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Retrovirus-mediated gene transfer to cystic fibrosis airway epithelial cells: effect of selectable marker sequences on long-term expression

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ABSTRACT

Retrovirus-mediated gene transfer offers the potential for stable long-term expression of transduced genes in host cells subsequent to integration of vector DNA into the host genome. Using a murine amphotropic retrovirus vector containing an interleukin-2 receptor (IL-2R) gene as a reporter and a neomycin phosphotransferase (*neo*^{r}) gene as a dominant selectable marker, we measured the efficiency of retrovirus-mediated gene transfer and the stability of transduced gene expression in a cystic fibrosis tracheal epithelial cell line (CFT1). The use of the IL-2R cell surface marker as a reporter of infection permitted both quantitation of vector gene expression and flow cytometric sorting of cells transduced with the vector. In initial studies, the optimal conditions for retrovirus-mediated gene transfer were determined. The presence of a polycation was required for optimal transduction efficiency. The efficiency of infection of CFT1 cells was increased by repetitive exposure to virus such that it was possible to transduce approximately 80% of the cells following three successive daily exposures. The long-term stability of expression of the non-selected IL-2R gene was also evaluated. A slow decline in the percentage of cells expressing IL-2R was seen with cells that were maintained under constant selection pressure for expression of the *neo*^{r} gene, which was expressed from an internal promoter. Similar results were obtained when cultures were selected initially for *neo*^{r} gene expression and maintained without selection thereafter. In contrast, stable expression was observed in CFT1 cells for at least one year following multiple infections in the absence of G418 selection. In conclusion, (i) transduction of foreign genes into human airway epithelial cells using an amphotropic retrovirus vector can be highly efficient in the presence of appropriate polycations and multiple exposures; and (ii) stable expression of a non-selected gene in these epithelial cells is better maintained without selection.

INTRODUCTION

Retrovirus-mediated gene transfer provides an efficient method of introducing genes into mammalian cells. Introduction of a normal cystic fibrosis conductance regulator (CFTR) cDNA into epithelial cells derived from the airways or other affected tissues of patients with cystic fibrosis (CF) has allowed the study of the complementation of the chloride transport defect and may possibly be used in the future for gene therapy of the disease (1–6).

Recently, efficient adenovirus-mediated gene transfer to cotton rat lung epithelia *in vivo* with transient expression of the normal CFTR cDNA has been reported (5). However, retroviruses remain attractive as vectors since they offer the opportunity for a 'cure' of CF airways disease consequent to integration into the host cell genome with subsequent stable long-term expression. Previously, we have used retroviruses to deliver normal CFTR cDNA to the CF airway epithelial cell line, CFT1 (4). It was shown that phenotypic correction of the Cl^- transport defect at the apical membrane was attained in cultures of polarized epithelial sheets. At least partial correction persisted in the cultures for periods of up to 6 months without selection for maintenance of expression of the vector sequences. In other studies, we performed a series of mixing experiments varying the proportion of corrected and uncorrected CFT1 cells in epithelial sheets (7). It was found that normal Cl^- transport function could be attained when only a minor fraction of cells was corrected, suggesting that a gene therapy approach for treatment of CF may be feasible.

A potential problem with using retrovirus vectors for direct gene transfer into the lung *in vivo* is likely to be infection efficiency since a number of studies have established that successful retrovirus infection requires cells actively traversing through the cell cycle. The fraction of cells cycling in the airways at any given time is low (0.1%–1.0%). Thus, if retroviruses are to be used for gene therapy of CF, it will be important to optimize gene transfer efficiency in order to maximize the infection of proliferating cells. In the work presented here, we used an amphotropic murine retrovirus vector containing the interleukin-2 receptor (IL-2R) as a reporter gene and a dominant selectable marker (*neo*^{r}) to address specific issues pertinent to

efficient gene transfer and long-term gene expression in cystic fibrosis airway epithelial cells.

METHODS

Cells and viruses

CFT1 cells are a cystic fibrosis airway epithelial cell line derived from a cystic fibrosis patient homozygous for the ΔF508 deletion (8). A single clone (clone 2) derived from this cell line was used in all experiments. CFT1 cells were maintained on 60-mm plastic tissue culture dishes and grown in serum-free, hormone supplemented medium (8). The cells were subcultured every 7−10 days.

