Rapid Titration of Retroviral Vectors Encoding Intracellular Antigens by Flow Cytometry

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Flow cytometry was used to detect cells infected with retroviral vectors encoding both simian virus 40 large T antigen and G418 resistance after indirect immunofluorescence staining using a T-antigen-specific monoclonal antibody and a fluorescein-conjugated secondary antibody. Titers of viral stocks determined by flow cytometry were equivalent to those determined by quantitation of G418-resistant colonies.

Transfer of a drug-resistant phenotype is often used to quantify infectious virus in defective retroviral vector preparations. The assay is time-consuming and limited to cell types that can expand clonally. This paper describes a rapid and precise biological method that overcomes these limitations. In this assay, flow cytometry is used to quantify infected cells that have been stained with antibodies against virus-encoded nuclear proteins.

Two viruses, both encoding simian virus 40 large T antigen (Tag) and G418 resistance and both based on the pZIP-NeoSV(X)1 vector (4), were used in these studies. Ψ -2 (14) cells producing SV40-6 virus (12) were provided by P. Jat. pZIPTX DNA (3) was provided by D. Livingston. High-titer viral stocks were generated by infection or transfection of PA317 cells (15) and repackaging in Ψ -2 cells, as described elsewhere (5, 16). G418-resistant Ψ -2 clones were expanded, and medium was collected 24 h after refeeding semiconfluent monolayers. Virus-containing medium was centrifuged at low speed to remove cellular debris, frozen at -70°C , and thawed before use in infection.

NIH 3T3 or BALB/c-3T3 (clone A31) cells were used as host cells for retroviral infections. Cells were grown on 10-cm plastic dishes treated with gelatin (9) in Dulbecco modified Eagle medium supplemented with 5% (vol/vol) fetal bovine serum and 5% calf serum. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Infections were performed by removing medium from cells seeded 16 to 31 h earlier at 5×10^5 per dish and replacing it with 2 ml of viral medium containing 4 μg of Polybrene per ml. Plates containing viral medium were rocked every 15 min. Virus was removed, and 10 ml of fresh medium was added after 2 h of incubation. To select G418-resistant colonies, 400 μg of G418 per ml was added 24 h postinfection.

The procedures for fixation and staining have been described in detail previously (11). Briefly, 10^6 cells were fixed with methanol, incubated with 1 μg of mouse monoclonal antibody PAb416 or PAb419 (6, 8) obtained commercially (Oncogene Science, Mineola, N.Y.), and then stained with 2.6 μg of goat anti-mouse immunoglobulin G F(ab')₂ fragments conjugated to fluorescein (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Cells were treated with RNase A (>0.04 Kunitz units) and then stained with 50 μg of propidium iodide per ml.

Fluorescence measurements were made on a flow cytometer (Cytofluorographs II; Ortho Instruments, Westwood, Mass.) by using the 488-nm line of an argon laser operating

at 200 to 300 mW. Green fluorescence was collected through a 530/20-nm-bandpass filter set, and red fluorescence was collected above 640 nm. Data acquisition was triggered on red fluorescence. A doublet discriminator (peak versus integrated signal) was used as the primary gate to eliminate cell aggregates. Immunofluorescence was logarithmically amplified. A minimum of 10,000 cells per sample were analyzed.

Figure 1 illustrates data obtained by flow cytometric analysis of stained cells. Bivariate contour plots of immunofluorescence versus DNA content are shown. Immunofluorescence can be divided into two populations: infected Tag-expressing cells and uninfected cells. Figure 1A demonstrates a plot for a mock-infected population showing background immunofluorescence, and Fig. 1B shows a plot for infected cells. The fraction of Tag-positive cells was calculated by setting a region around the positive population and integrating within the area. DNA staining was used both for doublet discrimination and to indicate the cell cycle distribution of each population. At 31 h postinfection, Tag-positive cells were present in all cell cycle compartments (G_1 , S, and $G_2 + M$) (Fig. 1B).

Tag-positive cells were routinely detected beginning 8 to 12 h postinfection (Fig. 2). The percentage of Tag-positive cells increased logarithmically until 24 to 30 h postinfection, at which time the percentage of Tag-staining cells in the population plateaued. In subsequent experiments, cells were fixed at least 24 h postinfection. Assuming that retroviral gene expression is not detected until after integration of the viral DNA has occurred, this logarithmic increase between 12 and 24 h may be related to cell growth rate. Since integration of retroviral DNA requires cellular DNA synthesis (21), one cell doubling must occur before all infected cells pass through an S phase and integrate viral DNA. Therefore, the doubling time of the target cell population should be considered for this assay.

