set of homing endonucleases.

The vector pLentiShuttle is schematically represented in Figure 2 and preferably comprises functional sites of retroviral and/or lentiviral origin adjacent its pair of homing endonuclease recognition sites. Preferably, these functional sites are grouped together to the 5'-terminus of the expression cassette. The functional sites comprise, starting at 5', the self inactivating (SIN) variant of the LTR of human immunodeficiency virus (HIV), a viral packaging site (Ψ),
as it is contained in a 3' deleted gag sequence of viral origin (GA), a splice donor site (SD), a Rev responsive element (RRE), a splice acceptor site (SA), and a poly purine tract (cPPT), resulting in improved nuclear import within transfected cells.

The SIN-variant, which is the preferred embodiment of the lentiviral LTR is characterized by deletions to disable the enhancer and promoter activities of the wild-type LTR as previously described in Zufferey et al., J. Virol., 72, 9873-9880 (1998) and US 5 994 136. Deletions of the 5'-LTR need to keep intact the cis-acting sequences required for reverse transcription as well as for integration (terminal di-nucleotide + att sequence). Arranged on the 3'-end of the homing endonuclease recognition sites is a 3'- LTR whose self-inactivating properties are activated upon integration into the DNA of the targeted animal host cell. In detail, the 5'-SIN represents a deletion of the U3 region, which deletion is duplicated into the 3'-LTR upon integration into the target's DNA, thus inevitably inactivating the promoter activity of the 3'-LTR.

An exemplary sequence of the inventive vector is given as Seq ID No 3, containing no expression cassette. Functional elements present on that vector, which represents a presently preferred embodiment include the Ampicillin resistance, an oriR, the SV40 promoter/enhancer element, the SV40 oriR, gpt (guanine phosphoribosyl transferase, a selection marker), a poly-adenylation site, a 5'-LTR (U3 and R/U5), an RNA packaging signal (Ψ), env (partially deleted), SD/SA sites, RRE and central DNA flap/cPPT, 3'-SIN-LTR (U3 partially deleted, R/U5) and restriction sites for I-Ceu I and PI-Sce I. These genetic elements may be deleted from the vector according to standard cloning procedures, resulting in a functional, although less preferred embodiment of the lentiviral vector, as long as the sequences encoding the lentiviral LTR-SIN regions framing the sequences intended for packaging into viral particles, the packaging signal and at least one homing endonuclease recognition sequence are contained or exchanged against other genetic elements providing their respective functions. Although the vector of Seq ID No 3 is preferred for receiving an expression cassette for transfer into cells, vectors functionally corresponding to Seq ID No 3 are included in the invention. Such vectors preferably hybridise under stringent conditions to Seq ID No 3, at least to its sequence portions 5368 to 161 and/or share at least 30, preferably at least 50 or at least 80 percent homology.
Packaging of lentivirus based vector into viral particles

In order to produce viral particles containing the virus based vector of the invention, its DNA has to be packaged appropriately. This packaging into viral particles can be performed according to established procedures, see e.g. US patent 601 35 16.

In general, the generation of viral particles containing the inventive lentivirus based vector carrying the desired expression cassette of a transgene can be achieved according to known methods. In a preferred method, only the inventive lentivirus based vector carries a packaging signal (Ψ) and SIN-LTR sequences necessary for integration, whereas the other components necessary for producing viral particles are encoded on at least two additional different plasmids. One of the additional plasmids can for example code for a viral gag and pol, directing synthesis of a group specific antigen and reverse transcriptase, respectively, as well as integrase and proteases necessary for maturation and reverse transcription (RNA dependent DNA polymerase), the other plasmid can code for the viral envelope proteins (env).

For instance, the HIV-env of the lentivirus HIV is replaced by vesicular stomatitis virus glycoprotein operably linked to promoter and/or enhancer sequences to render the viral particle competent for infection of virtually all cell types. Alternatively, target specificity can be assigned to the viral particle by using an env gene or other genes coding for envelope proteins or other surface proteins, respectively, with a desired cell specificity.

Viral particles are produced in a permissive helper cell line after co-transfection with the at least three plasmids and can then be recovered from the culture medium.