LISN is a Moloney murine leukemia virus (Mo-MuLV)-based retrovirus vector (4) that was derived from the LXSN vector (9). LISN contains a cDNA for the 55 kd α-subunit of the interleukin-2 receptor (IL-2R) under transcriptional control of the Mo-MuLV promoter and a *neo*^{r} gene under transcriptional control of an internal SV40 early promoter. The LISN vector was packaged in the retrovirus packaging cell line PA317 (10), which confers the amphotropic host range. The clonal cell line (LISN102) used in these experiments produces LISN with a titer of 2.0×10^6 G418-resistant colony forming units per milliliter using NIH3T3 cells as host when titered as described (9). This packaging cell line was found to be free of replication competent helper virus as determined by a marker rescue assay (11). PA317 cells were maintained in Dulbecco's modified Eagle's medium with 4.5 g of glucose per liter and supplemented with 10% fetal bovine serum.

Retrovirus infections

All retrovirus infections were performed by adding 1.0−1.5 ml virus-containing medium to cells on 60-mm plates. One day prior to infection, cells were seeded at 1.5×10^5 cells/60-mm plate. Unless otherwise noted, all infections were performed with virus in the presence of 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO). Protamine sulfate was obtained from Eli Lilly (Indianapolis, IN) and Lipofectin was obtained from Life Technologies, Inc. (Gaithersburg, MD). CFT1 cells undergoing selection for the presence of the *neo*^{r} gene were placed under G418 selection at 150 μg/ml active drug 2 days after infection.

DNA and RNA analysis

The isolation and analysis of chromosomal DNA and polyadenylated RNA from CFT1 cells was carried out as described previously (4).

Flow cytometry

Cells were detached from 60-mm plates by enzymatic digestion with 0.05% trypsin/EDTA and incubated in suspension at 4°C with a 1:1000 dilution of a mouse monoclonal anti-human IL-2R IgG antibody (a gift from Thomas Waldman, National Cancer Institute, Bethesda, MD) which recognizes a trypsin-resistant extracellular epitope. After washing with PBS, the cells were stained with a 1:400 dilution of goat anti-mouse IgG conjugated to phycoerythrin (Biomeda Corp., Foster City, CA). Flow cytometric analyses were performed with a FACS 440 cell sorter (Becton Dickinson, Mountain View, CA). Fluorescence excitation of labeled cells was provided by the 488 nm line of an Innova 90−5 argon laser (Coherent Inc., Palo Alto, CA) operating at an output of 150 mW. Fluorescence emission of phycoerythrin-labeled cells was collected through a 575/26-nm-bandpass filter. The resulting signals were processed through a logarithmic amplifier and stored on a DEC 11/73-based microcomputer (Digital Equipment Corporation, Maynard, MA). Fluorescent-labeled latex beads (Polysciences, Inc., Warrington, PA) were used to calibrate relative fluorescence intensities. Consort 40 software (Becton Dickinson, Mountain View, CA) was used for both data acquisition and subsequent data analysis. Uninfected cells stained with the fluorescent antibody provided the negative control. The percentage of cells expressing IL-2R in each sample was defined as the percentage of cells labeled with the fluorescent antibody appearing above the 99th percentile of fluorescence in the corresponding negative control. In some experiments, viable cells were sorted into IL-2R expressing and non-IL-2R expressing subpopulations which were then replated for immunofluorescence analysis and molecular studies.

Data analysis

All values are recorded as means ± SE. Determination of statistical significance was performed using the student's *t*-test. Significance was defined by $p<0.05$.