Virus dilution experiments were used to test for linearity of the assay and to determine the detection limit. Dose-response curves obtained from experiments of this type were linear with a slope of 1.00 ($r = 0.99$), indicating that Tag-positive cells were infected by single viruses and that the assay is linear over the 1,000-fold dilution range tested. At the highest dilution, Tag-positive cells were detected at a frequency of 10^{-4} . This endpoint defines the practical sensitivity of the current assay. This number can be improved by counting more cells; however, the sensitivity of the assay will vary depending on the antigen one is trying to detect and cellular background fluorescence. High background fluorescence results in a high threshold for positivity classification

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FIG. 1. Detection of infected cells by immunofluorescence staining and flow cytometry. NIH 3T3 cells were fixed 31 h postinfection, stained, and analyzed by flow cytometry. Mock-infected (A) and infected (B) cells are shown. Cells with a DNA content of G₁, S, or G₂ + M are indicated. In panel B, the region corresponding to Tag-positive cells is boxed. This region was set arbitrarily to include Tag-positive cells. Typically, this region included less than 0.5% of the mock-infected sample as background false-positives. In this sample, 25.8% of the cells in panel B are producing Tag.

and therefore decreased sensitivity. Background fluorescence depends on a number of factors including autofluorescence, nonspecific trapping of antibodies by the cell, affinity of antibody for antigen, cell fixation method, and specificity of the antigen-antibody reaction (10, 11). For the Tag system used here, background fluorescence is low (11) and antigen level is high; therefore, the documented 10^{-4} sensitivity is near the practical limit expected for the described protocol.

Under conditions in which antibodies are saturating, fluorescence intensity is proportional to antigen levels and, at relatively high levels, the antigen-positive population is visually distinct from the negative population (Fig. 1). At low antigen levels, the infected and uninfected populations overlap and more sophisticated analytic methods are used to estimate the maximum number of antigen-positive cells (10).

To test the reproducibility of the assay, six replicate plates were either mock infected or infected with 2 ml each of an identical viral stock. Values for the percentage of Tag-positive cells were 0.4 ± 0.2 for the mock-infected samples and 18.8 ± 0.6 for the infected samples. Therefore, the percentage of false-positive cells is low and variable (50% coefficient of variation), whereas estimation of the percentage of positive cells in infected samples is quite reproducible (3% coefficient of variation) and specific.

Virus titer obtained by flow cytometry was compared with

virus titer obtained by transfer of G418 resistance. In the vectors used here, both Tag and neomycin resistance RNAs are transcribed from the viral long terminal repeat. The Tag message is unspliced, while the neomycin resistance message is spliced. If both messages are present in all infected cells and if the protein yield is above the threshold level for their respective assays, quantification of the same viral stock by both methods will yield similar results. Table 1 shows that the two methods are equivalent.

Since Tag-positive cells in an infected population could be rapidly and precisely quantified by using the method described here, the efficiency of the infectious process was investigated to optimize the infection protocol and verify the utility of the assay. Two methods of Polybrene treatment (13, 20) were compared, and either 20 μ g of Polybrene per ml 1 h before infection or 4 μ g of Polybrene per ml during the 2-h infection produced similar infection efficiencies. The omission of Polybrene altogether reduced the efficiency by 95%, a finding similar to previously published data (18, 20).

The effect of target cell number was tested. An experiment in which a constant amount of virus was added to increasing numbers of cells per dish was performed. Adding virus to 3×10^5 cells optimized the percentage of infected cells, whereas the maximum number of infected cells was obtained with 1.4×10^6 cells. Therefore, significantly different strat-

FIG. 2. Time course of Tag expression after infection. Infected cells were fixed at various times postinfection, stained, and analyzed by flow cytometry. The data are from four separate experiments. The data are expressed as the percentage of the maximum (Max) number of Tag-positive cells for each experiment.

TABLE 1. Comparison of flow cytometry to drug-resistant colony formation^a

Experiment ^b	G418-resistant colonies	Cells/dish at time 0	% Infected cells		% Flow cytometry/% colony assay
			Colony assay	Flow cytometry	
1	1.96×10^5	7.13×10^5	27.5	12.8	0.47
2	1.21×10^4	4.56×10^5	2.7	2.7	1.00
3	9.85×10^4	4.56×10^5	21.6	27.0	1.25

^a For each experiment, two sets of plates containing NIH 3T3 cells were infected with dilutions of virus and at 30 h postinfection, one set was fixed, stained, and analyzed by flow cytometry. Medium containing G418 (400 μ g of G418 per ml) was added to the other set of plates at the time of fixation, and colonies were selected. The number of cells per dish at time 0 was determined by cell counts. In this way, the number of G418-resistant colonies obtained could be expressed as a percentage of the total cell number on the dish at time 0.

