Construction of a retrovirus capable of transducing and expressing genes in multipotential embryonic cells

(retroviral restriction/embryonal carcinoma cells/neo gene)

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ABSTRACT Retroviral gene expression is inhibited in embryonal carcinoma (EC) cells. We have constructed a recombinant retroviral vector that is capable of expressing the neomycin-resistance (neo) gene in EC cells. The critical modification that permits expression of the neo gene is the insertion of a composite simian virus 40 early gene–herpes simplex virus type 1 thymidine kinase gene (SVtk) promoter 3' to the viral first intron and 5' to the neo gene. When the SVtk promoter is deleted, the recombinant retrovirus is either unable or extremely inefficient at expressing the neo gene in EC cells.

Microinjection is the only method that has been used successfully to introduce DNA into cells of the embryo, and recently proper developmental regulation of microinjected genes has been obtained (1). An alternative to this technique would be to use viral vectors to transduce genes into embryonic cells. Retroviruses are an attractive choice for several reasons. The only structures required to produce recombinant genomic RNA (gRNA) from proviral DNA are grouped at the 5' and 3' termini of the provirus. These structures include: the long terminal repeats (LTR), which are required for integration; transcription of the gRNA and polyadenylation (3); and the first intron, which contains the packaging signal (4, 5). Furthermore, a transcomplementing cell line has been constructed that efficiently produces virions containing only the recombinant gRNA (5). Finally, the integrated recombinant virus can be reisolated by infecting the cell with a wild-type retrovirus (6).

However, although retroviruses can infect embryonic cells and integrate into the chromosome (7), there is a block in their gene expression in both embryos (8) and embryonal carcinoma (EC) cells (9). This block may be at the level of transcription (10) or RNA maturation, or both. An approach to overcome this block stems from our observation that the simian virus 40 (SV40) enhancer is functional in EC cells (11). In this paper, we describe the construction of a recombinant retroviral vector, based on the Moloney murine leukaemia virus (M-MuLV), in which the gag, pol (polymerase), and env (envelope) genes are replaced by the Tnl neomycin-resistance (neo) gene, under the transcriptional control of a composite SV40 early gene–herpes simplex type 1 (HSV-1) thymidine kinase gene (tk) promoter (SVtk) (11). We report that this retroviral vector can lead to the production of high titers of a virus that is capable of transmitting the neo gene into EC cells. Furthermore, the neo gene-encoded aminoglycoside 3'-phosphotransferase is stably expressed.

MATERIALS AND METHODS

Construction of Retroviral Vector. Description of the construction of the retroviral vectors will be presented elsewhere. Briefly, pSVtk-neoβ (11) was used to obtain the SVtk-neo and neo fragments. These fragments were then inserted between the Sal I and BamHI sites of pB6 (12), a plasmid derived from pMOV3 (13) (a proviral copy of M-MuLV). The Sal I site was constructed by inserting the M13mp8 polylinker (14) into the Pst I site at position 563 of the M-MuLV gRNA (15). The BamHI site, which is at position 6537 of the M-MuLV gRNA, is in the 5' end of the env gene.

Procedures used in the construction of these vectors are referenced in Rubenstein and Chappell (16). The enzymes used in the plasmid construction were obtained from Boehringer Mannheim.

Cell Culture, Virus Infection, and G418 Selection. Cell lines. All cells were grown in Dulbecco's modified Eagle's medium (DME medium) containing 10% fetal calf serum, penicillin, and streptomycin. The mouse EC cell lines utilized were LT-1, PCC4Azα (17), and PCC3 HPRT−/Ouab (18). The mouse differentiated cell lines were ϕ2 (5), NIH 3T3 (18), and NIH 3T6.

Virus production. ϕ2 cells containing recombinant retroviral DNA were placed in 6-cm-diameter culture dishes containing 4 ml of medium without G418. The cell density was approximately at 50% confluency. After incubating the cells at 37°C for 24 hr, the medium was removed, and then it was centrifuged at 3000 × g at 4°C for 15 min.