RESULTS

Use of IL-2R as a reporter gene

For assessment of gene transfer efficiency into the CFT1 airway epithelial cell line, the LISN retrovirus vector was used [(4), see Fig. 5 for a diagram]. This murine retrovirus-based vector contains two genes: (i) the α subunit of the interleukin-2 receptor (IL-2R) cDNA as a reporter gene (12) which is under transcriptional control of the promoter elements in the Moloney murine leukemia virus LTR; and (ii) the neomycin phosphotransferase gene (*neo*^{r}) which is under transcriptional control of an internal SV40 promoter. The fraction of cells expressing the IL-2R reporter gene was determined by flow cytometry of cells stained for IL-2R expression. CFT1 cells expressing IL-2R were found to have an average of nearly 100-fold greater fluorescence intensity than cells not expressing IL-2R (data not shown).

Effect of various polycations on gene transfer efficiency

Increased gene transfer efficiencies of CFT1 cells were achieved when retrovirus infections were carried out in the presence of polybrene, protamine sulfate, or the polycation liposome, Lipofectin. In the absence of polycations, the percentage of cells expressing IL-2R following a single 2 hr infection with LISN was low (4 ± 1%, Fig. 1). Gene transfer efficiency increased in a dose-dependent fashion for each polycation. Optimum polycation concentrations and gene transfer efficiencies were found to be: 2.0 μg/ml polybrene and 32 ± 5%, Fig. 1A; 15 μg/ml protamine sulfate and 15 ± 2%, Fig. 1B; and 20 μg/ml Lipofectin, 13 ± 4%, Fig. 1C.

As an index of toxicity by the polycations, we counted the total number of cells on each plate when LISN-infected cells were harvested for IL-2R-flow cytometric analysis and compared this number to the number of cells obtained from a plate of uninfected cells grown under the same conditions (Fig. 1D). Polybrene at 2−8 μg/ml and protamine sulfate at 15−20 μg/ml did not affect cell growth relative to the control plate of uninfected cells. In contrast, exposing cells to lipofectin at 20 μg/ml for the two hour infection period was associated with a 50% decrease in total cell number relative to untreated cells.

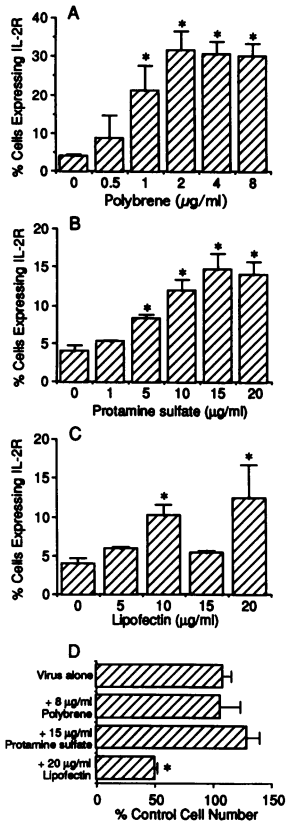

Figure 1. Effect of various polycations on efficiency of LISN infection of CFT1 cells. Cells were analyzed for IL-2R expression 48–72 hours following infection by LISN. In panels A–C, an asterisk indicates a significant difference from no added polycation. Panel D shows the viability of cells following a 2 hour exposure to CFT1 cells by LISN alone or plus the indicated polycation. Viability measurements were made 48 hours later and compared to uninfected control cells. An asterisk indicates significantly different from uninfected control cells.

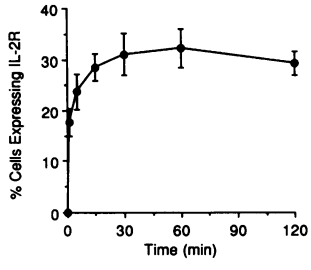

Figure 2. Length of infection period on gene transfer efficiency by LISN. Shown are the percentages of cells expressing IL-2R following infection of CFT1 cells in which the time of incubation of cells with LISN varied as follows: 1, 5, 15, 30, 60, and 120 minutes.

Length of infection period on gene transfer efficiency

Because the duration of infectious exposure may be brief with *in vivo* delivery of retrovirus vectors to the airways, we examined the effect of duration of exposure to LISN on gene transfer efficiency. As shown in Fig. 2, the maximum percentage of cells expressing IL-2R was achieved with incubation of LISN with cells for 30 minutes. Of note, significant gene transfer (approximately 50% of maximum) was observed with an incubation of LISN with cells for only one minute.

