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XENOTROPIC AND AMPHOTROPIC PSEUDOTYPED RECOMBINANT RETROVIRUS TO TRANSFER GENES INTO CELLS FROM VARIOUS SPECIES

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The ability to transfer genes into cells from different species with murine recombinant retroviruses was evaluated with the SVnls LacZ reporter gene. Mouse and cat packaging cell lines can be used to transfer amphotropic pseudotype, in human, mouse, cat, rabbit, sheep, horse and beef cells and with a very low efficiency in pig and avian cells. Xenotropic pseudotype recombinant retroviruses, produced in cat and rabbit packaging cell lines, transferred genes with the same efficiency as amphotropic retroviruses in human, cat, rabbit and sheep cells. In contrast to amphotropic retroviruses, xenotropic retroviruses infect beef, pig and horse cells with a high efficiency. These results emphasize the need to determine carefully the producer cell line (the type of helper virus and the species origin of the cell) for efficient transfer of genes in cells and embryos.

Gene transfer has proven to be of major interest for the production of transgenic mice (1) as of other mammals, particularly in projects for gene farming (2-5). However, the microinjection technique is very inefficient for producing transgenics in domestic animals (6-7). Therefore, techniques other than the microinjection of DNA into eggs are required for the production of a large number of transgenic farm animals.

Infection with recombinant retrovirus (RRV) offers advantages for introducing genes into cells and embryos. In particular, they allow the stable integration of a unique copy of the transgene in the genome, with no rearrangement of the DNA flanking the integration site. RRV can be packaged with viral proteins produced by a transcomplementing provirus (8-11). Murine RRV have a specific cell host range according to the pseudotype of envelope genes expressed by the packaging cell lines. Cells originating from non murine species can be infected with amphotropic and/or xenotropic pseudotype of M-MuLV (9-14).

In order to compare the efficiency of the different pseudotypes of RRV for gene transfer in animal cells, ecotropic, amphotropic and xenotropic pseudotypes of MMuLV SVnls LacZ (15) RRV have been produced. Our results show that the highest amphotropic RRV production is

Abbreviations used : M-Mu LV, Moloney murine leukemia virus; RRV, recombinant retrovirus; X-gal, 4-chloro-5-bromo-3-indolyl-β-D-galactoside; bcfu, blue colony forming unit.

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obtained in a cat cell line, and that at least one xenotropic pseudotype (X_{BMuX} (16)) is more efficient than the amphotropic ones on several animals and in particular on pig cells, a potentially interesting animal for models of human gene therapy (17). More generally, our results point out the necessity to adapt carefully the retrovirus producer cell line to the species in which retroviral infection of embryos (18), stem cells (19) or somatic cells in culture is desired.

MATERIALS & METHODS

Cell lines and culture

Primary cells were obtained by trypsinization of organ samples except for rabbit and sheep mammary cells which were isolated after collagenase digestion (20). Cell lines were from tisssue culture collections and are referenced in the legend of Table 2. Ecotropic RRV were produced in transcomplementing Ψ2 cells (8) and amphotropic RRV in Ψcr (11) and PA 12 cells (9-10). G355-5X_B, G355-5X_S, RL307X_B and RL307X_S cells produced the two xenotropic X_{BMuX} (X_B) and X_{SMuX} (X_S) viruses which were previously isolated from BALB/c and Swiss mice strains respectively (16).

Cells were cultured in Dulbecco's minimum essential medium (DMEM), high glucose (4.5 g/l), high sodium bicarbonate (4.4 g/l), supplemented with antibiotics (penicillin 1 x 10^5 I.U./l, streptomycin 0.1 g/l, gentamycin 10 mg/l), nystatin (20 mg/l) and foetal calf serum (FCS, 10%). Cells were maintained at 37°c in carbon dioxide (10%) in air. MMuLV viruses were handled inside biohazard laminar flow hoods.

Establishment of RRV producer cells

PA 12 and Ψcr mouse packaging cells were infected with the supernatant of Ψ2-21c cells (15) which produce an ecotropic SVnlsLacZ defective RRV, expressing the LacZ gene under the control of both SV40 and LTR promoter. The cells were cloned by limiting dilution and tested for LacZ expression and viral production . After cloning and selection of amphotropic RRV producers, the clones PA 12Ψ2.21c A4 (out of 37 tested clones) and ΨcrΨ2.21c F2 (out of 24 tested clones) were retained for further experiments. Both cell lines were found to be free of wild type virus when 2ml of viral stock were tested on a LacZ murine indicator cell line (D. Rocancourt and J.F. Nicolas, unpublished results).