^b Three separate viral stocks were used.

TABLE 2. Viral infection time and multiple applications of virus to NIH 3T3 cells

Infection procedure ^a	% Tag-positive cells (relative no.) ^b
1	9.0 (1.00)
2	11.1 (1.23)
4	14.7 (1.63)
6	16.2 (1.80)
1 + 1	14.7 (1.63)
2 + 2	19.1 (2.12)
2 + 2 + 2	23.9 (2.66)

^a Numbers separated by a plus indicate separate applications of fresh virus, and the number itself indicates the duration in hours of that viral application. For example, 2 + 2 + 2 indicates three consecutive viral applications, each lasting 2 h. All virus was identical (i.e., collected from the same producer cells at the same time) and was thawed immediately prior to applying to cells. Infections were terminated by removing virus medium and replacing it with medium containing no virus.

^b Data are normalized relative to the value for a single 1-h application of virus. Normalized data are given in parentheses.

egies should be used depending on the desired experimental outcome.

The effects of virus inoculum volume and virus concentration were tested in two experiments. In the first experiment, increasing amounts of virus were added to cells by increasing the volume of a viral stock added to plates (constant virus concentration). In the second experiment, the amount of virus added to cells was constant but virus concentration was changed by increasing the inoculum volume. Comparison of the data demonstrated that the concentration of virus has a greater effect on infection efficiency than does the total amount of virus. Therefore, a more efficient way to increase infection is to use more concentrated stock.

The effect of consecutive applications of fresh virus is demonstrated in Table 2. In these experiments, single or consecutive applications of fresh virus were added to cells for various times. When a single application of virus was added, the greatest number of cells were infected within the first hour. After 1 h the infection rate decreased. This suggests two possibilities. The first is that the virus has a short half-life once it is applied to cells and that the amount of infectious virus in contact with cells decreases over time. The second possibility is that the cells become less susceptible to viral infection. This would occur if cellular receptors for virus become saturated with defective viral particles. The retroviral vectors used here are not efficiently packaged into virions, since they lack a portion of the viral packaging signal (1, 2) and Ψ -2 cells producing these viruses are expected to produce empty virions that do not contain viral RNA (14). The multiple application experiment tests these possibilities. When two consecutive applications of virus are used in 2 h, 14.7% of cells become Tag positive, whereas 11.1% of cells are Tag positive after 2 h with a single virus application. Therefore, fresh virus can increase the infection rate, indicating that virus decay occurs with time. However, because two consecutive applications do not yield two times the 1 h infection level ($2 \times 9.0\% = 18\%$), cell susceptibility to infection also appears to play a role. However, here viral decay apparently predominates and multiple infections can be used to increase the percentage of infected cells.

Parameters of collection and handling of viral medium were tested. First, the seeding density of virus-producing packaging cells was found to have an effect on viral titer. The highest titer stocks were obtained from packaging cells that were plated at an initial density of 5×10^5 cells per 10-cm

dish. These titers were 11 and 59% higher than titers of stocks from cells plated at 10^6 and 2×10^6 cells per 10-cm dish, respectively. Second, the concentration of virus in stocks could be increased by simply reducing the volume of medium during the collection period. The highest titer stocks were obtained when 5 ml of medium per 10-cm dish was used to harvest virus. These titers were 29 and 41% higher than titers from stocks collected into 7.5 and 10 ml of medium per 10-cm dish, respectively. Finally, unfrozen viral medium was compared with medium stored at -70°C . Fresh, unfrozen medium has a twofold-higher titer than frozen medium. Loss of titer upon freezing could be partially abrogated by adding 10% dimethyl sulfoxide to the stock before freezing. Infection of cells with the dimethyl sulfoxide-containing stock resulted in the rounding up of target fibroblasts during infection; however, these cells subsequently attached and resumed growth after the removal of the viral stock.

Many of the factors reported above were combined to maximize infection efficiency. When 2.5×10^5 cells were infected with 2 ml of unfrozen virus 12 times over a 6-h period, 85.4% were scored as Tag positive. This is compared with 22.5% Tag-positive cells when 7.6×10^5 cells (obtained 16.75 h after plating 5×10^5 cells) were infected once with 2 ml of identical virus which had been frozen at -70°C .

