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Gene Transfer by Retrovirus Vectors Occurs Only in Cells That Are Actively Replicating at the Time of Infection

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Previous reports have shown that retrovirus infection is inhibited in nonreplicating (stationary-phase [hereafter called stationary]) cells. Infection of stationary cells was shown to occur when the cells were allowed to replicate at times up to a week after infection, suggesting that an unintegrated retrovirus could persist in a form that was competent to integrate after release of the block to replication. However, those studies were complicated by the use of replication-competent virus, which can spread in the infected cells. We have used a replication-defective retrovirus vector to compare the efficiency of gene transfer in stationary and replicating rat embryo fibroblasts. In agreement with previous results, gene transfer was inhibited 100-fold in stationary versus replicating cells. In contrast to previously reported results, the block to infection could not be relieved by stimulating stationary cells to divide at times from 6 h to 10 days after infection. Thus, for successful retroviral infection, the infected cells must be replicating at the time of infection. These results have important implications for the use of retroviral vectors for gene transfer.

Productive infection of cells with retroviruses is dependent on events associated with the host cell cycle. Infection of cells arrested in the nonreplicative phase of the cell cycle (stationary-phase [hereafter called stationary] cells) results in a block to virus infection that occurs prior to the production of unintegrated viral DNA (3, 4, 12). However, stationary cells infected with retroviruses can undergo productive infection if the cells are stimulated to divide from 1 day to 1 week after infection, suggesting that some stable viral intermediate can persist in stationary cells (2, 4, 6, 12). This intermediate may be viral RNA or a mixture of partially reverse-transcribed forms.

A complicating factor in these experiments is the use of replication-competent virus that can infect the small proportion of cells that still replicate in "stationary" cell cultures, and then spread to other cells after cell division is stimulated. Although experiments in which neutralizing antibody was used to limit the spread of virus after infection generally support the conclusions stated above (1), the possibility of spread of the virus between adjoining cells still exists. Definitive answers to these questions have important implications for understanding and treating retroviral disease, in which it is important to know whether retroviruses can persist in some form, even in nondividing cells. In addition, retroviral vectors are used increasingly for gene transfer, and it is important to know whether nondividing cells can be infected. For example, in experiments designed to transplant genetically modified hematopoietic cells, it would be useful to know why nonreplicating hematopoietic stem cells are so difficult to infect, since they must proliferate rapidly after being returned to irradiated animals and since retroviral intermediates should therefore persist and integrate.

We have used a replication-defective retroviral vector to examine these questions. The vector carries a *neo* gene, and infection was monitored by the production of G418-resistant colonies. No viral proteins are encoded by the vector, precluding spread of the vector in the infected cells. In support of previous observations, gene transfer was markedly reduced in stationary cells compared with that in dividing cells. In contrast to previous results, stimulation of stationary cells to divide as early as 6 h after infection did not result in an increased vector infection rate, showing that stable retrovirus intermediates are not formed in stationary cells.

MATERIALS AND METHODS

Retrovirus vector production. The retrovirus vector LN (9) contains the neomycin phosphotransferase gene (*neo*), which is transcribed by using the retroviral promoter. The vector was produced by using PA317 amphotropic retrovirus packaging cells (8). The virus was harvested from the vector-producing cell line PA317/LN c11 (9), filtered through a 0.45-μm-pore-size cellulose acetate filter, and stored at −70°C. The titer of the vector was about 10^7 CFU/ml, and no helper virus was detected (9).

Cell culture. 208F rat fibroblasts (10) and vector-producing PA317 cells were grown in Dulbecco modified Eagle medium with a high concentration of glucose (4.5 g/liter) and 10% fetal bovine serum (FBS). To prepare replicating cells, 208F cells were seeded at 5×10^5 cells per 10-cm-diameter dish and were used the next day. Stationary cells were prepared by seeding 208F cells at 10^6 cells per 6-cm-diameter dish and waiting 2 to 4 days until the cells were confluent. At confluence, the medium was changed to Dulbecco modified Eagle medium with a high glucose content (4.5 g/liter), 5% FBS, and 10^{-6} M dexamethasone sulfate (DEX). The medium was replaced every 3 days thereafter, and the cells were used 14 days after reaching confluence.

