



# Cationic lipid-mediated transfection of cells in culture requires mitotic activity

I Mortimer, P Tam, I MacLachlan, RW Graham, EG Saravolac and PB Joshi  
*Inex Pharmaceuticals Corporation, Burnaby, British Columbia, Canada*

Cationic lipid-based delivery systems such as lipoplexes or stabilized plasmid–lipid particles (SPLP) represent a safer alternative to viral systems for gene therapy applications. We studied the impact of cell cycle status on the efficiency of transfection of human ovarian carcinoma tumor cells using two cationic-lipid based delivery systems. Cells arrested in the G1 phase of the cell cycle by treatment with aphidicolin were compared with an asynchronous dividing population of cells. Treatment of the cells with aphidicolin had no effect on the rate of internalization of the lipid formulated DNA or on the level of gene expression observable in stably transfected cells. However, cells treated with

aphidicolin exhibited 20-fold lower reporter gene activity than asynchronous control cells upon incubation with lipoplexes. When cells arrested in the G1 phase were allowed to proceed through the cell cycle in the presence of the lipoplex or SPLP, transgene expression was found to coincide with the transition of cells from the G2/M phase into the G1 phase of the subsequent cell cycle. In addition, higher levels of reporter gene expression were observed when the cells were incubated with lipoplexes or SPLP during, or just before, mitosis. These results suggest that it may be possible to augment cationic lipid-mediated transfection by manipulating the cell cycle status of the target cells.

**Keywords:** gene therapy; cationic lipids; cell cycle

## Introduction

Nonviral transfection systems provide a safer alternative to viral systems for gene therapy. Although viral transfection systems cause efficient expression of transgenes within target cells, they suffer from several theoretical and practical disadvantages. They are difficult and hazardous to prepare, they have the potential to elicit an acute immune response upon interaction with pre-existing host antibodies against wild-type viruses and they have the ability to undergo recombination with wild-type viruses to generate viral particles with undefined properties. In addition, viral systems have a finite capacity for the size of foreign transgene due to packaging constraints. These features drastically limit the practical utility of viral systems for gene therapy.

In contrast, cationic lipid-based transfection systems are easy and safe to prepare, have low immunogenicity, and do not restrict the size of DNA that can be delivered.<sup>1–6</sup> Despite these clear advantages, nonviral transfection systems result in substantially less gene expression than that observed with viral systems. Most cationic lipid/DNA-based systems are taken up efficiently through an endocytosis pathway,<sup>7–10</sup> but the exact mechanisms of DNA release from the endosomes and the subsequent translocation to the nucleus are not clear.

One barrier to efficient nonviral transfection may be the nuclear translocation of cytoplasmic DNA.<sup>7</sup> It has been speculated that entry of exogenous DNA into the

nucleus occurs only in cells that are actively replicating and that the breakdown of the nuclear envelope during mitosis may be necessary for the nuclear uptake of exogenous DNA.<sup>7,11–14</sup> In contrast, certain viral vectors, such as the lentivirus HIV-1, have the ability to infect nondividing cells<sup>15–17</sup> and thus cellular infection with these vectors is independent of the cell cycle status of the target cell. The impact of cell cycle status in nonvirus-based transfection of mammalian cells is not well understood.

The goal of this study was to examine the relationship between cell cycle status and transfection efficiency using two distinct cationic lipid-based gene delivery systems: aggregates of cationic liposomes and plasmid DNA, termed lipoplexes,<sup>18</sup> and lipid encapsulated stabilized plasmid–lipid particles (SPLP).<sup>19</sup> The lipoplexes used in this study were prepared using the cationic lipid dioleoyldimethylammonium chloride (DODAC) in a 1:1 mixture with the fusogenic lipid 1,2-sn-dioleoylphosphatidylethanolamine (DOPE). The other cationic lipid transfection system examined here, SPLP, are fully encapsulated DNA particles, assembled using a detergent dialysis method.<sup>19</sup> The encapsulated system differs from lipoplexes in terms of its method of assembly, uniform morphology, smaller size (70–150 versus 500–700 nm) greater serum stability and ability to protect the plasmid from serum nucleases.<sup>19</sup> SPLPs are of potential interest in nonviral gene transfer due to their improved pharmacokinetic properties and the potential to add targeting ligands to the exterior surface.<sup>19</sup>

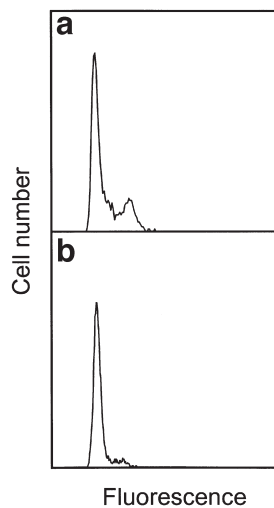
Understanding the relationship between cell cycle and transfection should provide insight into the mechanism of cationic lipid-mediated transfection which will enable the rational engineering of these systems for gene therapy applications.

## Results

### *Cells are efficiently blocked in the G1 phase after treatment with aphidicolin*

Numerous reagents and protocols are available that arrest cells in specific stages of the cell cycle. For the present study it was important to utilize reagents and conditions that would cause effective cell cycle arrest without adversely affecting cellular processes related to transfection, such as endocytosis, transcription and translation. Aphidicolin inhibits DNA polymerase  $\alpha$  and  $\delta$ , the two major polymerases involved in DNA synthesis during the S phase.<sup>20,21</sup> Aphidicolin should specifically arrest cells at the G1-S boundary without affecting processes involved in transfection. In an initial study, we optimized the conditions for employing aphidicolin to arrest human ovarian tumor cells, SK-OV-3 effectively. A typical subconfluent asynchronous cell culture contained approximately 75, 5 and 20% of cells in the G1, S and M phases, respectively, as measured by staining with propidium iodide (PI) followed by flow cytometry (Figure 1a). Treatment of cell cultures with aphidicolin at a final concentration of 5  $\mu\text{g}/\text{ml}$  for 16 h caused more than 95% of the cells to become arrested in the G1 phase of the cell cycle (Figure 1b). All subsequent experiments utilized these conditions for cell cycle arrest.

