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Protocol

Preparation of Trojan Horse Liposomes (THLs) for Gene Transfer across the Blood-Brain Barrier

William M. Pardridge

INTRODUCTION

Nonviral plasmid DNA is delivered to the brain via a transvascular route across the blood-brain barrier (BBB) following intravenous administration of DNA encapsulated within Trojan horse liposomes (THLs), also called PEGylated immunoliposomes (PILs). The liposome surface is covered with several thousand strands of polymer (e.g., polyethylene glycol [PEG]), and the tips of 1%-2% of the polymer strands are conjugated with a targeting monoclonal antibody that acts as a molecular Trojan horse (MTH). The MTH binds to a receptor (e.g., for transferrin or insulin) on the BBB and brain cell membrane, triggering receptor-mediated transcytosis of the THL across the BBB in vivo, and receptor-mediated endocytosis into brain cells beyond the BBB. The persistence of transgene expression in the brain is inversely related to the rate of degradation of the episomal plasmid DNA. THL technology enables an exogenous gene to be widely expressed in the majority of cells in adult brain (or other organs) within 1 d of a single intravenous administration. Applications of the THLs include tissue-specific gene expression with tissue-specific promoters, complete normalization of striatal tyrosine hydroxylase in experimental Parkinson's disease following intravenous tyrosine hydroxylase gene therapy, a 100% increase in survival time of mice with brain tumors following weekly intravenous antisense gene therapy using THLs, and a 90% increase in survival time with weekly intravenous RNA interference (RNAi) gene therapy in mice with intracranial brain tumors. This protocol describes the preparation of THLs for use in gene transfer in vitro or in vivo.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**!**>^{3}H-*N*-Succinimidyl propionate (NSP)

Alternatives for radiolabeling the MAb include the following (see Step 1):

- <**!**>*Iodo-Gen (Iodogen)*
- <**!**>*[^{125}I]Iodine*
- <**!**>*Chloramine-T is used in conjunction with [^{125}I]Iodine.*

<**!**>[α-^{32}P]dNTP and other reagents for nick translation

Antibodies, monoclonal, receptor-specific (MAbs) (see Discussion for details)

<**R**>BE buffer

<**!**>Chloroform

<**!**>EDTA

<**!**>Ellman's reagent (5,5′-dithiobis[2-nitrobenzoic acid])

Adapted from *Gene Transfer: Delivery and Expression of DNA and RNA* (ed. Friedmann and Rossi). CSHL Press, Cold Spring Harbor, NY, USA, 2007.
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Ethanol (optional; see Step 15)
<!>Exonuclease III
HEPES (0.05 M, pH 7.0)
Lipids (e.g., Avanti Polar Lipids) as follow (see Step 8):
- 1-Palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine, 760 Da (POPC)
- <!>Dimethyldioctadecylammonium bromide, 631 Da (DDAB)
- Distearoylphosphatidylethanolamine-polyethylene glycol 2000, 2748 Da (DSPE-PEG 2000)
- Distearoylphosphatidylethanolamine-polyethylene glycol 2000-maleimide, 2955 Da (DSPE-PEG 2000-maleimide)

<!>Nitrogen gas
<!>Pancreatic endonuclease I
Plasmid DNA

Maxipreparations of plasmid DNA generally start with 200 µg of plasmid DNA.

Traut's reagent (2-iminothiolane hydrochloride)
<!>Trichloroacetic acid (TCA)

Equipment

Bath sonicator
Column, Sepharose CL-4B (1.5- × 20-cm)
Equipment for nick translation
Extruder, hand-held (e.g., Liposofast hand-held extruder; Avestin)
Filter, Millex-GV, 0.22-µm (Millipore)
Filters, polycarbonate, pore size 100- and 400-nm (optionally, 50-nm pore size; see Step 20)
Gel-filtration chromatography apparatus
<!>Liquid nitrogen

<!>*A dry ice/ethanol bath can be used as an alternative (see Step 15).*

Particle size analyzer
Rocker platform
Rotary evaporator (optional; see Step 9)
Scintillation counter
Spectrophotometer
Tubes, glass, 12- × 75-mm
Vortex mixer
Waterbath preset to 37°C

METHOD

Radiolabeling

The number of MAb molecules conjugated to the THL must be determined as part of the quality control assessment of each THL production run.

