methods

Demethylation using the epigenetic modifier, 5-azacytidine, increases the efficiency of transient transfection of macrophages

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Abstract **This study was aimed at developing a method for high-efficiency transient transfection of macrophages. Seven methods were evaluated for transient transfection of murine macrophage RAW 264.7 cells. The highest transfection efficiency was achieved with DEAE-dextran, although the proportion of cells expressing the reporter gene did not exceed 20%. It was subsequently found that the cytomegalovirus plasmid promoter in these cells becomes methylated. When cells were treated with the methylation inhibitor 5-azacytidine, methylation of the plasmid promoter was abolished and a dose-dependent stimulation of reporter gene expression was observed with expression achieved in more than 80% of cells. Treatment of cells with 5-azacytidine also caused increased efficiency of transfection of macrophages with plasmids driven by RSV, SV40, and EF-1α promoters and transient transfection of human HepG2 cells. Inhibition of methylation also increased the amount and activity of sterol 27-hydroxylase (CYP27A1) detected in RAW 264.7 cells transfected with a CYP27A1 expression plasmid. Treatment of cells with 5-azacytidine alone did not affect either cholesterol efflux from nontransfected cells or expression of ABCA1 and CYP27A1. However, transfection with CYP27A1 led to a 2- to 4-fold increase of cholesterol efflux. We conclude that treatment with 5-azacytidine can be used for high-efficiency transient transfection of macrophages.**—Escher, G., A. Hoang, S. Georges, U. Tchoua, A. El-Osta, Z. Krozowski, and D. Sviridov. **Demethylation using the epigenetic modifier, 5-azacytidine, increases the efficiency of transient transfection of macrophages.** ***J. Lipid Res.*** **2005.** 46: **356–365.**

Supplementary key words cholesterol efflux • CYP27A1 • atherosclerosis • lipoproteins

Macrophages play an important role in host defense pathways and are also involved in a variety of diseases, including atherosclerosis (1, 2). The key role of macrophages in the development of atherosclerosis has made this cell type a versatile in vitro model of this disease (3). Transfection of macrophages is a powerful tool to study their function, and a number of methods have been described to achieve high levels of expression of different genes through transient transfection (4–6). These levels of expression are sufficiently high to study synthetic processes, when proteins are tagged or otherwise distinguished from host proteins. However, studying cell functions such as growth, lipoprotein binding, lipid uptake, and efflux requires not only high levels of gene expression but also for the gene to be expressed in a majority of cells, a high-efficiency transfection. High efficiency of transfection is also critical for a multiple gene transfection, as it requires that all transfected genes be expressed in the same population of cells. Viral and stable transfections offer adequate efficiency of DNA transfer; however, they are often labor-intensive and time-consuming. High-efficiency transient transfection of macrophages has proved to be difficult.

Here, we describe a method for high-efficiency transient transfection of RAW 264.7 mouse macrophages. We fortuitously found that the low efficiency of expression of transfected genes in macrophages is a consequence of methylation-mediated silencing of transfected genes rather than of low uptake of DNA into cells. To maximize the efficiency of macrophage transfection, we evaluated the DNA methylation inhibitor, 5-azacytidine, an epigenetic modifier often used to reactivate methylation-dependent transcriptionally silent genes (7). We demonstrated by methylation-specific PCR that 5-azacytidine prevents methylation of the promoter of transfected genes, and for the first time we achieved transient expression of a reporter protein in 80–100% of macrophage cells. The method was then used for the high-efficiency transient transfection of RAW 264.7 macrophages with sterol 27-hydroxylase (CYP27A1), which led to the stimulation of cholesterol efflux from these cells.

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Abbreviations: apoA-I, apolipoprotein A-I; CMV, cytomegalovirus; CYP27A1, sterol 27-hydroxylase; GFP, green fluorescent protein; LXR, liver X receptor.

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MATERIALS AND METHODS

Cells

RAW 264.7, HepG2, and CHOP (8) cells were grown in RPMI 1640 medium containing 10% FBS, 2 mmol/l L-glutamine, and penicillin/streptomycin (50 U/ml). The day before transfection, cells were plated on 12-well plates at a density of $\sim 0.6 \times 10^5$ cells per well.

Transient transfection

Transient transfection was performed on 12-well plates using 1 μg of plasmid DNA [cytomegalovirus (CMV)-LacZ or CMV-CYP27A1 tagged with myc (8)] and different transfection reagents. The methods were optimized using CHOP cells where they resulted in 80–90% transfection. CHOP cells were also used as a positive control during transfection.

DEAE-Dextran. Cells were incubated for 2 h in a 1 ml cocktail containing 10% FBS, 50 mmol/l Tris-HCl, pH 7.3, 0.35 mg/ml DEAE-dextran, and the plasmid DNA in Optimem. Cells were then washed with PBS and subjected to DMSO shock (10% DMSO in PBS) for 1 min. Cells were then washed and cultured for 48 h.

FuGene. Three microliters of FuGene 6 reagent (Roche) was added drop-wise to 97 μl of Optimem and incubated for 5 min at room temperature. One microgram of DNA was added to the FuGene/Optimem mix and incubated for 15 min at room temperature. Fresh medium was added to cells, the DNA/FuGene/Optimem mixture was added drop-wise, and cells were cultured for 48 h.

Effectene. One microgram of DNA was added to the final volume of 150 μl of DNA condensation buffer, mixed with 8 μl of Enhancer, and incubated for 5 min at room temperature. Twenty-five microliters of Effectene transfection reagent (Qiagen) was added to the DNA/Enhancer mixture and incubated for 10 min, allowing the complex to form. Cells were washed with PBS, 1 ml of fresh medium was added, followed by drop-wise addition of the transfection complex in 1 ml of medium. Cells were analyzed 48 h later.

Lipofectamine and Lipofectamine 2000. Plasmid DNA (1 μg) and 3 μl of Lipofectamine (Invitrogen) or 3 μl of Lipofectamine 2000 (Invitrogen) were diluted separately in 50 μl of Optimem. After 5 min, the diluted DNA was combined with the diluted transfection reagent for complex formation (20 min at room temperature). The mixture was then added to cells, and cells were cultured for 48 h.

