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Original Article

Gene Expression under *F8 Promoter* Driving In Mouse Hepatoma Cells: A Step towards Gene Therapy of Hemophilia

Pejman Fard-Esfahani[1], Mehdi Kadivar[1], Mojgan Allahyari[2], Fatemeh Mirkhani[2]

1. Dept. of Biochemistry, Pasteur Institute of Iran, Tehran, Iran
2. Dept. of Education, Pasteur Institute of Iran, Tehran, Iran

ABSTRACT

Background and Objectives: Significant progress has been made in treatment of hemophilia. Ex-vivo gene therapy is going popular due to the capability of this method in using isogenic cells for genetic manipulation and reintroducing them into same host after proliferation. Most gene therapy techniques use viral vectors, which usually harbor a strong and non-specific promoter (e.g. CMV early promoter) for driving the downstream gene. This may be a disadvantage due to uncontrollable nature of gene expression. In addition, considering the potentials of recently introduced stem cells as reservoirs and their potential to differentiate to other cell lines, uncontrolled expression may have unknown outcomes. To make gene therapy of hemophilia more resembling to the nature, we supposed *f8 promoter* might be a good candidate for driving downstream *f8* coding sequence.

Materials and Methods: To test our hypothesis, we designed and constructed a DNA construct by PCR, which harbors EGFP coding sequence downstream to mouse *f8 promoter* and transfected it to a mouse hepatoma cell line. Transfection was assayed qualitatively by fluorescent microscopy.

Results: Fluorescence was detected in transfected cells a sign of presence of EGFP.

Conclusion: *f8 promoter* can drive expression of downstream genes, a capability which and may have potential to be used in gene therapy of hemophilia. A conclusion that should be examined by further studies.

Keywords: Gene therapy, Hemophilia, Hepatoma cells

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Address communications to: Dr Pejman Fard-Esfahani, Department of Biochemistry, Pasteur Institute of Iran, Tehran, Iran
Email: fard-esfahani@pasteur.ac.ir

Introduction

Hemophilia A is an X chromosome-linked recessive genetic disorder, affecting 1 in 10,000 males, resulting in defective or deficient factor VIII (FVIII) molecules, which, in its severe form, is a life-threatening, hemorrhagic disease (1). FVIII plays an important role in the coagulation cascade. The conversion of factor X to factor Xa is accelerated by FVIII. Intravenous infusion of recombinant FVIII protein is an effective treatment of hemophilia A disorder especially in controlling bleeding episodes, but the requirement for frequent infusions, makes the treatment costly (2). A functional copy of a gene can be introduced into an organism to retrieve function of a defected endogenous gene. This approach may be the future strategy for curing of monogenetic inherited disorders. The most promising cure for hemophilia A disorders appears to be gene therapy, which delivers the functional factor VIII expression cassette directly to the patient (3). In spite of recent successes in site-specific correction of defective gene sequences, the focus of most gene therapy strategies to date is on gene addition rather than gene replacement (4). Several different gene delivery systems have been used in gene therapy of hemophilia, including retroviral vectors (5), adenoviral vectors (6, 7), adeno-associated viral vectors (8, 9), and non-viral gene delivery methods (10). In most of them, the CMV promoter is the driving sequence of choice for desired gene expression. Although the use of strong, nonspecific viral promoters such as the CMV promoter may have some advantages e.g. high-level expression, two disadvantages limit its usefulness. First, in a situation with possibly widespread of transfected cell, the uncontrolled expression may be hazardous. Second, other tissues might be harmed by expressed protein that normally is not expressed in them. None of these disadvantages are not applying to a system which the relevant promoter functions in tissue-specific manner.

f8 promoter is relatively liver specific and may be good candidate for expression of liver specific genes. To examine the potential capability of this gene in driving downstream genes, we designed and constructed a plasmid vector harboring mouse *f8 promoter* sequence, which was located upstream to EGFP coding sequence and transfected it into the mouse hepatoma cells line to evaluate any expression of the EGFP.

Materials and Methods

Vector design and construction: The sequence of mouse *f8 promoter* was extracted from *Mus musculus* Promoter Database (accession number: 84501) (MmPD) (11) (Fig. 1). Two forward (F1) and reverse primers (R1) were designed (Table 1) to proliferate mentioned *f8 promoter* segment from C57BL mouse genomic DNA by PCR. The coding sequence of EGFP was proliferated by PCR using IRES2-EGFP vector (Invitrogen) as template and conditions shown in Table 1. R1 and F2 primers were designed as to be carrying *Nco*I restriction digestion sites for fusing *f8 promoter* and EGFP coding sequences. The fused DNA was cloned into pJET1.2 plasmid using CloneJET^{TM} PCR cloning kit (Fermentas). The selected transformant was confirmed by DNA sequencing and plasmids were purified after propagation using Plasmid mini kit (Qiagen). Transfection was controlled by intact plasmid vector IRES2-EGFP (Invitrogen).

