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# Efficient Gene Transfer into Mammalian Cells with Cholesteryl-Spermidine

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The naturally occurring polyamine spermidine was covalently conjugated with cholesterol, resulting in a novel cationic compound that mediates efficient gene transfer into mammalian cells. Using reporter plasmids coding for firefly luciferase and $\beta$-galactosidase, a simple procedure was developed allowing highly reproducible and efficient transient and stable transfection of HuH-7 cells. Transfection efficiency could be further increased when a fusogenic peptide derived from the influenza virus hemagglutinin HA2 aminoterminal sequence was included in the cholesteryl-spermidine–DNA complex. Cholesteryl-spermidine (Transfectall) represents a novel cationic compound for efficient transfection of cultured cells *in vitro* and has the potential to be used for gene transfer *in vivo.* 

The ability to transfer and express heterologous genes in cultured cells is fundamental to studying the function and regulation of genes and their products *in vitro.* For this purpose, calcium phosphate precipitation (1), DEAE dextran (2), cationic liposomes (3) and electroporation (4) are widely used in many research laboratories. Moreover, substantial effort is being made to develop efficient and safe delivery systems for gene transfer *in vivo.* Although currently less efficient than viral gene transfer vectors, synthetic delivery systems are being considered increasingly for gene therapeutic applications because of their relative inertness, great carrier capacity, and the ease of large-scale production (5). In this regard, cationic liposomes are currently under investigation as vectors for gene therapy of malignant melanoma (6) and cystic fibrosis (7). Here, we describe a novel cationic compound based on the naturally occurring polyamine spermidine which allows highly efficient gene transfer into a variety of cultured cells *in vitro.* We found transfection using cholesteryl-spermidine to be simple, efficient and highly reproducible. In its structure, cholesteryl-spermidine is related to the cationic amphiphile 3$\beta$[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol (DC-chol), a cholesterol-derivative developed by the group of L. Huang (8) and to the lipospermines, notably dioctadecyl-amidoglycylspermine (DOGS), which were developed by the group of J.P. Behr (9). Similar to DOGS, cholesteryl-spermidine is not dependent on the presence of a helper lipid such as dioleylphosphatidylethanolamine and it is not expected to form liposomes under physiological conditions. In addition, compared to other synthetic gene delivery vehicles, cholesteryl-spermidine has the major advantage that it can be stored frozen and therefore has a virtually unlimited shelf-life.

## EXPERIMENTAL PROCEDURES

*Cholesteryl-spermidine and peptides.* The synthesis of spermidine-derivatives will be described in detail elsewhere (R.H.B. *et al.,* manuscript in preparation). Lyophilized cholesteryl-spermidine was reconstituted in water at a concentration of 10 mg/ml and stored in aliquots at −20°C. An influenza virus HA2 fusogenic peptide with the sequence GLFEA IAGFI

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Abbreviations: DC-chol, 3$\beta$[N-(N′,N′-dimethylaminoethane)carbamoyl]cholesterol; DOGS, dioctadecylamidoglycylspermine; MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline; RLU, relative light units.



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ENGWE GMIDG GG was used as an endosome-disrupting agent. A scrambled peptide with the sequence FGEGL EGIGM IDNIG EWGFA AG was used as a control. Peptides were reconstituted in 0.05 M ammonium bicarbonate at a concentration of 0.5 mg/ml and stored in aliquots at −70°C.

*Plasmid constructs.* The *Hin*dIII - X*ba*I fragment of pSP-luc+ (Promega, Madison, WI) was inserted into the *Hin*dIII-*Xba*I sites of pcDNA3 (Invitrogen, San Diego, CA) to yield plasmid pCMVluc+. This construct allows expression of the firefly luciferase gene under control of the cytomegalovirus intermediate early promoter and enhancer. Plasmid pSV$\beta$gal, containing the *E. coli lacZ* gene driven by the SV40 early promoter and enhancer, was obtained from Promega. Plasmids were grown in *E. coli* DH5$\alpha$ cells and DNA was prepared by alkaline lysis and purified by two rounds of CsCl-gradient ultracentrifugation according to a standard protocol (10).

