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Expression of Biologically Active Recombinant B-Domain-Deleted Human Factor VIII in Mammalian Cells

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Abstract

Hemophilia A is an X-linked recessive bleeding disorder, widely prevalent throughout the world, for which, replacement therapy is a current treatment done by infusion of either human plasma derived FVIII or recombinant FVIII. In order to produce a recombinant form of biologically active human coagulation factor VIII, a mammalian expression system is necessary for proper post-translational modifications. In this regard, two types of mammalian cell lines, COS7 and CHO, were transfected with a recombinant plasmid, constructed by insertion of a *Not*I restriction fragment containing B-domain-deleted cDNA of hFVIII in pcDNA3 plasmid, downstream of CMV promoter. By performing one-stage clotting assay as well as ELISA test on the conditioned media collected from transfected cells, we confirmed transient and stable expression of rhFVIII in the transfected COS7 and CHO cells, respectively. The presence of rhFVIII mRNA was also demonstrated by performing RT-PCR on total cellular RNA, extracted from the stably transfected CHO cells. The highest amount of produced active rhFVIII in the stably transfected CHO cells was estimated to be around 0.1 U/ml of the cultured media. By applying southern blotting experiment on the digested as well as high molecular weight chromosomal DNA, prepared from the stably transfected CHO cells, we have demonstrated the presence of the rhFVIII expressing plasmid in the CHO cells. The recombinant plasmid as well as the stable FVIII expressing cell line developed in this study has provided useful bases for further molecular studies of various important factors influencing the expression efficiency of rhFVIII.

Keywords: Recombinant human coagulation Factor VIII; Mammalian cell expression system; CHO and COS7; Hemophilia A

Introduction

Coagulation factor VIII (FVIII) is an integral component of the intrinsic pathway of blood coagulation cascade. Human FVIII (hFVIII) is a large glycoprotein of approximately 300 kDa, synthesized as a single-chain with the structural domains of A1-A2-B-A3-C1-C2 [1-4]. Upon secretion the precursor protein is

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proteolyticaly processed to generate heavy chain (A1-A2-A3-B) of 90-200 kDa and light chain (A3-C1-C2) of 80 kDa that are held together by metal ions. The B-domain is not required for procoagulant activity and released proteolyticaly upon activation by thrombin [5]. The step-wise hFVIII secretion-activation is outlined in Figure 1. Mutations in the hFVIII gene give rise to the genetic disease, Hemophilia A, a recessive bleeding disorder, affecting 1 in 10,000 males [6]. The X-linked hemophilia A is phenotypically characterized by recurrent bleeding episode. Replacement therapy is the most common treatment of hemophilia A, in which either human plasma derived FVIII or recombinant protein is infused. Viral safety has been a great concern in the replacement therapy of hemophilia during recent years. Therefore, the use of plasma-derived hFVIII was limited when it was proved to be associated with the possible transmission blood-borne pathogens such as hepatitis viruses, HIV and parvovirus B19 [7,8]. In this regard, the issue of transmission of prions, the proposed causative agents for Creutzfeldt-Jakob disease and bovine spongiform encephalopathy, has also been vigorously debated [9]. Characterization of FVIII gene structures and rapid development of recombinant DNA technology have facilitated a safe and abundant production of rhFVIII in mammalian cells for clinical use, without the risk of transmission of human pathogens thus eliminating the need for plasma-derived preparations [10]. Numerous forms of rhFVIII including several B-domain-deleted hFVIIIs (BDDhFVIII) have been expressed through eukaryotic expression systems [5,11]. The recombinant BDDhFVIII molecules are expressed at higher levels and show higher specific activity compared to the full-length hFVIII [12]. Human FVIIISQ is the smallest active form of recombinant hFVIII secreted as non-covalently associates of two chains of 90-kDa and 80-kDa. The FVIIISQ lacks the entire B-domain residing between amino acids Ser743 (S) and Gln1683 (Q) [13]. At present a number of preparations of rhFVIII are commercially available such as Kognate, Helixate (Bayer, USA) and Recombinate (Baxter, USA) as full-length forms of hFVIII and Refaco (Farmica, USA) and Kognate FS (Bayer-USA) as deleted forms [14].

In order to prodive an expression system for the production of active factor VIII, we constructed a recombinant plasmid being able to express the BDDhFVIII under CMV promoter in mammalin cell lines. In this paper we report the results obtained from the expression analysis of two cell-lines, namely COS7 and CHO, transfected separately with the rhFVIII expression plasmid.