In order to determine whether aphidicolin treatment affects cellular processes related to transgene expression, effects on DNA uptake and gene expression were analyzed. It had been shown previously that the uptake of lipoplexes prepared with fluorescently labeled plasmid DNA could be measured by flow cytometry.<sup>7,22,23</sup> We initially determined the extent of fluorescence detected by this assay that is due to cell surface-associated SPLP compared with internalized SPLP. Since internalization

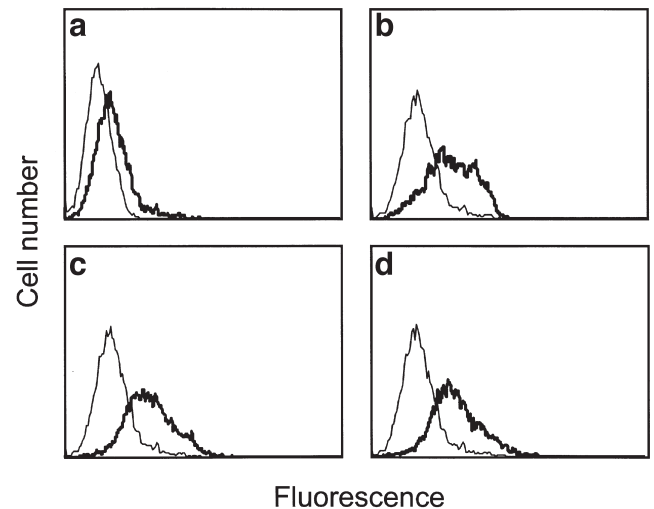


**Figure 1** Flow cytometry analysis of SK-OV-3 cells. Cells were treated for DNA content analysis as described in Materials and methods. Histograms of relative propidium iodide fluorescence versus number of cells are shown. (a) An asynchronous culture containing proliferating cells gives a characteristic trimodal profile. The peak to the left corresponds to cells in the G1 phase of the cell cycle with 2N amount of DNA; the peak to the right corresponds to cells in the G2 phase with 4N amount of DNA. The shoulder between the two peaks corresponds to the S phase in which the heterogeneous cells have a variable DNA content between 2N and 4N. (b) The aphidicolin-treated cultures were effectively synchronized, with more than 95% of the cells arrested in G1 phase of the cell cycle.

of cationic lipid-formulated DNA is via the energy-dependent process of endocytosis,<sup>7,8,10</sup> we hypothesized that incubating cells with formulated fluorescent plasmid at 4°C would permit interaction at the cell surface but prevent endocytosis. Any cell-associated fluorescence detected after this treatment followed by washing would thus represent formulated plasmid stuck at the cell surface.

Cells were thus incubated at 4°C for 1 h with SPLP containing YOYO-1-labeled plasmid and processed and analyzed by flow cytometry. Figure 2a shows that the method of processing cells utilized in this assay retains some cell surface-associated SPLP. A similar level of cell surface-associated fluorescence was also observed upon incubation of cells at 4°C with lipoplexes (not shown). In subsequent analysis to measure internalization, the fluorescence from cells incubated with YOYO-1-labeled SPLP at 4°C was used as a negative control.

Cells arrested with aphidicolin were incubated with SPLP containing YOYO-1-labeled plasmid DNA in the absence or continued presence of aphidicolin and were compared with control asynchronous cells that had not been exposed to aphidicolin. The rate of SPLP uptake per cell was determined by flow cytometry. Figure 2 shows that the SPLP were efficiently internalized in all cultures with more than 75% of the cells exhibiting fluorescence after 1 h of incubation. The level of fluorescence inside



**Figure 2** DNA uptake in SK-OV-3 cells. Histograms of relative YOYO-1 iodide/DNA fluorescence versus number of cells obtained by flow cytometry are shown. Plasmid DNA was labeled with YOYO-1 iodide and formulated as SPLP. SK-OV-3 cells were incubated with fluorescent SPLP for 1 h and processed for flow cytometry as described in Materials and methods. (a) Fluorescence after incubation of cells with SPLP at 4°C. The histogram on the left is due to autofluorescence from control untreated cells. The histogram on the right shows the fluorescence associated with cells after incubation at 4°C and hence represents fluorescence from surface-bound SPLP. After a 16-h incubation with aphidicolin to arrest cells in the G1 phase of the cell cycle, cultures were incubated with fluorescent SPLP for 1 h at 37°C in the continuous presence of aphidicolin (b) or in the absence of aphidicolin to remove the cell cycle arrest (c). An asynchronous culture that had never been exposed to aphidicolin (d) was used as a control. Cells were harvested as described in Materials and methods at the time-points indicated, and were analyzed by flow cytometry. The histogram to the left in panels b, c and d is the fluorescence profile of cells incubated with YOYO-1-labeled SPLP for 1 h at 4°C and represents a negative control. The increase in fluorescence relative to the 4°C control indicates internalization of SPLP.

