

Liposome Mediated DNA-transfer into Mammalian Cells

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We have investigated the interaction of mammalian cells with liposome encapsulated DNA. Tissue cultured mammalian cells were exposed to large, unilamellar phosphatidyl serine liposomes containing DNA molecules from different animal cells or prokaryotic organisms.

The liposomes bind rapidly to the surface and are taken up by the cells and significant proportion of the encapsulated DNA is transported to the nuclei.

Transient expression of the foreign genetic material could be detected in high percentage of the treated cells for a few days. During this period of time foreign DNA is present in both free and integrated form, however, the free form soon disappears.

Stable transformant cell colonies— with continuous expression of new gene(s)— were isolated under selective pressure with a frequency of approx. 10^{-5} .

Several techniques have been developed for the transfer of foreign DNA sequences into eukaryotic cells or protoplasts (McBride and Ozer, 1973; Pellicer et al., 1980; Graham and van der Eb, 1973; Fraley et al., 1980). One of the most promising methods uses artificial lipid vesicles (liposomes), providing protection against nucleases and delivering the DNA molecules into the cytoplasm by endocytosis or fusion with cellular plasmamembranes.

In this paper we report details of cell-liposome interaction, and the fate of foreign DNA sequences in the target cells. Furthermore, expression and physical state of the microinjected DNA will be discussed.

Materials and methods

Cell culture

Rat H56 tk⁻ cells (kindly provided by Dr. M. Weiss) and CHO-K1 pro⁻ chinese hamster ovary cells were maintained in F12 medium containing 5% calf serum. Mouse SL tk⁻ cells (obtained from Dr A. Venetianer) were grown in MEM supplemented with 5% calf serum.

Abbreviations: MEM: minimal essential medium; LUV: large, unilamellar vesicles; PBS: phosphate buffered saline; SSTKNa buffer: 0.34 M sucrose, 0.15 mM sperine, 0.5 mM spermidine, 15 mM tris (pH 8.0), 60 mM KCl, 15 mM NaCl; TCA: trichloroacetic acid; HAT medium: tissue culture medium, containing hypoxanthine, adenine and thymine; HSV-TK: thymidine kinase gene sequence of Herpes Simplex Virus.

DNA

DNA was purified from *E. coli*, lambda phage, cultured drosophila cells (KC cells), chick embryos, human embrionic liver of chicken red blood cells according to standard procedures.

Labelled DNA was purified from *E. coli* cells grown on a phosphate free medium supplemented with 5 mCi (200 MBq) ^{32}P orthophosphate. In vitro labelling of lambda phage particles or lambda DNA with ^{32}P was carried out according to Hunter and Greenwood (1971) and Commerford (1971), respectively.

Liposomes

Large unilamellar phosphatidyl serine vesicles were prepared according to Papahadjopoulos et al. (1975), purified by 1 M NaCl precipitation. Treatment of cells with liposomes was carried out as described earlier.

Uptake of liposome entrapped, labelled DNA by cultured cells. Cells treated with liposome entrapped, labelled DNA were washed with cold PBS thoroughly and suspended in SSTKNa buffer, containing 0.34 M sucrose, 0.15 mM spermine, 4 HCl, 0.5 mM spermidine. 3 HCl, 15 mM tris. HCl (pH 8.0), 60 mM KCl, 15 mM NaCl.

Radioactivity taken up by cells was determined by washing the cells on GF/C filters repeatedly with SSTKNa buffer, drying the filters and counting the radioactivity in toluene based scintillation cocktails. Isotope content of nuclei was measured similarly, except cells were lysed and washed with SSTKNa buffer containing 1% Triton X-100. For the determination of LUV encapsulated DNA, liposomes were digested with DNase I, washed and lysed with Triton X-100. DNA liberated from the vesicles was precipitated with cold 5% TCA, filtered and its radioactivity was determined as above.

Autoradiography

2×10^4 H56 cells grown on glass slides, rinsed in PBS were treated with liposomes containing radiolabelled lambda DNA for various times. The cells were washed with PBS, fixed in 3 : 1 (v/v) methanol : acetic acid followed by 5% cold TCA and repeated washings in distilled water.

H56 tk⁻ cells treated with Drosophila DNA encapsulated in liposomes were exposed to ^3H thymidine (10 MBq/ml, spec. act. 400 GBq/mM) one day after the liposome treatment for 24 hours. The cells were washed and fixed as above.

