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Gene Transfer into Mammalian Cells Using Targeted Filamentous Bacteriophage

Andrew Baird

INTRODUCTION

Phage vectors, because of their genetic simplicity, are uniquely suited to methods that use directed evolution to genetically optimize vectors for therapeutic gene delivery. Moreover, because phage production is restricted to strain-specific bacteria, the hosts are equally amenable to genetic engineering, modification, and even genetic selections to optimize yield, genetic stability, manufacture, and cost. The choice of targeting ligand determines the specificity of targeted phage transduction. Genetic targeting is limited to proteins that can be efficiently expressed and biologically active following secretion into the periplasmic space of the bacteria and subsequent incorporation into the phage particle. The capacity of phage to display a chosen targeting ligand must be determined empirically and optimized. Alternatively, the targeting ligand can be selected after display in a phage library. The orientation of the reporter gene relative to the phage structural genes can affect vector transduction efficiency. We have also targeted phage particles for gene delivery using an avidin-biotin linkage. This allows selection of ligands without concern for their ability to be displayed genetically. This protocol describes the use of targeted filamentous phage for gene delivery to mammalian cells. The final vector, although of low efficiency, is meant to serve as a starting point for a vector development platform that can use in vitro and in vivo techniques of combinatorial display to direct its evolution to high efficiency, high specificity, and eventually, safety in humans.

RELATED INFORMATION

Regarding reporter gene orientation, it was found that transduction efficiency of the MG4 phage vector (Kassner et al. 1999) in which the green fluorescent protein (GFP) cassette is in the antisense orientation relative to the phage sense strand is about threefold higher than the same vector containing the GFP cassette in the opposite orientation (MG3) (Larocca et al. 1999).

The phage particles are generally not toxic to mammalian cell lines. However, it is important that endotoxin be removed. DNase treatment is important to prevent nonspecific transfection of cells by any contaminating replicative form of the phage. Contamination by replicative-form phage DNA should be monitored before and after DNase treatment by evaluating the phage on an agarose gel. The timing, duration, and doses of genotoxic treatments must be optimized for each mammalian cell line used. Targeted phage will not produce infective phage particles after transfection of mammalian cells because bacterial promoters regulate all of the phage structural genes. Even if the phage proteins were expressed, the mechanism for phage packaging and the differences in the intracellular environment of mammalian cells

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versus bacteria make the probability of a productive infection negligible. Nevertheless, we recommend following the same biohazard safety precautions (Biosafety Level 2 [BSL-2]) for targeted phage as those used for nonreplication-competent adenoviral vectors.

METHOD

Transformation of Bacteria

Using standard molecular biology techniques, host bacteria are transformed with the replicative form of the recombinant phage vector that has been engineered to contain a mammalian expression cassette. In our studies, we have used cytome galovirus (CMV) promoter and growth hormone (GH) polyadenylation DNAs obtained from commercial sources.

1. Thaw 100 µL of competent cells on ice.
2. Add 1.7 µL of β-mercaptoethanol to cells and incubate on ice for 10 min.
3. Mix 50 ng of replicative-form phage DNA with cells and incubate for 30 min on ice.
4. Heat-shock bacteria for 45 sec by placing them at 42°C. Place bacteria on ice for 2 min.
5. Add 900 µL of SOC to cells and incubate with shaking at 250 rpm for 1 h at 37°C.
6. Spread cells on LB^+ plates and incubate overnight at 37°C.

Preparation of Targeted Phage Particles

Using standard phage display techniques, the desired ligand is engineered onto gIII so that the final particle will display a ligand-pIII fusion capable of targeting the phage to the mammalian cell.