Another amphotropic producer was established with the cat cell line G355-5. The cells were first infected by PA 12Ψ2.21c A4, and selected for LacZ expression. A $LacZ^+$ clone was then superinfected with a wild-type amphotropic virus produced by 1504 cells (13). The resulting cells, designated G355-5A LacZ, produce a mixture of wild and LacZ amphotropic viruses.

Xenotropic producers (G355-5X_B LacZ, G355-5X_S LacZ, RL307X_B LacZ, and RL307X_S LacZ) were obtained with the same protocole as G355-5A LacZ: G355-5 and RL307 cells selected for LacZ expression after PA 12Ψ2.21c A4 infection were superinfected with the wild-type xenotropic X_{BMuX} or X_{SMuX} retroviruses.

Production of retroviruses, infection and X-gal staining

When RRV producer cells were 75 % confluent, the medium was changed, and the following day, the supernatant containing virus was collected, filtered through a Millipore filter (0.22 µm), and used immediately for infection or kept frozen at -80°C. The day before infection, recipient cells were seeded at 10^4 cells/cm^2. At the time of infection, the medium was changed and an aliquot of RRV stock solution was added in medium supplemented with polybrene (5 µg/ml).

The β-galactosidase activity was tested 2 days after infection, by X-gal staining, as previously described (21). Titers were calculated from the linear portion of the titration curve, except for Ψcr and G355-5A LacZ producer cells, in which titration was obtained after infections with 1 ml of supernatant. Titers were the mean of at least 3 separate assays.

RESULTS

The defective MMuLV SVnlsLacZ RRV produced in the helper-free amphotropic PA 12 (8-10) and Ψcr (11) transcomplementing cell lines, were titrated on cells from different species

(Table 1). Their titers on human, mouse, rabbit, and cat cells ranged from 10^3 to 10^4 bcfu/ml. On horse cells the titer are around 10^2 bcfu/ml. For ovine, bovine porcine and avian cells the titers were about 10^1 bcfu/ml.

When the same LacZ RRV was produced in a non defective amphotropic helper cell line, in the G355-5A LacZ feline line, the titers were at least 10 fold higher as compared to the amphotropic defective producer. This applies to all cells tested whatever the species (except swine): the titer was 4.10^4 and 6.10^4 bcfu/ml for murine and feline cells, 4.10^3 and 1.10^3 bcfu/ml for ovine and bovine cells and 2.10^1 bcfu/ml for porcine cells (Table 1). The most

Table 1. Titers of M-MuLV LacZ ecotropic RRV produced by murine Ψ2.21c and amphotropic RRV produced by murine (PA 12 Ψ2.21c A4 and Ψcr-Ψ2.21c F2) or feline (G355-5A LacZ) packaging cells

Species			Ecotropic	Amphotropic		
Cell line	Origin	Cell-type	Ψ2.21c	PA12	Ψcr	G355-5A
Human						
MRC 5	Embryonic lung	F	< 1	1.10^3	n.d.	n.d.
HRT 18	Fibrosarcoma	F	< 1	5.10^2	n.d.	n.d.
Hep 2	Larynx carcinoma	E	< 1	1.10^3	n.d.	n.d.
Mouse						
3T3	Fœtus fibroblasts NIH	F	2.10^4	5.10^3	6.10^3	4.10^4
STO	Fœtus fibroblasts	F	5.10^3	3.10^3	n.d.	n.d.
Rabbit						
RL 307	Kidney	E	< 1	1.10^4	1.10^3	n.d.
PL	Lung (PC)	F	< 1	1.10^3	n.d.	n.d.
L 5174	Mammary gland (PC)	E	< 1	2.10^3	n.d.	n.d.
L 4278	Mammary gland (PC)	E	< 1	3.10^3	n.d.	n.d.
Cat						
G355-5	Lung	F	< 1	4.10^3	2.10^3	6.10^4
Sheep						
RFM	Fœtal spleen (SV)	F	< 1	3.10^1	2.10^1	4.10^3
PFM	Fœtal lung (PC)	F	< 1	1.10^0	n.d.	n.d.
B 4307	Mammary gland (PC)	E	< 1	1.10^2	n.d.	n.d.
Beef						
PV	Fœtal lung (PC)	F	< 1	1.10^1	1.10^1	1.10^3
MDBK	Kidney	E	< 1	< 1	n.d.	n.d.
Horse						
NBL 6	Skin	F	< 1	1.10^2	n.d.	n.d.
Pig						
PK 15	Kidney	E	< 1	2.10^1	1.10^0	2.10^1
ST	Fœtal testis	E	< 1	< 1	< 1	n.d.
RPTG 80	Fœtal kidney	E	< 1	< 1	n.d.	n.d.
Chicken						
CF	Fœtus trypsinized	F	< 1	5.10^1	< 1	n.d.
Quail						
QF	Fœtus trypsinized	F	< 1	2.10^1	n.d.	n.d.

