Construction of stable coxsackievirus and adenovirus receptor-expressing 3T3-L1 cells

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Abstract **3T3-L1 cells have been used as a model to study the differentiation and physiology of adipocytes. Exogenous expression of proteins in these cells offers the prospect of understanding the protein's function(s) in adipose tissue. Viral vectors, in particular, adenovirus, have proven to be a powerful means for introduction of genes into many cell types. However, we have previously shown that 3T3-L1 cells are inefficiently transduced by adenovirus (Orlicky, D. J., and J. Schaack. 2001. *J. Lipid Res.* 42: 460–466). To overcome the inefficient transduction, we have stably introduced the gene-encoding coxsackie and adenovirus receptor (CAR), which was modified by deletion of the region encoding the cytoplasmic tail, into 3T3-L1 cells. 3T3-L1 CARΔ1 cells are transduced approximately 100-fold more efficiently than parental 3T3-L1 cells. 3T3-L1 CARΔ1 cells should prove to be a useful tool for examination of exogenous protein expression in fat cells.**—Orlicky, D. J., J. DeGregori, and J. Schaack. **Construction of stable coxsackievirus and adenovirus receptor-expressing 3T3-L1 cells. *J. Lipid Res.* 2001.** 42: **910–915.**

Supplementary key words 3T3-L1 • adenovirus • transduction • adipose • adipocyte

The 3T3-L1 preadipocyte cell line is a useful model for the examination of mesenchymal cell differentiation into adipocytes (adipocyte conversion) and for examination of the cellular physiology of triacylglycerol vesicle formation. During the process of adipocyte conversion 3T3-L1 preadipocytes acquire many of the enzymatic and biochemical characteristics of adipocytes (1–3) and initiate the storage of energy in the form of triacylglycerol (TAG)-rich lipid droplets. Understanding the factors and mechanisms involved in adipocyte conversion and adipocyte lipid homeostasis is vital 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 involved is an important step in understanding the normal regulation of adipocytes or other lipogenic cells.

Two powerful methods exist for introducing genes transiently into cells to study the effects of expresssion of novel or modified proteins: transfection with naked DNA and transduction using recombinant viral vectors, particularly adenovirus vectors. Unfortunately, 3T3-L1 cells are inefficiently transfectable (D. J. Orlicky, unpublished observations) and inefficiently transducible by adenovirus (4). The efficiency of transduction by adenovirus can be dramatically increased by modifying the virions to promote their uptake (4). However, genetic modification of 3T3-L1 cells that would permit efficient transduction by unmodified adenovirus would offer more convenience.

Efficient infection by adenovirus is initiated by binding of the knob of the fiber protein to the coxsackie and adenovirus receptor (CAR) (5). Efficient internalization requires the interaction of the viral penton base with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins (6, 7). For at least some of the cell lines that are inefficiently infected with adenovirus, the initial binding of virions at the cell surface is limiting and exogenous expression of CAR dramatically increases the efficiency of infection or transduction. Exogenous expression of CAR in Chinese hamster ovary cells increased adenovirus transduction of these cells by nearly 100-fold (5). Furthermore, stable CAR expression in T lymphocyte cell lines (8) and in T-cells in vivo (9) led to vastly increased transduction efficiency by adenovirus in the absence of T-cell activation.

The cytoplasmic tail of CAR is not necessary for efficient transduction of adenovirus (8–10). Because the normal function of CAR is unknown, expression of a CAR allele, CARΔ1, that has the region encoding the cytoplasmic domain deleted reduces the possibility that CAR function will disrupt normal functions in cells that do not normally express CAR (8). We have taken advantage of the ability of CARΔ1 to promote transduction by adenovirus and have stably introduced the CARΔ1 gene via retroviral transduction into 3T3-L1 cells. 3T3-L1 cells that express CARΔ1

Abbreviations: ATCC, American Type Culture Collection; CAR, coxsackie and adenovirus receptor; GFP, green fluorescent protein; TAG, triacylglycerol, DMEM, Dulbecco's minimal essential medium; IBMX, isobutylmethylxanthine; MOI, multiplicity of infection.

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are transduced by adenovirus vectors approximately 100-fold more efficiently than the parental cells while maintaining a level of differentiation into fat cells that is only slightly reduced relative to the parental cells.

