Adenovirus-mediated gene transfer in primary murine adipocytes

Ann Vogel Hertzel,* Mark A. Sanders,† and David A. Bernlohr[1,*]

Department of Biochemistry, Molecular Biology, and Biophysics,* and Imaging Center,†
University of Minnesota, St. Paul, MN 55108

Abstract **The transfer of genes into primary murine adipocytes using an adenovirus system has been developed. A recombinant adenovirus was constructed (expressing green fluorescent protein [GFP] under the control of the strong cytomegalovirus [CMV] promoter and a luciferase reporter gene under the control of the weak adipocyte promoter keratinocyte lipid-binding protein [KLBP/FABP5]) and incubated with primary adipocytes from C57BL/6J mice. Analysis of infected cells by confocal microscopy detected GFP expression in both the cytoplasm and nucleus of adipocytes with a 64% efficiency of infection. To demonstrate the applicability of this method in the study of gene regulation, adenovirus-infected adipocytes exhibited significant levels of luciferase activity even from a weak promoter. TPA treatment of infected adipocytes increased luciferase activity, consistent with previous studies indicating that the KLBP/FABP5 gene is up-regulated by phorbol esters. These results provide an efficient, convenient, and sensitive method to transiently infect primary murine adipocytes, facilitating protein expression or permitting analysis of reporter gene activity from both viral and endogenous promoters.**—Hertzel, A. V., M. A. Sanders, and D. A. Bernlohr. **Adenovirus-mediated gene transfer in primary murine adipocytes.** ***J. Lipid Res.*** **2000.** 41: **1082–1086.**

Supplementary key words adipocytes • adenovirus • green fluorescent protein • reporter genes

Transient transfection of cells is widely used for many purposes including protein expression and analysis as well as promoter studies through the use of reporter constructs. Even though primary cells are typically the preferred cells to mimic the in vivo state, such cells are notoriously recalcitrant to transfection. This is especially true for primary murine adipocytes. Whereas adipocytes from inbred strains of mice harboring mutations in key genes related to obesity and insulin resistance have been used for biochemical analyses of lipid and carbohydrate metabolism (1, 2), studies of gene expression have lagged behind. The inability to efficiently introduce functional genes into murine adipocytes has limited the usefulness of such animal models. Although electroporation methods have been used to transfect primary rat adipocytes (3), this method has not been an efficient way to transfect primary murine adipocytes. An alternative method for efficient transfection of many resistant cells is through the use of adenovirus-mediated gene transfer. Therefore, a recombinant adenovirus was constructed to test this approach with primary murine adipocytes. In this article we report the methods necessary to achieve a high-efficiency transfer of genes into murine adipocytes by adenovirus-mediated gene transfer and, using this technique, we demonstrate that phorbol ester transcriptionally regulates the keratinocyte lipid-binding protein (KLBP/FABP5) gene.

MATERIALS AND METHODS

Primary adipocytes

Primary adipocytes were prepared from C57BL/6J mice as follows: gonadal fat pads were dissected from a minimum of three mice, minced, and digested with collagenase (type II collagenase, 1 mg/mL; Sigma, St. Louis, MO) for 1 h at 37°C in Krebs–Ringer–HEPES (KRH) buffer (4,5) supplemented with bovine serum albumin (BSA, 10 mg/mL) and 5 mM glucose. Adipocytes were isolated by repeated (three or four) washes with supplemented KRH buffer followed by centrifugation at 4,000 rpm for 10 min. The floating adipocytes were recovered and subjected to a final wash in Dulbecco's modified Eagle's medium (DMEM). The viability of the adipocytes was determined by trypan blue exclusion analysis. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Culture conditions

Adipocytes isolated in this manner were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in 1.5-mL Eppendorf tubes covered with aluminum foil. The cells were incubated at 37°C in a 5% CO_2 incubator without shaking and were viable for at least 18–24 h.