7. Inoculate phage-transformed bacteria into YT broth (2×) with ampicillin (60 µg/mL). Grow bacteria with shaking at 300 rpm overnight at 37°C.
8. Centrifuge bacterial culture at 6000g for 10 min at 4°C.
9. Save the supernatant and add 1/5 volume of cold 1.5 M NaCl/30% PEG. Mix well and incubate for 2 h on ice to precipitate phage.
10. Centrifuge at 15,000*g* for 30 min at 4°C to collect phage. Remove supernatant and all residual liquid.
11. Resuspend the phage pellet in PBS + 0.2 mM AEBSF and incubate for 30 min at 4°C.
12. Centrifuge at 20,000*g* for 20–30 min to remove debris.
13. Repeat Steps 9–11 if further concentration of phage is necessary.
14. Add $MgCl_2$ to 10 µM and 6 units of DNase I per milliliter of phage solution. Incubate for 20 min at room temperature. Stop the reaction by adding 10 µL of 0.5 M EDTA per milliliter of phage solution.

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15. Immediately add 1/5 volume of 1.5 M NaCl/30% PEG. Mix well and incubate for 2 h on ice to precipitate phage.
16. Centrifuge at 15,000g for 30 min at 4°C to collect the phage. Remove supernatant and all residual liquid.
17. Resuspend the phage pellet in PBS + 0.2 mM AEBSF. Incubate for 5–15 min at 37°C and then for 30 min at 4°C.
18. Centrifuge at 20,000g for 20–30 min to remove debris.
19. Filter phage through a 0.45-μm filter, freeze in 20% glycerol, and store at –70°C.

Endotoxin Removal by Triton X-114 Phase Partitioning

It is critical to remove endotoxin before using the particles in any cell or animal studies.

20. Add 100 μL of 10% Triton X-114 per milliliter of sample and incubate for 30 min on ice with occasional vortexing. Incubate for 10 min at 37°C.
21. Centrifuge at 14,000 rpm in a microcentrifuge for 10 min at room temperature. Save the aqueous (upper) phase.
22. Repeat phase partitioning (Steps 20 and 21) twice.

Quantification of Yields by Titering Phage for Plaque-Forming Units (pfu)

23. Prewarm LB plates at 37°C. Melt top agar (LB) and place in a 55°C water bath.
24. Grow F' bacteria (XLI-Blue) to OD_{600} = 0.5. Aliquot 300 μL of cells to Falcon 2059 tubes for each dilution to be tested.
25. Set up serial dilutions in PBS by starting with a 100-fold dilution (5 μL diluted in 500 μL of PBS) and repeating several times for desired dilution series. Add 100 μL from each dilution to bacterial cells.
26. Add 3 mL of top agar to each tube, briefly vortex, and pour on top of prewarmed LB plates. Allow top agar to harden and invert overnight at 37°C.
27. Count plaques. Determine titer in pfu/mL by multiplying the number of plaques by the dilution and volume plated.

Transduction of Cultured Cells

28. Seed cells in 1 mL of cell culture medium for GT in 12-well tissue-culture dishes. Incubate overnight at 37°C with 5% CO_2.

The seeding density is determined by growth rate. After an overnight incubation, the cells should be 25% confluent in the 12-well dishes. We seed PC-3 cells at 2 × 104 cells/well.

29. Remove cell culture medium for GT from the cells and replace with cell culture medium for GT containing phage. Incubate cells for 24–96 h at 37°C with 5% CO_2. If genotoxic treatments are being used, incubate for 40 h and proceed to Step 31.

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A typical dose of targeted phage for highest transduction efficiency is 1011 pfu/mL.

30 Harvest cells for reporter gene analysis:

- **i.** Remove the phage-containing culture medium and wash the cells with PBS.
- **ii.** Remove the cells from the culture dishes by adding 150 μL of 0.25% trypsin and incubating for 2–3 min at 37°C.
- **iii.** Once the cells have detached from the plate, add 350 μL of fixative buffer.
- **iv.** Analyze the cells by flow cytometry with an FITC filter set.

Genotoxic Treatments

The phage are added to cultures and genotoxic treatments are started 40 h later. Treatments should be optimized for each target cell line.