*Cell lines.* HuH-7 is a human hepatocellular carcinoma (11) and Sy5y a human neuroblastoma cell line (12). All other cell lines were obtained from the American Type Culture Collection (Rockville, MD). HuH-7, Hep G2 (human hepatoblastoma), LS 180 (human colon carcinoma), NIH/3T3 (mouse embryo), and COS-1 (SV40-transformed monkey kidney) cells were maintained in Eagle's Minimal Essential Medium (MEM; Mediatech, Washington, DC) supplemented with 10% heat-inactivated fetal bovine serum, non-essential amino acids, penicillin G, and streptomycin at 37°C in a humidified atmosphere of 7% $CO_2$. U-2 OS (human osteosarcoma), 293 (adenovirus-transformed human kidney), Sy5y, and MCF7 (human breast cancer) cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin G, and streptomycin.

*Transfection experiments.* Cells were grown to approximately 80% confluence in 6-well tissue culture plates. Typically, for one well 2 $\mu$g of DNA were diluted in 200 $\mu$l MEM and 2 to 20 $\mu$g of cholesteryl-spermidine were diluted in 200 $\mu$l MEM. The two solutions were combined, gently mixed, and incubated for 15 minutes at 20°C to allow complex formation. Finally, 200 $\mu$l MEM were added to the cholesteryl-spermidine-DNA complex and the mixture was overlayed onto the cells that had been rinsed with MEM or phosphate-buffered saline (PBS). Cells were incubated with the cholesteryl-spermidine-DNA complex for 2 to 4 hours at 37°C. Subsequently, cell monolayers were rinsed with MEM or PBS, and fresh culture medium was added to the cells. Transfections involving the influenza virus HA2 fusogenic peptide were performed similarly, with peptide being added to the cholesteryl-spermidine-DNA mixture at a final concentration of 10 $\mu$M after 10 minutes complex formation. When complex formation was allowed to proceed in water, DNA and cholesteryl-spermidine were diluted in water and an appropriate volume of 5-fold concentrated MEM was added to the mixture to give a final concentration of 1 × MEM before being overlayed onto the cells. Control transfections were performed using a commercially available calcium phosphate transfection kit (5′ → 3′, Inc., Boulder, CO).

*Reporter assays.* Luciferase assays were performed 24 hours posttransfection following a standard protocol (13). Relative light units were determined using a LKB-Wallac 1251 luminometer (Wallac, Turku, Finland). X-gal *in situ* staining was performed 48 hours posttransfection essentially as described (13). Briefly, cells grown and transfected on glass cover slides were rinsed with PBS, fixed for 5 minutes in 0.5% glutaraldehyde in PBS, rinsed three times with PBS, and incubated for 2 to 4 hours at 37°C in a solution containing 25 mM $K_3Fe(CN)_6$, 25 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 1.5 mM $MgCl_2$, and 1 mg/ml X-gal (5-Bromo-4-chloro-3-indolyl-phosphate) in PBS. Finally, slides were mounted in glycerol gelatin (Sigma, St. Louis, MO).

## RESULTS AND DISCUSSION

*Transfection of HuH-7 cells with spermidine derivatives.* A series of spermidine-derivatives monoalkylated at the $N^4$ secondary amine were prepared and tested for their ability to transfect HuH-7 cells. We envisioned that by virtue of electrostatic interaction the cationic spermidine backbone would bind to and condense the DNA (14) and that the nature of the alkylation at $N^4$ could be varied to provide a degree of lipophilicity that would allow transmembrane delivery of compacted DNA. Simple alkyl side chains such as octyl-, hexadecyl- and dodecyl-groups were not particularly effective in transfecting HuH-7 cells. However, attaching a $N^4$-(3$\beta$-(N-5-pentyl)carbamoyl)-cholesteryl lipophilic group to form cholesteryl-spermidine (Fig. 1a) gave excellent results and transfection using this compound was studied in detail. Remarkably, cholesteryl-spermidine could be reconstituted in water and subjected to multiple freeze-thaw cycles without loss of transfecting ability (data not illustrated). In the experiment shown in the upper panel of Fig. 1b, a constant amount of pCMVluc+ DNA was mixed with increasing amounts of cholesteryl-spermidine and transfection efficiency of the resulting complexes in HuH-7 cells was measured by luciferase assay. Transfection efficiency was dependent on the ratio of DNA to cholesteryl-spermidine with an optimum at ratios between 1:6 to 1:10. The lower panel of Fig. 1b demonstrates that transfection becomes efficient when the electrophoretic migration of complexed DNA in an agarose gel is completely retarded, i.e. the negative charge of the DNA is neutralized and the

