Polyethylenimine but Not Cationic Lipids Promotes Transgene Delivery to the Nucleus in Mammalian Cells*

Hélène Pollard‡, Jean-Serge Remy§, Gildas Loussouarn‡, Sophie Demolombe‡, Jean-Paul Behr§, and Denis Escande¶

From the ‡Laboratoire de Physiopathologie et de Pharmacologie Cellulaires et Moléculaires, INSERM CJF96–01, Hôpital Hotel-Dieu, Nantes and the §Laboratoire de Chimie Génétique, URA CNRS 1386, Faculté de Pharmacie, Illkirch, France

The β-galactosidase reporter gene, either free or complexed with various cationic vectors, was microinjected into mammalian cells. Cationic lipids but not polyethylenimine or polylysine prevent transgene expression when complexes are injected in the nucleus. Polyethylenimine and to a lesser extent polylysine, but not cationic lipids, enhance transgene expression when complexes are injected into the cytoplasm. This latter effect was independent of the polymer vector/cDNA ionic charge ratio, suggesting that nucleic acid compaction rather than surface charge was critical for efficient nuclear trafficking. Cell division was not required for nuclear entry. Finally, comparative transfection and microinjection experiments with various cell lines confirm that barriers to gene transfer vary with cell type. We conclude that polymers but not cationic lipids promote gene delivery from the cytoplasm to the nucleus and that transgene expression in the nucleus is prevented by complexation with cationic lipids but not with cationic polymers.

Despite their low efficiency in comparison to viral vectors (1–3), cationic vectors retain high attractiveness in gene therapy due to their theoretically excellent safety profile. Many research efforts are currently dedicated to optimizing transfection efficiency of nonviral vectors, and in this context, several novel chemical entities have recently being disclosed (4–6). However, rational (as opposed to empirical) chemical design of improved vectors requires a better knowledge of the multistage process by which nonviral vectors promote transgene expression in eucaryotic cells. Initial interaction of the synthetic vector with cDNA to form a complex (7–9) as well as delivery of the complex into the cell via an endocytosis pathway (9–13) have been studied in detail. By comparison, much less is known about intracellular trafficking of the transgene to the nucleus where it is transcribed. Release of cDNA from the endosomes is suggested to proceed by destabilization of the endosomal membrane (14). Zabner et al. (9) postulated that the cDNA must then dissociate from the cationic lipid vector before naked cDNA enters the nucleus. To this end, anionic lipids normally found on the cytoplasmic-facing monolayer of cell membrane can very potently displace cDNA from the cationic liposome/cDNA complex (14).

In this report, we demonstrate that synthetic polymers such as polyethylenimine (4, 15) and polylysine differ from cationic lipids inasmuch as (i) polymers promote gene delivery from the cytoplasm to the nucleus and (ii) transgene expression in the nucleus is prevented by complexation with cationic lipids but not with cationic polymers. Our findings provide new rationale for future developments targeted to improve the efficiency of nonviral vectors and highlight the in vitro superiority of cationic polymer vectors over cationic lipids.

EXPERIMENTAL PROCEDURES

Zeta Potential Measurements—Polyethylenimine (PEI/cDNA (1–4) zeta potential was determined using a Zetamaster 3000 (Malvern Instrument, Orsay, France) with the following specifications: sampling time, 30 s; three measurements per sample; viscosity, 1.01 centipoise; dielectric constant, 79; temperature, 20 °C.

Electron Microscopy—Carbon films were prepared by sublimation on freshly cleaved mica and recovered by flotation on Cu2+/Rh2+ grids (300 mesh, Touzard & Matignon, Courtaboeuf, France). After overnight drying, grids were kept on a blotting paper in a Petri dish. Immediately before sample addition, grids were glow-discharged (110 mV, 25 s). A drop (5 μL) of sample solution was left on the grid for 1 min. Complexes were negatively stained with 30 μL of aqueous uranylacetate (1% w/v) for 20 s, and excess liquid was removed with blotting paper. Observations were performed at 80 kV with a Philips EM 410 transmission electron microscope.

