Expression of recombinant alpha-1 antitrypsin in CHO and COS-7 cell lines using lentiviral vector

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Abstract
In this study, in order to facilitate and accelerate the production of eukaryotic protein alpha 1-antitrypsin (AAT) with correct post-translational modifications, a protein production system based on the transduction of CHO and COS-7 cells using lentiviral vectors was developed. Human *AAT* cDNA was cloned into a replication-defective lentiviral vector. The transgene *AAT-Jred* chimer was transferred to CHO and COS-7 cell lines using this vector and its expressions were visualized by fluorescent microscopy. The mRNA expression levels of the *AAT* genes were determined using Revearse Transcriptase-Polymerase Chain Reaction (RT-PCR) and its secretion into the medium by both cell types was determined using ELISA. The results show that by employing a lentiviral vector, efficient genetic loading of CHO and COS-7 cells with the *AAT* gene was achieved. In conclusion, by using a *Lentivirus*-based gene delivery system, large amounts of recombinant human AAT protein were expressed in both CHO and COS-7 cell lines. This expression system possesses key properties that ensure its application in the delivery of therapeutic genes into mammalian cultured cells.
Keywords: Alpha-1 antitrypsin; Transfection; Protein production; Lentiviral vector

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INTRODUCTION

Alpha-1-antitrypsin (AAT) is the major blood-borne inhibitor of neutrophil elastase; an enzyme released by neutrophils at sites of inflammation which can degrade all of the constituents of the pulmonary connective tissue matrix in (Perlmutter, 2006; Tamer and Chisti, 2001). In the event of AAT deficiency, elastase attacks and damages lung tissue, causing potential hereditary emphysema (Kumpalume *et al*., 2007). It may affect both the structure and the function of the lungs and can lead to premature death if left untreated (Francavilla *et al*., 2000). AAT deficiency can also lead to progressive liver dysfunction in children and can cause neonatal or juvenile liver disease (Fregonese *et al*., 2008; de Serres, 2002).

The only specific treatment for AAT deficiency available at present is augmentation therapy by intermittent intravenous administration of AAT. In AAT replacement therapy, each patient receives 4 g of AAT/week, i.e., 208 g/year (Kumpalume *et al*., 2007). The AAT for augmentation therapy is currently purified from donor blood and commercially sold as Prolastin. But this source is limited, expensive (approximately $130/g) and prone to contamination with infectious agents. The high cost of blood-derived AAT results from several factors, including a low concentration of AAT in blood, a complex purification

protocol, tests and processes that are needed to provide a reasonable degree of assurance of the product's freedom from blood-borne diseases (Tamer and Chisti, 2001). A less expensive supply of AAT from a recombinant producer can greatly reduce the cost of treating patients. Human recombinant AAT has been expressed in various yeasts, bacteria, animal and plant cells (Tamer and Chisti, 2001).

Production of recombinant proteins in bacteria is a relatively quick, efficient, and inexpensive procedure (Palomares *et al*., 2004). However, a major disadvantage of a prokaryotic expression system is that protein processing in bacteria occurs by a mechanism that differs significantly from that in mammalian cells (Gaillet *et al*., 2007). One of the main post-translational events is the attachment of complex sugar structures to the majority of secreted proteins. Glycoproteins mediate many diverse functions and the glycosylation pattern of secreted proteins influences their activity as well as their clearance from the body. The recombinant AAT molecules produced in bacteria lack carbohydrates. Consequently, their plasma half-life is markedly reduced, obviating the intravenous route for their administration (Garver *et al*., 1987). Since many bacteria have a high endotoxin content that may be pathogenic to humans (Knappskog *et al*., 2007), for patients, especially those who should be subjected to long term treatment with repeated injections of a recombinant protein, there is a high risk of transmission of toxins and infectious particles if the recombinant protein is produced in bacteria.