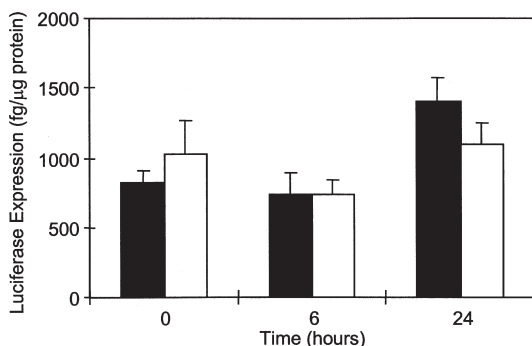
the cells reached a maximum within 1 h and remained at this level for at least 4 h (not shown). Thus, the rate of accumulation of fluorescently labeled DNA within cells arrested with aphidicolin was comparable to control cells that had not been exposed to aphidicolin. These results demonstrate that cell cycle arrest by aphidicolin treatment does not alter the internalization of SPLP by SK-OV-3 cells. Similar studies showed that the uptake of lipoplexes is also unaffected by aphidicolin treatment (not shown).

To determine the effect of aphidicolin-mediated cell cycle arrest on the transcriptional and translational machinery of the cell, a clonal HeLa cell line, HeLa-luc, stably transfected with a CMV-driven luciferase expression cassette (Song L, Inex Pharmaceuticals, unpublished) was analyzed. This cell line was engineered to express the gene encoding luciferase constitutively. If aphidicolin treatment down-regulates CMV-driven transcription or the host cells' ability to transport and translate the luciferase mRNA, we expected that aphidicolin-treated cells would display reduced luciferase activity compared with untreated cells. Figure 3 shows that treatment with aphidicolin did not significantly affect the level of luciferase gene expression compared with the untreated control cells.

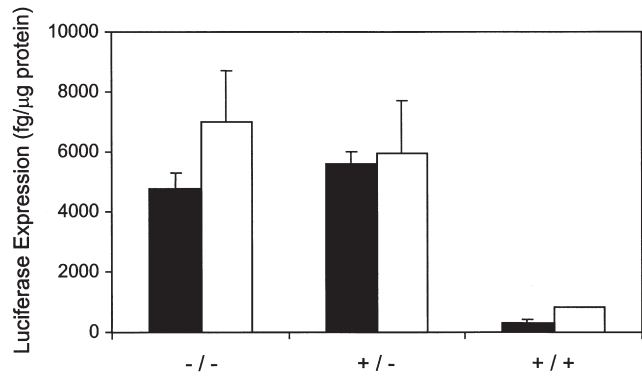
The results from these experiments suggest that aphidicolin treatment effectively arrests cells in the G1 phase of the cell cycle with negligible concomitant effects on the internalization of lipoplexes and on processes involved in gene expression.

*Cells arrested in the G1/S phase by aphidicolin are not transfected efficiently by lipoplexes or SPLP*

SK-OV-3 cells were synchronized in the G1 phase of the cell cycle by aphidicolin treatment and incubated with either lipoplexes or SPLP, either in the presence of aphidicolin, to continue the cell cycle arrest, or in the absence of aphidicolin, to release cells from cell cycle arrest. Luciferase activity was measured after 24 h of continuous exposure to lipoplexes or SPLP. Figure 4 shows that luciferase expression in cultures released from the cell cycle arrest did not differ significantly from that in control asynchronous cell cultures that had not been exposed to aphidicolin. The synchronous culture arrested in the G1 phase demonstrated 20-fold and 10-fold lower luciferase gene expression compared with control asynchronous



**Figure 3** Effect of aphidicolin on gene expression in HeLa-luc cells. HeLa-luc cells were arrested in the G1 phase by treatment with aphidicolin as described in Materials and methods. Luciferase gene expression was measured at 0, 6 and 24 h after arrest for both untreated cells (open bars) and cells blocked in G<sub>1</sub> (solid bars) of the cell cycle.



**Figure 4** Effect of cell cycle arrest on lipoplex- and SPLP-mediated transfection. Cells were arrested in G1 phase of cell cycle by treatment with aphidicolin as described. Cells were incubated with 0.5 μg plasmid formulated as lipoplexes (solid bars) or SPLP (open bars) and assayed for luciferase as described in Materials and methods. (-/-) Represents control cultures that had not been exposed to aphidicolin; (+/-) represents cultures that were synchronized with treatment with aphidicolin but then incubated with lipoplexes or SPLP in the absence of aphidicolin; and (+/+) represents cultures that were synchronized with treatment with aphidicolin and then incubated with lipoplexes or SPLP in the continued presence of aphidicolin. Luciferase activity was measured after 24 h of incubation. The data is presented as the mean of triplicate samples ± standard deviation.