Cellfectin. One microgram of plasmid DNA and 3 μl of Cellfectin (Invitrogen) were diluted separately in 100 μl of Optimem, combined, and incubated for 15 min at room temperature to allow complex formation. Cells were washed with PBS and incubated for 15 min in Optimem. The transfection cocktail was added to cells and after 6 h changed for a complete medium. Cells were analyzed 48 h later.

X-tremeGENE. Plasmid DNA was diluted in DNA dilution buffer, and 32 μl of X-tremeGENE (Roche) was diluted in Optimem. Reagents were combined, incubated for 10 min at room temperature, and added to cells in 500 μl of serum-free medium. After 4 h of incubation, 500 μl of RPMI containing 20% FCS was added, and cells were cultured for 48 h.

Where indicated, after transfection cells were incubated for 48 h with the indicated concentration of 5-azacytidine (Sigma) replenished daily. Cells were washed with ice-cold PBS, fixed with 4% formaldehyde for 5 min at 4°C, and stained for 30 min at 37°C in a staining solution containing 1 μg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal). For the purposes of this study, the efficiency of transfection was defined as a proportion of cells expressing a reporter gene [β-galactosidase or green fluorescent protein (GFP)].

Generation of recombinant adenovirus encoding GFP and CYP27A1

The recombinant adenovirus Ad5-CYP27 was generated by homologous recombination in HEK-293 cells using the AdEasy system (Quantum Biotechnologies). A human cDNA encoding CYP27 was subcloned into the shuttle vector pAdTrack by partial digestion using *Kpn*I and *Xba*I. A positive clone was linearized with *Pme*I and cotransformed with the adenoviral backbone pAdEasy-1 in *Escherichia coli* BJ5183 for homologous recombination. DNA from positive clones (Ad5-CYP27) was retransformed in DH5α for DNA amplification. The resulting adenovirus expressed the GFP and CYP27A1 both under the control of the CMV promoter.

For adenovirus production, 5 μg of the recombinant virus Ad5-CYP27 was digested with *Pac*I, extracted with phenol/chloroform, precipitated with ethanol, and transfected with 6 μl of Lipofectamine into the mammalian package cell line HEK-293. Virus formation was monitored by the production of GFP. Plaques were observed for 14 days after transfection. Cells were harvested at 2,700 *g* for 5 min at room temperature, and viruses were extracted by four cycles of freeze/thaw/vortex. Cells debris was removed by centrifugation at 7,700 *g* at room temperature for 5 min, and the supernatant containing the viruses was reused to infect a larger number of cells. Virus was purified by cesium chloride gradient centrifugation and dialyzed for 18 h at 4°C in a buffer containing 10 mmol/l Tris, pH 8.0, 2 mmol/l $MgCl_2$, and 4% sucrose.

RAW 264.7 cells were infected with the virus (multiplicity of infection = 6,200) and incubated for the indicated periods of time in the presence (treated) or absence (untreated) of 1 μmol/l 5-azacytidine. Cells were then washed and observed with a fluorescence microscope. The proportion of cells expressing GFP was counted in four wells.

Methylation studies

RAW 264.7 cells or CHOP cells were transfected with DEAE-dextran as described above and treated for 48 h with the indicated concentrations of 5-azacytidine. Cells were then harvested, and DNA was extracted with the tissue DNA extraction kit (Qiagen). Twenty micrograms of DNA was used for bisulfite conversion according to Clark et al. (9). Briefly, after digestion of the DNA with *Xba*I and DNA purification, alkaline denaturation was performed by incubating the DNA in 0.3 mol/l NaOH at 70°C for 15 min to obtain single-stranded DNA. For the deamination step, a saturated solution of sodium bisulfite was used. One hundred microliters of 1% hydroquinone solution in water was added to the saturated sodium bisulfite solution (4.55 g of sodium bisulfite dissolved in 9 ml of water, pH adjusted to 5.0), and 900 μl of this freshly prepared solution was added to the denatured DNA. The deamination reaction was performed overnight at 50°C in the dark. DNA was desalted using a DNA purification kit (Qiagen) and desulfonated in the presence of 0.3 mol/l NaOH at 37°C for 45 min. DNA solution was then neutralized, precipitated with ethanol, and resuspended in 200 μl of water.

Five microliters of bisulfite-treated DNA was prepared for hot-start PCR amplification using a pair of primers complementary to a region of the CMV promoter not containing methylation sites (oligos 1; 5′, TAT TGT TAT TAT TAT GGT GAT GTG G; 3′, ATT ACA ACA TTT TAA AAA ATC CCA TT) or a pair of primers complementary to a region of the CMV promoter that contains methylation sites (oligos 2; 5′, TTA TCG TTA TTA TTA TGG TGA TGC G; 3′, TAT TAC GAC ATT TTA AAA AAT CCC G). Amplification conditions involved initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 30 s, and elongation at 72°C for 2 min, with a final elongation at 72°C for 10 min. Five microliters of PCR products was run on a polyacrylamide gel, and the DNA was visualized using ethidium bromide.

Confocal microscopy

RAW 264.7 cells were grown on cover slips, transfected with CYP27A1 tagged with the myc epitope (8), and where indicated treated with 5-azacytidine. Cells were cultured for 48 h before immunostaining. Mitochondria were stained for 45 min at 37°C using 100 nmol/l Mitotracker Red (Molecular Probes). For immunostaining of CYP27A1, cells were washed with PBS, fixed with 4% formaldehyde, and quenched with 50 mmol/l NH_4Cl. Cells were then permeabilized with 0.5% Triton X-100, incubated with the monoclonal anti-myc antibody QE10 for 1 h, washed with PBS, and incubated in the dark with a secondary goat anti-mouse FITC-labeled antibody diluted 1:100 for 1 h. Cells were washed again with PBS and, after mounting onto glass slides, were studied using a Zeiss META confocal microscope.