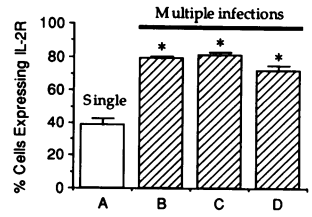

Figure 3. Effect of single versus multiple infections on the efficiency of gene transfer by LISN to CFT1 cells. An asterisk denotes significantly different from a single infection. Bar A shows the data for CFT1 cells infected once with LISN; Bar B, cells infected 2 hours daily for three consecutive days; Bar C, cells infected twice daily for three consecutive days; and Bar D, cells infected once daily for six consecutive days.

Effect of single versus multiple infections on gene transfer efficiency

We attempted to further increase gene transfer efficiency to CFT1 cells by performing multiple infections with LISN. Several different strategies for multiple infections were studied. As shown in Fig. 3, following a two hour exposure of CFT1 cells to LISN once daily for three consecutive days, approximately 79% of the cells expressed IL-2R (Bar B). This was about a two-fold greater gene transfer efficiency obtained following a single infection (Bar A). Doubling the number of infections per day but continuing infection for three days yielded similar results (approximately 81% of the cells expressed IL-2R, Bar C). Infection of CFT1 cells once daily for six consecutive days resulted in a similar increase in the efficiency of gene transfer (approximately 72% of cells expressed IL-2R, Bar D). Thus, each protocol using multiple infections significantly improved the efficiency of gene transfer over a single infection.

Using Southern blot analysis of DNA from cells sorted on the basis of IL-2R expression, we did not detect LISN proviral DNA in the ~20–30% of cells not expressing IL-2R following multiple exposures to the virus (see Fig. 6, lane 7 below). This suggests that most cells infected with LISN expressed IL-2R. Regardless of the protocol used, it was difficult to infect greater than 80% of the cells in a culture. We addressed the possibility that a sub-population of cells was intrinsically resistant to infection. In this experiment, IL-2R negative cells were isolated by cell sorting and infected with LISN. It was found that about 32% of the cells could be infected following a single 2 hour infection, a level similar to that obtained above (Fig. 3, Bar A). Thus, we conclude that the 20% resistant fraction is not enriched in cells that are intrinsically resistant to infection by amphotropic retroviruses.

Long-term IL-2R gene expression in cultures subjected to G418 selection

LISN-infected cells maintained under constant selection pressure for *neo*^{r} gene expression demonstrated a steady decline in the percentage of cells expressing IL-2R as determined by flow cytometry analysis (Fig. 4A). By passage 9 (approximately 75 days) following infection, the percentage of cells expressing IL-2R had dropped by 30%. A similar effect occurred in cultures maintained under selection pressure for only seven days following retrovirus mediated gene transfer. (Under these conditions

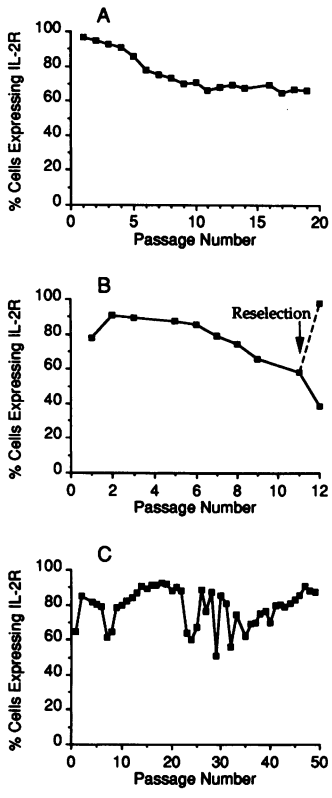

Figure 4. Long-term expression of IL-2R in LISN-infected CFT1 cells. Panel A, IL-2R expression in CFT1 cells maintained under constant G418 selection pressure following a single infection by LISN. Panel B, IL-2R expression of CFT1 cells that were selected with G418 for only seven days following a single infection by LISN, and then maintained in culture medium without G418. Panel C, IL-2R expression in CFT1 cells that were infected once daily for six consecutive days and never placed under G418 selection.