MATERIALS AND METHODS

Materials

All chemicals used were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

Cells and cell culture

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC CCL 92.1) and maintained in high-glucose Dulbecco's minimal essential medium (Life Technologies, Rockville, MD) supplemented with 10% FBS (Gemini Bio-Products, Woodland, CA) (complete medium referred to herein as DMEM). Adipocyte conversion was induced by treating 2-day post-confluent cultures with DMEM containing 0.25 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 10^{-7} M insulin for 48 h, then with DMEM containing 10^{-7} M insulin for the duration of the experiment, all as previously described (11). Culture medium was replaced with fresh medium every 2 days.

293 cells are transformed by and express high levels of the E1A and E1B proteins of adenovirus type 5 (12). 293 cells were grown in high-glucose DMEM supplemented with 10% bovine calf serum.

Anti-CAR immunohistochemistry

Cells grown on glass coverslips were stained for reactivity with the mouse monoclonal RmcB anti-human CAR antibody prior to fixation of the cells. Briefly, cells grown on glass cover slips were washed by rinsing in PBS and then preblocked with a solution of antibody diluent diluted 1:1 with normal cell culture medium. Antibody diluent is composed of PBS made 2% (w/v) with BSA plus 1% (v/v) of normal goat serum. Preblocked cover slips were incubated for 30 min at 37°C in an atmosphere made 5% in CO_2 with the anti-CAR antibody (hybridoma supernantant) diluted 1:1 with the pre-blocking solution. Next, the cover slip was washed with PBS and pre-blocked a second time. The cover slip was then incubated for 30 min at 37°C in an atmosphere made 5% in CO_2 with Alexa 488 goat anti-mouse antibody (Molecular Probes, Eugene, OR) diluted to 1:500 with the pre-blocking solution. The cover slip was washed with PBS, fixed for 10 min with 10% formalin made in PBS, washed again with PBS, and stained with Hoescht 33258 (Sigma) using 0.2 μg/ml in PBS.

Adenovirus vector

The virus AdCMV-GFP, which encodes green fluorescent protein (GFP) under the control of the cytomegalovirus major immediate early promoter (CMV promoter), was constructed using the system of He et al. (13) and grown and purified as previously described (4).

Retrovirus vector

The CARΔ1 retroviral vector (LXSN) encodes a version of CAR with its cytoplasmic tail deleted (8). Following transfection into the Phoenix 293 producer cell line, medium containing the packaged, infectious virus was collected.

Transduction procedures

Cells were transduced with the CARΔ1 retrovirus by addition of 4 ml of the retrovirus-containing medium to a T75 flask containing a low density culture of passage four 3T3-L1 cells. (3T3-L1 cells received from the ATTC are defined here as passage number one.) After 3 h, DMEM was added to the transduced cells. Between days 3 and 12 post transduction, cells were grown in the presence of 400-μg/ml Geneticin (G418, Gibco Laboratories, Grand Island, NY). During this time, cultures twice reached approximately 60% confluence and were split 1:5 to prevent terminal differentiation. On day 15 post-retroviral transduction, cells were transduced with 100 pfu/cell of AdCMV-GFP. After 24 h, the cells were removed from the T75 flasks by digestion with trypsin, washed twice with DMEM and once with PBS, then resuspended for sorting in 50:50 PBS:DMEM. Cells were sorted using a MoFlo flow cytometer from Cytomation (Fort Collins, CO) based on fluorescence from the adenovirus encoded GFP. Aliquots of the sorted cells were checked for purity on a Coulter Epics XL flow cytometer and found to be 99% or greater of the desired fluorescence. Aliquots of approximately 100 cells of each sorted population were grown out over the next 18 days and passaged four times (each 1:5), then examined to ensure no green fluorescence remained. Finally, populations were tested for their ability to be transduced by adenovirus and to differentiate. To perform this test, cells were grown in 35-mm culture dishes containing glass cover slips until confluent and then induced to differentiate. Two days later, cultures were transduced with purified AdCMV-GFP in 1.5 ml of medium. Twenty-four hours later the medium was replaced with 2 ml of medium per dish and cultured until 5 days post-induction of differentiation, when the dishes were evaluated for transduction and adipocyte conversion as described below.

Evaluation of 3T3-L1 cell adenovirus transduction and 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 (14) following fixation with 10% formalin fixative. Fixed cells were also stained with Hoescht 33258 as described above (under Anti-CAR Immunohistochemistry) to identify nuclei so transduction efficiencies could be determined.