Abbreviations: aP2/ALBP, adipocyte lipid-binding protein; CMV, cytomegalovirus; FABP, fatty acid-binding protein; GFP, green fluorescent protein; KLBP/FABP5, keratinocyte lipid-binding protein; MOI, multiplicity of infection; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate.

[1] To whom correspondence should be addressed.

Recombinant adenovirus

A recombinant adenovirus expressing both the green fluorescent protein (GFP; controlled by the cytomegalovirus [CMV] promoter) and firefly luciferase (controlled by the KLBP/FABP5 promoter; see ref. 6) was constructed by recombination in *Escherichia coli*, using the methods described by He and colleagues (7). Briefly, a 3.0-kb *Stu*I-to-*Nco*I fragment of murine KLBP/FABP5 (approximately bp −3.0 to +33 relative to transcription start) was cloned upstream of a promoterless luciferase gene in pGL3-Basic (Promega, Madison, WI). After removing the *Pac*I site by site-directed mutagenesis (see ref. 8; bp –450 from transcription start: TTAATTAA→TTAATTAT), the KLBP/FABP5-luciferase fragment (*Bam*HI-*Xho*I) was cloned into the *Bgl*II-*Xho*I sites of pADTrack (7). The resulting construct was recombined into pADEasy in *E. coli* BJ5183 cells, recreating the replication-deficient adenovirus genome. Linear constructs of the recombinant adenovirus were transfected (LipofectAMINE; GIBCO-BRL, Gaithersburg, MD) into 293 cells (American Type Culture Collection, Manassas, VA) to allow packaging and amplification of the adenovirus. Large-scale adenovirus preparations from twenty 10-cm plates of infected cells were propagated until approximately 50% of the cells lysed. The cells and media were collected and the remaining cells lysed by the addition of Nonidet P-40 (NP-40). The medium was centrifuged at 20,000 *g* for 10 min to pellet the debris. The supernatant was recovered, 10% polyethylene glycol (PEG) plus 1.25 M NaCl was added, and the mixture was incubated for 30 min on ice. The virus particles were precipitated by centrifugation at 20,000 *g* for 10 min. After resuspension in phosphate-buffered saline (PBS), the adenovirus was purified by double banding on a cesium chloride gradient. Finally, the adenovirus was dialyzed at 4°C against 20 mM HEPES, 150 mM NaCl (pH 7.3) and frozen in aliquots at –70°C.

Adenovirus infection

For an adenovirus infection, 2.5×10^5 primary adipocytes (approximately 0.25 mL, wet packed volume) in 0.25 mL of DMEM supplemented with 10% FBS were incubated with adenovirus (multiplicity of infection [MOI] of 50/cell) at 37°C in a 5% CO_2 incubator for 8–18 h.

Adenovirus titer

A limiting dilution plaque assay was used to determine the infective titer of the adenoviruses. Briefly, 293 cells were plated in six-well plates the day before infection. At approximately 50–80% confluence, 1 mL of viral dilution (in 1× Eagle's basal medium [GIBCO-BRL], 20 mM HEPES, 20 mM $MgCl_2$, 10% fetal bovine serum, and 1× penicillin–streptomycin per milliliter) was added to the cells and incubated for 4–6 h. The virus was removed and 2 mL of overlay (dilution medium plus 1% SeaPlaque low melting agarose [FMC Bioproducts, Rockland, ME]) per well was added. Every third day, 1 mL of overlay was added until plaques were visible and could be counted, typically around day 10.

Fluorescence microscopy

Living cells were immobilized on plastic coated with 0.01% polyethylenimine and were viewed with a Bio-Rad (Hercules, CA) MRC-1024 confocal microscope attached to a Nikon (Tokyo, Japan) Diaphot inverted microscope equipped with a 15-mW krypton/argon laser and fluorescein isothiocyanate (FITC) (excitation filter, 470–490 nm; barrier, 520–580 nm) and UV (excitation filter, 330–380 nm; barrier, 420 nm) filter sets. Preparations were also incubated with propidium iodide (1 μg/mL; Sigma). Digital images of the double-labeled preparations were sequentially collected using LaserSharp (Grapeview, WA) version 3.2 software.

