

Membrane transducing activity of recombinant Hoxc8 protein and its possible application as a gene delivery vector

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Abbreviations: *Antp*, antennapedia; GST, glutathione S-transferase; *Hox*, homeobox gene in mice related to the *Antennapedia* class gene of *Drosophila*; IPTG, isopropyl- β -D-thiogalactopyranoside; PPF, pig primary fetal fibroblast; PTD, protein transduction domain

Abstract

In order to examine whether the Hoxc8 protein can deliver nucleic acid into mammalian cells, we designed several Hoxc8-derived recombinant proteins to be synthesized as glutathione S-transferase (GST) fused forms in *E.coli* (GST-Hoxc8₁₋₂₄₂, containing a full length of Hoxc8; GST-Hoxc8₁₅₂₋₂₄₂, possessing a deletion of the acidic N-terminus of Hoxc8; GST-Hoxc8₁₄₉₋₂₀₈, which contained the homeodomain only). After labeling these proteins with Oregon 488, we examined their membrane transduction ability under the fluorescence microscope and verified that all three proteins showed similar transduction efficiency. The ability of the proteins to form *in vitro* protein-DNA complexes was analyzed on agarose gel; both GST-Hoxc8₁₋₂₄₂ and GST-Hoxc8₁₄₉₋₂₀₈ formed complexes. In contrast, the GST-Hoxc8₁₅₂₋₂₄₂ protein did not form a complex. The GST-Hoxc8₁₄₉₋₂₀₈ protein formed a complex with DNA at a mass ratio of 1 : 1 (DNA : protein), and GST-Hoxc8₁₋₂₄₂ formed a complex at a mass ratio of 1 : 5. When the DNA (pDsRed1-C1) and protein complexes were added to culture media containing mammalian cells, the cells uptook the complexes, which was indicated by red fluorescence expression under the fluorescent microscope. These results indicate that recombinant Hoxc8 derivatives that harbor a homeo-

domain are able to traverse the mammalian cellular membrane. DNA that is bound to the recombinant derivatives can be carried across the membrane as well. This process could be applied in the development of a useful delivery vector for gene therapy in the future.

Keywords: Antennapedia homeodomain protein; gene transfer techniques; gene therapy; HOXC8 protein, human; transfection

Introduction

The cell membrane acts as a barrier and has been shown to have poor permeability to large molecules. In addition, living cells do not actively import most macromolecules (Gros *et al.*, 2006). One of the main difficulties in the development of new drugs whose targets are located within host cells is membrane impermeability. This challenge is reflected in the fact that greater than 95% of all new therapeutics possesses poor pharmacokinetics. In general, the delivery of macromolecules utilizes lipids, electroporation, or viral vectors (Chae *et al.*, 2005; Neno *et al.*, 2006; Vinogradov, 2006; Cho *et al.*, 2007). However, these tools exhibit several limitations, including: (1) the inability to deliver to primary and non-dividing cells, (2) the requirement for optimization with each cell type, (3) low transduction level, (4) high cellular toxicity (Carriere *et al.*, 2003; Green *et al.*, 2003; Bachran *et al.*, 2005; Kuriyama *et al.*, 2006).

Because of these limitations, many alternative methods of macromolecule delivery have been generated through the observation of protein transduction domains (PTD), particularly PTDs derived from HIV-1 transcriptional regulator Tat (Schwarze *et al.*, 1999; Sihol *et al.*, 2002) and the *Drosophila* transcription factor Antennapedia (*Antp*) (Joliot *et al.*, 1991). These PTDs have been shown to mediate the rapid cellular delivery of proteins (Lundberg *et al.*, 2003) and peptides (Eum *et al.*, 2005) and have been observed to possess the ability to traverse the lipid bilayer of cells in a concentration dependent manner (Langedijk *et al.*, 2004). The exact mechanism of delivery is not fully understood, although the PTDs are generally known to undergo endocytosis (Drin *et al.*, 2003; Ignatovich *et al.*, 2003; Richard *et al.*, 2003; Trehin and Merkle, 2004). The importance of these proteins lies in their ability to uniformly transport large, biologically active molecules to a

population of mammalian cells growing under standard culture conditions (Snyder and Dowdy, 2004).

Hoxc8 is one of many homeobox genes that encode a set of master transcription factors (Lei *et al.*, 2007). It contains a similar homeodomain to that of *Antp* (Le Mouellic *et al.*, 1992). Homeobox genes encode protein domains of 60 amino acids, which are known to play important roles in defining the body plan of vertebrates (McGinnis and Krumlauf, 1992; Waltregny *et al.*, 2002). Homeodomain proteins belong to a class of transcription factors involved in multiple morphological processes. The homeodomain is composed of three α helices with one β turn between helices 2 and 3. The third helix is known to possess translocation properties (Derossi *et al.*, 1998).

It has been found that the C-terminus of *Hoxc8* (the acidic portion) contains many negatively charged cations such as glutamic acid and aspartic acid. Because DNA is highly negatively charged, when DNA and a construct that contains a homeodomain with an acidic C-terminus are combined in solution, a DNA-protein complex will not form due to the repelling effect between the molecules' negative charges (Sloots and Wels, 2005).

Although the entire homeoprotein can be used for delivering macromolecules, small poly-arginine (poly-R) and poly-lysine peptides have been shown to exhibit greater efficiency in delivering several peptides and proteins (Noguchi and Matsumoto, 2006).

