A New Potent hFIX Plasmid for Hemophilia B Gene Therapy

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Purpose. The purpose of this work was to construct and characterize a new potent hFIX plasmid, p2SV-hFIX, which has two hFIX expression units containing the SV40 promoter/enhancer for hemophilia B gene therapy.

Methods. p1SV-hFIX was constructed by insertion of amplified hFIX cDNA at the ECORI and XbaI sites of pSI expression vector containing simian virus 40 (SV40) promoter/enhancer. To construct p2SV-hFIX, the hFIX expression cassette was isolated from p1SV-hFIX by digestion with restriction enzymes, and the purified expression cassette was inserted at the BglII site of another p1SV-hFIX. The gene expression of p1SV-hFIX, p2SV-hFIX, and a plasmid containing a liver-specific apoE enhancer and alpha antitrypsin promoter, pAAV-hAAT-hFIX, were evaluated in various cell lines using polyethylenimine (PEI) as a gene carrier *in vitro*.

Results. The construction of p1SV-hFIX and p2SV-hFIX were confirmed by restriction enzyme studies. The transfection efficiency of p2SV-hFIX was 3.83-fold and 7.16-fold higher than that of pAAV-hAAT-hFIX in C2C12 and NIH3T3 cells, respectively. p2SV-hFIX also showed higher transfection efficiency than p1SV-hFIX in both cells.

Conclusions. In accordance with these results, p2SV-hFIX is a new potent hFIX plasmid that can be transfected in various cells. Systemic delivery of p2SV-hFIX via intravenous or intramuscular injection is feasible for treatment of hemophilia B.

KEY WORDS: Hemophilia B; Factor IX; non-viral gene therapy.

INTRODUCTION

Hemophilia B is an X chromosome-linked bleeding disorders caused by a deficiency or abnormality in the plasma coagulation protein factor IX. Hemophilia is classified as mild, moderate, or severe on the basis of circulating levels of clotting factor. Severe is defined as <1% of normal levels, moderate as 1–5%, and mild as >5%. Current treatment of hemophilia is based on protein replacement therapy by intravenous infusion of clotting factor concentrates. However, significant problems remain with replacement therapy, including the expense, the need for intravenous access, and risks of blood-borne disease transmission, which have fueled an interest in developing a gene-transfer approach to treatment.

Hemophilia is an ideal model for somatic gene therapy for inherited protein deficiencies because low levels of protein production will improve the quality of life of those afflicted with these diseases, several cell types are capable of producing biologically active coagulation factors, and precise regulation of gene expression is not required. The goal of gene therapy for hemophilia B is the sustained expression of factor IX at levels more than 1% of normal in a patient with severe disease.

Factor IX is normally produced in the liver and secreted into the blood. Therefore, there are many studies on expression of this gene delivered to the hepatocyte, and hepatocytes are currently a target that is being evaluated in the preclinical and clinical trials (1–3). Wang *et al.* reported long-term expression of above normal levels of FIX in immunocomepetent hemophilia B mice models after an intraportal injection of AAV vectors (4) encoding cDNA driven by a strong liver-specific enhancer and promoter. Le *et al.* also reported that high and stable levels of expression were achieved using a retroviral vector encoding cDNA driven by the liver-specific promoter, human alpha-antitrypsin promoter (5). However, there is a strong motivation for evaluating alternative target tissue because of the high prevalence of viral hepatitis (approximately 90%) among adults with severe hemophilia (6–7). Recently, skeletal muscle has received much attention as a target tissue for gene delivery (8,9). The transfer of foreign DNA into muscle cells by viral or non-viral vectors results in efficient and stable expression of a variety of transgenes (10–13).