Western blot, real-time RT-PCR, and enzyme activity

RAW 264.7 cells were lysed in RIPA buffer, and proteins were separated on a 12.5% SDS polyacrylamide gel followed by immunoblotting using the monoclonal anti-myc antibody QE10. Bands were visualized by chemiluminescence development and quantitated by densitometry.

Real-time RT-PCR for ABCA1 was performed as described previously (10). Real-time RT-PCR for CYP27A1 was performed using the Assay-on-Demand kit (Applied Biosystems, Foster City, CA). Quantities of mRNA were compared with 18S RNA and expressed in arbitrary units relative to the control.

Activity of CYP27A1 was assessed by conversion of [^{3}H]cholesterol into 27-hydroxycholesterol as described previously (8).

Cholesterol efflux

Apolipoprotein A-I (apoA-I) was isolated from human plasma as described previously (11). RAW 264.7 cells were grown on 12-well plates, transfected with 500 ng of plasmid (CYP27A1 or $pcDNA_3$), and where indicated treated for 48 h with 0.5 μmol/l 5-azacytidine. Cholesterol efflux experiments were performed as described previously (8, 12). Briefly, to label cellular cholesterol, cultures were incubated in serum-containing medium supplemented with [1α,2α(n)-^{3}H]cholesterol (Amersham Pharmacia Biotech; specific radioactivity 1.81 TBq/mmol, final radioactivity 74 KBq/ml) for 48 h in a CO_2 incubator. After labeling, cells were washed six times with PBS and further incubated for 18 h in serum-free medium containing 5-azacytidine where indicated. Cells were then washed and incubated for 0.5 or 2 h at 37°C in serum-free medium containing 30 μg/ml lipid-free apoA-I. The medium was then collected and centrifuged for 15 min at 4°C at 15,000 *g* to remove cellular debris, and the supernatant was counted. Cells were harvested using a cell scraper, dispensed in 0.5 ml of distilled water, and aliquots were counted. Cholesterol efflux was expressed as a percentage of labeled cholesterol transferred from cells to the medium.

Statistical analysis

All experiments were reproduced two to four times, and results of representative experiments are shown. Unless otherwise indicated, experimental groups consisted of quadruplicate cultures. Means ± SD are presented. The Student's *t*-test was used to determine the statistical significance of the differences.

RESULTS

Seven popular methods for DNA-mediated transient transfection were initially compared using RAW 264.7 cells. The methods tested were DEAE-dextran, FuGene, Effectene, Lipofectamine, Lipofectamine 2000, Cellfectin, and X-tremeGENE. The efficiency of transfection of RAW cells was tested using CMV-LacZ plasmid, which contains the bacterial β-galactosidase gene under the control of the CMV promoter in a $pcDNA_3$ plasmid. The efficiency of transfection was defined as a proportion of cells expressing β-galactosidase and was assessed microscopically after fixing and staining the cells with X-gal. The Lipofectamine method was the least efficient, with 0.1% of cells found to express the X-gal gene (**Fig. 1**). Other methods resulted in 5–10% of cells expressing the X-gal gene, with the DEAE-dextran method being marginally better than other methods (Fig. 1). The relatively better efficiency of the DEAE-dextran method is consistent with the findings of Mack et al. (13), and this method was chosen for further experiments.

To investigate whether the low efficiency of transfection is attributable to silencing of the CMV promoter by methylation of CpG island-rich sequences, we incubated cells for 48 h with different concentrations of 5-azacytidine after transfection. Increasing the amount of DNA from 0.1 μg to 1.0 μg per well increased the efficiency of transfection from 10% to 45% (**Fig. 2**). However, amounts of DNA greater than 0.5 μg led to changes in cell morphology and slowing of proliferation, most likely reflecting a toxic effect of large amounts of foreign DNA entering the cell. A dramatic effect was observed when cells were incubated with 5-azacytidine after transfection. Eighty percent of cells were positive for X-gal after incubation for 48 h in the presence of 1 μmol/l 5-azacytidine (Fig. 2). In some experiments, 5-azacytidine-induced demethylation increased transfection efficiency above 95%. We also evaluated different doses of 5-azacytidine: 0.5 and 1 μmol/l were almost equally effective (data not shown), whereas higher doses led to changes in cell morphology and detachment of a proportion of

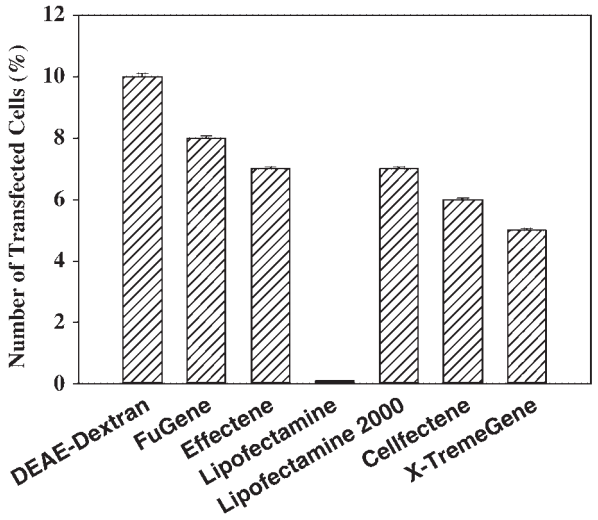


Fig. 1. Screening of different transfection methods in RAW 264.7 cells. RAW 264.7 cells were plated on 12-well plates, and seven different methods (described in Materials and Methods) were used to transfect 1 μg of the reporter plasmid cytomegalovirus (CMV)-LacZ. The number of transfected cells per 100 cells was counted in four wells. Means ± SD are presented.