Therefore, it was hypothesized that a homeodomain would be a more efficient delivery vector than a full sequence *Hoxc8* protein. In this study, we designed three different constructs and examined their membrane transduction activity: *Hoxc8*₁₋₂₄₂, which contained a full-length protein, *Hoxc8*₁₅₂₋₂₄₂, which contained the homeodomain with a partially deleted N-terminus and fully intact acidic C-terminus, and *Hoxc8*₁₄₉₋₂₀₈, which contained the homeodomain in a glutathione S-transferase (GST) fused form. We also analyzed the possibility of our constructs as delivery vectors for nucleic acids since the cationic homeodomain can electrostatically interact with DNA.

Materials and Methods

Plasmid construction

In order to construct the plasmid pGEX:*Hoxc8*₁₋₂₄₂, a 780 bp *Hoxc8* fragment was isolated from the plasmid pEGFP:C8 (Kwon *et al.*, 2003) after digestion with *Bam*HI and *Xho*I and then inserted into the same sites of the expression vector pGEX4T-1 (Amersham, Uppsala, Sweden). A 306 bp *Sal*I fragment was isolated from pGEX:*Hoxc8*₁₋₂₄₂ after

*Sal*I digestion and then cloned into the *Sal*I site in pGEX4T-1 to produce pGEX:*Hoxc8*₁₅₂₋₂₄₂. For pGEX:*Hoxc8*₁₄₉₋₂₀₈, PCR amplification was performed with *Hoxc8* homeodomain-specific primers (forward primer, 5'-GAGGATCCC-GGCGCAGCGGTTCG-3'; reverse primer, 5'-GGCTCGAGTTCTCCTTTTCC-AC-3'), which were designed to contain the *Bam*HI and *Xho*I sites, respectively (Bioneer, Daejeon, Republic of Korea). Following the PCR reaction (first denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s; annealing at 56°C for 30 s; extension at 72°C for 60 s; final extension at 72°C for 5 min), the amplified fragment was cloned into the pGEM-T Easy vector (Promega, Madison, WI). Then, the *Bam*HI and *Xho*I-containing portion of the 180 bp homeobox fragment was isolated and inserted into the same sites in the expression vector pGEX4T-1 to produce pGEX:*Hoxc8*₁₄₉₋₂₀₈.

Protein purification and SDS-PAGE

In order to purify the fusion proteins, each of the three plasmids - pGEX:*Hoxc8*₁₋₂₄₂, pGEX:*Hoxc8*₁₅₂₋₂₄₂, and pGEX:*Hoxc8*₁₄₉₋₂₀₈ - was transformed into the *E. coli* strain BL21 (DE3) and cultured in 2×YT media containing ampicillin (100 μ g/ml) at 37°C. When the OD₆₀₀ reached approximately 0.8, IPTG (Isopropyl-b-D-thiogalactopyranoside) was added to a final concentration of 500 nM, and the cells were incubated for another 3 h at 28°C. The cells were collected through centrifugation, cracked with a French Press (SLMOAMINCO, SLM instrument Inc., Urbana, IL), and then centrifuged at 12 krpm for 60 min. The supernatant was removed, incubated with glutathione sepharose beads (Sigma, St. Louis, MO) overnight, and then centrifuged for 10 min at 2 krpm. The precipitant was washed three times with ice cold PBS, added to reduced glutathione elution buffer (pH 8.0) containing 50 mM Tris and 10 mM reduced glutathione, and then incubated as per the manufacturer's suggestion. After centrifugation for 5 min at 1 krpm, the supernatant was removed, and the concentration of the purified protein was measured using Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). Purified proteins were denatured in sample buffers (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 100 mM DTT, and 0.1% bromophenol blue) by boiling for 10 min, and then resolved on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were stained with Coomassie blue.

Labeling proteins with Oregon 488

In order to effectively label proteins with Oregon 488 (Invitrogen, Carlsbad, CA), it was advised according to the manufacturer's instructions to use a protein

concentration of higher than 2.0 $\mu\text{g}/\mu\text{l}$. Therefore, purified proteins were concentrated to 4-10 $\mu\text{g}/\mu\text{l}$ using a centrifugal filter device (Amicon Bioseparations Centricon, Milipore, Bedford, MA). To do this, purified proteins were loaded onto the column of the centrifugal filter device and centrifuged for 2,000 g until the desired concentration of protein was obtained. About 5 mg of the protein were dissolved in 0.5 ml of 0.1 M sodium bicarbonate buffer. 100 μl of dye solution (0.5 mg of Oregon 488 dye dissolved in 0.5 ml of DMSO) was then added slowly while vortexing the protein solution. This final solution was incubated for an hour at room temperature with continuous stirring. The dye solution was freshly prepared immediately prior to starting the reaction because reactive compounds are not very stable in solution. The labeled proteins were purified using Sephadex G25 (Sigma).