Recombinant protein expression systems have also been developed using yeast and insect cells. However, even with these eukaryotic systems, post-translational modifications, such as glycosylation and processing of large protein precursors are not performed exactly as in mammalian cells (Gaillet *et al*., 2007; Andersen and Krummen, 2002). Glycoforms can significantly differ in their activity, physicochemical properties and pharmacokinetics. Although AAT does not require its carbohydrate side chains for activity, but the *in vivo* half-life of AAT expressed in yeast is 50-fold lower than that of plasma-derived AAT (Garver *et al*., 1987). Therefore, it would seem prudent to produce AAT in a mammalian expression system capable of making the appropriate posttranslational modifications. During the past two decades, cultured mammalian cells have become a widely used platform for producing recombinant therapeutic proteins (Wurm, 2004). Other major advantages of protein expression in mammalian cells are that proper posttranslational modifications, convey higher quality and efficacy to the protein compared with proteins produced by bacteria and yeast (Kwaks and Otte, 2006).

Chinese hamster ovary (CHO) and COS-7 cells [derived from cells being CV-1 (simian) in origin, and carrying the SV40 genetic material] are most frequently employed in the production of recombinant proteins for research and therapeutic applications. This popularity stems from the fact that CHO and COS-7 cells can be grown to very high densities and are capable of correctly folding and processing recombinant proteins (Butler, 2005; Wurm, 2004; Andersen and Krummen, 2002).

A great number of research concerning the introduction of the gene of interest into mammalian cells to achieve stable protein production has so far been carried out. There are a variety of ways of introducing foreign DNA into the nucleus of mammalian cells. These methods include calcium phosphate coprecipitation, electroporation, transfection with DEAE-dextran, lipofectamine and other chemical reagents (Ramos *et al*., 2002). Obtaining stable transfectants using any of these methods is slow, elaborate and time-consuming with low efficiency. None of the aforementioned transfection methods offer a simple, rapid and straightforward manner for production of milligram quantities of protein (Ramos *et al*., 2002; Lundstrom *et al*., 2001), which may be possible to achieve via viral transduction.

In this study, we aim to present a lentiviral system engineered for the expression of normal human AAT together with a tracking jred gene, and test its efficacy for transduction of CHO and COS-7 cells. The trasducted cells will be examined for secretion of AAT in the medium and the results help us to decide if these cells are useful as a bioreactor for producing AAT protein in convenient quality and with suitable post-translational modification which is needed for therapeutic purposes.

MATERIALS AND METHODS

Chemicals and reagents: Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM-F12), fetal bovine serum (FBS), penicillin-streptomycin, phos-

phate-buffered saline (PBS) and Trypsin-EDTA solution were obtained from Gibco BioCult (Paisley, UK). RNA extraction, cDNA synthesis and hot start PCR master mix kits were purchased from Qiagen (USA). L-glutamine and other reagents were from Sigma Aldrich Co. (USA). The ELISA quantitation kit for AAT was obtained from Genway Biotech Inc. (USA). AAT standard protein was purchased from Sigma Aldrich (USA). The transfer vector containing *Jred* expression cassettes and the puromycin-resistance gene were purchased from Open Biosystems Co (USA). Envelop plasmid pMD2G, coding for the broad range VSV-G envelope and the packaging plasmid psPAX2 were obtained from Addgenes Co. (USA).

CHO, COS-7, HEK 293T and HepG2 cell line cultures: CHO, COS-7, HEK 293T and human HepG2 hepatoma cells were obtained from the Pasteur Institute of Iran, Tehran. The HEK 293T, COS-7 and human HepG2 were maintained in DMEM, supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin, and 100 µg of streptomycin.

The CHO cells were maintained in DMEM-F12 medium supplemented with 10% (v/v) FBS, 2mM L-glutamine, 100 U/ml penicillin, and 100 µg of streptomycin. All cell lines were grown as a monolayer culture and maintained at 37°C in a humidified atmosphere of 5% CO_2, according to the supplier's instructions.

Construct: The target construct contained the puromycin-resistance gene and the *Jred* expression cassette under the control of the CMV promoter. *AAT* cDNA (1257 bp) was extracted from the HepG2 cells by RT-PCR. Total mRNA was isolated using the RNeasy Mini Kit protocol according to the manufacturer's instructions. Single-stranded cDNA was prepared from 1 µg of total RNA and 0.5 µg of oligo-$(dT)_{15}$ primer using the reverse transcription-PCR protocol of the First Strand cDNA Synthesis Kit.