31 Prepare cell culture medium for GT containing 10% FBS to use for heat shock and UV irradiation. Alternatively, add camptothecin to 1 μM and 100 μM or hydroxyurea to 10 mM and 100 mM.

32 Remove medium containing phage. Replace with one of the genotoxic media above or with cell culture medium for GT containing 10% FBS for heat shock or UV irradiation.

33 Incubate directly for 7 h at 37°C or 42°C (heat shock) or irradiate (10–100 J/m2).

34 Wash three times in PBS.

35 Add fresh cell culture medium for GT containing 10% FBS. Incubate an additional 24–48 h at 37°C.

36 Wash three times with PBS and evaluate by FACS analysis or by direct observation of GFP.

DISCUSSION

Phage-mediated gene transfer offers an alternative method of introducing genes into specific cell types because filamentous bacteriophage can be readily reengineered to transfer genes to mammalian cells by attaching a targeting ligand to the phage surface either noncovalently (Larocca et al. 1998) or genetically (Kassner et al. 1999; Larocca et al. 1999, 2001; Poul and Marks 1999; Mount et al. 2004). Successful gene transfer and subsequent protein expression is measured using a reporter gene such as GFP, neomycin phosphotransferase, or β-galactosidase. Theoretically, any gene with an appropriate mammalian transcriptional promoter and polyadenylation signal can be incorporated into a ligand-targeted phage vector. This combination of ligand retargeting and a mammalian expression cassette confers mammalian cell tropism. Once prepared, these modified phage act like nonproductive

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animal viral vectors that are propagated and manipulated genetically with all the conveniences of a phage vector.

Phage display is commonly used as a technique to identify peptides and proteins that can then be used as reagents themselves or used to retarget other particles (Kehoe and Kay 2005). By leaving the peptide moiety on the phage, a particle might be evolved and optimized for gene delivery. The molecular design of better phage is not new (Skiena 2001), and protein evolution in viral backgrounds is well described (Bamford et al. 2002; Baker et al. 2005; Briones and Bastolla 2005; Casjens 2005; Hambly and Suttle 2005). Molenaar et al. (2002), for example, showed that simply displaying peptides on phage could modify their natural pharmacokinetics when injected into mice. Indeed, the possibility of creating phage with altered pharmacokinetics when administered in vivo was first reported several years ago (Geier et al. 1973; Merril et al. 1996) and attributed to single-amino-acid changes in coat composition (Vitiello et al. 2005). Although their safety in humans (Bruttin and Brussow 2005) suggests that T4 phage may be an optimal starting point for directed evolution of a vector, we have focused on filamentous M13 phage (Larocca et al. 2002b), whereas other investigators have turned to similar approaches using mammalian viral systems (Spear et al. 2003; Perabo et al. 2006).

Gene transfer with phage, as with other mechanisms, is time- and dose-dependent. It is also specific for cell surface receptors. Transduced cells begin to appear at ~48 h after the addition of phage, and the percentage of cells expressing reporter gene increases with time. Phage is internalized through interaction of the targeting ligand with its cognate receptors on the cell surface. Accordingly, ligand-targeted phage transduction is inhibited by competition with the free ligand or with a neutralizing antireceptor antibody (Kassner et al. 1999; Larocca et al. 1999). Little or no transduction occurs in the absence of a targeting ligand because phage particles have no native tropism for mammalian cells. In addition, transduction occurs with concentrations of phage as low as ~100 phage/cell and increases up to the highest concentration tested ($\sim 1 \times 10^6$ phage/cell). At these higher doses, internalization of the ligand-targeted phage is highly efficient, and phage protein is detectable in almost all cells. Transduction efficiencies of up to 40% have been described, using ligand- and antibody-targeted phage/phagemid (Larocca et al. 1998; Kassner et al. 1999; Larocca et al. 1999; Poul and Marks 1999; Burg et al. 2002).

