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Amphotropic retroviruses with a hybrid long terminal repeat as a tool for gene therapy of cystic fibrosis

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We have made two retroviral vectors encoding the bacterial beta-galactosidase (lacZ) as a marker gene and a long terminal repeat (LTR) containing an enhancer of the polyoma F101 virus (△Mo+PyF101). One vector, L△Z, can be used as a test vector in grafting, lineage analysis and gene therapy studies. The other, L△SZ carries an additional unique cloning site in which a gene of interest can be cloned. Titration experiments showed that in human epithelial cell lines, L△Z produces a transcriptionally active integration more often than the commonly used BAG vector with the wild type LTR. Human epithelial cells in primary culture could be successfully infected. Our data suggest that gene therapy protocols requiring infection in situ, such as in the case of cystic fibrosis, will be hampered by the relatively low local titres that can be achieved at present. © 1992 Academic Press, Inc.

Retroviral vectors derived from the murine Moloney leukaemia virus are presently tested as vehicles for gene transfer in a variety of experimental systems. In particular, their possible use in gene therapy protocols has inspired recent developments (1-5)

Our study of the possibilities for gene therapy of cystic fibrosis prompted us to develop a set of amphotropic retroviral vectors that would be able to efficiently

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express a gene in human epithelial cells (6,7). We have constructed two lacZ encoding retroviral vectors, with a modified enhancer sequence in the LTR suitable for expression in different cell types. The performance in human epithelial cells of one of these vectors was tested and compared to the commonly used BAG vector in titration experiments. The possible use of amphotropic murine retroviral vectors in gene therapy of cystic fibrosis is discussed.

MATERIALS AND METHODS

Titration experiments

Immortalised human nasal epithelial cells (NCF3A) were cultured as described (8). NIH/3T3 cells, HeLa cells and primary human fibroblasts were cultured on DMEM with 10% calf serum, 0.15 g/l glutamine, and antibiotics. For titration experiments 10^5 cells were seeded on 6 cm dishes in medium containing 4.10^{-3} g/l polybrene. 24 hours later cells were infected with dilutions of virus stock harvested from producer cells grown to 70% confluence. Two hours later, one volume of fresh medium without polybrene was added. The next day, the medium was changed. At day three lacZ expressing cells were counted after X-Gal staining (9).

RESULTS AND DISCUSSION

Two retroviral vector constructs encoding lacZ and a hybrid LTR.

The retroviral vector plasmids pL△Z and pL△SZ are depicted schematically in figure 1. Due to the mechanism of replication of retroviruses, the virus produced from both vectors will have the modified LTR (△Mo + PyF101) at the 5' end of the inserted gene (1,10,11). In the modified LTR, the wild type enhancer sequence has been replaced by a mutated polyoma virus enhancer. This allows expression of the virus in embryocarcinoma cells in which the wild type LTR is silent. Furthermore, the modified LTR reduces the leukogenic potential of the retrovirus in mice (10). Both plasmids induced lacZ activity in NIH/3T3 cells in transient expression experiments and produced a lacZ expressing viral titre in packaging cells, showing that the lacZ sequence and LTR sequences are correctly inserted (not shown).

The L△Z vector (fig 1) can be used in a rapid test of infection with retroviruses and for labelling cells in grafting and lineage tracing experiments (12,13). The L△SZ vector has a unique XhoI site and can be used to clone genes of interest (fig 1). The lacZ gene makes rapid viable cell sorter selection and histological staining of infected primary human cells possible (M. Wilke unpublished data, 14). This allows expression and grafting experiments with primary cells when cloning is difficult or time-consuming.

