methods

Adenovirus transduction of 3T3-L1 cells

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Abstract 3T3-L1 cells offer an excellent model system for studies of differentiation and biochemistry of fat cells. However, these cells are limited in their utility by the low efficiency with which DNA can be introduced by transfection. Gene delivery by viral vectors, particularly adenovirus, has proven a powerful means for introduction of genes into certain cell types. Furthermore, adenovirus transduction has been used to study mechanisms involved in the differentiation of 3T3-L1 cells into mature fat cells. We show in this study that 3T3-L1 cells are inefficiently transduced by adenovirus. The potential advantages offered by adenovirus transduction led us to examine methods designed to enhance transduction of 3T3-L1 cells by adenovirus. Of these methods, polylysine-mediated enhancement demonstrates considerable promise because it permits up to 100% of cells to be transduced and because it does not inhibit differentiation of 3T3-L1 cells.—Orlicky D. J., and J. Schaack. **Adenovirus transduction of 3T3-L1 cells. *J. Lipid Res.* 2001.** 42: **460–466.**

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Efficient introduction of genes into tissue culture cells offers a powerful method for studies of control processes within cells. Not all cell lines can be efficiently transfected using naked DNA. However, virally mediated introduction of genes permits vastly greater efficiencies of introduction of genes of interest. In particular, adenovirus has become a popular and powerful tool for transduction of tissue culture cells [e.g., Mazu et al. (1)]. In certain cell lines, adenovirus vectors made replication-defective by deletion of the E1 region can be used to deliver genes to virtually 100% of the cells in culture with little or no adverse effect on the transduced cells. This efficiency permits cells to be studied in bulk and allows determination of control mechanisms to be derived from biochemical studies.

The 3T3-L1 preadipocyte cell line can differentiate into mature, lipid droplet-containing adipocytes when stimulated with an appropriate hormonal regimen. During the process of adipocyte conversion, these preadipocytes lose their primitive mesenchymal character, assume a rounded morphology, and acquire many of the enzymatic and biochemical characteristics of adipocytes (2–4). Adipocytes play a key role in providing energy in times of demand and the storage of energy in the form of triacylglycerol-rich lipid droplets. Understanding the factors and mechanisms involved in adipocyte lipid homeostasis is key to understanding conditions such as obesity, lipoatrophies, and non-insulin-dependent diabetes, as well as hypertension and coronary artery disease. Therefore, identification of the proteins and their activities associated with lipid droplet accumulation is an important step in understanding the normal regulation of adipocytes or other lipogenic cells. The adenovirus vehicle could be of great use in these studies if a means were found to increase its transduction efficiency in the 3T3-L1 cell line.

MATERIALS AND METHODS

Materials

All chemicals used were purchased from Sigma (St. Louis, MO) unless otherwise noted. In particular, polyethylenimine was purchased from Aldrich (Milwaukee, WI), LipofectAMINE and Dulbecco's modified Eagle's medium [DMEM] were purchased from Life Technologies (Rockville, MD), and Superfect was from Qiagen (Valencia, CA). The polylysine used here was poly-L-lysine hydrobromide, molecular weight 30,000–70,000, from Sigma.

Cells and cell culture

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC CCL 92.1) and maintained in high glucose DMEM supplemented with 10% fetal bovine serum. Adipocyte conversion was induced by treating 2-day postconfluent cultures with DMEM containing 0.25 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 10^{-7} M insulin for 48 h, and then with DMEM containing 10^{-7} M insulin for the duration of the experiment, all as previously described (5). Culture medium was replaced with fresh medium every 2 days.

Abbreviations: CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PFU, plaque-forming units.

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293 cells were transformed by and expressed high levels of the E1A and E1B proteins of adenovirus type 5 (Ad5) (6). 293 cells were grown in high glucose DMEM.

