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Liposome-mediated Transfer of Simian Virus 40 DNA and Minichromosome into Mammalian Cells

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SUMMARY

We have investigated the use of liposomes as carriers for the transfer of simian virus 40 (SV40) DNA into mammalian cells. The amount of DNA entrapped in liposomes was dependent on the input DNA concentration and lipid composition. DNA remained intact after liposome encapsulation and was resistant to deoxyribonuclease digestion. Combined transfer to and expression of liposome-entrapped SV40 DNA in monkey kidney cells was assayed by infectious plaque formation. Negatively-charged liposomes containing phosphatidylserine were more effective in DNA transfer and expression than neutral liposomes. The inclusion of carrier salmon sperm DNA inhibited liposome-entrapped SV40 DNA infectivity. Infectivity of liposome-entrapped DNA was directly related to both liposome DNA concentration and number of vesicles added. Liposome-entrapped SV40 minichromosome was 20-fold more infective than free minichromosome, but only 20% more infective than liposome-entrapped SV40 DNA. Thus, the presence of hyperacetylated histones on the DNA failed to enhance liposome-mediated DNA transfer appreciably. Incubation of cells with various modulators of endocytosis implicated the endocytotic pathway in the mechanism of liposome-mediated DNA transfer. These studies show that liposomes are suitable carriers for the introduction of viral DNA and chromatin into mammalian cells.

INTRODUCTION

Introduction of nucleic acids into mammalian cells has permitted experimental approaches to a number of fundamental problems concerning gene structure and regulation. Current techniques for nucleic acid transfer include direct microinjection (Graessman et al., 1979; Anderson et al., 1980; Capecchi, 1980), presentation of DNA complexed with polycations such as DEAE-dextran (Pagano, 1969) or calcium phosphate precipitates (Graham & van der Eb, 1973), use of hybrid virus-vectors (Hamer et al., 1979; Mulligan et al., 1979; Shimotohno & Temin, 1981) or cell fusion with nucleic acid-loaded red cell ghosts (Straus & Raskas, 1980). Each of these methods has certain advantages and limitations. Recently, haemoglobin mRNA (Ostro et al., 1978; Dimitriadis, 1978), poliovirus RNA (Wilson et al., 1979) and tobacco mosaic virus RNA (Fukunaga et al., 1981) have been entrapped in phospholipid vesicles (liposomes) and introduced into cells. In addition, liposome-entrapped mitotic chromosomes have been used to transfer the hypoxanthine-guanine phosphoribosyltransferase gene into cells at a significantly higher rate than with free mitotic chromosomes (Mukherjee et al., 1978).

Although several reports have described methods to entrap ‘naked’ DNA in liposomes (Hoffman et al., 1978; Dimitriadis, 1979; Lurquin, 1979), transfer and subsequent expression of such DNA in mammalian cells has only recently been demonstrated. Liposome-mediated transfer and expression of viral DNA (Fraley et al., 1980; Straus et al., 1981), E. coli plasmid β-lactamase gene (Wong et al., 1980), and herpes simplex virus thymidine kinase gene (Schaefer-Ridder et al., 1982) have been reported.
In the present investigation, simian virus 40 (SV40) DNA was entrapped in phospholipid vesicles and used to transfect monkey kidney cells. Furthermore, we show that the SV40 minichromosome can be similarly transferred into cells. These studies suggest that liposome-mediated DNA transfer may be a suitable alternative to other methods for the introduction of viral DNA and chromatin into cells.

**METHODS**

*Cells.* African green monkey kidney cells (CV-1), obtained from Dr Janice Chou, were routinely grown in Eagle’s minimal essential medium (HEM Research, Inc.) supplemented with 10% foetal bovine serum.

*Chemicals.* Phosphatidylcholine and phosphatidylserine were purchased from Analabs or Grand Island Biological Company. Diethyl ether was a product of Mallinckrodt. Restriction endonucleases were obtained from Bethesda Research Laboratories. All other chemicals were from Sigma Chemical Company.