Cell Cultures—COS-7 cells, pancreatic epithelioid CFPAC-1 from a ΔF508/ΔF508 cystic fibrosis patient and human A549 lung carcinoma cells obtained from the ATCC (Rockville, MD) were cultured as previously indicated (16).

Polycondensations and Plasmids—PEI (25 kDa) and polylysine were used as 10 mM monomer aqueous stock solution. Diocetyldecylamidoglycyl spermine (Transfectam®, Promega, Madison, WI) was used as a 2 mM ethanolic solution. N-(1-(2,3-dioleoloyloxy)propyl)-N,N,N-trimethylammonium methysulphate (DOTAP, Boehringer Mannheim) was used in aqueous solution at 1 mg/ml. A pCMV-LacZ plasmid in which the β-galactosidase cDNA was established under the control of the CMV promoter was used as a reporter gene. Alternatively, we used a pcMV-cystic fibrosis transmembrane conductance regulator (CFTR) plasmid (a kind gift from J. Ricardo, Lisbon, Portugal) in which a CMV promoter drove expression of the CFTR protein.

Cell Transfection—Adherent cells were seeded in 24-well plates (Life Technologies, Inc.). Amounts and volumes given below refer to a single well. Two μg of pCMV-LacZ were diluted into 50 μl of a 150 mM NaCl solution. The desired amount of 25-kD PEI was diluted into 50 μl of 150 mM NaCl, gently vortexed, spun down (500 rpm for 5 s), and then incubated for 10 min. PEI was used at PEI/cDNA ratios between 2 and 10 equivalents (PEI/cDNA ratio calculated on the basis of PEI nitrogen/cDNA phosphate; see Ref. 15). The cationic vector was added to the plasmid solution, mixed, vortexed, spun down, and incubated for 10 min. Two μg of pcMV-LacZ were diluted into 50 μl of 150 mM NaCl solution. The desired amount of 25-kDa PEI was diluted into 50 μl of 150 mM NaCl, gently vortexed, spun down (500 rpm for 5 s), and then incubated for 10 min. PEI was used at PEI/cDNA ratios between 2 and 10 equivalents (PEI/cDNA ratio calculated on the basis of PEI nitrogen/cDNA phosphate; see Ref. 15). The cationic vector was added to the plasmid solution, mixed, vortexed, spun down, and incubated for 10 min.

* This work was supported by Association Française de Lutte contre la Mucoviscidose (AFLM) and Center et Réseaux de Thérapie Génique (CRTG) grants. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Laboratoire de Physiopathologie et de Pharmacologie Cellulaires et Moléculaires, INSERM CJF96–01, Bât HNB, Hôpital Hotel-Dieu, BP 1005, 44093 Nantes, France. Tel.: 240.41.29.49; Fax: 240.08.75.23; E-mail: denis.escande@sante.univ-nantes.fr.
Polyethylenimine Promotes Transgene Delivery

10 min. Twenty-four h post-transfection, cells were fixed for 15 min with 0.5% formaldehyde and then analyzed histochimically using X-gal (17). All experiments were done in duplicate.

Intracellular Plasmid Microinjections—Cells were microinjected at day 1 post-plating. In this procedure, we used the Eppendorf microinjector 5234 system, the micromanipulator 5171 system, and a Nikon Diaphot inverted microscope. Nuclear or intracytoplasmic injections were performed with the Z (depth) limit option using a 0.3-s injection time and 30–80-hectopascal injection pressure. Injection pipettes (internal diameter 0.5 ± 0.2 μm) were pulled from borosilicate glass capillaries. Plasmids were diluted at a final concentration ranging from 0.01 to 10 μg/ml in an injection buffer made of 50 mM HEPES, 50 mM NaOH, 40 mM NaCl, pH 7.4. 0.5% fluorescein isothiocyanate-dextran (150 kDa) was added to the injection medium. The plasmid solution was injected into the cells, and the exact number of nuclearly or cytoplasmically injected cells was counted. Twenty-four h post-injection, cells were fixed and analyzed for exogenous β-galactosidase expression. Di-octadecylaminodiglycyl spermine was used at six charge equivalents (six cationic amino groups per phosphate group). For polylysine, we used 0.6 μl of polylysine 10 mM/μg of plasmid. This mixture yields a polylysine/cDNA ratio of 2, corresponding to a PEI/cDNA ratio of 5 on the basis of theionic charges of the complexes. In a separate set of experiments, we observed that polylysine transfection efficiency peaked at 2 equivalents. For DOTAP, we used 6 μl of DOTAP/μg of plasmid.