Fixed cells were treated with 3% paraformaldehyde in PBS, pH 7.2, for a total of 4 min at a limit temperature of 37°C in a Pelco model 3450 microwave oven (Ted Pella, Redding, CA). The samples were permeabilized with ~20°C methanol for 5 min, collected, and allowed to adhere to 12-mm coverslips coated with 0.01% polyethylenimine (Sigma). The coverslips were rinsed in several changes of PBS and incubated in 5% normal goat serum, 5% glycerol, 1% cold water fish gelatin in PBS for 2 min at 37°C under the same microwave conditions. Blocked cells were treated with primary antibody or preimmune serum diluted 1:1,000 for a total of 15 min at 37°C. Specimens were washed in several changes of PBS and subsequently treated with Texas Red-conjugated secondary goat anti-rabbit IgG (Molecular Probes, Eugene, OR) diluted 1:500 for a total of 15 min at 37°C. The labeled coverslip cultures were mounted in 20% glycerol and 1% *n*-propyl gallate in PBS (pH 7.8). Preparations were viewed with a Nikon Eclipse E800 photomicroscope equipped with bright-field, differential interference contrast (DIC), phase, and fluorescence optics including 100-W mercury lamp epifluorescence illumination viewed with standard UV (excitation filter, 330–380 nm; barrier, 420 nm), FITC, GFP (excitation filter, 470–490 nm; barrier, 520–580 nm) and Texas Red, Rhodamine (excitation filter, 510–560 nm; barrier, 570–620 nm) filter sets. Digital images were collected with a CoolCam liquid-cooled, three-chip color CCD camera (Cool Camera Company, Decatur, GA).

TPA treatment

Primary murine adipocytes were isolated and infected with KLBP/FABP5-luciferase-containing adenovirus (50 MOI/cell) and pCMV-β-galactosidase adenovirus (50 MOI/cell). The infected cells were incubated with or without 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 100 ng/mL in dimethyl sulfoxide [DMSO]; Sigma) in DMEM (phenol-red free) supplemented with 10% fetal bovine serum for 8 h. The cells were washed twice with PBS by flotation after centrifugation at 1,200 *g* for 5 min.

Luciferase activity

Primary murine adipocytes were prepared and infected as described above. After 8 h of incubation, the cells were washed twice with PBS and lysed with 100 μL of 1× lysis buffer (Promega). The lysate was centrifuged for 2 min and 5 μL of supernatant was added to 25 μL of luciferin substrate and assayed in a Lumat LB9507 luminometer (EG&G Berthold, Wildbad, Germany) for 10 s. Each experiment was repeated a minimum of three times with similar results.

β-Galactosidase activity

The lysate (see above) was centrifuged for 2 min and 70 μL of supernatant was added to 70 μL of 2× β-galactosidase substrate and incubated for 3 h at 37°C. The optical density (OD) at 420 nm was measured and used to normalize the infection efficiency.

RESULTS AND DISCUSSION

An efficient method of introducing DNA into primary murine adipocytes, using either cationic lipids or electroporation techniques, has not been reported. Previous methods have been devised for in vitro transfection of adipocytes from rats by electroporation (3) and, more recently, for ex vivo and in vivo transfection of human adipocytes (9). To develop a high-level expression method for murine adipocytes, the infection of primary cells with recombinant adenovirus was undertaken. The recombi-

nant adenovirus was created as described by He and colleagues (7), a method based on recombination in *E. coli* resulting in efficient generation of a functional adenovirus. By cloning a KLBP/FABP5 promoter upstream of a firefly luciferase gene into the adenoviral vector pADTrack, the resulting recombinant adenovirus will coexpress the luciferase gene from a weak adipocyte promoter, as well as green fluorescence protein (GFP) from a constitutively active CMV promoter.

Large-scale preparations of the recombinant adenovirus were obtained and were purified on cesium chloride gradients. To determine the titer of infective adenovirus, a limiting dilution plaque assay was utilized. Various dilutions of adenovirus were added to 293 cells followed by an agarose overlay. By counting plaques from three independent experiments, the KLBP/FABP5-luciferase adenovirus titer was determined to be 2×10^9/mL.