Autoradiography was carried out by dipping the slides into L4 Ilford autoradiographic emulsion (diluted 1 : 1 in dist. water). After 1–20 days of exposure the slides were developed stained and photographed.

DNA preparation from liposome-treated cells

CHO-K1, H56 tk⁻ and SL tk⁻ cells were treated with liposome-entrapped HSV-TK/pBR322 or with a mixture of empty liposome and non-encapsulated HSV-TK/pBR322. For two days cells were maintained in non-selective medium which was then replaced by selective, HAT medium (Maitland and McDougall,

1977). After 1, 2, 4 or 6 days of incubation the cells were harvested and high molecular weight DNA was prepared as described by Pellicer et al. (1978). Restriction endonuclease cleavage of DNA with Bgl II was performed in the presence of 0.01 % sodium azide at 37 °C overnight. The enzyme-to-DNA ratio was 1.5 units/ μg of DNA and usually 20 μg of DNA was digested.

Filter hybridization

Cell DNA was tested for the presence of HSV-TK/pBR322 either by spot or Southern blot hybridization. Spot hybridization of non-digested DNA was carried out exactly as described by Brandsma and Miller (1980). ^{32}P -labelled HSV-TK probe was prepared by nick translation (Rigby et al., 1977) with a specific activity of $1-2 \times 10^8$ cpm/ μg . For blot hybridization 20 μg of Bgl II digested DNAs were fractionated by electrophoresis on 0.8 % horizontal agarose gel and transferred to nitrocellulose filter essentially as described by Ketner and Kelly (1976). Annealing with labelled probe was carried out according to Wahl et al. (1979).

Results

Uptake and intracellular distribution of liposome-entrapped DNA

Uptake and transport of liposome-entrapped DNA molecules by cells were studied by the use of [^{32}P]-labelled *Escherichia coli* DNA encapsulated in LUV. Radioactivity was determined in the whole cells or in the nuclei 60 min after the addition of liposomes to cells. Table 1 shows that radioactivity measured in the cells or nuclei was practically the same, independently of the doses of liposomes. [^{32}P]-labelled DNA found in the nuclei represented about 93 % of total activity taken up by cells which suggests rapid transport of DNA from cytoplasm into the nucleus. (Similar results were found using chicken leukemia cells and labelled DNA entrapped in liposomes Duda and Travniček [unpublished].)

Table 1

Uptake of liposome-entrapped, ^{32}P -labelled DNA by cells

Liposome + (μl)	Radioactivity (cpm) in		
	cells	nuclei	
100	10.35×10^3	9.87×10^3	(95.4 %)*
50	7.90×10^3	7.31×10^3	(92.5 %)*
20	5.31×10^3	4.96×10^3	(93.5 %)*
10	2.72×10^3	2.38×10^3	(87.5 %)*
5	1.44×10^3	1.39×10^3	(96.5 %)*

[†] in 1 ml of liposome suspension 2.135×10^6 cpm of ^{32}P -labelled DNA was encapsulated. Liposomes were added to H 56 cells and incubated for 60 min at 37 °C.

* Radioactivity of nuclei as compared to radioactivity of whole cells in percentage.

Intracellular distribution of radiolabelled DNA during liposome treatment was followed by autoradiography (Fig. 1). Cultured cells were treated for various times with ^{125}I -labelled, λ DNA encapsulated in LUV. 0.5 min after the addition of liposomes to cells, the plasma membrane was surrounded with LUV-entrapped, labelled DNA (Fig. 1a) 10 min later DNA was found within the cells showing an almost homogenous intracellular distribution (Fig. 1b), while an additional 10 min of incubation resulted in the accumulation of labelled DNA in the nuclear or perinuclear region of the cells (Fig. 1c). These results demonstrate that liposome-entrapped DNA molecules were taken up by cells, emptied into the cytoplasm and a significant fraction of DNA was transported into the nucleus within 20 min.

The persistence and stability of the microinjected DNA

To test the presence and stability of foreign DNA, cloned DNA molecules were introduced into cultured cells by liposomes. The presence of foreign DNA was detected by nucleic acid spot hybridization after a longer period of incubation. tk⁻ mouse and rat cells were treated with liposome-entrapped HSV-TK gene inserted in pBR322, incubated for two days in non-selective and then in selective (HAT) medium. High molecular weight DNA was isolated from cells at various times of cultivation and 1 μg from each was assayed for the presence of foreign DNA by nick translated [^{32}P]-HSV-TK/pBR322 probe. Spot hybridization of the samples (Fig. 2) showed the presence of foreign DNA in all cells treated with liposome-entrapped DNA but no hybridization was detected with DNA isolated from control cells.