Determination of Injected Volume—The volume of the solution intracellularly microinjected was determined using radiolabeled probes. 0.5% fluorescein isothiocyanate-dextran supplemented with 99Tc (80 μCi/μl) or 111In (50 μCi/μl) was injected into the nucleus of 100 cells. Immediately after injection, cells were washed in phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 1 mM CaCl2, 1 mM MgCl2, pH 7.4. Cells were trypsinized and suspended into 1.5 ml of culture medium, and the radioactivity of the solution in cpm was determined. Different solutions of known volumes and radioactive concentrations were used as internal standards. In COS-7 cells, 8735 ± 823 cpm/ml was measured in the cell lysate (n = four experiments) using 99Tc. This value corresponded to an estimated injection volume of 923 ± 191 fl/cell (range: 387 to 1402 fl/cell). A comparable estimate was also obtained using 111In. Thus, for a pCMV-LacZ plasmid concentration of 100 μg/ml, the number of injected cDNA copies was in the order of 10,000/cell. This value is 1 log lower than the number of cells per Petri dish exhibiting X-gal blue staining (Fig. 1B) divided by the number of injected cells exhibiting fluorescein isothiocyanate-dextran fluorescence. Fig. 2A summarizes data obtained after intranuclear injection and shows that the injection efficiency increased according to a sigmoidal relation of the number of injected cDNA copies increased in the range 10^1–10^3 copies/cell. Fig. 2A (right panel) shows that transgene expression was comparable in the absence or presence of PEI, irrespective of the PEI/cDNA ratio used, i.e., of the ionic charge of the PEI/cDNA complexes. Data obtained with intracytoplasmic microinjections are summarized on Fig. 2B. As previously reported (20–22), intracytoplasmic injection of naked cDNA led to a low expression of the reporter gene (<15% of the cells), yet only at injected cDNA copies/cell greater than 10^3 (Fig. 2B). Remarkably, cDNA complexation with PEI increased transgene expression (Fig. 2B). Comparable expressions (40–50%) were observed after injection of 10^4 copies in the cytoplasm and 10^5 copies in the nucleus. Increased expression when cDNA was complexed with PEI was noticed only for PEI/cDNA ratio higher or equal to 2 (Fig. 2B) and may be a consequence of the electrostatic charge and/or of the morphology of the complexed DNA. Zeta potential (an indication of surface charge) measurements of PEI/cDNA complexes performed with increasing amounts of PEI showed that particles become neutral at a PEI/cDNA ratio around 3.5. The morphology of PEI/cDNA complexes may be inferred from a combination of centrifugation and electron microscopy experiments. Complexes formed at a PEI/cDNA ratio of 1 cannot be pelleted (<5%, 10 min, 11,000 × g), presumably because of extensive DNA looping out (Fig. 3), whereas for PEI/cDNA ratio 2–10, complexes were compact and centrifugable (>90%). We next explored whether in cytoplasmic injection experiments, the reporter gene was necessarily reaching the nucleus at the time of cell division. To this goal, isolated cells (<10% confluency) were injected in the cytoplasm with pCMV-LacZ (10^4 cDNA copies/cell) complexed with 5 equivalents of PEI (n = 4 assays; 107 cells). If plasmid cDNA only had access to the nucleus during the transient breakdown of the nuclear membrane that is associated with mitosis, then one would expect to see only clusters of at least 2 cells expressing the transgene. We observed that 20 cells out of 40 expressing the transgene were isolated cells (Fig. 4), indicating that cell mitosis was not an absolute requirement for transgene expression.
Polyethylenimine Promotes Transgene Delivery

Fig. 2. Efficiency of intranuclear (A) and cytoplasmic (B) cDNA microinjections in COS-7 cells. In A and B (left panels) dose-effect relations with naked pCMV-LacZ cDNA (open symbols) or with PEI-cDNA complexes at 5 equivalents (filled symbols) are shown. A variable number of injected plasmid copies as indicated on the x axis was used. Data are mean ± S.E., with n between 178 and 650. Right panels show the effects of varying the PEI/cDNA ratio on injection efficiency. Cells were injected with 10,000 plasmid copies. Stars indicate significant difference, with p < 0.05 (*) or p < 0.001 (**) between cells injected with naked DNA and cells injected with DNA complexed with PEI.