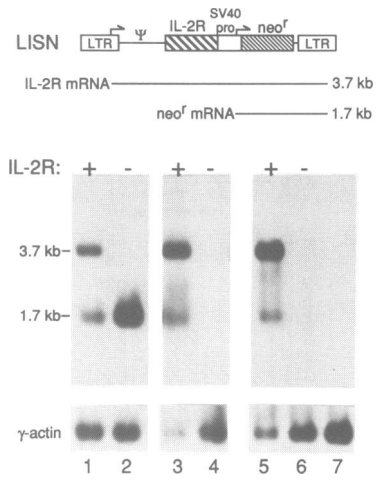

Figure 5. RNA analysis of cells sorted on the basis of IL-2R expression. At the top of the figure is a diagram showing the structure and transcription pattern from the LISN retrovirus vector. Structural features include the long terminal repeats (LTR), the sequence required for packaging the full-length RNA into virions (Ψ), the interleukin-2 receptor (IL-2R) α-subunit cDNA, the SV40 early region promoter, and the *neo*^{r} gene which confers G418 resistance. Arrows indicate transcription start sites. About 2−5 μg polyadenylated RNA from CFT1 cells was fractionated on a formaldehyde-1.2% agarose gel, transferred to nitrocellulose and hybridized to a nick-translated ^{32}P-probe, derived using a gel purified 3.6 kbp Kpn I -digested pLISN fragment (see Fig. 6) as the template. The symbols at the top of the gel refer to IL-2R expressing (+) or non-expressing cells (−). Lanes 1 and 2, CFT1 cells under continuous G418 selection. Cells were sorted by flow cytometry and analyzed at passage 17; Lanes 3 and 4, CFT1 cells selected for 1 week after infection and subsequently maintained without G418. Cells were sorted and analyzed at passage 14; Lanes 5 and 6, CFT1 cells infected once daily for 6 days and never exposed to G418. Cells were sorted and analyzed at passage 15. Lane 7, uninfected CFT1 cells. The sizes of the major RNA species are indicated. They were estimated by comparing the autoradiogram to RNA markers (Life Technologies Inc., Gaithersburg, MD) run in an adjacent lane and visualized by ethidium bromide staining. To normalize for differences in RNA loading and transfer efficiencies, the blots were re-hybridized with a human actin probe.

selection was incomplete). In this case the percentage of cells expressing IL-2R was stable through six passages (~50 days). After this period, the number of cells expressing IL-2R demonstrated a steady decline (Fig. 4B). Re-selection with G418, however, restored the percentage of cells expressing IL-2R to virtually 100%.

Stable, long-term IL-2R gene expression is maintained in multiply-infected cultures with no selection

In contrast to the above results, cultures multiply infected but never placed under selection have exhibited relatively stable, long-term IL-2R expression through at least 49 passages (> 1 year, Fig. 4C). The somewhat cyclical pattern of IL-2R expression in the culture remains unexplained and may reflect fluctuation in growth rates of sub-populations of cells in the culture.

RNA analysis of long-term cultures

In order to understand the decline in IL-2R expression in cultures kept under continuous G418 selection pressure (Fig. 4A), we analyzed LISN-specific RNA from polyclonal CFT1 cultures sorted on the basis of IL-2R expression. Northern blot analysis of RNA from both IL-2R expressing and non-IL-2R expressing cells, sorted at passage 17, revealed detectable levels of a 1.7 kbp subgenomic *neo*^{r} mRNA transcript (Fig. 5, lanes 1 and 2). The full-length 3.7 kb RNA transcript which would serve as message for the IL-2R gene was easily detected in the IL-2R expressing cells but was not detected in the non-IL-2R fraction (compare lanes 1 and 2). The lack of IL-2R mRNA in these cells thus accounts for the lack of IL-2R expression.

In contrast, there was no evidence of LISN-related sequences from the non-IL-2R expressing cells sorted from cultures G418-selected for only a week following infection, or not placed under selection at all (Fig. 5, lanes 4 and 6). This suggests that only under continuous selection does a sub-population of cells expressing only the 1.7 kb RNA grow to sufficient quantities to be detected. As predicted, both the 3.7 kb and 1.7 kb RNA transcripts were detected in cells sorting positive for IL-2R expression from cultures selected for only a week or in cultures not exposed to G418 (Fig. 5, lanes 3 and 5).