1. Label the ∈-amino group of external lysine moieties on the MAb with NSP.

   Alternatively, radiolabel with [^{125}I]iodine and chloramine T or Iodogen. Discard the ^{125}I-labeled MAb after 1 mo. The ^{3}H-labeled MAb can be used for 6-12 mo if stored at -20°C.

2. Measure the TCA precipitability of the radiolabeled MAb at the beginning of each THL production run.

3. Purify any MAb that has a TCA precipitation of <95% by gel-filtration chromatography.

4. Radiolabel an aliquot of the plasmid DNA with ^{32}P using nick translation.

   This is used to measure the amount of DNA encapsulated in the interior of the THL as part of the quality control for each THL production run. Discard the ^{32}P-labeled plasmid DNA after 10 d of storage at 4°C.

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MAb Thiolation

5. To a 12- × 75-mm glass tube, add:

MAb	3.0 mg (i.e., 20 nmol)
Radiolabeled MAb (from Step 3)	2 µCi
BE buffer	~200 µL

6. Add 800 or 1200 nmol of Traut's reagent.

   Use the 40:1 molar ratio if targeting a rat IgG (e.g., 8D3 MAb), and a 60:1 molar ratio if the targeting MAb is a mouse IgG2a isotype (e.g., OX26 or 83-14 MAb). The intent is to add 1-1.5 thiol groups per MAb.

7. Confirm the number of thiol groups added per MAb with Ellman's reagent and a spectrophotometric assay.

   If the number of thiol groups added per MAb is >1.5, intermolecular MAb cross-linking can occur. If the number of thiol groups added per MAb is <1.0, then the efficiency of conjugation of the MAb to liposome will be reduced.

Liposome Production and Extrusion

8. To a 12- × 75-mm glass tube containing 1 mL of chloroform, add:

POPC	18.6 µmol
DDAB	0.6 µmol
DSPE-PEG 2000	0.6 µmol
DSPE-PEG 2000-maleimide	0.2 µmol

9. Completely evaporate the lipid under a stream of nitrogen gas while vortexing continuously to produce a thin lipid layer film.

   Alternatively, use a rotary evaporator. This thin lipid layer should be formed carefully.

10. Hydrate the lipids by adding 300 µL of 0.05 M HEPES so that the lipids are ~100 mM.

    The hydration and subsequent vortexing of the lipids should be done carefully and without interruption.

11. Place the hydrated lipid preparation in a bath sonicator for 2 min.

12. Vortex at maximum speed for 1 min.

13. Determine the diameter of the liposome with a particle size analyzer that employs quasi-elastic light-scattering measurements.

    Following hydration, vortexing, and bath sonication, the size of vesicles should be 500-1000 nm. Monitor the size of the liposome throughout the procedure.

14. Add the plasmid DNA (100-250 µg) with ~1 µCi of ^{32}P-labeled plasmid DNA (from Step 4) to the hydrated lipids.

15. Freeze the mixture in liquid nitrogen for 5 min.

    Alternatively, use a dry ice/ethanol bath.

16. Thaw the lipids for 10 min at 37°C.

17. Repeat Steps 15-16 five to seven times.

    The plasmid DNA is encapsulated in large liposomes with the repeat freeze/thaw cycles. Check the sample for excess air bubbles, which should be discharged; their presence can impair the subsequent extrusion process.

18. Add HEPES buffer to a final volume of ~500 µL (i.e., 40 mM lipid).

19. To reduce the size of the liposomes, pass the lipid/DNA mixture through two stacked polycarbonate filters with a pore size of 400 nm in a hand-held extruder. Repeat five times.

20. Disassemble the extruder. Place two stacked polycarbonate filters with a pore size of 100 nm in it. Pass the lipid/DNA mixture through the 100-nm filters five times.

    The optimal diameter of the liposomes at this point should be 80-120 nm. It might be necessary to pass the mixture through two filters with a 50-nm pore size to achieve this.