Virus infection. One milliliter of virus stock, containing 5 μg of Polybrene (Sigma) per ml was added to 5 × 10^6 cells in 6-cm plates. The samples were incubated at 37°C for 2 hr, and then the medium was replaced with fresh medium. One day after the viral infection (or DNA transformation, see next section), various numbers of cells (between 5 × 10^6 and 2.5 × 10^7) were transferred to 10-cm plates. One day after this transfer, the medium was replaced with medium containing 500 μg of G418 (geneticin from Gibco) per ml and was changed every 2 days until only G418-resistant cells were present, at which point the medium was changed every 3 days. The G418-resistant colonies were counted at 10–14 days after the beginning of the G418 selection. Individual clones were obtained by sucking them up into a 2-ml pipette and transferring them into 1-cm culture wells.

DNA Transformation. Plasmid DNA was transfected into cells by the technique of Graham and van der Eb (19). The calcium phosphate precipitate was formed with 20 μg of DNA in a volume of 500 μl. In acute transfections and in most of the stable transfections, 6.6 μg of the plasmid pCH110 was added to the other DNA as an internal control for the efficiency of transfection. This plasmid expresses β-galactosidase (20). The DNA precipitates were added to a 6-cm plate containing 5 × 10^5 cells. Then, after incubating the

Abbreviations: LTR, long terminal repeat; EC, embryonal carcinoma; neo, neomycin-resistance gene; M-MuLV, Moloney murine leukaemia virus; HSV-1, herpes simplex virus type 1; gRNA, genomic RNA; env, envelope gene; pol, polymerase gene; SV40, simian virus 40; tk, thymidine kinase gene; SVtk, composite of SV40 early gene promoter–HSV-1 tk promoter.

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cells for 30 min at 22°C, 5 ml of medium was added. One day later, the medium was changed; then, after an additional 24 hr, protein extracts were prepared from the cells. For stable transformation experiments, the cells were diluted into 10-
cm plates containing fresh medium after the initial 24 hr (see the previous section).

Assay of Protein Extracts for Aminoglycoside 3'-Phosphotransferase and β-Galactosidase. Cells were washed twice using TS buffer (138 mM NaCl/5 mM KCl/0.7 mM Na₂HPO₄/25 mM Tris base/0.7 mM CaCl₂/1.0 mM MgCl₂, pH 7.4). Then, after adding 200 μl of lysis buffer (85% TS buffer/15% glycerol containing 10 mM dithiothreitol), the cells were scraped off the plates and sonicated (Ultrasonics W-375) for 2 min at 4°C at 100% power output and 50% duty cycle settings. Next, 1/10th vol of 0.5% deoxycholate/1.0% Nonidet P-40 was added. The cellular debris was pelleted by centrifugation for 10 min in a Beckman Microfuge, and the supernatant was saved. The protein concentration of the extract was assayed by the Bradford procedure (21). The activities of β-galactosidase and of aminoglycoside 3'-phosphotransferase were assayed as described, respectively (22, 23).