Labeling and autoradiography of cells. Cells were labeled by exposure to medium containing 10 μCi of [*methyl*-^{3}H]thymidine (50 to 80 Ci/mmol) per ml for up to 48 h. After labeling, the plates were rinsed with 3 ml of cold phosphate-buffered saline and fixed with ethanol-acetic acid (3:1) for 30 min. The cells were rinsed twice with water, and 3 ml of cold 5% trichloroacetic acid was added for 5 to 10 min. Next, 3 ml of 70% ethanol was added for 10 min and then removed, and 3 ml of 100% ethanol was added for 5 min. The plates were allowed to air dry. Autoradiography of the cells was performed with Kodak Nuclear Track Emulsion (NTB-2).

DNA extraction and purification. Unintegrated viral DNA

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was prepared by a modification of the Hirt (5) fractionation procedure. Cells were washed once with phosphate-buffered saline, and 3.8 ml of TNE (1 mM EDTA, 100 mM NaCl, 10 mM Tris, pH 8.0) plus 0.5% sodium dodecyl sulfate was added directly to the plate. Lysed cells were scraped into 5-ml ultracentrifuge tubes, and proteinase K was added to yield a final concentration of 250 μg/ml. The samples were gently mixed and left at room temperature for 10 min. The sodium dodecyl sulfate concentration was increased to 0.85%, the concentration of NaCl was increased to 1 M, and the samples were incubated at 4°C for 24 h. Samples were centrifuged at 42,000 × *g* for 75 min, and the pellet was discarded. RNase A was added to yield a final concentration of 10 μg/ml, and the samples were incubated at 37°C for 15 min. Proteinase K was added to a final concentration of 100 μg/ml, and the incubation was continued for an additional 15 min at 37°C. Samples were then extracted twice with phenol-chloroform (1:1) and once with chloroform-isoamyl alcohol (25:1). Sodium acetate (2 M, 0.1 volume) was added, and the DNA was precipitated with 2 volumes of 100% ethanol. DNA was pelleted by centrifugation at 8,500 rpm in a Beckman HB-4 swinging bucket rotor for 20 min, washed with 70% ethanol, lyophilized, and suspended in water. Total DNA was prepared from cells by standard methods.

RESULTS

Conditions required for inhibition of cell division. 208F rat embryo fibroblasts form a contact-inhibited monolayer that can persist for at least a month without degeneration or cell overgrowth, even in the presence of serum. We measured the rate of 208F cell division at various times after the cells reached confluence by determining the percentage of labeled cell nuclei after incubating the cells with [^{3}H]thymidine for 24 h. The cells were fed every 3 days during the experiment. When the cells were fed with medium containing 5% FBS, the division rate was ≥3% per day at times up to 20 days after the cells reached confluence. Feeding the cells with medium containing 5% FBS and 10^{-7} M DEX further reduced the division rate, which stabilized 10 days after the cells reached confluence, at a rate of 1 to 2% of the cells replicating per day. In contrast, 90% of the cell nuclei in 208F cells growing at low density in medium plus 10% FBS were labeled after 24 h, indicating a high division rate. Thus, for subsequent experiments involving stationary cells, we used 208F cells cultured for 14 days after reaching confluence, and the cells were fed every 3 days with medium containing 5% FBS and 10^{-7} M DEX. These stationary cells appeared healthy, and trypsinization and replating of the cells resulted in rapid resumption of cell growth with little apparent cell death.

Retrovirus vector gene transduction is markedly reduced in stationary cells. We used a replication-defective retroviral vector that expresses the *neo* gene to infect replicating and stationary 208F cells. At several times after infection, the cells were trypsinized and plated in the presence and absence of G418 to measure the percentage of G418-resistant colonies induced by virus infection. The culture medium in all cultures that had not already been trypsinized was replaced at 24 h, which should have removed most of the residual virus. At all time points, the infection efficiency in stationary cells was at least 100-fold lower than that in replicating cells (Fig. 1). Because up to 2% of the cells in the stationary cultures were still replicating, the low transduction frequency observed in the stationary cells can be entirely explained by the infection of the few cells that were still replicating in those cultures. In both stationary and dividing cells, the frequency of G418-resistant colonies increased as a function of time at early time points, presumably reflecting differences in the time of exposure to the virus (and thus the number of infection events) and the time available for *neo* gene expression prior to selection. The infection efficiency reached a plateau at about 2 days after infection of both replicating and stationary cells, reflecting the inability of the retrovirus vector to spread and transduce additional cells.