One method of packaging using such additional plasmids encoding the genes lacking on the lentiviral vector is described in Example 3.

In the alternative, lentiviral constructs can be packaged in cell lines which stably express the proteins necessary for packaging, for example the envelope proteins and group specific antigen as well as reverse transcriptase.

The present invention is now described in the following examples which are not intended to limit the scope of the disclosure or claims.
Example 1: Cloning of an expression cassette in pBShuttle

For cloning an expression cassette to be flanked by two recognition sites for homing endonucleases, namely I-Ceu I and PI-Sce I (Figure 1 a), Seq ID No 4 and 5, respectively), pBShuttle-CMV was used that already contained the CMV promoter/enhancer element (designated as CMV). Further regulatory elements used were the spleen focus forming virus promoter (designated as SFFV), the phosphoglycerate kinase promoter (designated as PGK), the SV40 promoter/enhancer (designated as SV40-P/E) and the Woodchuck hepatitis virus posttranscriptional regulatory element (designated as WPRE). As structural genes, *Aequorea victoria* derived enhanced green fluorescent protein (designated as eGFP) and the humanized *Renilla reniformis* derived green fluorescent protein (designated as hrGFP) as well as the improved *Discosoma sp.* DsRed variant RedStar (Knop et al., 2002, Biotechniques 33, 3: 592) were introduced 3’ to CMV.

Structural genes hrGFP and RedStar as well as the regulatory element WPRE were introduced into the Nhe I – Sba I – Xho I – Not I – Kpn I – Afl II- multiple cloning site of pBShuttle CMV as follows: hrGFP was recovered as the Not I / Kpn I restriction fragment from plasmid phrGFP-1 (Stratagene, LaJolla, USA) and RedStar as the Xba I / Xho I restriction fragment form plasmid p415-Gal-1RedStar (gift from M. Knop, Max-Planck-Institut für Biochemie, Martinsried, Germany). The regulatory element WPRE was subcloned from the vector pHRR (Zufferey et al., ) into pCITE-2a (+) (Novagen, Darmstadt, Germany) and amplified by PCR using forward primer 5’-CTGGTACCTTGATGCCCTGAC-3’ (Kpn I site underlined) (Seq ID No 6) and reverse primer 5’-ATACTTAAGCCGAATTCGAGCTCC-3’ (Afl II site underlined) (Seq ID No 7), then digested with Kpn I and Afl II and ligated into respective restriction sites in pBShuttle.

Further expression cassettes comprising the SFFV promoter and reporter genes were constructed as follows: An Mfe I / Xho I fragment was excised from pShuttle CMV to eliminate the CMV promoter sequence and an EcoR I / Sal I fragment with compatible overhangs containing the SFFV promoter and eGFP was ligated into the Mfe I / Xho I cut pShuttle, resulting in pShuttle SFFV eGFP WPRE. In pShuttle CMV hrGFP WPRE an Mfe I / BamH I fragment with the CMV promoter was replaced by an EcoR I / BamH I fragment with compatible overhangs containing the SFFV promoter, resulting in pShuttle SFFV hrGFP WPRE. A further derivative of the latter constructed contained a PGK promoter (gift from F.
Stewart, EMBL Heidelberg, Germany) in the place of the SFFV promoter, the result being named pShuttle PGK hrGFP WPRE.

All cloning procedures were carried out according to standard procedures, transformations were done with electrocompetent E. coli Top10 (Invitrogen Life Technologies), unless indicated otherwise.

**Example 2: Cloning of an expression cassette from pBShuttle into pLentiShuttle**

Expression cassettes constructed in pBShuttle were subcloned into pLentiShuttle by digestion with homing endonucleases I-Ceu I and PI-Sce I (NewEngland Biolabs, Beverley, MA, USA) and ligation into equally digested pLentiShuttle. Because pLentiShuttle contains lentiviral LTRs, this last cloning step was performed in ElectroMax Stbl 4 competent cells (Invitrogen Life Technologies) according to the manufacturer's instructions in order to prevent recombination events. The lentivirus based vectors containing expression cassettes are schematically represented in Figure 2.