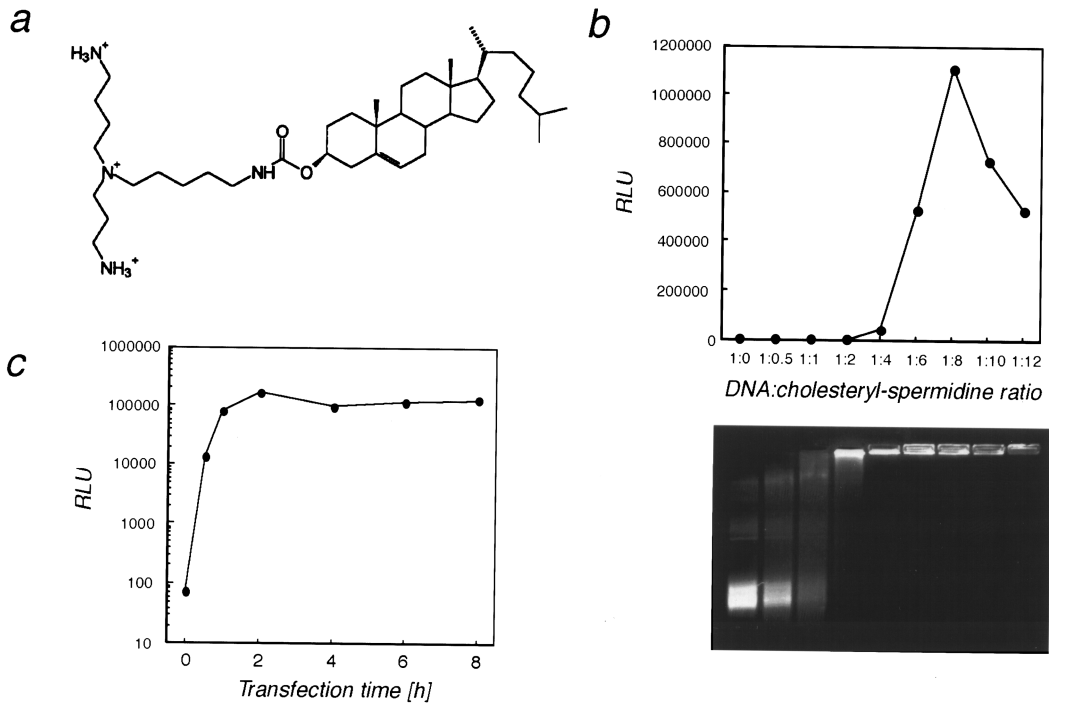


**FIG. 1.** Cholesteryl-spermidine mediates efficient gene transfer into HuH-7 cells. ***a,*** Chemical structure of cholesteryl-spermidine. ***b,*** Upper panel: Dependence of transfection efficiency on DNA:cholesteryl-spermidine ratio. 2 $\mu$g pCMVluc+ was complexed with increasing amounts (1:0 to 1:12 weight ratio) of cholesteryl-spermidine in MEM and incubated for 4 hours with HuH-7 cells as described in Experimental Procedures. luciferase activity in cell lysates is expressed as RlU in approximately 5000 cells. lower panel: Electrophoretic mobility retardation assay. 0.5 $\mu$g pCMVluc+ diluted in 25 $\mu$l MEM was mixed with cholesteryl-spermidine prediluted in 25 $\mu$l MEM at the same ratios as in *b* and incubated for 15 minutes at 20°C. Subsequently, samples were analyzed by 1% agarose gel electrophoresis. *c,* Time course. 2$\mu$g pCMVluc+ was complexed with 12 $\mu$g cholesteryl-spermidine in MEM and incubated with HuH-7 cells for various amounts of time. Subsequently, cell monolayers were rinsed with MEM and fresh culture medium was added to the wells. luciferase activity in cell lysates is expressed as RlU in approximately 5000 cells.