PC : Primary cells , SV : SV40 transformed cells, F : Fibroblast, E : Epithelial,
< 1 : no bcfu / 1 ml supernatant, n.d. : Not determined.

noticeable difference between the viral production of G355-5A LacZ and PA 12 or Ψcr LacZ clones lies in the 100 fold increased potential of the G355-5A LacZ virus to infect bovine and ovine cells. These results revealed three classes of species for amphotropic infection : human, mouse, rabbit and cat cells (highly infectable); sheep, beef and horse cells (moderately infectable); and finally pig and avian cells which are very poorly infectable.

At the present time no helper free xenotropic transcomplementing line exists, so the study of xenotropic host range and infectivity was performed with SVnlsLacZ RRV produced in presence of wild type virus. The results obtained with the xenotropic producer lines, G355-5X_B LacZ (bearing a BALB/c xenotropic virus with B tropism), G355-5-X_S LacZ (bearing a Swiss xenotropic virus with NB tropism) (Table 2) were compared to those obtained in the same

Table 2. Titers of M-MuLV LacZ xenotropic RRV produced by cat (G355-5X_B LacZ and G355-5X_S LacZ) and by rabbit (RL 307X_B LacZ and RL 307X_S LacZ) packaging cells

Species			RRV			
Cell line	Origin	Cell type	G355-5 LacZ X_B	G355-5 LacZ X_S	RL 307 LacZ X_B	RL 307 LacZ X_S
Human						
Hep 2	Embryonic lung	E	3.10^4	2.10^3	5.10^2	n.d.
Mouse						
3T3	Fœtus fibroblasts NIH	F	< 1	< 1	< 1	< 1
Rabbit						
RL 307	Kidney	E	5.10^4	3.10^4	4.10^3	6.10^2
PL	Lung (PC)	F	1.10^3	n.d.	1.10^1	n.d.
Cat						
G355-5	Lung	F	1.10^5	6.10^3	2.10^4	2.10^3
Sheep						
RFM	Fœtal spleen (SV)	F	4.10^3	n.d.	5.10^2	n.d.
PFM	Fœtal lung (PC)	F	5.10^4	n.d.	4.10^3	n.d.
Goat						
PFC	Fœtal lung (PC)	F	7.10^3	n.d.	7.10^2	n.d.
Beef						
PV	Fœtal lung (PC)	F	4.10^4	1.10^4	3.10^2	3.10^1
Horse						
NBL 6	Skin	F	4.10^4	3.10^3	3.10^3	n.d.
Pig						
ST	Fœtal testis	E	1.10^4	n.d.	1.10^3	n.d.
RPTG 80	Fœtal kidney	E	1.10^2	5.10^1	1.10^2	3.10^1

PC : Primary cells , SV : SV40 transformed cells, F : Fibroblast, E : Epithelial,
< 1 : no bcfu / 1 ml supernatant, n.d. : Not determined.

context with G355-5A LacZ (Table 1) which also produce wild type virus. The titers obtained with xenotropic SVnlsLacZ RRV are slightly higher than those obtained with the amphotropic RRV on human, rabbit and ovine cells. The main difference is that cat, bovine and porcine cells are respectively 25, 50 and 200 fold more infectable by the xenotropic RRV. Human and horse cells are respectively 30 and 200 fold more infectable by the xenotropic G355-5X_B LacZ RRV than by the amphotropic PA 12 LacZ. Titers obtained from feline cells (xenotropic G355-5X_B and X_S LacZ RRV) are 10 to 100 fold higher than those obtained from rabbit cells (xenotropic RL307X_B and X_S LacZ RRV) on all animal cells tested. Finally in both species, xenotropic RRV pseudotyped by BALB/c xenotropic virus have higher titers than RRV pseudotyped by Swiss xenotropic virus (Table 2).

Host range results obtained with the amphotropic and xenotropic RRV also show that in spite of some differences in the level of infectability (in pig and sheep, Table 1), the M-MuLV derived vectors are species, but not tissue, specific. For example, amphotropic LacZ RRV infects kidney epithelial (RL307), mammary epithelial (L 5174, L 4278) and lung fibroblasts (PL) rabbit cells (Table 1) and xenotropic LacZ RRV infect testis (ST) and kidney (RPTG80) epithelial pig cells (Table 2). The differences in titers are 10 to 100 fold.