The physical state of the microinjected DNA, i.e. whether it was integrated into the host genome or remained in a free form, was studied by Southern blot hybridization. DNA extracted from liposome-treated tk⁻ rat cells was digested with Bgl II restriction endonuclease cleaving the circular HSV-TK/pBR322 molecule only at a single site. DNA fragments, separated by gel electrophoresis and transferred to nitrocellulose filters were hybridized to highly labelled ^{32}P -HSV-TK/pBR322 probe (Fig. 3). Fragments equivalent to or larger than the liposome-injected DNA were present in the cells one or two days after the liposome treatment, corresponding to the free and probably to the integrated forms of plasmid DNA. A few days later the pattern of fragments has changed suggesting the disappearance of the free form, leaving only integrated sequences in the nuclei.

Expression of genes of liposome-injected total genomic DNA in mammalian cells

Gene expression of total genomic DNA sequences extracted from *Drosophila melanogaster*, chicken red blood cells and human embryonic liver was investigated.

Expression of *Drosophila* DNA entrapped in liposomes was tested in tk⁻ mouse cells by autoradiography. Cells were treated with 'empty' and DNA-loaded liposomes and then incubated in HAT medium containing [^3H]-thymidine. After one day of cultivation (allowing time for phenotypic expression), the autoradiography of control and DNA-treated cells showed a striking difference in [^3H]-thymidine incorporation (Fig. 4). In cells treated with empty liposomes no radio-

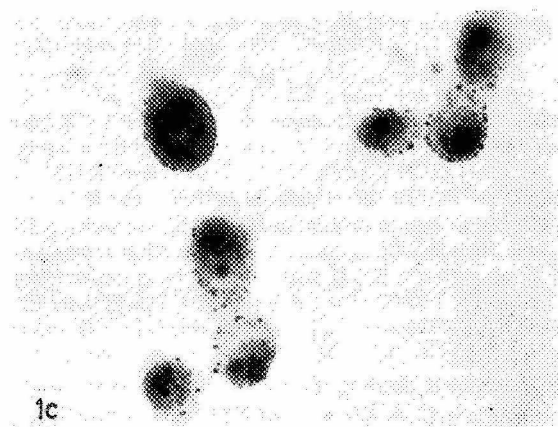
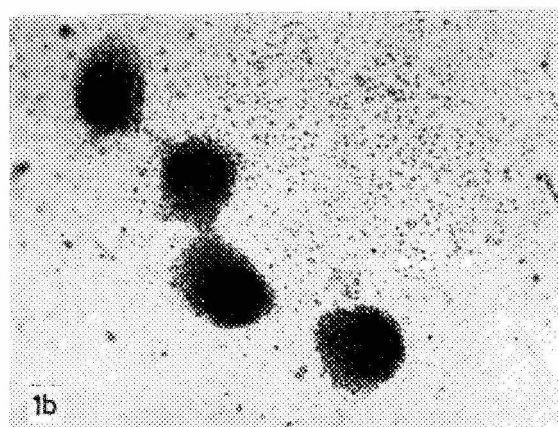
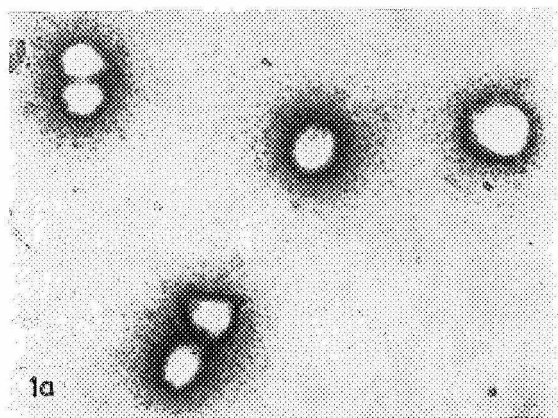


Fig. 1. Cell-liposoma interactions and intracellular distribution of ^{125}I labelled DNA followed by autoradiography CHO-K1 hamster cells were treated with liposomes entrapping ^{125}I labelled lambda phage DNA. Incubation times were: a, 0.5 min; b, 10 min; c, 20 min

