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Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA

(transfection/lipospermine/intermediate pituitary/gene expression)

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ABSTRACT **A general and efficient transfection procedure, based on compacted lipopolyamine-coated plasmids, has been developed. The active species is obtained by simple addition of excess synthetic lipospermine solution to the DNA and binds within minutes to the cell membrane. This technique has been developed on endocrine cells of the intermediate lobe of the pituitary as a general tool for physiological work on primary cells; it is not toxic and does not interfere with physiological regulations in melanotrope cells. A variety of eukaryotic cell cultures also have been transfected with success for transient and stable expression.**

A number of new techniques have been devised recently to introduce foreign DNA into eukaryotic cells (refs. 1–8 and references therein). Yet the most commonly used techniques are still DNA/calcium phosphate coprecipitation (5, 9) or methods based on the use of high molecular weight polycations (e.g., dextran) (10). Although extensively used for cell-line transfection, these methods suffer from high variability of efficiency or of cell survival and need to be adapted to each cell line. Liposomes, which offer the potential advantages of targeting and lower toxicity have been used for gene transfer (1, 2), but efficiency of such methods is variable also, and DNA encapsulation is time consuming. Recently, it has been reported that positively charged liposomes containing the membrane fusion-promoting lipid distearoyl phosphatidylethanolamine can be used for efficient gene transfer into eukaryotic cells (3). An alternative approach would be to bind DNA onto the external layer of liposomes. We have shown (11) that liposomes containing lipids with nucleic acid-binding headgroups form discrete complexes with DNA by wrapping it around the unilamellar 800– to 1000-Å vesicles ("nanoparticles") in a way reminiscent of the structure of the nucleosome.

In this report, we describe a transfection method based on compacted cationic lipid-coated plasmid DNA. To this end, we synthetized a series of lipospermines whose headgroups interact strongly with DNA and eventually coat it with a cationic lipid layer, thus promoting the binding to cell membranes. This technique, combining simplicity, high efficiency, and absence of any detectable toxic side effects, was developed as a general tool for physiological studies in primary cultures where gene transfer techniques are currently not available. We show by using the bacterial chloramphenicol acetyltransferase (CAT) gene as a marker that this technique allows gene transfer into a variety of primary and other cultures. Further, we show in pituitary endocrine cells, that gene transfer by this method does not alter membrane-located receptors or physiological regulations of hormone biosynthesis. Moreover, the use of fully biodegradable lipopolyamines as DNA carriers should make it possible to extend this method for gene transfer *in vivo*.

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MATERIALS AND METHODS

Synthesis of Lipospermines. A functionalized L-5-carboxyspermine derivative leaving intact ammonium residues (12) was synthesized from L-ornithine through biscyanoethylation followed by reduction of the dinitrile; amino groups were protected as *t*-butoxycarbonyl (Boc) derivatives. In order for the lipid headgroup to be able to anchor itself in the minor groove of DNA, a spacer arm was required (11) between the hydrocarbon chains and the polyamine. Therefore, a glycine residue was linked to dioctadecylamine (benzyloxycarbonylglycyl-*p*-nitrophenol at 1 equiv plus triethylamine at 1.1 equiv in CH_2Cl_2 for 5 hr; H_2, 10% Pd/C in CH_2Cl_2/EtOH for 1 hr; 87% overall yield). See Fig. 1 for schematic diagram of synthesis.

L-5-Carboxytetrabutoxycarbonylspermine ($Boc_4SperCO_2H$) was coupled with glycyldioctadecylamide in the presence of dicyclohexylcarbodiimide (DDC at 1.1 equiv in CH_2Cl_2 for 12 hr; 90% yield after chromatography on silica) and deprotected in trifluoroacetic acid (10 min at room temperature) to give dioctadecylamidoglycylspermine (DOGS)·4 trifluoroacetic acid.

$Boc_4SperCO_2H$ was activated as the *N*-hydroxysuccinimide ester (1.1 equiv plus DCC at 1.1 equiv for 12 hr in CH_2Cl_2/tetrahydrofuran) and treated with dipalmitoyl phosphatidylethanolamine (1 equiv plus triethylamine at 1 equiv in $CHCl_3$/EtOH for 12 hr at 40°C, 55% yield after chromatography on silica). The resulting compound was deprotected in trifluoroacetic acid-containing CH_2Cl_2 to give dipalmitoyl phosphatidylethanolamidospermine (DPPES)·4 trifluoroacetic acid.