To test the ability of the adenovirus to infect primary adipocytes, murine gonadal fat pads were dissected and digested with collagenase to release the adipocytes. The fat cells were washed and maintained in medium to which infective adenovirus particles were added. After incubation with the adenovirus (MOI of 50) for 18 h, confocal microscopy was used to assess expression of the green fluorescent protein (**Fig. 1**). Propidium iodide staining (red) of nuclei facilitated total cell observation. The results indicate successful adenoviral infection based on the intense green fluorescence from the GFP (Fig. 1A), unlike the uninfected control cells (Fig. 1B). The yellow color demonstrates the overlap of propidium iodide staining and GFP expression in the nucleus. There was no difference seen if the adipocytes were derived from epididymal fat pads, ovarian fat pads, or a mixture of both.

To show unequivocally that the cells infected were indeed adipocytes and not contaminating stromal vascular cells, immunofluorescence microscopy was performed on adenovirus-infected primary cells, using an antibody directed toward the fat cell-specific adipocyte lipid-binding protein (ALBP/aP2). Primary adipocytes were fixed and hybridized with anti-ALBP/aP2 antibody followed by a Texas Red-conjugated goat anti-rabbit IgG. As shown in **Fig. 2C–F**, the cells exhibited green fluorescence (Fig. 2D and E) as well as red staining (Fig. 2D and F), indicating positive identification as an adipocyte. In addition, the light-phase image (Fig. 2C) shows the nucleus being pushed to the top edge of the cell, typical of adipocytes that contain large quantities of triglycerides. Uninfected control cells (Fig. 2A and B) showed a lack of green fluorescence (Fig. 2B), and infected cells (Fig. 2G and H) treated with preimmune sera showed no red staining (Fig. 2H).

To determine the percent efficiency of adenovirus infection, individual cells that stained red for the ALBP/aP2 antibody were evaluated for green fluorescence. On average, 64% of the ALBP/aP2-expressing cells were green, indicating successful adenovirus infection. Small and large cells were infected equally, indicating that the size of the adipocyte was irrelevant to the likelihood of being infected. The infections were repeated three times, giving similar results. Additional experiments using a reduced level of adenovirus per cell (MOI of 15) yielded a lower percentage (35%) of cells infected. With an MOI of 150, a higher percentage (75%) of cells infected was achieved; however, the cell integrity was significantly compromised