Continuous selection pressure selects for cells with rearranged proviruses

The lack of a full-length 3.7 kb RNA transcript in the non-IL-2R expressing population of cultures grown under continuous G418 selection (Fig. 5, lane 2) suggested that either transcription from the upstream LTR was attenuated in this cell population or, alternatively, the proviral DNA in this population was rearranged. To distinguish between these possibilities, chromosomal DNA from polyclonal cultures sorted on the basis of IL-2R expression was digested with restriction endonucleases and the resulting

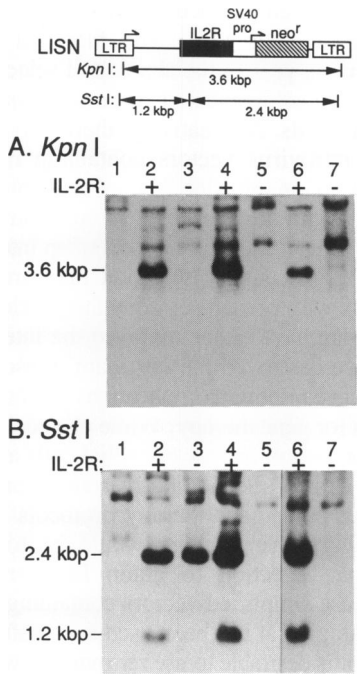

Figure 6. DNA analysis of cells sorted on the basis of IL-2R expression. At the top of the figure is a diagram showing a *Kpn* I and *Sst* I restriction endonuclease map of the LISN retrovirus vector. About 10 μg chromosomal DNA from CFT1 cells was digested with either *Kpn* I (Panel A) or *Sst* I (Panel B), fractionated on a 1% agarose gel, transferred to nitrocellulose and hybridized to a nick-translated ^{32}P-probe, derived using a gel purified 3.6 kbp *Kpn* I-digested pLISN fragment as the template. The symbols at the top of the gel refer to IL-2R expressing (+) or non-expressing (−) cells. Lane 1, uninfected CFT1 cells. The hybridizing bands are due to cross-hybridization of the probe with endogenous IL-2R sequences; Lanes 2 and 3, CFT1 cells under continuous G418 selection. Cells were sorted by flow cytometry and analyzed at passage 17; Lanes 4 and 5, CFT1 cells selected for 1 week after infection and subsequently maintained without G418. Cells were sorted and analyzed at passage 14; Lanes 6 and 7, CFT1 cells infected once daily for 6 days and never exposed to G418. Cells were sorted and analyzed at passage 15. The sizes of the LISN-specific DNA species are indicated. They were estimated by comparing the autoradiogram to Hind III-digested bacteriophage lambda DNA markers (Life Technologies Inc., Gaithersburg, MD) run in an adjacent lane and visualized by ethidium bromide staining.

DNA fragments were subjected to gel electrophoresis and Southern blot analysis using a full-length LISN probe (Fig. 6). Digestion with *Kpn* I, which cleaves LISN proviral DNA in each LTR, was predicted to yield a 3.6 kbp DNA fragment. The 3.6 kbp fragment was detected in DNA from IL-2R expressing cells (Fig. 6A, lane 2) but was not detected in DNA from IL-2R non-expressing cells grown under continuous G418 selection (Fig. 6A, lane 3).

Further analysis was performed using *Sst* I, which cleaves at an internal site, in addition to once in each LTR. Cleavage with *Sst* I is predicted to yield a 1.2 kbp fragment containing 5′-LISN sequences and a 2.4 kbp fragment containing 3′-LISN sequences including all of the *neo*^{r} gene. As predicted, both fragments were present in *Sst* I-digested DNA from the sorted IL-2R expressing cells (Fig. 6B, lane 2). In contrast, only the 2.4 kbp fragment was detected in DNA from IL-2R non-expressing cells kept under continuous G418 selection (Fig. 6B, lane 3). These results suggest that the IL-2R non-expressing cells from the culture kept under continuous G418 selection do not contain an intact LISN provirus. Specifically, sequences from the 5′ one-third of the provirus in this cell population appear to have been altered. Although the *neo*^{r} gene appears to be intact, we do not know if the *neo*^{r}-related 1.7 kb RNA transcript observed in these cultures (Fig. 5, lane 2) arises by transcription from an intact SV40 promoter. Also, because a polyclonal population of cells was studied, we would not have detected heterogeneously sized fragments containing the 5′ LTR sequences in this assay, so we do not know the status of these sequences.