The lipopolyamines were pure as judged by 200-MHz ^1H NMR, and the spectra were in agreement with their structure. DOGS $\delta(CD_3OD)$: 0.9[t, $(CH_3)_2$], 1.3[m, $2\times(CH_2)_{15}$], 1.4–1.7(m, $2\times CH_2CH_2NCO$), 1.8–2.2(m, $4\times CH_2CH_2N^+$), 3.0–3.2(m, $5\times CH_2N^+$), 3.35(t, $2\times CH_2NCO$), 4.0(t, CHN^+), 4.15(s, $COCH_2N^2H$). DPPES $\delta(CDCl_3$ 1:CD_3OD 1): 0.85[t, $(CH_3)_2$], 1.3[m, $2\times(CH_2)_{12}$], 1.5–1.65(m, $2\times CH_2CO_2$), 1.8–2.1(m, $4\times CH_2CH_2N^+$), 2.3(tt, $2\times CH_2CH_2CO_2$), 2.9–3.1(m,

Abbreviations: CAT, chloramphenicol acetyltransferase; Boc, *t*-butoxycarbonyl; $Boc_4SperCO_2H$, L-5-carboxytetrabutoxycarbonylspermine; DOGS, dioctadecylamidoglycylspermine; DPPES, dipalmitoyl phosphatidylethanolamidospermine; POMC, proopiomelanocortin; DODAC, dioctadecyldimethylammonium chloride.

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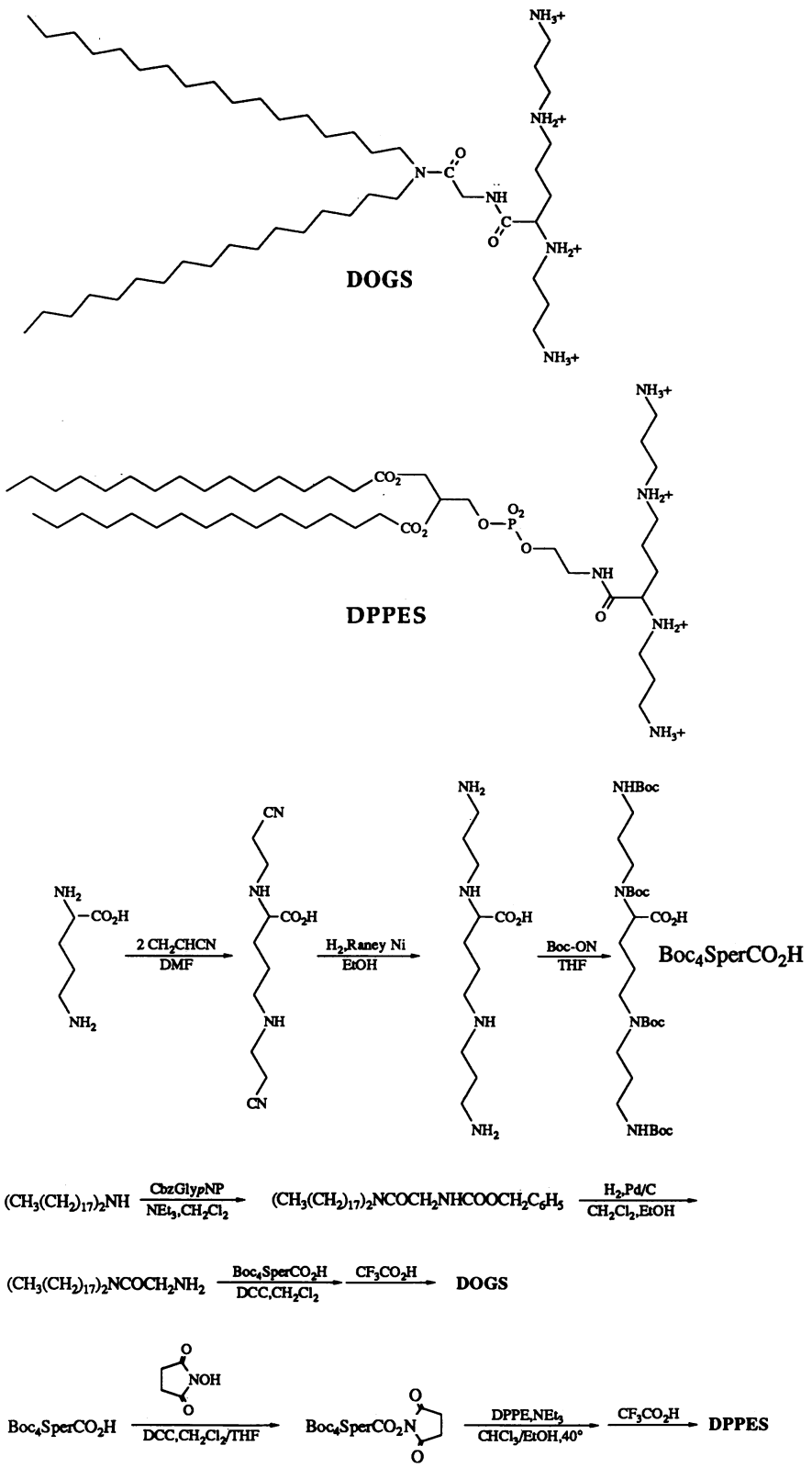

