DNA, isolated from Simian virus 40 (SV40), has been encapsulated in large (0.4-μm diameter) unilamellar phospholipid vesicles. The procedure used for liposome preparation encapsulated the SV40 DNA at high efficiency (30 to 50% entrapment) and did not alter the physical or biological properties of the DNA molecules. The biological activity of the liposome-entrapped viral DNA was determined by plaque assays on a permissive monkey cell line. The infectivity of liposome-entrapped SV40 DNA was enhanced at least 100-fold over that of free naked DNA. Importantly, the infectivity of vesicle-entrapped DNA was resistant to DNase digestion, dependent on the amount of DNA encapsulated per vesicle and on the vesicle lipid composition. Liposomes composed of phosphatidylserine were the most efficient for delivery of DNA to cells (1.8 × 10⁶ plaque-forming units/pg of DNA). Following the incubation of DNA-containing liposomes with cells, their infectivity could be enhanced an additional 10- to 200-fold by exposing the cells to high concentrations of polyethylene glycol or glycerol. Under these conditions the infectivity of liposome-encapsulated SV40 DNA (3 × 10⁶ plaque-forming units/pg) was comparable with values reported using the calcium phosphate method. In addition to providing a sensitive assay for monitoring and optimizing the delivery of vesicle contents to cells, the liposome-mediated delivery of nucleic acids may have potential for increasing the efficiency of DNA delivery to cells and for extending the number of cell types which can be transformed or transfected.

Recent demonstrations of the entrapment of biologically active RNA molecules within liposomes (1-4) have suggested that phospholipid vesicles may have considerable utility as vehicles for introducing nucleic acids into cells. The logical extension of the work described above is to the area of DNA delivery to cells. Although several studies have reported on the encapsulation of DNA within liposomes (5,6), the criterion of maintenance of biological activity has been met in only a few cases. Mukherjee et al. (7) reported that lipochromosomes were transferred to cells at 10-fold higher efficiency than naked chromosomes; however, the nature of the association of the chromosomes with lipid vesicles was not clear in this study. Fraley et al. (8) encapsulated the Escherichia coli plasmid, pBR322, in phospholipid vesicles and found that incubation of the liposome-encapsulated pBR322 DNA with *E. coli* cells in the presence of high levels of DNase resulted in the appearance of tetracycline-resistant colonies; under these conditions transformation by free DNA was negligible.

Apart from the obvious genetic implications of liposome-mediated DNA delivery to cells, it should be emphasized that the expression of foreign DNA in cells would provide an unequivocal and sensitive biological assay for monitoring the delivery of liposomal contents to cells. In an attempt to develop such an assay and to examine the potential of liposomes to deliver DNA to mammalian cells, we have encapsulated the DNA from SV40 (9-12) together with its potential for use as a transducing vector make it an attractive model system for study.

**MATERIALS AND METHODS**

**Lipids and Other Materials**—The sources, purity, and storage of the phospholipids used in this study have been reported previously (13). [3H]Poly(A) (25 to 89 Ci/mol) was obtained from Amersham. [3H]Poly(A) was a gift of Dr. L. Rall (University of California, San Francisco). DEAE-dextran (M, = 50,000) was purchased from Pharmacia. Polyethylene glycol (M, = 1,500) was obtained from BDH Chemicals, Ltd. (Poole, England). All other chemicals were of the highest purity available.

**Preparation of Phospholipid Vesicles**—REV liposomes were prepared by the method of Soska and Papahadjopoulos (14) with the following modifications for DNA encapsulation. Phospholipid (10 μmol) was added to a screw-cap test tube (13 × 100 mm, with Teflon liner) and the solvent was removed under reduced pressure by rotary evaporation. The lipid was redissolved in 1.0 mL of diethyl or isopropyl ether. These solvents were redistilled over sodium bisulfite immediately before use to remove any peroxides. 0.33 mL of sterile aqueous buffer (2 mM N-[Tris(hydroxymethyl)methyl]-2-amino]ethanesulfonic acid, 2 mM L-histidine, 100 mM sodium chloride, 0.1 mM EDTA (pH 7.4)) containing 1 to 100 μg of purified SV40 DNA and 0.1 μCi of either [3H]-labeled pBR322 or [3H]poly(A) was added and the resulting two-phase system was sonicated briefly (10 to 15 s) by placing tubes in a bath-type sonicator (Laboratory Supplies, Long Island, N. Y.) to form a one-phase dispersion. The mixture was placed on a rotary evaporator and the ether was removed under reduced pressure as previously described (14).