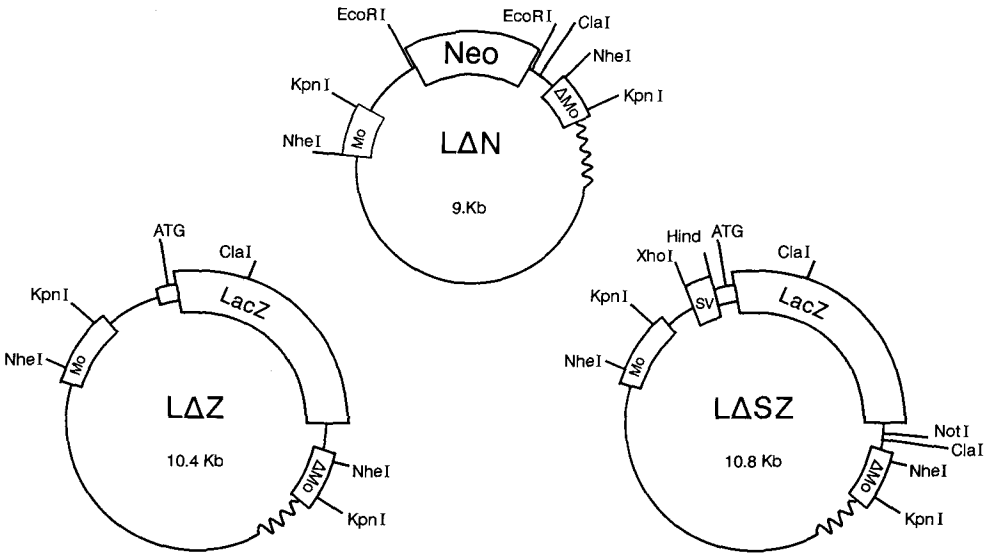

Fig.1. Schematic representation of vector constructs. pL△Z was made by ligation of the 3 kb HindIII-XbaI fragment from pSDK-Z (kindly donated by Janet Rossant, Mt Sinai Hosp., Toronto), which encodes lacZ preceded by a Shine-Dalgarno-Kozak sequence, to the 7.2 kB EcoRI fragment from pL△N after blunting the sticky ends with Klenow polymerase. pL△SZ is a vector in which lacZ is transcribed from an SV40 early promoter. It contains the △Mo + PyF101 LTR and the modified LTR-psi region from the LXSN vector kindly provided by Dusty Miller (25). Genes cloned into the unique XhoI site will be transcribed from the LTR. Complete maps and cloning strategies are available at request. Mo: wild type murine moloney LTR, △Mo: △Mo + PyF101 LTR, Neo: Neomycin phosphotransferase gene, LacZ: bacterial beta-galactosidase gene, SV: SV40 early promoter, kb: kilobases, ATG: box indicates translation start sequence.

A high titre amphotropic producer of L△Z.

Ecotropic producers of L△Z were made by co-transfection of GP + E86 packaging cells (15) with pL△Z and pSV2neo (in a ratio of 20:1) using the lipofection method (16).

To obtain an amphotropic producer of L△Z we used a co-culture ("ping-pong") method which reportedly results in extremely high titres due to repeated proviral integration in packaging cells (18,19). A pool of 77 clones producing ecotropic L△Z was mixed with the amphotropic packaging cell line GP + *env*AM12 (17) (optimal ratio 1:1, data not shown), and co-cultured in non-selective medium for six passages. Amphotropic virus producing cell lines were isolated by selection with hygromycinB (100 μg/ml). Six clones out of twelve were lacZ positive and produced a high titre of active viral particles as shown by infection and lacZ staining of NIH/3T3 cells. The commercially available BAG vector has a neomycin

Table 1. Titre of amphotropic virus with different cell types

Exp.		LΔZ		BAG		LΔZ-BAG
I	NIH/3T3	6.8	(0.58)	7.2	(0.49)	-0.4
I	HeLa	6.0	(0.16)	3.0	(0.08)	3
II	NIH/3T3	5.0	(0.13)	4.5	(0.15)	0.5
II	HeLa	3.5	(0.15)	1.5	(0.04)	2.0
II	NCF3A	4.2	(0.12)	1.9	(0.19)	2.3

NIH/3T3, HeLa and NCF3A (immortalised, CF nasal epithelial) cells were infected with 5 or 6 dilutions of amphotropic virus stocks in parallel experiments in triplicate. Titres are defined as the dilution at which one single lacZ positive cell would be expected in this assay. This value and its standard error is found by regression analysis, fitting the titration curve to the expression $Y = e^{(t-a\cdot X)}$ where Y = the number of blue cells, X = Log(dilution) a = slope and t = titre. Experiment I was performed with a low passage producer (PP2 for LΔZ and AM12-BAG for BAG), experiment II after eight more passages of both producers. The Data are expressed in the table as -log(titre) (Standard Error). The last column represents the difference between the log titre LΔZ and log titre BAG, i.e. the log of the ratio of both titres.

resistance marker transcribed from a SV40 promoter and a lacZ gene transcribed from a wild type Mo-LTR (13). An amphotropic producer of BAG was made by infection of GP+*env*AM12 cells with ecotropic BAG virus. In this case, only 4 of 74 neomycin resistant clones produced virus as measured by lacZ staining of NIH/3T3 cells, only one having a high titre (table 1, fig 2). Our results confirm therefore, that the co-culture method producers with much higher frequency than a conventional approach. However, we did not observe titres higher than 10^7 as was claimed by Bodine et al (19). A comparable result was obtained by Lynch et al (20).

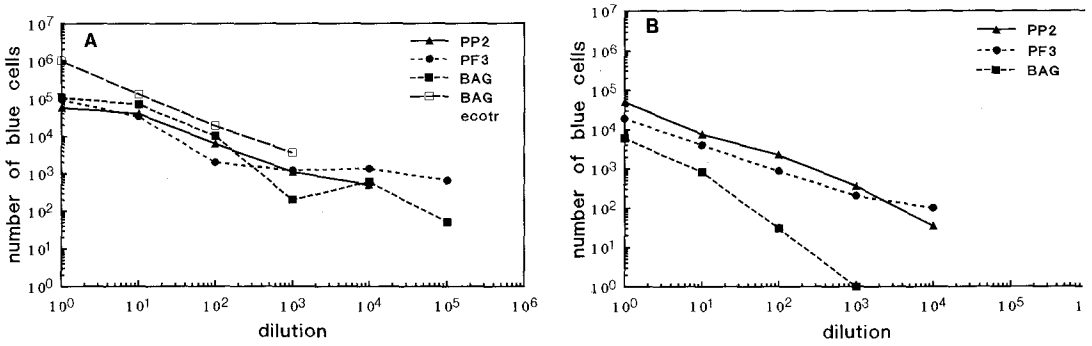

Fig. 2. Titration of virus on NIH/3T3 and HeLa cells. Titrations were performed as described in the legend of table 1. 2A: Titration on NIH/3T3 cells. 2B: Titration on HeLa cells. ecotropic BAG, amphotropic BAG (producer AM12-BAG), amphotropic LΔZ (producer PP2), amphotropic LΔZ (producer PF3). All producers tested were free of helper virus activity.