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21. Incubate the liposomes with pancreatic endonuclease I and exonuclease III for 60 min at 37°C.
If the extrusion process was performed correctly, ~40%-60% of the DNA is encapsulated in 100-nm liposomes, and the remainder is exteriorized. This exteriorized DNA can be toxic in vivo and must be removed. Nuclease digestion is therefore recommended (Monnard et al. 1997).

22. Stop the reaction by adding EDTA to a final concentration of 20 mM.
The removal of external DNA with a strong anion-exchange column such as DEAE is not recommended, because the column does not remove anionic DNA that is electrostatically attached to the cationic lipid residues on the surface of the liposome. The use of a DEAE column will produce a THL preparation with significant exteriorized DNA that can elicit toxic and inflammatory reactions in vivo (Norman et al. 2000).

Conjugation and Gel-Filtration Column Chromatography

23. Mix the nuclease-treated PEGylated liposomes (from Step 22) with the thiolated MAb (from Step 7).
24. Cap the solution. Gas with nitrogen. Rock slowly overnight at room temperature.
25. Apply the preparation to a 1.5- × 20-cm column of Sepharose CL-4B equilibrated with 0.05 M HEPES.
26. Elute the column at room temperature with the HEPES buffer at 1 mL/min. Collect 1-mL fractions (Huwyler et al. 1996).
27. Count an aliquot of each fraction for both ^{3}H and ^{32}P radioactivity. For ^{3}H/^{32}P double-isotope liquid scintillation counting, count the ^{3}H in a window of 0-16 keV and the ^{32}P in a window of 16-1700 keV.
28. Determine the percent of DNA encapsulation from the amount of ^{32}P radioactivity in the first (i.e., liposome) peak off the CL-4B column.
29. Calculate the number of MAb molecules conjugated per individual THL from the ^{3}H radioactivity in the same peak.
The THL peak off the column migrates at about fraction 10, a 10-mL elution volume. The unconjugated MAb elution volume is ~25 mL, and the nuclease-digested nucleic acid elutes at 30-35 mL. There is no spillover of ^{3}H into the ^{32}P channel, but there is an ~2% spillover of ^{32}P into the ^{3}H channel; this should be accounted for in the calculations of ^{3}H and ^{32}P radioactivity.
30. Before using the THLs, sterilize them by passage through a 0.22-µm Millipore Millex-GV filter.
This does not alter their structural integrity (Zhang et al. 2002a).
31. Apply the THLs to cells in culture or inject into animals within 24 h of production, preferably on the same day as elution from the CL-4B column.
THLs can be stored overnight at 4°C.

DISCUSSION

Nonviral forms of gene transfer generally employ polyplexes of a cationic polymer and anionic DNA. These polyplexes, which form small stable structures in water or media of low ionic strength, form micron-size aggregates in physiological saline (Plank et al. 1999; Simberg et al. 2003). This aggregation underlies the high potency of the structures in cell culture but their limited efficacy in animals following intravenous injection. The microparticles trigger uptake by cells in culture via phagocytosis, which produces expression of the transgene in tissue culture (Matsui et al. 1997; Niidome et al. 1997). However, in vivo, the aggregates are rapidly sequestered within the first vascular bed encountered after an intravenous injection, that is, the pulmonary circulation. They embolize in the pulmonary capillaries, which is why gene expression in the lung is log orders of magnitude greater than in peripheral tissues (Hong et al. 1997; Song et al. 1997). The intravenous administration of cationic polyplexes does not result in any gene expression in the brain.

THLs and cationic liposomes have markedly different molecular formulations. Cationic liposomes form sandwich-like structures with anionic DNA, and the DNA is exposed to any surrounding nuclease (Simberg et al. 2001). In contrast, in THLs, a single supercoiled plasmid DNA is encapsulated in the interior of a 100-nm liposome, which contains a small amount of cationic lipid, but has a net anionic lipid charge (Pardridge 2003). This encapsulation renders the plasmid DNA insensitive to the ubiquitous endo- and exonucleases present in vivo (Shi and Pardridge 2000). Plasmid DNA must be engineered to incorporate the appropriate promoter and 3′-untranslated region (UTR). Some promoters will

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enable transgene expression in cell culture following transfection with cationic lipids. However, cationic polyplexes are injurious to cells with a narrow therapeutic index and can activate transcription pathways such that weak promoters permit transcription of the transgene in cell culture (Kofler et al. 1998). Such a promoter might not drive transgene expression in vivo following delivery with THLs. Moreover, certain 3′ UTRs could contain a sequence that enables translation repression, thus blocking transgene expression in vivo (Luikenhuis et al. 2004).