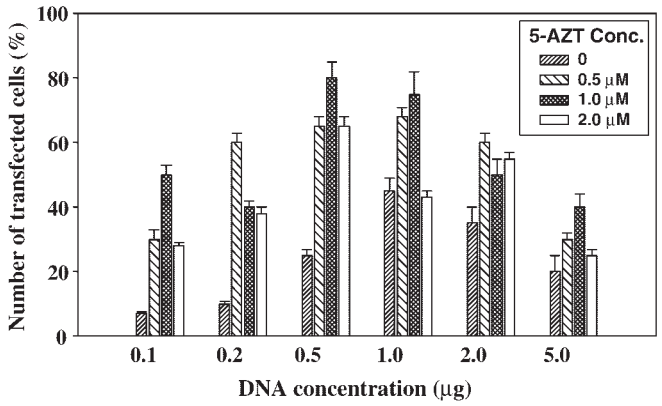
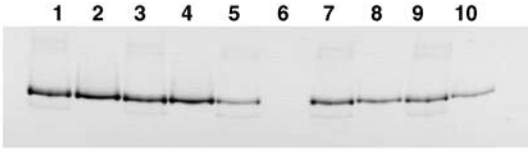


Fig. 2. Effect of DNA concentration and demethylation on transfection efficiency in RAW 264.7 cells. RAW cells were plated on 12-well plates and transfected with increasing concentrations of the reporter plasmid CMV-LacZ. After transfection, cells were treated with the indicated concentrations of 5-azacytidine for 48 h. The number of transfected cells per 100 cells was counted in four wells. Means ± SD are presented. 5-AZT Conc., 5-azacytidine concentration.



Source	CMV		CHOP		RAW		RAW		RAW	
5'azacytidine	-		-		-		0.3 μM		1.0 μM	
Oligo 1	+		+		+		+		+	
Oligo 2		+		+		+		+		+

Fig. 3. Methylation status of the CMV promoter after treatment of cells with 5-azacytidine. Pure plasmid (lanes 1, 2) or DNA was isolated from transfected CHOP cells (lanes 3, 4) or RAW cells untreated (lanes 5, 6) or treated with 0.3 μM (lanes 7, 8) or 1.0 μM (lanes 9, 10) 5-azacytidine. DNA was treated as described in Materials and Methods and amplified by PCR using oligos 1 (directed against a region of the CMV promoter not containing methylation sites) or oligos 2 (directed against a region of the CMV promoter that contains methylation sites).

cells, possibly indicating a toxic effect of high doses of 5-azacytidine. Therefore, 0.5 μg of DNA and 1–0.5 μmol/l 5-azacytine were used for further experiments.

To demonstrate that 5-azacytidine acted by demethylation of the CMV promoter, methylation status of the CMV promoter was tested by methylation-specific PCR. The methylation status of six CpG sites located within the CpG island of the CMV promoter was examined by bisulfite genomic treatment to establish whether any CpG sites were methylated (9). This method is based on a selective deamination of cytosine to uracil by treatment with bisulfite and subsequent amplification by PCR using specific primers. Two pairs of oligonucleotide sequences were selected to serve as primers in the PCR reaction. Oligos 1 were designed to be complementary to the region of the CMV promoter that does not contain methylation sites and amplify PCR products independently of methylation. Oligos 2 were designed to be complementary to the region of the CMV promoter that contains methylation sites and would not amplify PCR products if methylation occurs (see Materials and Methods). When plasmid itself was tested after bisulfite conversion, amplification by PCR occurred with both oligos 1 and oligos 2, indicating that the original plasmid DNA is not methylated (**Fig. 3**, lanes 1, 2). This was also true for DNA isolated from transfected CHOP cells, indicating that there is no methylation of the CMV promoter in these cells (Fig. 3, lanes 3, 4). When DNA was isolated from RAW 264.7 cells transfected as described above and not treated with 5-azacytidine, there was no signal when oligos 2 were used, whereas a PCR product was formed with oligos 1, indicating methylation of the CMV promoter in these cells (Fig. 3, lanes 5, 6). When cells were treated with 5-azacytidine, methylation of the CMV promoter was abolished, because with both sets of primers a PCR product was formed (Fig. 3, lanes 7–10). As expected, no bands were found with both primers in nontransfected cells (data not shown). Thus, treatment of RAW 264.7 cells with 5-azacytidine abolished methylation of the CMV promoter, therefore suppressing the silencing of a gene after transfection.

To test whether methylation of the CMV promoter also causes low efficiency of transfection by other methods, we treated RAW cells with 5-azacytidine after transfection by three other methods. Treatment with 5-azacytidine only slightly increased the efficiency of transfection of macrophages by FuGene and had no effect on cells transfected with Lipofectamine (**Fig. 4A**). However, when the cells were infected with adenovirus, treatment with 5-azacytidine resulted in up to a 5-fold improvement of the efficiency of transfection (Fig. 4B).

To test whether promoter methylation is a cause of low efficiency of transfection with plasmids driven by non-CMV promoters, we tested transfection with plasmids in which CMV promoter was substituted with either one of two viral promoters, RSV and SV40, or a mammalian promoter, EF-1α. These promoters are frequently used for overexpression of heterologous genes and together with the CMV promoter represent a vast majority of the promoters used for heterologous gene overexpression. The basal efficiency of expression driven by these promoters was lower than from plasmids driven by the CMV promoter, consistent with these promoters being weaker promoters compared with CMV. However, treatment of macrophages with 5-azacytidine caused 11-, 8-, and 5-fold increases in the number of transfected cells for plasmids driven by RSV, SV40, and EF-1α promoter, respectively (**Fig. 5A**).

To determine whether or not methylation of CMV promoter is a unique phenomenon in mouse macrophages, we tested the effect of 5-azacytidine on transient transfection of human hepatoma HepG2 cells. When different transfection methods were compared without treatment with 5-azacytidine, the highest efficiency of transfection was observed with the FuGene method (data not shown). Treatment with 5-azacytidine more than doubled the number of transfected HepG2 cells (Fig. 5B).