The cDNA was then amplified by PCR using the following primers; forward 5′-TACT CG AGATGCCGTCTTCTGTCTCG -3′ and reverse 5′-ACACGCG T TTATTTTTGGGTGGGATTC-3′, and ligated into *Xho*I and *Mlu*I sites of the PlexJred transfer vector. PCR parameters for amplification of the *AAT* genes (1257 bp) were as follows: an initial denaturation step of 5 min at 95°C followed by 32 cycles consisting of 45 s of denaturation at 94°C, 45 s of annealing at 55°C and a 1 min extension at 72°C, with a final extension step of 7 min at 72°C. Fragments introduced into the transfer vector were confirmed by digestion and the cycle sequencing method.

Production of *lentivirus* vectors: Viral particles were produced as previously reported and used to infect CHO and COS-7 cells. The lentiviral vector was generated by calcium phosphate-mediated transfection of HEK 293T cells (Sena-Esteves *et al*., 2004). Briefly, 5×10^6 293T cells were used to inoculate 10 ml of DMEM medium in 10 cm^2 plates (Nunc) and transfected the following day with 21 µg of transfer vector plasmid (Plex-Jred) carrying the *AAT* gene, 10.5 µg of the pMD2G plasmid and 21 µg of the psPAX2 plasmid. Medium was removed at approximately 14-16 h post-transfection and replaced with 10 ml of fresh preheated virus collecting medium. Supernatants were collected on post-transfection days 2 and 3 and filtered through a 0.4 µm pore size filter and concentrated 10-fold using an Amicon ultra centrifugal filter device (Millipore, Billerica, MA). The titer of the concentrated vectors was checked with HEK 293T cells.

Vector titration: Titers of the *Jred*-expressing virus were determined by infection of HEK 293T cells using serial dilutions of concentrated virus stock. 4×10^5 293T cells/2ml of media/well were plated in 5 wells of a 6 well plate, 4 wells for virus plus one well for a negative control. Serial dilutions of the virus were made in 1.5 ml eppendorf tubes (0.5 µl, 1 µl, 2 µl, 25 µl and 50 µl volumes of the virus were each added to 2ml of medium); these were then added to the pre-plated 293 T cells. After 72 h, the *Jred*-expressing cells (4×10^5) were quantified by Fluorescent Activated Cell Sorting (FACS) for each dilution to determine transducing units (TU) per ml. Titers of virus were calculated as followes:

Cell number × 2 (doubling factor in 24 h) × % Jred$^+$ cell × 1000/µl virus.

Lentiviral vector transduction: The goal of this study was to create a delivery system to stably express *AAT* in CHO and COS-7 cells using lentiviral vectors. The human *AAT* gene was introduced into a self-inactivating lentiviral vector expressing *Jred* under the control of the CMV promoter. Jred serves as an internal transfection control by marking all cells that

