rent flow through these areas. It is even possible that this small current represents interference from current flow through chloride cells (see above). In any case, it is clear that nonchloride cells offer high resistance to ionic flow. In contrast, chloride cells represent sites of much higher conductance. The rectified nature of the chloride cell *I/V* relationship is similar to that observed for the entire opercular membrane (*8*), demonstrating that *I/V* characteristics of chloride cells determine these parameters for the entire tissue. The chloride cell conductance ($G^{cc}$) begins to decrease at $-20 \pm 3$ mV ($N$ = five tissues) and $+76 \pm 10$ mV ($N = 5$). These voltage-dependent decreases of $G^{cc}$ may be caused by conductance or transport pathways that are voltage-sensitive or saturable, or both. However, their exact cause and anatomical location are unknown. Probe-measured $G^{cc}$ from five cells in five tissues, determined from the slopes of the *I/V* relationships over the linear range, are similar for 2- and 3-week seawater-adapted fish, 0.05 and 0.04 microsiemens (μS), respectively. These values compare well with indirect estimates based on total tissue conductance and the total number of chloride cells, that is 0.09 and 0.15 μS for 2- and 3-week seawater-adapted fish, respectively (*7*). Adaptation of fish to seawater for 7 weeks appears to result in an enhancement of probe-measured $G^{cc}$ (0.35 to 1.14 μS).

We have provided direct evidence that chloride cells are the only significant electrogenic and conductive elements in the tilapia opercular membrane. The large negative current densities observed over chloride cells represent net chloride extrusion by these cells under short-circuit conditions since transepithelial $I_{sc}$ appears to be carried exclusively by chloride (*7*). The basis of the branchial salt extrusion mechanism is also an active chloride secretion process (*10*). Nonchloride cells do not appear to be involved in net electrogenic ion transport and, in view of their high resistance, probably serve as relatively impermeable barriers to passive conductive ionic flow. These results validate the cumulative correlative data that have strongly implicated the chloride cell as the one responsible for branchial salt secretion (*1*, *2*). Furthermore, it is now clear that Ussing chamber studies of opercular membrane electrophysiology under the conditions of our experiments represent electrophysiological studies of chloride cell biology. The vibrating probe technique should have successful application in other epithelia in which the heterogeneous nature of the cell types comprising the epithelium has made the assignment of transport functions ambiguous.

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## Liposomes as Gene Carriers: Efficient Transformation of Mouse L Cells by Thymidine Kinase Gene

Abstract. *Stable transformation of mouse L cells deficient in thymidine kinase was achieved by liposome-mediated transfer of a recombinant plasmid carrying the thymidine kinase gene. Ten percent of the recipient cells expressed thymidine kinase activity. The transformed phenotype (for example, 200 out of $10^6$ cells) was stable under selective and nonselective conditions. The liposome technique is compared with other methods currently used for gene transfer.*

A variety of techniques for introducing nucleic acids into eukaryotic cells in vitro has been developed. These techniques include incubation of the recipient cells with coprecipitates of DNA and calcium phosphate (*1*); direct injection of genes into the nucleus of the recipient cell (*2*); and use of viral vectors to carry genes into cells (*3*). Because these methods have some limitations, the use of liposomes as vehicles for gene transfer has been investigated. Liposomes could offer certain advantages, such as simplicity, low toxicity, and higher efficiency, and they could, perhaps, be used in vivo.

Recently, viral SV40 DNA (*4*) and a prokaryotic gene coding for β-lactamase (*5*) were introduced into mammalian cells by liposomes and their expression has been shown. However, SV40 DNA is replicated extrachromosomally in permissive cells, and it is not known whether the liposome-mediated transfer of eukaryotic genes will lead to stably transformed cell lines. We have studied this question and have further evaluated the liposome method of gene transfer using the thymidine kinase gene as a model system. The transfer of this gene to mutant mouse cells deficient in thymidine kinase was previously accomplished with the calcium phosphate method (*6*). A newly acquired thymidine kinase activity can be assayed immediately, the transformed cells can be selected by a special culture medium, and the covalent integration of the TK gene in chromosomal DNA is a prerequisite for stable expression (*7*). We report here the liposome-mediated delivery of the TK gene to $LTK^-$ cells and the stable expression of thymidine kinase in transformants. We found that the efficiency of transformation is comparable to that of other transfection methods.