complex has a slight net positive charge. Fig. 1c shows that transfections with cholesteryl-spermidine can be performed with short incubation periods of complexed DNA with the cells. Maximal transfection efficiency was reached after only two hours. In most experiments we therefore limited incubation of DNA-cholesteryl-spermidine complexes with the cells to 2 to 4 hours.

*Effect of influenza virus HA2 fusogenic peptide.* Influenza virus hemagglutinin undergoes a major conformational change at the acidic pH of the endosome, thus exposing at the aminoterminus of the HA2 subunit a hydrophobic sequence of 25 amino acids that are essential for membrane fusion (15). In addition, protonation of the acidic residues E11, E15, and D19 of the fusogenic peptide may allow transition to an $\alpha$-helical configuration that promotes membrane binding and destabilization (16). To allow escape from the endolysosomal degradation pathway and to augment gene transfer, the HA2 fusogenic peptide has been included in polylysine-based compounds thought to enter the cell via receptor-mediated endocytosis by the transferrin receptor (17) and the asialoglycoprotein receptor (18). The mechanism of uptake of cationic lipid-compacted DNA by the cell is not completely understood. There is recent evidence to suggest, however, that a significant proportion of such complexes enter the cell through endocytosis (19). Similarly, uptake of cholesteryl-spermidine-compacted DNA into cells may depend on direct fusion with the plasma membrane, but also on endocytosis and possibly phagocytosis. In this regard, addition of the

fusogenic peptide to DNA-cholesteryl-spermidine complexes increased transfection efficiency by 10- to 50-fold when complexes were formed in water but only 1.5- to 5-fold when complexes were formed in MEM, as shown in Fig. 2a. Analogous results were obtained when pSV$\beta$gal was used as a reporter construct and transfected cells were stained *in situ* for $\beta$-galactosidase activity, as shown in Fig. 2b. Remarkably, 40 to 50% of HuH-7 cells could be transfected by this method (Fig.