Adenovirus vectors

The virus AdCMV-GFP, which encodes green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) major immediate-early promoter, was constructed using the system of He et al. (7). The viral chromosome was constructed via recombination between the plasmids pAdEasy1 and pAdTrack-CMV in *Escherichia coli* BJ5183 (7). BJ5183 cells that had been transformed with pAdEasy 1 and made competent were transformed with *Pme*I-digested pAdTrack-CMV to generate pAdCMV-GFP. A 6-cm dish of 293 cells at approximately 75% confluence was transfected with 6 μg of *Pac*I-digested AdCMV-GFP plasmid DNA, using calcium phosphate precipitation (8). After 24 h, the cells were fed fresh medium. Incubation was continued for an additional 6 days. Virus was released from the cells by freezing and thawing and plaque purified by infection of 293 cell-containing plates at various dilutions followed by overlaying with medium in 1% Noble agar. After incubation for 7 days, plaques positive for GFP as determined by fluorescence were picked and the virus was amplified in 293 cells. In addition to AdCMV-GFP, the virus Ad5*dl*327$_{Bst}$β-gal, which encodes *E. coli* β-galactosidase (LacZ) under the control of the CMV promoter (9), was used in certain experiments.

Growth and purification of adenovirus vectors

Adenovirus vectors were used to infect thirty to one hundred 10-cm dishes of 293 cells and incubated for 48–72 h until maximal cytopathic effect was apparent. Cells were harvested by centrifugation, and virus was released by three cycles of freezing and thawing followed by pelleting of cell debris. The supernatant was collected. The cell pellet was extracted two additional times by resuspension in 2 ml of phosphate-buffered saline (PBS) with freezing and thawing after the first resuspension. Cell debris was pelleted and the supernatants combined. The virion-containing supernatants were layered over steps of 1 ml of 1.40-g/ml and 2 ml of 1.25-g/ml CsCl in PBS and centrifuged for 50 min at 36,000 rpm, using an SW40 rotor. The virions, which band at the interface between the 1.25- and 1.40-g/ml CsCl steps, were collected by side puncture and rebanded isopycnically in 1.35-g/ml CsCl in PBS, using an NVT100 rotor for 3 h at 75,000 rpm. Virus bands were collected by side puncture and freed of CsCl by dialysis against three changes of 135 mM NaCl, 10 mM Tris-HCl (pH 7.9), 1 mM $MgCl_2$, and 50% (v/v) glycerol for 1 h each at 4°C. Virus particle concentrations were determined spectrophotometrically, with one A_{260} unit considered equal to 10^{12} particles. The virus stock was plaque titered with 293 cells. Various dilutions of virus were used to infect 6-cm plates of 293 cells. The plates were overlaid with Noble agar containing medium and serum 16 h after infection. The plates were stained with neutral red after 7 days and plaques were counted on day 8. The ratio of particles to plaque-forming units (PFU) was determined to be 96:1, which was rounded to 100:1 because of greater error in plaque titration relative to particle determination.

Transduction protocols

The basic transduction method used here was the addition of CsCl-banded, purified AdCMV-GFP in 1.0 ml of serum-free medium to PBS-washed cultures in a 35-mm culture dish. After an incubation of 1.5 h, 1.5 ml of DMEM was added to the culture. At 24 h the medium was changed to complete medium. The culture was maintained until 4 days posttransduction and then fixed with formalin, stained with Nile Red, and checked for GFP-containing cells.

Polylysine-assisted transduction involved the addition of polylysine to the serum-free medium prior to the addition of AdCMV-GFP, and then static, room temperature incubation of this mixture for 100 min prior to adding it to the PBS-washed cell culture. Again, after incubation of the culture being transduced for 1.5 h, 1.5 ml of DMEM was added to the culture. Cholesterol-assisted transduction was performed as reported (10). LipofectAMINE-assisted transduction was performed as suggested by Life Technologies for LipofectAMINE-assisted plasmid transfection, except that AdCMV-GFP was substituted for the plasmid. Similarly, the Superfect-assisted transduction was performed as suggested by Qiagen for Superfect-assisted plasmid transfection, except that AdCMV-GFP was substituted for the plasmid.

In those experiments using the virus Ad5*dl*327$_{Bst}$β-gal, LacZ expression was detected by a colorimetric assay based on hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) after fixation with paraformaldehyde (11).

Evaluation of adipocyte conversion

Lipid accumulation in 3T3-L1 cells was identified by staining with Nile Red to detect lipid droplets, as described by Greenspan, Mayer, and Fowler (12), after fixation with 10% formalin fixative.