Fig. 1- Selected mouse *f8 promoter* sequence

Table 1- Primers sequences and PCR conditions

	Sequence	PCR conditions	Segment length
Forward primer (*f8 promoter*)	AACTCGAGTTACAGCTGAGTAATAAATATTCC	94^{oC} 4 min, (94^{oC} 1 min, 57^{oC} 1 min, 72^{oC} 2 min)X30 72^{oC} 7 min.	1110 bp
Reverse primer (*f8 promoter*)	CCACCATGGCTTTTATTACTAGAAAATG		
Forward primer (EGFP)	AACCATGGTGAGCAAGGG	94^{oC} 4 min, (94^{oC} 1 min, 60^{oC} 1 min, 72^{oC} 2 min)X30 72^{oC} 7 min.	960 bp
Reverse primer (EGFP)	AAATCGATATACATTGATGAGTTTGGAC		

Cell culture and transfection: Mouse hepatoma cells, Hepa 1-6 (NCBI code: C517, ATCC Number: CRL1830) were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10%FBS. Then the cells were plated onto 25 cm^2 tissue culture flasks at a concentration of 1000 cells/cm^2 and cultures were incubated at 37^{o}C, 5% CO_2. The medum were removed every 3 days. DNA transfection was carried out by PolyFect Transfection Reagent (Qiagen) using manufacturer protocol. Confirmation of transfection was done by fluorescent microscopy.

Results

Vector Construction: A fused DNA was constructed using mouse *f8 promoter* sequence derived from the mouse promoter database and EGFP coding sequence. The downstream (3' end) primer of mouse *f8 promoter* and upstream primer (5' end) of EGFP coding sequence were designed to have *Nco*I restriction sites. Two PCR products were treated by this enzyme and ligated by T4 DNA ligase, indeed the start codon (ATG) of EGFP gene was reconstructed in the

recognition site of this restriction enzyme. There were tree possible types of ligates: Tail to tail ligation of *f8 promoter* sequence; head to head ligation of EGFP coding sequence and tail of *f8 promoter* sequence to the head of EGFP coding sequence, the correct one. After then the ligation product was subjected to blunt end cloning. Some of the resultant clones were screened by plasmid extraction and restriction enzyme treatment. To confirm the clone, the insert was sequenced and the construct was confirmed (Fig. 2).

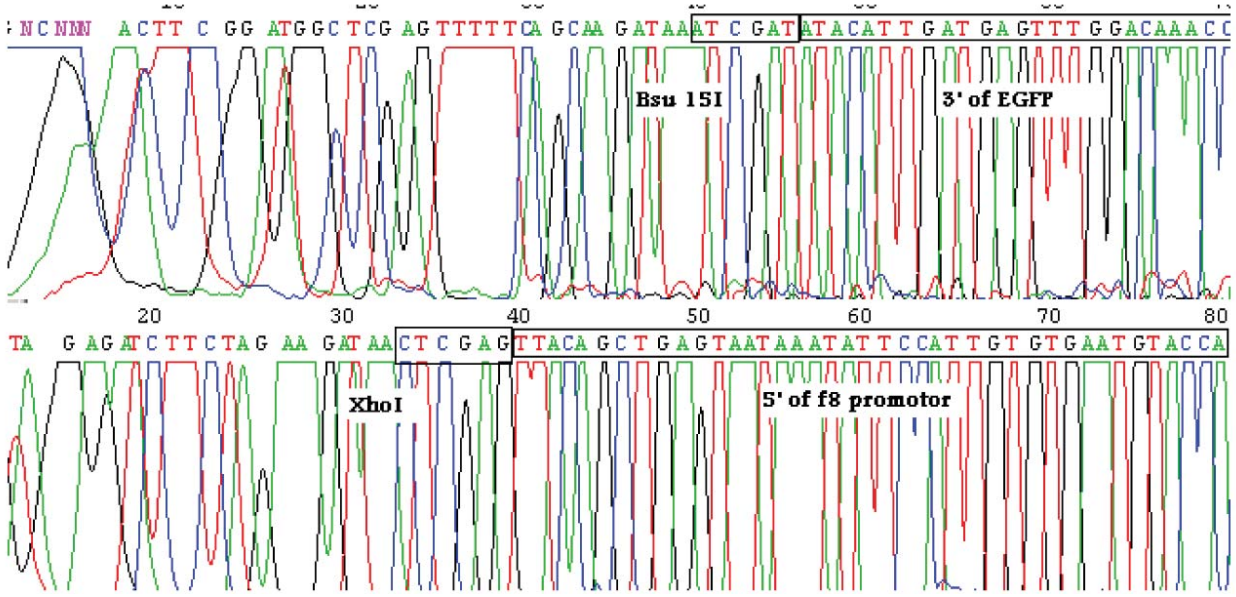

Fig. 2- Partial sequencing of 3' (up) and 5' (down) of insert in plasmid pJET1.2. The restriction enzyme sites (*Bsu15*I and *Xho*I) are entrance site of insert into the vector (small boxes)