Cultures that had been G418-selected for only one week, and cultures multiply-infected and never exposed to G418 were also sorted on the basis of IL-2R expression and subjected to Southern analysis. In each case, only the DNA from the IL-2R positive fraction of cells was shown to contain LISN-specific DNA of the predicted sizes following digestion with either *Kpn* I or *Sst* I (Fig. 6A and 6B, compare lane 4 with lane 5 and lane 6 with lane 7. These results suggest that the rearrangement events described above are not frequent enough to be detected by Southern analysis in the absence of continuous G418 selection.

DISCUSSION

We have demonstrated efficient gene transfer with stable long-term expression in human CFT1 cells, a transformed cystic fibrosis airway epithelial cell line. We have also addressed issues potentially affecting the efficiency of gene transfer *in vivo* and patterns of long-term gene expression that might occur *in vivo*. An immortalized cell line was chosen for these studies because of the need for prolonged longevity of cells and because preliminary studies demonstrated similar patterns of gene transfer efficiency in both CFT1 cells and in primary airway epithelial cells (data not shown).

Optimizing gene transfer to airway epithelial cells

Polycations have long been used to enhance the infection efficiency of retroviruses, presumably by increasing virus adsorption and/or penetration through a charge-mediated mechanism (13). Polybrene has been the polycation most frequently used in previous studies. However, recent reports have suggested that protamine sulfate (14) and cationic liposomes (15) may also be useful to enhance entry of retrovirus vectors into host cells. Although infection efficiency of CFT1 cells was increased with all three agents, polybrene was the superior polycation (Fig. 1). Of these three polycations, only lipofectin at 20 μg/ml was associated with acute cell toxicity as indexed by an inhibition of cell growth.

A potential problem of retrovirus-mediated gene delivery for gene therapy of cystic fibrosis is the length of time of the effective exposure of retrovirus to the airway epithelium. This period may be limited by either neutralizing substances in the airway surface liquid or perhaps by the rate of mucociliary clearance. We measured the time period for optimal incubation of virus and cells *in vitro* and found it to be approximately 30 minutes (Fig. 2). An unexpected finding was that a one minute exposure yielded about 50% of the maximal gene transfer efficiency; in part this may be related to the fact that we used undiluted high-titer virus for these experiments. Previous studies have not generally examined such brief periods of exposure to retroviruses, although gene transfer efficiencies of up to 5% have been noted to occur *in vivo* in mouse hepatocytes following a ten minute infusion of a retrovirus vector directly into the portal circulation (16).

Because the fraction of replicating airway cells in the mature human lung is low, it may be necessary to perform multiple infections to achieve the efficiency required to treat CF. We

therefore tested whether multiple infections further increased infection efficiency (Fig. 3). Our findings of increased transduction efficiencies following multiple infections are consistent with the findings of others using amphotropic vectors with bone marrow cells and fibroblasts as targets for infection (17, 18).