Data analysis

Experiments presented here were performed from two to five times each. For each coverslip, 300–600 cells were assessed for GFP fluorescence and lipid droplets at a magnification of ×400. The percentage of GFP-positive cells is reported as the percentage of transduced cells. The results demonstrated excellent reproducibly. However, because of slight modifications in the protocol as the methods were refined, these experiments are not grouped together. In each case, figures show the results of one typical experiment. Color photographic exposures of the green (GFP) and red (Nile Red) fluorescence were made with Eastman Kodak (Rochester, NY) 320T film and commercial E6 developing solution.

RESULTS

Several methods were examined for their ability to enhance transduction by AdCMV-GFP. The basic method of transduction used here involved addition of subsaturating quantities of AdCMV-GFP to PBS-washed 3T3-L1 cultures in the presence of serum-free medium. This basic method yielded a low percentage of GFP-expressing cells (**Table 1**). Subsaturating concentrations of AdCMV-GFP were used in these experiments to allow easy detection of improvements in the basic transduction method. Altering this method of transduction by increasing the concentration of AdCMV-GFP (decreasing the volume of serum-free medium), increasing the time of transduction, or increasing the multiplicity of infection (MOI) failed to significantly increase the percentage of cells expressing GFP.

Next, additions of various agents useful in increasing transduction efficiencies with other cultured cell lines were examined. Of the agents tested, LipofectAMINE, Superfect, polylysine, and cholesterol all were able to significantly increase the transduction efficiency of the 3T3-L1 cells, whereas polyethylenimine was not able to do so.

The four agents identified above as having a positive

TABLE 1. Sumary of transduction methods

Method[a]	Transduction Efficiency	Effect on Differentiation
	%	
Basic	0.1	None
Increased concentration	0.1	None
Increased time	0.1	None
Increased MOI	1–3	None
Polyethylenimine addition	0.2	Inhibits
LipofectAMINE addition	8–12	Inhibits
Superfect addition	35–45	Inhibits
Polylysine addition	25–35	None
Cholesterol addition	30–35	None

[a] The basic transduction method involved use of CsCl-banded, purified AdCMV-GFP [MOI of 300 PFU/cell; this is a subsaturating dose of adenovirus that was used to permit the different methods to be compared accurately] added in serum-free medium to PBS-washed cultures. After an incubation of 1.5 h, whole medium was added to the culture. At 24 h the medium was changed to complete medium. The culture was maintained until 4 days posttransduction, fixed with formalin, stained with Nile Red, and checked for GFP-containing cells, all as indicated in Materials and Methods. Alterations of the basic method included increasing the concentration of AdCMV-GFP (decreasing the volume of serum-free medium during the transduction from 1.0 to 0.30 ml), increasing the time AdCMV-GFP plus serum-free medium were on the culture from 1.5 to 4.0 h, or increasing the MOI from 300 to 2,500 PFU/cell. Alternatively, other reagents such as polyethylenimine, LipofectAMINE, Superfect, polylysine, or cholesterol were mixed with the virus and added to cells as indicated in Materials and Methods. The effect on differentiation of the culture is reported to be none if there was no change in the percentage of Nile Red-positive cells when compared with cultures that had not been treated with either adenovirus or transduction-enhancing agents. In the experiments performed here more than 90% of the cells differentiated.

effect on the transduction efficiency were examined further to determine an optimal concentration of each to facilitate uptake and expression of AdCMV-GFP. This experiment was performed with AdCMV-GFP at an MOI of 300 PFU/ml. **Figure 1A** shows that the percentage of GFP-expressing cells increased as the concentration of polylysine increased until a maximum value (here 25–35%) was reached between 0.3 and 1.0 μg of polylysine per milliliter of serum-free medium. At concentrations of polylysine of 0.00–1.0 μg/ml, there was no effect on differentiation. An equal percentage of transduced cells underwent differentiation compared with untransduced cells (in these experiments more than 90% of both transduced and untransduced cells differentiated). Increasing the concentration of polylysine beyond 1.0 μg/ml actually decreased the percentage of GFP-expressing cells, and 10 μg/ml and above decreased the differentiation of the cells substantially. Figure 1B shows a parallel experiment utilizing cholesterol. Again, a concentration-dependent effect was observed and up to 30–35% of the cells were transduced at a cholesterol concentration of 10 μg/ml. Unlike polylysine, there was no observable decrease in transduction or differentiation at 10 μg/ml, the maximal concentration of cholesterol used as suggested by Worgall et al. (10). Figure 1C shows a parallel experiment using LipofectAMINE. Although LipofectAMINE could clearly increase the transduction efficiency, it did not do so to the same extent as polylysine, cholesterol, or Superfect. Furthermore, LipofectAMINE, even at 6 μl per reaction, inhibited differentiation of the 3T3-L1 cells. 3T3-L1 cells transduced in the presence of LipofectAMINE had a spread-out, nonnormal morphology after transduction. Figure 1D shows a parallel experiment using Superfect. A large increase in transduction efficiency was seen when utilizing small quantities of Superfect. However, less than 30% of the cells transduced with Superfect differentiated into Nile Red-positive, lipid droplet-containing cells by 5 days postdifferentiation induction, in contrast to 90% of the untransduced cells.