*DNA.* SV40 DNA was either obtained from Bethesda Research Laboratories or isolated from intact virions purified as described (Khoury & Lai, 1979). Purified virions were dissociated by incubation at 50 °C for 30 min in 1% sarkosyl, extracted twice with phenol : chloroform (1:1), and the DNA precipitated with 2.5 vol. of cold ethanol. Both sources of SV40 DNA yielded preparation greater than 80% form I upon agarose gel electrophoresis. Lambda DNA and 3H-labelled φX174 DNA were obtained from Bethesda Research Laboratories.

*Liposome preparation.* Sterile technique was used throughout the procedure. SV40 DNA and tracer labelled φX174 DNA were precipitated with ethanol prior to use. This DNA was redisolved in 1.0 ml of 0.14 M- NaCl–0.01 M-HEPES, pH 7.2. Liposomes were made by the method of reverse-phase evaporation (Szoka & Papahadjopoulos, 1978) with minor modifications. Routinely, 88 μmol total lipid consisting of phosphatidylcholine : cholesterol : phosphatidylserine (molar ratio 7:2:2) dissolved in chloroform was dried onto the surface of a 50 ml round-bottom flask under a stream of N2 by rotating the flask in a 30 °C water bath. This flask was placed at 4 °C and lipids were redissolved in 3.0 ml diethyl ether. The DNA solution was then added, the flask flushed with N2 and sealed with a rubber stopper. Phase dispersion was accomplished by shaking the stoppered flask vigorously by hand for 1 min. The flask was then immediately placed on the rotary evaporator in the 30 °C water bath and the ether evaporated under vacuum. After 5 to 10 min, 2 ml of NaCl-HEPES was added and evaporation resumed for 45 min. Liposomes were diluted with 10 ml of the same buffer, washed twice by centrifugation at 20,000 g for 30 minutes and resuspended in fresh buffer. In some cases, liposomes were further treated with deoxyribonuclease I (50 μg/ml) at 37 °C for 30 min followed by repeated washing. To estimate the amount of radioactive DNA entrapped, aliquots of the washed liposomes were solubilized in Hydrofluor (National Diagnostics) and counted. The amount of phospholipid was determined for each preparation (Bartlett, 1959). Liposomes were usually used for transfer experiments on the day of preparation, but occasionally were stored up to 2 weeks at 4 °C under N2.

*Liposome incubation and plaque assay.* CV-1 cells were grown in 100 mm diameter culture dishes. As the cells became confluent, 1 or 2 days prior to liposome addition, medium was changed to modified Eagle’s enriched with 3 times the usual concentration of glucose and amino acids, buffered with 0.05 M-Tricine, containing 4% foetal bovine serum, penicillin, streptomycin, and Fungizone (Chou & Martin, 1974).

Medium was decanted and the monolayer was washed once with 5 ml phosphate-buffered saline. Then, 0.3 ml liposomes in NaCl–HEPES was added and the dish tilted to distribute liposomes over the monolayer. Dishes were placed in a humidified incubator at 37 °C for 45 min with redistribution of the liposomes over the monolayer at least once during the incubation. If cells were to be post-treated with glycerol, then after the 45 min 2 ml of 30% (v/v) glycerol in phosphate-buffered saline was added without removal of liposomes and incubation continued at room temperature for 4 min (Fraley et al., 1980). Post-treatment with other agents was performed in a similar fashion. For prior incubation with chemicals, proteins or other agents, the appropriate substance was dissolved in NaCl–HEPES and 0.2 ml added to the monolayer for 10 min prior to liposome addition. After incubation with liposomes, cells were washed twice with 5 ml phosphate-buffered saline and overlaid with medium containing 1-2% agar. After 13 to 16 days, cells were overlaid with phosphate-buffered saline containing agar and 0.04% neutral red dye. Plaques were counted the following day.

*Nuclease digestions.* Liposome and DNA digestions with restriction endonucleases were performed using the buffers recommended by the supplier in a final volume of 0.1 ml at a ratio of 5 units enzyme/μg DNA for 2 h at 37 °C. For deoxyribonuclease I digestion, liposome-entrapped DNA was resuspended in 2 ml 10 mM-HEPES, 5 mM-CaCl2, 5 mM-MgCl2, 0.14 M-NaCl pH 7.4, and digested with 50 μg/ml deoxyribonuclease I at 37 °C for 30 min. All reactions were stopped by the addition of EDTA (25 mM final concentration). Lipid was removed from liposomes by two extractions with 2 vol. chloroform : methanol (3:1). DNA was precipitated by addition of 3 vol. of ice-cold ethanol and incubation overnight at –20 °C.