Data analysis

Two separate sorting experiments were performed yielding the two sorted groups each time. Transduction efficiency and ad-

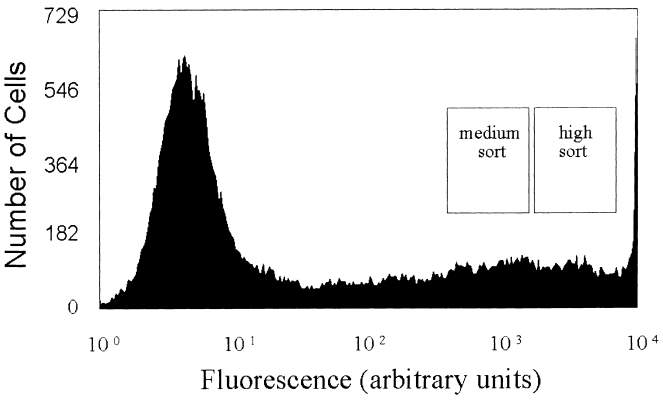

Fig. 1. Green fluorescent profile of the AdCMV-GFP transduced, CARΔ1-expressing 3T3-L1 cell population prior to sorting. Following stable integration of the CARΔ1 encoding retrovirus, and 24 h after transduction with the green fluorescent protein expressing AdCMV-GFP adenovirus, cells were prepared for flow cytometry analysis and sorting. Shown is the profile of green fluorescence emitted from the unsorted population as well as the windows that were used during the subsequent sorting procedure. Arbitrary fluorescent units are plotted on a logarithmic scale.

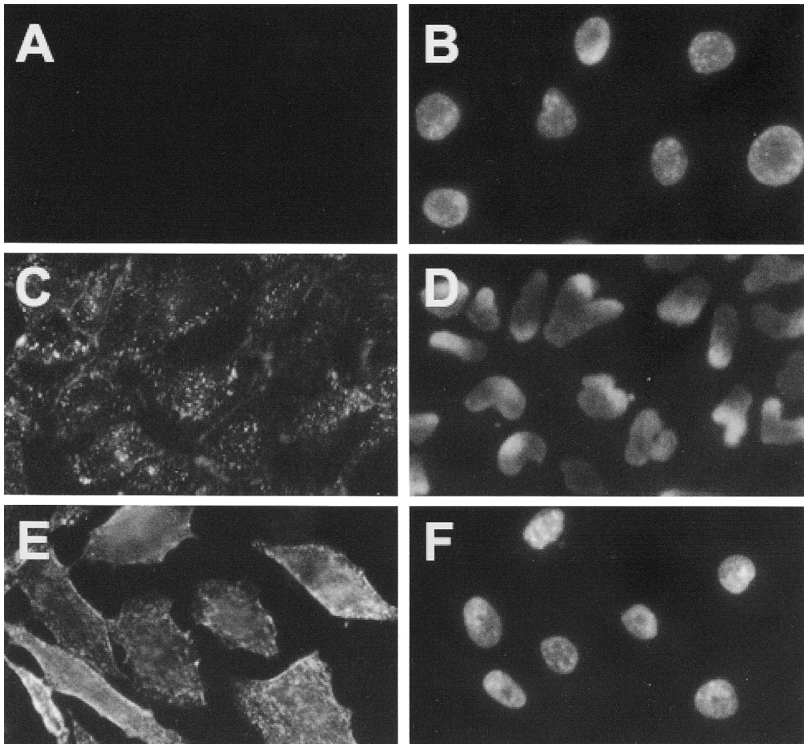

Fig. 2. Immunohistochemical staining for CAR. Parental 3T3-L1 cells (A, B), 293 cells (C, D), and 3T3-L1 CARΔ1 high sort cells (E, F) were stained for the expression of CAR (A, C, E) and the presence of DNA (B, D, F). Corresponding pairs of photomicrographs reveal the presence of abundant CAR reactivity in the 293 and 3T3-L1 CARΔ1 cells but not in parental 3T3-L1 cells. Magnification approximately 100×.

ipocyte conversion assessments were performed in duplicate on each population from each of these sorts. For each cover slip 300–600 cells were assessed for GFP fluorescence and lipid droplets at a magnification of 400×. The percentage of GFP positive cells is reported to be the percentage of transduced cells. The percentage of lipid-containing cells is reported to be the percentage of differentiated cells. The results demonstrated excellent reproducibility. However, owing to slight modifications in the protocol, these experiments are not grouped together. In each case, figures show the results from one experiment. Color photographic exposures of the green (GFP), red (Nile Red), and blue (Hoescht 33,258) fluorescence were made using Kodak 320T film and commercial E6 developing.