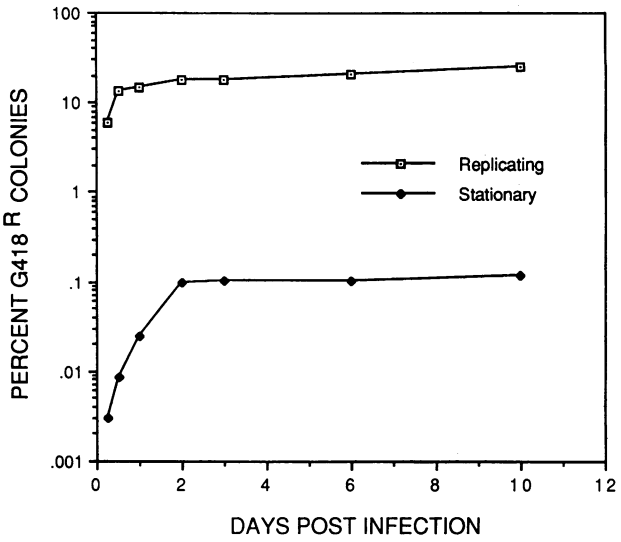

FIG. 1. Vector infection efficiency in stationary and in replicating 208F cells. Stationary and replicating 208F cells were prepared as described in Materials and Methods. The cells were infected with LN virus in the presence of 4 μg of Polybrene per ml at a multiplicity of infection of >2 CFU per cell. At the indicated times postinfection, the cells were treated with trypsin and seeded in the presence and absence of G418 (1 mg of active compound per ml) at a range of dilutions from 1:10 to 1:10,000. Virus-containing medium was replaced with fresh medium 24 h after infection, and every 3 days thereafter, in cultures that had not yet been trypsinized for assay. The percentage of cells that gave rise to G418-resistant colonies was calculated by dividing the number of colonies that grew in the presence of G418 by the number that grew in the absence of G418 (after correction for different dilutions) and expressing this ratio as a percentage. Results are the averages of two separate experiments.

Since the replicating 208F cells in the experiment described above were grown in the absence of DEX, we were concerned that DEX might directly inhibit virus infection, independent of the growth status of the cells. However, replicating cells grown at low density in medium plus 5% FBS and 10^{-6} M DEX were just as infectible (16% G418-resistant colonies when the cells were trypsinized and plated into selective medium 2 days after infection) as cells grown in 10% FBS (Fig. 1). In addition, the marked difference in infectibility between dividing and stationary cells was also seen when the stationary cells were fed every 3 days with medium containing 5% FBS without DEX (data not shown). The absolute rate of infection in stationary cells was slightly higher in cultures without DEX than in cultures with DEX, but this can be explained by the slightly increased rate of division in cultures without DEX. Thus, DEX appears to have no direct effect on infection efficiency in stationary or dividing cells, but it does serve to reduce the background division rate in stationary cell cultures.

Block to infection in stationary cells not relieved by stimu-

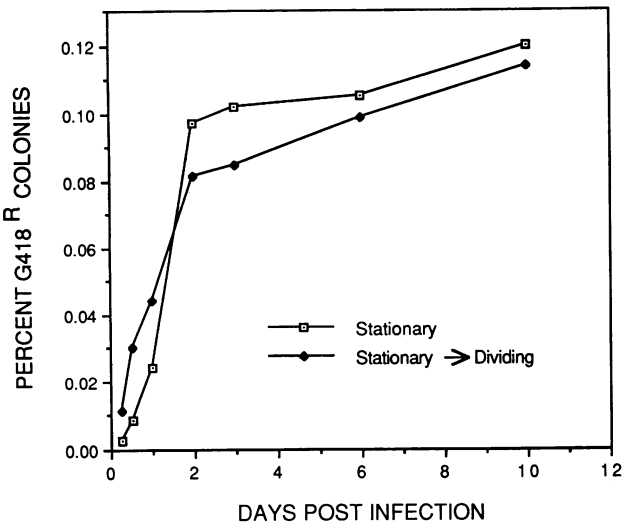

FIG. 2. Gene transfer frequency in stationary cells with or without growth stimulation after infection. Stationary cells were prepared and infected as described in the legend to Fig. 1. At the indicated times after infection, the cells were trypsinized and either assayed immediately for the percentage of G418-resistant cells (⊡) or split 1:10 into medium containing 10% FBS, allowed to grow for 2 days, and then assayed for the percentage of G418-resistant cells (◆). The percentage of cells that gave rise to G418-resistant colonies was calculated as described in the legend to Fig. 1. Results are the averages of two separate experiments.

