Suspension packaging cell lines for retroviral vectors

The invention concerns suspension packaging cell lines for retroviral vectors and their use for producing infectious replication-incompetent retroviral vectors.

Viruses are composed of a genome coding for the viral genes and a virus capsid which, as in the case of the retroviruses, can be surrounded by a membrane envelope.

With retroviruses one distinguishes between the cis elements 5'-LTR, 3'-LTR, packaging sequence Psi, + and – strand primer binding site and the trans elements gag (capsid proteins), pol (reverse transcriptase) and env (envelope proteins). The trans elements can be replaced by foreign genes on the viral genome; however, in this case the virus is dependent on so-called helper viruses which can either be complete infectious viruses or non-infectious viruses e.g. viruses without packaging sequences. Cells which contain such non-infectious helper viruses and/or helper genes of one or various helper viruses that are present transiently, episomally or integrated into the genome are referred to as packaging cell lines. Hence, packaging cell lines do not release infectious, replication-competent virus particles. If the helper genes are derived from different helper viruses then the vectors that are formed are referred to as chimeric retroviral vectors; this process is referred to as pseudotyping. Such chimeric packaging systems are usually used e.g. GP+env Am12 (Markowitz, D.G. et al. in Trans. Assoc. Am. Physicians 101 (1988) 212 – 218) carries the gag-pol genes of the ecotropic MoMuLV and the env gene of the amphotropic 4070 Ampho virus. It is even possible that a mixture of env genes from several viruses (even with genetically modified env genes) are present in the packaging cell lines.

Packaging cell lines can also contain the incomplete viral helper genome in a fragmented form in which case they are referred to as packaging cell lines with a split up virus genome and these are a variant with greater safety against undesired release of infectious, replication-competent viruses by recombination events.

However, if they are transfected or transduced with a retroviral vector which, in addition to the cis elements, can also carry foreign genes, then these virus genomes are packaged and released as infectious but not replication-competent retroviral vectors. This is referred to as a retrovirus producer cell line.

All previously produced retroviral packaging cell lines are based on adherent cells i.e. on cells which grow adhering to surfaces:

The packaging cell lines GP+E86 and GP+envAm12 based on adherent NIH3T3 cells are described by Markowitz, D.G. et al. in Trans. Assoc. Am. Physicians 101 (1988) 212 – 218. In Mol. and Cell Biol. 6 (1986) 2895 – 2902 Miller, A.D. et al. describe the packaging cell line PA317 based on adherent NIH3T3 cells. Miller, A.D. et al. describe in J. Virol. 65 (1991) 2220 – 2224 the GALV packaging cell line PG13 based on NIH3T3 cells. Danos, O. et al describe in PNAS USA 85 (1988) 6460 – 6464 the packaging cell lines ψCre and ψCrip based on NIH3T3 cells.

Rigg, R.J. et al. describe in Virology 218 (1996) 290 – 295 the packaging cell line Propack-A which is based on a human cell line. Cosset, F.-L. et al. describe in J. Virol. 69 (1995) 7430 – 7436 the packaging cell lines FLY A13 and FLY RD18 which are based on a human fibrosarcoma line (HT10 80). Packaging cell lines based on D17 and other adherent dog cell lines are described in WO92/05266.

The majority of the generally described cell lines also grow adherently. An advantage of adherent cell lines is that they are easy to handle. Thus adherent cells are relatively easy to clone and viruses, especially retroviruses can be used i.e. a direct clonal selection of the cells after transfection or transduction is possible, individual colonies can be easily recognized, counted and isolated by picking, dead cells simply become detached and can therefore not be mistaken for living cells, in order to change the medium it is only necessary to simply remove the supernatant by pipette and add new medium. Seeding and keeping the cells is usually not critical since adherent cells form colonies of cells which stimulate each other in the tissue unit. When transferring the cells an additional trypsin treatment is necessary for adherent cells in order to detach them which is usually not critical.

