A hyperthermostable bacterial histone-like protein as an efficient mediator for transfection of eukaryotic cells

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Gene delivery has shown potential in a variety of applications, including basic research, therapies for inborn genetic defects, cancer, AIDS, tissue engineering[1,2], and vaccination[3]. Most available systems have serious drawbacks, such as safety hazards[4], inefficiency under in vivo–like conditions, and expensive production. When using naked DNA[2,5], for instance, a large amount of ultrapure DNA has to be applied as a result of degradation by nucleases[6]. Similarly, the use of eukaryotic histones[7,8], synthetic peptides, or peptide nucleic acids[9,10] may be limited by high production costs. We have demonstrated a biotechnologically feasible and economical approach for gene delivery using the histone-like protein from the hyperthermostable eubacterium *Thermotoga maritima*, TmHU[11,12] as an efficient gene transfer reagent. HU can be easily isolated from recombinant *Escherichia coli*, is extraordinarily stable, and protects dsDNA from thermal denaturation[12]. This study demonstrates its use as an inexpensive tool for gene delivery.

We established a protocol for TmHU-mediated transient transfection in vitro, transfecting the *E. coli lacZ* gene into NIH 3T3 cells. The protocol was optimized for several critical parameters, such as the TmHU:DNA ratio and the conditions of preincubation. The optimal TmHU:DNA ratio was 12.5:1 (wt/wt), corresponding to a five- to sixfold net positive charge excess at physiological pH. Upon heating the reaction mixture at 95°C for 40 min and adding calcium to a final concentration of 2 mM, a dense, compact precipitate formed, accompanied by a 100-fold increase in transfection efficiency and a higher reproducibility. In the absence of calcium, the observed aggregates were larger, had a lower density, and floated in the medium. The optimized system routinely yielded about 1×10^4 X-gal positive cells/µg DNA, the number of transfected cells being roughly proportional to the amount of reaction mixture used. A control experiment without TmHU yielded only 3 X-gal positive cells/µg DNA, demonstrating that the transfection was not due to DNA–calcium phosphate coprecipitation. The DEAE-dextran method (Stratagene, La Jolla, CA) and lipofection using the cationic lipid Tfx-50 (Promega) yielded about 3×10^2 and 3.3×10^3 X-gal positive cells/µg DNA, respectively. Transfecting several cell lines with TmHU, we achieved enhancements of 33-fold (NIH 3T3), 120-fold (U251), and 6-fold (A431), as compared with DEAE-dextran mediated transfection (Fig. 1). 293T cells could not be transfected with DEAE-dextran (the cells detached) but were very efficiently transfected with TmHU. These results (summarized in Fig. 1) demonstrate the general applicability of the system.

Addition of endosomolytical agents (e.g., chloroquine) under several conditions did not improve transfection by TmHU but in fact was often detrimental. We also observed neither a decrease in transfection efficiency when cells grew confluent, nor inhibition of transfection by serum. All transient transfections with TmHU were therefore performed with confluent cultures and in the presence of serum.

Since these features point toward the feasibility of an in vivo application, we tested this potential, injecting TmHU–DNA complexes into the thigh muscles of female BALB/c mice. We were able to increase transient expression of the luciferase reporter gene by an average of eightfold compared to naked DNA injection (Fig. 2A). Interestingly, the conditions for efficient transfection in vivo differed considerably from those determined in vitro, with precipitate formation seemingly decreasing efficiency, probably because of reduced diffusion. The optimal TmHU:DNA ratio for in vivo transfection was determined to be 1.2:1 (wt/wt).

In order to evaluate toxicity, we monitored the number of viable 293T cells after 24 h incubation with various concentrations of either TmHU or the cationic lipid Lipofectin (GIBCO BRL). Whereas incubation with even low amounts of Lipofectin caused considerable cell death, an effect of TmHU was observed only at much higher concentrations (Fig. 2B), the determined toxicity of 1 mg/ml TmHU corresponding roughly to that of 10 µg/ml Lipofectin. Considering that only 1.2 µg TmHU were used to transfect 1 µg DNA in vivo, but usually a three- to fourfold mass excess of Lipofectin over DNA is used, we conclude that TmHU is at least two orders of magnitude less toxic.

The protocol optimized for transient transfection was also used to stably transfect NIH 3T3 cells with the neomycin resistance gene in vitro. TmHU-mediated gene transfer in the presence or absence of calcium yielded colonies of stable transfectants after selection with G418 (541 and 224 colonies, respectively). These yields are comparable to those of the standard calcium phosphate precipitation method.