FIG. 1. Schemes for the synthesis of the L-5-carboxyspermine headgroup and for the lipopolyamines DOGS and DPPES. THF, tetrahydrofuran; CbzGly*p*NP, benzyloxycarbonylglycyl-*p*-nitrophenol; NEt_3, triethylamine.

$5\times CH_2N^+$), 3.2(bm, CH_2N^2HCO), 3.75–4.05(m, CHN^+, $2\times CH_2OP$), 4.15–4.40($2\times$dd, CO_2CH_2), 5.20(OCH).

Plasmids. Plasmids were propagated and purified by standard techniques (13). The CAT expression vector (a gift from B. Wasylyk, Strasbourg) that was used throughout the study on melanotrophs (pituitary cells that elaborate melanocyte-stimulating hormone) was derived from plasmid pCAT-8$^+$ (14) by inserting a 100-base-pair fragment (*Bam*HI–*Xba* I) containing four copies of the API-binding consensus sequence (pCAT-4XB).

Cell Culture and Treatment. Porcine intermediate lobe cells were prepared as described (15) [after enzymatic dispersal,

intermediate lobe cells were resuspended in Dulbecco's modified Eagle's medium (DMEM); 0.5–1 × 10^6 cells per 500 μl]. The lipopolyamine stock solution was obtained by 1:9 dilution of a 20 mM ethanolic solution with water. The DNA–lipid complex, freshly obtained by adding 250 μl of DNA solution to 250 μl of lipopolyamine solution in DMEM medium, was added to the cell suspension for various times and concentrations (see legends to Figs. 1–5). Except for the 24- and 48-hr treatment, cells were kept in suspension in an humidified 95% O_2/5% CO_2 incubator at 37°C. After the transfection periods, cells were washed twice and plated. After 48 hr, cells were washed in phosphate-buffered saline, and plates were frozen until CAT activity or other parameters were measured. Intermediate lobe cells were also subjected to standard calcium phosphate precipitation or dextran transfection assays.

CAT Assay. CAT activity was determined by the method of Gorman *et al.* (16). Cells were suspended in 100 μl of 200 mM Tris·HCl (pH 7.4). After several freeze/thaw cycles, 50μl of the supernatant was added to 40 μl of Tris·HCl (pH 7.4) containing ^{14}C-labeled chloramphenicol (0.1 μCi; specific activity, 47 μCi/mmol; 1 Ci = 37 GBq). After 5 min at 37°C, the reaction was initiated by adding 20 μl of 4 mM acetyl CoA. After 1 hr at 37°C, chloramphenicol and its acetylated derivatives were extracted with ethyl acetate, separated by TLC, and autoradiographed. Autoradiograms were analyzed by an LKB UltroScan densitometer.

Evaluation of Toxicity and Physiological Parameters. Total RNA synthesis was measured by [^3H]uridine incorporation as reported (17). Proopiomelanocortin (POMC) gene expression was evaluated by measuring steady-state levels of POMC mRNA with a radiolabeled DNA complementary to the full-length sequence of porcine POMC mRNA (18) (gift from J. Drouin, Montreal). POMC mRNA measurements by the dot-blot hybridization technique have been reported (17, 19).