Cells growing in suspension require a different spectrum of methods for cloning and for transduction experiments. Hence, clonal selection requires the use of immobilizing additives such as soft agars, a dilution series of the cells referred to as limited dilution until theoretically only 1 cell per vessel is present or the use of a surface marker or a fluorescent marker (such as GFP) which enables the direct selection of recombinant cells by means of an FAC sorter. Living and dying cells can only be differentiated by staining. A change of medium always requires a centrifugation step.

Cell lines growing in suspension as retroviral packaging cell lines have not been previously known. Also no experiments have been described up to now in which established packaging cell lines growing adherently have been brought into suspension.

Surprisingly it has turned out that it is indeed possible to adapt adherent packaging cell lines to growth in suspension and that the resulting suspension packaging cell lines are able to produce viruses with high titres. Moreover, it was demonstrated that it is also possible to produce cell lines growing in suspension that produce retroviruses from classical suspension cell lines such as the human erythroleukemia cell line K562, the rat hepatoma line N1-S1 and the rat lymphoma line C58 by cotransfection of retroviral packaging gene plasmids with retroviral vector plasmids i.e. these cell lines release retroviral vector particles which are able to transduce other cells.

The technical procedure for producing suspension packaging cell lines is similar to methods described many times for adherent cells by introducing helper sequences (in one piece or preferably split up) which alone are not able to form an infectious virus except for the already mentioned difference that suspension cells require other cloning and culture techniques.

Once a suspension packaging cell line has been produced, it can be converted into a suspension-producer cell line which releases infectious but replication-incompetent retroviral vectors by transduction with a compatible retroviral vector (e.g. an amphotropic packaging cell line containing a vector derived from an ecotropic packaging cell line), and preferably by transfection with a retroviral vector plasmid.

Mammalian cell lines growing completely in suspension as retroviral packaging cell lines have not been known previously. Also no method has previously been known in which established packaging cell lines growing adherently can be brought completely into suspension.

The object of the invention is to provide packaging cell lines for retroviral vectors growing in suspension and methods for their production and use.

The invention concerns a packaging cell line for retroviruses which grows completely in suspension which is preferably transduced with a retroviral vector.

A further subject matter of the invention is a method for producing retroviral vectors characterized in that a packaging cell line growing in suspension which is transduced with the said vector, is cultured in suspension, the cells are separated from the cell supernatant and the vector is isolated from the supernatant.

A packaging cell line is understood as a mammalian cell line which contains the genes gag, pol and env (helper genes) necessary for the production of viral particles but not functionally active packaging sequences (ψ). Such a cell line cannot alone form a virus particle containing genomic RNA.

A functionally active packaging sequence is understood as the region of a retroviral genome which functionally causes the packaging of the RNA genome. Its function can be deactivated by complete or partial deletion or by mutation.

According to the invention a retroviral packaging cell line growing completely in suspension (no insoluble matrix (vessel wall or microcarrier to which the cells adhere) is required for growth even for long periods e.g. days) is preferably produced from a retroviral packaging cell line growing adherently by the stepwise adaptation of such an adherent packaging cell line to growth in suspension over a period of at least three months. The steps comprise a continuous reduction of the FCS content in the medium until it is free from serum, repeated treatment of the cells with trypsin and/or DNAse, the use of medium for suspension cell lines (for example DMF024A), preferably tissue culture flasks for suspension cells and hybridoma cells (Sarstedt), continuous shaking of the culture flasks on a shaker and a cell density between 5×10^4 and 1×10^6 cells/ml (hence it is necessary to split the cells much more frequently than in a comparable adherent culture).