The REV liposomes were sized to 0.4 μm by passage through a 0.4-μm Unipore filter as described by Olson et al. (15). The liposome-encapsulated SV40 DNA was separated from unencapsulated material by flotation on Ficoll gradients. The REV preparation (0.5 mL) was mixed with 1.0 mL of 30% (w/v) Ficoll (in the above buffer) and transferred to a sterile cellulose nitrate centrifuge tube (Beckman SW 50.1 rotor). Three milliliters of 10% (w/v) Ficoll and 1.0 mL of buffer

1 The abbreviations used were: SV40, Simian virus 40; PS, phosphatidylserine; REV, reverse evaporation vesicle; MeSO₃, dimethyl sulfoxide; PEG, polyethylene glycol; pfu, plaque-forming unit.
were gently layered consecutively onto the sample and the step gradient was centrifuged (30 min at 100,000 x g). The liposome-entrapped DNA was banded at the interface between buffer and 10% Ficoll and was well separated from unentrapped DNA, which remained in the densest Ficoll fraction. The vesicle band was collected and analyzed for lipid phosphate (16) following lipid extraction (17). DNA encapsulation was determined by monitoring an aliquot (50 μl) for radioactivity in a Triton/toluene-based scintillant. Typically, 30 to 40% of the applied SV40 DNA and 80 to 90% of the phospholipid were recovered at the 0/10% Ficoll interface. Prior treatment of the DNA-containing vesicles with 20 to 50 μg of DNase did not alter the recoveries on the gradients, indicating that the vesicle-associated SV40 DNA was entrapped within the liposomes and was not accessible to DNase digestion. Control experiments in which buffer-loaded PS vesicles were mixed with SV40 DNA prior to centrifugation indicated that DNA does not bind to the vesicle surface.

Cell Culture and Preparation of SV40 DNA—CV-1P, an established cell line of African green monkey kidney cells, were cultured as described by Mertz and Berg (18) using Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. Viral DNA was obtained from CV-1 cells infected at low multiplicity with wild type SV40 virus. The viral DNA was extracted (19) and purified by CaCl2-ethidium bromide centrifugation as previously described (18).

Plaque Assays—Plaque assays using purified viral DNA were performed as described by Mertz and Berg (18). The infectivity of the SV40 DNA in the presence of DEAE-dextran was typically 5 x 105 pfu/μg of DNA, and the reproducibility of this value was within 30% from experiment to experiment. Incubations of cells with liposome-entrapped SV40 DNA were carried out in a similar manner. Tris-buffered saline (0.2 ml) (20), containing between 0.1 and 1 μg of SV40 DNA and 0.93 to 400 nmol of phospholipid, was added to the washed monolayers and the plates were incubated for 30 min at 37°C. Increasing the incubation period did not further enhance infectivity. Following the incubation, the monolayers were overlaid with 1.0 ml of Tris-buffered saline containing 0 to 40% (w/v) glycerol for 4 min at 25°C. The monolayers were then washed twice with Tris-buffered saline and overlaid with agar medium as above.

RESULTS

Encapsulation of SV40 DNA in Liposomes—A variety of different methods have been used for the encapsulation of nucleic acids in phospholipid vesicles (1-8). While these methods have succeeded in entrapping nucleic acids in liposomes, they suffer from several experimental disadvantages including: 1) low efficiency of encapsulation, 2) the requirement for large amounts of RNA or DNA, and 3) the limitation of the procedure to one type of phospholipid. These deficiencies become important considerations when attempting to encapsulate small quantities of valuable DNA or when examining the influence of vesicle lipid composition on cellular delivery.

In this respect, the reverse-phase evaporation (REV) procedure (14) appears well suited for the encapsulation of DNA molecules. This method produces large (0.4- to 0.5-μm diameter) unilamellar liposomes which can encapsulate 40 to 50% of the aqueous buffer, importantly, high molecular weight compounds are also encapsulated at these high efficiencies. In addition, REV liposomes can be made from nearly all phospholipids or lipid mixtures and the procedure can be scaled down for using small aqueous volumes (0.05 ml) when sample availability is limited.