DISCUSSION

Transgenic mice have been obtained either by microinjection of DNA into fertilized eggs (reviewed in 3) or by retroviral infection (18, 22-24). Any attempts to introduce genes by retroviral infection of embryos of other species should take into account that it is dependent on the infectibility of their stem cells. This infectibility is largely dependent on the expression of the receptor of the murine retrovirus and on the properties of the viral stocks as determined by the parameters of the titration curves. In vitro models must therefore be used initially to select an appropriate RRV producer cell.

Our results clearly demonstrate that there is a wide variation in the efficiency of infection depending on both the nature of the helper virus and the origin of the producer cell lines. Two classes of helper virus have been used : two amphotropic viruses 1504A (13) and 4070A (11) and two xenotropic viruses X_{BMuX} and X_{SMuX} (16). The amphotropic virus was used either as a defective helper virus in PA12 and Ψcr cells (where 4070A amphotropic enveloppe has been recombined with a MMuLV NB tropic *gag-pol* gene), or as a helper virus (strain 1504A) in a cat cell line, G355-5. The amphotropic viruses produced in helper free lines were efficient for infection of human, mouse, rabbit and feline cells (relative efficiency of 0.2 to 1 compared to mouse cells), inefficient for ovine, bovine, equine, porcine and avian cells (relative efficiency of 0.01 to 0.001) (Table1). The amphotropic virus produced with wild type virus in feline cells was also very efficient on mouse, feline and rabbit (relative efficiency of 1). It was more efficient than the helper free stocks on ovine, bovine (relative efficiency of 0.1) and porcine cells (relative efficiency of 0.01). This difference is probably due to the properties of stocks of RRV as suggested by their higher titers (10 fold) on mouse and feline cells compared to those of helper free stocks. Further experiments will show whether the difference in "quality" is due to differences in the enveloppe gene or other transcomplementation functions between the viral

strains 4070A and 1504A or if it is due to the species origin of the producer line (cat rather than mouse). Therefore, our results indicate that the amphotropic pseudotype is an appropriate RRV candidate for gene transfer in cells of a number of mammals including human, with the noticeable exception of pig.

To obtain efficient transfer in pig cells (10^3 to 10^4 bcfu/ml) we had to use a B-tropic xenotropic RRV produced in the cat or rabbit cell lines (Table 2). In addition, xenotropic RRV infect human and rabbit cells at least as efficiently , and cat cells more efficiently than amphotropic RRV; and every non murine cells tested were infectable by xenotropic RRV. Therefore, this suggests that the xenotropic pseudotype is potentially very effective for RRV infection of animal somatic cells. The developement of a wild-type free xenotropic packaging line would be of great interest, it could also provide an alternative strategy for gene therapy .

To evaluate more specifically this last point primary cells from rabbit, sheep, and beef were infected. As a general rule, they gave titers only slightly lower (by a factor of 3 to 20) than cell lines. As this observation is independent from the pseudotype used, a likely explanation would be that it is related to their longer doubling time. It should also be noticed that variations in titers were observed between cells from different tissues of a given species (see examples for rabbit, sheep and pig). It probably reflects differencies in the level of synthesis or accessibility of the viral receptor(s).

In addition, higher titers than those reported here could probably be obtained with RRV with different structures. In the M-MuLV SVnls LacZ RRV the reporter molecule is at the first Pst I site in the virus (15). It has been reported that increasing the size to include a portion of the *gag* gene augmented packaging efficiency of the vector RNA (25).

Our results differ in some way from those summarized in Teich's review (12) : we found that bovine, ovine and porcine cells, but also the avian chicken and quail cells are infectable by amphotropic LacZ RRV (Table 1); xenotropic LacZ RRV infect ovine and porcine cells (Table 2). The basis of these differences probably lies in the techniques used. In the studies reported by Teich, the infectability was determined by a rescue method, which requires in addition to infection, viral replication and production. A low infectability in such conditions may be undetectable; for example titers of LacZ amphotropic RRV on pig cells is only 20 bcfu/ml, and might be too low to allow detection by the rescue method. Our technique relies only on infection and proviral integration, the ensuing LacZ expression is driven by the SV40 promoter. The infectability of bovine cells of embryonic origin by amphotropic viruses was also recently established with Neo RRV (26).

Altogether our results illustrate that an efficient system for producing RRV is not necessarily based on the murine cells usually employed. In future studies, cell lines from various species must be tested to identify the optimal viral producer for the infection of a given species. For instance, the most favorable producer cell line for infection of pig cells was obtained from cat. Having the producer cells in hand already constitutes a valuable material to further characterize the infectability of embryonic stem cells established in culture as well as preimplantation embryos from a number of species .

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