The cell line according to the invention which grows completely in suspension and is transfected with a retroviral vector plasmid or transduced with a retroviral vector is preferably produced by transfecting a suspension cell line with a retroviral vector plasmid or transducing it with a retroviral vector, selecting the transfected or transduced cells by means of a co-transfected or co-transduced surface marker by limited dilution and/or the addition of immobilizing additives and isolating the suspension cells according to the invention selected in this manner. The suspension cell line is preferably a retroviral packaging cell line. In order to produce a retroviral packaging cell line which grows in suspension, a retroviral packaging cell line which grows adherently and is transfected with nucleic acids which code for helper sequences and with a retroviral vector plasmid or is transduced with a retroviral vector and is cultured in a serum-containing medium is preferably converted into a cell line growing in suspension by continuous reduction of the serum content in the medium until it is essentially free of serum, repeated treatment of the cells with trypsin and DNAse to detach the cells from the carrier during which the cell density in suspension is maintained in a range between 5×10^4 to 1×10^6 cells/ml. This process is particularly preferably carried out over a long period preferably for at least three

months. In a further preferred embodiment the transfected or transduced cells are selected by means of a co-transfected or co-transduced surface marker by limited dilution and/or the addition of immobilizing additives.

It has surprisingly turned out that packaging cell lines growing completely in suspension that are treated in this manner are still able to produce retroviruses despite the massive long-term manipulation over several months after they have been transfected with retroviral vector plasmids or transduced with retroviral vectors.

It is also surprising that such packaging cell lines growing completely in suspension can be cloned by transfection with retroviral vector plasmids or transduction with retroviral vectors by isolation steps such as limited dilution in which the cells are diluted until practically only single cells per vessel are present in suspension or by the use of immobilizing additives such as an overlay agar or methylcelluloses (Metho Cult H4100 / Stem Cell Technologies) or cell sorting by means of a cell surface marker, and retroviral producer cell lines which grow completely in suspension are formed from this which are able to produce retroviral vectors to the same extent as adherent retroviral producer cell lines.

Retroviral producer cell lines produced in this manner allow a much simpler large-scale culture compared to adherent retroviral producer cell lines in stirred fermenters or high density dialysis culture methods.

In addition the methods for isolating the vector viruses also differ. Whereas in the case of adherent retroviral producer cell lines the culture supernatants containing retroviruses only have to be removed and a sterile filtration step, although not absolutely necessary, is nevertheless always carried out for safety reasons, in the case of retroviral producer cell lines growing completely in suspension the much smaller, lighter retroviral vector particles are separated from the larger heavier producer cell lines by a filtration and centrifugation step or at least two filtration steps.

Retroviral vectors which contain a therapeutic gene are used as retroviral vector plasmids or retroviral vectors. These vectors contain no packaging sequences like gag, pol, env in order to prevent release of replication-competent retroviral vectors during production in the suspension producer cell line. The therapeutically relevant gene is usually inserted between 5'-LTR and 3'-LTR and it is expedient that selection genes such as the neomycin or hygromycin resistance gene are present.

A replication-incompetent (replication-deficient) vector is understood as a vector which contains no retroviral gene functions (gag, env, pol) and hence cannot independently form virus particles. However, the vector usually contains a packaging function (psi). In order to form infectious retroviral particles a packaging cell line which contains the gene functions gag, env and pol in a stable form (as an episome or integrated into the genome) is transduced with a replication-deficient DNA vector. RNA transcripts are formed which are packaged into the virus particles as a result of the gag and env functions. These virus particles are replication-deficient because the RNA genome that they contain carries no retroviral gene functions.

Abbreviations

MoMuLV	moloney murine leukaemia virus
FCS	foetal calf serum
Δ LNGFR	low affinity nerve growth factor receptor in which the intracellular domain is deleted (cf. WO 95/06723)
SV40-P/E	promoter/enhancer unit of SV40
neoR	neomycin resistance gene
pBR 322	E. coli base vector plasmid
T 25	cell culture flask size
CMV-P/E	promoter/enhhancer unit of CMV (cf. e.g. EP-B 0 173 177)
TK-P	herpes simplex thymidine kinase promoter
poly-A	polyadenylation site
hygR	hygromycin resistance gene
gpt	gpt selection gene
pUC19	base vector
pUC20	base vector
5'LTR MoMuLV	5'LTR from MoMuLV
cfu	colony forming units

The invention is further elucidated by the following examples and publications the scope of which is derived from the patent claims. The described methods are to be understood as examples which still describe the subject matter of the invention even after modifications.