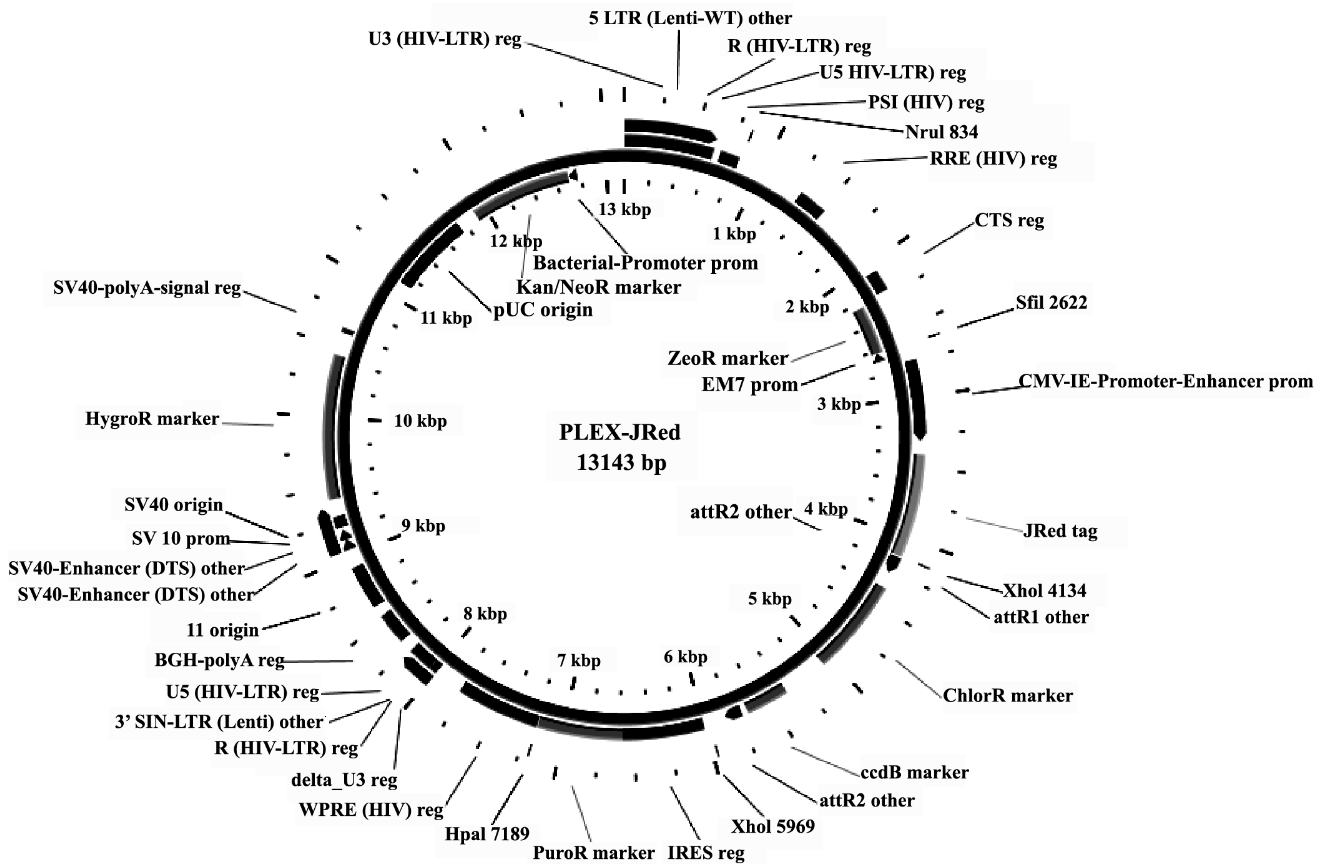

Figure 1. Schematic representation of the Plex-Jred transfer vector map. *AAT* gene was ligated into *Xho*I and *Mlu*I sites of PlexJred.

receive the target plasmid having the *AAT* gene. 2×10^4 CHO and COS-7 cell/cm^2 in 24-well culture plates were transduced with 10-fold concentrated lentiviral vectors (2.8×10^7 TU/ml). A single round of transduction was carried out at various multiplicities of infection (MOI of 5-50) for 6 h in the presence of polybrene (8 µg/ml). After this period, virus supernatants were removed and 1 ml of fresh medium was added to the remaining cells. *Jred* expression was visualized using a fluorescence microscope (TE-2000, Nikon, Japan) 3 days post-transduction. The plated cultures were maintained at 37°C and 5% CO_2 in the medium permanently containing puromycin (1 µg/ml). Several clones expressing *AAT* were then picked for propagation. Supernatants were continuously collected from these expanded clones during 15 days and stored frozen (at -70°C) until assayed for ELISA.

