Entrapment of high-molecular-mass DNA molecules in liposomes for the genetic transformation of animal cells

József SZELEI and Ernő DUDA

Institute of Biochemistry, MTA Biological Center, Szeged, 6701 Hungary

We modified the Ca/EDTA procedure for the production of liposomes [Papahadjopoulos, Vail, Jacobson & Poste (1975) *Biochim. Biophys. Acta* 394, 483–491] to entrap intact DNA molecules of very high molecular mass into large unilamellar phospholipid vesicles. The use of DNA–protein complexes and phage particles instead of naked linear DNA increases the efficiency of entrapment and protects the integrity of DNA molecules. We investigated the interaction of mammalian cells with liposome-encapsulated recombinant λ bacteriophages carrying marker genes. The liposomes bind surprisingly fast to the cellular surface and are taken up by the cells. A significant proportion of the encapsulated DNA is transported to and soon located in or around the nuclei. Experiments prove that these liposomes can be used for the genetic transformation of mammalian cells.

INTRODUCTION

The use of liposomes for the genetic transformation of tissue-cultured animals cells (Mukherjee *et al.*, 1978; Wong *et al.*, 1980; Kondorosi & Duda, 1980; Schaefer-Ridder *et al.*, 1982) seemed to be a promising alternative to capillary micro-injection (Diacumakos, 1973; Graessmann *et al.*, 1977) and calcium phosphate coprecipitation of DNA (Graham & van der Eb, 1973; Wiegler *et al.*, 1977).

Liposomes are not toxic to cells, and the fusion of plasma membranes and phosphatidylserine liposomes usually takes place spontaneously, without the use of harmful chemicals (Wilson *et al.*, 1979; Fraley *et al.*, 1980, 1981; Wong *et al.*, 1980). Incorporation of pH-sensitive lipids and fusogenic viral polypeptides into the liposomal membrane is also reported to enhance the fusogenic properties of liposomes (White & Helenius, 1980; Düzgüneş *et al.*, 1985; Kondorosi & Duda, 1982).

Still, liposome-mediated gene transfer of mammalian cells has not become a widespread method, since procedures for DNA entrapment and transformation are not effective. There are two procedures used for the entrapment of DNA molecules, the Ca/EDTA method (Papahadjopoulos *et al.*, 1975) and the reverse-phase-evaporation method (Szoka & Papahadjopoulos, 1978). We have found that the first method encapsulates large DNA molecules very inefficiently, whereas the other uses sonication or other dispersive techniques, known to shear high-molecular-mass DNA.

Since a high percentage of the eukaryotic genes are larger than 10 kb, neither of the above procedures seems to be adequate for the preparation of liposomes used for the transformation of animal cells with these genes.

Here we report a technique for the entrapment of cosmid or recombinant phage DNA packaged *in vitro* or *in vivo* in the form of phage head particles into phosphatidylserine vesicles. These liposomes contain high concentrations of very large DNA molecules, and seem to be ideal vehicles for the genetic transformation

of tissue-cultured mammalian cells or even cells of living organisms.

MATERIALS AND METHODS

Cell cultures

HeLa, CHO-K1 and mouse L cells were grown in Dulbecco's modified minimal essential medium (Serva, Heidelberg, Germany) with 10% (v/v) newborn-calf serum (HUMAN, Budapest, Hungary). For the transformation experiments approx. 10^5 cells were seeded per 60 mm-diam. Petri dish, 1 day before the experiments. Selection for transformants started 48 h after transformation, when cells were transferred into 170 mm-diam. Petri dishes. Isolation of genetically transformed cells and colonies were carried out 2–3 weeks after transformation, by standard techniques (Puck & Kao, 1968).

Liposomes

Large unilamellar phosphatidylserine vesicles (containing 25–50 mol% of cholesterol to increase vesicle size; Kirby *et al.*, 1980) and to stabilize liposomes in the presence of proteins (Hoekstra & Scherphof, 1979) were prepared as described by Papahadjopoulos *et al.* (1975), except that a suspension of phage particles was added to the pelleted cochleate cylinders, followed by EDTA in NHTE buffer (0.1 M-NaCl, 2 mM-histidine 2 mM-Tes, 0.4 mM-EDTA). Liposomes were purified and freed from unsequestered DNA molecules by ultracentrifugation. Treatment of cells with liposomes was carried out as described by Somlyai *et al.* (1985).