Selection and long-term expression

Previously, using a retrovirus vector of similar design, we reported long-term (6 months) correction of the chloride permeability defect in G418-selected clones of CFT1 cells expressing the CFTR cDNA (4). In that study, we observed some attenuation of provirus gene expression occurring at the transcriptional level. In those experiments, CFT1 cells were initially selected with G418 and subsequently maintained in continuous culture without further selection. In the current study, we observed attenuation of gene expression of the non-selected gene that involved an entirely different mechanism. In this case, long-term culture of polyclonal CFT1 cells under continuous selection for the expression of the dominant *neo*^{r} selectable marker resulted in the outgrowth of cells containing rearranged (presumably deleted) proviruses. We suspect that the outgrowth of cells with rearranged proviruses is due to a selective growth advantage of these cells during continuous G418 selection. We did not detect cells with rearranged proviruses during brief (7 day) G418 selection or in the absence of selection, suggesting that proviral rearrangements have occurred in only a minor fraction of integrations (Fig. 6). It is likely that the rearrangements arose shortly after gene transfer, since previous studies have shown that deletions and other rearrangements of retrovirus DNA are frequently detected shortly after infection [see (19) and references cited therein]. It is unlikely that the rearranged virus was present in the initial virus stock, since the virus was derived from a clonal packaging cell line containing a single intact LISN provirus (data not shown).

The selection process appeared to result in cells expressing higher levels of the *neo*^{r} mRNA, as indicated by the relatively high levels of the *neo*^{r} mRNA species in the IL-2R non-expressing cells sorted from cultures under continuous selection (Fig. 5, compare lane 1 with lane 2). Also, it is significant that the relative ratio of *neo*^{r} mRNA to full length RNA was noticeably higher in G418-selected versus non-selected cultures (Fig. 5, compare lanes 1 and 3 to lane 5). This result may suggest that integration at chromosomal loci favoring transcription initiation from the internal SV40 promoter in the LISN construct results in a selective growth advantage of these cells when placed under G418 selection. These results are reminiscent of studies by Emerman and Temin (20–22) using spleen necrosis virus vectors or MuLV-based vectors containing two dominant selectable markers, each using a separate promoter. In those studies, it was also found that the relative expression of a selectable marker was directly related to whether or not it was selected for.

While dominant selectable markers, such as *neo*^{r}, are useful elements to include in the construction of retrovirus vectors, a number of laboratories have cited potential problems associated with *neo*^{r} sequences or with the G418 selection process itself. Decreased provirus stability has been associated with selection of *neo*^{r}-containing vectors (23, 24). Decreased long-term expression of retrovirus vector sequences in hematopoietic cells *in vivo* (25) and *in vitro* (26, 27) has been attributed, in part, to the presence of the *neo*^{r} gene. Sequences within the *neo*^{r} coding region have been claimed to act as repressor sequences in some eukaryotic cells (26). The fact that we observed expression for over a year in the absence of selection (Fig. 4C) suggests that these *neo*^{r}-associated repressor sequences are not dominant in CFT1 cells. Alternatively, there may be an intrinsic design flaw in retrovirus vectors containing more than one promoter. Transcription initiation from a weak internal promoter may be at odds with transcription from a strong upstream promoter. Sub-optimal transcription rates of an internal dominant selectable marker gene under selection pressure may lead to selection for cells with proviruses containing deletions or other rearrangements that have either removed the internal promoter sequences or have destroyed the upstream transcriptional unit.

These results have important implications for the use of similar two gene vectors for gene therapy. While protocols using *ex vivo* infection and selection of airway epithelial cells are unlikely for treatment of CF, similar selection for rearranged proviruses could also take place in other gene therapy protocols that use G418 selection. An alternative strategy would be to use multiple infections without selection to attain long-term expression (Fig. 4C), or to use simplified vectors containing only the gene of interest, a strategy that has been used successfully by others, e.g. (28–30). If it is desirable to use retroviruses with a dominant selectable marker, the strategy of using bicistronic retrovirus vectors might yield more stable vectors (31–33). As an alternative to using a dominant selectable marker, it may be possible to include a gene encoding a 'passive' cell surface marker, such as IL-2R, to tag infected cells which can subsequently be isolated by cell sorting.

In summary, transduction of foreign genes into airway epithelial cells using a retrovirus vector can be highly efficient when presented to cells with appropriate polycations and multiple infections. Moreover, long-term stable expression of foreign genes is possible in immortalized CF airway epithelial cells despite frequent passaging of cells. Given the much lower rate of cell proliferation of airway epithelial cells *in vivo*, stable retrovirus expression of transduced genes may be attainable and if gene transfer can be made sufficiently efficient (7), gene therapy will offer long-term therapeutic promise for CF patients.

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