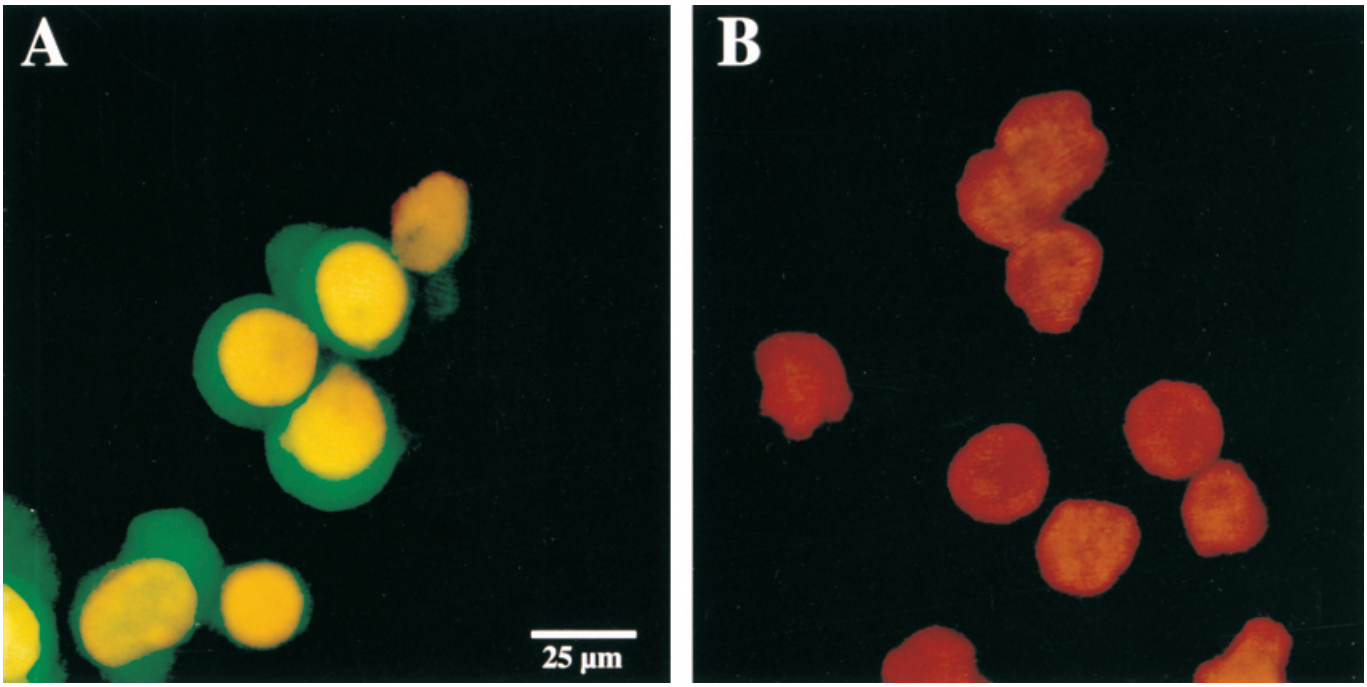

Fig. 1. Adenovirus-mediated gene transfer into primary murine adipocytes. Confocal laser-scanning image of infected living adipocytes (A) and uninfected control cells (B), with fluorescence of GFP and labeling with propidium iodide. The green fluorescence indicates adenovirus infection and the red propidium iodide stains nuclei. The experiment was repeated two times.