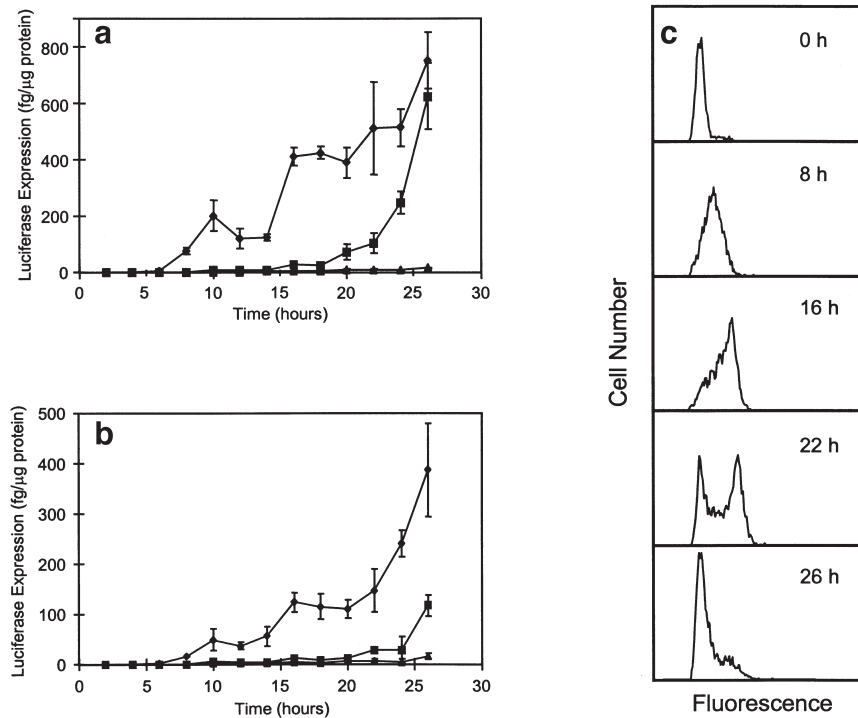
cultures when incubated with lipoplexes or SPLP, respectively. Cell cycle analysis of the synchronous culture revealed that approximately 95% of the cells remained arrested in the G1 phase at the end of the 24 h incubation with either lipid formulation (not shown). The cell cycle status profile of the culture released from cell cycle arrest mirrored that of the control asynchronous culture (not shown). These results suggest that aphidicolin treatment and the concomitant cell cycle arrest severely inhibits cationic lipid-mediated transfection at a certain, as yet unidentified, point during the transfection process.

*Cationic lipid-mediated transfection efficiency is dependent on cell cycle phase*

In order to gain further understanding of lipid-mediated transfection processes in proliferating and non-proliferating cells, we measured and correlated the kinetics of cell cycle status and luciferase gene expression in cultures that were cell cycle-arrested by treatment with aphidicolin, cultures which had been released from cell cycle arrest, and control cultures that had not been exposed to aphidicolin. The cell cycle status and luciferase activity of each culture were determined at 2 h intervals after the initiation of incubation with the lipoplexes or SPLP.

Figure 5 shows the correlation between cell cycle status of cultures from which the cell cycle arrest had been removed and the kinetics of luciferase gene expression after incubation with either lipoplexes (Figure 5a) or SPLP (Figure 5b). Incubation with either lipid formulation in cell cycle-arrested cultures resulted in extremely low levels of luciferase activity throughout the course of the experiment. In these cultures, 95% of the cells remained arrested in the G1 phase throughout the course of the experiment (not shown).

The control asynchronous cultures, which had not been exposed to aphidicolin, began to exhibit significant luciferase activity after 6–8 h of incubation with either



**Figure 5** Correlation of kinetics of luciferase gene expression in aphidicolin-treated cells allowed to progress through the cell cycle. SK-OV-3 cells were arrested as described in Materials and methods. At time ( $t = 0$ ), cells synchronized by treatment with aphidicolin were incubated with  $0.5 \mu\text{g}$  of plasmid DNA formulated as either lipoplexes (a) or SPLP (b) in the absence (+/-) (■) or continued presence (++) (▲) of aphidicolin. Asynchronous cultures that had never been exposed to aphidicolin (-/-) (◆) were used as controls. Samples were harvested at 2 h intervals and analyzed for luciferase activity as well as cell cycle status (c). Analysis of cell cycle status was determined at the time-points shown, as described in Materials and methods. Luciferase expression is expressed as the mean of triplicate samples  $\pm$  standard deviation.

lipid formulation. The levels of luciferase activity progressively increased and reached a maximum at 26 h when the experiment was terminated. At this time, an approximately 40-fold difference in luciferase activity was observed between control asynchronous and synchronous cultures incubated with lipoplexes. An approximately 20-fold difference was observed between control asynchronous and synchronous cultures when incubated with SPLP. The cell cycle profile of the control cultures remained unchanged and characteristic of a proliferating asynchronous culture throughout the course of the experiment (not shown).

We observed significant differences in luciferase expression between similarly treated cultures from experiment to experiment. For example, Figure 4 shows that the asynchronized culture yielded 5–7 pg of luciferase per microgram of protein whereas Figure 5 shows that the asynchronous cultures yielded 400–800 fg of luciferase per microgram of protein after incubation with the same amount of plasmid for identical length of incubation. We attribute these differences to either batch to batch variations of the components of lipoplex and SPLP and/or variations in the transfectability of different cultures of the same cell lines due to differences in passage number and growth kinetics. However, the differences in the relative trends of transfection between differently treated cultures (eg asynchronous *versus* aphidicolin treated cultures) were routinely observed.

Cultures which had been released from cell cycle arrest exhibited a delayed onset of luciferase expression compared with the control asynchronous culture when incu-

bated with either lipoplexes or SPLP; exposure to lipoplexes resulted in significant luciferase expression only after 16 h. Exposure to SPLP resulted in luciferase expression even later, at 20–22 h.

Analysis of the cell cycle status of these treatment groups revealed that by 6–8 h after the removal of aphidicolin, cells had resumed their progression through the cell cycle and had accumulated in the S phase (Figure 5c, 8 h). By 16 h, approximately 85% of the cells in these cultures had progressed through the S phase and were in the G2/M phase (Figure 5c, 16 h). Within 22 h, approximately 45% of the cells had undergone mitotic division and had progressed into the G1 phase of the subsequent cell cycle (Figure 5c, 22 h). Thus, higher luciferase activity in cultures incubated with either lipid formulation appeared to coincide with the passage of cells into the G2/M phase and into the G1 phase of the subsequent cell cycle. Continued incubation with either lipoplexes or SPLP in cultures which had been released from cell cycle arrest resulted in a progressive increase in luciferase levels and a concomitant increase in the number of cells in the G1 phase (Figure 5c, 26 h). This increase corresponded to an accumulation of cells that had undergone a mitotic division.