For the preparation of DNA-loaded liposomes (*8*) we used the method of reverse phase evaporation, which yields large unilamellar vesicles with high efficiency of entrapment (*9*). As one of the lipid components we chose phosphatidylserine, since a high percentage of negatively charged phospholipid favors the binding capacity of liposomes to cells and their cellular uptake (*10*); as another component we used cholesterol, since a high content of cholesterol increases the size of the vesicles (*11*), reduces liposomal leakage, and stabilizes the vesicles in the presence of serum proteins (*12*). Short sonication of the two-phase system of lipid-ether and DNA-buffer produces inverted micelles which, by evaporation of ether, reassemble into lipo-


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somes. Negative stain electron micrographs showed more than 90 percent of the lipid in the vesicle suspension to be in the form of (predominantly unilamellar) particles, 400 to 700 nm in diameter.

The recombinant plasmid, pAGO, carrying an active TK gene (2 kb of the Pvu II fragment of HSV I) inserted in the Pvu II site of pBR322 (*13*) was used for transformation experiments. To determine the entrapment efficiency, we labeled the DNA to be encapsulated with $^{32}P$ (*14*). The liposome suspension was digested with deoxyribonuclease I to degrade nonentrapped DNA, and the liposomes were separated from digested DNA by chromatography on Sepharose 4B. The radioactivity of the DNA entrapped in the liposomes was then monitored. Routinely, we achieved entrapment efficiencies of 10 to 20 percent. To check that the encapsulated DNA was intact, we disrupted the liposome fraction isolated after chromatography with sodium dodecyl sulfate. We found that the liberated DNA was identical in its electrophoretic profile with authentic DNA.

In most experiments the ratio of DNA to lipid was 2 μg of entrapped pAGO per micromole of phospholipid. We used this ratio on the basis of the calculation that at least one DNA molecule should be present per vesicle. The DNA-loaded liposomes were stable for at least 4 weeks in the cold. For transfer, a portion of the vesicle suspension was incubated with LTK$^-$ cells (*15*). The thymidine kinase activity present in the transformed cells was determined 24 hours later by measuring the capacity of the cells to incorporate [$^3$H]thymidine into nuclear DNA using autoradiographic analysis (Fig. 1) (*16, 17*). We routinely found that approximately $10^5$ cells, that is, 10 percent of the total cells, were TK-positive when 500 ng of entrapped DNA per dish or $10^6$ cells, respectively, were added. When cells were incubated with 500 ng of free DNA per dish, only a few positive cells were detected by this assay.

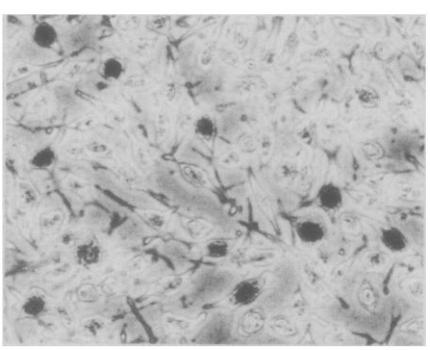

Fig. 1. Autoradiographic analysis of thymidine kinase activity present in LTK$^-$ cells after treatment with pAGO-loaded liposomes. Liposomes prepared from 250 nmole of phospholipids encapsulating 500 ng of pAGO DNA were added to $10^6$ LTK$^-$ cells (*8, 15, 16*). For the calculation several thousand TK$^+$ cells were counted in ten different areas of the dish.

Table 1. Efficiency of different transfection methods. Liposome-mediated transfection is described in the text; liposomes prepared from 250 nmole of phospholipids encapsulating 500 ng of pAGO DNA were added to $10^6$ LTK$^-$ cells (*8, 15*). The calcium phosphate method was carried out as described by Wigler and co-workers (*6, 7*) with 50 ng of pAGO per $10^6$ cells. The TK$^+$ cells were detected by autoradiographic analysis. Colonies were counted after 10 to 12 days in HAT medium.

| Methods | Percentage of TK$^+$ cells | Colonies per $10^6$ cells |
|---|---|---|
| Liposomes | 10 | 200 |
| Calcium phosphate | 3 | 500 |
| DEAE-dextran | 0.1 to 1 | 0 |
| Microinjection | 50 to 100 | (200 to 1000)* |

*Data were calculated from (*19*) for $10^6$ cells.

