COMMUNICATION TO THE EDITOR

Cell Cycle Dependence of Retroviral Transduction: An Issue of Overlapping Time Scales

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Abstract: Recombinant retroviruses are currently used as gene delivery vehicles for the purpose of gene therapy. It is generally believed that the efficiency of retroviral transduction depends on the cell cycle status of the target cells. However, it has been reported that this is not the case for the transduction of human and murine fibroblasts, in contrast to other cell types such as lymphocytes. The predictions of a mathematical model that we constructed, offer an explanation of this contradiction, based on the dynamics of the underlying processes of target cell growth and the intracellular decay of retroviral vectors. The model suggests that the utility of synchronization experiments, that are usually employed to study cell cycle specificity, is severely limited when the time scales of the above kinetic events are comparable to each other. The predictions of the model also suggest the use of retroviral vectors as cell cycle markers, as an alternative way to detect cell cycle dependence of retroviral transduction. This method obviates the need for cell synchronization and therefore, it does not perturb the cell cycle or interfere with the life cycle of retroviral vectors. Moreover, it does not depend on the intracellular stability of retroviral vectors. Our results show that in contrast to previously reported results, transduction of murine fibroblasts is cell cycle dependent, and they are consistent with the current notion that mitosis is the phase that confers transduction susceptibility. *Biotechnol Bioeng* **58:** 272–281, 1998.
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INTRODUCTION

Gene therapy is a new therapeutic modality that holds significant potential for the treatment of genetic and other disorders that have major impact on cell metabolism. It is defined as the introduction of genes into target cells for the purpose of treating human disease. Most metabolic diseases result either from the absence of an enzyme, or the presence of a defective enzyme that produces a disturbance in a metabolic pathway. Such metabolic abnormalities can be corrected in principle, by delivery of a functional gene into a target cell. Expression of the gene then results in RNA and protein synthesis with the desired biochemical function.

Recombinant retroviruses are currently the vehicle of choice for the transfer of genes into mammalian cells (Anderson, 1992; Crystal, 1995; Hodgson, 1995; Miller, 1992; Mulligan, 1993). It is generally believed that cell division is a requirement for successful replication of oncoretroviruses (Bukrinsky et al., 1992; Chen and Temin, 1982; Fritsch and Temin, 1977; Harel, 1981; Roe et al., 1993; Springett et al., 1989; Varmus et al., 1977; Yoshikura, 1970), but not of lentiviruses such as HIV-1 (Bukrinsky et al., 1992; Lewis and Emerman, 1994). Studies with replication-defective retrovirus have shown that gene transfer was inhibited 100-fold in nonreplicating (confluent) vs. replicating cells (Miller et al., 1990). Moreover, the transduction block could not be relieved by stimulating cells to divide at times from 6 h to 10 d after transduction, suggesting that intracellular transduction intermediates are short-lived. The block to retroviral transduction of nonreplicating cells occurs primarily before the production of unintegrated viral DNA. Thus, for successful retroviral transduction, the target cells must be actively replicating at the time of transduction (Miller et al., 1990). Further support for this finding comes from experiments with hematopoietic cells, in which gene transfer efficiency is improved when they are prestimulated with growth factors (Lim et al., 1989). Others (Crooks and Kohn, 1993) have shown that addition of interleukin-3 (IL-3), IL-6, and Steel factor in cultures of human $CD34^+$ and primitive $CD34^+$ $CD38^-$ bone marrow pro-

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genitor cells, increase virus binding to cell surface receptors. The authors proposed that certain cytokines affect the number and/or affinity of amphotropic receptors, through a proliferation-associated phenomenon, rather than by inducing differentiation.

More recent studies suggest that retroviral integration may depend on mitosis (Lewis and Emerman, 1994; Roe et al., 1993). In situ hybridization showed a sharp increase in the nuclear accumulation of viral DNA when cells traverse through mitosis. The authors hypothesized that viral integration complexes enter the nucleus only after the nuclear envelope is transiently disassembled during mitosis (Roe et al., 1993). Others reported that retroviral transduction of T-lymphocytes depends on the cell cycle status of the target cells (Springett et al., 1989). In contrast, for human and murine fibroblasts the transduction efficiency was found to be cell cycle independent (Springett et al., 1989), suggesting that the efficiency of retroviral transduction depends on the cell cycle status of the target cells, for some cell types but not others.

We have developed a mathematical model that describes the kinetics of retroviral transduction along with the cell cycle kinetics of the target cells (Andreadis and Palsson, 1996). The model is based on the time-maturity formalism proposed by Rubinow (Rubinow, 1968), and describes the dependence of intracellular events of the retroviral lifecycle on different phases of the cell cycle. The predictions of the model suggested that synchronization methods cannot detect cell cycle specificity of retroviral transduction, when the intracellular half-life of recombinant retroviruses is comparable to, or greater than, the doubling time of the target cells. Direct measurements show that the intracellular half-life of recombinant retroviruses is in the range of 5.5–7.5 h (Andreadis et al., 1997).

Our analysis points to the importance of the dynamic interplay of the underlying processes and their time scales. The time scales of the cell growth and intracellular retroviral decay determine the suitability of synchronization experiments to detect cell cycle dependence of transduction. Our purpose in this communication is to provide a theoretical framework that explains experimental results which are based on synchronization methods, and propose an alternative method for the study of cell cycle specificity of transduction.

The method that we propose makes use of the recombinant retrovirus as a cell cycle marker to detect cell cycle distributions of transduced cells. The outcome of the experiments does not depend on the intracellular stability of recombinant retroviruses, because this method effectively ''decouples'' the processes of target cell growth and intracellular decay of retroviruses. Our results show that retroviral transduction of NIH-3T3 murine fibroblasts is cell cycle dependent, and are consistent with the current notion that mitosis is the phase that confers transduction susceptibility.