Cell culture and intracellular delivery of protein and nucleic acid

PPFF (pig primary fetal fibroblast), COS-7 (SV40-transformed African green monkey kidney cells), and T98G cells (Myeloma cells obtained from the brain) were cultured in DMEM (Welgene, Daegu, Korea) containing 10% FBS and 5% penicillin-streptomycin at 37°C in a 5% CO₂ atmosphere. To analyze protein transduction, cells were seeded on a 24-well plate at a density of 1×10^5 cells/well. After incubating for 24 h, the medium was replaced with fresh medium containing fusion proteins (10 nM, 100 nM, 500 nM, or 1 mM) and then incubated again (0, 5 min, 10 min, 15 min, 30 min, 1 h, or 2 h). A trypsin treatment was added, which removed proteins that were electro-statically bound to the cell surface. Protein transduction was analyzed under a fluorescent microscope (Olympus IX70, Olympus, Melille, NY). To analyze the transduction of nucleic acid complexed with recombinant Hoxc8 protein, cells were incubated in the presence of a medium containing the protein-DNA (pDsRed1-C1 plasmid) complex for 24 h. Fresh medium was added, and the cells were incubated 24 h further. After cell viability analysis with the light microscope, the cells were maintained for another 24 h to allow for protein expression. The cells were washed three times with PBS, fixed in 1.5% formaldehyde solution in PBS for 20 min and then permeabilized with 100% methanol. After washing with PBS, cells were stained with DAPI, and analyzed under the fluorescence microscope.

Protein-nucleic acid complex analysis using agarose gel retardation

The complex formation between the recombinant

Hoxc8 protein and the nucleic acid (a reporter plasmid DNA) was investigated using a gel retardation on agarose gel. Increasing amounts of protein (0.1 to 2 μg) were incubated with 0.1 μg of nucleic acid (pEGFP-C1 plasmid), not exceeding 20 μl in total volume. Glycerol was then added to 30%. The mixture was left at room temperature for 1 h, and then the electrophoretic mobility of the resulting protein-DNA complexes was analyzed on 1% agarose gel containing ethidium bromide.

Results

Production of Hoxc8 fusion protein and its fusion derivatives

In order to examine whether the Hoxc8 protein can deliver double-stranded DNA into mammalian cells, several Hoxc8-derived fusion proteins were prepared by cloning into the plasmid pGEX4T-1 (Figure 1). The plasmids pGEX:Hoxc8₁₋₂₄₂, pGEX:Hoxc8₁₅₂₋₂₄₂, and pGEX:Hoxc8₁₄₉₋₂₀₈ were constructed as described in Materials and Methods (Figure 1A). *E. coli* cells containing each plasmid were cultured in 2 \times YT media in the presence of 0.5 mM IPTG at 28°C in order to produce the fusion proteins. To confirm whether the resultant fusion proteins matched the original design, each protein was partially purified using GST-agarose beads and analyzed on a polyacrylamide gel containing SDS. As shown in Figure 1C, the fusion proteins GST-Hoxc8₁₋₂₄₂, GST-Hoxc8₁₅₂₋₂₄₂, and GST-Hoxc8₁₄₉₋₂₀₈ were 54-, 38- and 35 kDa, respectively, matching their original design.

Cellular translocation of GST-fused Hoxc8 derivatives; GST-Hoxc8₁₋₂₄₂, GST-Hoxc8₁₅₂₋₂₄₂, and GST-Hoxc8₁₄₉₋₂₀₈

In order to analyze the translocation efficiency of GST-fused Hoxc8 derivatives, each protein, in addition to GST alone, was labeled with Oregon 488 as described in the Materials and Methods. When the labeled GST-Hoxc8₁₋₂₄₂ was added to the culture media, green fluorescence was detected inside of all PPFF cells as shown in Figure 2 (A and B), indicating that the labeled protein entered the cell through the lipid bilayer; this was expected based on previous results (Lee *et al.*, 2006). In contrast, no fluorescence or very low levels of fluorescence were detected in the cells treated with Oregon 488 dye (Figure 2C) and GST protein (Figure 2D). Although a small amount of GST was found to enter the cell, the translocation efficiency of the GST-Hoxc8₁₋₂₄₂ was far greater than that of GST alone.

The cellular internalization of proteins was tested using 500 nM of Oregon 488-labeled GST-Hoxc8₁₋₂₄₂