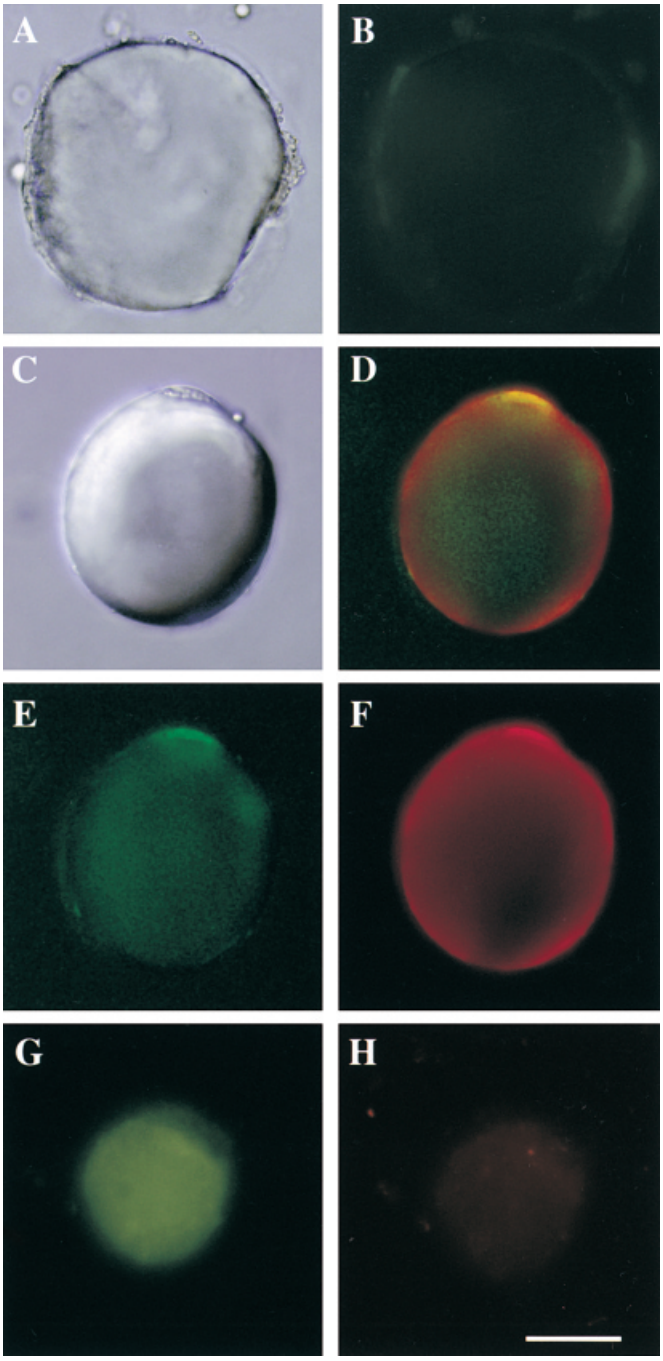

Fig. 2. Adenovirus-mediated gene transfer into primary murine adipocytes. Confocal laser-scanning image of fixed infected adipocytes (C–H) and uninfected control cells (A and B). The cells in (C–F) were treated with the adipocyte-specific antibody, ALBP/aP2, whereas the cells in (G) and (H) were hybridized with preimmune sera. (A) and (C) are light-phase images; (B), (D), (E), and (G) show GFP levels; (D), (F), and (H) indicate antibody binding. The scale bar represents 20 μm. The experiment was repeated a minimum of three times.

as judged by trypan blue exclusion and fluorescence microscopy.

Because the GFP was being expressed from a strong CMV promoter, an additional experiment was performed to assess the effectiveness of this method in the examination of low-expressing genes. Primary murine adipocytes were infected with the adenovirus (MOI of 50) that contained a luciferase reporter construct driven by a weak adipocyte promoter, the keratinocyte lipid-binding protein (KLBP/FABP5) promoter (6). After 8–18 h, the resulting cell extract was assayed for luciferase activity (**Fig. 3**). The levels of luciferase driven by the forward promoter reached approximately 30-fold over background whereas uninfected cells had luciferase levels comparable to background readings. There were no significant differences if the cells were incubated between 8 and 18 h. Incubation times of less than 8 h resulted in low levels of expression. All other transfection methods attempted (LipofectAMINE, electroporation) on primary murine adipocytes yielded levels equivalent to background levels (data not shown). These results demonstrate the ability to monitor even weak promoter activity through reporter genes in primary murine adipocytes, using an adenovirus-mediated gene transfer technique.

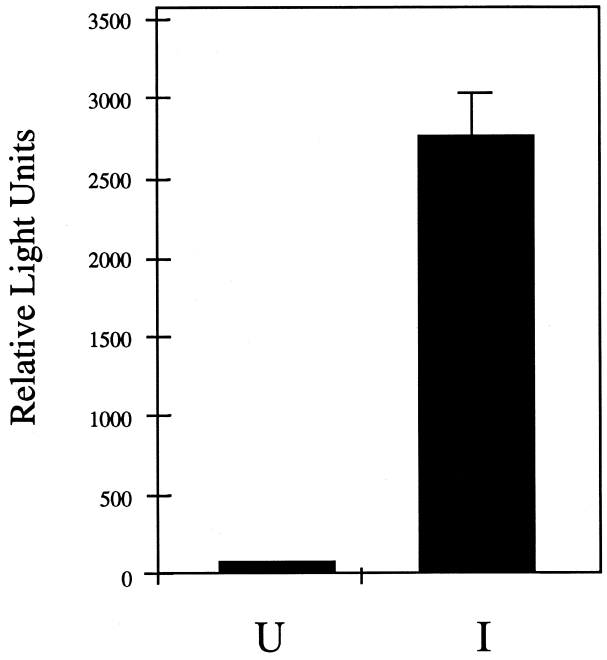

Fig. 3. The KLBP/FABP5-luciferase reporter assay of adenovirus-infected adipocytes. Luciferase activity was measured from infected or control primary adipocytes. Levels of activity from 10-s counts were plotted as the average ± SEM of three separate experiments. The results are statistically significant ($P < 0.05$) by the paired Student *t* test. I, Infected cells; U, uninfected cells.