MATERIALS AND METHODS

Cell Culture

Target NIH-3T3 fibroblast cells were seeded at 3,500 cells/cm^2 in either 6-well plates (Costar, Cambridge, MA) or 10 cm tissue culture dishes at least 24 h prior to exposure to retroviral supernatant. Cells were grown in Dulbecco Modified Eagles Medium (DMEM) with 10% calf serum supplement (Gibco) at 37°C and 5% CO_2. The amphotropic retrovirus packaging cell line was kindly provided by Dr. J. Wilson, and was produced by transfecting the pMFG vector containing the LacZ gene under the Moloney Murine Leukemia Virus (MMuLV) LTR promoter, into ψCRIP packaging cells (Danos and Mulligan, 1988). The packaging cell line was grown under the same conditions as the target cells.

Retrovirus Supernatant

Packaging cells were grown nearly to confluence in DMEM with 10% calf serum supplement. Fresh medium was added to the cells 24 h before the virus supernatant was harvested and filtered through 0.45 μm pore-sized filters (Gelman, Ann Arbor, MI). The filtered viral supernatant was immediately aliquoted and stored at –80°C until use.

Transduction

Prior to exposure of cells to the viral supernatant, polybrene was added at concentration 13.2 μg/mL (as indicated in each experiment) to promote viral entry into the target cells. The time of exposure of cells to the virus was 2–4 h (as indicated in each experiment). After the virus was removed, cells were washed twice with HBSS to remove any unbound virus, and fresh medium was added to the culture. Cells were allowed to grow for 48–72 h before they were stained for flow cytometry.

Hoescht-Labeling

Exponentially growing cells were suspended by use of trypsin, and Hoescht-33342 (DNA label for staining viable cells) was added at a final concentration of 5 μg/mL of cell culture medium for 30 min, while cells were maintained at 37°C and 5% CO_2.

Flow Cytometry

FDG/PI Staining

The product of the transferred LacZ gene reacts with d-β-D-galacto-pyranoside conjugated with FITC (FDG) to form a product readily detectable with flow cytometry. NIH-3T3 cells were prepared as follows. Each sample was washed twice with HBSS, the cells were detached from the substrate with trypsin (0.3 mL), and resuspended in fresh media.

Then, the cells were centrifuged for 10 min at 1000 rpm, resuspended in 20 μL of medium, and placed in a water bath at 37°C for 5 min. Immediately after, 70–100 μL of a hypotonic solution of 2 m*M* FDG was added to each tube, and the tubes were placed in the water bath for 90 sec. The tubes were then immediately placed on ice, and 500 μL of cold (4°C) PBS with 1% (w/v) bovine serum albumin (BSA) and 1 μg/mL of propidium iodide (PI) was added. Samples were left on ice in the dark for at least 1 h before they were filtered and processed for flow cytometry. Only viable cells (PI negative) were used to obtain the fraction of transduced cells. Identification of $PI^{-}FDG^{+}$ cells was based on two control samples: one stained with FDG but without PI, and the other stained with PI but without FDG.

To obtain DNA histograms, after ethanol fixation cells were resuspended in HBSS containing 20 μg/mL PI and 30 μg/mL DNase-free RNase, for at least 30 min at 37°C. The FDG/PI samples were evaluated on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) excluding debris and aggregates from analysis, using standard doublet discrimination (DDM) procedures.

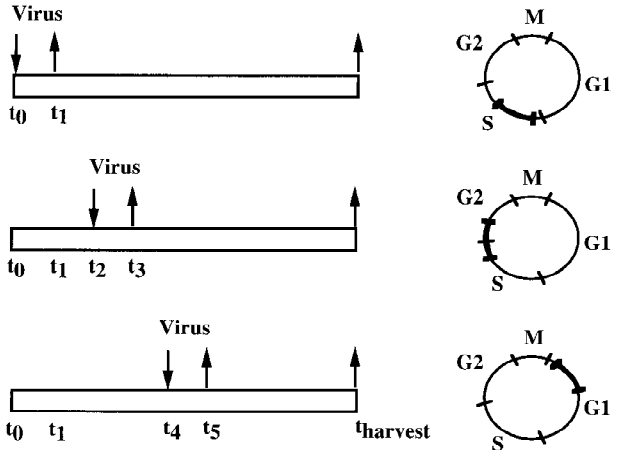

Figure 1. Schematic protocol of transduction of cells synchronized by serum depletion. Each bar represents a synchronized cell population. All samples are allowed to enter the cell cycle simultaneously (t_0). The virus is introduced at different times (t_0,t_2,t_4), when each sample is positioned at different phases of the cycle, as depicted by the heavy lines on the cycle next to each bar. The first two arrows in each bar represent the beginning and end of the time period of exposure of cells to the virus. All samples are exposed to the virus for identical time periods, $t_1 - t_0 = t_3 - t_2 = t_5 - t_4$. Then, they are allowed to grow for 3 d before they are harvested (last arrow pointing up), and the fraction of transduced cells is measured by flow cytometry.

RESULTS

Cell Synchronization by Serum Depletion

To establish the role of the cell cycle status of target cells on the efficiency of retroviral transduction, serum deprivation was initially used as a method of cell synchronization. Serum-containing medium was removed from the cell cultures and medium without serum added for 24 h. This resulted in cell cultures with 75–80% of the cells in G_1 phase of the cell cycle, as shown by flow cytometric analysis of the DNA content of the cells. After 24 h of serum depletion, the cultures were supplied with serum-containing medium, and cells were allowed to grow. At different times following the addition of serum, duplicate samples of cells were exposed to the virus for 4 h. Therefore, at the time of exposure to the virus, cells in different samples were located at different phases of the cell cycle. This procedure is schematically shown in Figure 1. If retroviral transduction were cell cycle dependent, various samples located at different positions in the cell cycle would show different levels of transduction. The efficiency of transduction would show an oscillatory behavior for at least two cell generations (Cooper, 1991), that is the time that cells still remain synchronous.