We further tested whether the prior formation of TmHU–DNA complexes could enhance the efficiency of lipofection. A total of

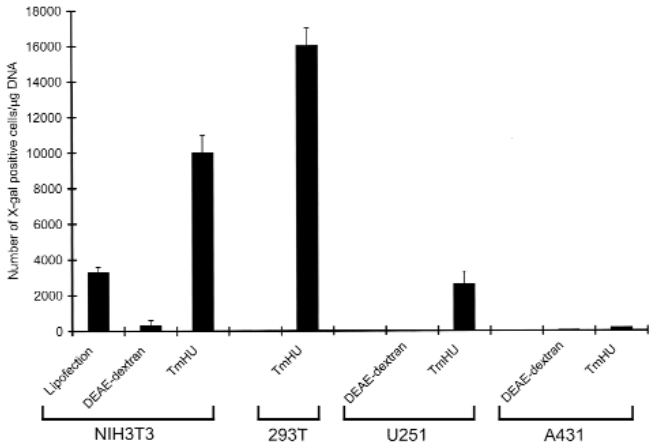

Fig. 1. Transient TmHU-mediated transfection in various cell lines in vitro in comparison with other transfection methods. NIH 3T3, 293T, U251, and A431 confluent cells were transfected with pEli92 plasmid DNA using the optimized protocol for TmHU-mediated transfection, the DEAE-dextran method (Stratagene) or lipofection (Tfx-50, Promega), respectively, and the number of X-gal positive cells was counted 48 h after transfection.

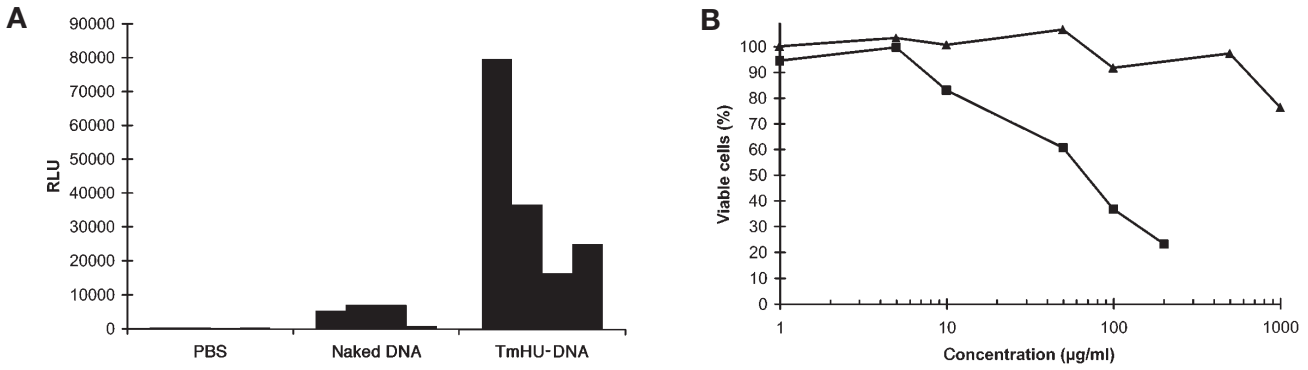

Fig. 2. Feasibility of TmHU-mediated transfection in vivo. (A) Intramuscular injections of PBS, naked DNA, and TmHU–DNA complexes show a significant enhancement upon addition of TmHU. The plasmid transfected encodes luciferase, and the results of single-injection experiments are shown (RLU, relative light units). (B) Semilogarithmic plot of the number of viable 293T cells after incubation (24 h) with increasing concentrations of TmHU (▲) or Lipofectin (■). TmHU is considerably less toxic than Lipofectin in this assay.

16 different experiments was set up using 1 µg of plasmid DNA and varying amounts of TmHU and of the cationic lipid Tfx-50. As demonstrated in Figure 3, the optimal condition of lipofection in the absence of TmHU was a charge ratio of 2:1, resulting in 3.3×10^3 X-gal positive cells/µg DNA. Preincubation with TmHU increased the transfection efficiency significantly, yielding up to 1.6×10^4 X-gal positive cells/µg DNA (3:1 charge ratio, 6.25 µg TmHU). This yield was significantly higher than the sum of yields obtained using either TmHU or cationic lipid alone, indicating a synergistic effect. Unlike the optimized protocol for TmHU-mediated transfection, heating the TmHU–DNA mixture before adding it to the cationic lipid decreased the transfection efficiency to about 30%.