To assess the possibility of using 5-azacytidine in functional

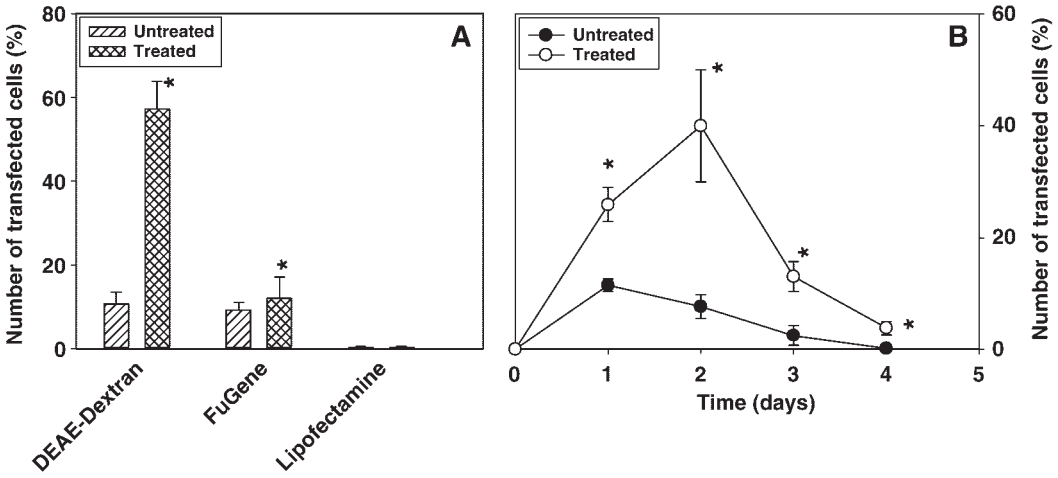


Fig. 4. Effect of demethylation on the efficiency of different transfection methods. RAW 264.7 cells were plated on 12-well plates, and different methods (described in Materials and Methods) were used to transfect 1 μg of the reporter plasmid CMV-LacZ (A) or adenovirus containing green fluorescent protein and sterol 27-hydroxylase (CYP27A1) under the control of the CMV promoter (B). Cells were incubated for 48 h (A) or the indicated periods of time (B) in the presence (treated) or absence (untreated) of 1 μmol/l 5-azacytidine. The number of transfected cells per 100 cells was counted in four wells. Means ± SD are presented. A: Transient transfection. B: Time course of gene expression after transfection with adenovirus (multiplicity of infection = 6,200). * $P < 0.01$ versus untreated cells.

studies, we investigated the effect of transfection of RAW 264.7 cells with CYP27A1 on cholesterol efflux. CYP27A1 stimulated cholesterol efflux when transfected with high efficiency into CHOP cells (8). Studying cholesterol efflux requires that the majority of cells in the culture respond to treatment, because even large increases in cholesterol efflux from a small proportion of cells are difficult to detect. Because macrophages may contain endogenous CYP27A1 (14), the transfected protein was tagged with C-myc peptide. When analyzed on a Western blot, treatment with 0.5 and 1 μmol/l 5-azacytidine significantly increased the amount of tagged CYP27A1 expressed in RAW 264.7 cells after transfection (**Fig. 6A**). Further increasing the concentration of 5-azacytidine to 2 μmol/l decreased the amount of CYP27A1, possibly as a result of a toxic effect. The activity of CYP27A1, assessed as the conversion of [^{3}H]cholesterol into [^{3}H]27-hydroxycholesterol, was significantly higher in cells transfected with CYP27A1 and treated with 1 μmol/l 5-azacytidine (Fig. 6B).

The effect of 5-azacytidine on the expression of CYP27A1 was further investigated using confocal microscopy. CYP27A1 is a mitochondrial enzyme, and to assess possible colocalization of transfected CYP27A1 with mitochondria, the latter were labeled with Mitotracker Red. Transfected cells

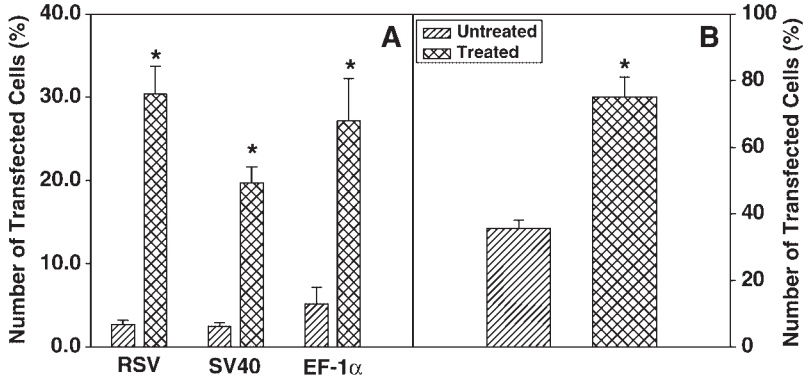


Fig. 5. Effect of demethylation on the efficiency of transfection with different plasmids and in different cells. A: RAW 264.7 cells were plated on 12-well plates transfected using the DEAE-dextran method and 1 μg of the reporter plasmid CMV-LacZ, RSV-LacZ, SV40-LacZ, or EF-1α-Lac-Z. Cells were incubated for 48 h in the presence (treated) or absence (untreated) of 0.5 μmol/l 5-azacytidine. The number of transfected cells per 100 cells was counted in four wells. Means ± SD are presented. B: HepG2 cells were plated on 12-well plates transfected using the FuGene method and 1 μg of the reporter plasmid CMV-LacZ. Cells were incubated for 48 h in the presence (treated) or absence (untreated) of 0.5 μmol/l 5-azacytidine. The number of transfected cells per 100 cells was counted in four wells. Means ± SD are presented. * $P < 0.01$ versus untreated cells.