Difference in efficacy between amphotropic and ecotropic viruses

lacZ expression rates of near 100% are routinely observed with ecotropic BAG virus when NIH/3T3 cells are infected at an apparent multiplicity of more than 10 (fig 2A). However, with amphotropic viruses we did not observe this. The titration curves level off at about 10% infection. The reason for this apparent difference in efficacy between ecotropic and amphotropic viruses is not clear. Given the fact that all other factors are constant, it may reflect a limited availability in NIH/3T3 cells of the receptor for the amphotropic *env* protein compared to the receptor for the ecotropic *env* protein.

L⌂Z producers have a high titre on human epithelial cell lines.

Titration of the amphotropic L⌂Z producers PP2 and PF3 showed titres in the order of 10^6-10^7 on NIH/3T3 cells (mouse fibroblast). The titre of the BAG producer AM12-BAG was in the same range (Fig 2A, table 1). However, when HeLa cells, a human epithelial cell line, were infected in a parallel experiment, a different pattern emerged. First, all titres were lower on HeLa cells compared to NIH/3T3 cells. Second, the titre of the BAG producer was more than hundred fold lower than the titres of the L⌂Z producers PP2 and PF3 (table 1, fig 2B). After both producers were grown for eight passages under selective pressure, the titres obtained were considerably lower. This shows that it is not advisable to culture amphotropic high titre producing cell lines for many passages (table 1). As soon as a cell line is established and expanded, a large number of samples should be frozen and used for a limited period only. Also in this experiment, however, the titre of the BAG producer on HeLa cells was much lower than the L⌂Z titre, while the titres on NIH/3T3 cells were comparable (table 1). Moreover, when immortalised human nasal epithelial cells (8) were used the same result was found (table 1). Apparently, the L⌂Z virus produces more active integrations in human epithelial cells than the BAG virus. The difference in performance of BAG and L⌂Z probably reflects a relatively low average transcriptional activity of the wild type Moloney virus LTR in human cells, resulting in a lower count of lacZ expressing cells.

L⌂Z expression is stable in human cells in culture.

As the wild type LTR is often subject to progressive inactivation by methylation (21), we have tested the stability of lacZ expression by the L⌂Z vector. The percentage of lacZ positive primary human fibroblasts or immortalised epithelial

cells (NCF3A) does not change significantly during several passages after infection with the LΔZ virus (four and thirteen respectively). This indicates that transcription from the ΔMo+PyF101 LTR is stable in these cells, under these conditions.

Primary human epithelial cells in culture can be infected with LΔZ virus.

Human keratinocytes (22) and nasal epithelial cells (23) were infected at a multiplicity of 10 (calculated from the titre on NIH/3T3 cells), using the conditions described in the legend of table 1. This resulted in 0.5 % (± 0.1 , N=4) lacZ expressing cells in both cases. Infection with the amphotropic BAG virus resulted in 0.02 % positive cells. This confirms the observation that the LΔZ vector performs better in human epithelial cells than BAG. Infection frequencies increase with repeated infections and when cells are co-cultured with producers (M. Wilke, unpublished data). The relatively low infection frequencies with LΔZ of human primary epithelial cells in culture may reflect the relatively low mitotic activity of these cells under our conditions (24).

Gene therapy of cystic fibrosis with murine retroviral vectors.

Gene therapy of cystic fibrosis with retroviral vectors would require efficient infection of lung epithelial cells in situ (6). It should be noted that murine retroviral vectors can only integrate in proliferating cells (24). Therefore, the long term effect of the infection procedure would depend on the percentage of proliferating stem cells that can be infected. The LΔZ vector appears to fulfil the necessary requirements of a test vector as we could show its stable expression in human epithelial cells in culture. Our titration data show that with the state of the art technology it is impossible to obtain titres sufficient for a high multiplicity of infection during in vivo infection: one would need more than 100 litre of viral stock to infect a human lung with a multiplicity of 1). This, combined with the low mitotic activity of bronchial epithelial cells would lead us to predict a low rate of infection when viral stocks are applied to the surface of the lung. Indeed, preliminary experiments with LΔZ virus applied to mouse lungs have confirmed this assumption, no lacZ expressing cells could be detected after application of 0.1 ml LΔZ virus (ca 10^6 pfu) to mouse trachea (B. Bout unpublished data). We conclude that for efficient in vivo infection with amphotropic murine retroviruses new methods of vector delivery will have to be developed.

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