The results of this experiment are shown in Figure 2. The efficiency of transduction is plotted as a function of the time of exposure of cells to the virus. Initially, the transduction efficiency increases with time until it reaches a maximum, where it remains almost unchanged for a period of 12–16 h. At later times, it decreases continuously to low levels of transduction. The initial increase in transduction supports the idea of cell cycle dependence. However, there are two points that should be mentioned here. First, the behavior is not repeated for cells exposed to the virus in the second cell generation (Cooper, 1991), that is transduction does not follow an oscillatory behavior. Therefore, the initial increase could be attributed to a cell cycle perturbation, or to the fact that cells do not start cycling immediately after serum is added to the culture. Rather, there is a delay period that is responsible for the initial low transduction efficiencies. Although this argues for a requirement for cell replication in order for transduction to occur effectively, it does not necessarily mean that the process of retrovirus transduction is cell cycle dependent. The latter means that a particu-

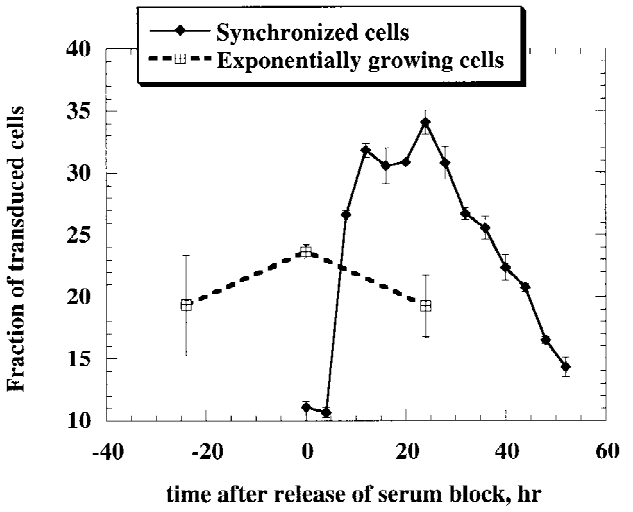

Figure 2. Cell synchronization by serum depletion. Fraction of transduced cells as a function of the time of exposure of synchronized cells to the virus.

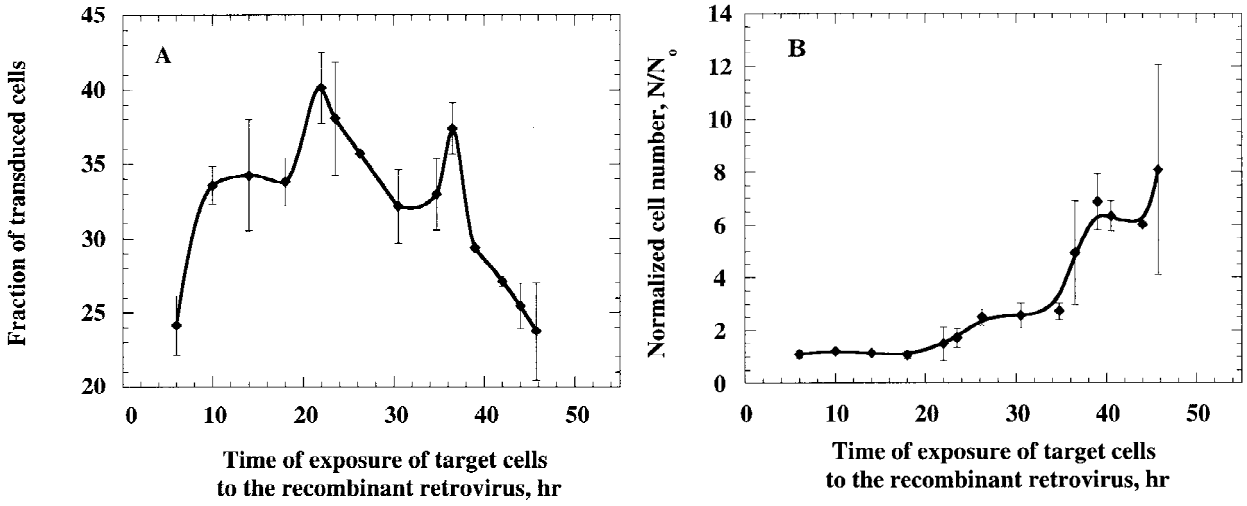

Figure 3. Cell synchronization by cell sorting using flow cytometry. Cells were labeled with Hoescht, and G_1 cells were sorted and exposed to the virus for 4 h, at various times following the cell sort. (**A**) Fraction of transduced cells as a function of the time of exposure to the virus. (**B**) Number of cells normalized to the initial cell number, as a function of the time of exposure to the retrovirus. The kinetic pattern of cell growth is characteristic of a synchronized cell population.