Example 1 **Adaptation of adherent retroviral packaging cell lines to growth in suspension**

The adherent packaging cell lines FLY A13 (amphotropic env from MoMuLV) and FLY RD18 (env from cat endogenous virus RD114) (Cosset, F.-L. et al., J. Virol. 69 (1995) 7430 – 7436) which are both based on the human fibrosarcoma line HT1080, were adapted to suspension growth by the following process steps:

a) Adaptation of the adherent packaging cell lines to growth in suspension:

I. Continuous and slow FCS reduction in the medium; duration ca. 5 months; 10 % FCS ->5 % ->2.5 % ->2 % ->1 % ->0.5 % ->serum free

II. Use of a medium for suspension cell lines

III. Use of culture flasks for suspension cell lines (Sarstedt)

IV. Continuous gentle shaking of the culture flasks on a shaker

V. The maintenance requires a frequent detachment of the clumps of cells that form by trypsin and

DNAse treatment and frequent splitting so that they remain in suspension. The cell density is always a critical factor (5.10^4 – 1.10^6 cells/ml) i.e. it must not be too high or too diluted.

b) Determination of the retrovirus titre of transient cultures

The packaging cell lines growing in suspension were compared with the corresponding packaging cell lines growing adherently with regard to production properties.

For this all cell lines were transfected with the retroviral vector plasmid and the retrovirus titre of the transient cultures was determined.

Materials:

*retroviral vector pLΔNSN (6853 bp)
[-5'LTR MoMuLV – ψ – ΔLNGFR – SV40-P/E – neoR – 3'LTR MoMuLV – pBR322 – plasmid part-]

*transfection reagent: DOSPER; in each case 20 µg DOSPER/mixture
(manufacturer: Boehringer Mannheim GmbH, GER)

*plasmid DNA conc.: 5 µg DNA/mixture/plasmid

Methods:

1st day: seed cells for the transfection:
adherent cells: $6x10^5$ cells/dish in 60 mm petri dishes
suspension cells: $3x10^5$ cells/ml; in culture flasks for suspension cells (T25, Sarstedt, upright); total of 2 ml.
incubation overnight at 37°C, 5 % CO_2, suspension cells are shaken gently.

2nd day: change of medium (by centrifugation and resuspension in the case of suspension cells), DNA/DOSPER mixture is prepared and added dropwise onto the cells (100 µl DNA/DOSPER mixture in each case).
incubation for 5-6 h, 37°C, 5 % CO_2. subsequently addition of medium.

3rd day: the supernatant is removed 24 h after the transfection, centrifuged (only in the case of suspension cell lines!), filtered (0.45 µm filter) and titrated on NIH3T3 cells (G418 selection).
titration result ca. 10 – 14 days later

Titre:

adherent cell lines	FLY A13:	$8x10^5$ cfu/ml
		$2x10^5$ cfu/ml
	FLY RD18:	previously not determined
cell lines brought into suspension:	FLY A13:	$2.4x10^3$ cfu/ml
	FLY RD18:	$1x10^2$ cfu/ml

Result:

The packaging cell lines brought into suspension are in principle able to produce recombinant retroviruses. Differences in the titre are due to the different experimental conditions in the transfection and in the titre determination e.g. different transfection efficiencies in suspension cells and adherent cells and differences in the stability and infectiousness of retroviruses in FCS-free and FCS-containing medium.

Example 2: **Retrovirus production after transient triple transfection of the two helper plasmids and the retroviral vector plasmid into suspension cell lines**

An adherent cell line and several cell lines growing in suspension were compared in transient triple transfection preparations with regard to their ability to form infectious retroviral vectors.