With either lipoplexes or SPLP, the increase in luciferase activity appeared to occur in two phases. In cells incubated with lipoplexes, the first phase occurred between 16 and 22 h and represented a slower rate of increase in luciferase expression than the second phase, between 22 and 26 h (Figure 5a). In cells incubated with SPLP, the first phase occurred between 20 and 24 h and

a second phase representing a faster rate of increase in luciferase expression occurred between 24 and 26 h (Figure 5b). The reason for the observed differences between lipoplex and SPLP incubated cells in the onset and duration of each phase is not known.

The second phase of luciferase expression corresponds to an increased number of cells in the G1 phase due to an increased number of cells that had undergone cell division upon completion of mitosis. Therefore, these experiments demonstrated that the increase in luciferase expression in cultures which had been released from cell cycle arrest paralleled the increase in the number of cells undergoing mitosis.

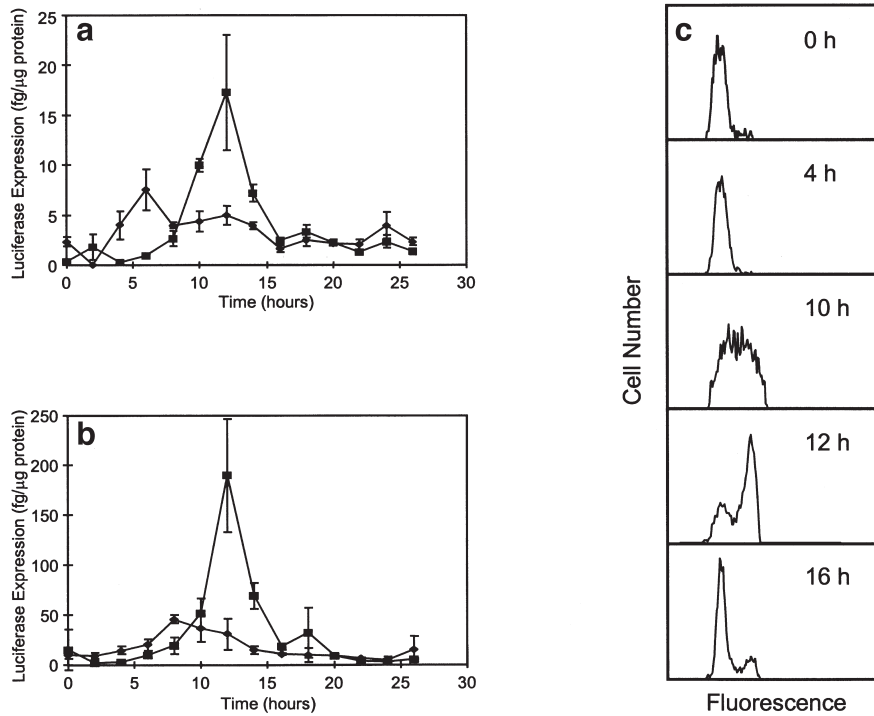
In order to ascertain the relationship between mitosis and lipid-mediated transfection, an experiment was conducted in which cells were incubated transiently, rather than continuously, with either lipoplexes or SPLP. Cells synchronized in the G1 phase by aphidicolin were released from cell cycle arrest by washing the cells with phosphate-buffered saline and replenishing the cells with fresh, aphidicolin-free media ( $t = 0$ ). At 2 h intervals thereafter (ie  $t = 2, 4$  or  $6$  etc), these cultures were exposed to lipoplexes or SPLP for 1 h after which the medium was removed and the incubation continued for a further 5 h in media free of aphidicolin and lipid formulation. The cells were then harvested and analyzed for luciferase activity. An asynchronous control culture that had not been exposed to aphidicolin was transfected and analyzed in parallel.

Figure 6a and b show the transfection profiles of cultures incubated with lipoplexes and SPLP, respectively.

With either formulation, luciferase expression was observed to increase in cultures which had been released from cell cycle arrest, beginning at approximately 10 h after the removal of aphidicolin and reaching a maximum at 12 h. Compared with the control asynchronous cultures, the cultures which had been released from cell cycle arrest exhibited three-fold and eight-fold increases in luciferase expression with lipoplexes and SPLP, respectively.

Analysis of the cell cycle status of the cultures which had been released from cell cycle arrest indicated that progression of cells from the G2/M phase into the G1 phase of the subsequent cell cycle occurred at 10 h after the removal of aphidicolin (Figure 6c, 10 h). The peak in luciferase expression at 12 h coincided with the highest number of cells transiting from the G2/M phase into the G1 phase of the subsequent cell cycle corresponding to the highest number of cells that had undergone mitosis (Figure 6c, 12 h). After this time, the luciferase activities subsided and by 16 h had reverted to levels comparable to those in the control cultures. This decline in luciferase activity coincided with the reduction in the number of mitotic cells entering the G1 phase of the subsequent cell cycle.

These results indicate that luciferase gene expression in cultures exposed to cationic lipid-mediated transfection systems was dependent on the number of cells undergoing mitotic cell division. A 1-h exposure of cultures to these transfection systems was sufficient to elicit detectable luciferase gene expression if it coincided with cells undergoing mitotic division.