RT-PCR reaction: Total mRNA was isolated from transduced CHO and COS-7 cells using the RNeasy Mini Kit protocol, according to the manufacturer's instructions. DNase I digestion was also performed to minimize the possibility of genomic DNA contamination. Single-stranded cDNA was prepared from 1 µg of total RNA and 0.5 µg of oligo-$(dT)_{15}$ primer using the reverse transcription-PCR protocol of the First Strand cDNA Synthesis Kit. cDNA from the reverse transcription reaction was then used in the subsequent PCR reactions with 0.2 µmol/l of the forward primer 5′-AGAGACCCTTTGAAGTCAAGGACACC-3′ and 0.2 µmol/l of the reverse primer 5′-TTCAGGGGTGCCTCCTCTGTGACC-3′. PCR parameters for amplification of the *AAT* genes were as follows: an initial denaturation step of 5 min at 95°C followed by 32 cycles consisting of 95°C/45s (denaturation), 55°C/45s (annealing) and 72°C/60s (extension), with a final extension of 10 min at 72°C. The amplified PCR fragment of 400 bp was electrophoresed on 2% (w/v) agarose gel and visualized with ethidium bromide staining. The PCR mixture without the template (cDNA) was used as a negative control.

Enzyme linked immunosorbent assay (ELISA): An ELISA quantitation kit with a detection range of 7.5-500 ng/ml was used to measure the secreted human AAT concentration in the culture medium according to the protocol provided by the manufacturer. The purified human AAT protein was used as standard. AAT concentration in the culture medium was measured on day 7 and 14 in stable transfected cell cultures. Microplates were coated with anti-hAAT (1:312) in 0.05M bicarbonate buffer (pH 9.6) for 60 min at 4°C. Standards and culture extracts containing hAAT, FBS, and DMEM supplemented with 10% (v/v) FBS (basal media) were added to each related wells and then plate was incubated at 37°C for 1 h. After blocking, a detection procedure was performed using the second antibody, peroxidase conjugated chicken anti-hAAT, (1: 3000) followed by tetramethylbenzidine (TMB) detection on an ELISA reader at 450 nm. Samples from the two separate cultures were analyzed in triplicate for each condition. The absorbance of the basal medium was subtracted from each sample's absorbance, and AAT concentration was determined from a standard curve prepared from different dilutions of human AAT (Table 1).

RESULTS

Cloning of *AAT* gene into the transfer vector: *AAT* cDNA (1257bp) was extracted from HepG2 by RT-PCR and was successfully ligated into *Xho*I and *Mlu*I sites of the PlexJred transfer vector (Fig. 1). Introduced fragments were confirmed by digestion, PCR and the cycle sequencing methods (Fig. 2).

Vector titration: Vector titers were derived from quantitative FACS analysis of HEK 293T cells. The concentrations of vector particles were in the range of 2×10^7 to 9×10^7 viral particles/ml which is typically obtained with this vector system.

CHO and COS-7 cells' transduction efficiency: The transduction conditions were optimized by using different multiplicities of infection. After 72 h, percentages of the transduced cells were analyzed by direct fluorescence, using an inverted fluorescence microscope. The percentage of Jred-positive cells to the total CHO and COS-7 cells were close to 90% at an MOI of 20 and 35, respectively (Figs. 3A, B).

Quantification of *AAT* gene expression by RT-PCR: RT-PCR was used to determine the expression of the *AAT* gene in transduced CHO and COS-7 cell lines. The expression of the *AAT* gene in both cells lines was confirmed using RT-PCR. The HepG2 cell line was used as a positive control (Fig. 4).

ELISA: The concentration of AAT in both cell line cultures was measured on day 7 and 14 by the sandwich ELISA procedure with a detection range of 7.5-

Table 1. Alpha-1 antitrypsin standard concentrations and their values.