RESULTS AND DISCUSSION

3T3-L1 cells were initially tested for CAR immunoreactivity and found to be negative. Following transduction with the CARΔ1-expressing retrovirus, the cells were transduced with AdCMV-GFP, analyzed for green fluorescence, and

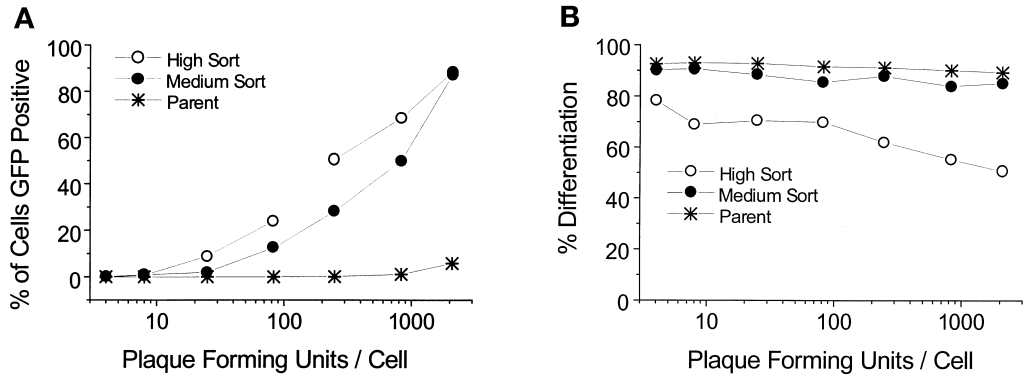

Fig. 3. A: Adenovirus transduction analysis. Following selection and growth of the high and medium expresser cell populations, these cells as well as the parental 3T3-L1 cells were examined for efficiency of transduction by adenovirus as a function of adenovirus concentration. The average of two values (cover slips) for each experimental condition is presented. Cells expressing GFP were assumed to be transduced by adenovirus. B: Differentiation following adenovirus transduction. Both the high and medium expresser cell populations, as well as the parental 3T3-L1 cell line, were examined for their ability to accumulate Nile Red positive TAG vesicles after transduction with adenovirus on the second day following induction of differentiation. Cells expressing Nile Red positive TAG vesicles were assumed to have differentiated. The average of two values (cover slips) for each experimental condition is presented.

sorted to obtain two populations. **Figure 1** shows the green fluorescent expression profile of the AdCMV-GFP transduced, CARΔ1-expressing 3T3-L1 cell population that was sorted by flow cytometry. Approximately 60% of the CARΔ1 retrovirus transduced population had little or no demonstrable green fluorescence (less than 20 arbitrary units). The two populations sorted for further analysis are also indicated. Sorting in this manner selected for cells expressing a medium amount of green fluorescence (approximately 400–1,500 arbitrary units) and higher amount of green fluorescence (approximately 1,600–7,000 arbitrary units). The cells expressing the very highest amount of green fluorescence were not tested further for efficiency of transduction. Microscopic inspection of the very high expressing population revealed intensely green shrunken cells.

Following growth from approximately 100 cells including four passages in T75 culture vessels, the high and medium sort cell populations as well as the parent 3T3-L1 cell lines were examined immunohistochemically for the presence of CAR (**Fig. 2**). The 3T3-L1 parent cell line demonstrated no detectable CAR. However, note that the mouse monoclonal RmcB anti-human CAR antibody does not recognize mouse CAR as robustly as it does human CAR (8). Nonetheless, the complete absence of anti-CAR staining coupled with the inability of adenovirus to transduce 3T3-L1 cells (4) suggests that CAR is not present on 3T3-L1 cells. Also included in Fig. 2 are anti-CAR-stained 293 cells and the CARΔ1-transduced, high-sort 3T3-L1 cell population, both of which stained with anti-CAR serum and both of which are efficiently transduced by adenovirus. Hoescht staining of cell nuclei is included to identify all cells.