Previously, we evaluated the transfection efficiency of pAAV-hAAT-hFIX, which has liver-specific apoE enhancer and human alpha-antitrypsin promoter using Gal-PEG-PEI (GPP) as a hepatocyte targeting gene carrier. However, the FIX level in plasma was approximately 20 ng/ml, which is lower than the minimum therapeutic level (50 ng/ml) of hFIX after intravenous and intraportal injection of pAAV-hAAT-FIX/GPP complexes in rats (data are not reported). Therefore, we designed a new, more potent FIX gene expression cassette containing a simian virus 40 (SV40) enhancer/promoter that encodes two copies of hFIX cDNA to produce high level gene expression. In general, viral promoters such as the cytomegalovirus (CMV) early promoter, Rous sarcoma virus long terminal repeats (RSV-LTR), and the simian virus 40 (SV40) early promoter are much stronger than tissue- or cell-specific promoter**.** Here, we report the construction and characterization of p2SV-hFIX plasmid and evaluation of hFIX gene expression in various cell lines.

MATERIALS AND METHODS

Construction and Preparation of p2SV-hFIX Plasmid

pSI mammalian expression vector was purchased from Promega (Madison, WI, USA), and the FIX cDNA was kindly provided by Dr. Katherin A. High, The Children's Hospital of Philadelphia (Philadelphia, PA, USA). To introduce EcoRI and XbaI sites at the end of the cDNA, the hFIX cDNA was amplified by PCR. The primers sequences used were hFIX forward, 5′-GGAATTCATGCAGCGCGTGAACATGAT-3′; hFIX backward, 5′- GCTCTAGATCATTAAGTGAGCTTTGTTT-3′. The EcoRI and XbaI sites are underlined. The PCR reaction consists of 95°C for 2 min, 35 cycles at 95°C for 1 min, 61°C for 1 min, and 72°C for 2 min, followed by an extension of 5 min at 72°C; the length

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of the expected product was 1836 bp. The hFIX cDNA was digested by EcoRI and XbaI and inserted at the EcoRI and XbaI sites of pSI (Promega), resulting in construction of p1SV-hFIX. To construct p2SV-hFIX, the expression cassette was isolated from p1SV-hFIX by digestion with restriction enzymes, and the purified hFIX expression cassette was inserted at the BglII site of another p1SV-hFIX, resulting in construction of p2SV-hFIX. The construction of p2SV-hFIX was confirmed by restriction enzyme studies and DNA sequencing. The plasmid was amplified in an *Escherichia coli* host strain, DH 5 alpha, and purified using Plasmid Maxi Purification Kit Qiagen (Valencia, CA, USA). The plasmid purity and integrity were confirmed by 0.7% agarose gel electrophoresis, and DNA concentration was measured by the absorbance at 260 nm. The optical density ratio at 260–280 nm of the plasmid was in the range 1.6–1.8.

Cell Culture and *in Vitro* Transfection

HepG2 (human hepatocarcinoma cells), C2C12 (myoblast cells), and NIH3T3 (fibroblast cells) were grown in Dulbecco's Minimum Essential Medium (DMEM), supplemented with 10% fetal bovine serum (FBS, heat-inactivated at 56°C for 30 min), and treated with barium sulfate to remove the endogenous bovine factor IX (14) and 15 μg/ml of vitamin K1 (15) at 37°C under humidified 5% CO_2.

The cultured HepG2 cells were seeded on a 12-well plate at an initial density of 1 to 1.5 × 105 cells/well 24-h prior to transfection. All experiments were done in triplicate. To prepare pAAV-hAAT-hFIX/PEI and p2SV-hFIX/PEI complexes, 4 μg of plasmid DNA (25 μg /ml in DMEM) were mixed with PEI (Mw = 25 kDa, Aldrich (Milwaukee, WI, USA)) dissolved in DMEM at the same volume at N/P ratio of 5. After incubation of complexes for 30 min at 37°C, 320 μl of the complex solutions containing 4 μg of plasmids and 680 μl of FBS-free DMEM were added to the wells. After 4 h transfection time, the medium was removed and replaced by fresh growth medium containing 10% FBS. The cells were incubated for an additional 48 h, the media were collected for the assay of hFIX, and the cells were washed twice with 1 ml of Ca_2^+, Mg_2^+-free phosphate-buffered saline (PBS). The cells were incubated for 0.5 h on ice with 100 μl of Lysis Reagent 1X (Promega). The lysate was centrifuged for 2 min at 14,000 rpm at room temperature, and supernatant was collected for protein assay.