Polylysine and cholesterol both dramatically enhanced transduction without affecting differentiation. However, because of potential complications in the use of cholesterol with fat cells, only the polylysine-assisted transduction method was studied further. The polylysine-assisted method of transduction was experimentally manipulated in an attempt to optimize it further. The basic method involved mixing polylysine into serum-free medium, adding AdCMV-GFP, and allowing this mixture to incubate (first incubation period). After the first incubation period and after washing the cells with PBS the polylysine-adenovirus mixture was added to the culture and allowed to incubate for a second period of time. After the second incubation period, DMEM was added to the culture and the culture was incubated for 24 h, and then the medium was removed and the cells were fed fresh DMEM. Several parts of this procedure were examined and found to affect the outcome. The time interval of the first incubation period should be at least 1 h, with maximal transduction apparent after 100 min. Decreasing the temperature of the incubation or agitation of the mixture reduced the efficiency of transduction. The second incubation period should also be longer than 1 h, with maximal transduction apparent after 100 min. The data shown in Fig. 1A were obtained by this optimized method.

The percentage of GFP-expressing cells was then examined as a function of increasing MOI while using the optimized polylysine-assisted transduction method (**Fig. 2**). This experiment was performed at three concentrations of polylysine: 0.00, 0.03, and 0.5 μg/ml. Interestingly, regardless of the polylysine concentration, the percentage of GFP-expressing cells did not increase appreciably until an MOI of approximately 85 PFU/cell was used. At the optimal concentration of polylysine (0.5 μg in 1.0 ml of serum-free medium), almost 100% of the cells could be made to express GFP by increasing the MOI to approximately 2,000 PFU/cell. When 2,000 PFU/cell was used in the absence of polylysine, only 1–2% of the cells expressed GFP. Results similar to those shown in Fig. 1A have also been obtained with Ad5*dl*327$_{Bst}$β-gal and visualizing the transduced cells with a colorimetric reaction utilizing X-Gal (data not shown). In the experiments reported in Fig. 2 the percentage of differentiated cells expressing GFP was similar to the percentage of differentiated cells not expressing GFP, suggesting neither polylysine, the adenoviral vector, nor expression of GFP had a noticeable effect on differentiation of the cells.