Liposomes are rapidly cleared by the reticuloendothelial system in vivo owing to rapid absorption of serum proteins at their surface. However, this protein absorption can be minimized by conjugating several thousand strands of polymer, for example, PEG 2000, to the surface of the liposome (Papahadjopoulos et al. 1991). Unfortunately, PEGylated liposomes with encapsulated DNA also allow only minimal gene transfer across cell membranes in vivo because the PEGylated liposome is poorly recognized by cell-membrane-internalizing mechanisms, other than phagocytic uptake mechanisms in liver and spleen. To overcome this shortcoming, PEGylated liposomes are converted to THLs by conjugating the tips of 1%-2% of the polymeric strands with a receptor-specific molecular Trojan horse (MTH) (Fig. 1A). The MTH binds a cell membrane receptor, triggering internalization. The degree of internalization is a function of the receptor specificity; when an IgG isotype control antibody replaces the receptor-specific targeting MAb on the THL, gene expression in vivo is not observed (Shi et al. 2001). An electron micrograph of a THL is shown in Figure 1B.

The receptor-specific MAbs that have been used to deliver plasmid DNA to adult rats, mice, and Rhesus monkeys are species-specific (Pardridge 2001). Gene delivery to the mouse uses the 8D3 rat MAb to the mouse transferrin receptor, which is not active in rats, whereas delivery to rats uses the OX26 murine MAb to the rat transferrin receptor. These antibodies are not active in primates or humans. Gene delivery to old world primates, but not to new world primates, uses the 83-14 murine MAb to the human insulin receptor (HIR). This MAb cannot be used in humans, owing to immune reactions, but a genetically engineered form whose activity is identical to that of the original can be used in humans. Because 1-3 mg of purified MAb is used in each THL production run, the hybridoma secreting the MAb must be obtained so that adequate amounts of MAb can be produced with either ascites or large volumes of serum-free conditioned medium. It is likely that MAbs to receptors other than the blood-brain barrier (BBB) transferrin or insulin receptor could be used, but these have not been validated. The MAb to the receptor must be an "endocytosing antibody," because not all receptor MAbs enable endocytosis into the cell following surface membrane binding to the receptor.

Tissue-Specific Expression of Reporter Genes in Brain In Vivo

The THL is the only formulation, viral or nonviral, that enables expression of a transgene throughout the brain following a single intravenous administration. In the example shown in Figure 2, a β-galactosidase expression plasmid was encapsulated in THLs targeted with the 8D3 MAb and injected intravenously in adult mice; 48 h later, the brain was removed and β-galactosidase histochemistry was performed on frozen sections. The transgene was expressed in all parts of the brain, including the brain stem (Fig. 2A; Zhu et al. 2004), and in all parts of the retinal pigmented epithelium, but not in the photoreceptor cells (Fig. 2B). Gene expression was not observed in the photoreceptor layer because there are few transferrin receptors in the outer nuclear layer of the photoreceptor cell (Zhu

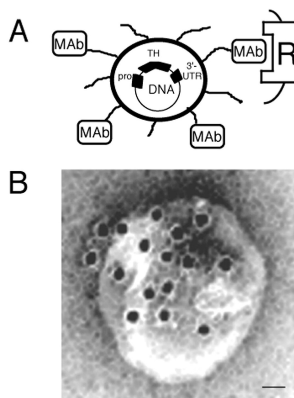

FIGURE 1. (*A*) Diagram of a supercoiled expression plasmid DNA encapsulated in an 85-nm PIL targeted to a cell membrane receptor (R) with a receptor-specific, endocytosing monoclonal antibody (MAb). Tissue-specific expression of the plasmid is regulated by the promoter (pro), which is inserted 5′ of the gene, in this case, tyrosine hydroxylase (TH). (*B*) Transmission electron microscopy of a PIL. The MAb molecule tethered to the tips of the 2000-Da PEG is bound by a conjugate of 10-nm gold and a secondary antibody. The position of the gold particles shows the relationship of the PEG-extended MAb and the liposome. Magnification, 20 nm. (Reprinted, with permission, from Zhang et al. 2003a.)