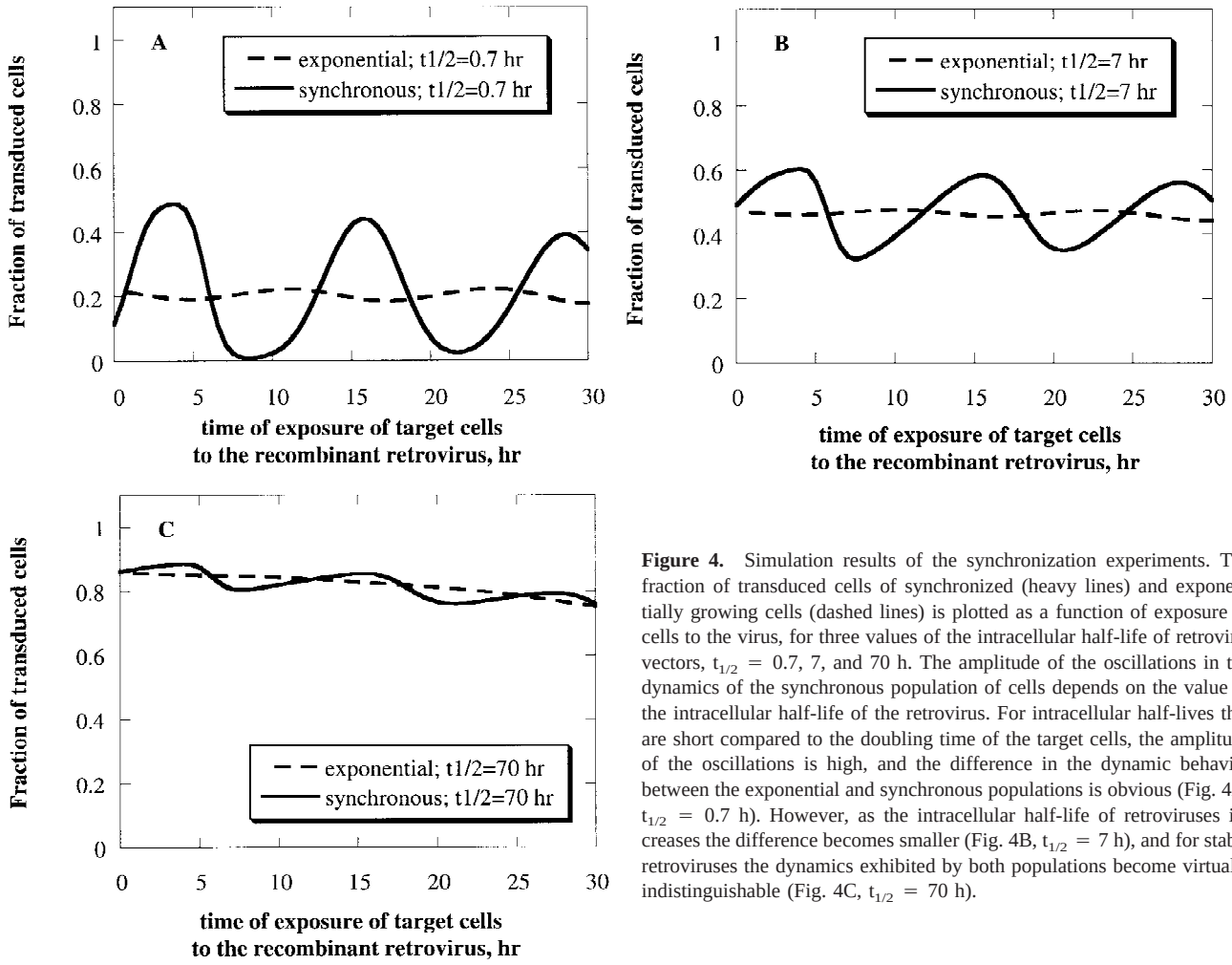

Figure 4. Simulation results of the synchronization experiments. The fraction of transduced cells of synchronized (heavy lines) and exponentially growing cells (dashed lines) is plotted as a function of exposure of cells to the virus, for three values of the intracellular half-life of retroviral vectors, $t_{1/2}$ = 0.7, 7, and 70 h. The amplitude of the oscillations in the dynamics of the synchronous population of cells depends on the value of the intracellular half-life of the retrovirus. For intracellular half-lives that are short compared to the doubling time of the target cells, the amplitude of the oscillations is high, and the difference in the dynamic behavior between the exponential and synchronous populations is obvious (Fig. 4A, $t_{1/2}$ = 0.7 h). However, as the intracellular half-life of retroviruses increases the difference becomes smaller (Fig. 4B, $t_{1/2}$ = 7 h), and for stable retroviruses the dynamics exhibited by both populations become virtually indistinguishable (Fig. 4C, $t_{1/2}$ = 70 h).

lar biochemical event in the viral life cycle occurs only at a particular phase of the cell cycle and therefore, it is a stronger statement. To our knowledge, in all cell cycle studies, synchronization experiments stop at t = 16 to t = 20 h after the cell cycle block is removed and therefore, the long-term behavior of transduction is not captured. Second, the decrease of the efficiency of transduction after t = 32 h is not expected. At these time points, cells lose their synchrony and obtain an exponential distribution. Therefore, the transduction efficiency should decrease and then stay constant for all later times, and equal to the transduction efficiency of an exponential cell population. Currently, the reasons for this behavior are unknown. They probably could be attributed to long-term effects of serum depletion on cell metabolism and perhaps, on the retroviral life cycle.

Cell Synchronization by Flow Cytometric Cell Sorting

To avoid the unknown effects of serum starvation on cell metabolism, flow cytometry was used to sort cells in a particular phase of the cell cycle; the above protocol (Fig. 1) was repeated to investigate cell cycle specificity of retroviral transduction. NIH-3T3 cells were sorted in G_1 phase of the cell cycle with a flow cytometer. Multiple parallel cultures were established from the sorted cell population, and they were exposed to the virus at different times after plating. For each exposure time, the fraction of transduced cells (in duplicate samples) was determined by flow cytometry, and the number of cells counted (in separate parallel duplicate samples) to determine the synchrony of growth.

A clear difference in transduction efficiencies between the base level t = 0 and the maximum t = 22 h was observed (Fig. 3A). At t = 0 h transduction is very low, probably because of the delay period between the time that cells were plated on the growth surface and the time that they started cycling. During this period, the majority of cells were not actively cycling and therefore, they could not be transduced productively. At t = 22 h there was a peak in transduction efficiency that corresponded to the time just prior to the first division (Fig. 3B). A second peak in transduction efficiency at t = 36.5 h was observed, and it corresponded to the time just prior to the second cell division. The periodic behavior in the transduction frequency supports the result that retroviral transduction is cell cycle specific. It is also consistent with the current consensus that mitosis is the cell cycle phase that confers transduction susceptibility, because both maxima of transduction efficiency correspond to the time just prior to cell division. However, the difference in transduction efficiencies between the peaks and the valleys was too small to allow for definite conclusions about cell cycle dependence (Fig. 3A). Given the small differences in the peaks and valleys, alternative strategies are clearly needed to distinguish the relative importance of cell cycle on retroviral transduction.

