ENCAPSULATION OF HIGH MOLECULAR WEIGHT DNA IN LARGE UNILAMELLAR PHOSPHOLIPID VESICLES

Dependence on the size of the DNA

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1. Introduction

Liposomes, phospholipid vesicles, have been used for the past several years as a gentle and simple means of introducing into mammalian cells biologically active agents for which the cell surface membrane is not permeable. Multilamellar vesicles and small unilamellar vesicles have been used as carriers for small molecules, drugs and enzymes (reviewed in [1]). A preparation similar to multilamellar vesicles has also been used to entrap DNA [2] and as a carrier for chromosomes [3].

Recently two procedures have been described for the preparation of large unilamellar vesicles [4,5]. Using these techniques, globin mRNA was introduced into differentiated mammalian cells resulting in the production of a globin-like protein [6,7]. In addition, after encapsulation intact poliovirus and purified poliovirus RNA were shown to be infectious in cells normally resistant to infection with poliovirus [8,9].

We report here that large unilamellar phosphatidylserine vesicles (LUV) are capable of encapsulating fragments of phage T7 DNA, prepared by digestion with restriction endonucleases with mol. wt $0.27–14.19 \times 10^6$. The encapsulated DNA is resistant to nuclease digestion. The efficiency of encapsulation of DNA is shown to be dependent upon the size of the DNA molecules.

2. Materials and methods

Phage T7 DNA was prepared as in [10]. $^{32}PO_4$ was added to the medium to give 10–25 μCi/ml final conc. The DNA had spec. act. $\sim 2.6 \times 10^5$ cpm/μg DNA. The concentration of DNA was determined spectrophotometrically. Restriction endonuclease fragments of T7 DNA were prepared using *Mbo*I (*Moraxella bovis*, Biolabs) and *Hpa*I (*Haemophilus parainfluenzae*, Bethesda Res. Labs) according to [11]. The digestions were stopped after 60 min by the addition of 1/10 vol. 0.1 M EDTA (pH 7.0) and the fragments were used immediately for encapsulation.

Encapsulation of DNA fragments in LUV was achieved by a slight modification of the method in [4]. DNA fragments were added to a pellet of cochleates prepared from small unilamellar phosphatidylserine (PS, Avanti Biochemicals) vesicles [4] and the suspension was mixed gently by hand. EDTA (0.15 M, pH 9.5) was added to the suspension until it became opalescent and non-particulate, at a final pH 7.0–8.0 [7]. Again, mixing was done only by hand not with a mechanical device. The vesicle–DNA suspension was allowed to stand at 37°C for 60 min, diluted to 10 ml final vol. with TES buffer (2 mM *N*-Tris [hydroxymethyl]methyl-2-aminoethane sulfonic acid, 2 mM histidine, 100 mM NaCl (pH 7.4) and centrifuged at 48 000 × *g* at 20°C for 20 min. The pellet was resuspended with TES buffer to 150 μl final vol.

To test the nuclease sensitivity of encapsulated DNA, 1.35 ml Dulbecco's Ca^{2+} and Mg^{2+}-free phosphate-buffered saline made 0.55 mM in Ca^{2+} and 10 μl micrococcal nuclease (Worthington Biochemicals,

Table 1
DNase resistance of vesicle-associated DNA

	*Mbo*I fragments (μg DNA)		*Hpa*I fragments (μg DNA)	
	Control	Encapsulated	Control	Encapsulated
Initial suspension	19.26	26.24	28.8	43.8
After centrifugation	0.095	0.56	–	–
After nuclease treatment	0.077	0.26	0.029	1.55
Fraction remaining	0.04%	1.0%	0.11%	3.54%

DNA fragments were added to a pellet of cochleates in a ratio of ~5 μg DNA/μmol phosphatidylserine, encapsulated, washed by centrifugation and treated with micrococcal nuclease as in section 2. Phosphatidylserine concentration was determined by the procedure [13]