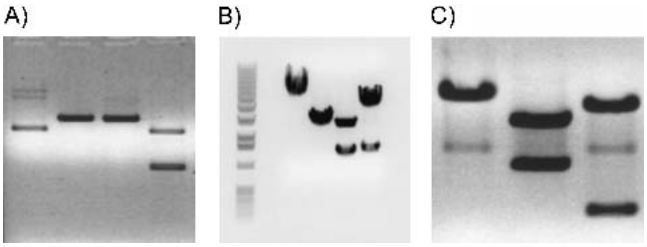

Fig. 2. Enzyme digestion and gel electrophoresis. (A) The construction of p1SV-hFIX was confirmed by digestion using EcoRI and NotI. Lane 1, untreated; lane 2, EcoRI; lane 3, NotI; and lane 4, EcoRI and NotI. (B) The isolation of expression cassette from p1SV-hFIX was determined by digestion using BglII, BamHI, and PVUI. Lane 1, BglII; lane 2, BglII and BamHI; lane 3, BglII, BamHI, and PVUI; lane 4, PVUI. (C) The construction of p2SV-hFIX was assessed by digestion using BglII, EcoRI, and PVUI. Lane 1, BglII; lane 2, EcoRI; and lane 3, PVUI.

Human Factor IX Assay

The hFIX concentrations were determined by ELISA assay (Affinity Biologicals, Hamilton, Ontario, Canada). Human factor IX was obtained from Enzyme Research laboratories (Swansea, UK). The linear range of the standard curve was from 0.5 to 100 ng/ml. As the capture antibody, affinity purified sheep anti-FIX was used at a dilution of 1:100 for coating ELISA plates, and a horseradish peroxidase labeled affinity purified sheep anti-FIX was used as the detecting antibody at dilution of 1:100 to detect captured FIX. The production of hFIX was expressed as nanogram of hFIX per milligram protein. Protein concentration was measured by BCA protein assay (Pierce Rockford, IL, USA).

RESULTS AND DISCUSSION

Construction and Characterization of p2SV-hFIX

We constructed p2SV-hFIX, which has two hFIX expression units containing the SV 40 promoter/enhancer (Fig. 1). The amplified cDNA was digested by EcoRI and XbaI, and the resulting fragment was purified by 0.7% agarose gel electrophoresis and elution. The hFIX cDNA was inserted at the EcoRI and XbaI sites of pSI (Promega), resulting in construction of p1SV-hFIX. The plasmid was amplified in *E. coli* host