Phages, cosmids

Recombinant-DNA work was carried out by standard procedures (Maniatis *et al.*, 1982). Phages, mixtures for packaging *in vitro* and most of the restriction endonucleases were purified in our Institute. Plasmid pAG60 (Colbere-Garapin *et al.*, 1981), containing the *neo* gene of the aminoglycoside phosphotransferase

enzyme, was generously given by A. C. Garapin (Institut Pasteur, Paris, France). Phage λ gtWES.neo was constructed by inserting a linearized (partially digested with *Eco*RI) pAG60 molecule between the 'arms' of *Eco*RI-digested λ gtWES Δ B-phage DNA.

Autoradiography

Labelling *in vitro* of λ -phage particles or λ -phage DNA with 125 I was carried out as described by Hunter & Greenwood (1964) and Commerford (1971) respectively.

For the autoradiography CHO-K1 cells were grown on glass slides, treated with liposomes containing 125 I-labelled λ -phage DNA (or radioactive λ -phage particles, as controls) packaged *in vitro* for various times. After treatment the cells were washed with phosphate-buffered saline (0.14 M-NaCl/0.027 M-KCl/0.01 M-phosphate buffer, pH 7.2), rinsed with methanol/chloroform (3:1, v/v), fixed in methanol/acetic acid (3:1, v/v) followed by cold 5% trichloroacetic acid, and repeatedly washed in distilled water.

Autoradiography was carried out by dipping the slides into Ilford L4 autoradiographic emulsion, diluted 1:1 with distilled water.

Hybridization

Cell DNA was tested for the presence of transforming sequences by either spot or Southern-blot hybridization. Spot hybridization of non-digested DNA was carried out exactly as described by Brandsma & Miller (1980). DNA was fractionated by electrophoresis on horizontal agarose gels, transferred to nitrocellulose filters and hybridized with nick-translated radioactive probes in accordance with standard procedures (Maniatis *et al.*, 1982).

RESULTS

It is known that large unilamellar vesicles (LUVs) encapsidate DNA with rather poor efficiency; however, there are no reports about the relationship between molecular size and the effectiveness of encapsidation of DNA molecules. We used DNA molecules of different sizes (bacteriophage λ DNA and its fragments produced by various restriction endonucleases), LUVs and vesicles produced by reverse-phase evaporation (REVs) to investigate this question.

Fig. 1 shows the results: LUVs entrap DNA with decreasing efficiency as the size of the molecule increases. Practically no DNA with molecular mass over 10 kb is encapsidated. On the other hand, REVs do not show any preference for low-molecular-mass DNA, and encapsidate DNA with a fair efficiency. However, gel electrophoresis of the entrapped DNA reveals degradation of the macromolecules. Since preparation of REVs starts with production of microscopic droplets of the aqueous phase (shearing a viscous DNA solution), disintegration of DNA molecules is not surprising.

We thought that LUVs are inefficient, since the diameter of the vesicles is too small to accommodate long DNA molecules. (During the formation of REVs DNA must fold within the aqueous droplet, since it is insoluble in the surrounding organic phase.) If that is true, the use of DNA-protein complexes of more compact structure, e.g. chromatin-like structures assembled *in vitro* or large DNA molecules packaged *in vitro* or *in vivo* into phage heads (probably the most compact form of DNA), would increase the ratio of entrapped molecules significantly.

Fig. 1. Relationship of DNA fragment size and efficiency of entrapment into different liposomes

LUVs (○): 10 μ g of λ CI857Sam7-phage DNA was digested with *Hind*III or *Sal*I endonuclease, and samples containing 32 P-labelled DNA (nick-translated to low sp. radioactivity of 10^6 c.p.m./ μ g) were mixed with the precipitate of preformed cochleate cylinders (10 μ mol of total lipid, 50 mol % phosphatidylserine, with 50 mol % cholesterol). Then 200 mM-EDTA in NHTE buffer was added dropwise with gentle shaking until the precipitate cleared up. The suspension was incubated at 37 °C for 30 min. REVs (●): similar samples of labelled DNA solutions were dispersed in 3 ml of chloroform/methanol (3:1, v/v, containing 5 μ mol of phosphatidylserine and 2.5 μ mol of cholesterol) by vortex-mixing and brief sonication. The organic phase was removed in a rotary evaporator. The resulting liposome suspension was diluted with NHTE buffer. Free DNA was separated from liposomes by repeated centrifugations, and radioactivity was measured in dried samples on filter paper in toluene-based scintillation mixtures.