RESULTS

CAT Expression in Melanotrope Cells. CAT expression 48 hr after transfection of about 2 × 10^5 cells is given in Figs. 2 and 3. Fig. 2 shows the gradual increase of CAT activity with increasing amounts of DNA. A detectable signal was obtained with as little as 10 ng of DNA (see Fig. 2 *Inset*). Contact-time dependency of the transfection is illustrated in Fig. 3 and shows that nearly maximal efficiency was obtained when the transfection medium containing the plasmid–lipospermine complex was removed, and cells were plated as early as 10 min after mixing. Cell washing and plating after 5 min (the shortest transfection duration studied) resulted in 60–70% of maximal efficiency, thus reflecting the rapid

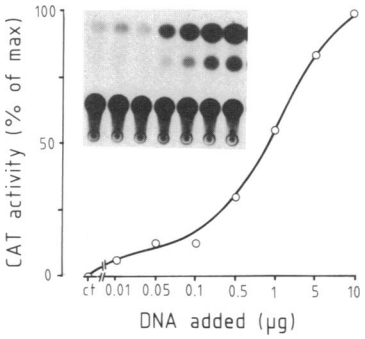

FIG. 2. Melanotrophs were transfected by increasing concentrations of pCAT-4XB–DOGS complex with a 3× excess of lipopolyamine charges. After dispersal, cells were washed and incubated 2 × 10^5 cells per 1 ml of DMEM in Eppendorf tubes for 30 min, washed twice, and plated. CAT assay was performed after 48 hr. ct, Control cells without DNA added.

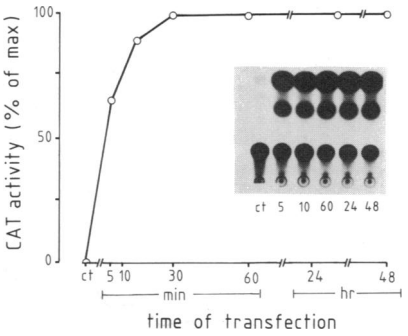

FIG. 3. Time course of the transfection efficiency was studied on 2 × 10^5 cells incubated with 5 μg of DNA–DOGS complex with an excess (3×) of cationic charges. After the indicated time period, cells were washed twice with DMEM. Experiments up to 60 min were performed in Eppendorf tubes before plating. Cells for the 24- and 48-hr time points were plated immediately, and the DNA–DOGS complex was added to the culture dish. ct, Control cells without DNA added.

association of the DNA–lipospermine complex with the cell membranes.

In a second set of experiments, 2 × 10^5 cells were transfected for 30 min with 5 μg of DNA and increasing amounts of DOGS. Fig. 4 shows the variation of transfection efficiency with the ratio of cationic lipid charge to anionic DNA charge; DOGS (assumed +4 cationic charge) and the commercially available dioctadecyl dimethylammonium chloride DODAC (+1 cationic charge) were tested. For both lipids, transfection started to take place around neutrality, suggesting a common mode of action requiring a net positive charge for the DNA–lipid complexes. In these conditions, DOGS-mediated transfection is very efficient and independent of the excess of lipid, whereas DODAC promotes lower CAT activity [in agreement with other work (3)], which quickly decreases at higher lipid ratios because of toxic side effects (see below).

To gain further insight into the structural requirements of the lipid as well as into the transfection mechanism, various parent compounds were compared with DOGS at concentrations giving 3× excess cationic charges (or at the same concentration for zwitterionic compounds); the following order of CAT expression was found (data not shown): DOGS ≈ DPPES > DODAC; stearylamine, spermine, sonicated dispersions of phosphatidylethanolamine, and egg yolk lecithin either alone or loaded with 20 mol % of DOGS showed no transfection ability. Furthermore, DOGS-mediated transfection efficiency was only slightly decreased by a 5× excess of spermine, highlighting the strong cooperative nature of the lipospermine–DNA interaction.

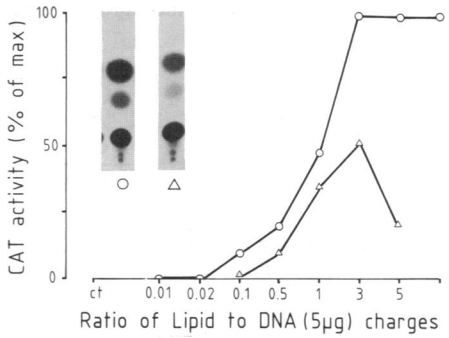

FIG. 4. Cells (2 × 10^5) were transfected for 30 min with 5 μg of DNA. The CAT vector was mixed with increasing amounts of DOGS (○) or DODAC (△). Transfection starts around the neutrality point of the complexes. (*Inset*) Data comparing the maximal efficiency of DOGS and DODAC complexes. ct, Control cells without lipid added.