RESULTS

Structural Features of the Retroviral Vectors. Fig. 1 shows the structure of two M-MuLV-derived retroviral vectors. There are a number of features of these molecules that warrant description. (i) There are two 492-base-pair LTRs that are essential for transcription of the genomic RNA and integration of the provirus (2, 3). (ii) 3' to the 5' LTR are 418 base pairs (up to position 563 of the gRNA; see ref. 15) that contain M-MuLV's first intron (15) and the putative packaging signal of the genomic RNA (5). (iii) The following Sal I recognition site is derived from the M13mp8 bacterial phage polylinker (14). (iv) Between this Sal I site and the BamHI site (position 6537 in the M-MuLV gRNA) is the 1100-base-
pair neo gene. The neo gene can confer G418 resistance to mammalian cells (11, 24). One of the plasmids, pM-MuLV-SVtk-neo has the SV40 early gene promoter and the HSV-1 tk promoter [600 base pairs (11)] 5' to the neo gene. (v) Following the neo gene are 1.237 base pairs corresponding to the 3' end of the M-MuLV env gene, followed by the 3' LTR, which contains the polyadenylation signal (15). (vi) Flanking both LTRs are sequences derived from the mouse chromosome (200 base pairs 5' and 800 base pairs 3') (12). (vii) The remaining 4.4 kilobases of the plasmid are sequences corresponding to a rearranged copy of pBR322, which expresses the ampicillin-resistance gene. Therefore, the neo gene in pM-MuLV-neo is expected to be under the transcrip-
tional control of only the LTR, whereas in pM-MuLV-SVtk-
neo, the neo gene is expected to be under the transcriptional control of the LTR, the SV40 early gene promoter, and the HSV-1 tk promoter.

The Retroviral Vector Containing the Internal SVtk Promoter Expresses the neo Gene in EC Cells. Transient expression. To test whether unintegrated copies of a retroviral vector can express the neo gene, we transfected pM-MuLV-SVtk-neo DNA into fibroblast cells (NIH 3T6) and EC cells (LT-1). The recombinant retroviral DNA led to the production of detectable quantities of aminoglycoside 3'-phosphotransferase in both cell lines, although pM-MuLV-SVtk-neo was expressed 5-fold more efficiently in the NIH 3T6 cells than in the LT-1 cells (Fig. 2).

Stable transformants. Next we tested whether the recombinant retroviral vectors could stably express the neo gene in EC cells (presumably after chromosomal integration). We transfected LT-1 cells with pM-MuLV-neo or pM-MuLV-SVtk-neo DNA. Table 1 reports the efficiency of producing G418-resistant clones and shows that only the retroviral vector containing the SVtk composite promoter had the ability to produce G418-resistant LT-1 cells, although both plasmids were equally efficient at transforming differentiated cells (see next section). Next, to learn whether adjacent retroviral sequences have an effect on the expression of the SVtk-neo gene in EC cells, we compared the efficiency of transformation of pM-MuLV-SVtk-neo to that of pSVtk-neo, a nonretroviral plasmid that also uses the SVtk promoter to express the neo gene. These two plasmids were essentially equally efficient at conferring G418 resistance to LT-1 cells (Table 1). Therefore, it is unlikely that a mutation is required for pM-MuLV-SVtk-neo to be transcriptionally active in EC cells.

Production of Viruses from a Recombinant Retroviral Plasmid Containing an Internal SVtk-neo Transcription Unit. To produce virions carrying gRNA encoded by pM-MuLV-neo or pM-MuLV-SVtk-neo, we transfected these plasmids into 3T3 cells, a retrovirus transcomplementing cell line that does not produce M-MuLV but can package recombinant retroviruses (5). After the plasmid transfection, cells expressing the neo gene were selected by adding G418 to their culture medium. The efficiency of obtaining G418-resistant clones was approximately 10⁻³ for both plasmids (Table 1).

The transformed clones were mixed, and their supernatant was collected. We tested whether this supernatant contained retroviruses carrying the neo gene by exposing NIH 3T3 cells to the supernatant and then selecting for cells capable of growing in G418. The supernatants from the 3T3 cells carrying the pM-MuLV-neo or the pM-MuLV-SVtk-neo plasmids had a titer of ~8 × 10⁵ G418-resistant colony-forming

![Diagram of the two retroviral vectors that express the neo gene.](image-url)

Fig. 1. Diagram of the two retroviral vectors that express the neo gene. ☐, M-MuLV LTR; ☐, M-MuLV sequences; ☐, neo gene (the arrow corresponds to the orientation of the coding strand of the gene); ☐, mouse chromosomal DNA; ☐, pBR322 sequences, ☐, SVtk promoter; *, Sal I site in pM-MuLV-neo was destroyed during the construction of this molecule.
units (Table 1). Therefore, ψ2 cells containing these retroviral vectors are efficient producers of viros that can transfer the neo gene into differentiated cells. Furthermore, the internal SVtk promoter does not substantially change the efficiency of producing these recombinant retrovirus (Table 1). Finally, both M-MuLV-neo and M-MuLV-SVtk-neo expressed the same level of aminoglycoside 3'-phosphotransferase in infected NIH 3T3 cells (Fig. 2).