In order to demonstrate that the expression of structural genes as transgenes after transduction into target cells is exclusively driven by promoters within the expression cassette, instead of possibly remaining regulatory elements of the SIN-LTR or by promoter sequences present at the integration sites of lentiviruses, an expression cassette devoid of promoter sequences containing hrGFP WPRE was introduced into pLentiShuttle as a negative control. It was found that no expression occured in HEK293T cells when only the lentiviral sequences of pLentiShuttle and the structural gene were present.

**Example 3: Preparation of viral particles from virus based vector pLentiSIN**

Human kidney 293T cells (5 x 10⁷) were plated in pre-coated poly-L-Lysine culture flasks (T175, Sigma Chemical Co.) in Iscoves modified Dulbecco's culture medium DMEM (Gibco BRL) including 10% fetal calf serum and 2 mMol L-glutamine (Gibco, BRL), penicillin (100 U/mL) and streptomycin (100 mg/mL) and placed under 5% CO₂ in an incubator. Following 3 days culture at 37 °C, transient transfection was carried at 60-70 % confluence by the Calcium phosphate method (Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour) out using 20 µg of lentivirus vector DNA, 13 µg packaging plasmid pCMVΔ8.91 coding for the Gag, Pol, Rev, and Tat proteins as well as 7 µg of plasmid pMD.G coding for the envelope proteins for pseudotyping the lentiviral
particles with VSV.G. Protein G of VSV (vesicular stomatitis virus) enables unspecific entry into virtually all cell types, independent of their cell cycle status or specific receptor sites.

Medium was changed after 8 hours continued culture against DMEM containing 10 mM sodium butyrate (Upstate Biotechnology) for 20 hours. Following that, cells were changed to serum-free DMEM containing 2mMol L-glutamine, penicillin (100 U/mL) and the supernatant medium was harvested every 24 hours for 2 days by exchange against fresh medium, pooled, and filtered through a 0.45 μm pore size filter. The resultant fluid represents the viral particle stock.

Samples of the culture medium of the transfected 293T cells were tested during 3 weeks of culture for absence of replication-competent virus by monitoring for the presence of p24 antigen expression using ELISA. No replication-competent virus was detected.

**Example 4: Determination of titers of viral particles produced from virus based vector pLentiShuttle**

Titers of unconcentrated supernatants from the cell culture for producing viral particles according to Example 3 were determined in a biological assay using triplicate serial dilutions of the vector stocks in presence of 8 μg/mL Polybrene (Sigma, Deisenhofen, Germany) on 1 x 10^5 293T cells per well in 24-well plates. Medium was replaced after 16 hours incubation at 37 °C in 5% CO₂ by viral particle suspensions. After 72 hours continued incubation, infected cells were washed twice in PBS, detached and analysed, in the case of GFP or RedStar by flow cytometry using a FACS Calibur (Becton Dickinson, Heidelberg, Germany) flow cytometer. Non-infected HEK293 cells were used as negative controls, data were analyzed with the analytical computer programmes WinMDI 2.8 and GraphPad Prism (version 3.02 for Windows, GraphPad Software, San Diego, USA). Based on the flow-cytometrical data, titers were back-calculated to the initial cell number (1 x 10^5/well) and 1 mL vector supernatant. As the actual cell number / well during infection is significantly higher than the initially seeded cell number per well 16 hours prior to infection, which number was used for the calculation, the actual titers are underestimated.

**Example 5: Titers of pLentiShuttle in comparison to conventional viral vectors**

The effects of the expression cassette cloned into the multiple cloning site of pBShuttle when present in pLentiShuttle were estimated in a comparison with conventional lentiviral vectors.
containing the same expression cassette. The conventional vectors were pHR SINcPPT SFFV eGFP WPRE (pHR' SINcPPT SEW) and pHR' SINcPPT CMV eGFP WPRE (pHR' SINcPPT CEW) belonging to the so-called advanced generation lentiviral vectors. These comparative vectors do not contain a homing endonuclease site. The results depicted in Figure 3 demonstrate that the efficiency of packaging and infection of the inventive vectors is not compromised by the presence of homing endonuclease restriction sites.