To prove this point, we tried to encapsidate large-molecular-mass complexes of DNA molecules with spermidine, histons and polycations. All these complexes showed improved encapsidation. In parallel experiments we found that by using bacteriophage particles the DNA content of the liposomes preparation can be increased by two orders of magnitude (Table 1).

Actually, the percentage of entrapment was so high as to be unusual even with low-molecular-mass molecules. We tried several approaches to prove the validity of these results. Radiolabelling and re-isolation of the DNA from the liposomes showed 35–45% entrapment. DNAase and proteinase treatment of the liposome suspension left 40–45% of the bacteriophage DNA (and proteins) intact.

We concluded that phage particles either serve as initiation centres for liposome formation or fuse with the vesicles produced [either way, according to electron-microscopic pictures (not shown) the entrapped phages lose most of their structure]. The exact mechanism of the phage-DNA entrapment was not further investigated.

Uptake and intracellular distribution of liposome-entrapped phage particles and DNA

We studied the interaction of tissue-cultured cells with the liposomes encapsidating phage DNA by using radiolabelled particles. Intracellular distribution of radiolabelled DNA during liposome treatment was studied by autoradiography (Fig. 2). Cultured cells were treated

Table 1. Entrapment of phage heads into LUVs

Bacteriophage suspensions of λ cI857Sam7 (48.5 kb), containing 0.2–5.0 mg of phage particles/ml (a), or λ gtWES.neo (42.1 kb), containing 0.6–4.0 mg of phage particles/ml (c), were mixed with the precipitate of preformed cochleate cylinders (10 μ mol of total lipid; 50 mol % phosphatidylserine with 50 mol % cholesterol). Then 200 mM-EDTA in NHTE buffer was added dropwise with gentle shaking until the precipitate cleared. The suspension was incubated at 37 °C for 30 min. Liposomes and phage particles were separated by gradient centrifugation. Empty liposomes (LUVs) of the same composition were mixed with the same λ cI857Sam7-phage suspensions as controls (b). After 30 min incubation at 37 °C, liposomes and phage particles were separated. DNA and proteins of liposome-entrapped phage particles were purified and quantified after protease/DNAase digestion. Plasmid pAG60 encapsulated in REVs (d, as detailed in Fig. 1) served as a control in transformation experiments (see Table 2).

Liposomes	Encapsulation (%)	No. of expts.
(a) λ cI857Sam7 in LUVs	6–12	5
(b) λ cI857Sam7 with LUVs	1–3	3
(c) λ gtWES.neo in LUVs	50–59	4
(d) pAG60 in REVs	17–25	3

for various times with 125 I-labelled λ -phage particles or DNA encapsulated in LUVs. The interaction of cells and liposomes took place surprisingly quickly; 0.5 min after the addition of liposomes to cells, the plasma membrane was surrounded with vesicles (Fig. 2a). By 5–15 min later DNA was found within the cells, showing an almost homogeneous intracellular distribution, and incubation for an additional 10 min resulted in the accumulation of labelled DNA in the nuclear or perinuclear region of the cells (Fig. 2c). These results demonstrate that liposome-entrapped DNA molecules were taken up by the cells, emptied into the cytoplasm, and a significant fraction of DNA was transported to the nucleus within 20 min.

Spot and Southern-hybridization experiments with DNA isolated from cells 2–10 days after liposome treatment proved the presence of a fraction of phage DNA molecules, partly in undigested, free, form (during the first 6 days, in decreasing amounts) or in integrated form (from day 4).