Last, the efficiency of transduction using the optimized

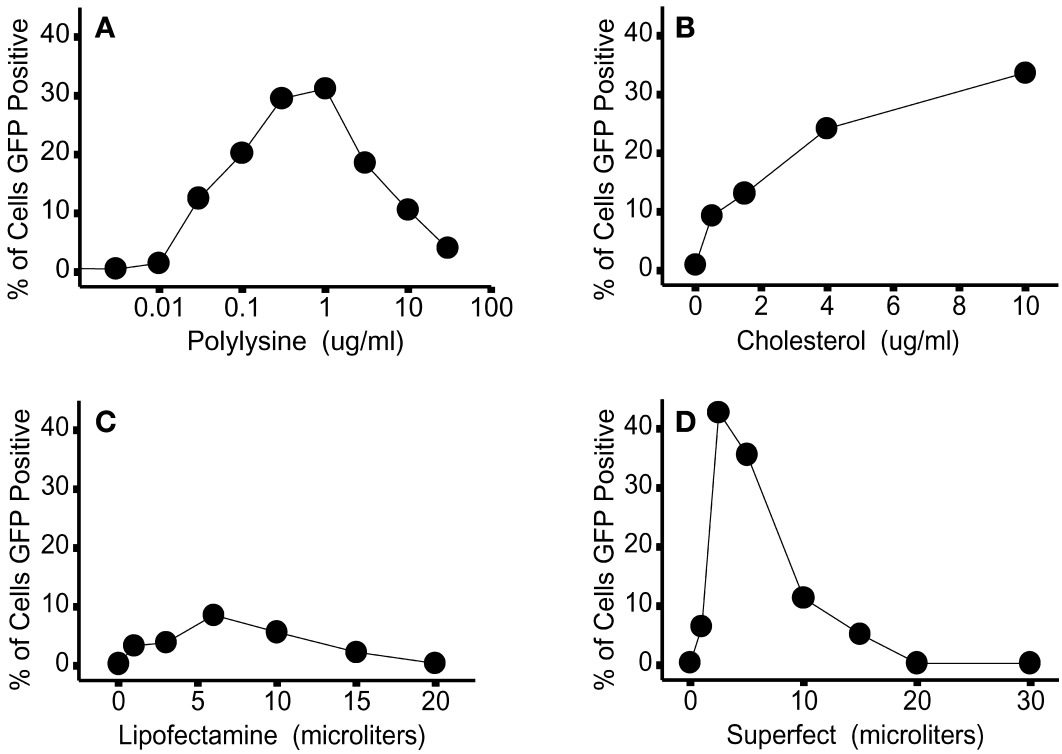

Fig. 1. Transduction as a function of agent concentration. Cultures of 3T3-L1 cells, on the first day after induction of differentiation, were transduced with AdCMV-GFP (MOI of 300 PFU/cell), using the (A) polylysine-assisted protocol, (B) cholesterol-assisted protocol, (C), LipofectAMINE-assisted protocol, or (D) Superfect-assisted protocol. The cultures were fixed and stained on day 5 after induction. The efficiency of transduction is equated with the percentage of cells expressing GFP. Note that the abscissa in (A) only is a logarithmic scale.

polylysine-assisted method was examined as a function of the time after the induction of differentiation at which the 3T3-L1 cells were transduced (**Fig. 3**). Cells were transduced either 0, 1, 2, 3, 4, or 5 days after the induction of differentiation. The target cell cultures had visible lipid droplets by 4 days after the induction took place. In all cases, the cells were examined for the presence of GFP 4 days after transduction. The percentage of cells transduced was fairly constant with transduction during the first few days after induction of differentiation, and increased slightly thereafter. In this experiment it was clear that both the undifferentiated (0 days after differentiation, Nile Red-negative cells) and differentiating 3T3-L1 cells (Nile Red-positive cells) could be transduced by the

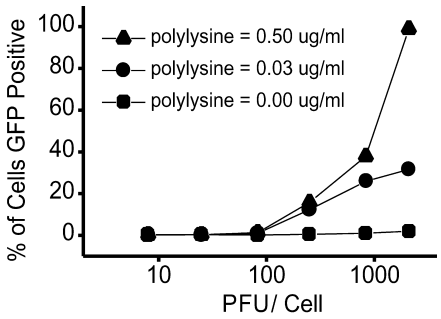

Fig. 2. Polylysine-assisted transduction as a function of MOI. Cultures of 3T3-L1 cells were transduced on day 1 after induction of differentiation with various MOIs (listed in PFU/cell) of AdCMV-GFP, using the polylysine-assisted protocol. Three concentrations of polylysine were used: 0.00, 0.03, and 0.5 μg/ml. On day 5 after differentiation induction the cultures were fixed and stained. The efficiency of transduction is equated with the percentage of cells expressing GFP.

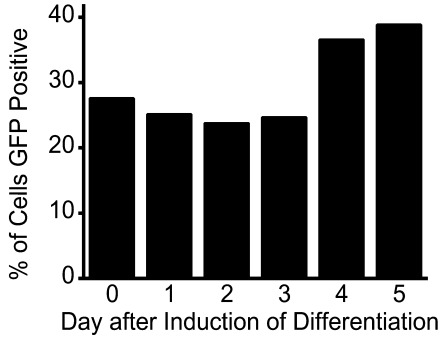

Fig. 3. Polylysine-assisted transduction as a function of time after induction of differentiation. Cultures of 3T3-L1 cells were transduced with AdCMV-GFP, using the polylysine-assisted protocol, on various days after induction of differentiation (MOI of 300 PFU/cell, polylysine concentration of 0.5 μg/ml). The cultures were then fixed and stained 4 days after they were transduced. Data are reported relative to the day of transduction. The efficiency of transduction is equated with the percentage of cells expressing GFP.