**Figure 6** Determination of the period most amenable to transfection in aphidicolin-treated cells allowed to progress through the cell cycle. Cultures that had been synchronized by treatment with aphidicolin were allowed to proceed through the cell cycle by replacing the media with aphidicolin-free media (at  $t = 0$ ) (+) and were compared for luciferase gene expression with asynchronous cells that had never been exposed to aphidicolin (◆). At 2 h intervals ( $t = 2, 4, 6$  etc), the cultures were incubated with  $0.5 \mu\text{g}$  plasmid DNA formulated as either lipoplexes (a) or SPLPs (b) in the absence of aphidicolin. The cultures were analyzed for their cell cycle status at the time of incubation with the formulated DNA (c). After 1 h of incubation, the formulations were removed and the cells cultured for a further 5 h in the absence of formulation and aphidicolin and were analyzed for luciferase activity. Luciferase expression is expressed as the mean of triplicate samples  $\pm$  standard deviation.

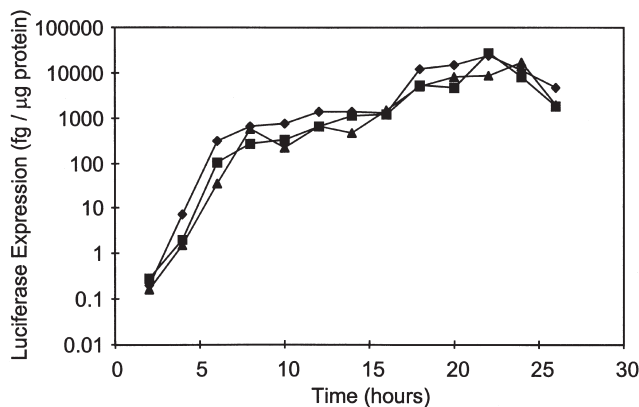
### Cell cycle arrest does not affect transfection efficiency of cytoplasmic expression systems

In order to determine if transfection systems that do not require nuclear delivery of the exogenous nucleic acids are dependent on cell division, we investigated the relationship between transfection and cell proliferation in the alphavirus, Semliki Forest virus (SFV). Alphaviruses are enveloped viruses that contain a sense-strand RNA genome. The genomic RNA serves as a substrate for cytoplasmic translation and replication upon infection. Infection with replication-defective SFV vectors is therefore not dependent on nuclear delivery of the genetic material.

The virus, SFV-luc, used in this study contained the gene encoding luciferase in place of the viral structural genes. SK-OV-3 cells were arrested in the G1 phase before infection with SFV-luc as described in Materials and methods. The kinetics of luciferase gene expression were determined and compared with synchronous cell cycle-arrested cultures, cultures which had been released from cell cycle arrest and control asynchronous cultures. There was no significant difference in the pattern of gene expression between the three types of cell cultures tested (Figure 7). This observation suggests that transfection systems that do not require entry into the nucleus function independently of the cell cycle. The results from this experiment also support the earlier observation (see Figures 2 and 3) that aphidicolin-mediated cell cycle arrest does not affect internalization by endocytotic pathways and translational machinery.

### Discussion

We have shown that SK-OV-3 tumor cells that were synchronized in the G1 phase of the cell cycle by aphidicolin treatment were not efficiently transfected by lipid-based systems until they were released from cell cycle arrest and allowed to progress through the cell cycle. In cultures in which the aphidicolin block was removed, the



**Figure 7** Semliki Forest virus-mediated gene expression. SK-OV-3 cells were arrested as described in Materials and methods. At time  $t = 0$ , arrested cells were released from cell cycle arrest by the addition of fresh media without aphidicolin; arrested cells were supplemented with aphidicolin in fresh growth media. At this time, all cells were infected with SFV-luc at a multiplicity of infection of 0.5 as described in Materials and methods. Luciferase expression was measured every 2 h for untreated cells (◆), cells released from aphidicolin arrest (■), and cells that remained arrested throughout the experiment (▲). Luciferase expression is expressed as the mean of triplicate samples.

cells were most amenable to transfection when they underwent mitosis soon after exposure to the transfecting reagents. The correlation between transfectability and mitosis was observed with both lipoplexes and SPLP, systems which differ in their assembly, structure and stability.<sup>18</sup>

It is well known that gene transfer using retrovirus-based systems, such as murine leukemia virus (MLV) requires cell proliferation for expression of the exogenous genes.<sup>24,25</sup> In contrast, lentiviral and adenovirus-based gene delivery systems have been shown to transduce non-proliferating cells.<sup>15-17,26</sup> Reports describing the role of cell division in cationic lipid-based transfection systems have only recently begun to appear in the literature. Wilke *et al*<sup>11</sup> showed that LLC-PK1 cells that were prevented from proliferating by growth in serum-free media were less efficiently transfected with lipofectin (DOTMA:DOPE) than control proliferating cells grown in media containing serum. They also showed that NIH 3T3 cells which were arrested in the S phase by exposure to thymidine were not transfected efficiently until the block was removed and the cells were allowed to progress through the cell cycle. Fasbender *et al*<sup>13</sup> have recently shown that immature human epithelial cells undergoing mitosis (identified by labeling with BrdU) are much more likely to express a lipoplex (DMRIE:DOPE)-delivered transgene than cells in other stages of the cell cycle. Similarly, Jiang *et al*<sup>14</sup> have also recently demonstrated that primary normal human bronchial epithelial cells are less amenable to lipoplex (GL-67:DOPE)-mediated transfection when arrested by treatment with aphidicolin. The results from our study are consistent with their observations correlating cell proliferation and transgene expression. It therefore appears that the correlation between cell proliferation and transfection is a common feature of cationic lipid-mediated transfection systems.