Standards	Concentration (ng/ml)	value (OD_{450})	SD	CV%
St01	500	1.78 1.76	0.016	0.9
St02	250	1.35 1.36	0.011	0.8
St03	125	0.917 0.903	0.021	2.2
St04	62.5	0.556 0.559	0.004	0.8
St05	31.25	0.297 0.287	0.014	4.8
St06	15.625	0.15 0.151	0.001	0.9
St07	7.813	0.078 0.077	0.001	1.8

SD: standard deviation
CV: Central variation

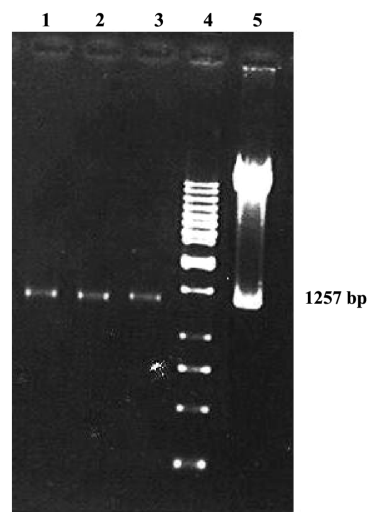

Figure 2. The *AAT* gene introduced into the plex-Jred vector was confirmed by PCR and digestion. Lanes 1, 2, 3 are PCR product for AAT (1257bp), In addition to sequencing a 1257 bp bond was confirmed AAT gene was cloned into transfer vector correctly ;lane 4 DNA ladder (1Kb) and lane 5 digestion of the plex-Jred vector containing the *AAT* gene.

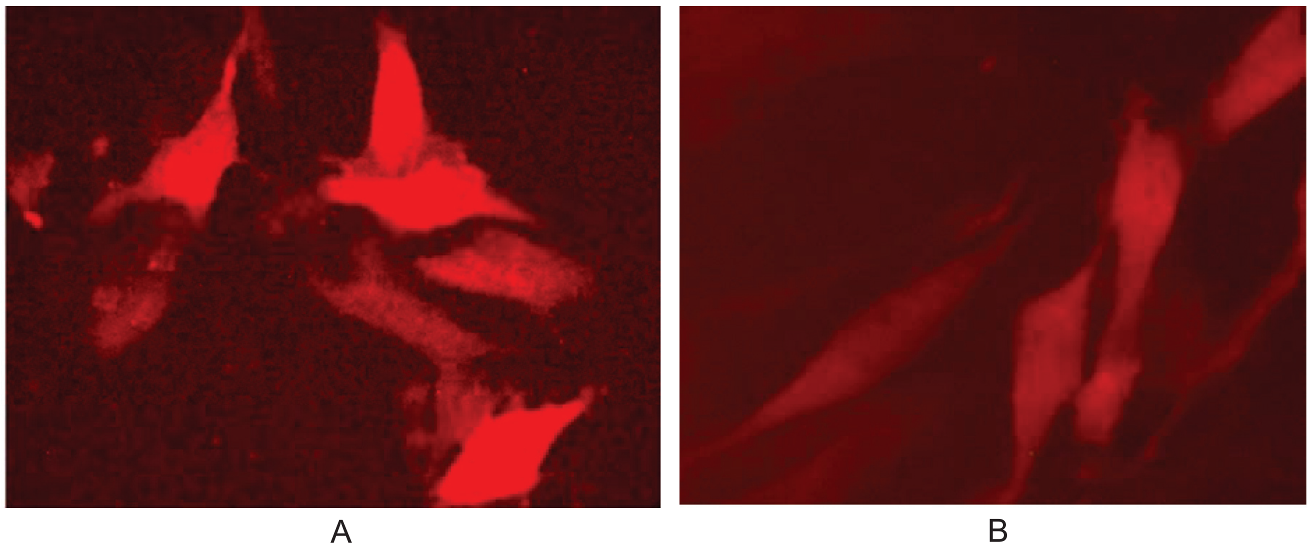

Figure 3. A: *AAT* expression in the CHO cell line.Transfected CHO cells expressing *AAT* are red in color. V: *AAT* expression in the COS-7 cell line. Transfected COS-7 cells *AAT* are red in color.

500 ng/ml, using purified hAAT protein as a standard. The absorbance of FBS was 0.0012. It showed that there was no reaction between the human AAT antibody and bovine AAT in FBS.