Genetic transformation of HeLa and mouse L cells

Plasmid pAG60, a shuttle vector containing the *neo* gene of transposon Tn5 under the influence of the herpes-simplex viral thymidine kinase promoter, is routinely used for kinetic transformation of animal cells. This plasmid was used for the construction of recombinant bacteriophages, replacing the middle *Eco*RI fragment of λ gtWES Δ B with the entire plasmid. The resulting phage has a genome of 42174 bp. The presence of Ca^{2+} ions and EDTA during the liposome formation destabilizes λ phage and recombinant phages with a wild-type-sized genome. It was shown previously (Harrison *et al.*, 1973) that phage mutants with slightly smaller genome size tolerate the absence of bivalent cations and the presence of EDTA better; consequently the DNA in our phages

Fig. 2. Cell-liposome interaction and intracellular distribution of the liposome-entrapped phage DNA

CHO-K1 cells (1×10^4 – 2×10^4) were grown on glass slides, rinsed in phosphate-buffered saline, and then treated with liposomes containing λ -phage particles packaged *in vitro* containing 125 I-labelled DNA. Approx. 10^4 liposomes (containing 5–800 phage particles) were added per cell. Incubation was carried out in Dulbecco's medium without serum for 0.5 min (a), 8 min (b) and 20 min (c) at 37 °C. After treatment the cells were washed with phosphate-buffered saline, rinsed with methanol/chloroform (3:1, v/v), fixed in methanol/acetic acid (3:1, v/v) and cold 5% trichloroacetic acid, followed by repeated washings in distilled water. Autoradiography was carried out by dipping the slides into Ilford L4 autoradiographic emulsion, diluted 1:1 with distilled water. After exposure for 1–20 days the slides were developed, stained and photographed.

Table 2. Genetic transformation of HeLa cells

(1) With a λ gtWES.neo phage suspension at 4 mg/ml, 52–59% of the recombinant particles became sequestered into liposomes; 10 μ mol of lipid (phosphatidylserine and cholesterol, 50 mol%, each) was used, which entrapped a total of 220 μ g of phage DNA (in the form of phage particles). To study dependence of the transformation efficiency on the ratio of empty to loaded liposomes, we prepared another batch of liposomes (2), with lower DNA content (35.2 mg of DNA/10 μ mol of lipid). (3) *neo* gene containing plasmid-pAG60 DNA was entrapped into REVs (see Fig. 1). (4) λ gtWES.neo phages were added to preformed LUVs and incubated at 37 °C for 5 min in the presence of 20 mM-EDTA in NHTE buffer. Transformation of the cells was carried out as described in the Materials and methods section.

Liposome batch	Plasmid pAG60 (μ g/500 nmol of lipid)	Frequency of transformation
(1)	1.65	5.1×10^{-4}
(2)	0.27	2.2×10^{-4}
(3)	0.74	3.0×10^{-5}
(4)	0.50	5.0×10^{-6}

and cosmid is constructed to be shorter than the wild-type λ -phage DNA.

We used the original pAG60 plasmid entrapped in REVs (these vesicles are the most efficient in entrapping naked DNA molecules) for the transformation of HeLa cells. In the parallel experiment we used *neo* gene containing recombinant λ phage (λ gtWES.neo) particles encapsidated in LUVs for transformation.

The experiment proved that our liposomes can be used for the transformation of tissue-cultured cells with very good results. Actually, they are at least as effective in transforming HeLa and mouse L cells as is calcium phosphate co-precipitation, and much better than conventional DNA-liposomes. It is not just the presence of liposomes and phages that is needed for effective transformation: preformed liposomes, mixed with recombinant λ phages (and incubated with 20 mM-EDTA for 5 min at 37 °C) proved to be inefficient in transforming cells (Table 2).

DISCUSSION

We have developed a technique for the introduction of eukaryotic gene-sized DNA fragments into tissue-cultured mammalian cells. This method is a modification of the Ca/EDTA procedure described by Papahadjopoulos *et al.* (1975) for the production of LUVs. Our procedure is based on the fact that bacteriophage- λ heads are entrapped very efficiently into liposomes. Since cosmid-size DNA molecules packaged *in vivo* or *in vitro* into phage heads represent the most compact form of DNA, these vesicles contain high-molecular-mass DNA molecules in concentrations unusually high in liposomes. In fact, these liposome preparations contain 2–3 orders of magnitude higher concentrations of DNA than do conventional liposome preparations.