Theoretical Explanation of Experimental Results

To explain the above results, we used a mathematical model to simulate the synchronization experiments described (Andreadis and Palsson, 1996). The model describes the kinetics of retroviral transduction, along with the kinetics of the cell cycle. It accounts for the events of retrovirus binding and entry into the cell, intracellular inactivation, and integration of retroviral DNA into the genome of the target cells. Three different cell populations are defined, and rate law equations are written for each one of them—non-transduced, virus carrier, and transduced cells. Cells that contain virus in their cytoplasm but not an integrated provirus are called *virus carrier cells.* Integration can then take place to produce transduced cells. Carrier cells can either become *transduced,* if the virus integrates during mitosis, or *non-transduced* cells, if the viral intermediates degrade be-

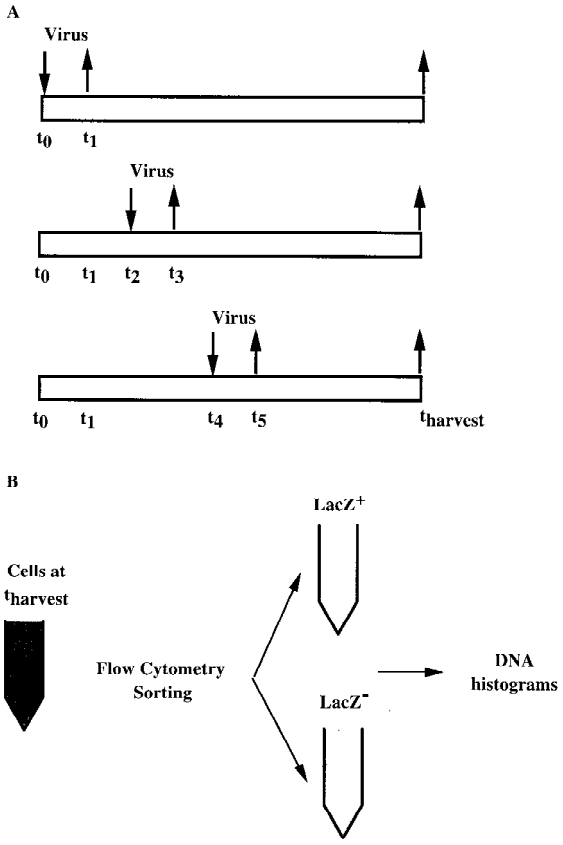

Figure 5. Schematic protocol of the experiment that uses the virus as a cell cycle marker, to study cell cycle dependence of retroviral transduction. Each bar represents an exponentially growing cell population. Parallel cultures of exponentially growing cells were transduced at various times. The first two arrows in each bar represent the beginning and end of the time period of exposure of cells to the virus. The last arrow represents the end of the experiment when cells are harvested and processed for analysis to determine the transduction efficiency. All cultures were harvested simultaneously and the $LacZ^+$ (transduced) and $LacZ^-$ (non-transduced) cells are sorted and stained with PI; the DNA histograms were obtained by flow cytometry. Thus, DNA distributions of transduced cells were determined at different times following virus internalization.