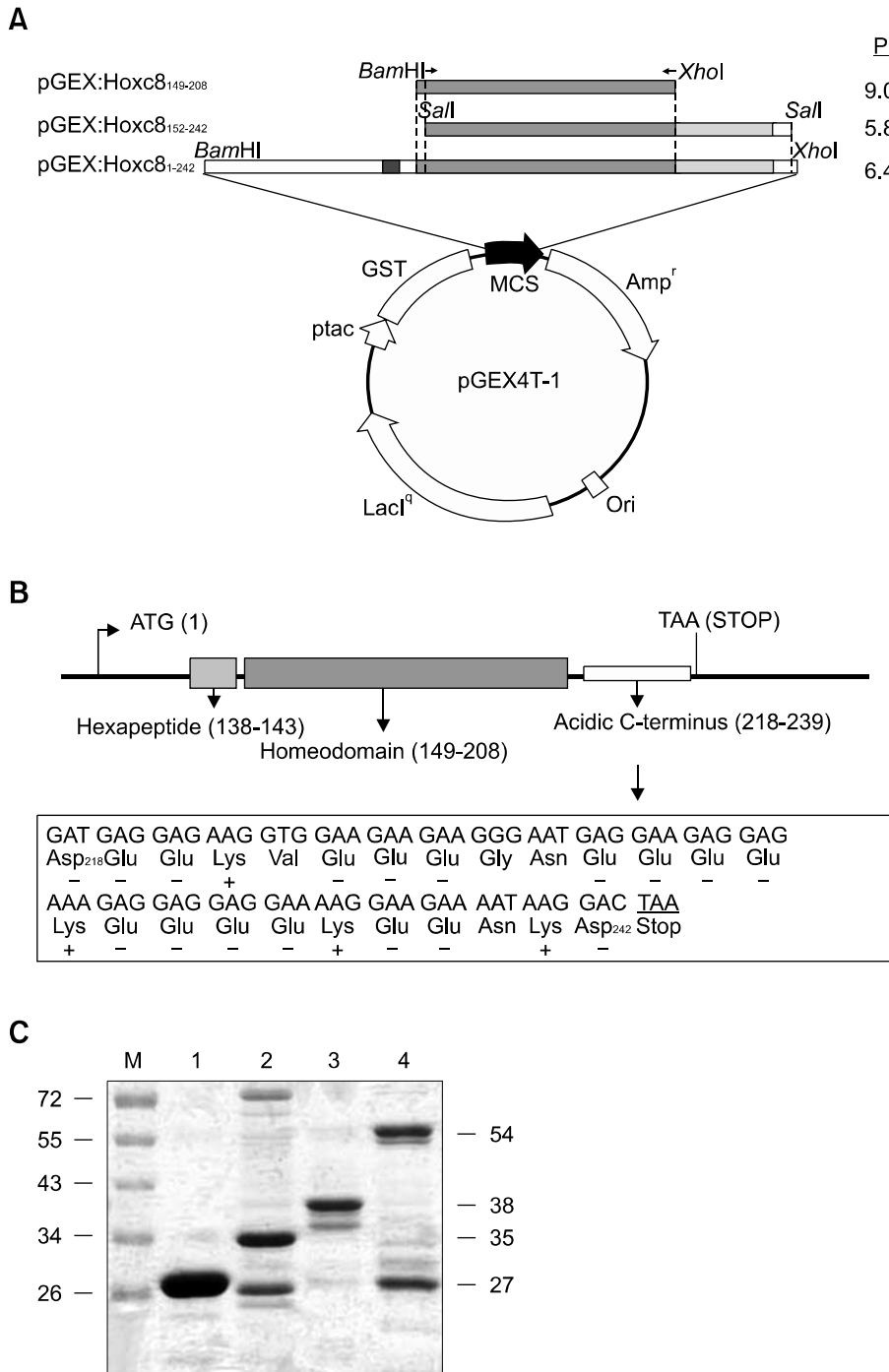


Figure 1. Schematic drawing of plasmids and SDS-PAGE gel showing partially purified proteins of GST:Hoxc8₁₋₂₄₂, GST:Hoxc8₁₅₂₋₂₄₂ and GST:Hoxc8₁₄₉₋₂₀₈. (A) The full length Hoxc8 gene was inserted into the *Bam*HI and *Xho*I sites located in the multi cloning sites (MCS) of the pGEX4T-1 vector, creating pGEX:Hoxc8₁₋₂₄₂, in which the Hoxc8 protein was expressed as a glutathione-S-transferase (GST)-fused form under the control of the *tac* promoter (*ptac*) and operator in the presence of an inducer (IPTG). The construct pGEX:Hoxc8₁₅₂₋₂₄₂ was obtained by digesting pGEX:Hoxc8₁₋₂₄₂ with *Sal*I endonuclease and ligating into the same site in the pGEX4T-1 vector. With pGEX:Hoxc8₁₄₉₋₂₀₈, a homeodomain fragment was amplified using PCR in the presence of a homeodomain specific primer set that contained *Bam*HI and *Xho*I sites at each end (*Bam*HI, 5'-GAGGATCCCGCGCAGCGGTTCG; *Xho*I, 5'-GGCTCGAGTTCTCCTTTT-TCCAC). The fragment was then cloned into the same sites in the pGEX4T-1 vector after digestion with *Bam*HI and *Xho*I. Each restriction site (*Bam*HI, *Xho*I, and *Sal*I) that was used for cloning, the replication origin (*ori*), the ampicillin resistance gene (*Amp*^r), and the Lac repressor gene (*LacI*^q) are marked on the map. The calculated PI of each GST-fusion protein was written on the right. (B) Schematic illustration of the Hoxc8 protein indicating the hexapeptide, the homeodomain, and the acidic C-terminus with the amino acid position in numbers. At the bottom, the amino acid residues from AA₂₁₈ through AA₂₄₂ are shown along with the stop codon, TAA. (C) SDS-PAGE gel showing partially purified proteins of GST:Hoxc8₁₋₂₄₂, GST:Hoxc8₁₅₂₋₂₄₂ and GST:Hoxc8₁₄₉₋₂₀₈. Each GST-fused protein was partially purified using glutathione agarose beads, analyzed on the 10% polyacrylamide gel containing SDS, and then detected by Coomassie staining. Lanes 1 to 4 indicate GST, GST:Hoxc8₁₄₉₋₂₀₈, GST:Hoxc8₁₅₂₋₂₄₂, and GST:Hoxc8₁₋₂₄₂, respectively. The molecular weights of marker (M) and partially purified proteins are written on the left and right in kDa, respectively.

protein following different incubation times (5 min, 10 min, 15 min, 30 min, 1 h, and 2 h). The green fluorescence intensity in the cell gradually increased with time until reaching a plateau at 1 h of incubation (Figure 2E).