Cochleate cylinders turn into liposomes only in the presence of a large excess of EDTA, but λ -phage particles

do not tolerate the absence of bivalent ions. The stability of the phage particles in these circumstances is increased by decreasing the size of the genome of the recombinant phages. Still, entrapped phage particles lose their structure during liposome formation. As a consequence no cellular process is needed to free the transforming DNA molecules; they are released into the cytoplasm upon fusion with the target cell membranes. Since most of the vesicles are taken up by endocytosis, which could lead to the digestion of the DNA, incorporation of fusogenic proteins or lipids (Kondorosi & Duda, 1982; Düzgüneş *et al.*, 1985) into the liposomal membrane increases the efficiency of transformation. Chloroquine and ammonium salts, known to inhibit lysosomal activity, can also be used to increase the survival rate of introduced DNA molecules.

Liposomes prepared by our procedure can be used for effective transformation of animal cells, resulting in transformation frequencies equal to or higher than with the routinely used calcium phosphate co-precipitation method. The procedure is very mild to the cells, providing an excellent way to transform sensitive cell lines or cells of living organisms.

REFERENCES

- Brandsma, J. & Miller, G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6851–6855
- Colbere-Garapin, F., Horodniceanu, F., Kourilsky, P. & Garapin, A. C. (1981) *J. Mol. Biol.* **150**, 1–14
- Commerford, S. L. (1971) *Biochemistry* **10**, 1993–1999
- Diacumakos, E. G. (1973) *Methods Cell Biol.* **7**, 287–291
- Düzgüneş, N., Straubinger, R. M., Baldwin, P. A., Friend, D. S. & Papahadjopoulos, D. (1985) *Biochemistry* **24**, 3091–3098
- Fraley, R., Subramani, S., Berg, P. & Papahadjopoulos, D. (1980) *J. Biol. Chem.* **255**, 10431–10435
- Fraley, R., Straubinger, R. M., Rule, G., Springer, E. L. & Papahadjopoulos, D. (1981) *Biochemistry* **20**, 6978–6987
- Graessmann, A., Graessmann, M. & Müller, C. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4831–4834
- Graham, F. L. & van der Eb, A. J. (1973) *Virology* **52**, 456–467
- Harrison, D. P., Brown, D. T. & Bode, V. C. (1973) *J. Mol. Biol.* **79**, 437–449
- Hoekstra, D. & Scherphof, G. (1979) *Biochim. Biophys. Acta* **551**, 109–121
- Hunter, W. & Greenwood, F. (1964) *Biochem. J.* **91**, 43–56
- Kirby, C., Clarke, J. & Gregoriadis, G. (1980) *Biochem. J.* **186**, 591–597
- Kondorosi, É. & Duda, E. (1980) *FEBS Lett.* **120**, 37–40
- Kondorosi, É. & Duda, E. (1982) *Biochem. Biophys. Res. Commun.* **107**, 367–373
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Mukherjee, A., Orloff, S., Butler, J., Triche, T., Lalley, P. & Schulman, J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1361–1365
- Papahadjopoulos, D., Vail, W., Jacobson, K. & Poste, G. (1975) *Biochim. Biophys. Acta* **394**, 483–491
- Puck, T. T. & Kao, F. T. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **60**, 561–568
- Schaefer-Ridder, M., Wang, Y. & Hofschneider, P. H. (1982) *Science* **215**, 166–168

- Somlyai, G., Kondorosi, É., Karikó, K. & Duda, E. (1985) *Acta Biochim. Biophys. Acad. Sci. Hung.* **20**, 203–212
- Szoka, F. & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4194–4198
- White, J. & Helenius, A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3273–3277
- Wiegler, M., Silverstein, S., Lee, S. L., Pellicer, A., Cheng, T. & Axel, R. (1977) *Cell* **11**, 223–232
- Wilson, T., Papahadjopoulos, D. & Taber, R. (1979) *Cell* **17**, 77–84
- Wong, T. K., Nicolau, C. & Hofschneider, P. H. (1980) *Gene* **10**, 87–94

Received 4 October 1988/20 December 1988; accepted 28 December 1988