*Agarose gel electrophoresis.* DNA was dissolved in sample buffer (pH 8) consisting of 0.04 M-Tris–HCl, 0.013 M-sodium acetate, 0.002 M-EDTA, and 20% (v/v) glycerol, with bromophenol blue as marker. Electrophoresis was performed for 6 to 8 h at 20 mA/gel in water-cooled vertical slab gels consisting of 0.8% or 1.2% agarose in 0.4-
Fig. 1. Entrapment of DNA in negatively-charged liposomes. Liposomes composed of phosphatidylcholine:cholesterol:phosphatidylserine (7:2:2) were prepared using different concentrations of lambda DNA.

Fig. 2. Digestion of liposome-entrapped lambda DNA with restriction endonuclease HindIII. Liposome-entrapped lambda DNA was subjected to digestion with HindIII, lipid was extracted, and DNA electrophoresed in a 0.8% agarose gel followed by staining with ethidium bromide. The following DNA samples are shown: input lambda DNA (lane 1); liposome-entrapped lambda DNA (lane 2); free lambda DNA digested with HindIII (lane 3); free lambda DNA plus empty liposomes digested with HindIII (lane 4); liposome-entrapped lambda DNA digested with HindIII (lane 5); liposome-trapped lambda DNA plus 1% Triton X-100 digested with HindIII (lane 6).

RESULTS

Entrapment of DNA in liposomes

Using a minor modification of the technique of reverse-phase evaporation (Szoka & Papahadjopoulos, 1978), we entrapped DNA in neutral liposomes composed of phosphatidylcholine:cholesterol (molar ratio 7:2). The amount of input DNA entrapped increased with increasing amounts of lipid with a maximum entrapment of 24 ± 2% (n = 16) input DNA when 80 to 90 μmol total lipid was used. Additional lipid failed to increase the amount of DNA entrapped under the conditions described above.

In contrast to neutral liposomes, negatively-charged vesicles composed of phosphatidylcholine:cholesterol:phosphatidylserine (7:2:2) were somewhat less efficient in entrapping DNA. With 80 to 90 μmol lipid, only 17 ± 1% (n = 20) of input DNA was recovered with liposomes. The percentage of DNA entrapped was independent of the input DNA concentration over a wide range (Fig. 1). Thus, there was a direct relationship between the concentration of input DNA and the concentration of vesicle-associated DNA.
The physical state of liposome-entrapped DNA was examined by agarose gel electrophoresis. A typical gel is shown in Fig. 2. High molecular weight lambda DNA entrapped in liposomes migrated identically to input DNA (Fig. 2, lanes 1 and 2). Upon incubation of liposome-entrapped lambda DNA with restriction endonuclease HindIII, the DNA was found to remain largely resistant to digestion although a minor fraction was cleaved (Fig. 2, lane 5). This nuclease-resistant DNA was largely digested when 1% Triton X-100 was added to the reaction (Fig. 2, lane 6). In contrast, free DNA added to empty liposomes was entirely susceptible to digestion in the absence of detergent. Analogous results were obtained for both neutral and negatively-charged liposomes with restriction endonuclease BamHI or deoxyribonuclease I (data not shown). Furthermore, examination by fluorescence microscopy of vesicles entrapping an ethidium bromide-DNA complex revealed homogeneous pale staining within the vesicles. These combined results are consistent with the majority of the DNA being enclosed within the lipid vesicles.