Expression Kinetics in Transfected and in Injected Cells—

COS-7 were transfected with pCMV-LacZ complexed with PEI. Eight h post-transfection, a low percentage of cells showed β-galactosidase activity (Fig. 5, left panel). At 12 and 24 h post-transfection, ~20 and 60% of the cells expressed the transgene, respectively. By contrast, 4 h post-injection of naked or complexed cDNA into the nucleus, a high percentage of cells already expressed the transgene. In cells injected into the cytoplasm with naked cDNA, at least 12 h of incubation were needed to detect transgene expression. Eight h post-injection in the cytoplasm of PEI-cDNA complexes, a detectable proportion of cells stained blue. Prolonging the incubation period from 24 to 48 h only slightly increased expression efficiency. Expression kinetics were also followed using a pCMV-CFTR plasmid and a SPQ fluorescence assay. Measurements of membrane permeability to halide under cAMP stimulation was used to detect functional CFTR proteins in relation to post-injection time (Fig. 5, right panel). Direct intranuclear PEI/cDNA injection in COS-7 cells led to the expression of a CFTR-related conductance within 4 h, i.e. within the same time frame as β-galactosidase expression. Comparable kinetics were observed with nuclear injection of naked cDNA. Microinjection of PEI-pCMV-CFTR in the cytoplasm markedly delayed transgene expression in comparison to nuclear injections, with detectable transgene expression 12 h post-injection. We concluded that (i) exogenous transcription and translation are fast processes and (ii) transgene expression kinetics after intracytoplasmic injection and transfection were comparable, pointing at plasmid intracellular trafficking to the nucleus as the rate-limiting step.

Transfection and Injection Efficiencies in COS-7, CFPAC-1, and A549 Epithelial Cell Lines—

Transfection efficiency was markedly variable between different cell lines, since it was much greater in COS-7 than in CFPAC-1 or A549 cells (Fig. 6A). Consistent with previous studies using PEI (4), transfection efficiency was optimal at a PEI/cDNA ratio of at least 5 equivalents, i.e. when complexes were positively charged. Nuclear injection of cDNA-PEI led to comparable expression for all cells (Fig. 6B), suggesting similar transcription levels. By contrast, the efficiency after cytoplasmic injections markedly varied with the cell line, with the highest efficiency in COS-7 cells and the lowest in A549 cells.

Expression Efficiency after Injection with Various Cationic Vectors—

Finally, we investigated the influence of the vector’s molecular nature on transgene expression. In agreement with previous reports (9, 22), neither cytoplasmic nor nuclear injection of cDNA complexed with cationic lipids such as dioctadecylamidoglycyl spermine or DOTAP produced blue stained cells (Fig. 7). By contrast, nuclear and cytoplasmic injections of cDNA complexed with polylysine produced consistent expression of the reporter gene albeit with a lower efficacy than with PEI (p < 0.001 in both cases).

DISCUSSION

Our results demonstrate that cDNA complexation with cationic polymers promotes gene accessibility to the nucleus, a property that is not observed with cationic lipids. When 10,000 naked cDNA copies were injected in the cytoplasm, only 13% of the cells expressed the transgene. A comparable expression efficiency was obtained when less than 10 copies of naked cDNA were directly injected into the nucleus. This suggests that less than 1/1000 naked cDNA copies injected in the cytoplasm were effectively trafficked to the nucleus, an estimate that is in agreement with previous observations (9, 18). A similar calculation shows that 1/100 cDNA-PEI complexes were
Polyethylenimine Promotes Transgene Delivery