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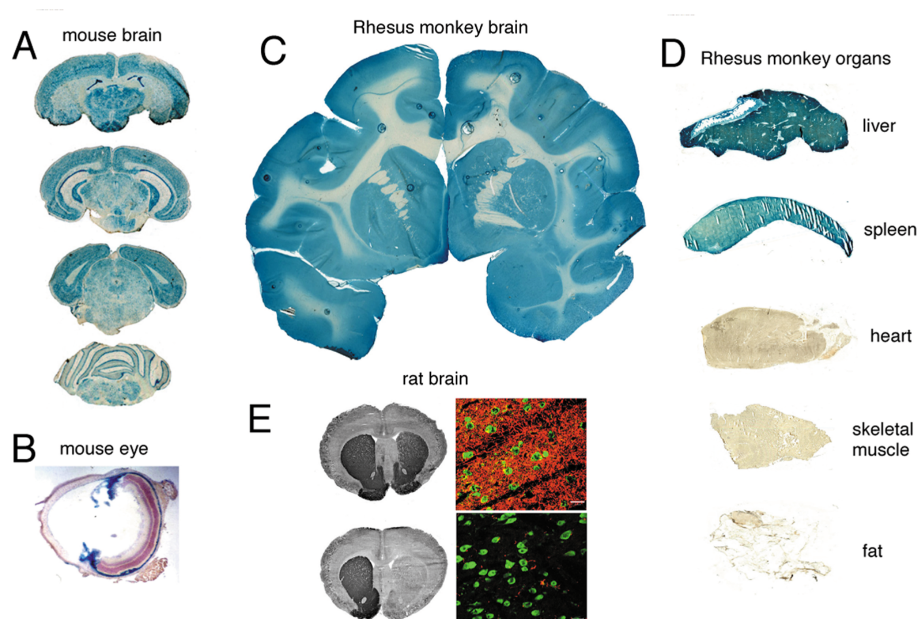

FIGURE 2. (*A*,*B*) β-Galactosidase histochemistry of brain (*A*) and eye (*B*) of the adult mouse removed 48 h after the intravenous injection of a β-galactosidase expression plasmid encapsulated in PILs targeted with the 8D3 rat MAb to the mouse transferrin receptor. (*C*,*D*) β-Galactosidase histochemistry of brain (*C*) and peripheral organs (*D*) of the adult Rhesus monkey removed 48 h after the intravenous injection of a β-galactosidase expression plasmid encapsulated in PILs targeted with the 83-14 murine MAb to the HIR. There is no gene expression in heart, skeletal muscle, or fat. (*E*) Tyrosine hydroxylase immunocytochemistry (*left*) or confocal microscopy (*right*) of rat brain removed 72 h after the intravenous injection of a tyrosine hydroxylase expression plasmid encapsulated in PILs targeted with either the OX26 mouse MAb to the rat transferrin receptor (*top*) or a mouse IgG2a isotype control antibody (*bottom*). Three weeks before gene administration, the rats were injected with 6-hydroxydopamine into the medial forebrain bundle on the right side, causing a complete loss of immunoreactive tyrosine hydroxylase in the striatum ipsilateral to the neurotoxin lesion. Gene therapy with PILs targeted with the transferrin receptor MAb causes a complete normalization of striatal tyrosine hydroxylase (*top*). There is no restoration of striatal tyrosine hydroxylase if the tyrosine hydroxylase expression plasmid is encapsulated in PILs targeted with an isotype control antibody that does not target a BBB receptor (*bottom*). None of the sections in panels *A*, *C*, or *D* are counterstained. (*A*: Reprinted, with permission, from Zhu et al. 2004; *B*: reprinted, with permission, from Zhu et al. 2002 [© Association for Research in Vision and Ophthalmology]; *C*, *D*: reprinted, with permission, from Zhang et al. 2003c [© Elsevier]; *E*: reprinted, with permission, from Zhang et al. 2004a.) (For color figure, see doi: 10.1101/pdb.prot5407 online at www.cshprotocols.org.)

et al. 2002). Insulin receptor is expressed in the outer nuclear layer, and when the transgene was targeted with the HIR MAb, gene expression in the photoreceptor cells was observed (Zhang et al. 2003b).

