Recombinant Retroviruses Encoding Cell Surface Antigens as Selectable Markers

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Recombinant retroviruses are frequently used in the transfer and analysis of genes. This report describes new retrovirus vectors that incorporate a cDNA copy of a cell surface antigen to function as a selectable marker. By using techniques based on quantitative cell surface immunofluorescence, these vectors allow the rapid detection and isolation of infected cells. These vectors also allow the rapid detection of packaging cell lines producing large amounts of recombinant retroviruses. Potential applications of these vectors are demonstrated.

Retrovirus-mediated gene transfer has been widely used to study the expression of a variety of genes (2-5, 7, 13). Advantages to the use of recombinant retroviruses to achieve gene transfer include the ability to generate high-titer virus stocks which efficiently transfer genetic material; the unique integration mechanism of retroviruses, which ensures unrearranged representation of the recombinant sequence in the host genome; and the ability to establish conditions whereby recipient cells receive only a single copy of the genetic material to be transferred. Disadvantages to the use of recombinant retroviruses include the refractoriness of certain sequences to passage as a retrovirus intermediate; the low levels of expression seen in certain cell types; and the limited amount of genetic material which can be incorporated into a recombinant retrovirus.

Many recombinant retrovirus vectors have been designed to contain a cDNA encoding a dominant selectable marker (2-5, 7, 10). Infected cells are then grown under the appropriate selection conditions to generate cells which express the selectable marker. In this report we describe a series of retrovirus vectors in which cell surface antigens are used as the selectable marker. These vectors allow the rapid detection of cell lines producing high titers of recombinant retroviruses, the ability to detect and isolate infected cells in a short period of time, and the ability to isolate populations of infected cells expressing different amounts of the recombinant retrovirus.

The structure of the recombinant retroviruses used in these studies is outlined in Fig. 1. The retrovirus vector depicted in Fig. 1A contains the cDNA encoding the human transferrin receptor (8) as the selectable marker and the Tn5 *neo* gene as the gene whose transfer is being studied. To generate cell lines producing recombinant retroviruses, the plasmid shown in Fig. 1A was mixed with PSV₂HIS, an expression vector containing the histidinol dehydrogenase cDNA (S. Hartman and R. C. Mulligan, manuscript in preparation) in a 10:1 molar ratio and transfected into ψ 2 cells (9). Colonies were generated in medium containing histidinol (11), and each colony was screened for quantitative cell surface expression of the human transferrin receptor by labeling with fluorescein-conjugated mouse anti-transferrin receptor monoclonal antibody (Becton Dickinson) and analysis on a Becton Dickinson FACS Analyzer flow cytometer, as described previously (1). Representative colonies

with different levels of cell surface expression of the transferrin receptor were assayed for quantitative virus production by infection of 3T3 cells followed by selection for Tn5 *neo* gene expression in G418. As shown in Fig. 2, there was a strong quantitative correlation of cell surface expression of the selectable marker (transferrin receptor) with virus production. Furthermore, in the absence of any selective pressure, these cell lines showed stable levels of cell surface antigen expression and virus production for at least 6 months. These results indicate that cell surface immunofluorescence can be used as a rapid screening procedure to detect ψ 2 cell lines producing large amounts of recombinant retroviruses.

The correlation of transferrin receptor expression as detected by immunofluorescence and virus production demonstrated in Fig. 2 suggest that high-titer virus producers can be isolated from a population of ψ 2 cells by fluorescence-activated cell sorting. To test this hypothesis, ψ 2 cells were cotransfected as described above, and a population of histidinol-resistant cells was generated. This population was then subjected to three sequential cycles of cell sorting, with each sort isolating the 10% of the population with the brightest cell surface immunofluorescence for the transferrin receptor. As is demonstrated in Fig. 3, each sequential sort generated a population of cells with greater cell surface transferrin immunofluorescence and higher virus titer. The low initial virus production by the population (prior to sorting) presumably resulted from the cotransfection protocol used to generate these cells. Since no selection for proviral genes was used, many of these cells either do not contain or do not stably express high levels of the plasmid DNA. With sequential cell sorting, there are then increments in both cell surface expression and virus production.