In order to test the optimum fusion protein concentration for cellular transduction, 10 nM, 100 nM, 500 nM and 1 μM of Oregon 488-labeled full

length Hoxc8 fusion proteins (GST-Hoxc8₁₋₂₄₂) were added to the T98G cells. As shown in Figure 3 (A-D), the intensity seemed to reach a peak at 500 nM of proteins after 1 h of incubation. When the transduction efficiency of the other two fusion proteins, GST-Hoxc8₁₅₂₋₂₄₂ and GST-Hoxc8₁₄₉₋₂₀₈, were analyzed in the same way, both showed similar results (Figure 3E-L), indicating that all three pro-

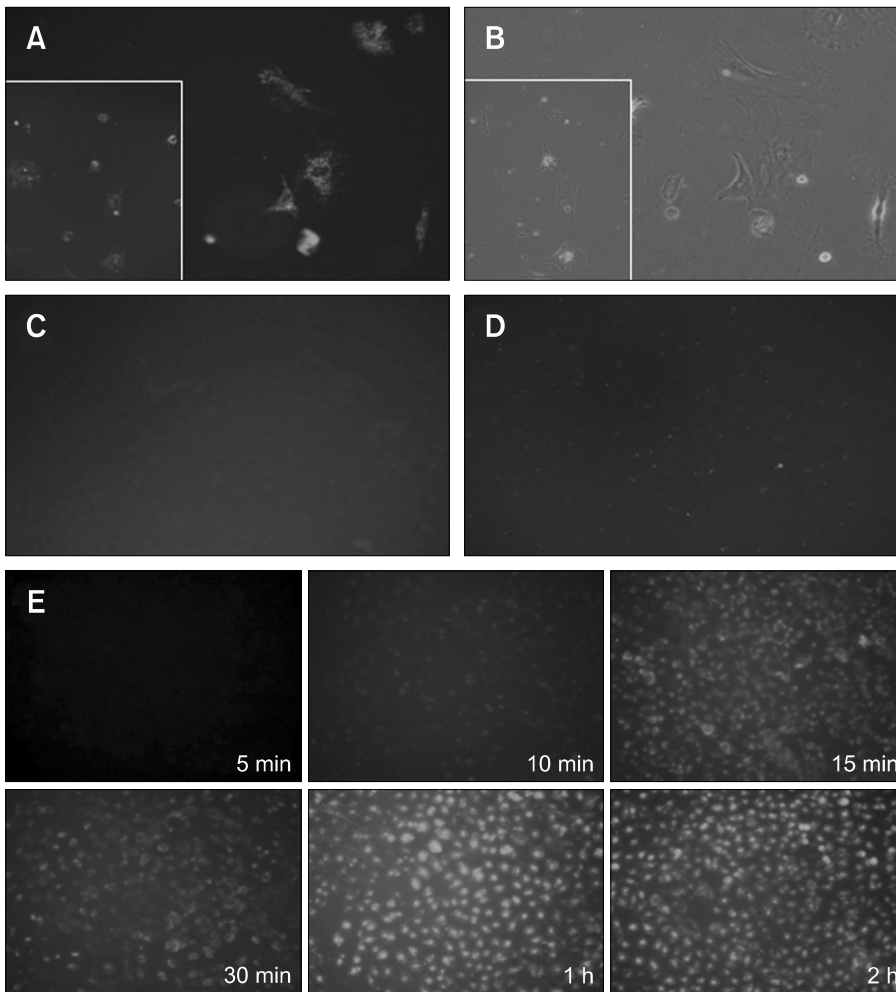


Figure 2. Cellular transduction of Oregon 488-labeled GST:Hoxc8₁₋₂₄₂ in PPF cells. PPF cells were cultured in the presence of Oregon 488-labeled GST:Hoxc8₁₋₂₄₂ protein (500 nM) as described in Materials and Methods and analyzed under the fluorescence (A) and light microscope (B). Oregon 488 alone (C) and Oregon 488-labeled GST protein (D) were included as controls. (E) Time course of protein (Oregon 488-labeled GST:Hoxc8₁₋₂₄₂) transduction. After adding the fusion protein GST:Hoxc8₁₋₂₄₂ labeled with Oregon 488 for 5 min, 10 min, 15 min, 30 min, 1 h, or 2 h, cells were washed with PBS and then fluorescence microscopy was taken.

teins have similar transduction efficiencies.

Formation of DNA-protein complex

In order to test whether the Hoxc8 protein could be applicable to the development of delivery vector for nucleic acid, the protein and DNA interaction was investigated using a gel retardation experiment. As described in Materials and Methods, different concentrations of GST-Hoxc8 fusion proteins were incubated with the same amount of DNA. GST-Hoxc8₁₋₂₄₂ seemed to bind well with DNA (Figure 4); most of the DNA complexed with the proteins in a ratio of 1 : 15 (mass ratio of DNA : protein). In the case of GST-Hoxc8₁₅₂₋₂₄₂ having PI value of 5.8, the formation of DNA-protein complexes was inhibited. In contrast, GST-Hoxc8₁₄₉₋₂₀₈ containing only the homeodomain (PI, 9.0) regained the ability to form DNA-protein complexes. Even with small amounts of GST-Hoxc8₁₄₉₋₂₀₈ protein (at the mass ratio of 1 : 1), high amounts of protein-DNA complex were

formed; about 50% of DNA formed complex with this protein at this mass ratio (Figure 4, lane 2). As controls, BSA and GST proteins did not form complexes with DNA at any concentration, even when 20 × excess proteins were added.