Previously, the KLBP/FABP5 gene was identified through its upregulation by TPA treatment (10). To demonstrate that a regulated gene could be monitored by this method, adipocytes were infected with the KLBP/FABP5-luciferase adenovirus along with the pCMV-β-galactosidase control adenovirus and were treated with TPA for 8 h (**Fig. 4**). The cells were washed, lysed, and the luciferase and β-galactosidase activities were measured. The luciferase activity was normalized for infection efficiency (as indicated by the β-galactosidase levels). The reporter analysis showed a 1.85-fold increase in luciferase activity due to TPA treatment.

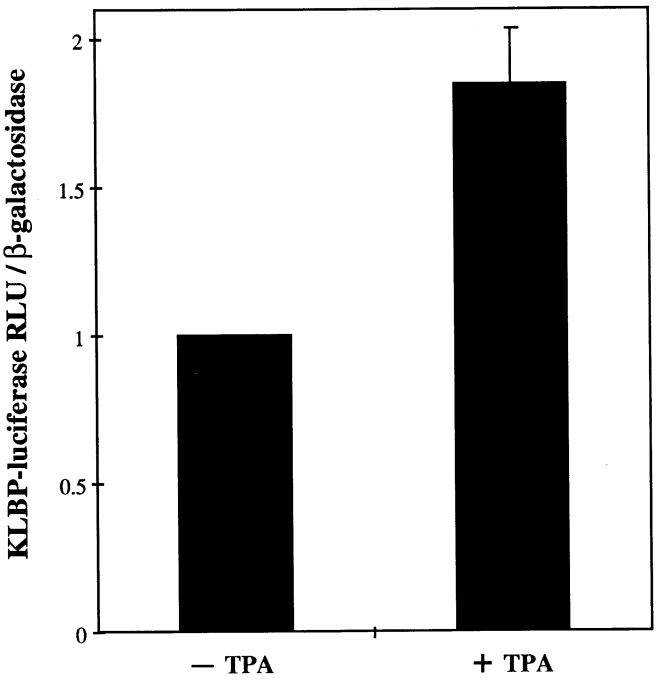

Fig. 4. TPA treatment of adenovirus-infected primary murine adipocytes. Primary adipocytes were treated with or without TPA at 100 ng/mL for 8 h. The cells were washed, lysed, and assayed for luciferase and β-galactosidase activities. The data represent the average ± SEM of three experiments using pooled adipocytes from a minimum of four mice. The results are statistically significant ($P < 0.05$) by the paired Student *t* test.

Although this technique should prove valuable, it must be taken into account that cells in culture do not always faithfully maintain the protein expression pattern of the cells in vivo. The metabolic state of the cells in vitro may be altered as compared with the cells in vivo. It is consequently important to verify that the specific effect/mRNA/protein studied holds true through the culturing process. One example in which protein concentrations change during adipocyte culturing involves the glucose transporters GLUT4 and GLUT1. On culturing primary rat adipocytes, GLUT4 mRNA levels decrease 20-fold and GLUT1 mRNA levels increase 70-fold (11). Because of this alteration in mRNA, insulin-stimulated glucose transport is reduced. Therefore, it is necessary to determine that the results obtained are accurate and not simply a result of the in vitro incubation.

The method presented here results in efficient gene transfer in primary murine adipocytes, permitting expanded studies of lipid metabolism and control of gene expression. Because most transgenic animals are mice, the ability to introduce DNA into murine adipocytes is invaluable. Currently, studies of mice with mutations connected to obesity and insulin resistance have included mostly biochemical analyses of lipid and carbohydrate metabolism in fat cells. With the methods described here, studies of gene expression and identification of the alterations in specific gene expression responsible for the biochemical consequences will be possible, allowing a greater understanding of adipocyte cell function.

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