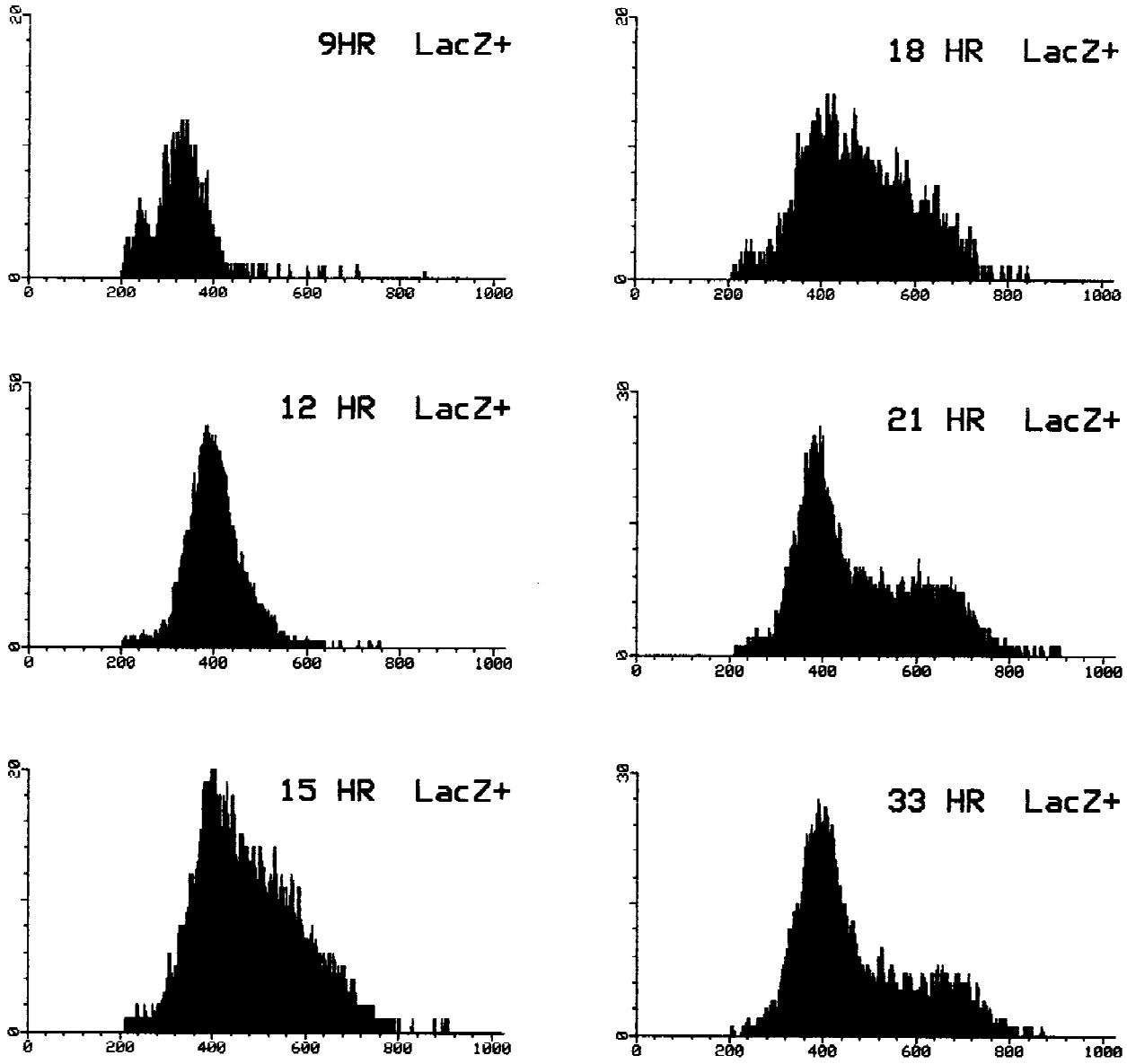

Figure 6A. Time sequence of DNA histograms of transduced cells at various times following short-term exposure to the virus. The number of cells is plotted as a function of the fluorescence intensity. The first peak represents cells with unreplicated DNA (one arbitrary unit) and belong in G_1 phase. The second peak represents cells with fully replicated DNA (two arbitrary units), and are G_2-or M-phase cells. The intermediate population consists of S-phase cells, with an intermediate amount of DNA. The fraction of transduced cells was determined with flow cytometry by detecting the cells that express β-galactosidase. Both the $LacZ^+$ and $LacZ^-$, transduced and non-transduced subpopulations, respectively, were sorted; and DNA stained with propidium iodide (PI) to determine their DNA distributions. A parallel unsorted sample was stained with PI as control, to verify that the total population was exponentially growing. Chicken red blood cells were added to all samples and served as a control.

fore integration of DNA into the host chromosomes. Simulations were carried out for synchronous and exponentially growing cell populations, for different values of the intracellular half-life of the virus. The intracellular stability of retroviral vectors is defined as their ability to integrate and express the marker or therapeutic gene as a function of the time spent inside the cell, that is the time between virus internalization and DNA integration.

In Figure 4, the efficiency of gene transfer is plotted as a function of the time of exposure of cells to the retrovirus, t_0, for three values of the intracellular half-life of the virus, $t_{1/2}$. For intracellular retroviral half-lives that are short compared to the doubling time of the target cells, $t_{1/2} = 0.7$ h (the doubling time of NIH-3T3 cells is 12–14 h), the dynamic behavior of the transduced synchronized cell populations is clearly different from the behavior of transduced exponentially growing cells (Fig. 4A). The transduction efficiency of a synchronized cell population exhibits an oscillatory behavior, while that of the exponential population is constant. As the virus becomes more stable compared to the doubling time of the cells, $t_{1/2} = 7$ h, the amplitude of the oscillations of the synchronized cell population decreases

(Fig. 4B). For long intracellular retroviral half-lives, $t_{1/2}$ = 70 h, the amplitude of oscillations of the synchronized cell populations is so small, that the dynamics of transduction become virtually indistinguishable from the transduction dynamics of exponentially growing cells (Fig. 4C). Therefore, the predictions of the mathematical model suggest that intracellular retroviral half-lives comparable to, or longer than, the doubling time of the cells severely limit the utility of synchronization studies, because cells close to, and far from, the susceptible phase are transduced with similar probabilities and exhibit similar transduction efficiencies.

Furthermore, in real experiments, synchronization is never perfect and a certain fraction of the population escapes the block, thus reducing the differences in transduction between different subpopulations. Consequently, the amplitude of the oscillations of the synchronized cells decreases. Moreover, all induction synchronization methods make use of chemicals such as aphidicolin, or they prevent cells from cycling by nutrient depletion. These chemicals have toxic effects on cells; and serum starvation has profound, yet unknown effects on cell metabolism (Cooper, 1991). Therefore, use of these methods may cause perturbations of the cell cycle (Cooper, 1991), and may interfere with events in the life cycle of recombinant retroviruses, thus biasing the experimental results.

Recombinant Retrovirus Can Be Used As a Cell Cycle Marker to Determine Cell Cycle Dependence of Retroviral Transduction

To overcome the problems of cell synchronization and dependence of the results on the intracellular stability of retroviruses, an alternative approach was pursued. The virus can be used as a cell cycle marker that labels cell subpopulations which go through the phase that confers transduction susceptibility. The DNA distribution of transduced cells—the labeled cells—monitored over time, can be used to determine cell cycle dependence of retroviral transduction. If there is no cell cycle dependence, then cells at all stages of the cell cycle have the same probability of being transduced. Therefore, the transduced population should exhibit DNA distributions characteristic of exponentially growing cells at all time points. In contrast, if mitosis is required for viral DNA integration (Roe et al., 1993), then shortly after exposure to the virus, the transduced cells should be in G_1 phase of the cell cycle.

Parallel cultures of exponentially growing cells were transduced at different times. Exposure of cells to the virus was short (1.5 h), so that the cells did not progress significantly in the cell cycle during this period. All cultures were harvested simultaneously, and thus DNA distributions of transduced cells were determined at different times following virus internalization (Fig. 5). The time difference between the end of the experiment, $t_{harvest}$, and the time of exposure to the virus represents the time period that the cells were allowed to grow after virus internalization into the cell cytoplasm. The fraction of transduced cells was determined with flow cytometry by detecting the cells that express β-galactosidase. Both the $LacZ^+$ and $LacZ^-$, transduced and non-transduced subpopulations, respectively, were sorted and DNA stained with propidium iodide (PI) to determine their DNA distributions (Fig. 5). A parallel unsorted sample was stained with PI as control, to verify that the total population was exponentially growing.