Intracellular delivery of DNA complexed with murine Hoxc8 homeoprotein

In order to test whether the Hoxc8 protein could act as a nucleic acid delivery vector for future therapeutic purposes, the protein-DNA complexes were added to COS-7 cells in fresh DMEM medium containing 10% FBS as described in Materials and Methods. For easy observation of delivered DNA, pDsRed1-C1 plasmid encoding red fluorescent protein was used. And the fusion protein GST-Hoxc8₁₄₉₋₂₀₈ showing the most efficient complex formation with DNA was chosen as cargo protein of DNA. pDsRed1-C1 DNA (1 μg) complexed with GST-Hoxc8₁₄₉₋₂₀₈ at a mass ratio of 1 : 16 (DNA :

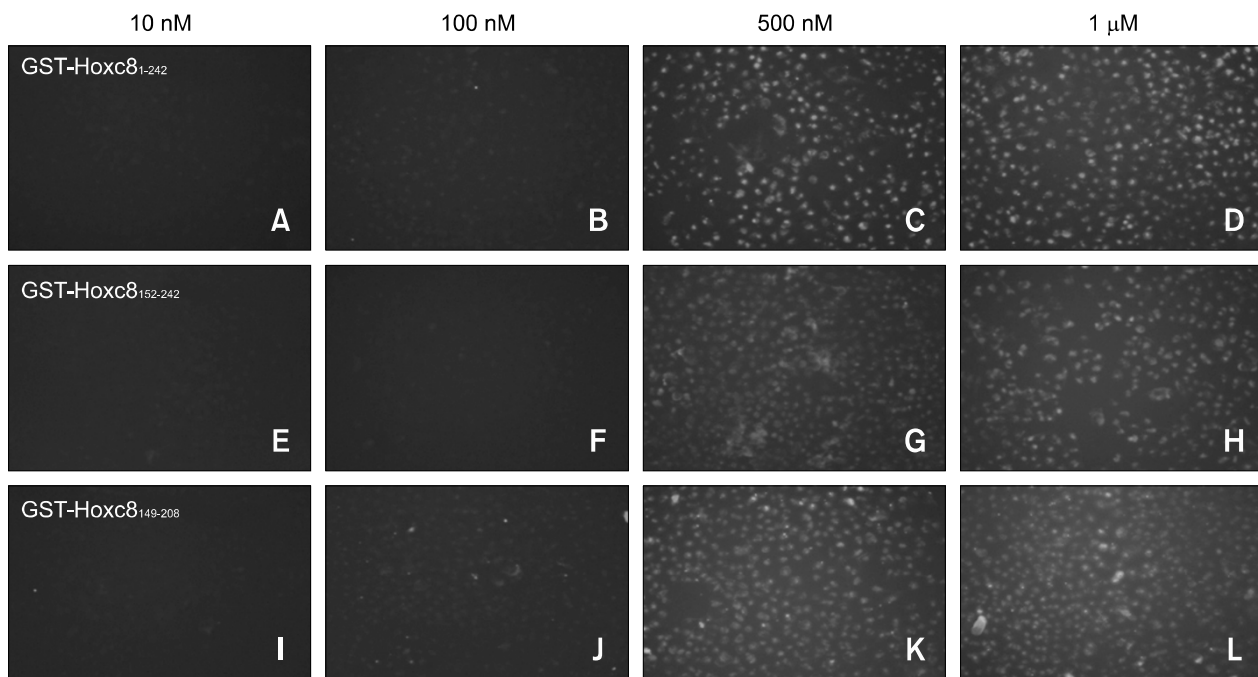


Figure 3. Fluorescence microscopy showing the cellular transduction of each recombinant fusion protein labeled with Oregon 488. Each labeled protein of GST:Hoxc8¹⁻²⁴² (A-D), GST:Hoxc8¹⁵²⁻²⁴² (E-H), or GST:Hoxc8¹⁴⁹⁻²⁰⁸ (I-L) was added to the media containing T98G cells at different concentrations; 10 nM (A, E, and I), 100 nM (B, F, and J), 500 nM (C, G, and K) and 1 μM (D, H, and L). The protein transduction was then analyzed under the fluorescent microscope.