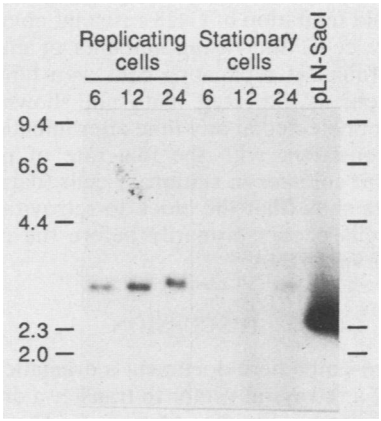

FIG. 3. Quantitation of unintegrated viral DNA after infection of replicating and stationary cells. Replicating cells (10^6 cells per 10-cm-diameter dish) were infected with 200 μl of virus per dish, and stationary cells (5×10^6 cells per 6-cm dish) were infected with 1 ml of virus per dish, to give a multiplicity of infection of about 2 in both cases. At 6, 12, and 24 h after infection (indicated at the top of the figure), unintegrated viral DNA was prepared from individual dishes, as described in Materials and Methods, and analyzed by Southern blotting using a *neo* probe. The majority of the unintegrated DNA observed appears to be linear DNA having the expected size of 2.96 kilobase pairs. *Sac*I-digested pLN retroviral vector DNA was also analyzed (rightmost lane) and has the expected size of 2.36 kilobase pairs. We hypothesize that the faint bands seen at about 5.2 kilobase pairs in several lanes in replicating and stationary cells represent closed circular viral DNA (not supercoiled). All of the low-molecular-weight DNA from a dish was analyzed in each lane, so the number of infected cells analyzed is fivefold higher in the lanes for stationary cells than in those for replicating cells. All samples were analyzed in parallel after electrophoresis in the same gel. The exposure time for autoradiography was 1 week for all lanes. The positions of *Hin*dIII-cut bacteriophage λ DNA size markers are indicated on the sides of the figure. Sizes (in kilobase pairs) are indicated at the left.

lating the cells to divide after infection. It was possible that exposure of stationary cells to retrovirus results in production in infected cells of an inactive intermediate form of the virus which can complete the process of infection if the cells are subsequently stimulated to divide. To test this possibility, stationary cells were infected with the retroviral vector carrying the *neo* gene. At various times after infection, the cells were trypsinized and either assayed directly for the percentage of G418-resistant colonies or assayed for G418-resistant colonies after 2 days of growth at low density in nonselective medium. Allowing the cells to grow prior to selection had no significant effect on the percentage of G418-resistant colonies (Fig. 2). Thus, stimulation of stationary cells to grow as early as 6 h after exposure to virus does not result in a significant increase in the percentage of G418-resistant colonies, showing that whatever infection intermediates that might exist are short-lived. Cells must be actively replicating at the time of exposure to virus for successful infection to occur.

After trypsinization and replating of the stationary cells at low density, there was an obvious increase in the number of cells in the culture plates over the following 48 h, as determined by microscopic observation. To prove that the cells resumed growing, the cells were labeled with [^{3}H]thymidine during the 2-day period following replating. By 16 h after replating of the cells, 11% of the nuclei were labeled, and by 48 h after replating, 86% of the nuclei were labeled, showing that the cells resumed division.