Since we have successfully used large unilamellar PS vesicles made by the Ca2+-EDTA chelation procedure to deliver entrapped poliovirus (21) or polio RNA (3) to cells, our initial studies with SV40 DNA were also performed with liposomes made from PS. Liposomes containing SV40 DNA were separated from free DNA by flotation on discontinuous Ficoll gradients as described under "Materials and Methods." These gradients provide a rapid and quantitative separation of vesicles from DNA (and other large macromolecules) that is not easily achieved by molecular sieve chromatography or velocity centrifugation.

The encapsulated SV40 DNA was extracted from PS vesicles and the infectivity of the liposome-encapsulated SV40 DNA was determined by plaque assays on CV-1P cells and was compared to control SV40 DNA (Table I). The infectivity of control and liposome-extracted SV40 DNA, using the DEAE-dextran procedure, were identical (lines 1 and 2). This result was consistent with those obtained from agarose gel electrophoresis in which the profile of the liposome-extracted SV40 DNA was indistinguishable from control DNA. The incubation of 10 ng of liposome-encapsulated SV40 DNA with CV-1P cells resulted in a low, but detectable, frequency of infection (1.5 x 103 pfu/μg, line 3). In contrast, naked SV40 DNA was generated.

### Table I

<table>
<thead>
<tr>
<th>DNA preparation</th>
<th>DNA*</th>
<th>DNase*</th>
<th>pfu/μg of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Control DNA (DEAE-dextran)</td>
<td>0.01</td>
<td>10×</td>
<td>5.0 x 10²</td>
</tr>
<tr>
<td>2) Extracted DNA (DEAE-dextran)</td>
<td>0.01</td>
<td>10×</td>
<td>4.3 x 10²</td>
</tr>
<tr>
<td>3) Liposome-encapsulated DNA</td>
<td>10</td>
<td>10×</td>
<td>1.3 x 10²</td>
</tr>
<tr>
<td>4) Free DNA</td>
<td>100</td>
<td>10×</td>
<td>&lt;10³</td>
</tr>
<tr>
<td>5) Free DNA + empty liposomes</td>
<td>100</td>
<td>10×</td>
<td>&lt;10³</td>
</tr>
</tbody>
</table>

* The amount of SV40 DNA added per plate.
+ Indicates incubations that were performed in the presence of DNase I (10 μg/plate).
+ The infectivity of various DNA preparations.

![Fig. 1. Effect of glycerol concentration on the enhancement of SV40 DNA infectivity. Following incubation with free or encapsulated DNA, the monolayers were treated for 4.0 min with 1.0 ml of a glycerol solution (0 to 40%) (w/v) in Tris-buffered saline buffer, a, infectivity of SV40 DNA (0.001 or 0.01 ng added) using the DEAE-dextran procedure; b, infectivity of SV40 DNA encapsulated in PS:cholesterol liposomes (0.35 μg of DNA encapsulated/μmol of phospholipid, 0.1 to 10 ng added); and c, infectivity of naked SV40 DNA (10 to 100 ng added).](image-url)
Liposome-mediated Delivery of DNA to Cells

DNA (100 ng) was not infectious (<10 pfu/µg, line 4) when incubated with cells under the same conditions. Adding buffer-loaded vesicles to naked SV40 DNA (106 ng) did not increase its infectivity (line 5). The infectivity of the liposome-encapsulated SV40 DNA was not reduced by the addition of DNase during the incubation (line 3), whereas the infectivity of free DNA (extracted from the liposomes) using the DEAE-dextran method was very sensitive to DNase digestion (line 1 and 2).

Optimization of Liposome-mediated Delivery of SV40 DNA — Although the SV40 DNA encapsulated in PS vesicles was at least 2 orders of magnitude more infectious than free DNA, the delivery via liposomes was still much less efficient (~0.1%) than the DEAE-dextran method. Since a number of different vesicle lipid compositions have been reported to promote liposome-mediated delivery of molecules to cells (22), we examined whether vesicles formed from other phospholipids or lipid mixtures would be more efficient in delivering SV40 DNA to cells than pure PS vesicles. Vesicles made from egg phosphatidylcholine, a neutral phospholipid, were at least 10-fold less efficient than the negatively charged PS vesicles in promoting DNA delivery. Including 10 mol % stearylamine in the egg phosphatidylcholine vesicles, which imposes a positive surface charge, lowered infectivity even further. Surprisingly, the infectivity of SV40 DNA encapsulated in a variety of other negatively charged lipid vesicles, including those composed of phosphatidylglycerol, azolectin, or dicetyl phosphate, was much lower (3–40-fold) than observed for PS vesicles. It should be noted that the relative encapsulation of SV40 DNA in these various preparations was similar, so that the large differences observed in infectivity must reflect differences in the extent of cell binding or delivery. Including 50 mol % cholesterol in the PS vesicles resulted in a 50% enhancement in infectivity which probably reflects a decrease in the extent of cell-mediated vesicle leakage compared to pure PS vesicles.2 PS-cholesterol vesicles were used in all subsequent studies to further optimize DNA delivery because of their reduced leakiness and superior storage properties (DNA-containing liposomes have been stored under argon at 4°C for up to 8 weeks without detectable loss of infectivity).