Viral Transduction of the neo Gene into EC Cells. We next tested whether the M-MuLV-neo and the M-MuLV-SVtk-neo viruses could confer G418 resistance to EC cells. Three EC cell lines were studied, each having different developmental properties. LT-1 is a nullipotent cell line, whereas PCC3 and PCC4 are multipotent (17). These EC cells were grown in the virus-containing medium, and the cells were then transferred into medium containing G418. In contrast to the result obtained with differentiated cells (NIH 3T3), only the M-MuLV-SVtk-neo virus was capable of conferring stable G418 resistance to all three types of EC cells. The M-MuLV-neo virus was either completely unable to transduce an active neo gene (when infecting LT-1 cells) or very inefficient relative to the p-MuLV-SVtk-neo virus (when infecting PCC4 or PCC3 cells) as shown in Table 1.

We isolated individual G418-resistant EC clones, all of which maintained their typical EC morphology. To prove that the neo gene is actually expressed in these cells, we assayed cellular extracts for aminoglycoside 3'-phosphotransferase. The neo gene product was expressed in all of the G418-resistant cells infected with the M-MuLV-SVtk-neo virus (Fig. 2). On the other hand, the G418-resistant PCC3 cells derived from infection with the M-MuLV-neo virus contained at least 40-fold less aminoglycoside 3'-phosphotransferase than did EC cells infected with the M-MuLV-SVtk-neo virus or differentiated cells infected with either the M-MuLV-neo or M-MuLV-SVtk-neo virus.

Therefore, the addition of the internal SVtk promoter 5' to the neo gene confers upon retroviruses the ability to transduce and stably express the neo gene in nullipotent and multipotent EC cells. Furthermore, the few G418-resistant EC cells transduced by M-MuLV-neo produced less aminoglycoside 3'-phosphotransferase by a factor of 40 than did M-MuLV-neo-transduced differentiated cells.

**DISCUSSION**

Retroviral gene expression is inhibited in EC cells due to a block in transcription (10, 26-28) or RNA maturation, or both. We have constructed a recombinant retrovirus that overcomes this restriction. By inserting the SVtk composite promoter after the M-MuLV first intron, and 5' to the neo gene (Fig. 1), the retrovirus (M-MuLV-SVtk-neo) was capable of introducing and expressing the neo gene in all EC cell lines tested. Each of these EC cell lines has characteristics of different stages of embryonic development (17), suggesting that maintaining which expression is necessary for successful transduction of the neo gene into EC cells.

**Table 1. Efficiency of producing G418-resistant cells by using the recombinant retroviruses**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Virus infection, G418* cfu/ml</th>
<th>pM-MuLV-neo</th>
<th>pM-MuLV-SVtk-neo</th>
<th>pSVtk-neoβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-MuLV-neo</td>
<td>M-MuLV-SVtk-neo</td>
<td>DNA transfection*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ψ2</td>
<td>NIH 3T3</td>
<td>9 x 10^4</td>
<td>6.5 x 10^4</td>
<td>11 x 10^-4</td>
</tr>
<tr>
<td>LT-1</td>
<td>≤1</td>
<td>2 x 10^2</td>
<td>≤2 x 10^-6</td>
<td>3 x 10^-5</td>
</tr>
<tr>
<td>PCC4</td>
<td>10</td>
<td>2.8 x 10^2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PCC3</td>
<td>4 x 10^2</td>
<td>2 x 10^1</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*The efficiency of producing G418* colonies is reported as the ratio of the number of G418* colonies divided by the number of cells initially transduced.