Figure 2 shows the transformation efficiency when the amount of DNA per vesicle was kept constant. If more than $2 \times 10^4$ vesicles per cell were added (that is, > 500 ng of pAGO per $10^6$ cells) the efficiency reached a maximum. With a lower (one-tenth) DNA to vesicle ratio the plateau of the curve could still be observed (data not shown) and thus appears to coincide with saturation of the available liposome binding sites on the cell surface.

To select for stable transformants we incubated cell monolayers treated with DNA-loaded liposomes with HAT medium (hypoxanthine, aminopterin, thymidine) under standard conditions (*6*). An average of 200 (minimum 140, maximum 300) colonies per $10^6$ cells per 500 ng of entrapped DNA was calculated from ten consecutive experiments. To test for the stability of TK expression we isolated 20 clones after growing the cells for 2 weeks in HAT medium. We have successfully propagated these clones for more than 50 generations. The TK$^+$ phenotype was also conserved under nonselective conditions, because the plating efficiency of the cloned TK$^+$ cell lines did not show any differences in normal and HAT medium, even after several shifts from selective to nonselective medium and vice versa.

Only those cells which have the TK gene integrated in chromosomal DNA survive in HAT medium (*7*). Therefore, the stable integration of liposome-encapsulated TK gene into the chromosomal DNA of LTK$^-$ cells can be assumed. The fact that 0.2 percent of TK$^+$cells, that is, 0.02 percent of the total cells, stably express TK activity (Table 1) makes the liposome technique superior to the method of polycation treatment (DEAE-dextran) where only transient expression of TK activity could be observed (*18*). This proportion is similar to the percentage of stable colonies reported for the method of microinjection (Table 1). However, the liposome technique is simpler and much faster. To obtain, for example, 200 stable transformants by microinjection (*19*) at least 200,000 cells have to be injected.

The percentage of stably transformed TK$^+$cells obtained with DNA-loaded liposomes is similar to that obtained with the calcium phosphate technique. We applied a tenfold higher amount of DNA (500 ng per dish) to get maximum efficiency (Table 1). Reduction of the DNA to vesicle ratio to one-tenth with a constant vesicle to cell ratio did not change the transformation ratio considerably; we still found 100 colonies when 50 ng were added per dish. However, reducing the vesicle to cell ratio to one-tenth with a constant DNA to vesicle ratio decreased the transformation efficiency (Fig. 2).

Coencapsulation of calf thymus DNA (50 ng of pAGO and 500 ng of calf thymus DNA) did not affect the transformation efficiency. That calf thymus DNA can be omitted makes the liposome technique superior to the calcium phosphate technique where carrier DNA may cause problems in the interpretation of gene transfer experiments. That the lipo-

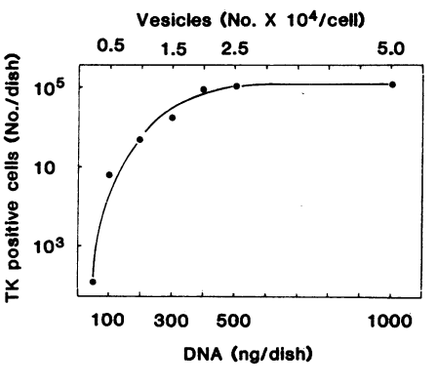


Fig. 2. Dose response for transfection of LTK$^-$ cells with liposome-entrapped pAGO. The liposomes were prepared with phosphatidylserine and cholesterol (1:1 molar ratio). The DNA to lipid ratio was 2 μg of entrapped pAGO per micromole of phospholipid, corresponding to $10^{11}$ vesicles with an average diameter size of 0.5 μm; $10^4$ cells were incubated with different amounts of vesicles.

some suspension remains active even after weeks of storage is advantageous for serial experiments.

Lipid vesicles are already important in medicine as drug carriers in vivo (*20*), and it is possible that liposome-mediated gene transfer might become applicable to experiments in vivo.