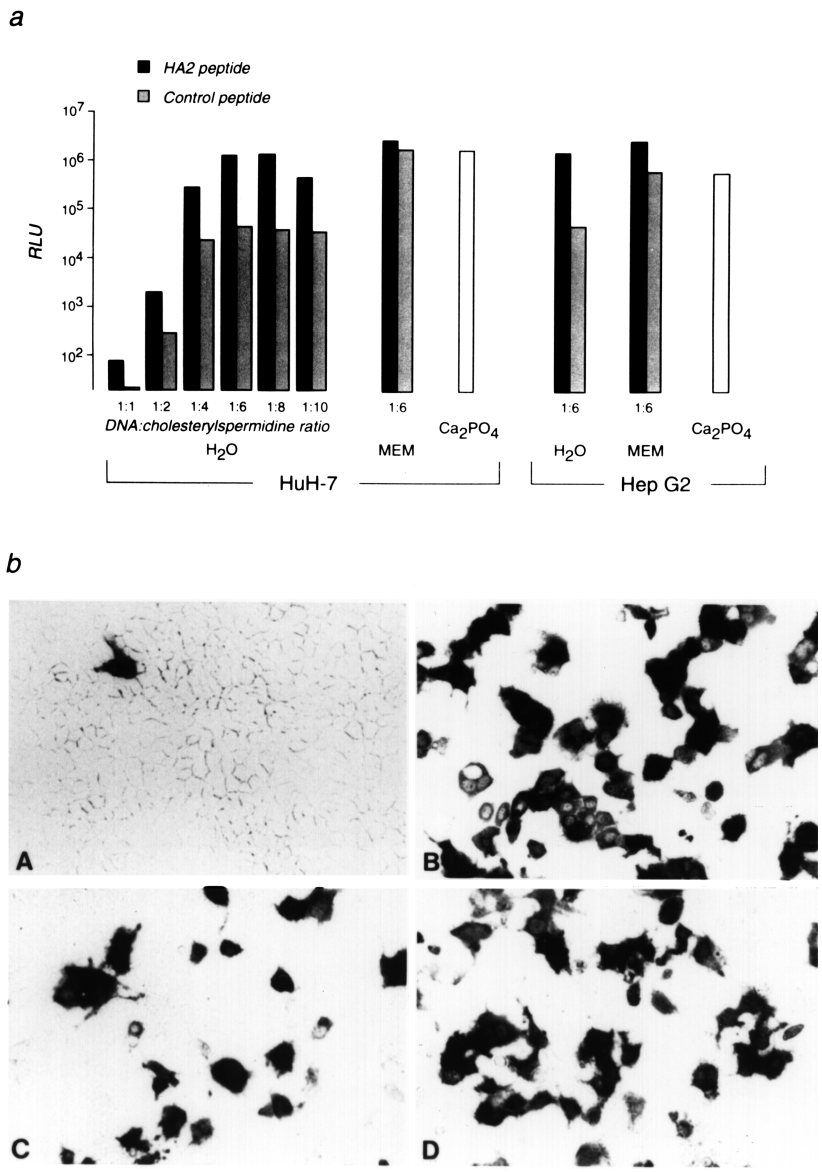


**FIG. 2.** Influence of influenza virus HA2 fusogenic peptide on transfection efficiency. ***a,*** Transfection efficiency in HuH-7 and Hep G2 cells. 2 $\mu$g pCMVluc+ was complexed in water or in MEM with increasing amounts or with a fixed amount of 12 $\mu$g cholesteryl-spermidine and incubated with HuH-7 or Hep G2 cells in the presence of 10 $\mu$M HA2 peptide or scrambled control peptide. luciferase activity in cell lysates is expressed as RlU in approximately 30,000 cells. Mean values of duplicate determinations are shown. ***b,*** *In situ* X-gal staining. 4 $\mu$g pSV$\beta$gal was complexed in water (**A** and **B**) or MEM (**C** and **D**) with 24 $\mu$g cholesteryl-spermidine and incubated for 4 hours with HuH-7 cells in presence of 10 $\mu$M scrambled control (**A** and **C**) or HA2 peptide (**B** and **D**). X-gal staining was performed 48 hours posttransfection as described in Experimental Procedures.