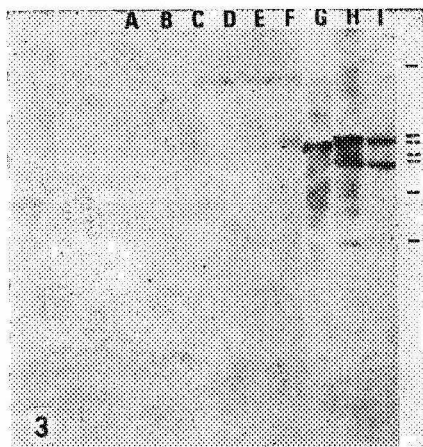
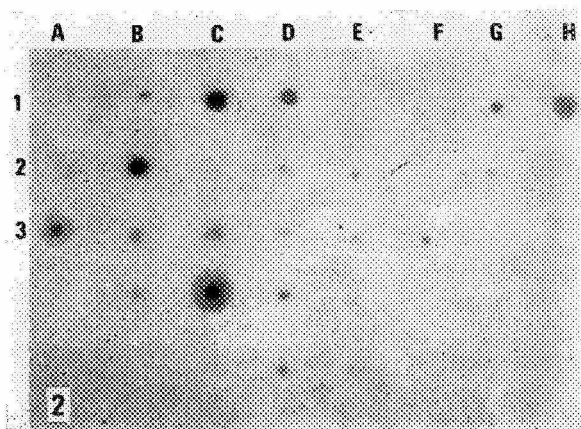


Fig. 2. Spot hybridization of DNAs derived from liposome-treated cells. ^{32}P -labelled HSV-TK/pBR322 was used as a probe. 2A—2G, DNA from SLTK cells 1, 2, 3, 4, 5, 6, 8 and 9 days after treatment with liposomes containing HSV-TK/pBR322; 3B—3E, DNA from H56 cells 2, 4, 6, and 8 days after treatment with liposomes containing HSV-TK/pBR322; 1A, untreated SLTK; 1C, untreated H56; 2H, SLTK and 3A, H56 treated with a mixture of empty liposomes and HSV-TK/pBR322; 1B, HSV-TK/pBR322

Fig. 3. Presence of HSV-TK/pBR322 sequences in cells treated with liposome-entrapped HSV-TK/pBR322. The hybridization profiles of 20 μg of Bgl II-cleaved DNA from tk^- mouse cells (lane A) and cells treated with a mixture of empty liposomes and HSV-TK/pBR322 (lane B) are shown along with the Bgl II patterns of DNAs derived from cells treated with liposome-entrapped HSV-TK/pBR322 for 1 day (lane C), 2 days (lane E), 4 days (lane F) and 6 days (lane D). HSV-TK/pBR322 and markers are shown in lane G and lane H-I, respectively

activity was found while a high amount of ^3H -thymidine was incorporated into the DNA of cells microinjected with *Drosophila* DNA (Fig. 4b). Incorporation of ^3H -thymidine reflects thymidine kinase enzyme activity, probably due to the

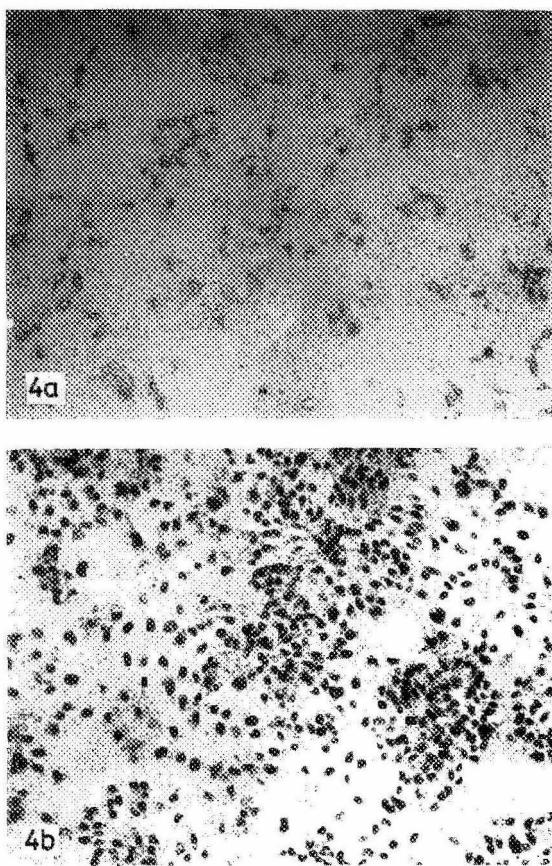


Fig. 4. Thymidine kinase gene expression in treated cells tk^- mouse cells were treated with *Drosophila* DNA containing liposomes and one day later thymidine kinase activity was detected on the basis of 3H -thymidine incorporation. a, cells treated with "empty" liposomes; b, cells treated with liposomes containing *Drosophila* DNA

transient expression of *Drosophila* *tk* gene. No stable tk^+ transformants were produced after a 3 weeks period of incubation in HAT medium.