The high- and medium-sort cell populations as well as the parent 3T3-L1 cell line were examined for transduction by adenovirus (**Fig. 3A**). Both of the sorted popula-

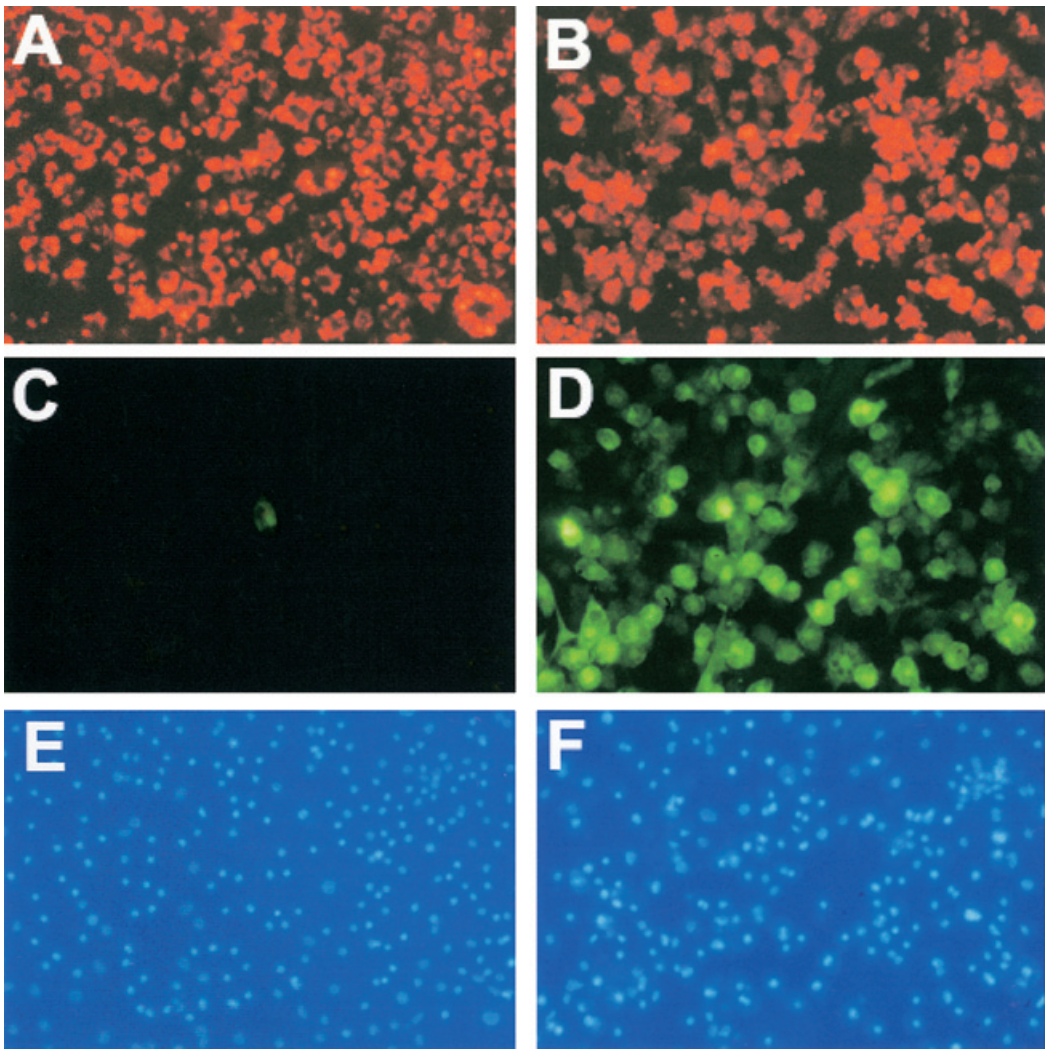

Fig. 4. Transduction of parental 3T3-L1 and medium level CARΔ1-expressing 3T3-L1 cells. Parental 3T3-L1 cells (A, C, E) and CARΔ1 expressing 3T3-L1 cells (B, D, F) were transduced with Ad-GFP at an MOI of 600 pfu/cell on the second day post-induction of differentiation and harvested on the fifth day post-induction of differentiation. Following fixation and staining with Nile Red and Hoescht, corresponding regions of each population were photographed for TAG accumulation (red fluorescence, A and B), adenovirus transduction (green fluorescence, C and D), and nuclei (blue fluorescence, E and F). Magnification approximately 100×.

tions of CARΔ1-expressing 3T3-L1 cells were efficiently transduced by adenovirus, with the group labeled high expressers showing a greater percentage of positive cells at any given multiplicity of infection (MOI). The parent 3T3-L1 cell cultures did not show more than 1% GFP positive cells until an MOI greater than 2,000 pfu/cell was used.