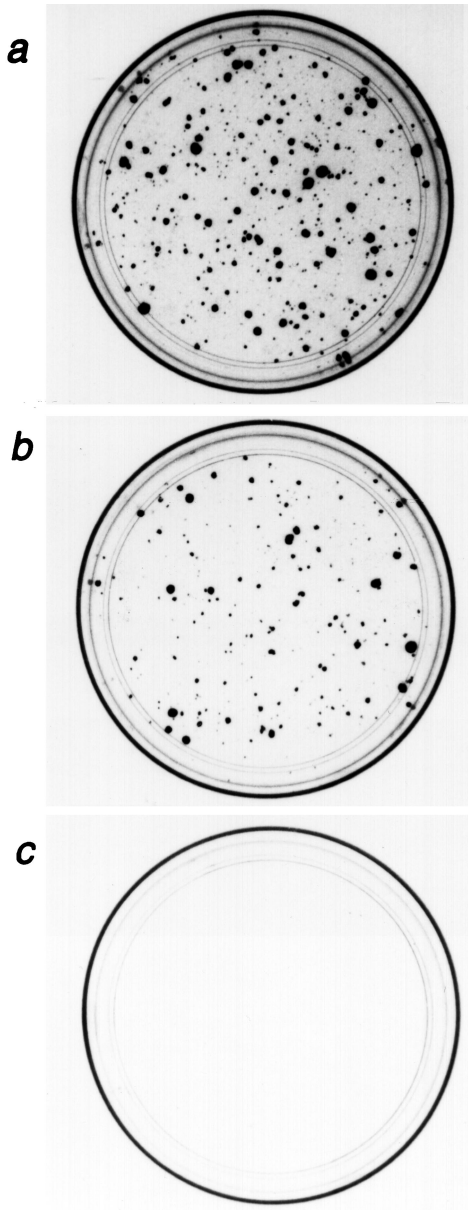


**FIG. 3.** Stable transfection using cholesteryl-spermidine. 80% confluent HuH-7 cells in 100 mm dishes were incubated with 10 $\mu$g pCMVluc+ complexed with 60 $\mu$g cholesteryl-spermidine in a volume of 2 ml MEM (***a***) or with the same amount of DNA in the form of a calcium phosphate precipitate (***b***). pCMVluc+ contains a neomycin-resistance cassette allowing selection of stably transfected clones in culture medium containing G418.Nontransfected HuH-7 cells served as negative controls (***c***). Two days posttransfection, cells were trypsinized, and approximately 500,000 cells were seeded into new 100 mm dishes and allowed to attach. Subsequently, cells were cultured for two weeks in medium containing 200 $\mu$g/ml G418. To visualize G418-resistant colonies, culture dishes were stained with Brilliant Blue R-250.

2bB). A possible explanation for the difference between the behaviour of complexes formed in water vs. MEM is that endocytosis may be more important as an uptake route for the former. Indeed, preliminary data using photon correlation spectroscopy indicates that complexes formed in water are much smaller than complexes formed in MEM. Also, we observed the influence of the

HA2 peptide to be greater in cell lines such as HepG2 which may be less phagocytotic than HuH-7 cells and in which endocytosis may therefore be more important as a route of uptake.

*Transfection of other cell lines.* A broad range of cell lines including NIH/3T3, COS-1, U-2 OS, FOCUS, 293, MCF7, and Sy5y have been successfully transfected without further optimization of the protocol described above. However, transfection conditions, particularly the cell density, the amount of plasmid DNA, the DNA to cholesteryl-spermidine ratio, and the incubation time with the complex are important parameters to consider with this approach and may be further optimized for each cell line. Nevertheless, employing the protocol optimized for HuH-7 cells in the cell lines mentioned above, luciferase activity was at least as high and in most cases higher than that achieved using calcium phosphate precipitation even in the absence of HA2 fusogenic peptide. This was particularly noteworthy in the case of Sy5y (transfection efficiency using cholesteryl-spermidine $17.3 \pm 5.6$ [$n = 3$] times higher than using calcium phosphate precipitation) and MCF7 cells (transfection efficiency $5.9 \pm 4.7$ [$n = 7$] times higher). Both these cell lines are very difficult to transfect by calcium phosphate precipitation.

*Stable transfection using cholesteryl-spermidine.* The results shown in Fig. 3 demonstrate that cholesteryl-spermidine can also be used for stable transfection. Using cholesteryl-spermidine without HA2 peptide, a stable transfection efficiency of $3.7 \times 10^{-4}$ was achieved in HuH-7 cells with plasmid pCMVluc+. With the same amount of DNA, an efficiency of $2.5 \times 10^{-4}$ was achieved using calcium phosphate precipitation and no colonies survived in nontransfected cells (negative control).

In conclusion, cholesteryl-spermidine represents a novel cationic compound for efficient transient and stable transfection of a variety of cultured cells *in vitro.* It will be interesting to pursue the implementation of this gene delivery system *in vivo.* In this regard, we found that plasmid DNA is partially protected from nuclease digestion by cholesteryl-spermidine (R.H.B., unpublished data). Moreover, the flexibility of conjugating other side chains to the spermidine backbone may facilitate the development of modular gene transfer systems carrying ligands for cell-specific delivery, endosomal release and nuclear localization functions. Indeed, targeting of DOGS to the hepatocyte-specific asialoglycoprotein receptor was recently reported (20). The successful implementation of such synthetic virus-like gene transfer systems *in vivo* will ultimately depend on a better understanding of the intracellular fate of the complex and on improved control of the size and overall charge of compound-DNA complexes.

Cholesteryl-spermidine is an active ingredient in Transfectall brand of transfection agent.

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