**Transfer and expression of liposome-entrapped SV40 DNA**

In order to test for transfer and expression of DNA in mammalian cells, we entrapped SV40 DNA into liposomes and then incubated them with monkey kidney cells. The ability of this DNA to induce infectious plaque formation established successful transfer. Using neutral vesicles composed of phosphatidylcholine : cholesterol (7 : 2), we were unable to induce plaque formation. Since negatively-charged liposomes are reported to fuse with cell membranes more effectively than neutral vesicles (Poste et al., 1980), we constructed liposomes composed of phosphatidylcholine : cholesterol : phosphatidylserine (7 : 2 : 2). Incubation of these negatively-charged vesicles containing SV40 DNA with monkey kidney cells consistently resulted in plaque formation (data not shown). Free DNA alone or empty liposomes failed to induce plaques. Liposome-entrapped DNA induced plaques even when treated after liposome formation with deoxyribonuclease.

Two lines of evidence suggest that the observed plaque formation was due to DNA and not due to contaminating virus particles. First, free DNA alone at the concentration used failed to induce plaques. Secondly, when SV40 DNA was digested with Proteinase K or various restriction endonucleases known to cleave this DNA and then tested for plaque formation, there was a direct correlation between the physical integrity of SV40 DNA and infectivity. As shown in Fig. 3, Proteinase K digestion, which decreases virion infectivity by greater than four orders of magnitude (unpublished experiments), had no effect on infectivity of liposome-entrapped SV40 DNA, while plaque formation was only decreased by those endonucleases that cleaved SV40 DNA. EcoRI, which cleaves SV40 DNA at one site and linearizes the circular SV40 DNA molecule (i.e. converts Form I and II DNA to Form III), was virtually as efficient in suppressing infectivity as HindIII, which cleaves this DNA at multiple sites. XbaI fails to cleave SV40 DNA and did not affect infectivity. Thus, we conclude that the observed infectivity was actually due to viral DNA.

The effects of various cell treatments on the frequency of liposome-mediated DNA transfer was examined. As reported by Fraley et al. (1980), post-treatment of cells with 30% glycerol stimulated plaque formation 10- to 50-fold. The following post-treatments failed to consistently enhance plaque formation: polyethylene glycol (40%, mol. wt. 6000), lysolecithin (10 to 80 μg/ml), CaCl₂ (125 mM), or dimethyl sulphoxide (10 to 20%). Centrifugation of the vesicles directly onto the cell monolayer (2000 g, 5 min) also had no effect on plaque number.

Since the addition of carrier DNA greatly enhances viral DNA infectivity using the calcium phosphate technique (Graham & van der Eb, 1973), it was of interest to examine what effect carrier DNA would have on liposome-mediated DNA transfer. Liposomes were therefore prepared with a constant amount of SV40 DNA and increasing concentrations of carrier salmon sperm DNA. The inclusion of a 2- or 20-fold excess of carrier DNA over SV40 DNA inhibited plaque formation by 51% and 75%, respectively. The liposome preparations contained vesicles quite heterogeneous in size, with the largest vesicles up to several microns in diameter. Liposomes can be sized by extrusion through polycarbonate membranes having a uniform pore diameter (Olson et al., 1979). Extrusion
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Fig. 3. Evidence that infectivity is due to SV40 DNA. SV40 DNA (5 μg) was digested with the following enzymes: none (lane 1); Proteinase K, 50 μg/ml (lane 2); HindIII, 25 units (lane 3); EcoRI, 25 units (lane 4); XbaI, 25 units (lane 5). Aliquots of DNA were electrophoresed in a 1.2% agarose gel and stained with ethidium bromide. The remaining DNA was entrapped in liposomes and tested for infectivity. Symbols to the left of the gel indicate the positions of forms I, II, and III SV40 DNA.

Table 1. Effect of endocytosis modulators on infectivity of liposome-entrapped SV40 DNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Plaques (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100*</td>
</tr>
<tr>
<td>Colcemid</td>
<td>1 μg/ml</td>
<td>83</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>10 μg/ml</td>
<td>38</td>
</tr>
<tr>
<td>Monodansylcadaverine</td>
<td>100 μM</td>
<td>52</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>10 mM</td>
<td>51</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.05 μg/ml</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>0.5 μg/ml</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>5.0 μg/ml</td>
<td>135</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>5.0 μg/ml</td>
<td>90</td>
</tr>
</tbody>
</table>

* Control standard deviation ± 9%.

through membranes of pore diameter 3.0 μm and 1.0 μm resulted in no significant alteration in efficiency of plaque formation by negatively-charged DNA liposomes (data not shown). Thus, vesicles greater than 1 μm in diameter were not exclusively responsible for DNA transfer.