Stable transformants of targeted cells can be isolated from phage-transduced cells using G418 drug selection, with sustained gene expression for several months. A kinetic analysis of the fate of particles reveals that the coat is metabolized within hours of internalization but that phage DNA can be recovered from cells long afterward. For this reason, it may be possible to optimize intracellular trafficking. Genotoxic treatments, which are thought to increase double-stranded DNA synthesis, improve transduction efficiency of single-stranded phage (Burg et al. 2002). Further improvements in transduction efficiency are possible by incorporating peptides into the phage coat protein that facilitate trafficking in the cell and by applying directed molecular evolution to genetically select improved phage from combinatorial libraries (Kassner et al. 1999; Larocca et al. 1999, 2001, 2002a,b).

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Phage vectors are simple and convenient to produce in bacteria, can be specifically targeted to cells, and have the potential to be evolved genetically for specific applications. In addition, filamentous phage have an inherent capacity to package large DNA inserts because they are not limited in size by a pre-formed capsid but instead form their protein coat as they are extruded from bacteria. We have successfully transduced cells with phage vectors approaching 10 kb in length, including both the targeting ligand sequence and the mammalian expression cassette. For larger gene inserts that tend to be unstable in phage, we have recently engineered phagemid vectors that are much simpler and smaller (~6 kb). Phagemid vectors contain no phage DNA sequences except the origin of replication and are therefore prepared in bacteria by rescue with helper phage (Smith and Scott 1993; Kehoe and Kay 2005) or by reengineering the host itself. The simple genetics of filamentous phage vectors make them particularly adaptable for a wide variety of targeted gene-transfer applications (Larocca et al. 2001; Mount et al. 2004).

The flexible structure of the pIII coat protein is well suited for displaying a variety of biologically active peptide and protein sequences while retaining the structural integrity of the phage particle (Smith 1985; Smith and Scott 1993; Kehoe and Kay 2005). For example, biologically active hormones, cytokines, and growth factors have been displayed on phage (Bass et al. 1990; Gram et al. 1993; Saggio et al. 1995; Buchli et al. 1997; Merlin et al. 1997; Souriau et al. 1997; Vispo et al. 1997). To date, phage-mediated gene delivery has been performed with targeting ligands on pIII, but fusion to pVIII provides similarly targeted phage/phagemid (Petrenko and Smith 2000; Mount et al. 2004; Kehoe and Kay 2005). In fact, phage displaying multiple copies of a peptide on the major coat protein are rapidly internalized into mammalian cells (Ivanenkov et al. 1999). There are many examples of active pIII fusion proteins, but not all ligands are equally displayed. Differences in the ability of the fusion proteins to be secreted into the bacterial periplasmic space for packaging into the phage particle can significantly affect surface display. Thus, although many ligands are functional when displayed on phage, insufficient display is a limitation that should be considered when identifying new ligands that can target phage for gene delivery to cells. Alternatively, noncovalent display of the targeting ligand (Larocca et al. 1998) can be used when genetic display is not applicable.

Many types of ligands can be used for phage gene delivery, including those identified from phage libraries. For example, Poul and Marks (1999) have targeted M13 phage using an anti-Her2 single-chain antibody. Whereas ligand selection is often performed by panning phage on cells for binding and internalization (Barry et al. 1996; Pereira et al. 1997; Watters et al. 1997), we developed novel selection strategies called LIVE (ligand identification via expression) and SNAAP (selection of nucleic-acid-amplified phage) that directly select for ligands that bind and internalize into target cells for gene delivery (Kassner et al. 1999; Burg et al. 2004). Repeated rounds of phage transfection and recovery from transfected cells select gene targeting ligands. In these systems, a functional ligand can be enriched a million-fold after three to four rounds of selection.