Based on ELISA the supernatant showed that CHO and COS-7 cells were actively expressing and secreting AAT protein into the medium. CHO and COS-7 cells were secreting the AAT protein at approximately 59.05 ± 8.27 ng/ml/10^5 cells, 58.13 ± 7.65 ng/ml/10^5 cells (mean: 58.59± 1.23 ng/ml/10^5 cells); 70.76 ± 1.38 ng/ml/10^5 cells and 75.41±2.56 ng/ml/10^5 cells (mean: 73.08±3.28 ng/ml/10^5 cells), on day 7 and 14, respectively. Concentration of standards and culture extracts containing hAAT are shown in Tables 1 and 2.

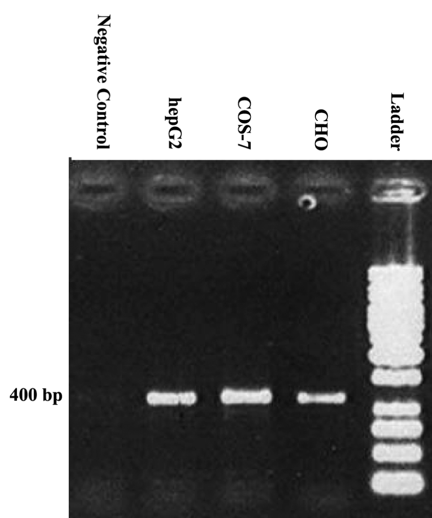

Figure 4. RT-PCR analysis of the *AAT* gene expression in COS-7 and CHO cells. The HepG2 cell line was used as a positive control. Right to left; line 1: DNA ladder (100bp): RT-PCR AAT in CHO (line 2), in COS-7 (line 3) and in hepG2 (line 4). Line 5: negative control.

DISCUSSION

AAT deficiency is a common genetic disorder that primarily affects the lungs and liver and can lead to emphysema and cirrhosis (Mulgrew *et al*., 2007). The lack of AAT in the lungs, permits uninhibited proteolytic damage to the connective tissue matrix of the lung causing the destruction of its alveolar walls and leading to the early onset of pulmonary emphysema (Perlmutter *et al*., 2007). Between 2-18% of the patients with emphysema are alpha-1 antitrypsin deficient. The condition is also associated with risks of developing hepatic disease (Fregonese *et al*., 2008; de Serres, 2002).

Intravenous AAT administration may be an option for the patients characterized by a severe diminution in plasma levels of AAT. The levels of AAT in serum, bronchoalveolar lavage, and the pulmonary interstitium required to provide protection against proteolytic lung destruction can be achieved by intermittent infusions of plasma-purified AAT (Mulgrew *et al*., 2007). As predicted, AAT deficiency occurs in 1 in 2500 people and 15-25% of these require replacement therapy (Kumpalume *et al*., 2007).

On the other hand, in normal humans, more than 2

Table 2. Concentration (ng/ml) of AAT secreted by CHO and COS-7 cells into the media as measured by ELISA.

Assays	Day 7		Day 14	
	CHO	COS-7	CHO	COS-7
1	50.00	71.46	58.21	76.02
2	66.22	71.68	50.45	72.60
3	60.95	69.17	65.75	77.62
Mean ±SD	59.05 ±8.27	70.76±1.38	58.13±7.65	75.41±2.56

g of AAT is synthesized daily, resulting in a serum concentration of 2 mg/ml. Since AAT normally circulates at this concentration and has a half-life of 6 days, considerable quantities (4 g/week/patient) would be required for replacement therapy of afflicted individuals. Such large amounts of protein will be available only if recombinant DNA technology is used for production (Archibald *et al*., 1990). In fact, the normal human *AAT* cDNA has so far been used to transform *Escherichia coli* and yeast (Garver *et al*., 1987).

Although production of recombinant proteins in bacteria is a relatively quick, efficient, and inexpensive procedure but commonly used bacterial hosts do not glycosylate their proteins (Fussenegger *et al*., 1999). As an alternative to bacteria, yeast can be used for the purpose of recombinant protein production. They grow more rapidly, are cheaper to maintain and are easier to handle in the laboratory than mammalian cell cultures. However, even with these eukaryotic systems, post-translational modifications, such as glycosylation and processing of large protein precursors, are not performed exactly as in mammalian cells (Gaillet *et al*., 2007; Butler, 2005; Andersen and Krummen, 2002). Yeasts glycosylate proteins containing high mannose oligosaccharide structures and are thus not suitable for injection into humans. Similarly, baculoviruses and plant expression systems synthesize carbohydrates that are undesirable for pharmaceutical applications (Fussenegger *et al*., 1999).