Cell lines used:

adherent cell line (control)	NIH3T3 (mouse fibrosarcoma)
suspension cell lines:	K562 (human erythroleukaemia cell line)
	N1-S1 (rat hepatoma)
	C58 (rat lymphoma)

Material:

a) Helper plasmids:

*gag-pol expression plasmid pGAPOGPT (10202 bp)
[-CMV-P/E-gag-pol-polyA-SV40-P/E-gpt-polyA-pUC19-plasmid part-]

*ENV expression plasmid pENVCHT (7933 bp)
[-TK-P-hygR-polyA-CMV-P/E-env (amphotropic)-IpolyA-pUC20-plasmid part-]

b) retroviral vector plasmid:

*retroviral vector pLΔNSN (6853 bp)
[-5'LTR MoMuLV - ψ - ΔLNGFR – SV40-P/E – neoR – 3'-LTR MoMuLV – pBR322-plasmid part-]

*transfection reagent: DOSPER (BM); 20 µg DOSPER/mixture in each case

*plasmid DNA conc.: 5 µg DNA/mixture/plasmid

Methods:

1st day: seed cells for the transfection:

adherent cells (NIH3T3): $6x10^5$ cells/dish in 60 mm petri dishes

suspension cells: $3x10^5$ cells/ml; in T25 culture flasks (for suspension cells; upright); total of 2 ml,

incubation overnight at 37°C, 5 % CO_2, suspension cells are shaken gently.

2nd day: change of medium (by centrifugation and resuspension in the case of suspension cells), DNA/DOSPER mixture is prepared and added dropwise onto the cells (100 µl DNA/DOSPER mix in each case).

incubation for 5-6 h, 37°C, 5 % CO_2. subsequently addition of medium.

3rd day: the supernatant is removed 24 h after the triple transfection, centrifuged (only in the case of suspension cell lines!), filtered (0.45 µm filter) and titrated on NIH3T3 cells (G418 selection).

titration result ca. 10 – 14 days later

Titration result on NIH3T3 (number of G418 resistant colonies):

NIH3T3 (adherent control cells): 3 colonies per 1.4 ml virus supernatant (titre: 2 cfu/ml)

K562: 6 colonies per 0.8 ml virus supernatant (titre: 7.5 cfu/ml)

N1-S1: 6 colonies per 2 ml virus supernatant (titre: 3 cfu/ml)

C58: 1 colony per 2 ml virus supernatant (titre: 0.5 cfu/ml)

List of References

Cosset, F.-L. et al., J. Virol. 69 (1995) 7430-7436

Danos, O. et al., PNAS USA 85 (1988) 6460-6464

Markowitz, D.G. et al., Trans. Assoc. Am. Physicians 101 (1988) 212-218

Miller, A.D. et al., J. Virol. 65 (1991) 2220-2224

Miller, A.D. et al., Mol. and Cell Biol. 6 (1986) 2895-2902

Rigg, R.J. et al., Virology 218 (1996) 290-295

WO 92/05266

Patent Claims

1. Packaging cell line growing completely in suspension.

2. Packaging cell line growing completely in suspension which is transfected with a retroviral vector plasmid or transduced with a retroviral vector.

3. Process for the production of a packaging cell line growing completely in suspension which is transfected with a retroviral vector plasmid or transduced with a retroviral vector, wherein

   a suspension cell line is transfected with a retroviral vector plasmid or transduced with a retroviral vector,

   the transfected or transduced cells are selected by means of a co-transfected or co-transduced surface marker, by limited dilution and/or the addition of immobilizing additives,

   the suspension cells selected in this manner are isolated.

4. Process for the production of a packaging cell line growing in suspension, wherein

   a packaging cell line growing adherently which is transfected with nucleic acids that code for helper sequences and with a retroviral vector plasmid or is transduced with a retroviral vector and is cultured in a serum-containing medium is converted into a cell line growing in suspension by

   continuous reduction of the serum content in the medium until it is free of serum, repeated treatment of the cells with trypsin and DNAse to detach the cells from the support during which the cell density in the suspension is maintained in a range between 5×10^4 and 1×10^6 cells/ml.

5. Process as claimed in claim 4, wherein the process is carried out over a period of at least three months.

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6. Process as claimed in claim 4 or 5, wherein the transfected or transduced cells are selected by means of a co-transfected or co-transduced surface marker by limited dilution and/or the addition of immobilizing additives.