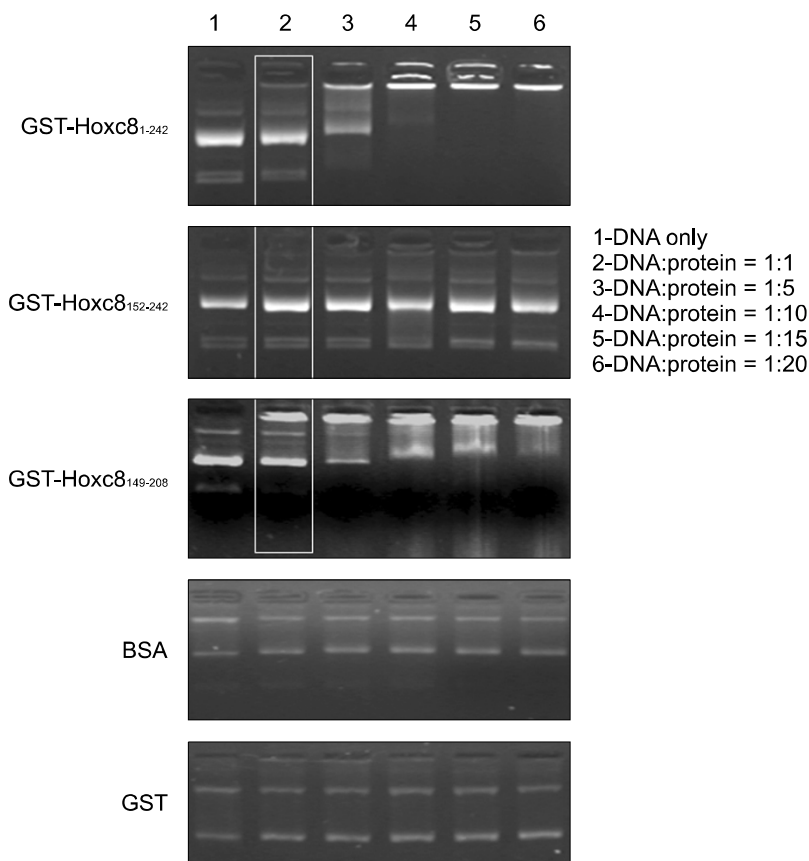


Figure 4. Gel retardation experiment for DNA-protein complex. Different amounts of each protein (GST:Hoxc8¹⁻²⁴², GST:Hoxc8¹⁵²⁻²⁴², GST:Hoxc8¹⁴⁹⁻²⁰⁸, BSA, and GST) were mixed with 0.1 μg of pEGFP-C1 plasmid in 20 μl of reaction volume and were kept at room temperature for 1 h. After electrophoresis of the reaction mixtures on a 1% agarose gel containing ethidium bromide, photography was taken under the UV lamp.

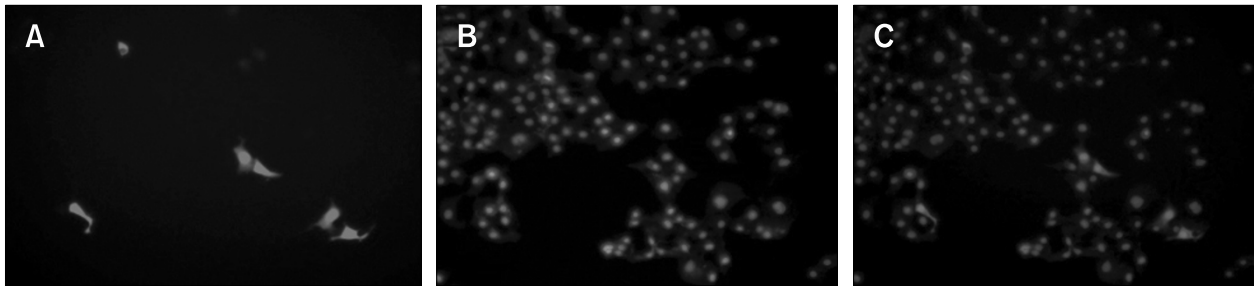


Figure 5. Intracellular delivery of DNA complexed with the recombinant murine Hoxc8 homeoprotein. The recombinant fusion protein GST:Hoxc8₁₄₉₋₂₀₈ complexed with pDsRed1-C1 plasmid was transfected into the COS-7 cells and then analyzed the red fluorescence under the fluorescence microscope as described in the Materials and Methods. (A) Red color indicates the expression of red protein encoded in the pDsRed1-C1 DNA transfected into the cells along with the GST:Hoxc8₁₄₉₋₂₀₈ protein. (B) and (C) are the DAPI staining of (A) and merged photo of (A) and (B), respectively.

protein) was applied to a well (12-well plate), incubated for 24 h, and then analyzed under the fluorescence microscope for red fluorescence. As shown in Figure 5, the red fluorescence protein turned out to be expressed and detected in the cells indicating that the murine Hoxc8 homeoprotein GST:Hoxc8₁₄₉₋₂₀₈ delivered the nucleic acid (pDsRed1-C1 plasmid) into COS-7 cells.

Discussion

Protein transduction domains (PTD) have received much attention since being reported to traverse biological membranes. The homeodomain of Antennapedia, which acts as a PTD, is well known and well studied. In this study, we show that the homeodomain of Hoxc8 can traverse mammalian cell membranes. According to Derossi *et al.* (1996), the third helix of the Antennapedia homeodomain is essential for cellular internalization of Antennapedia. Derossi *et al.* (1996) found that one mutant, which had two hydrophobic residues (tryptophan48 and a phenylalanine49) deleted from the third helix of the homeodomain, lost its translocating ability. Using synthesized peptides, they found that a peptide of 16 amino acid residues, which corresponded to amino acids 43-58 of the homeodomain (penetratin-1 or 'third helix'), possessed translocation properties comparable to that of the entire homeodomain.

We have previously reported that murine Hoxc8 homeoprotein can traverse the cellular membrane efficiently (Lee *et al.*, 2006). We have found that the Hoxc8 protein contains many glutamic acid and aspartic acid residues at the C-terminus (Figure 1B), which gives the protein a negatively charged property. When PTDs are constituted with basic amino acids such as poly-Arg or poly-Lys, they seem to be more effectively transduced into cells. Therefore, in

this study, we constructed several Hoxc8 deletion derivatives that contained homeodomains and analyzed their transduction activity alone or with nucleic acids. We generated three types of GST-Hoxc8 fusion proteins: a protein that contained a full length Hoxc8 (GST-Hoxc8₁₋₂₄₂), a protein that contained a homeodomain, which possessed a negatively charged C-term (GST-Hoxc8₁₅₂₋₂₄₂), and a protein that contained a homeodomain with a deleted C-term (GST-Hoxc8₁₄₉₋₂₀₈) (Figure 1). With GST-Hoxc8₁₅₂₋₂₄₂, 3 amino acids were missing at the beginning of the homeodomain. These missing amino acids were not considered to be of serious importance because it has been suggested that the first few amino acids of the homeodomain are not important, while the last helix is crucial (Roy and Sen, 2005).