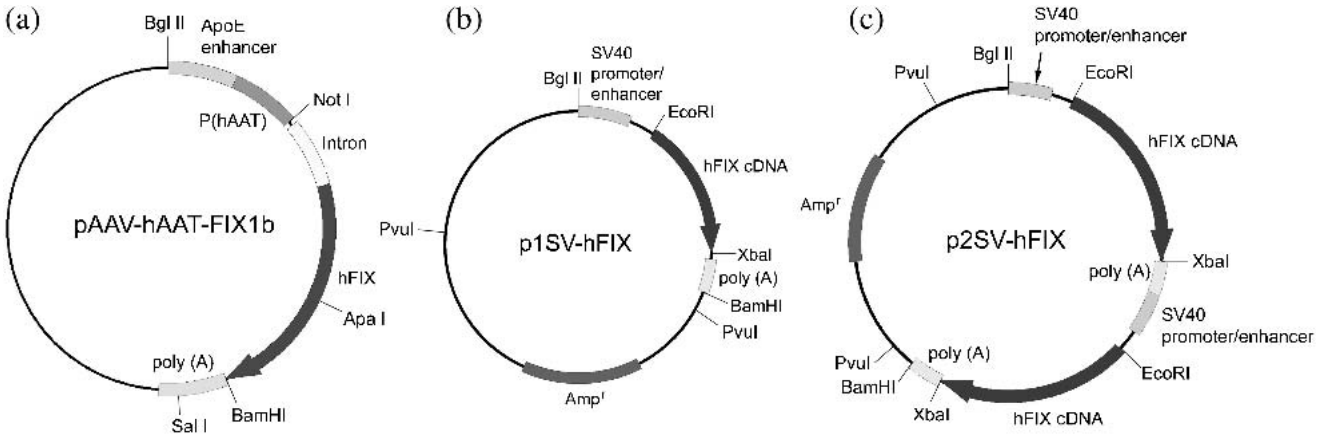

Fig. 1. Construction of hFIX plasmids: (a) pAAV-hAAT-hFIX, (b) p1SV-hFIX, and (c) p2SV-hFIX.

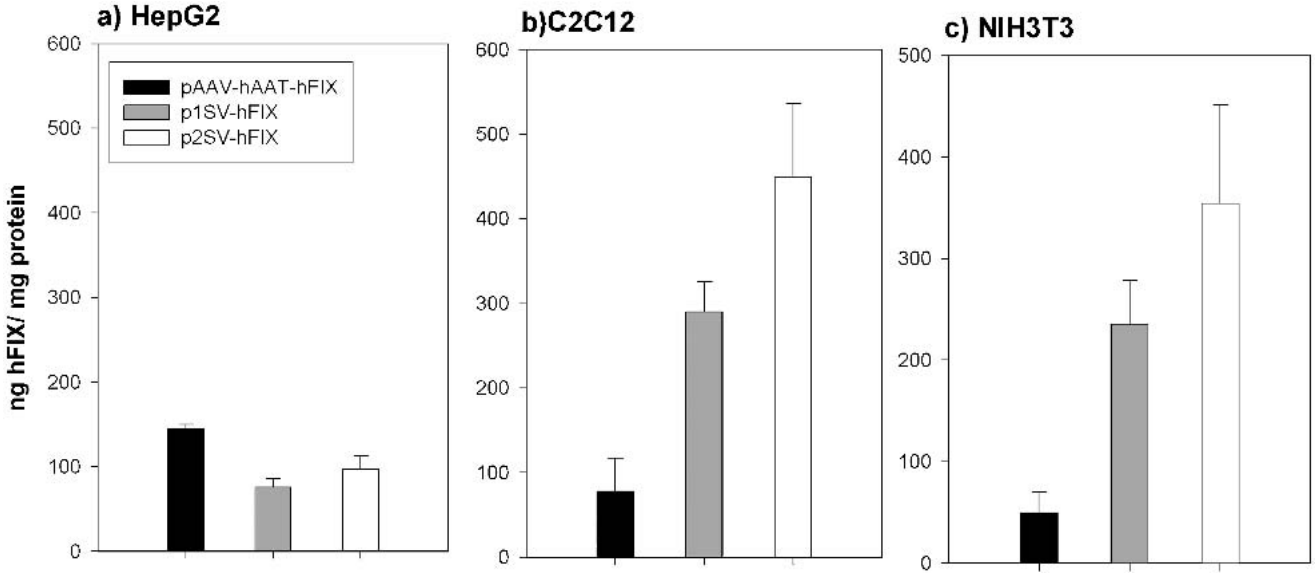

Fig. 3. *In vitro* transfection efficiency of various hFIX plasmids in different cell lines. pAAV-hAAT–hFIX/PEI, p1SV-hFIX, and p2SV-hFIX/PEI complex at N/P of 5/1 was transfected to HepG2 (human hepatocellular carconoma cell), C2C12 (mouse myoblast cell), and NIH3T3 (mouse fibroblast cell), respectively. hFIX produced is expressed as ng/mg total protein. Each column represents the mean ± SD (n = 3).