The use of genes as drugs is still limited by the lack of low-cost, yet efficient and nonhazardous vectors and vehicles. In this study, we present a gene transfer agent that may offer an alternative to present systems. We have shown that TmHU is an efficient carrier of heterologous DNA into various eukaryotic cells and an enhancer of another transfection method (lipofection). Using the production capabilities of modern fermentation and the simplicity of TmHU purification[12], large amounts of the protein can be produced easily and efficiently to provide sufficient material for large-scale in vitro or in vivo transfections. The system should be feasible for applications that do not require targeting to a defined cellular population, because the uptake of the protein itself seems to be independent of cell type (data not shown).

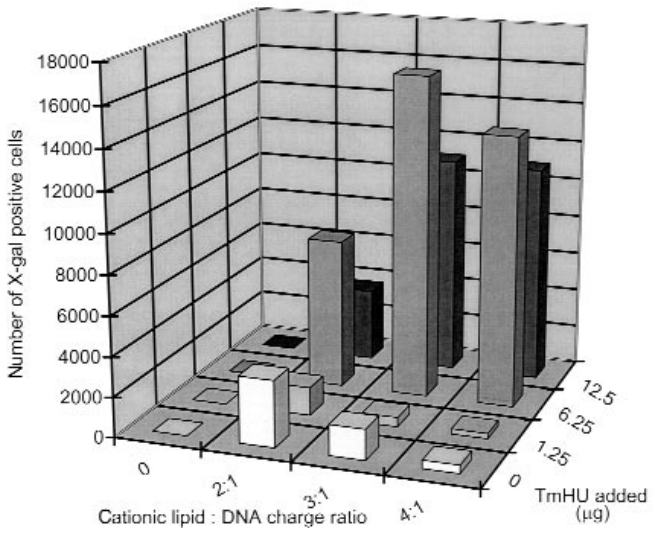

Fig. 3. Enhancement of the efficiency of lipofection by prior incubation of DNA with TmHU. 1 µg of pEli92 plasmid DNA was incubated with various amounts of TmHU and lipid, and the mixture applied to NIH 3T3 cells. The number of X-gal positive cells was counted after 48 h.

In preliminary toxicity studies, TmHU proved to be significantly less toxic than Lipofectin, and no adverse effects have been observed in our in vivo experiments thus far. One important point that has not been addressed yet is the potential immunogenicity of TmHU; the bacterial origin of the protein might elicit an immune response that could be detrimental for repeated injections. Relevant studies will have to be performed to investigate these potential hazards. In addition, as shown in this work, the formation of large aggregates that is inherent to this technique may not always be favorable and may sometimes even pose a potential danger in vivo (e.g., in intravenous applications).

TmHU, a bacterial protein, can mediate transfection of eukaryotic cells with high efficiency because it is a polycation, like many transfection reagents. It binds DNA nonspecifically and protects it efficiently from DNase digestion (data not shown). At a high TmHU:DNA ratio, the surplus positive charges facilitate binding of the TmHU–DNA complex to the negatively charged cell membrane. Furthermore, considering that some cationic amphiphilic peptides are known to cause endosomal disruption[13], a fraction of TmHU molecules or partially denatured molecules may destabilize the endosomal membrane, inducing uptake into the cytosol. The heating pulse may help in partially unfolding TmHU molecules, thus enhancing membrane translocation by exposure of normally buried hydrophobic groups, similar to urea-denatured fusion constructs containing HIV-1 Tat[14]. In addition, we have obtained evidence that, similar to other protein domains[14,15], native TmHU also facilitates the transfer of proteins fused to it, indicating an intrinsic ability of TmHU to cross the cell membrane (unpublished data). The presence of calcium might further enhance endosomolysis, as has been postulated earlier[8]. However, we also observed that calcium caused compaction of the heat-induced precipitates containing TmHU and DNA, thus improving delivery to the cells; it has been demonstrated that this has profound effects on transfection performance[16].

Interestingly, using the program PSORT[17], we could identify two composite nuclear localization signals (NLS) in TmHU. The program estimated a probability of 98% for nuclear localization of the protein in eukaryotic cells. Although it appears to be purely coincidental that a bacterial protein has a eukaryotic NLS, it is intriguing with respect to the high efficiency of transient and stable gene delivery.

Experimental protocol

TmHU purification. TmHU was expressed and purified as described[12]. Briefly, most proteins from the *E. coli* cell lysate were precipitated by heating at 80°C; TmHU remained in the supernatant, was then applied to a Poros HS column (PerSeptive Biosystems, Framingham, MA) and eluted using a linear salt gradient. TmHU, purified with a yield of 20 mg/L culture medium, was homogeneous as determined by SDS–PAGE and UV/VIS absorption spectroscopy.