A variety of agents or incubation conditions (3, 5, 23–28) have been used to enhance the extent of vesicle fusion or to promote the uptake of DNA into cells. Several of these treatments were tested to determine their effectiveness in enhancing the infectivity of liposome-encapsulated SV40 DNA. The most successful of these treatments was washing the cells with either Me₃SO (25%, 4 min), PEG (44%, 90 a), or glycerol, following the 30-min incubation of vesicles with cells, which increased SV40 infectivity an additional 10- to 200-fold. We were unable to enhance DNA delivery by increasing the concentration or length of exposure to either Me₃SO or PEG; however, increasing the glycerol concentration in the Tris-buffered saline buffer markedly increased infectivity (Fig. 1). Following a 40% glycerol wash, the infectivity of the SV40 DNA-containing liposomes was ~2 to 3 × 10⁵ pfu/µg. The glycerol treatment also produced an increase in the infectivity of SV40 DNA (Fig. 1) which has never been reported before; however, only slight (~2-fold) enhancement was found with the DEAE-dextran procedure. It should be noted that at the higher glycerol concentrations (35 to 40%), depending on the age and confluency of the monolayers, cells were occasionally observed to detach from the plates.

The effect of altering the DNA content of the liposomes on SV40 infectivity was examined (Fig. 2) using liposome preparations with varying ratios (0.002 to 2) of DNA molecules/vesicle. It is apparent that the number of plaque-forming units/plate increases linearly with increasing concentrations of added liposomes. The saturation in the curves, occurring between 50 and 100 nmol of added phospholipid, was also observed for liposome-encapsulated polio RNA (3). This observation suggests that above this lipid concentration (vesicle/cell ratio = 10⁶), the liposomes have saturated all the available binding sites on the cell surface. As was also observed for polio RNA encapsulated in liposomes (3), the specific infectivity of the liposomes (plaque-forming units/µmol of phospholipid) increases with higher ratios of DNA/vesicle (Fig. 2, inset); this result emphasizes the need for a highly efficient encapsulation procedure in order to minimize the competition by “empty” liposomes. The fact that plaques can be observed at 0.1 nmol of added phospholipid (1 to 2 vesicles/cell, assuming 10 to 20% of the liposomes bind to cells) suggests that a single DNA molecule can initiate infection, albeit at a low probability.

DISCUSSION

A previous study from this laboratory has demonstrated that large unilamellar PS vesicles are capable of encapsulating poliovirus RNA and delivering it efficiently to both primate and nonprimate cells (3). The biological activity of the liposome-encapsulated polio RNA was high (1 to 2 × 10⁶ pfu/µg) and the data suggested that infection could be initiated by a single RNA molecule. A logical extension of these experiments is the encapsulation of DNA molecules in liposomes. As is the case with polio RNA, the encapsulation of SV40 DNA provides a sensitive and unambiguous assay system for monitoring the introduction of liposomal contents into cells, thereby providing an excellent model system for studying and optimizing the interaction of liposomes with cultured cells. In addition, the delivery of DNA molecules by liposomes offers the potential for increasing the efficiency of DNA delivery to cells as well as extending recombinant DNA technology to cells which are inefficiently transfected using current techniques for DNA delivery.