In conclusion, this paper describes a rapid and precise technique for titration of retroviral vector stocks encoding intracellular antigens. Although the results in this paper have been obtained after staining for Tag, a nuclear protein, the assay is applicable to proteins localized elsewhere in the cell. Previously, others have used antiserum against whole virus in combination with flow cytometry to assay nonrecombinant murine leukemia virus (7). More recently, investigators have described flow cytometric detection of cells infected with retroviruses encoding surface antigens (19) and the cytoplasmic enzyme β -galactosidase (17). Hence, the method described here is a general one for the detection and quantification of retroviruses encoding genes for many proteins.

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LITERATURE CITED

- Armentano, D., S.-F. Yu, P. W. Kantoff, T. von Ruden, W. F. Anderson, and E. Gilboa. 1987. Effect of internal viral sequences on the utility of retroviral vectors. *J. Virol.* **61**:1647-1650.
- Bender, M. A., T. D. Palmer, R. E. Gelin, and A. D. Miller. 1987. Evidence that the packaging signal of Moloney murine leukemia virus extends into the *gag* region. *J. Virol.* **61**:1639-1646.
- Brown, M., M. McCormack, K. G. Zinn, M. P. Farrell, I. Bikel, and D. M. Livingstone. 1986. A recombinant murine retrovirus for simian virus 40 large T cDNA transforms mouse fibroblasts to anchorage-independent growth. *J. Virol.* **60**:290-293.
- Cepko, C. L., B. E. Roberts, and R. C. Mulligan. 1984. Construction and applications of a highly transmissible murine retrovirus shuttle vector. *Cell* **37**:1053-1062.
- Chang, S. M. W., K. Wager-Smith, T. Y. Tsao, J. Henkel-Tigges, S. Vaishnav, and C. T. Caskey. 1987. Construction of a defective retrovirus containing the human hypoxanthine phosphoribosyltransferase cDNA and its expression in cultured cells and mouse bone marrow. *Mol. Cell. Biol.* **7**:854-863.
- Crawford, L., and E. Harlow. 1982. Uniform nomenclature for monoclonal antibodies directed against virus-coded proteins of simian virus 40 and polyoma virus. *J. Virol.* **41**:709.

7. Hand, R. E., Jr., R. W. Tennant, W.-K. Yang, and G. C. Lavelle. 1978. Immunofluorescent analysis of murine leukemia virus-infected cells by flow microfluorometry. *J. Immunol. Methods* **23**:175-186.
8. Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson. 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. *J. Virol.* **39**:861-869.
9. Hogan, B., F. Constantini, and E. Lacy. 1986. Manipulating the mouse embryo: a laboratory manual, p. 262. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. Jacobberger, J. W. 1989. Cell cycle expression of nuclear proteins. In A. Yen (ed.), *Flow cytometry: advanced research and clinical applications*. CRC Press, Inc., Boca Raton, Fla.
11. Jacobberger, J. W., D. Fogelman, and J. M. Lehman. 1986. Analysis of intracellular antigens by flow cytometry. *Cytometry* **7**:356-364.
12. Jat, P. S., C. L. Cepko, R. C. Mulligan, and P. A. Sharp. 1986. Recombinant retroviruses encoding simian virus 40 large T antigen and polyomavirus large and middle T antigens. *Mol. Cell. Biol.* **6**:1204-1217.
13. Linney, E., S. D. Neill, and D. S. Prestridge. 1987. Retroviral vector gene expression in F9 embryonal carcinoma cells. *J. Virol.* **61**:3248-3253.
14. Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**:153-159.
15. Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* **6**:2895-2902.
16. Miller, A. D., D. R. Trauber, and C. Buttimore. 1986. Factors involved in production of helper virus-free retrovirus vectors. *Somatic Cell Mol. Genet.* **12**:175-183.
17. Nolan, G. P., S. Fiering, J.-F. Nicolas, and L. A. Herzenberg. 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β -D-galactosidase activity after transduction of *Escherichia coli lacZ*. *Proc. Natl. Acad. Sci. USA* **85**:2603-2607.
18. Notter, M. F. D., J. F. Leary, and P. C. Balduzzi. 1982. Adsorption of Rous sarcoma virus to genetically susceptible and resistant chicken cells studied by laser flow cytometry. *J. Virol.* **41**:958-964.
19. Strair, R. K., M. J. Towle, and B. R. Smith. 1988. Recombinant retroviruses encoding cell surface antigens as selectable markers. *J. Virol.* **62**:4756-4759.
20. Toyoshima, K., and P. K. Vogt. 1969. Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. *Virology* **38**:414-426.
21. Varmus, H. E., T. Padgett, S. Heasley, G. Simon, and J. M. Bishop. 1977. Cellular functions are required for the synthesis and integration of avian sarcoma virus-specific DNA. *Cell* **11**:307-319.