The 3T3-L1 CARΔ1 cells were next tested for ability to undergo differentiation into adipocytes as a function of the amount of adenovirus transducing vector used. More than 89% of the parental 3T3-L1 cells differentiated in the presence of 0–2,083 pfu/cell adenovirus. Medium expresser cells were only slightly impaired in their ability to differentiate; approximately 90% differentiated in the absence of transduction by adenovirus and 85% differentiated at an MOI of 2,083 pfu/cell (Fig. 3B). However, high expresser cells were much more impaired in their ability to differentiate; approximately 78% differentiated in the absence of transduction by adenovirus and 50% differentiated at an MOI of 2,083 pfu/cell. **Figure 4** demonstrates GFP expression and differentiation following transduction of 3T3-L1 parental cells and 3T3-L1 CARΔ1 medium expresser cells following transduction at an MOI of 600 pfu/cell.

The inefficient differentiation of the high expresser cells may have several explanations. It was not possible to accurately quantitate the relative amounts of CARΔ1 present on the high and moderate expresser cells owing to the inability of the antiserum to recognize denatured proteins in Western blots. However, it is likely that the cells that expressed higher amounts of GFP at the time of the cytometry sorting also expressed higher levels of CARΔ1. Although expression of CARΔ1 had no apparent effect on the physiology of T-cells (8), it is possible that it does adversely affect 3T3-L1 cells. It is also probable that the cells expressing higher amounts of GFP at the time of cytometer sorting contained more copies of the GFP-encoding adenovirus and this may have led to long-lasting adverse effects on the cells. Whatever the explanation, this differentiation experiment suggests that the medium expresser cells created here are better suited for studies of cellular control mechanisms.

In the experiments described herein, cells were transduced on the second day following induction of differentiation, much as transductions might be performed to examine effects of exogenous gene expression on the accumulation of TAG-containing vesicles. If instead transduction was on the third day following induction of differentiation, the difference between the high and medium expressers was not as pronounced, whereas if transduction was on the first day, the differences were increased (data not shown).

Stable expression of CARΔ1 enhanced the efficiency of transduction of 3T3-L1 cells by adenovirus by a factor of approximately 100. This increase in efficiency is similar to that observed in other poorly transducible cell lines modified to express CAR (8). However, the efficiency of transduction of 3T3-L1 CARΔ1 cells remains below that of cell lines that are efficiently infected or transduced, such as 293 or HeLa cells. This difference may reflect the contribution of other cellular proteins, including vitronetin-binding integrins, to the adenovirus infectious process. Such proteins may be present at suboptimal levels in 3T3-L1 cells. It may be possible to further increase the efficiency of transduction of 3T3-L1 cells after determination of the factor-limiting transduction in 3T3-L1 CARΔ1 cells. However, the modifications described here make it possible to use adenovirus vectors to study regulation of processes involved in fat cell differentiation. Initial experiments with adenovirus encoded GLUT4/HA demonstrate that transduction of 3T3-L1 CARΔ1 cells with a relevant gene also occurs at high efficiency (data not shown).

The goal of the experiments described here was to produce a variant of the 3T3-L1 cell line useful for expression of exogenous normal and mutant genes, thus allowing examination of the effects of the encoded proteins on adipocyte differentiation and/or adipocyte physiology. Although other investigators have used adenovirus in experiments designed to exogenously express proteins of interest in 3T3-L1 cells (e.g., 15–19), we have shown that 3T3-L1 cells are only poorly transduced by adenovirus (4). As a first step toward a solution to this problem, we and others (4, 20, 21) have examined the effects of various transduction-enhancing agents. In an attempt to obviate the potential problems introduced by transduction enhancing agents, we have now created a population of 3T3-L1 cells stably expressing a variant of CAR and selected cells based on efficiency of transduction by adenovirus as well as ability to differentiate. These cells should be of use in the study of adipocyte differentiation and the accumulation of TAG.

We acknowledge the financial support of the American Heart Association (CWGS-44-97 to D.J.O.), the support of NIH grant HL58344 to J.S. and NIH grant RO1 CA77314 to J.D., and a Scholar Award from the Leukemia and Lymphoma Society to J.D. We also acknowledge the Colorado Cancer Center and the excellent assistance of Karen Helm, Pat Schor, and Mike Ashton of the Colorado Cancer Flow Cytometry Core, which is supported by National Institutes of Health Grant 2 P30 CA46934-09. We thank Robert Evans for helpful discussions and use of equipment, and J. Bergelson and R. Finberg for their generous gift of the RmcB monoclonal antibody.

Manuscript received 27 November 2000 and in revised form 6 February 2001.

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