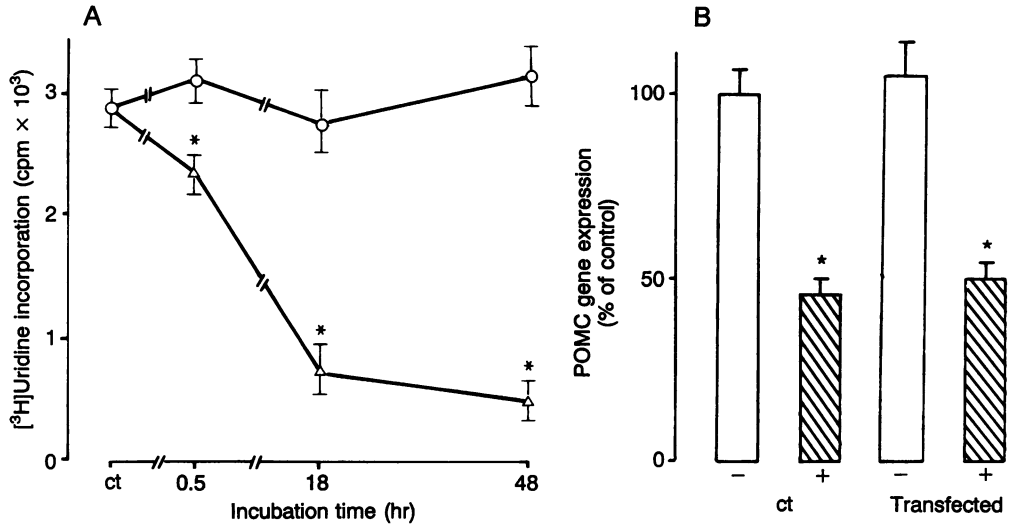

FIG. 5. (*A*) Melanotrophs (5×10^4) were incubated for various time periods with either DNA (5 μg)–DOGS (○) or DNA (5 μg)–DODAC (△ complexes ($3 \times$ excess lipid charges). After 48 hr, [^{3}H]uridine (1 μCi/ml) was added, and trichloroacetic acid-precipitable material (total nucleic acid synthesis) was measured after an additional 2 hr. In contrast to the DNA–DOGS complex, overnight treatment (18 hr) with DNA–DODAC resulted in almost complete cell death as reflected by a severe decrease in [^{3}H]uridine incorporation. Control cells (ct) received no DNA–lipid complex. (*B*) Native (lanes ct) or transfected melanotrophs were plated onto costar 24-multiwell dishes and treated with 10 nM bromocryptine (lanes +) for 48 hr. POMC mRNA was evaluated by dot-blot hydridization to a single-stranded porcine POMC probe. Transfection with the pCAT-4XB vector did not alter significantly basal POMC mRNA levels or the ability of bromocriptine to inhibit POMC gene expression (n = 6; *, $P \leq 0.01$).

Evaluation of the Toxicity and Unspecific Alteration of Physiology. Toxicity was evaluated by measuring total RNA synthesis in melanotrophs maintained in the presence of the DNA–lipid complex for up to 48 hr. Clearly (Fig. 5*A*) the DNA–DOGS complex did not alter trichloroacetic-precipitable [^{3}H]uridine-labeled material when measured after 2 days of culture, indicating that no noticeable cell death occurred. This is in sharp contrast with the results obtained with the DNA–DODAC complex, which produced severe cell death when cells were treated for more than 30 min. This could be a reason for the lower and variable CAT expression obtained with this compound (Fig. 3). Moreover, DOGS transfection did not alter the main physiological regulation of the melanotrope cell (dopaminergic D_2 inhibition). This was shown by measuring the inhibition of the D_2 receptor against bromocryptine on POMC gene expression as shown (20). After transfection, a 48-hr chronic treatment with 10 nM-bromocryptine induced a decrease in POMC mRNA levels comparable to untransfected cells.

Generalization of the Method. A broad range of cell lines of diverse origin (including LMTK, Ras4, CHO, F9, BU4, S49, HeLa, and AtT-20 cells) as well as primary cultures (including anterior pituitary, chromaffin cells, and neurons from the central nervous system) have been efficiently transfected without further optimization of the method by using either a 30-min transfection step or by leaving in contact with the DNA–DOGS complex for 48 hr.