Similarly, a β-galactosidase expression plasmid was encapsulated in THLs targeted with the 83-14 MAb (Zhang et al. 2003c). Forty-eight hours after an intravenous injection in the adult Rhesus monkey, there was global expression of the transgene in the brain (Fig. 2C) and widespread expression in liver and spleen, but not in heart, skeletal muscle, or fat (Fig. 2D). The THL must traverse two barriers before entering the nucleus: a vascular endothelial barrier (in the brain, the BBB) and the plasma membrane of the parenchymal cells of the target organ. The insulin receptor is present on both barriers in brain and eye, but only on the parenchymal cells in heart, skeletal muscle, and fat, thus explaining the distribution of gene expression. Insulin receptor is not expressed on the vascular barrier in liver or spleen, but the THLs freely cross the porous vascular barrier in these organs, which are perfused by a sinusoidal capillary bed. In contrast, heart, skeletal muscle, and fat are perfused by a capillary bed with a continuous endothelial barrier. New targeting MAbs that enable transport through the vascular endothelial barrier in peripheral tissues must be discovered before THLs can be used to target genes to these tissues.

The delivery of genes to the brain with THLs that target the BBB transferrin or insulin receptor can result in ectopic expression of the transgene in peripheral tissues because of the expression of the transferrin or insulin receptor in those organs. However, if the transgene is incorporated in an

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expression plasmid under the influence of a brain-specific promoter, such as the 5′-flanking sequence of the glial fibrillary acidic protein gene, ectopic expression can be eliminated in the mouse (Shi et al. 2001). Similarly, transgene expression in the brain or peripheral tissues is not observed following the intravenous injection of THLs carrying a gene under the influence of the 5′-flanking sequence of the rhodopsin gene, although gene expression in ocular structures is observed in the adult Rhesus monkey (Zhang et al. 2003b). Thus, the combination of tissue-specific gene promoters and THLs enables highly localized expression of transgenes.

Intravenous Gene Therapy with THLs

THLs were produced that carried an expression plasmid encoding a 700-nt antisense RNA against the human epidermal growth factor receptor (EGFR) (Zhang et al. 2002b). Mice with intracranial human brain tumors were treated with weekly intravenous injections of THLs that were doubly targeted with two MAbs. The rat 8D3 MAb to the mouse transferrin receptor delivered the THL across the mouse BBB of the brain tumor, and the murine 83-14 MAb to the HIR delivered the THL across the brain tumor cell plasma membrane. Weekly intravenous injections of the THLs produced a 100% increase in survival time (Zhang et al. 2002b).

Similarly, mice with intracranial brain tumors were treated with THLs targeted with the 8D3 and 83-14 MAbs that encapsulated an expression plasmid encoding a short hairpin RNA that knocks down the EGFR via RNAi (Zhang et al. 2004b). Weekly intravenous RNAi gene therapy produced a 90% increase in survival time in adult subacute combined immunodeficiency mice with intracranial human brain tumors. This is the first demonstration that the survival time in experimental tumors can be prolonged with intravenous RNAi gene therapy.

In adult rats with experimental Parkinson's disease, THLs carrying a tyrosine hydroxylase expression plasmid produced a 100% normalization of striatal tyrosine hydroxylase (Fig. 2E, top). If the THL carrying the tyrosine hydroxylase expression plasmid was targeted with a mouse IgG2a isotype control antibody, there was no restoration of striatal tyrosine hydroxylase (Fig. 2E, bottom; Zhang et al. 2004a).

THLs could be used to deliver plasmids containing the inverted terminal repeats that allow for permanent yet random integration of the host genome. However, this is not desirable, owing to the insertional mutagenesis that follows random integration of the host genome. The use of plasmid DNA that functions episomally allows for reversible gene therapy, and episomal gene therapy can be given at repeat intervals, similar to other medicines.

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