In Figure 3, titers are compared for packaging in HEK 293T cells, titration was performed on the same cell line in parallel. It is demonstrated that the inventive vectors are packaged equally well, namely pHR' SIN cPPT SFFV eGFP WPRE (pHR' SIN cPPT SEW) \((5.3 \times 10^6 \pm 1.9 \times 10^5)\) was packaged essentially as effective as the inventive vector carrying the same expression cassette, pLentiShuttle SFFV eGFP WPRE \((1.9 \times 10^6 \pm 2.3 \times 10^5)\) and pLentiShuttle SFFV hrGFP WPRE \((3.0 \times 10^6 \pm 1.6 \times 10^5)\). Comparative vector pHR' SIN cPPT CMV eGFP WPRE (pHR' SIN cPPT CEW) \((3.5 \times 10^6 \pm 2.0 \times 10^5)\), pLentiShuttle CMV hrGFP WPRE \((3 \times 10^6 \pm 4.2 \times 10^5)\) and pLentiShuttle PGK hrGFP WPRE \((3.1 \times 10^6 \pm 1.1 \times 10^5)\) showed titers in the same range. Inventive vector pLentiShuttle CMV RedStar WPRE \((7.7 \times 10^6 \pm 9.2 \times 10^5)\) yielded a titer about 40 times lower than the corresponding hrGFP containing pLentiShuttle CMV hrGFP WPRE. Elimination of the promoter element CMV gave no transgenic cells positive for GFP.

**Example 6: Expression of the transgene from pLentiShuttle**

For flow cytometrical determination of expression levels in human HEK293T cells, these were grown in complete DMEM at 37 °C, 5 % CO\(_2\) with 4μg/mL protamine sulfate (Sigma) and infected with an MOI of 1. 100μM deoxynucleoside triphosphates (Amersham Biosciences, Freiburg, Germany) were added to enhance reverse transcription of the viral genome (Zhang et al., 1995, J. Virol. 69, No. 6: 3929). After 48 hours, cells were washed twice with PBS, detached and resuspended in PBS for flow-cytometrical measurements.

Results demonstrate that expression activities in neonatal rat cardiomyocytes and rat mesenchymal stem cells infected with inventive pLentiShuttle containing the expression cassette SFFV hrGFP WPRE, PGK hrGFP WPRE, CMV RedStar WPRE and CMV hrGFP WPRE, and a comparative vector containing identical expression cassettes, respectively, essentially coincide. The elements used were promoters CMV (cytomegalovirus) or SFFV (spleen focus forming virus) with the structural gene eGFP (enhanced green fluorescent
protein) or hrGFP (humanized recombinant green fluorescent protein) with both control and inventive variants being enhanced by the WPRE sequence (post-transcriptional regulatory element of woodchuck hepatitis virus).

The results of GFP expression levels are depicted in Figure 4, showing the FACS analysis of HEK293T cells transduced with an MOI of 1 using conventional vectors pHRSIN cPPT SFFV eGFP WPRE (Figure 4 a), bold grey line) and pHRSIN cPPT CMV eGFP WPRE (Figure 4 b), bold grey line) in comparison to pLentiShuttle SFFV hrGFP WPRE (Figure 4 a), thin black line) and pLentiShuttle CMV hrGFP WPRE (Figure 4 a), thin black line), respectively. It is seen that the lines overlap, indicating quite similar expression levels. Accordingly, no negative influence of the additional sequence portions comprising the homing endonuclease restriction sites are observed.

The results obtained for measuring red fluorescence cannot be compared directly to the values obtained for green fluorescence, however, the flow-cytometrical data demonstrate bright red fluorescence produced by inventive lentiviral vectors containing expression cassettes for RedStar as well.

**Example 7: Infection of differentiated cells by viral particles containing vector pLentiShuttle**

Viral particles obtained from transduced human HEK293T cells were effectively used to infect rat fibroblasts and rat cardiomyocytes, murine pneumonic epithelial cells and their progenitor cells, as well as adult human endothelial progenitor cells and bone marrow derived mesenchymal stem cells. For infection, an MOI of 1 to 10 was used, obtaining an efficiency of infection as measured by fluorescence of up to 100%, depending on the fluorescent variant gene employed.