Comparison with Other Transfection Methods. Quantitative comparison of our method with the classical calcium phosphate coprecipitation or dextran techniques was not possible in primary cultures of melanotrophs because of the deleterious effects of these approaches. Therefore, transfection efficiencies of the lipospermine and the calcium phosphate methods were compared in established cell lines for either transient or stable transfection. CHO and Ras4 cell lines were transfected (pSV2-cat) over 10 times more efficiently, and the calcium phosphate-resistant lymphoid cell line S49 could be transfected successfully. For stable transformation, the same conclusions hold: CHO (transfection frequencies of 10^{-3} for DOGS and 10^{-4} for calcium phosphate); S49 (10^{-4} and 0, respectively, for DOGS and calcium phosphate).

DISCUSSION

In this paper we report a simple, reproducible, and highly efficient method for introducing and expressing foreign DNA in mammalian primary and other cells. The reason for this success must rely on the original amphiphilic and binding properties of the lipopolyamine. When dispersed in water (either by sonication or dilution of an ethanolic solution), DOGS (as well as DPPES and DODAC) forms unilamellar vesicles of 800–1000 Å, as measured by photon-correlation spectroscopy. These nanoparticles are unstable in ionic media and will aggregate cooperatively with plasmids since the spermine headgroup has a strong (10^5–10^7 M^{-1}) affinity for DNA (21). However, the high dilutions (1–5 nM) of both the multimolecular and macromolecular species avoid reticulation and favor the formation of discrete (11) plasmid–lipid associations in which the former is compacted and eventually coated with an excess cationic lipid layer; such particles should repel each other. This picture is in agreement with experiments showing the need for an excess of cationic charges (see Fig. 4) and the unability of stearylamine, a micelle former, and DOGS/egg yolk lecithin small unilamellar vesicles to mediate transfection. Thus, the present technique is not a liposome-mediated or a polycationic coprecipitation procedure but rather involves *cationic lipid-coated plasmids*.

Since natural lipid headgroups are either zwitterionic or anionic, such masked "cationic" plasmids should associate spontaneously and quickly (see Fig. 3) with the cell membrane through a cooperative ionic interaction and subsequently should be internalized by endocytosis. The wide applicability of this technique is in agreement with such a pathway and contrasts with most other techniques that apply essentially to phagocytic cell lines.

Although the lipopolyamine-mediated method works better than conventional ones with established cell lines, its major interest comes from its capability to transfect sensitive primary cultures where other approaches cannot be used. For instance, we currently are using this method for *in situ* hybrid arrest experiments: in chromaffin cells, expression of c–Fos protein is efficiently blocked by introducing an antisense

expression vector to this oncogen. Indeed, a general decrease of c–Fos immunoreactivity is seen, indicating that cells are uniformly transfected. This results from the high efficiency and the lack of toxicity even under chronic exposure to the complex. A further advantage of the present method is the noninterference with normal physiological processes. In melanotrophs, we show that the cells were still responsive to their physiological regulator dopamine and responded to chronic agonist treatment with a decrease of POMC mRNA levels (Fig. 4). In peripheral neurons, transfection did not modify normal cell growth (neurite outgrowth) or electrical characteristics. Indeed, a patch-clamp analysis showed that transfected neurons express the tetrodotoxin-sensitive Na^+ current and the three described Ca^{2+} currents, which were identical in amplitude and activation/inactivation characteristics to currents recorded from normal nodose ganglia neurons (A. Feltz and J.-P.L., unpublished observation).

Taken together, these observations indicate that this technique will allow the use of gene transfer technology to study physiological problems in normal primary cells—approaches that up to now have been limited to permanent cell lines. An example would be the analysis of cell-type promoter specificity in the heterogeneous cell population of the brain. A nucleic acid transfer technique (expression vectors or oligonucleotides) based on the use of DOGS or related molecules could be a further step toward genetic therapy or selective antiviral targetting, especially in conjunction with lipoantibodies.

The CAT expression vector containing the AP1 consensus sequences and the POMC probe were generous gifts from Dr. B. Wasylyck (Strasbourg) and Dr. J. Drouin (Montreal), respectively. We warmly thank Dr. F. Schweighoffer (Rhône Poulenc Santé, Vitry, France) for quantitation of the CHO, Ras4, and S49 transfection efficiencies and Dr. P. Feltz for helpful discussions. This work was supported in part by the Institut National de la Santé et de la Recherche Médicale (Grant 876013) and by the Association pour la Recherche sur le Cancer (Grant 6089 to J.-P.L.)

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