Several mechanisms could be contributing to this increment in surface protein expression and virus titer. Each cell sort could be generating populations of cells with greater amounts of helper virus, the end result being a population whose increased cell surface expression and virus production were secondary to high titers of helper virus. Alternatively, each sort could be selecting for cells with high levels of cell surface antigen expression and virus production that were present in the initial population. Since the data presented in Fig. 2 indicate the presence of cells with high levels of transferrin receptor expression and virus production in the initial population, we believe that the progressive isolation of these initially present cells by sequential sorting is the

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FIG. 1. Plasmids used to generate recombinant retroviruses. The starting plasmids used were Dol (7) and pHISBg11 (11). The human transferrin receptor cDNA (TR) was a 2.8-kilobase *Bam*HI-*Bgl*II fragment from pCD-TR1 (9). The Leu-1 cDNA was a 2.3-kilobase *Eco*RI fragment (6). Plasmids depicted in panels B and D have been used in similar experiments; however, a quantitative assessment of virus production and transfer has not been undertaken with these plasmids. LTR, Long terminal repeat; SV40 Pro., simian virus 40 promoter.

most likely explanation for our results. Moreover, assays for helper virus, performed by detecting the rescue of an integrated recombinant retroviral genome containing the hygromycin resistance gene by supernatants from each of the sorted populations, failed to reveal any increment in helper virus with each sequential sort (data not shown). In addition, levels of transferrin receptor expression and virus production by these populations were stable for a minimum of 6 months.

Retroviruses contained in the supernatants of high-titer virus-producer cell lines similar to the ones described above have been used to infect both immortalized cells in culture, such as NIH 3T3 cells, and primary cells, such as baby rat kidney (BRK) cells. In both cases, cells expressing the cell surface antigen marker can be detected by immunofluorescence as early as 3 days after infection. In the experiment depicted in Fig. 4, either NIH 3T3 cells (Fig. 4A) or BRK cells (Fig. 4B) were infected with a recombinant retrovirus containing the cDNA encoding the lymphocyte antigen Leu-1 (6) as the selectable marker (Fig. 1C). Three days after infection, cells were assayed for expression of the Leu-1 antigen by immunofluorescence and flow cytometry with phycoerythrin-conjugated anti-Leu-1 murine monoclonal antibody (Becton Dickinson). As can be seen in Fig. 4,

FIG. 2. Relationship between cell surface immunofluorescence and retrovirus production. ψ 2 cells were transfected and selected as described in the text and previously (11). Quantitative expression of the human transferrin receptor cDNA was determined by labeling with fluorescein-conjugated anti-transferrin receptor antibody (Becton Dickinson). Flow cytometric analysis was performed as described previously (1). Six colonies with different levels of mean relative immunofluorescence were assayed for the number of recombinant retroviruses produced per milliliter of cell supernatant as described in the text and previously (11).

FIG. 3. Selection of cells producing large amounts of recombinant retroviruses by cell sorting. (A) ψ 2 cells were cotransfected with the plasmid depicted in Fig. 1A and pSV₂HIS. A population of histidinol-resistant cells was tested for cell surface expression of the human transferrin receptor (TFR) by immunofluorescence as described in the text and in reference 1. The same population was assayed for virus production as described in the text. Relative mean immunofluorescence of this population was 23 units (arbitrary units); virus titer is shown in the upper right corner of each panel. (B) The population shown in panel A was labeled with anti-transferrin receptor antibody, and cell sorting for the brightest 10% of the population was undertaken. The sorted cells were then expanded and analyzed as described for panel A. Mean immunofluorescence of this population was 36 units. (C) The population shown in panel B was labeled, sorted, and analyzed as described above. Mean immunofluorescence of the resultant cell population was 78 units. (D) The population shown in panel C was labeled, analyzed, and sorted as described above. The resultant cell population had a mean immunofluorescence of 165 units.

infection of both cell types was detectable, although much more efficient infection and expression occurred in NIH 3T3 cells.

FIG. 4. Infection of NIH 3T3 cells and BRK cells with recombinant retroviruses containing the Leu-1 cDNA as a selectable marker. (A) NIH 3T3 cells were infected with recombinant retroviruses generated from the plasmid schematically shown in Fig. 1C by previously published techniques (11). Three days after infection, cells were labeled with phycoerythrin-conjugated mouse anti-Leu-1 monoclonal antibody and analyzed as described in the text. A similar analysis on mock-infected (control) NIH 3T3 cells is also shown. (B) BRK cells were infected as described above and in reference 11 except that Polybrene (2 μ g/ml) was used during the infection. Cells were analyzed as described for panel A.