When infecting target cells, for example human endothelial progenitor cells (huEPC) or human mesenchymal stem cells (huMSC), a multiplicity of infection (MOI) of 10 in serum free medium XVVI-10 (Bio Whittaker, UK) was used at 37 °C in 5% CO₂ atmosphere. Infection was carried out by gently shaking (100 rpm) for 5 hours at 37 °C in presence of 4 μg/mL protamine sulfate and 100 μM deoxyribonucleoside triphosphates to enhance reverse transcription of the viral genome (REV). Following that, viral particle containing supernatant was removed and the cells were washed twice in 1 x PBS (phosphate buffered saline, pH 7.4).
Cells were cultured for further seven to ten days at a density of $1 \times 10^5$ cells/well in glass bottom chamber slides coated with polylysine-ornithine.

The flow-cytometrical results of the transduction of neonatal rat cardiomyocytes (Figures 5 a, c, and e) and rat mesenchymal stem cells (Figures 5 b, d, f, g, and h) are depicted in Figure 5. For transduction, comparative vector pHRSIN cPPT CMV EGFP WPRE (Figures 5 a and b) and inventive vectors pLentiShuttle SFFV hrGFP WPRE (Figures 5 c and d), pLentiShuttle PGK hrGFP WPRE (Figures 5 e and f), pLentiShuttle CMV RedStar WPRE (Figure 5 g), and pLentiShuttle CMV hrGFP WPRE (Figure 5 h) were used. Transductions were carried out using an MOI of 5 according to Example 4. Dotted lines depict background fluorescence, the percentages given indicate the percentages of transduced cells expressing the fluorescent reporter genes GFP and RedStar, respectively.

These results demonstrate that the transduction using the inventive lentiviral vector leads to similar fluorescence intensities, i.e. expression of the transgene GFP as a comparative conventional lentiviral vector. Also, a single round of transduction with an MOI of 5 resulted in similar transduction rates ranging from 30% to 36% for inventive vectors pLentiShuttle and 35% for comparative vector pHRSIN in cardiomyocytes as indicated by the percentages of cells positive for GFP expression. In rat mesenchymal stem cells, transduction rates were from 23% to 56% for inventive vectors and 36% for comparative vectors, respectively. The transduction using an inventive RedStar containing lentiviral vector resulted in 48% transduced cells expressing RedStar.

**Example 8: Exemplary lentiviral constructs of pLentiShuttle**

In order to use the inventive lentiviral vector, termed pLentiShuttle, further structural and regulatory genes were expressed using different combinations with promoter and enhancer sequences. The following table lists constructs which were cloned using construction of the expression cassette in pShuttle and subsequent transfer of the expression cassette via restriction/ligation of the two flanking homing endonucleases I-Ceu I and PstI-See I. The lentiviral vector sequence corresponds to that of Seq ID No 3, the arrangement of the genetic elements from 5' to 3' corresponds to the order of these elements as given in the respective vector designation.
Production of lentiviral particles was obtained in HEK293T cells, for expression cardiomyocytes, pneumocytes and stem cells were used.

**Table: Lentiviral Vector constructs pLentiShuttle containing expression cassettes:**

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Nkx2.5, Crescent, and GATA4 are transcription factors
Claims

1. Lentivirus based DNA vector comprising a bacterial selection marker, a bacterial origin of replication and recognition sequences for endonucleases suitable for receiving a transgene, the recognition sequences for endonucleases arranged between two lentivirus-derived LTR sequences, characterized in that the vector comprises, arranged between two lentivirus-derived LTR sequences, the following elements:
   a) a lentiviral packaging signal ($\Psi$),
   b) at least one homing endonuclease recognition site for receiving at least one expression cassette comprising a transgene.

2. DNA vector according to claim 1, characterized in that the vector further comprises arranged between two lentivirus-derived LTR sequences
   c) a splice donor sequence, and
   d) a splice acceptor sequence.

3. DNA vector according to claim 1 or 2, characterized in that the vector further comprises arranged between two lentivirus-derived LTR sequences
   e) a Rev-responsive element.

4. DNA vector according to one of the preceding claims, characterized in that at least one of the LTRs carries a deletion making it a self-inactivating LTR (SIN).