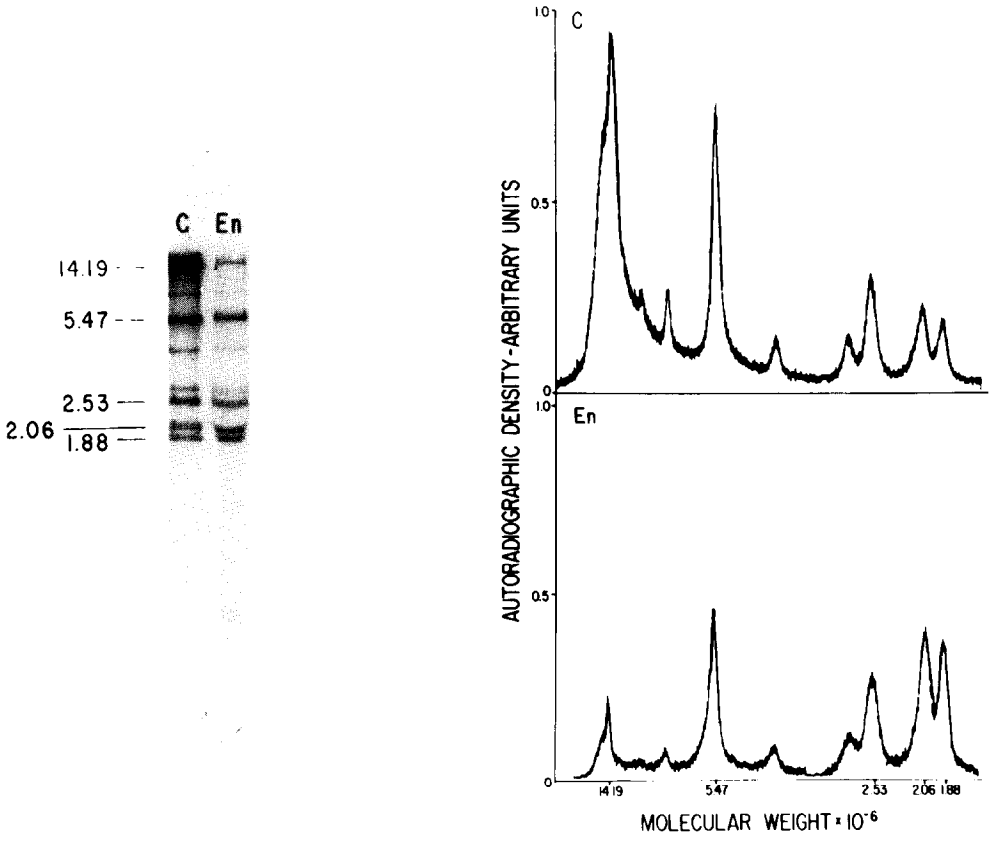

Fig.1. Encapsulation of *Mbo*I fragments of T7 DNA. DNA fragments were separated by horizontal slab gel electrophoresis through 0.7% agarose (see section 2). Autoradiographs of fragments before (C) and after (En) encapsulation and nuclease treatment are shown along with the corresponding microdensitometer tracings of each autoradiograph.

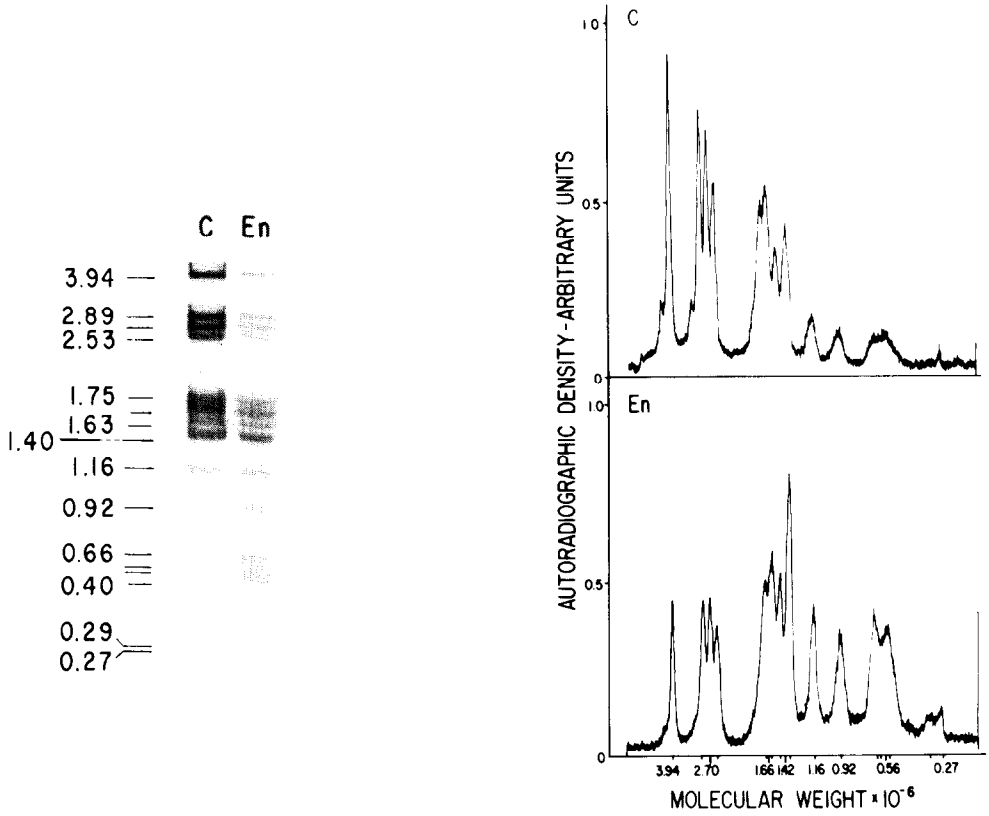

Fig.2. Encapsulation of *Hpa*I fragments of T7 DNA. DNA fragments were separated by electrophoresis through 1.0% agarose and analyzed as in fig.1. (C) Controls before encapsulation. (En) Fragments remaining vesicle associated.