The experimental results show that almost all transduced cells show up in G_1 phase of the cell cycle shortly after exposure to the virus (fraction of G_1 cells ~95%), and thus support cell-cycle dependence of transduction (Figs. 6A,B). Furthermore, they are consistent with the result that M phase confers transduction susceptibility (Roe et al., 1993).

The changes in the DNA distribution of transduced cells over time depends on the intracellular stability of the vector (Andreadis et al., 1997). This is explained with a schematic in Figure 7. The top two panels (A,E) show the exponential distribution of target cells. The hatched region is mitosis that confers transduction susceptibility (Roe et al., 1993). The double-hatched area represents the population of cells that are susceptible to retroviral transduction, and depends on the intracellular stability of recombinant retroviruses. If the internalized virus is very unstable, then only cells that are very close to the permissible phase can be transduced (small double-hatched area, Fig. 7A). Viruses present in cells that are in other phases of the cell cycle are more likely to degrade than to integrate. Therefore, the initially transduced cells will move through the cycle as if they were synchronous, since no additional transduced cells enter G_1 (Figs. 7B,C,D at times t_1,t_2,t_3). In contrast, if the vector is stable, then all cells are equally likely to be transduced (wide double-hatched area, Fig. 7E). As time goes on, transduced cells will move to subsequent phases of the cycle, while more newly transduced cells will show up in G_1 (Figs. 7F,G at times t_1,t_2). Therefore, transduced cells will quickly obtain a DNA distribution characteristic of exponentially growing cells (Fig. 7H at time t_3). This argument has been

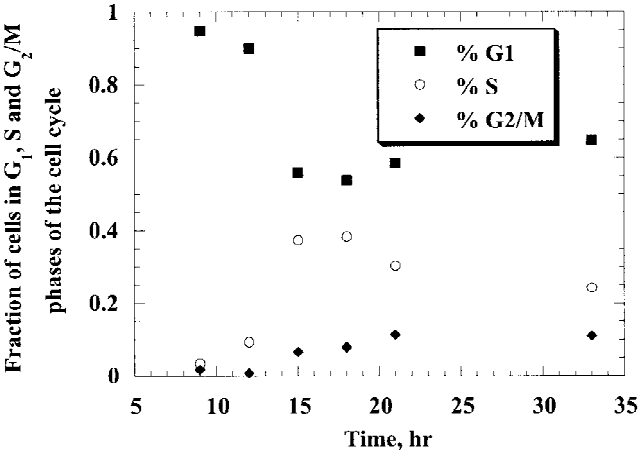

Figure 6B. Fraction of G_1- and S-phase cells in the population of transduced cells, at different times after exposure of cells to the virus. The fractions of cells in the phases of the cell cycle were obtained by analysis of DNA histograms with the software MODFIT.

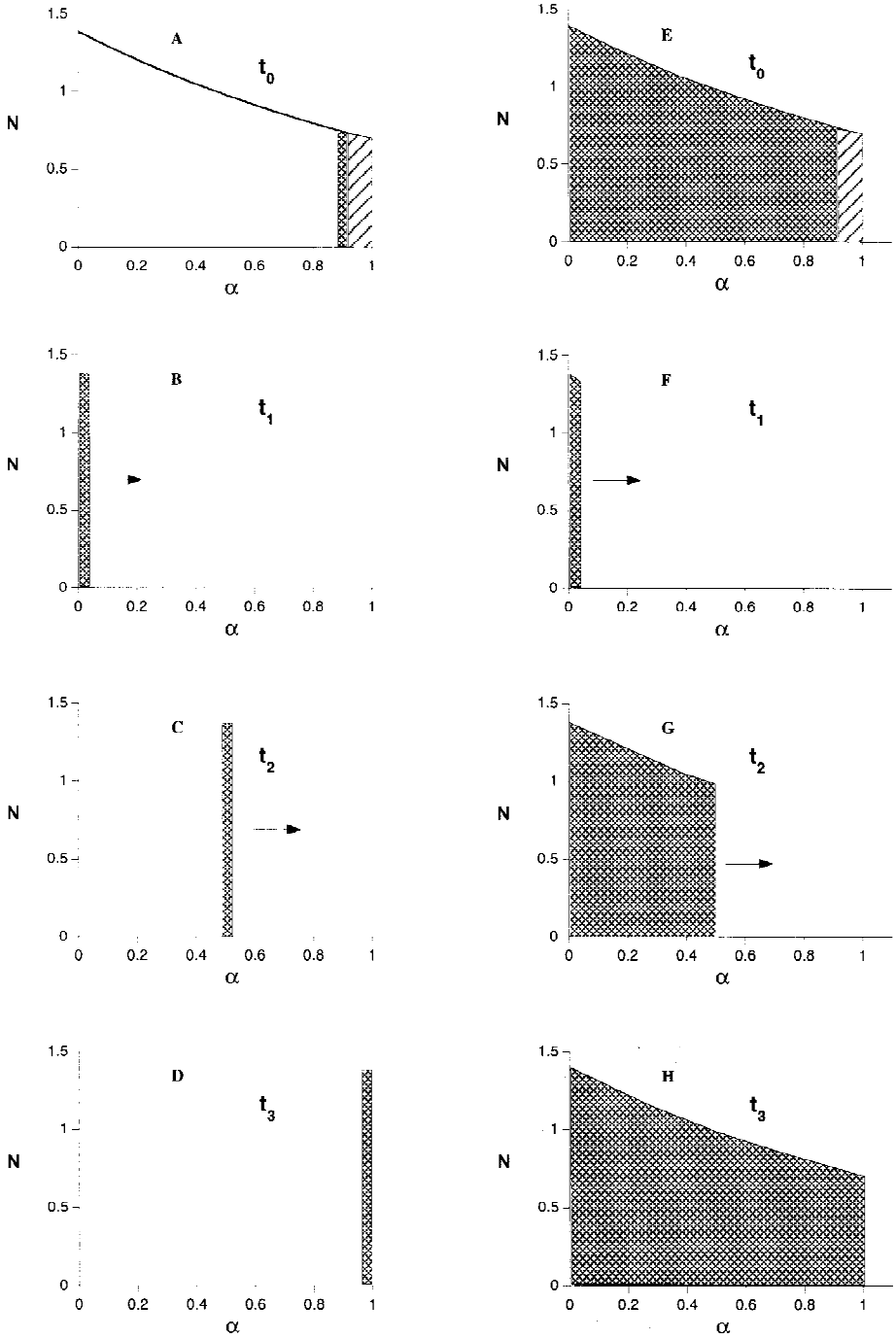

Figure 7. Schematic explanation of cell cycle (maturity) distributions of transduced cells, at various times after exposure of cells to the virus. For details see text.