5. DNA vector according to claim 4, characterized in that the self-inactivating property is effected by a deletion of the U3 gene.

6. DNA vector according to one of the preceding claims, characterized in that the vector comprises
   f) a poly purine tract (cPPT) between its LTR sequences.

7. DNA vector according to one of the preceding claims, characterized in that the vector comprises
   g) the post-transcriptional regulatory element of woodchuck hepatitis virus (WPRE) between its LTR sequences.
8. DNA vector according to one of the preceding claims, characterized in that the vector comprises two different homing endonuclease recognition sequences for receiving at least one expression cassette.

9. DNA vector according to claim 8, characterized in that the recognition sequences for homing endonucleases are selected from the group comprising the recognition sequences for I-Ceu I and PI-Sce I.

10. DNA vector comprising the Sequence ID No 3 or a functional homolog or derivative thereof.

11. DNA vector according to claim 10, characterized in that the functional homolog or derivative hybridizes under stringent conditions to Sequence ID No 3 and/or is at least 30% homologous to Sequence ID No 3.

12. DNA vector according to one of the preceding claims, characterized in that the vector comprises at least one expression cassette integrated into the at least one homing endonuclease recognition site.

13. DNA vector according to one of the preceding claims, characterized in that the expression cassette comprises at least one sequence selected from the group consisting of structural genes, regulatory sequences suitable for expressing a structural gene, accessory sequences suitable for influencing a regulatory sequence, and sequences coding for anti-sense RNA, ribozymes, and siRNAs.

14. Viral particle useful for transfecting animal cells \textit{in vivo} or \textit{in vitro}, characterized in that the viral particle comprises a DNA vector according to one of the preceding claims.

15. Method for producing viral particles useful for infecting animal cells \textit{in vivo} or \textit{in vitro} by transfecting permissive animal cells, characterized by the use of a DNA vector according to one of claims 1 to 13.
16. Pharmaceutical composition, characterized by a content of a DNA vector according to one of claims 1 to 3 or a viral particle according to claim 14.

17. Method for introducing a transgene into animal cells in vivo or in vitro, characterized by the use of a DNA vector according to one of claims 1 to 13 or a viral particle according to claim 14.

18. Cloning system for producing a DNA vector useful for transfecting animal cells, characterized by
   I. a first bacterial vector comprising at least one cloning site, framed by recognition sequences for homing endonucleases, and
   II. a second lentivirus based bacterial shuttle vector according to claims 1 to 13.

19. Cloning system according to claim 18, characterized in that the at least one cloning site of the first bacterial vector is replaced by a multiple cloning site.

20. Method for producing viral particles useful for infecting animal cells in vivo or in vitro by transfecting permissive animal cells, characterized by the use of a cloning system according to one of claims 18 to 19.

21. Method for producing a DNA vector useful for transfecting animal cells, characterized by the use of a cloning system according to one of claims 18 to 19.

22. Animal cells or animals, producible by infection with a viral particle according to claim 14.

23. Animal cells according to claim 22, characterized in that the animal cells are stem cells, progenitor cells or differentiated cells.
Figure 4

Counts

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<110> Medizinische Hochschule Hannover
       Martin, Ulrich

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example for inventive vector plentiShuttle without expression cas sette

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Artificial Sequence

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Reverse Primer

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/867

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X Further documents are listed in the continuation of box C.

X Patent family members are listed in annex.

* Special categories of cited documents:
  
  "A" document defining the general state of the art which is not considered to be of particular relevance
  
  "E" earlier document but published on or after the international filing date
  
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  
  "C" document referring to an oral disclosure, use, exhibition or other means
  
  "P" document published prior to the international filing date but later than the priority date claimed

"X*" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"*" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"*Y*" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"*X*" document member of the same patent family

Date of the actual completion of the international search

28 January 2005

Date of mailing of the international search report

16/02/2005

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patenttsaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016

Authorized officer

Sommer, B

Form PCT/ISA/210 (second sheet) (January 2004)
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<td>WO 00/30687 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY; THE UN) 2 June 2000 (2000-06-02) claims; figures _____</td>
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**INTERNATIONAL SEARCH REPORT**

**Box II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. X Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
   
   Although claim 17 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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