Our study also provides additional insight into the kinetics and mechanism of this process. When synchronized cells were allowed to proceed through the cell cycle at the time of incubation with lipoplexes or SPLP, an initial delay in the onset of gene expression was observed. This lag coincided with the progression of cells from G1 into the S phase. The appearance and subsequent increase in transgene expression coincided with an increase in the number of cells completing mitosis and progressing into the G1 phase of the subsequent cell cycle. The reduced transgene expression after lipid-mediated transfection in non-mitotic cells was shown not to be due to effects on the internalization of the lipid-formulated transgene or reduced activity of the gene expression machinery. In the experiments in which we transiently incubated the cells with either SPLP or lipoplexes at different time-points after the removal of the aphidicolin block, we observed that transfection efficiency could be augmented three- to eight-fold if the incubation coincided with the transition of the maximum number of cells from the G2/M phase into the G1 phase of the subsequent cell cycle.

The close correlation between the onset of transgene expression and mitosis suggest that event(s) occurring during mitosis somehow augment transfection, possibly by aiding the delivery of the plasmid into the nucleus. This hypothesis is supported by the observation that SFV-mediated transfection, a process which is not dependent on the delivery of the transgene to the nucleus, is unaffected by cell cycle arrest. It has been speculated that

the delivery of DNA to the nucleus is facilitated by the breakdown of the nuclear membrane.<sup>11–14</sup> Our demonstration of a correlation between mitotic activity and transgene expression add support to this hypothesis. Wilke *et al*<sup>11</sup> have also shown that cells in which naked plasmid DNA was injected directly into the nucleus yielded approximately five-fold greater transgene expression than cells in which the plasmid was injected into the cytoplasm. Using similar microinjection techniques, Jiang *et al*<sup>14</sup> have shown that plasmid DNA injected directly into the cytoplasm of VERO cells within aphidicolin-treated cultures resulted in approximately 10-fold fewer cells expressing the transgene compared with control untreated cultures. These results suggest that intracellular pools of plasmid DNA are not transported efficiently into the nucleus and that nuclear entry may represent a potential rate-limiting step in lipid-mediated gene transfer.

Cell cultures that had been subjected to cell cycle arrest throughout the transfection period exhibited a low but significant level of transgene expression following incubation with lipid-formulated plasmid. It is possible that this level of gene expression is derived from the small percentage (5–10%) of cells that evade cell cycle arrest. Alternatively, it is possible that inefficient nuclear delivery processes independent of the cell cycle exist which facilitate transfection in cell cycle arrested cells. Indeed, Dowty *et al*<sup>7</sup> have shown that plasmid DNA microinjected into the cytosol of post-mitotic rat myotubules was capable of yielding transgene expression. From their studies, cell-cycle independent nuclear delivery of plasmid DNA clearly exists in certain cell types.

The dependence of lipid-mediated transfection on cell proliferation has important implications for the utility of these systems in gene therapy protocols. Tissues consisting of non-proliferating cells, such as muscle and neural tissue, would theoretically not be amenable to efficient lipid-mediated transfection. On the other hand, neoplastic tissue and targets that are composed of rapidly proliferating cells will theoretically be more amenable to transfection. Even in these tissues, however, the fraction of cells undergoing mitosis is less than 20% (Joshi *et al*, unpublished) at a given time, thus limiting the number of cells within the tissue which are amenable to transfection. The duration of the mitotic phase is cell type-specific but is typically 1 h. Therefore, even with formulations that are effectively internalized and released into the cytoplasm, the window of opportunity within a typical cell cycle of approximately 24 h to enter the nucleus is limited to 1 h during the mitotic breakdown of the nuclear membrane. Using microinjection techniques, Lechardeur *et al*<sup>10</sup> have found that naked plasmid DNA has a rapid turnover ( $t_{1/2}$  = approximately 90 min) when directly injected into the cytoplasm. This suggests that if the release of the exogenous DNA into the cytoplasm does not coincide with breakdown of the nuclear membrane (and the subsequent entry of the plasmid into the nucleus), then the DNA dose available for entry into the nucleus at the next mitotic division will be drastically reduced due to degradation in the cytoplasm.

Taken together, these observations provide clues to explain the inefficiency of lipid-mediated transfection. These observations also encourage the rational design of lipid-based systems to include nuclear-targeting elements to permit the entry of plasmid DNA in non-mitotic cells

and/or the development of strategies which increase the window of opportunity for the entry of plasmid into the nucleus by manipulation of the cell cycle status of target cells.

## Materials and methods

### Cell culture

SK-OV-3 cells (human ovarian tumor cells, ATCC HTB-77) were cultured in RPMI 1640 media (StemCell Technologies, Vancouver, Canada) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY, USA). HeLa-luc cells were cultured in Dulbecco's modified Eagle medium (StemCell Technologies) supplemented with 10% fetal bovine serum and 400  $\mu$ g/ml Geneticin (Life Technologies, Gaithersburg, MD, USA).

### Reagents

Supercoiled plasmid DNA was purified using a modified alkaline lysis procedure followed by purification using a CsCl/EtBr gradient.<sup>28</sup> For lipid-mediated transfection studies, an expression plasmid, pCMVluc18 (P Tam, unpublished) in which the *Photinus pyralis* luciferase gene was under the control of the CMV promoter was used. The HeLa-luc cell line was created by stable integration of a CMV luc expression cassette into the genome of the HeLa cells. Aphidicolin was obtained from Sigma (St Louis, MO, USA). DODAC was obtained from Dr Steven Ansell (Inex Pharmaceuticals) and 1-O-[2'-(w-methoxypropyl)ethyleneglycol)succinoyl]-2-N-octoyl-sphingosine (PEG-CerC<sub>8</sub>) was obtained from Dr Zhao Wang (Inex Pharmaceuticals). DOPE was obtained from Northern Lipids (Vancouver, Canada).