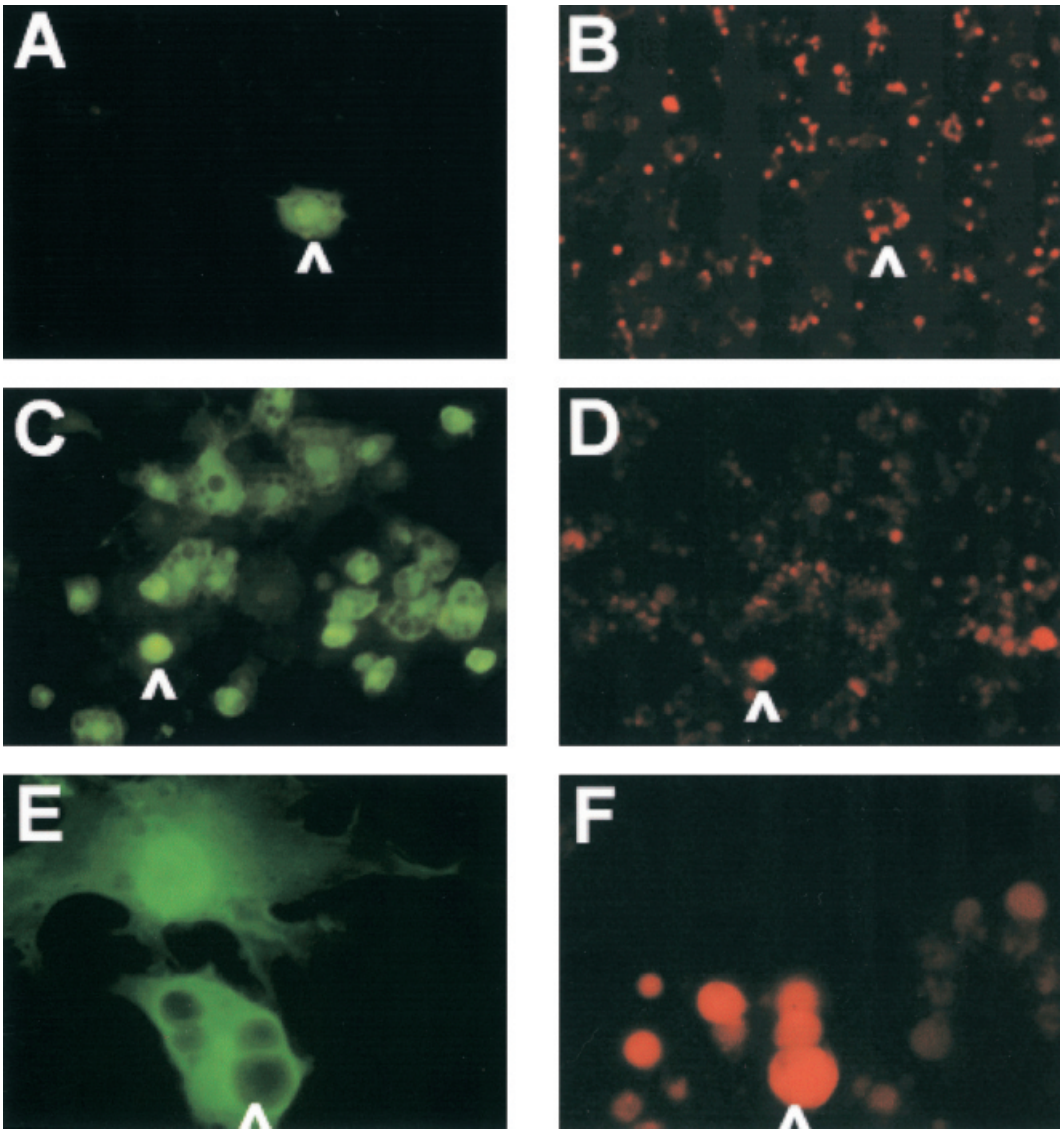

Fig. 4. Correlation of GFP fluorescence and lipid droplet presence. Cultures of 3T3-L1 cells were transduced on day 1 after induction of differentiation with AdCMV-GFP (MOI of 500 PFU/cell), using the polylysine-assisted protocol. In A (green fluorescence) and B (Nile Red fluorescence), no polylysine was present during transduction. In C (green fluorescence) and D (Nile Red fluorescence), polylysine at 0.5 μg/ml was used. On day 5 after differentiation induction the cultures were fixed and stained with Nile Red. In the absence of polylysine few cells expressed GFP. In contrast, in the presence of polylysine many cells expressed the transduced gene GFP. E (green fluorescence) and F (Nile Red fluorescence) are higher magnifications of cells from the cultures transduced with adenovirus and incubated in the presence of polylysine at 0.5 μg/ml. Four cells are present in E and F; the upper cell has been transduced but is nondifferentiated and the bottom middle cell has been transduced and has differentiated (contains lipid droplets). In addition, the cells flanking the bottom middle cell contain lipid droplets, but have not been transduced. This pair of micrographs demonstrates that cells transduced by the polylysine method are able to form both lipid droplet-containing (differentiating) and -noncontaining (nondifferentiating) cells. Arrowheads are provided to help align respective pairs of photomicrographs. Original magnification: (A–D) ×100; (E and F) ×400.

polylysine-assisted method. Again, both Nile Red-positive and Nile Red-negative cells were seen to express GFP (**Fig. 4**). Also, the percentage of differentiated cells was not affected by transduction with adenovirus; the percentages of differentiated and undifferentiated cells positive for GFP were similar, indicating that adenovirus transduction did not affect differentiation when polylysine was used.

DISCUSSION

Exogenous expression of proteins or mutant versions of proteins is particularly useful for biochemical activity studies. While simple, transfection protocols are not always efficient enough to yield a cell population with a high percentage of the cells expressing the experimental protein. Adenoviral transduction is a useful alternative

that provides acceptable efficiencies of gene expression. Unfortunately, adenovirus does not transduce all cell types equally well. The 3T3-L1 cell line is inefficiently transduced with adenovirus. In this report, several transduction-enhancing methods have been evaluated in the 3T3-L1 cell line. Evaluation was based on positive effects on transduction and negative effects on the growth and differentiation of the cell line.