Transfection: The constructed vector was transfected into Mouse hepatoma cell line by dendrimers. These highly branched spherical molecules terminate at charged amino groups radiate from a central core molecule and absorb negative charge molecules like DNA (12). After transfection, fluorescent microscopy detected fluorescence in transfected cells (Fig. 3) which indirectly shows expression of EGFP gene by action of *f8 promoter*. Simultaneously, for checking the accuracy of transfection the same cell line was transfected by empty IRES2-EGFP vector and the EGFP fluorescence was detected (data not shown).

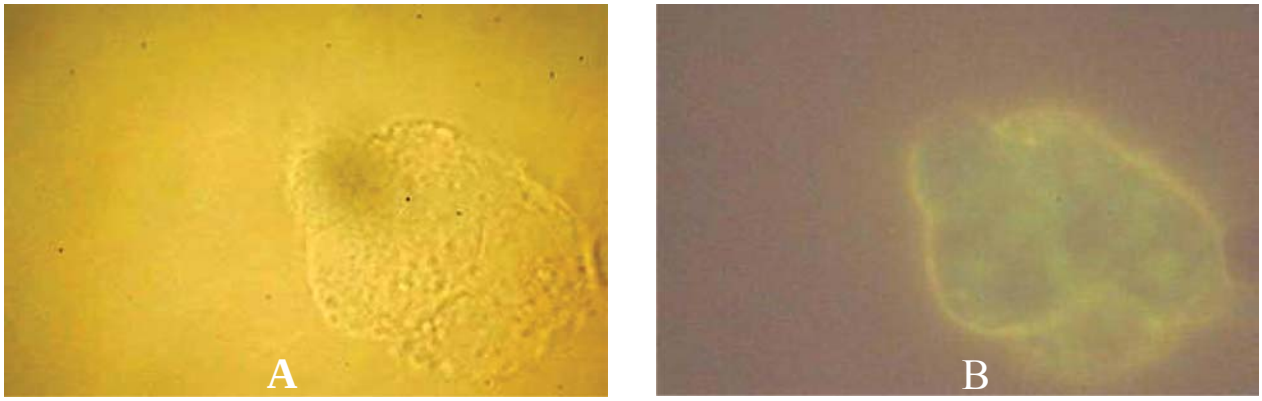

Fig. 3- Fluorescence microscopy of transfected hepatoma cells: (A) conventional light and (B) fluorescent light. (Leitz, 250x, FL 540 nm)

Discussion

Somatic gene therapy for hemophilia A has been challenging to investigators. There are reports of using different strategies to replace enough factor VIII in hemophilia A patients, but achieving a continues high serum level of the factor VIII is the main obstacle (13). The main difference of these strategies is type of vector for transfecting target cells. Each of them has advantages and disadvantages. For example with genetically modified adenovirus, which was one of the first pioneer vectors to be utilized for gene therapy of hemophilia A, the viral proteins evoke cytotoxic responses and halt production of the factor by killing vector harboring cells. By usage of second-generation vectors, where many viral genes have been deleted, cell toxicity has been lowered and factor VIII expression has been prolonged. In factor VIII knockout mice, such a vector mediated a high level of factor VIII expression with minimal toxicity (6). The common feature of many of these vectors is usage of a potent mammalian virus promoter (e.g. CMV early promoter) upstream to domain B deficient coding sequence of *f8* gene. One of the newly announced strategies is stem cell *ex vivo* gene therapy (14) which uses genetically modified adult stem cells and re-injects them into the organism. Theoretically, the adult stem cells can be differentiated to many specialized cell lines, so the presence of abnormal protein within unrelated specialized cells may cause unexpected outcome. Assuming that some of the stem cells may differentiate to hepatic cells (15), the expression of factor VIII gene under *f8 promoter* control may be more resemble to normal.

In this study, the driving capacity of mouse *f8 promoter* for expressing EGFP coding sequence was investigated. The promoter sequences were retrieved from *Mus musculus* Promoter Database (MmPD, above). Considering *f8 gene* expresses in adult liver mouse cells (16), we hoped that our construct would be expressed in hepatoma cells. The results showed that green fluorescence appeared about 48 hours after transfection. The results were assessed qualitatively since the only consideration at this point was capability of mouse *f8 promoter* in driving downstream gene.

Conclusion

For utilization of this promoter in future stem cell gene therapy, the driving potential of it can be assessed in adult stem cells by transfecting the construct into such cells (e.g. hematopoeitic stem cells).

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