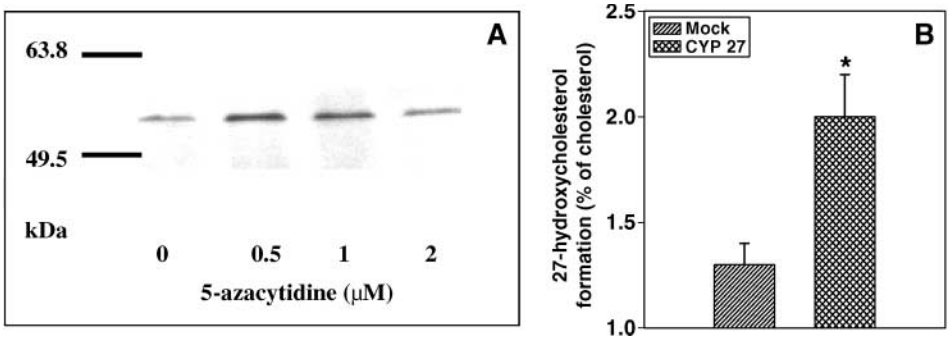


Fig. 6. Expression and activity of CYP27A1 in RAW 264.7 cells after treatment with 5-azacytidine. A: Western blot of lysates of RAW 264.7 cells transfected with pcDNA$_3$2M-CYP27A1-myc and untreated (lane 1) or treated for 48 h with 0.5 (lane 2), 1.0 (lane 3), or 2.0 (lane 4) μmol/l 5-azacytidine. B: Activity of CYP27A1 in mock-transfected RAW 264.7 cells and cells transfected with CYP27A1 and treated with 1 μmol/l 5-azacytidine. Cells were labeled with [^{3}H]cholesterol as described in Materials and Methods, and lipids were extracted and separated using thin-layer chromatography. The activity of CYP27A1 was assessed as the proportion of [^{3}H]cholesterol converted into [^{3}H]27-hydroxycholesterol. Means ± SD are presented. * $P < 0.01$ versus controls.

were incubated with or without 5-azacytidine. Mitochondria were stained with Mitotracker Red just before immunostaining with monoclonal anti-myc antibody and FITC-labeled anti-mouse IgG antibody. Without treatment with 5-azacytidine, there were very few cells stained with anti-myc antibody (green) (**Fig. 7A**), although they were carrying mitochondrial staining (red) (Fig. 7B). When cells were treated with 5-azacytidine, all cells that were carrying mitochondria label (Fig. 7D) were also stained with anti-myc antibody (Fig. 7C). There was a nearly total overlap of red and green staining, with most cells stained yellow when both staining patterns overlapped (Fig. 7E).

When cholesterol efflux from RAW 264.7 to apoA-I was assessed, there was no statistically significant effect of 5-azacytidine (**Fig. 8A**). Cholesterol efflux was then stimulated by treating cells with the liver X receptor (LXR) agonist TO-901317 (1 μmol/l), which induces expression of ABCA1, a key element in the cholesterol efflux pathway (15). Although cholesterol efflux tripled after treatment of cells with TO-901317, there was no difference in the efflux from cells treated with or without 5-azacytidine (Fig. 8A). To evaluate whether treatment with 5-azacytidine affects the expression of key cellular proteins responsible for cholesterol efflux, expression of ABCA1 was assessed by real-time RT-PCR. No effect of 5-azacytine on the expression of ABCA1 was found, whereas treatment with TO-901317 increased ABCA1 expression 10-fold (Fig. 8B). Lack of changes in ABCA1 expression strongly indicates a lack of the effect of 5-azacytidine on the expression of LXR, a key regulator of a number of genes of lipid metabolism, including ABC transporters (16). We also assessed the effect of 5-azacytidine on another putative regulator of the cholesterol efflux pathway, CYP27A1 (8). There was no effect of 5-azacytdine on CYP27A1 expression in RAW cells (Fig. 8C). Thus, it is unlikely that 5-azacytidine itself affects cholesterol metabolism in macrophages. We also assessed a pattern of digestion of cellular DNA with two restriction enzymes, *Msp*I, which cuts sites not susceptible to methylation, and *Hpa*II, which cut sites susceptible to methylation. We found no difference in the DNA digestion pattern by either enzyme when cells were treated with or without 1 μmol/l 5-azacytidine (data not shown). This finding indicates that there are no gross changes in DNA methylation in RAW 264.7 cells after treatment with 5-azacytidine. However, a more sensitive, gene-specific approach is required to more fully understand the effects of 5-azacytidine on the genome of host cells.

When RAW 264.7 cells were transiently transfected with CYP27A1 and treated with 5-azacytidine, cholesterol efflux to apoA-I was 2- to 4-fold higher compared with mock-transfected cells also treated with 5-azacytidine (**Fig. 9A**). There was a 3-fold increase in expression of ABCA1 as a result of transfection with CYP27A1 (Fig. 9B). This is consistent with 27-hydroxycholesterol and possibly another product of CYP27A1, cholestenoic acid, being ligands of the LXR (17). This observation contrasts with our previous finding that overexpression of CYP27A1 induces cholesterol efflux without affecting ABCA1 levels in CHOP cells (8), indicating possible tissue-specific differences in regulation of the cholesterol efflux pathway. There was no increase of cholesterol efflux from cells transfected with CYP27A1 but not treated with 5-azacytidine (data not shown).

DISCUSSION

In vitro gene delivery permits the study of gene function in various cell types through the insertion of foreign genes or the alteration of existing genes and their expression patterns under a variety of physiological stimuli. A critical parameter for success is the selection of an appropriate cell line. The macrophage cell lines are a classic cell type often used to investigate the mechanism of atherosclerosis (18, 19). However, there remains an inherent problem, as genes are transcriptionally silenced in many cell types, which poses a major obstacle to transgene delivery (20, 21).