In order to determine whether endocytosis rather than liposome–cell membrane fusion was involved in liposome-mediated DNA transfer, cells were treated with various modulators of endocytosis some time before, and during, liposome incubation. As shown in Table 1, several inhibitors of endocytosis (cytochalasin B, monodansylcadaverine) or of phagosome–lysosome fusion (NH₄Cl) substantially but not completely reduced plaque formation. Bovine serum albumin or colcemid, which have little effect on endocytosis, were poor inhibitors of plaque formation. Fibronectin, a stimulator of endocytosis, increased plaque formation by 35% at the highest concentration tested (5 μg/ml).

As shown in Fig. 4, plaque formation by liposome-entrapped SV40 DNA was dose-dependent. The maximal number of plaques was produced when approximately 20 to 80 nmol of lipid was added per dish. In some experiments, plaque formation was inhibited at greater than
800 nmol lipid/dish. The maximal frequency of plaques was clearly dependent on the DNA concentration per liposome; liposomes containing higher amounts of DNA were more infective than those with lower amounts of DNA. Moreover, the absolute infectivity as determined from the linear portion of the dose–response curve was also directly related to liposome DNA concentration from 34 to 570 ng SV40 DNA/μmol lipid (Fig. 5). However, when expressed in relation to the amount of DNA added, there was a striking uniformity in infectivity when comparing liposome preparations containing at least 10-fold differences in DNA concentration. That is, all liposome preparations gave approximately 10⁵ plaque-forming units/μg DNA.

### Liposome-mediated transfer of the SV40 minichromosome

Within the intact SV40 virion the viral DNA is complexed with nucleosomes consisting of hyperacetylated histones H2a, H2b, H3 and H4 (Meinke et al., 1975). It was of interest to determine whether this nucleoprotein complex would be more efficient in liposome-mediated DNA transfer than naked DNA. Accordingly, the SV40 minichromosome was isolated by virion dissociation (Christiansen et al., 1977) and entrapped in liposomes. After liposome solubilization with Triton X-100, this minichromosome showed the same typical micrococcal nuclease digestion pattern as unentrapped minichromosome indicating that liposome-

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**Table 2. Infectivity of liposome-entrapped SV40 minichromosome**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Plaque-forming units per μg DNA (× 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free minichromosome</td>
<td>0.6</td>
</tr>
<tr>
<td>Free minichromosome + DNase*</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Liposome-entrapped minichromosome†</td>
<td>12</td>
</tr>
<tr>
<td>Liposome-entrapped DNA†</td>
<td>10</td>
</tr>
</tbody>
</table>

* 50 μg/ml DNase I.
† Treated with 50 μg/ml DNase I after liposome formation.

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**Fig. 4.** Dose-dependent relationship between the amount of vesicle lipid added and number of plaques. Each point represents the number of plaques obtained using liposome preparations containing 34 (○), 138 (□) or 570 (△) ng SV40 DNA/μmol lipid. Cells were post-treated with 30% glycerol.

**Fig. 5.** Relationship between liposome DNA concentration and infectivity of liposome-entrapped SV40 DNA. Each point represents the calculated infectivity obtained from the linear portion of infectivity curves, similar to those in Fig. 4, at 1 nmol lipid/dish.
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entrapment did not appreciably alter the basic chromatin structure (data not shown). As shown in Table 2, liposome-entrapped SV40 minichromosome was 20-fold more infective than unentrapped minichromosome. However, liposome-entrapped minichromosome was only 20% more infective than liposome-entrapped SV40 DNA. Thus, the presence of nucleosomes and VP3 on SV40 DNA had little effect on the efficiency of liposome-mediated DNA transfer.