In order to investigate protein transduction with the fluorescence microscope, the proteins were labeled with Oregon 488. The Oregon 488 dye alone could not cross the cell membrane (Figure 2C). As expected, the GST-Hoxc8₁₋₂₄₂ protein traversed the cellular membrane efficiently (Figure 2A and B). The GST alone showed a very weak signal (Figure 2D), which was not surprising because GST itself has been reported to be a PTD (Namiki *et al.*, 2003). Our previous work has also shown GST to possess low cell traversing activity (Lee *et al.*, 2006). Although more research is needed to prove that the Hoxc8 protein alone is an effective penetrating protein, the protein itself could be as effective as GST-Hoxc8. In our laboratory, a 16 amino acid derivative of the Hoxc8 homeodomain has been shown to have PTD activity (Data not shown).

When the three different GST-Hoxc8 fusion proteins were tested to find the optimum concentration for transduction activity, all three proteins (GST-Hoxc8₁₋₂₄₂, GST-Hoxc8₁₅₂₋₂₄₂, and GST-Hoxc8₁₄₉₋₂₀₈) seemed similar, showing the highest efficiency at 500 nM concentration after 1 h incubation. Further-

more, all of them were internalized into almost 100% of the cells, showing even more transduction efficiency similarities. The sub-cellular localization seemed to be in both the cytoplasm and the nucleus in all cases (Figure 2 and 3).

When complexed to DNA, each of the three proteins had very different complex formation efficiencies. GST-Hoxc8₁₅₂₋₂₄₂ did not form a complex with DNA, like the control proteins BSA and GST both did. This could be due to the negatively charged C-term of Hoxc8, which could have repelled the acidic DNA. In fact, GST-Hoxc8₁₅₂₋₂₄₂ is an acidic protein with a theoretical PI of 5.8; BSA has a theoretical PI of 5.82. In contrast, both GST-Hoxc8₁₋₂₄₂ and GST-Hoxc8₁₄₉₋₂₀₈ formed complexes with DNA (Figure 4). The PI of GST-Hoxc8₁₋₂₄₂ and GST-Hoxc8₁₄₉₋₂₀₈ is 6.37 and 9.0, respectively. Although the GST-Hoxc8₁₋₂₄₂ contained an acidic C-term, it was still able to complex with DNA. GST-Hoxc8₁₋₂₄₂ contained a full length Hoxc8 protein, which is a well known transcription factor that can bind to DNA through a helix-turn-helix DNA binding motif. Regional specificity for binding seemed to be enough to form a complex with DNA even though GST-Hoxc8₁₋₂₄₂ is acidic as a whole. This could be partially explained by the fact that the PI of GST itself is 10.84. However, the GST alone did not form a complex with DNA, meaning that the positive charge itself is not sufficient for DNA binding. The basic protein GST-Hoxc8₁₄₉₋₂₀₈, which contained only the homeodomain, made a complex with acidic DNA more efficiently than the other fusion proteins, as was expected from another report's results (Unamalai *et al.*, 2004).

In this study, we found that GST-Hoxc8 fusion proteins not only traversed the mammalian cell membrane, but also delivered nucleic acids into cells (Figure 5), although the transfection efficiency was rather low; less than 5% of the cells expressed the red fluorescence protein (Figure 5). This result is still interesting since it is the first time to show that Hoxc8 PTD can deliver DNA into the cell. In the case of Tat PTD, it also formed a complex with DNA very well. However, the efficiency of gene transfer was reported to be extremely low, almost null unless poly lysine was fused to the Tat PTD (Tat-pK), in which case about 5% of the cells expressed foreign gene (Hashida *et al.*, 2004). In order to express foreign genes, DNA must be translocated across the cell membrane and then transported into the nucleus. Recently Doyle and Chan (2007) have reported that the intracellular localization of DNA influenced the exogenous gene expression in mammalian cells: DNAs complexed with PEI (polyethylenimine) were found to be located in the close proximity to the nucleus and expressed highly,

whereas DNAs complexed with PEI conjugated with polyarginine PTD were found to be widely distributed throughout the cytoplasm and negligible gene expression was noted although polyarginine PTD enhanced the membrane transductional properties of PEI. Although the intracellular localization of the plasmid DNA (pDsRed1-C1) was not examined here, the DNA could be located in somewhere unfavorable for expression. Harada *et al.* (2006) also reported that the transfection efficiency of Tat-pK was easily influenced by several factors, such as pH or temperature of medium, preincubation period, and quantity of DNA. Not only these we can not exclude the possibility of DNA degradation before and/or after entering the cell. Thus far, there have been many reports on PTD as a delivery vector for proteins though, not many cases have been described for nucleic acid delivery (Morris *et al.*, 2000, 2004; Mukherjee *et al.*, 2005). Although the transfection efficiency was not as effective as commercialized lipofectamine or viral vectors (Harada *et al.*, 2006), using PTDs in combination with other gene delivery systems could enhance the transfection efficiency of PTDs in the future.

In conclusion, the homeodomain of Hoxc8 has high membrane traversing ability in mammalian cells. Viral vectors are mostly used in gene therapy, though they have been reported to have high toxicities and cause immune reactions. However, no alternatives are yet available. PTDs like Hoxc8 could be a promising tool for delivering macromolecular nucleic acids such as oligonucleotides or DNA/siRNA in research or in clinical use. The results presented here could be a step in the development of safe and tissue-specific vectors for gene therapy.

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