Transient expression of *Drosophila* DNA was demonstrated also in CHO-K1 pro^- cells by prolonged survival of cells in proline free medium (Fig. 5). Cells treated with empty liposomes showed typical survival curves while cells microinjected with DNA showed a somewhat increased life-time.

While the temporary expression of microinjected genes could be detected in a large fraction of treated cells, stable genetic transformation of recipient cells occurred only at a rather low frequency. Transformant colonies were produced only if cells were microinjected with avian or mammalian genomic DNA. 200 μg high molecular weight DNA purified from chicken red blood cells or human em-

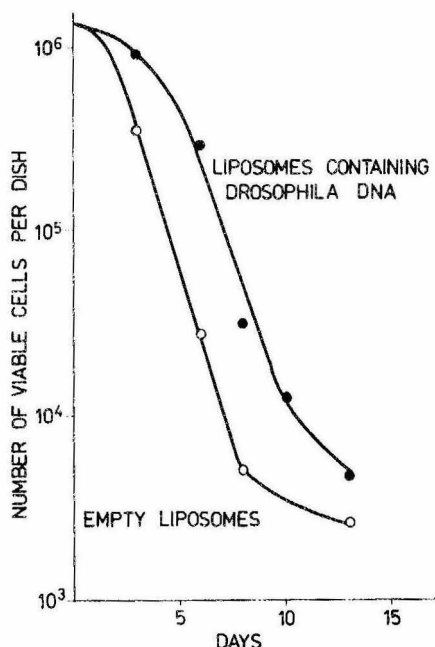


Fig. 5. Survival of CHO-K1 pro⁻ cells in proline deficient medium after liposome mediated DNA transfer. Cells were treated with "empty" and Drosophila DNA containing liposomes (for one hour) and kept on medium lacking proline. Transient gene expression in DNA treated cells increased survival time with 2–3 days

bryonic liver were encapsulated in liposomes prepared from 1.9 μmol phosphatidyl serine per ml and 100 μl of liposome suspension were added to each petri dish containing 5×10^6 CHO-K1 pro⁻, or mouse or rat tk⁻ cells. After cultivation of the cells in proline free or HAT medium, respectively, pro⁺ and tk⁺ colonies were observed at a frequency of approximately 10^{-5} (above rate of 10^{-7} to 4×10^{-7} found in case of untreated, control cells).

Discussion

The potential usefulness of liposome-entrapped genetic material for transformation of cultured cells highly depends upon the uptake of encapsulated material and on the fate of microinjected nucleic acids within the cells. We have demonstrated that liposome-entrapped DNA molecules from different sources were effectively taken up by cells and transported from the cytoplasm of cells into the nucleus. 20 min after the addition of liposomes to cells a high proportion of foreign DNA accumulated in the nuclear and perinuclear parts of the cells, and 40 min later approximately 93% of the labelled foreign DNA was localized within the

nucleus. The use of cloned DNA molecules demonstrated the survival of microinjected DNA molecules for at least for 8 days. The foreign DNA sequences seemed to be integrated, having molecular weights higher than the introduced plasmid DNA. (The presence and state of foreign DNA were investigated only in a relative short period of incubation since 8 days after the liposome treatment almost all cells died, providing insufficient amounts of cells for extraction of host DNA.)

Temporary expression of foreign genomic DNA was observed in a great proportion of liposome-treated cells; however, stable genetic transformation of mammalian cells produced by avian or mammalian DNA was a relatively rare event. The fact that no transformant colonies were observed after treatment with *Drosophila* DNA is in agreement with the results of DNA-mediated transformation of mammalian cells, although our data indicated a transient expression of *Drosophila* genes.

On the basis of our results we assume that nucleic acids entrapped in liposomes can be applied for transformation of cultured cells and liposome-mediated microinjection of DNA provides an alternative, probably generally applicable technique for introduction of specific genes into cultured cells.

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