Transient transfection with TmHU. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. In the optimized transfection protocol, 25 µg TmHU in 50 µl PBS were mixed with 2 µg plasmid DNA (pEli92) containing the *E. coli lacZ* gene under the control of the cytomegalovirus (CMV) immediate early promoter and 5 µl of 20 mM $CaCl_2$. The mixture was incubated at 95°C for 40 min, and subsequently at room temperature for another 20 min. After the addition of 350 µl DMEM with 10% FBS, the transfection mixture replaced the culture medium of confluent cells seeded into 12-well plates. The mixture was removed after 4 h of incubation and replaced with fresh medium. Cells were fixed and stained with X-gal 48 h after transfection using standard protocols.

Intramuscular injection of TmHU–DNA complexes. Fifty microliters of one of the following were injected in quadruplicate into the quadriceps femoris muscles of six-week-old female BALB/c mice: phosphate-buffered saline (PBS); 5 µg plasmid DNA (pGV-C2 encoding luciferase as a reporter gene) in 50 µl PBS; or 5 µg plasmid DNA + 6 µg TmHU in 50 µl PBS[6]. One week after application, the mice were killed, the muscles homogenized, and luciferase expression quantified (PicaGene kit; Toyo Ink Products, Tokyo, Japan). To avoid extensive aggregate formation, the calcium addition and heat incubation were excluded.

Evaluation of toxicity. 293T cells were seeded at a density of 2×10^4 cells/well into 96-well plates. On the next day, cells were overlaid with a mixture consisting of four parts growth medium and one part PBS containing one of the following: TmHU (1–1,000 µg/ml); Lipofectin (1–200 µg/ml); or staurosporin (0.2–200 µM). Twenty-four hours after application, the medium was exchanged with medium containing 20% MTS/PMS (Promega, Madison, WI) and viability assayed by recording the absorption at 490 nm. Medium containing 20% PBS without any further additive showed no effect in the period observed (100% viability), whereas a concentration of 200 µM staurosporin was found to kill all cells (0% viability).

Stable transfection with TmHU. The established protocol for transient transfection in the presence and absence of calcium was used. After transfection of the plasmid pSV2neo (12 µg) to cells seeded in a 60 mm dish and culture for two days, cells were split into three 100 mm dishes and incubated for an additional 12 h. Subsequently, the medium was changed to selective medium containing the antibiotic G418 (1 mg/ml). After two weeks (medium change every three to four days), colonies of G418-resistant cells were stained with methylene blue.

Lipofection in the presence of TmHU. NIH 3T3 cells were seeded one day before lipofection at a cell density of 3×10^4 cells/well in a 24-well plate. Subsequently, 1 µg plasmid DNA (pEli92) was incubated with 0–12.5 µg TmHU in a total volume of 195 µl DMEM. After 1 h at 37°C, Tfx-50 (Promega) was added (0–6 µl corresponding to a 0 to 4:1 cationic lipid:DNA charge ratio), leading to 16 lipofection mixtures. The mixtures were vortexed and incubated for 15 min at room temperature. The culture medium in the wells was aspirated and the cells were incubated with the lipofection mixtures for 1 h, then 1 ml DMEM with 10% FBS was gently overlaid. After the culture of cells for 48 h, X-gal staining was performed.

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Rapid genotyping by MALDI-monitored nuclease selection from probe libraries

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Data on five single-nucleotide polymorphisms (SNPs) per gene are estimated to allow association of disease risks or pharmacogenetic parameters with individual genes[1]. Efficient technologies for rapidly detecting SNPs will therefore facilitate the mining of genomic information[2]. Known methods for SNP analysis include restriction-fragment-length polymorphism polymerase chain reaction (PCR), allele-specific oligomer hybridization, oligomer-specific ligation assays, minisequencing, direct sequencing, fluorescence-detected 5′-exonuclease assays, and hybridization with PNA probes[3–6]. Detection by mass spectrometry (MS) offers speed and high resolution[7,8]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) can detect primer extension products[9–11], mass-tagged oligonucleotides[12], DNA created by restriction endonuclease cleavage[13], and genomic DNA[14]. We have previously reported MALDI-TOF-monitored nuclease selections of modified oligonucleotides with increased affinity for targets[15]. Here we use nuclease selections for genotyping by treating DNA to be analyzed with oligonucleotide probes representing known genotypes and digesting probes that are not complementary to the DNA. With phosphodiesterase I, the target-bound, complementary probe is largely refractory to nuclease attack and its peak persists in mass spectra (Fig. 1A). In optimized assays, both alleles of a heterozygote were genotyped with six nonamer DNA probes (≥125 fmol each) and asymmetrically amplified DNA from exon 10 of the cystic fibrosis transmembrane regulatory gene (CFTR).