Differentiated (NIH 3T3) or EC (LT-1, PCC4, and PCC3) cells were infected with virus produced by ψ2 cells containing either pM-MuLV-neo or pM-MuLV-SVtk-neo. The table reports the number of G418-resistant (G418*) colony-forming units (cfu)/ml of ψ2 supernatant. Efficiency of producing G418* cells was also measured in differentiated (ψ2) or EC (LT-1) cells that were transinfected with pM-MuLV-neo, pM-MuLV-SVtk-neo, or pSVtk-neoβ DNA. Note that the data are not corrected for the cloning efficiency of the EC cells, which is approximately 1/10 that of the NIH 3T3 cells (unpublished results).

The efficiency of producing G418* colonies is reported as the ratio of the number of G418* colonies divided by the number of cells initially transduced.
that this retroviral vector may be suitable for introducing and expressing genes in embryos.

On the other hand, the retrovirus without the SV\textit{k} promoter (M-MuLV-neo) is unable or extremely inefficient at expressing the \textit{neo} gene in EC cells. For instance, by either DNA transformation or viral infection (Table 1), pM-MuLV-neo was unable to confer stable \textit{neo} gene expression to LT-1 cells. In contrast to LT-1 cells, PCC4 and PCC3 cells were transformed to G418 resistance by the M-MuLV-neo retrovirus, although at an extremely low efficiency (10 and 400 G418\textsuperscript{c} cfu/ml, respectively, Table 1). These G418-resistant cells maintain EC morphology and multipotency. Although they are resistant to G418, their level of aminoglycoside 3'-phosphotransferase expression is lower by at least a factor of 40 than in the EC cells that had been infected with M-MuLV-SV\textit{k}-neo (Fig. 2). Therefore, the retroviral promoter and splicing are probably functioning in these cells but at a reduced efficiency. One of the characteristics of PCC3 cells is their ability to differentiate in culture. For this reason, it is likely that within the PCC3 cell population, cells at different states of differentiation coexist (29). We hypothesize that a subpopulation of PCC3 cells have the ability to express, although inefficiently, genes under the transcriptional control of the retroviral LTR.

The fact that the internal SV\textit{k} promoter did not significantly change the amount of virus produced by the \textit{\&} cells containing pM-MuLV-SV\textit{k}-neo (Table 1) shows that a second promoter, which is in tandem with the LTR, is not necessarily deleterious to the propagation of a retrovirus. Other workers also have been able to propagate retroviruses containing internal promoters (30–32). We have extended this result to a retroviral vector containing the \textit{Escherichia coli lacZ} gene under the transcriptional control of the SV40 early promoter. Furthermore, it is likely that the internal SV\textit{k} composite promoter is the only active promoter when the provirus is integrated in EC cells. In that case, by replacing the SV\textit{k} promoter with the 5' region of developmentally regulated genes, one can use this system to introduce new genes into embryonic cells in order to study the control of gene expression during embryogenesis.

We wish to transduce an antiparallel copy of a gene into a cell. We (25) and another group (33) have presented evidence that antiparallel mRNA can inhibit the expression of the complementary mRNA in eukaryotic cells. The inhibition of a developmentally important gene by expressing an antiparallel copy of that gene has the potential of being a powerful technique to study development.

Note Added in Proof. Cocultivation of 5 × 10\textsuperscript{5} LT-1 cells with 2 × 10\textsuperscript{3} mitomycin-treated (overnight with 2 μg/ml) M-MuLV-SV\textit{k}-neo-producing \textit{\&} cells for 1 week transforms at least 10% of the LT-1 cells to G418 resistance. Furthermore, PCC3 cells transduced with the M-MuLV-SV\textit{k}-neo virus stably express the \textit{neo} gene product when grown in medium without G418 for 70 generations.

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