### Preparation of lipoplexes

Lipoplexes were prepared as follows: all reagents were sterile and cooled to 4°C before complex preparation. A solution containing 62  $\mu$ m DODAC:DOPE large unilamellar vesicles (LUVs) and a solution containing 10  $\mu$ g/ml plasmid DNA were prepared in distilled water. The DNA was added to an equal volume of lipid dropwise, while vortexing. The solution was incubated on ice for 30 min before transfection. The resulting lipoplexes contained a final DNA concentration of 0.5  $\mu$ g/100  $\mu$ l and the cationic lipid:DNA (+:–) charge ratio was 1:1.

### Preparation of stable plasmid–lipid particles (SPLP)

Plasmid pCMVluc18 was encapsulated as SPLP using the detergent dialysis method<sup>19</sup> (Saravolac *et al*, in preparation). The SPLP contained 42.5:42.5:15 mol% of DOPE:DODAC:PEG-CerC<sub>8</sub>, at a lipid:DNA ratio of 15:1 (w/w). SPLP were separated from empty liposomes by centrifugation through a discontinuous sucrose gradient.<sup>19</sup>

### Cell cycle arrest and transfection conditions

SK-OV-3 or HeLa-luc cells were seeded at a density of 30 000 cells per well in a 24-well plate in growth media 24 h before treatment with aphidicolin. Aphidicolin was reconstituted in phosphate-buffered saline (PBS) containing 0.5% dimethylsulfoxide. Cells were incubated with aphidicolin at a final concentration of 5  $\mu$ g/ml in 1 ml of media per well for 16 h before transfection. Transfection was accomplished by the addition of lipoplex or SPLP equivalent to a dose of 0.5  $\mu$ g plasmid DNA to each well.

### Semliki Forest virus-luciferase

Luciferase cDNA was cloned into the multiple cloning site of pSFV1-3. Recombinant virus encoding luciferase was prepared according to the protocol described by Liljestrom and Garoff.<sup>29</sup> Before infection of SK-OV-3 cells, virus particles were activated in 0.5 mg/ml chymotrypsin for 30 min at room temperature. Chymotrypsin was inactivated by the addition of aprotinin to a final concentration of 0.67 mg/ml. Activated virus at a multiplicity of infection of 0.5 was added directly to the cells in growth media.

### Assessment of cell cycle status

The cell cycle status of cultures was determined by flow cytometry of permeabilized cells that were stained with propidium iodide (PI). Before harvesting, media was aspirated and the cells were washed twice with 2 ml PBS. After washing, two to three drops of pre-warmed trypsin-EDTA (0.25% trypsin, 1 mM EDTA; LifeTechnologies) were added to each well and the plate was incubated at 37°C until the cells had detached from the plate. Trypsin was inactivated by the addition of 1.5 ml of cold media. The samples were transferred to 6 ml polystyrene tubes (Becton Dickinson, San Jose, CA, USA) and centrifuged at 1000 *g* for 5 min (Beckman, Palo Alto, CA, USA; GS-6R, rotor GH3.8). Cells were fixed and permeabilized by incubation for 10 min in 70% methanol (pre-chilled to -70°C). Samples were then centrifuged at 1000 *g* for 5 min and the cells were incubated in 0.5 ml of 150 µg/ml PI (Molecular Probes, Eugene, OR, USA) in PBS for 30 min on ice in the dark before analysis by flow cytometry. 5000 cells were analyzed in the FL2-A channel ( $\lambda_{\text{emission}} = 585 \pm 21$  nm) of a FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an Ar-ion laser ( $\lambda_{\text{excitation}} = 488$  nm).

For the plasmid DNA uptake experiments, the method previously described,<sup>7,22,23</sup> using fluorescently labeled DNA and flow cytometry, was used with the following modifications. Plasmid DNA was fluorescently labeled by incubation with YOYO-1 iodide (Molecular Probes) at a dye:base pair ratio of 100:1 for 10 min at 4°C. The sample was then dialyzed twice against autoclaved deionized water.

SPLP and lipoplexes were prepared using YOYO-1 labeled plasmid as described above and incubated with cells. The cells were washed three times with PBS to remove noninternalized SPLP or lipoplexes and detached from the wells by trypsin, as described above. The cells were washed three times in PBS before analysis by flow cytometry. Fluorescence was measured in the FL1 channel ( $\lambda_{\text{emission}} = 530 \pm 15$  nm) of a FACSort flow cytometer (Becton Dickinson) equipped with an Ar-ion laser ( $\lambda_{\text{excitation}} = 488$  nm). Data was collected from 10 000 cells and analyzed for fluorescence using CellQuest software (Becton Dickinson). Analysis by fluorescence microscopy showed that most of the fluorescence associated with the cells was intracellular and associated with subcellular organelles.

### Luciferase and protein assays

Luciferase assays were performed using the luciferase assay system kit (Promega) according to the manufacturer's protocol. Cell samples were washed twice with PBS, lysed by incubation with 200 µl of lysis buffer (0.1% triton in 250 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4) for 20 min. 20 µl of

cell lysate was assayed (in duplicate) using an ML3000 microtiter plate luminometer (Dynex Technologies, Chantilly, VA, USA). A standard curve was determined using purified luciferase (Boehringer Mannheim, Laval, PQ, Canada). Cell lysate was assayed for protein content using the bicinchoninic acid (BCA) colorimetric method (Pierce, Rockford, IL, USA) according to the manufacturer's protocol.

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