Highly glycosylated proteins, require the post-transcriptional metabolic machinery only available in mammalian cells (Butler, 2005). In particular, proper post-translational modifications convey higher quality and efficacy to the protein when compared to proteins produced by bacteria and yeast (Kwaks and Otte, 2006; Wurm, 2004). The two major hosts for these processes are COS-7 and CHO cells, which are becoming increasingly important for the production of human recombinant proteins, especially in the pharmaceutical field (Liu and Chen, 2007; Yoon *et al*., 2006; Blasey *et al*., 2000).

There are many of ways of introducing foreign DNA into the nucleus of mammalian cells. Among a variety of expression systems that have been developed to overproduce proteins in mammalian cells, viral vectors are particularly powerful tools because they have inbuilt mechanisms to subvert the cellular machinery in their favor (Yang *et al*., 2000). Viral delivery of genes is also faster, less expensive and capable of integrating transgenes into cells (Stoll *et al*., 2001). They have been widely used to introduce cDNA into cells in culture (Berns *et al*., 2008). Accordingly, lentiviral delivery of gene has the same advantages, such as rapid and stable transgene expression (Yang *et al*., 2000), less expensive and able to integrate transgenes into the cells. It provides a suitable vehicle to stably infect a high percentage of cells and thereby express the protein of interest (Yang *et al*., 2000; Naldini *et al*., 1996).

Lentivirus-derived vectors have been demonstrated to achieve an efficient and stable gene transfer in dividing and nondividing cells (Stoll *et al*., 2001). Such an efficient gene transfer is attributed to the Vesicular somatitis virus glycoprotein protein (VSVG), which also enables broad cell type tropism of the recombinant virus. Integration of the transgene into the host cell genome leads to the stable transduction of cells. As the transfection yield using lentiviral vectors is high, it permits the synthesis of milligram quantities of recombinant proteins in mammalian cells in a timely manner without the need for generating stable transfectants (Yang *et al*., 2000).

In this study, a human *AAT* (hAAT) gene was introduced into a self-inactivating lentiviral vector expressing *Jred* under the control of the CMV promoter. The human *AAT* gene was then transferred into CHO and COS-7 cell lines using this lentiviral vector. The reporter gene was made to attach to the N-terminal of

the *AAT* gene in the transfer vector. The use of the AAT- Jred fusion protein enables direct assessment of the abundance of new AAT expression in these cells. It serves as an internal transfection control by marking all cells that receive the target plasmid having the gene of interest. Fusion of the AAT protein to *Jred* also permits visualization of the expressed AAT protein by fluorescence microscopy. Because expression of the AAT protein is linked to the expression of the Jred protein, this system thus greatly facilitates the monitoring process of growth, cell infectivity and recombinant protein production in the host cells. The Jred protein then dissociates from the fusion protein after dissociation of the signal peptide from the AAT N-terminal, however, this does not affect the function of the AAT protein. ELISA showed that stable CHO and COS-7 cells transduced with hAAT gene were active for AAT protein expression and secreting AAT at approximately 58.59 ± 1.23 ng/ml/10^5 cells and 73 ± 3.2 ng/ml/10^5 cells into the medium.

These data show that CHO and COS-7 cells transduced with the normal *AAT* gene have an efficient ability to express the AAT protein and possess key properties that ensure their employment as platforms to produce AAT and other therapeutic proteins in eukaryotic cell lines. This study takes an alternative approach to utilizing recombinant DNA technology for expression of AAT by introducing a normal human *AAT* cDNA into the mammalian genome with a lentiviral vector. The results of this study strongly support the promising potential of lentiviral vectors for long-term expression and secretion of proteins of interest in target cells.

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