**Fig. 5. Transgene expression kinetics after transfection or microinjection in COS-7 cells.** In the left panel, COS-7 cells were either injected into the nucleus (circles) or the cytoplasm (triangles) with naked pCMV-LacZ cDNA (injected cDNA copies, 10,000 per cell (open symbols)) or with pCMV-LacZ complexed with 5 equivalents of PEI (filled symbols) or transfected with pCMV-LacZ (2 μg) complexed with 5 equivalents of PEI (crossed symbols). After various durations of incubation as indicated on the x-axis, cells were revealed for β-galactosidase activity and counted. * denotes p < 0.05; ** p < 0.01; and *** p < 0.001 versus cells injected with naked cDNA. ○, nuclear naked; ●, nuclear PEI; △, transfected; ▲, cytoplasm naked; ▼, cytoplasm PEI. The right panel shows pCMV-CFTR expression kinetics after direct microinjection of pCMV-CFTR in COS-7 cells assayed with SPQ fluorescence microscopy. The rate of SPQ dequenching under nitrate application in the presence of forskolin (10 μM), which reflects the cell permeability to halide upon cAMP stimulation, is plotted against time elapsed after nuclear or cytoplasmic injections with pCMV-CFTR complexed with PEI (filled symbols) or nuclear injections with naked pCMV-CFTR (open circles). ** denotes p < 0.001 versus cells injected in the cytoplasm with cDNA-PEI complexes. Injected copy number: 10,000 in all cases. Data are mean ± S.E. of 6–12 different cells.

**Fig. 6. Transfection and injection efficiencies in COS-7 (black bars), CFPAC-1 (gray bars), or A549 cells (empty bars). A, cells were transfected with pCMV-LacZ complexed with PEI at variable equivalents. B, comparison of injection efficiencies in different cell lines after intranuclear or intracytoplasmic injections of cDNA-PEI complexes at 5 equivalents. Injected copy number: 10,000 in all cases. ** denotes p < 0.01 versus intracytoplasmically injected COS-7 cells.

**Fig. 7. Percentage of X-gal-positive cells after injection of cDNA pCMV-LacZ plasmid complexed with different vectors.** Intracellular (filled bars) or intracytoplasmic (empty bars) injections of COS-7 cells were performed with pCMV-LacZ (copy number: 10,000/cell) complexed with 5 equivalents of PEI or 6 equivalents of dioctadecylamido glycyl spermine. Polylysine and DOTAP were complexed to the plasmid as described under “Experimental Procedures.” Data are mean ± S.E. n, the number of injected cell per experimental condition, varied from 325 to 470. DOGS, dioctadecyl amido glycyl spermine.

trafficked to the nucleus and thus that cDNA complexation increased 10-fold the access of the transgene to the nucleus. Our results also show that in opposition to cationic lipids, cationic polymers when injected in the nucleus do not prevent gene expression and thus likely disassemble from cDNA in the nucleus, a process that is supposedly fast since expression kinetics were very similar with coated or uncoated cDNA. We have also showed that rupture of the nuclear envelope as occurs during cell division was not an absolute requirement for penetration of cDNA-PEI complexes into the nucleus. This observation is in agreement with efficient in vitro (23) and in vivo (24) PEI-mediated transfections of post-mitotic cells such as neurons. In addition, we found that compaction of plasmid DNA into spherical particles rather than the ionic strength of the cDNA-PEI complexes appeared as a critical factor for transgene trafficking to the nucleus. Finally, expression kinetics after intracytoplasmic injection of cDNA-PEI complexes or classical PEI-mediated cell transfection were comparable, suggesting trafficking through the nuclear envelope as the rate-limiting step. The simplest explanation to account for our results as a whole is that plasmids coated with polymers but not with cationic lipids are targeted to the nucleus. However, alternative mechanisms could also very well explain our observations, including improved intracytoplasmic mobility or polymer-related protection of cDNA from endogenous nuclease degradation. Current programs in our laboratory are dedicated to evaluating this latter hypothesis. Transfection efficiency with PEI markedly varied depending on the cell line, with low levels of expression in CFPAC-1 or A549 epithelial cells. Intracytoplasmic injection of cDNA-PEI complexes in CFPAC-1 and in A549 cells led a lower level of expression as compared with COS-7 cells, although this did not reach significance in CFPAC-1 cells. These results suggest that intracellular barriers to plasmid trafficking quantitatively varies with cell type.