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15. Mouse $LTK^-$ cells were seeded 24 hours before transformation ($5 \times 10^5$ cells per 10-cm petri dish). Before adding the liposome suspension the medium ws changed to Dulbecco's minimum essential medium (DMEM) without serum. Ten to five hundred nanomoles of phospholipid encapsulating 25 to 1000 ng of pAGO were added directly into the medium. After incubation for 30 minutes at 37°C, the cells were treated with 25 percent glycerol for 4 minutes; 24 hours later the cells were incubated either with [$^3$H]thymidine for autoradiographic analysis or with HAT medium as described (*6*) for selection of stable transformants.
16. For the autoradiographic assay cells were exposed to [$^3$H]thymidine (25 μCi per milliliter of DMEM containing 10 percent fetal calf serum) overnight, fixed with methanol, extracted with 5 percent trichloroacetic acid and overlaid with Kodak NTB-2 emulsion (*17*). The dishes were developed 48 to 72 hours later, and $TK^+$ positive cells (showing radioactivity in the nucleus) were counted under a Leitz light microscope.
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## Group B Erythrocytes Enzymatically Converted to Group O Survive Normally in A, B, and O Individuals

Abstract. *With an α-galactosidase, B erythrocytes can be converted to blood group O under conditions that neither impair their viability in vitro nor affect their ability to survive normally after transfusion to individuals of groups O, A, and B. Such an approach has the potential for producing enzymatically converted group O cells for use in transfusion therapy. It should also be possible to convert A cells to group O by using the appropriate* α-N-*acetylgalactosaminidase.*

Individuals of blood groups A, B, and O expresss A and H, B and H, and H antigens, respectively, on their erythrocytes. The antigenic molecule consists of one or more straight or branched carbohydrate chains attached to ceramide or a peptide backbone, which in turn is embedded in the lipid bilayer of the cell membrane. Blood group specificity is determined by the nature and linkage of the monosaccharides at the ends of the carbohydrate chains. For H antigenic activity the immunodominant sugar fucose is bound in an α-1,2-glycosidic linkage to the penultimate galactose residue. Blood group A or B specificity occurs when *N*-acetylgalactosamine or galactose is attached in an α-1,3 linkage to the same residue as fucose (*1*). Given such structures, it is possible to convert A or B antigenic determinants into H by using specific exoglycosidases to hydrolyze only the *N*-acetylgalactosamine or galactose linked through a terminal α-glycoside, thus producing group O cells. Our interest is in effecting this conversion under conditions that yield cells of transfusable quality. Using an α-galactosidase previously reported to remove group B activity (*2*), we converted B erythrocytes to group O and transfused the converted cells back to the donor and to O and A individuals, where they survived normally.

To achieve this, we have developed treatment conditions that maintain the integrity and viability of the erythrocyte while providing reasonable enzyme kinetics. For these studies coffee bean α-galactosidase (E.C. 3.2.1.22) (Boehringer Mannheim) was obtained and purified to yield a specific activity of the order of 25 U (*3*) per milligram of protein. No detectable sialidase activity was found and proteases were present in only trace amounts. Contaminating exoglycosidases, when present, were at levels never greater than $2 \times 10^{-3}$ percent of the specific activity of α-galactosidase.

We have extensively studied enzymatically converted cells in vitro (*4*). No more than 0.5 to 1 percent of cells are lysed by the conversion process, and fragility tests demonstrate that enzyme treatment neither increases the cells' susceptibility to osmotic shock nor renders any part of the cell population more fragile. Adenosine 5′-triphosphate and 2,3-diphosphoglycerate concentrations as well as partial oxygen pressures and oxygen dissociation curves remain normal during the treatment to remove B antigenicity and there is an increase in methemoglobin formation of only 1 to 3 percent. These results indicate that cell deformability and normal oxygen binding and exchange are unimpaired. Under the light microscope, treated cells in phosphate-buffered saline (*p*H 7.3) are seen as a mixture of discocytes and spiculated discocytes in about equal numbers. They do not exhibit any membrane stickiness, since after centrifugation they resuspend in the same manner as untreated cells and are not agglutinated by their own serum or by the serum of A or B individuals.

We have searched for changes in membrane structure which could result from enzyme treatment. For example, we tested the membrane surface for perturbation caused by the removal of terminally α-linked galactose residues from group B antigens, the $P_1$ antigen (if present), and trihexosyl ceramide, which is a surface constituent of all erythrocytes. We used as markers acetylcholinesterase, which is bound to the outer membrane surface (*5*), and cholesterol, which is involved in the maintenance of proper membrane fluidity (*6*). Their levels were unchanged following enzyme conversion. Converted cells were also subjected to antigen profiling as another measure of membrane alteration. Among the antigens tested for were A, B, H, Rh (D, C, and E), MNS, $P_1$, Lewis, Kell, Lu-


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