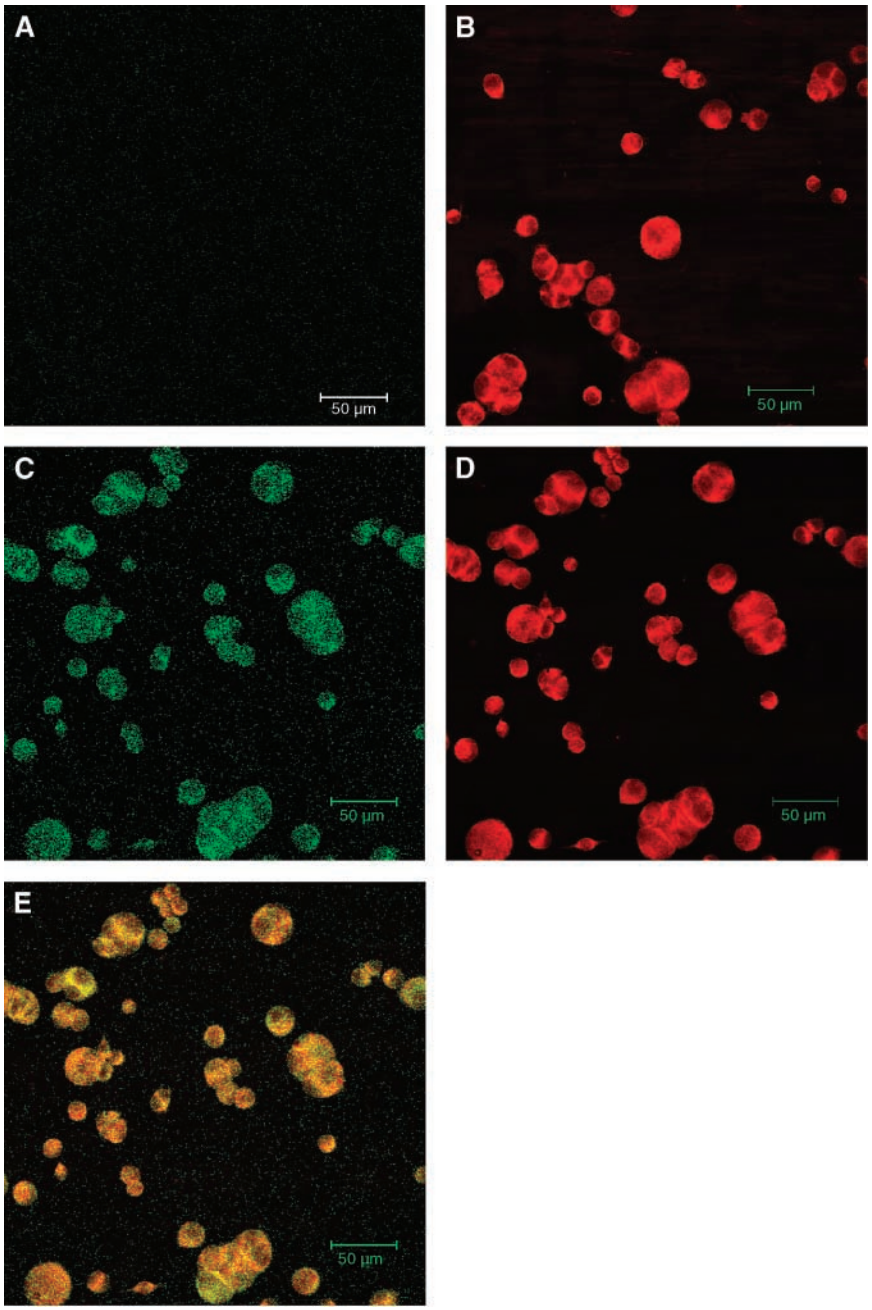


Fig. 7. Confocal microscopy of RAW 264.7 cells after transfection with CYP27A1 and treatment with 5-azacytidine. After transfection, RAW 264.7 cells untreated (A, B) or treated with 0.5 μmol/l 5-azacytidine (C, D, E) were immunostained with anti-myc antibody and prepared for confocal microscopy as described in Materials and Methods. Mitochondria were stained with Mitotracker Red. A, C: Green fluorescence (FITC) reflecting staining of CYP27A1. B, D: Red fluorescence (Mitotracker Red) reflecting staining of mitochondria. E: Overlay of C and D. Bars = 50 μm.

The major finding of this study is that abolishing methylation of the plasmid promoter after transient transfection of macrophages results in a dramatic increase in the proportion of cells expressing heterologous protein. Transient transfection is a powerful tool for rapid screening of the effect of the expression of a chosen gene in a cell. In contrast to stable transfection, transient transfection does not require a time-consuming cloning process, and preparing a plasmid is easier and faster than a viral infection. Transient transfection is therefore an essential tool when many genes have to be tried in a high-throughput assay and for multiple gene transfections. The common problem, however, is that certain cell types are difficult to transfect with efficiency sufficiently high for the functional studies. The macrophage is one of these cell types.

Low efficiency of transfection can be caused by either low competency of transferring DNA into the cell or rapid silencing of the plasmid promoter. Seven different meth-

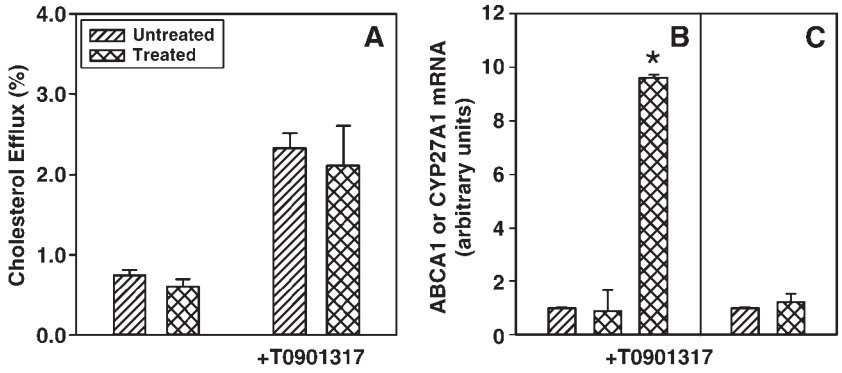


Fig. 8. Effect of 5-azacytidine on ABCA1 expression and cholesterol efflux from RAW 264.7 cells. A: RAW 264.7 cells were labeled with [^{3}H]cholesterol as described in Materials and Methods and incubated for 48 h in the presence (treated) or absence (untreated) of 0.5 μmol/l 5-azacytidine. This was followed by incubation of cells for 18 h in the absence or presence of the liver X receptor agonist T-O901317 (final concentration, 1 μmol/l). Cells were then incubated with human lipid-free apolipoprotein A-I (apoA-I; final concentration, 30 μg/ml) or serum-free medium alone for 2 h at 37°C in a CO_2 incubator. Medium was collected, cells were washed, and the amount of radioactivity in the cells and medium was determined by liquid scintillation spectrometry. B: Real-time RT-PCR of ABCA1 mRNA isolated from RAW 264.7 cells incubated for 48 h in the presence (treated) or absence (untreated) of 0.5 μmol/l 5-azacytidine or with 0.5 μmol/l 5-azacytidine and 1 μmol/l T-O901317. * $P < 0.001$ versus control. C: Real-time RT-PCR of CYP27A1 mRNA isolated from RAW 264.7 cells incubated for 48 h in the presence (treated) or absence (untreated) of 0.5 μmol/l 5-azacytidine. Means ± SD are presented.

ods were tested in this study, but none of them produced the transfection efficiency required for functional studies. We hypothesized that the problem might not be only a delivery of DNA into the cells but also a silencing of heterologous gene expression.