Nuclear transport in mammalian cells is a critical limiting step in gene transfer using nonviral vectors (9, 18). Our data are in line with a reasonable scheme proposed by Xu and Szoka...
postulating that during transfection, cDNA is released from cationic lipids in the cytoplasm and is then trafficked uncoated by an inefficient mechanism into the nucleus. The present work suggests that the sequence leading to transfection with cationic polymers differs from the latter scheme inasmuch as coated cDNA may penetrate post-mitotic nuclei where it is most likely released by competitive interaction with genomic DNA (25). Our data also suggest that cDNA compaction rather than the ionic charge of cDNA complexes improves nuclear targeting. Altogether these findings provide a novel mechanistic basis for rational vector design.

Acknowledgments—We thank Dr. A. Faivre for his kind help with injection volume measurement. We thank B. Le Ray for her expertise with cell culture and M. J. Louerat for plasmid amplification.

REFERENCES
1. Behr, J. P. (1994) Bioconjugate Chem. 5, 382–389
2. Gao, X., and Huang, L. (1995) Gene Ther. 2, 710–722
3. Crystal, R. G. (1995) Science 270, 404–410
4. Boussif, O., Lezoualc’h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7297–7301
5. Goldman, C., Sorocceanu, L., Smith, N., Gillespie, G. Y., Shaw, W., Burgess, S., Bilbao, G., and Curiel, D. T. (1997) Nat. Biotechnol. 15, 1–6
6. Vigneron, J. P., Oudrhiri, N., Pasquet, M., Vergely, L., Bradley, J. C., Basseville, M., Lehn, P., and Lehn, J. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9652–9656
7. Gershon, H., Ghirlando, R., Guttman, S. B., and Minsky, A. (1993) Biochemistry 32, 7143–7151
8. Felgner, J. H., Kumar, R., Sriramarao, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M., and Felgner, P. L. (1994) J. Biol. Chem. 269, 2550–2561
9. Zabner, J., Fashender, A. J., Moninger, T., Poellinger, K. A., and Welsh, M. J. (1995) J. Biol. Chem. 270, 18997–19007
10. Legendre, J. Y., and Szoka, F. C. (1992) Pharm. Res. (N. Y.) 9, 1235–1242
11. Behr, J. P. (1993) Acc. Chem. Res. 26, 274–278
12. Zhou, X., and Huang, L. (1994) Biochim. Biophys. Acta 1189, 195–203
13. Wu, L., and Collins, D. (1995) Biochim. Biophys. Acta 1235, 296–304
14. Xu, Y., and Szoka, F. C. (1996) Biochemistry 35, 5616–5623
15. Boussif, O., Zanta, M. A., and Behr, J. P. (1996) Gene Ther. 3, 1074–1080
16. Mohammad-Panah, R., Demolombe, S., Richet, D., Leblais, V., Loussouarn, G., Pollard, H., Baro, I., and Escande, D. (1998) Am. J. Physiol., in press
17. Lim, K., and Chae, C. B. (1989) BioTechniques 7, 576–579
18. Labat-Moleur, F., Steffan, A. M., Brissin, C., Perron, H., Feugeas, O., Forstenberger, P., Oberling, F., Brambilla, E., and Behr, J. P. (1996) Gene Ther. 3, 1010–1017
19. Minaschev, G., and Bereiter-Hahn, J. (1989) Exp. Cell Res. 183, 434–442
20. Capecechi, M. R. (1980) Cell 22, 479–488
21. Dowty, M. E., Williams, P., Zhang, G., Haggstrom, J. E., and Wolf, J. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4572–4576
22. Wilke, M., Fortunati, E., Van Der Broek, M., Hoogerveen, A., and Scholte, B. J. (1996) Gene Ther. 3, 1133–1142
23. Sola, B., Staedel, C., Remy, J. S., Bahr, A., and Behr, J. P. (1997) J. Neurosci. Methods 71, 183–186
24. Abdallah, B., Hassan, A., Benoist, C., Goulou, D., Behr, J. P., and Demeneix, B. A. (1996) Hum. Gene Ther. 7, 1947–1954
25. Remy, J. S., Kichler, A., Mordvinov, V., Schuber, F., and Behr, J. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1744–1748