10 000 units/ml) were added to the resuspended pellets and the mixture was incubated at 37°C for 30 min. The nuclease digestion was stopped by the addition of 50 μl, 0.1 M EDTA (pH 7.4). The mixture was diluted to 10 ml with TES buffer and centrifuged at 48 000 × *g* for 20 min at 20°C.

For analysis of encapsulated DNA, the pellets were resuspended in 100 μl TES buffer and lysed with Triton X-100 (5% final conc.). The amount of each DNA fragment present before and after encapsulation was quantitated by horizontal agarose slab gel electrophoresis [11]. The gels were dried onto Whatman 3 MM filter paper using a Hoefer Scientific Instruments model SE540 slab gel dryer. An autoradiogram of the dried gel was made using Kodak XR-5 X-ray film. The autoradiogram was scanned using a Joyce Loebl Microdensitometer 3CS and the area under each peak determined using a Joyce Loebl MK2 electronic integrator. Only bands within the range linear for autoradiographic density versus radioactive cpm were used for quantitation. Molecular weights were assigned to the bands according to [11].

It is important to be aware of the biohazard potential of genetic material encapsulated in lipid vesicles capable of fusing with mammalian cells.

3. Results and discussion

The encapsulation of restriction endonuclease fragments of DNA results in a population of fragments that remain associated with the vesicles and are resistant to digestion with micrococcal nuclease. DNA added to preformed vesicles is almost completely degraded by nuclease treatment and is not vesicle-associated (table 1).

The vesicle-associated DNA can be recovered by lysing the vesicles with Triton X-100. The patterns of DNA fragments we obtained from phage T7 DNA after restriction with *Mbo*I and *Hpa*I restriction endonucleases were analogous to those in [11]. Analysis of the recovered fragments by agarose gel electrophoresis shows that:

(1) Fragments of every size class, 0.27–14.19 × 10^6 mol. wt, have been encapsulated;
(2) No breakage of the DNA has occurred during the encapsulation process (fig.1,2).

The amount of a specific DNA fragment that becomes vesicle associated is dependent upon the size of the fragment (fig.3). Fragments up to ~1 × 10^6 mol. wt are encapsulated at an efficiency of ~8–10%, approximately equivalent to the encapsulation of a small molecule, e.g., sucrose, in our conditions. Beyond that point the efficiency of encapsulation drops as the molecular weight of the DNA increases.

Our working hypothesis is that the size of the DNA is the factor determining the efficiency of encapsulation. It may be possible, therefore, to increase the efficiency of encapsulation of very large DNA by condensing the DNA, perhaps through the use of histones or poly-cationic compounds.

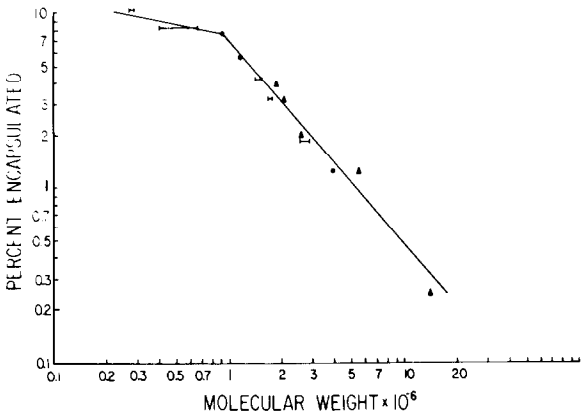

Fig.3. Size dependency of encapsulation of DNA. The μg of each size DNA fragment encapsulated was calculated from the known specific activity of the DNA, the amount of radioactivity remaining vesicle associated after nuclease treatment, and the autoradiographic density of each band in an autoradiogram relative to the total, expressed as % of the amount of each fragment present in the initial encapsulation mixture. (▲) *Mbo*I fragments; (●) *Hpa*I fragments of a single size; (|–|) *Hpa*I fragments of various sizes which cannot be resolved into single species and were evaluated as a group.

However, the number of molecules of, e.g., the T7 *Mbo*I fragment of 2.06 × 10^6 mol. wt encapsulated by 6 μmol PS in the form of cochleates is ~1.7 × 10^{10}. The number of LUV/μmol phosphatidylserine is ~1 × 10^{11} [8]. Thus, even at an efficiency of encapsulation of 3% for this size DNA, ~1 vesicle in every 35 contains this fragment of DNA. Up to 1000 LUV can become associated with a target cell without disturbing the viability of the cell [8,12]. It is, therefore, theoretically possible to introduce at least one fragment of this size class into every cell in the population.

The biological activity of DNA and DNA fragments introduced into mammalian cells with this technique is being investigated.

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