Filamentous phage-mediated gene transfer, like that of adeno-associated virus (AAV), involves the introduction of single-stranded DNA that must be converted to double-stranded DNA for transgene expression. Accordingly, phage-mediated transduction can be increased

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by the same genotoxic treatments that enhance AAV efficiency (Yalkinoglu et al. 1988), such as camptothecin, hydroxyurea, heat shock, and UV irradiation. The degree of enhancement varies among cell lines, presumably because of individual differences in response to genotoxic stress. We obtained maximum enhancement of phage-mediated transduction (with minimal toxicity) on COS-1 and PC-3 cells using 5–10 μM camptothecin, 40 mM hydroxyurea, 50 J/m^2 UV irradiation, or 7-h heat shock at 42.5°C.

Recombinant M13 phage vectors are adapted for gene transfer to mammalian cells by inserting a mammalian expression cassette into the intergenic region of the phage genome (Larocca et al. 1999). The modified phage vector contains the GFP gene expression cassette from pEGFP-N1 (Clontech) that encodes a mutagenized GFP (Cormack et al. 1996) which is optimized for visualization by fluorescent microscopy or FACS. It also contains the simian virus 40 (SV40) origin of replication from pEGFP-N1. Any phage vector that can be engineered for phage display (i.e., fUSE5 [Scott and Smith 1990], fAFF1 [Cwirla et al. 1990], and M13East [Giebel et al. 1995]) can be adapted for gene delivery in this manner, including phagemid vectors. When phagemids are rescued with helper phage, both wild-type pIII and the ligand-pIII fusion protein are incorporated into the phagemid particle (Kehoe and Kay 2005), resulting in monovalent display of the targeting ligand. Monovalent display, however, is sometimes less optimal because the number of ligands on the phage surface can significantly affect binding and internalization (Becerril et al. 1999). In this case, the system can be adapted for multivalent display by rescuing the phagemid with a gene-III-deleted helper phage as described by Rakonjac et al. (1997).

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MATERIALS

RECIPES: Please see the end of this article for recipes indicated by <R>.

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

β-mercaptoethanol

Camptothecin (Sigma-Aldrich), 10 mM in dimethyl sulfoxide (DMSO)

Store at –20°C.

Hydroxyurea (Sigma-Aldrich H8627; 1.0 M in PBS) can be used as an alternative genotoxic treatment (see Step 31). Store at –20°C.

<R>Cell culture medium for GT

DNase I (Invitrogen 18068-015)

EDTA (0.5 M)

<R>Fixative buffer

Glycerol (20%)

Host F′ bacterial strain (Stratagene 200249 XLI-Blue Competent Cells; Agilent Technologies)

<R>LB agar for GT

Use this to prepare LB plates and top agar. Also use it to prepare LB+ plates, to which 60 μg/mL ampicillin has been added.

$MgCl_2$ (1 M)

PC-3 cell line (NCI cell bank) and e.g., HT1229 (ATTC)

Phosphate-buffered saline (PBS)

Also prepare PBS + 0.2 mM AEBSF (4-[2-aminoethyl]-benzenesulfonyl fluoride; Roche Applied Science 11585916001).

Replicative-form M13 phage DNA

SOC medium (Invitrogen 15544034)

Sodium chloride (NaCl; 1.5 M)/30% polyethylene glycol (PEG) 8000

Targeted phage particles containing reporter gene (GFP)

Triton X-114 (10%)

Trypsin (0.25%) (Invitrogen 25200-056)

<R>YT broth (2×) with ampicillin

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Equipment

Centrifuge bottles

Cryovials

Fluorescence-activated cell sorter (FACS)/Flow cytometer with a fluorescein isothiocyanate (FITC) filter set

Incubators preset to 37°C and 42°C

Microcentrifuge (Eppendorf)

Shaking platform

Spectrophotometer

Syringe filters (0.45 μm)

Syringes

Tissue-culture dishes (12 well)

Tubes (sterile; Falcon 2059)

Ultraviolet (UV) irradiation (e.g., Stratalinker UV cross-linker, Stratagene or similar)

Vortex mixer

Water bath preset to 55°C