verified by the predictions of the mathematical model that describes the process of retroviral transduction along with the kinetics of cell growth (Andreadis and Palsson, 1996).

Thus, the time sequence of DNA histograms can provide an order of magnitude estimate of the intracellular stability of the virus. Transduced cells obtained an exponential distribution (fraction of G_1 cells ~50–55%) in almost 12 h (Figs. 6A,B) (starting at $t = 9$ with all transduced cells in G_1, the DNA distributions were exponential at $t = 21$ h, when the fraction of mitotic cells reaches a steady level). Although these results do not represent a direct measurement, they suggest that the intracellular half-life of recombinant retroviruses is not much shorter than the doubling time of NIH-3T3 cells. However, a half-life that is comparable to, or longer than, the doubling time would also be consistent with the results. To determine the intracellular stability of recombinant retroviruses, two new experimental methods were developed in our laboratory. The results show

that MuLV-derived retroviral vectors decay intracellularly with a half-life that ranges between 5.5–7.5 h (Andreadis et al., 1997).

The fact that the DNA distributions change with time (Figs. 6A,B) excludes the possibility that transduced cells were cell cycle arrested in G_1, due to virus transduction. Had this been the case, all cells would be in G_1 phase for all time points after transduction. Cells move through the cell cycle and eventually, they obtain an exponential distribution, thus ruling out the possibility that retroviral transduction caused cell cycle arrest.

We studied the cell cycle dependence of retroviral transduction, by a method that views the recombinant retrovirus as a cell cycle marker to determine the DNA distributions of transduced cells. This method eliminates the difficulties inherent to cell cycle synchronization experiments. It also determines cell cycle dependence irrespective of the intracellular stability of retroviruses. Therefore, it can be used to study cell cycle dependence of transduction of slowly, as well as rapidly, growing target cells. The results show that transduction of murine fibroblasts by murine-based retroviral vectors is cell cycle dependent, and are consistent with the current notion that mitosis is the phase that confers transduction susceptibility.

DISCUSSION

Retroviral transduction is generally believed to be dependent on the cell cycle status of the target cells (Lewis and Emerman, 1994; Miller et al., 1990; Roe et al., 1993; Springett et al., 1989). However, in contrast to T-lymphocytes, transduction of human and murine fibroblasts by retroviral vectors has been reported to be cell cycle independent (Springett et al., 1989). The theoretical predictions of a mathematical model that describes the process of retroviral transduction showed that synchronization experiments depend on the intracellular half-life of retroviral vectors. They cannot be used to study cell cycle dependence, when the intracellular half-life of recombinant retroviruses is comparable to, or longer than, the time scale of target cell growth. In this case, the transduction efficiency of synchronous and exponential cell populations exhibit virtually indistinguishable dynamics. Moreover, synchronization experiments are limited by two additional factors. First, synchronization is never perfect, because a significant fraction of cells escapes the block, and proceeds through the cycle. Second, synchronization methods perturb the cell cycle and cell metabolism, and may interfere with events of the life cycle of retroviral vectors. Therefore, intracellular viral half-lives comparable to, and not only longer than the doubling time can impose limitations on the utility of synchronization experiments. If a synchronized population shows a behavior that cannot be clearly distinguished from that of an exponential population, then an experiment may suggest that transduction is not cell cycle dependent. While in reality, this result would just reflect the inability of the synchronization approach to determine cell cycle specificity, for long intracellular viral half-lives.

In the new approach, the virus is thought of as a cell cycle marker that labels cells only when they traverse mitosis. Therefore, the cell cycle distributions of transduced cells can be used to answer the question of cell cycle dependence of retrovirus-mediated gene transfer. This experimental method is relatively simple, and it obviates the need for cell synchronization. Furthermore, it provides an estimate of the intracellular stability of the viral vectors. The transduced cells obtain an exponential distribution in almost 12 h. This suggests that the intracellular half-life of the virus is not much shorter than the doubling time of the target cells. This prediction has been verified by direct measurements of the intracellular half-life of MuLV-derived retroviral vectors in NIH-3T3 cells, which showed that retroviral intracellular half-life ranges between 5.5–7.5 h (Andreadis et al., 1997).

In this communication, we provide a framework to explain the experimental results of the studies of cell cycle dependence of retroviral vectors. Our analysis demonstrates that synchronization methods are not suitable to study cell cycle dependence of retroviral transduction, because of the overlapping time scales of intracellular retroviral decay and the target cell growth. To overcome this limitation, an alternative method is proposed. It is based on the cell cycle distributions of the transduced target cells and does not depend on the time scale of retroviral decay. The results show that retroviral transduction of murine NIH-3T3 cells is cell cycle dependent, and support the finding that mitosis confers transduction susceptibility. They also suggest that intracellular events may limit the efficiency of gene transfer, because only a fraction of cells with internalized virions will become transduced. The dynamic interplay between cell-cycle and retroviral life-cycle events shows the importance of systemic kinetic factors, that determine the efficiency of retrovirus-mediated gene transfer (Palsson and Andreadis, 1997; Chuck et al., 1996), especially for slowly growing target cells such as the stem cells of the hematopoietic system.

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