The infectivity of the liposome-encapsulated SV40 DNA in

---

2 R. Fraley, R. Straubinger, and D. Pašahadipoupolos, manuscript in preparation.
this study is ~100-fold less efficient than was found for encapsulated polio RNA under similar conditions (Ref. 3 and Footnote 2). This large difference may reflect in part the additional requirement of delivery to the nucleus in the case of SV40 DNA in order for it to replicate and register as a "positive" in our assay. The lower infectivity of SV40 DNA encapsulated in liposomes (compared to polio RNA in liposomes) could also be due to fewer cells being infected, especially since different cell lines have been used in the two studies. A variety of phospholipid compositions, incubation conditions, and putative fusogenic agents were examined for their ability to increase SV40 infectivity. The most successful combination was DNA entrapment in PS vesicles containing 50 mol % cholesterol and postincubation exposure to high concentrations of glycerol which increased infectivity 200-fold (up to 1 to 3 \times 10^7 pfu/µg). It should be emphasized that while these values for infectivity are less than can be obtained using the DEAE-dextran procedure, they are comparable or better than values for SV40 DNA infectivity reported using the calcium phosphate technique (20). It should also be noted that the large differences we have observed between various liposomes with and without glycerol have not been reported before in the area of liposome-cell interactions.

Despite the fact that glycerol, sucrose, or Me2SO treatments have been shown to stimulate DNA infectivity or expression in a number of cell lines (24, 26), very little is known regarding their mechanism of action. No increases are detected in the extent of vesicle binding to cells following the glycerol wash, which indicates that the enhancement must be due to an increased efficiency of DNA delivery or expression. However, since a similar enhancement by glycerol is observed in the infectivity of liposome-encapsulated polio RNA, the latter possibility is unlikely as is the possibility that the glycerol treatments may be promoting simply delivery to the nucleus. While high concentrations of glycerol are known to enhance membrane fusion (30), the observation that the infectivity of naked SV40 DNA was also increased to a similar degree by the glycerol treatments suggests that a mechanism involving a stimulation of endocytosis and/or an increase in membrane permeability (either plasma or lysosomal) should also be considered. It must be emphasized that the majority of the liposomes which interact with cells remain absorbed to the cell surface (22, 31); therefore, treatments which increase the chance for internalization of these vesicles would be expected to greatly enhance delivery. At high concentrations of added liposomes (~10^6 vesicles/cell), both vesicle binding and plaque formation are observed to plateau (Fig. 2). The saturability of infection or transformation with increasing DNA concentrations has been thought to be due to a competence effect whereby only a subpopulation of the cells is capable of taking up or processing DNA (32). It is interesting to note, however, that the apparent level of saturation observed with other carrier systems, such as calcium phosphate, can be substantially increased by modifying the properties of the carrier DNA complex (32, 34). Our own results show that the final level of infectivity at saturation depends critically on the specific DNA content of the liposomes and that increasing the number of DNA molecules/liposome results in a higher value for saturation. These results support an earlier study using microinjection techniques (35) that indicated that the frequency of SV40 DNA infection or transformation is proportional to the quantity of DNA molecules introduced into the cell and are more consistent with the interpretation that all cells may become "competent" when a sufficient number of DNA molecules are delivered.

Since only a small fraction of the added liposomes bind to cells under our incubation conditions, subsequent attempts to increase liposome-mediated delivery of encapsulated molecules (and SV40 DNA infectivity) will be directed at increasing the amount of liposomes bound to the cell. This may be achieved through the covalent attachment to liposomes of antibody molecules which bind specifically to cellular antigens (36). Alternatively, the intrinsic infectivity of DNA may be altered by complexing it with basic proteins or polycations prior to liposome encapsulation. Importantly, the use of liposomes as DNA carriers affords the flexibility to undertake these experimental approaches, whereas other commonly used techniques for gene delivery (e.g., calcium phosphate and polycations) do not. The SV40 system described here will be extremely useful as a model system for further optimizing conditions for maximal delivery of DNA and other molecules to mammalian cells. We are optimistic that the efficiency of liposome-mediated DNA delivery to cells can be optimized further to provide a useful alternative to other existing techniques in even the most efficient cell systems. Presently, an important application of liposomes as vehicles for introducing DNA into cells may be found in those situations where standard techniques for gene delivery are either inefficient or inapplicable, e.g., suspension cells, in vivo gene delivery, plant cells, etc.; under these conditions, even a low efficiency of DNA delivery mediated by liposomes would be extremely useful. In this respect, our results using liposomes to deliver the large tumor-inducing Agrobacterium tumefaciens plasmid to Nicotiana protoplasts indicate that liposomes will have considerable value in the field of plant cell genetics.\(^3\)

Acknowledgment—We thank Dr. L. Rall for help in the preparation of this manuscript.

REFERENCES

5. Lurquin, P. F. (1979) Nucl. Acids Res. 6, 3773-3784

---

Liposome-mediated Delivery of DNA to Cells