strain, DH 5 alpha, and purified using Plasmid Maxi Purification Kit (Qiagen). The construction of p1SV-hFIX was confirmed by restriction enzyme studies. Lane 1 shows circular p1SV-hFIX plasmid, and lanes 2 and 3 show linear plasmid (5.3 kb) cut by EcoRI and NotI, respectively. Lane 4 shows the hFIX cDNA (1.6 kb) cut by EcoRI/NotI (Fig. 2a). To construct p2SV-hFIX, SV40 promoter/enhancer and hFIX expression cassette was isolated from p1SV-hFIX by digestion with Bgl II, BamHI, and PvuI. To isolate hFIX expression cassette from p1SV-hFIX, restriction enzyme digestion assay was performed using BglII, BamHI, and PvuI. Lane 1 shows linear plasmid of p1SV-hFIX (5.3 kb) cut by BglII, and lane 2 shows two fragments (2.64 and 2.66 kb) cut by BglII/BamHI. Lane 3 shows three fragments (2.64, 1.35, and 1.27 kb) cut by BglII/BamHI/PvuI, and lane 4 shows two fragments (3.95 and 1.35 kb) cut by PvuI (Fig. 2b). The hFIX gene expression cassette (2.64 kb) was isolated from p1SV-hFIX by digestion with BglII/BamHI/PvuI, and the purified hFIX expression unit was inserted at the BglII site of another p1SV-hFIX, resulting in construction of p2SV-hFIX. The construction of p2SV-hFIX was assessed by digestion using BglII, EcoRI, and PVUI. Lane 1 shows the linear p2SV-hFIX plasmid (7.9 kb) cut by BglII, and lane 2 shows two fragments (2.68 and 5.26 kb) cut by EcoRI. Lane 3 shows two fragments (6.59 and 1.35) cut by PvuI (Fig. 2c).

Expression of hFIX After Transfection of p2SV-hFIX/PEI Complexes

The transfection efficiency of p2SV-hFIX was evaluated in different cell lines (Fig. 3). p2SV-hFIX/PEI complex, pISU-hFIX/PEI complex, and pAAV-hAAT-hFIX/PEI complex at N/P of 5/1 were transfected to HepG2 (human hepatocarcinoma cell), C2C12 (mouse myoblast cell), and NIH3T3 (fibroblast cell). The amount of pDNA was fixed 4 μg/well in 12-well microassay plate. The cells were incubated at 37°C in the presence of the complex for 4 h with FBS-free DMEM medium followed by replacement of 1 ml of fresh DMEM medium containing 10% FBS and Vt K1. After 44 h further incubation, the growth medium was subjected to ELISA. The transfection efficiency of p2SV-HFIX was compared to pAAV-hAAT-hFIX, which has liver-specific apoE enhancer and alpha antitrysin promoter using PEI as a gene carrier. In HepG2 cells, pAAV-hAAT-hFIX showed 1.5 folds higher gene expression efficiency than p2SV-hFIX because pAAV-hAAT-hFIX has liver-specific apoE enhancer and alpha antitrysin promoter. However, p2SV-hFIX showed higher transfection efficiency in C2C12 and NIH3T3 cells than pAAV-hAAT-hFIX because SV 40 promoter/enhancer supports strong and constitutive expression in many cell types. The transfection efficiency of p2SV-hFIX was 3.83-fold and 7.16-fold higher than that of pAAV-hAAT-hFIX in C2C12 and NIH3T3 cells, respectively. p2SV-hFIX also showed higher transfection efficiency than p1SV-hFIX, which encoded one copy of hFIX cDNA in C2C12 and NIH3T3 cells. These results demonstrated that p2SV-hFIX is a new potent hFIX plasmid in many cell lines without cell specificity. Because p2SV-hFIX can be transfected in various cells, systemic delivery via intravenous or intramuscular injection is feasible.

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