The use of adenovirus transduction-enhancing agents is not new. A number of reports have made use of reagents designed to facilitate the adherence and/or uptake of the adenovirus particles and have suggested means for how some of them may work [e.g., see refs. (13–17)]. Two of these reports made use of adenovirus transduction-enhancing reagents (polyethylenimine and LipofectAMINE) with other fat cell lines (16, 17). However, of the numerous studies utilizing adenoviral vectors with 3T3-L1 cells that have been reported, none employed any agents to facilitate transduction [e.g., see refs. (18–22)]. Not surprisingly, some of these previous reports indicate using much higher MOIs to achieve results than are seen to be necessary here. One previous report detailed using a high MOI of polylysine-conjugated adenovirus virions to facilitate uptake of plasmid DNA into primary cultures from mouse epididymal fat pads (23).

In this study, four of the examined transduction-enhancing agents were found to be effective at increasing adenovirus transduction of 3T3-L1 cells. Unfortunately, LipofectAMINE and Superfect also inhibited differentiation when used at the concentrations required to affect transduction. LipofectAMINE also adversely affected the morphology of the cells. Of the two agents that augment transduction without inhibiting differentiation, cholesterol was not pursued further because of its possible effect on the lipid vesicles and lipid metabolism that are of key interest in these cells.

It is of interest that the positively charged polylysine molecule works well to enhance transduction in the 3T3-L1 cell line, considering that this cell line is well known for its production of negatively charged glycosaminoglycans. In light of this, it can be hypothesized that a complex of adenovirus and polylysine forms and is important for adherence of the adenovirus to the cells. The requirement for a long, nonagitated incubation of polylysine and adenovirus further suggests formation of the complex for uptake. In the 3T3-L1 cell line, the polylysine may allow cellular binding of adenovirus in a manner that facilitates its uptake, mimicking the function of the coxsackie and adenovirus receptor.

In summary, polylysine-mediated enhancement offers a simple, inexpensive, and effective method for dramatically increasing transduction of 3T3-L1 cells by adenovirus. With this dramatic enhancement of transduction, adenovirus vectors encoding a variety of proteins involved in metabolism in fat cells will be useful in the study of 3T3-L1 cell biochemistry.

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