We also determined that the cells were infectible with fresh virus during the period following stimulation of stationary cells to divide. In these experiments, dishes of stationary cells were infected with the retroviral vector carrying the *neo* gene. At 6 h after infection, the cells were trypsinized and replated at a 1:10 dilution in fresh medium. At 16 h after the replating, some of the cells were exposed to additional virus carrying the *neo* gene. At 48 h after the replating, all of the cultures were assayed for the percentage of G418-resistant cells. Without secondary exposure to virus, 0.018% of the cells formed G418-resistant colonies, a result consistent with the data in Fig. 2. When cells were exposed to fresh virus after release from the block to replication, 13% of the cells formed G418-resistant colonies, showing that stationary cells that have been stimulated to divide are indeed infectible during the period of renewed growth, at rates similar to those of cells that have been maintained in log-phase growth (Fig. 1).

Block to infection in nonreplicating cells occurs before formation of unintegrated viral DNA. Low-molecular-weight DNA from replicating and stationary cells infected with the retrovirus vector carrying the *neo* gene was analyzed by Southern blotting, using a *neo* probe to detect unintegrated viral DNAs. Bands of the size expected of linear unintegrated viral DNA (2.96 kilobase pairs) were detected in DNAs from both replicating and stationary cells, but the levels were much reduced in stationary cells (Fig. 3). Note that DNA from five times as many infected stationary cells as infected replicating cells was analyzed, so the real difference in unintegrated DNA is larger than is apparent in Fig. 3. By comparing autoradiographs of different exposure times and correcting for the difference in the numbers of cells analyzed, we estimated that the unintegrated DNA signal was reduced about 50-fold at 6 and 12 h after infection and about 25-fold at 24 h after infection. This reduction is similar

to the 100-fold inhibition of G418-resistant colony formation in stationary cells. Increasing amounts of integrated viral DNA were found in replicating cells as a function of time after infection, as expected (data not shown). Integrated DNA was not detected at any time after infection of stationary cells, consistent with the low rate of production of G418-resistant colonies in stationary cells (data not shown). These results show that the block to retroviral infection in stationary cells occurs primarily before the production of unintegrated viral DNA.

DISCUSSION

Results presented here document a dramatic difference in the ability of a retroviral vector to transfer a drug resistance gene to stationary or dividing fibroblasts. The infection rate was 100-fold lower in stationary cells, and virus infection was blocked at a stage before the formation of the unintegrated viral DNA. We have not determined the exact stage of the block, but previous studies suggest that the block to retroviral infection occurs after virus entry into the cell, because incomplete products of reverse transcription can be detected in infected stationary cells (12). The low rate of infection in stationary cells corresponds with the percentage of cells that are still dividing in these cultures (about 1 to 2% per day), suggesting that even this low rate of infection is due to contaminating dividing cells and that nondividing cells are not infectible.

The block to retroviral vector infection of stationary cells was not released by stimulating the cells to divide at any time from 6 h to 10 days after infection, in contrast to previous results obtained by using replication-competent viruses. Since a replication-competent virus can productively infect the few cells that are still dividing in stationary cultures, a plausible explanation is that viral DNA synthesis and virus production in the previous studies were due to the spread of viruses to uninfected cells and not to persistence of intermediate retroviral forms in stationary cells exposed to the virus. Such spread could occur even in the presence of neutralizing antibodies, by spread between adjoining cells. This conclusion is strengthened by the finding that the majority of unintegrated viral DNA is newly synthesized in stationary cells that have been stimulated to divide (3, 4, 12), consistent with reinfection of cells with newly formed virus.

In contrast to investigators who have employed serum starvation or mitogen-depleted serum for the generation of stationary cells [see above], we have been able to generate stationary cells by using medium containing a normal level of serum and 10^{-6} M DEX. These conditions allow indefinite growth of cells plated at low density and do not inhibit retrovirus vector infection. In contrast, serum starvation ultimately leads to cell death and may have other unpredictable effects on the cells.

We have found that retroviral vectors infect only cells that are replicating at the time of infection. This finding has important implications for the use of retroviral vectors for gene transfer, particularly in the case of gene therapy, in which target cells may not be replicating or may replicate slowly. Thus, for efficient gene transfer, the cells should be stimulated to divide before infection. Support for this prediction comes from experiments with hematopoietic cells, in which prestimulation with growth factors prior to infection appears to improve gene transfer efficiency (7). Results found here are also consistent with the finding that infection efficiency in some cell types is cell cycle dependent, even in replicating cells (11), suggesting that the process of retroviral infection is critically dependent on the cell cycle status of the infected cell.

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