FIG. 5. Fractionation of infected cells into populations with different levels of expression of the recombinant retrovirus. (A) NIH 3T3 cells were infected with retroviruses generated from the plasmid depicted in Fig. 1C. Infected cells and control cells were labeled and analyzed as described in the text and in the legend to Fig. 4. This population was fractionated into cells with low levels of Leu-1 surface expression (B) and cells with high levels of Leu-1 expression (C) by immunofluorescent labeling and cell sorting. (D) Cellular RNA (20 μ g) from the starting population shown in panel A, the population with low Leu-1 expression shown in panel B, and the population with high Leu-1 expression shown in panel C were electrophoresed in formaldehyde-agarose, transferred to nitrocellulose, and hybridized to 32 P-labeled plasmid DNA (Fig. 1C). Lane 1, RNA from the population with low surface expression of Leu-1; lane 2, RNA from the starting population; lane 3, RNA from the population with high levels of Leu-1 surface expression.

The presence of a selectable marker which can be quantitated by cell surface immunofluorescence allows attempts to fractionate infected cells into populations with different levels of expression of the cDNA being studied. For example, in the experiment depicted in Fig. 5, cell sorting was used to fractionate a population of cells (Fig. 5A) infected with the recombinant retrovirus containing the Leu-1 antigen cDNA as the selectable marker and the Tn5 *neo* gene as the cDNA whose expression was being studied (shown schematically in Fig. 1C). Distinct populations of cells with low levels of Leu-1 expression (Fig. 5B) and high levels of Leu-1 expression (Fig. 5C) were generated. RNA was prepared from the starting cell population as well as the populations with low and high levels of expression and analyzed for relative amounts of retroviral RNA by electrophoresis in formaldehyde-agarose, transfer to nitrocellulose, and hybridization to 32 P-labeled retroviral DNA (Fig. 1C). The results of this analysis are shown in Fig. 5D. Two RNA species were detected; the larger RNA corresponded to full-length retroviral transcripts initiating at the 5' long terminal repeat promoter, and the smaller transcript represented RNA initiating at the internal simian virus 40 promoter. The cells with low levels of surface Leu-1 expression (Fig. 5B) contained smaller amounts of these RNAs (lane 1) than were present in the unfractionated cell population (lane 2) and the cells with high levels of Leu-1 expression (lane 3). Since all three populations contained an equivalent percentage of G418-resistant cells (approximately 85% plating efficiency in G418), we do not believe that the differences

detected in the RNA analysis can be accounted for by differing amounts of contamination of the cell populations with uninfected cells. In addition, the pattern of RNA analysis did not significantly change after growth of these populations in G418 (data not shown).

A variety of techniques have been used to express exogenous genes or cDNAs in animal cells. In many cases a dominant-acting selectable marker encoding drug resistance is used to isolate cells which have received and are expressing exogenous DNA. This report describes the use of cDNAs encoding cell surface antigens as selectable markers in recombinant retroviruses. These vectors allow the rapid selection of cell lines producing high titers of recombinant retroviruses, the rapid detection and quantitation of gene transfer and expression into both immortalized (3T3) and primary (BRK) cells, and the ability to select cells with various levels of expression. The rapidity of selection may make these vectors particularly applicable to the study of exogenous genes in primary cells with limited growth potential *in vitro*. Furthermore, the ability to select only cells with high levels of expression may make these vectors useful in attempts to achieve somatic cell gene therapy, provided that the surface marker used is not immunogenic in the animal into which the cells are transferred. For example, bone marrow myeloid and hematopoietic progenitor cells do not express the Leu-1 surface membrane protein, while T-lymphocyte populations do. It may therefore be possible to infect such progenitor cells with the vector and obtain constitutive production of the marker, which, since it is normally present in the host but on different cell populations, would not be immunogenic. Nonetheless, cells derived from such infected progenitors, such as myeloid cells, which do not normally express Leu-1, would be identifiable by virtue of their simultaneously expressing a myeloid tissue-specific antigen and the Leu-1 antigen.

The use of cell surface immunofluorescence as a means of detecting and isolating infected cells has the added advantage of not requiring that selective pressure be applied to the cell. Whether this will prove to be a useful attribute of these vectors remains to be determined. Theoretically, however, the absence of selective pressure may diminish the selection and amplification of clones with rearrangements and other genetic alterations which might dissociate levels of expression of the selectable marker from levels of expression of the gene to be transferred. Supporting such a hypothesis is the fact that we have never observed cells with high levels of expression of the surface antigen which did not also produce correspondingly high levels of the adjacent gene. Furthermore, the use of immunofluorescence to detect infection may make these vectors useful in cell lineage studies (12) or in studies in which recombinant retroviruses are used to study promoter function (11). It should also be noted that cell-sorting techniques to allow expansion from single cells rather than populations of cells can be used when pure clones of cells are required.

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