There are several mechanisms for how genes may be silenced, with methylation of the gene promoter being one of the most common phenomena (22–24). Demethylation is often used to overcome gene silencing (25). Promoters of heterologous genes may also be methylated, and demethylation and/or histone deacetylation has been used to increase the efficiency of gene expression after stable (20, 26) or viral (27–29) transfections. To our knowledge, however, demethylation has never been previously used to improve the efficiency of gene expression after transient transfection. It was earlier demonstrated that the CMV promoter, a promoter most widely used for transfection, may be silenced in mouse (30) and human (26, 29) cells after viral transfection or in transgenic zebrafish (31).

We used demethylation of the CMV promoter to achieve

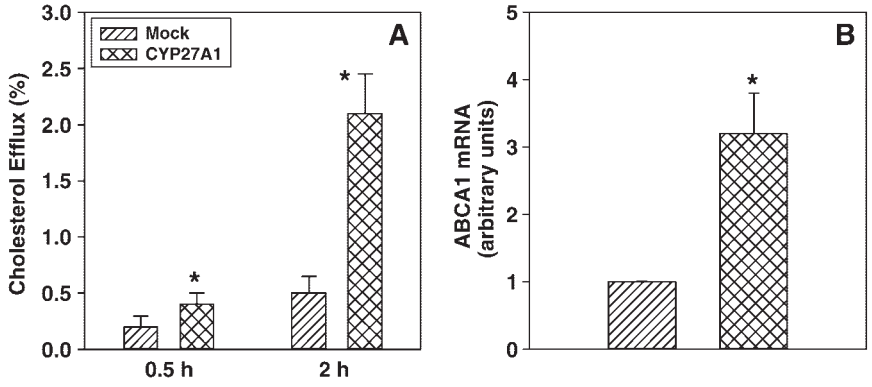


Fig. 9. ABCA1 expression and cholesterol efflux from RAW 264.7 cells after transfection with CYP27A1. A: Transfected and mock-transfected RAW 264.7 cells were labeled with [^{3}H]cholesterol as described in Materials and Methods and incubated with lipid-free apoA-I (final concentration, 30 μg/ml) or serum-free medium alone for 0.5 or 2 h at 37°C in a CO_2 incubator. Medium was collected, cells were washed, and the amount of radioactivity in the cells and medium was determined by liquid scintillation spectrometry. Cholesterol efflux is expressed as the percentage of labeled cholesterol moved from cells to medium (i.e., radioactivity in the medium/radioactivity in the medium + radioactivity in the cells). Background values (i.e., cholesterol efflux to the medium alone) were subtracted. Means ± SD of quadruplicate determinations are shown. B: Real-time RT-PCR of ABCA1 mRNA isolated from RAW 264.7 cells mock-transfected and transfected with CYP27A1 and treated with 0.5 μmol/l 5-azacytidine. * $P < 0.01$ versus mock-transfected cells.

for the first time the 80–100% efficiency of transient transfection of mouse macrophage RAW 264.7 cells. We found that the CMV promoter is indeed methylated in transiently transfected cells and that the demethylation agent, 5-azacytidine, abolished methylation and restored gene expression. The effectiveness of this method was confirmed in a functional study. High-efficiency transfection of RAW 264.7 cells with CYP27A1 led to the expected increase of cholesterol efflux, and the effect was not seen with low-efficiency transfection without treatment with 5-azacytidine. The phenomenon was not limited to the CMV promoter or mouse macrophages: 5-azacydine significantly increased the efficiency of transfection with plasmids with other viral and mammalian promoters as well as the transient transfection efficiency of human hepatoma cells. Thus, prevention of silencing of a plasmid promoter may be an effective tool for achieving high-efficiency transient transfection in a variety of circumstances.

A potential downside of this method is that abolishing methylation may also affect the expression of the host genes. The expression of a number of genes is affected by methylation-demethylation in a variety of cells (for review, see 22), although little is known about gene methylation in macrophages. Thus, demethylation can potentially affect host pathways if they are regulated by this mechanism. Demethylation could be a cause of a toxic effect observed at high concentrations of 5-azacytidine. However, at low concentrations, 5-azacytidine had no effect on cell viability, morphology, or growth. 5-Azacytidine also had no effect on cholesterol efflux from activated or nonactivated cells and the expression of ABCA1, CYP27A1, and LXR. Thus, it is unlikely that 5-azacytidine affects cholesterol efflux in macrophages. 5-Azacytidine also did not affect DNA digestion pattern after treatment with the methylation-sensitive enzyme *Hpa*II. Nevertheless, the possibility cannot be completely ruled out that some genes are methylated in RAW 264.7 cells and treatment with 5-azacytidine affects their expression. It is therefore important to examine the effect of 5-azacytidine on a specific pathway investigated in a particular study before adopting the technique.

It is important to recognize that although the term "transfection" was used throughout this paper, treatment with 5-azacytidine does not directly affect the transfer of DNA into cells. Rather, it prevents gene silencing after successful DNA transfer has been achieved, highlighting that the competency of DNA delivery still remains an important issue in transient transfection. Accordingly, demethylation was less effective with those methods of transient transfection that did not result in the delivery of sufficient quantities of DNA into the cells. Thus, both DNA delivery and plasmid silencing need to be considered when high-efficiency transfection cannot be achieved in a particular cell type.

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