DISCUSSION

We have demonstrated that SV40 DNA and minichromosome can be entrapped in liposomes, transferred to mammalian cells and subsequently expressed. The technique of liposome preparation we used, reverse-phase evaporation (Szoka & Papahadjopoulos, 1978), has been reported to produce large, unilamellar vesicles. These entrap greater quantities of input DNA than multilamellar vesicles (Hoffman et al., 1978; Lurquin, 1979) or unilamellar vesicles produced by other methods (Dimitriadis, 1979). Additionally, DNA molecules as large as bacteriophage lambda (approx. 50 kb) are entrapped unsheared into these liposomes and with an efficiency as great as the much smaller SV40 DNA molecule (5.2 kb).

In our experiments, liposome-entrapped DNA was protected from digestion with deoxyribonucleases. We also observed that the lipid composition of the SV40 DNA vesicles had a marked effect on infectivity. Neutral liposomes composed only of phosphatidylcholine and cholesterol were ineffective compared to negatively-charged vesicles composed of the same lipids with phosphatidyserine. Other negatively-charged lipids have been reported to be less efficient than phosphatidyserine for enhancing liposome-mediated DNA transfer (Fraley et al., 1980) possibly because phosphatidyserine-containing vesicles fuse with cell membranes more readily than do vesicles composed of other lipids (Poste et al., 1980).

The detailed mechanism of liposome-mediated DNA transfer is not yet fully established. Liposomes are believed to interact with cells in several ways: endocytotic uptake, direct fusion, and stable adsorption onto the plasma membrane (Pagano & Weinstein, 1978). Although modulators of endocytosis influenced the frequency of plaque formation in our experiments, direct fusion of liposomes with the plasma membrane also remains a possible, perhaps major, mechanism for DNA entry into the cells.

The inclusion of carrier salmon sperm DNA in liposomes containing SV40 DNA inhibited plaque formation in a roughly dose-dependent manner. Since it is unlikely that carrier DNA would alter vesicle fusion or uptake, it seems more probable that some intracellular process is being affected. If cellular deoxyribonuclease activity were a major factor limiting SV40 DNA expression, the presence of competing carrier DNA should either enhance or have no effect on the probability of SV40 DNA expression due to competition for nuclease active sites. Although alternate explanations exist, intact SV40 DNA molecules may fail to reach the nucleus in the presence of competing DNA molecules, or carrier DNA, once inside the nucleus, may compete with SV40 DNA for the host transcription system.

The frequency of plaque formation was dependent on both the number of vesicles added per dish and the DNA content per vesicle. The presence of saturation kinetics for lipid concentration implies that there is a rate-limiting process which most likely reflects either vesicle binding to the cell surface or vesicle internalization. A direct relationship between virus DNA content per vesicle and vesicle infectivity was found. No evidence of saturation of this process was observed with the amounts of DNA used. The data suggest that the DNA molecule has a characteristic probability, less than unity, of ultimately being expressed after transfer into the cell, but the rate of DNA entry into the cell is a major limitation of frequency of plaque formation.

The liposome-mediated transfer of a soluble chromation complex has not been previously reported. Surprisingly, the SV40 minichromosome was not appreciably more infective than SV40 DNA after entrapment in liposomes. We conclude that the histones and viral capsid protein VP3 which are present in this minichromosome preparation (Christiansen et al., 1977) have little direct effect on the frequency of liposome-mediated DNA transfer. It is yet to be determined whether transcription of the transferred minichromosome and DNA is similar.

The specific DNA infectivity achieved using liposome-mediated DNA transfer (10^5 plaque-
forming units/µg DNA) is similar to the value obtained in another study using liposomes with 30% glycerol post-treatment (Fraley et al., 1980). It compares favourably with that reported using the calcium phosphate technique (Graham & van der Eb, 1973) which requires the use of carrier DNA. Although higher specific DNA infectivity may be obtained using the techniques of direct microinjection or hybrid virus vectors, the liposome method may prove more useful since it is simple, requires no elaborate equipment, and can be used to treat a large number of cells. Moreover, since it provides the versatility to transfer nucleoprotein complexes as well as naked DNA, the liposome method may allow one to examine the biological effects of nucleic acid–protein interactions. With liposomes, still higher infectivity may be achievable by increasing vesicle binding to the cell surface through the use of antibody-coated vesicles (Leserman et al., 1981) or lectin-mediated binding (Szoka et al., 1981).

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