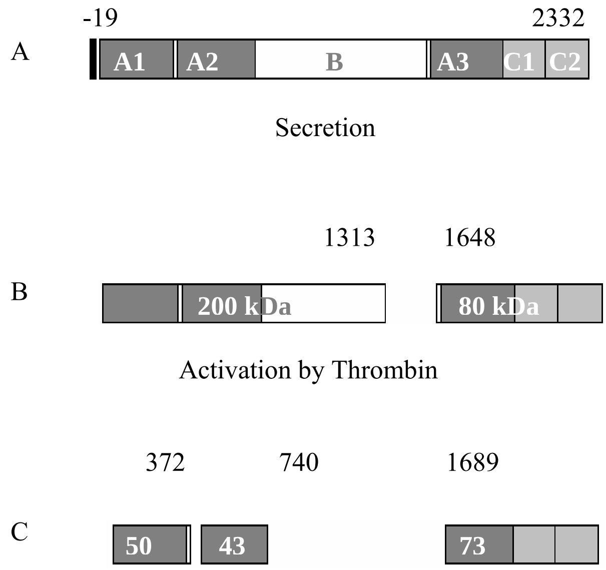


Figure 1. Domain-structure of hFVIII, before secretion (A) and after secretion (B) and after activation (C). hFVIII is synthesized as a 2351-residue single-chain precursor from which a 19-residue signal peptide is cleaved upon translocation into the lumen of the endoplasmic reticulum and consists of the structural domains A1-A2-B-A3-C1-C2. Upon secretion the precursor protein is proteolytically processed to generate heavy chain (A1-A2-A3-B) of 90-200 kDa and light chain (A3-C1-C2) of 80 kDa that are held together by metal ions. The B domain is proteolytically released upon activation by thrombin and is not required for procoagulant activity.

Materials and Methods

Bacterial Strains, Mammalian Cell Lines, Plasmids, and Primers

DH5α (*strategene-USA*) strain of *Escherichia coli* was used for sub-cloning steps. Two mammalian cell-lines, COS7 (African green monkey cells) and CHO (Chinese hamster Ovaries cells) (*Pasteur Institute-Iran*), were used as hosts for the expression of hFVIII. Plasmid pGEM3-hFVIII was kindly provided by John H. MacVey (MRC- Clinical Science Center in Imperial College School of Medicine, London, UK) and used as a source for the B-domain-less hFVIII cDNA. Plasmid pcDNA3 (*Invitrogen-USA)* was used for the construction of the hFVIII-expressing plasmid. The expression plasmid contains neomycin-resistance (neo) selectable marker, allowing neomycin selection of stable transformants of mammalian host cells. The oligonucleotides (synthesized by *MWG-Germany*) used for polymerase chain reaction (PCR) and sequencing are listed in Table 1.

Table 1. Activity measurement of the purified rhFVIII form conditioned medium by two-step ion-exchange chromatography. The activity of the purified fractions of SP- and Q-sepharose chromatography was lower than that of the concentrated medium.

Sample	Test		
	Protein concentration mg/ml	Clotting activity%	ELISA%
Conditioned medium (C.M.)	2.5	9	10
Concentrated C.M.	7.5	120	110
Conditioned medium of untransfected cells	3.2	0	0
Normal citrated plasma	7	100	100
Frac. B of SP-sepharose	1.2	23	23
Frac. B of Q-sepharose	0.8	5	-

Media, Enzymes, Chemicals and Kits

Luria-Bertani (LB) [10 g/l Bacto-tryptone, 5 g/l Bacto yeast extract, and 10 g/l NaCl, pH 7.0 with NaOH, purchased from *Merk*-Germany] was used as the bacterial culture medium, and ampicillin (100 mg/ml) was added when required to maintain selection pressure. Enzymes *Not*I, *Bgl*II, Taq DNA polymerase and T4 DNA ligase were purchased from the *Roche*-Germany. AMV-reverse transcriptase was purchased from (*Fermentas*). Geneticin (G-418) and the transfection reagent, FuGENE6, were obtained from *Roche*-Germany. Alkaline lysis method was applied for plasmid DNA preparations [16]. The commercially prepared columns (*Roche*-Germany) were used for the purification of DNA. The COS7 cells were grown in Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco-BRL Llife Technology), 100 U/ml penicillin G and 100 μg/ml streptomycin (*Sigma-Germany*). The CHO cells were grown in Hams-F12 (Gibco-BRL Llife Technology). ELISA kit for measuring hFVIII antigen (Asserachrom VIIIc: Ag) and deficient FVIII plasma were purchased from *Diagnostica Stago*-France. Citrated normal pooled-plasma (kindly provided by Kamran Atarodi in the quality control unit of iranian blood transfusion organization) was used as a standard in coagulation test. Dig- DNA- labeling and Dig-nucleic acid detection kits and the positive charged membrane (Roche-*Germany*) were used in southern blotting experiment. RNX^{TM} (plus) RNA isolation kit (*Cinnagen-Iran*) was used for isolation of the total cellular RNA.

Construction of the Recombinant pcDNA3-FVIII Plasmid

DNA manipulation techniques, such as plasmid DNA isolation, DNA digestion and sub-clonings were performed according to standard methods [15]. To construct the recombinant plasmid, a *Not*I restriction fragment, originated from the pGEM3-hFVIII plasmid containing the FVIIISQ coding sequence, was purified from agarose gel and sub-cloned in the *Not*I site of pcDNA3 plasmid. The recombinant plasmid was transferred into the DH5α strain of *Escherichia coli* and transformants were isolated on ampicillin containing LB medium and subjected to further use. The recombinant plasmids, carrying the insert with proper orientation, were selected by restriction analysis of the DNA obtained from the transformants. The recombinant plasmid was also verified through PCR, using specific primers followed by complete sequencing of the cloned fragment, using AB1 373A automated sequencer (*MWG-Germany*).

The PCRs were carried out with the temperature profile of a 3-minute period at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C followed by a final extension for 5 min at 72°C. The PCR-amplified products were analyzed by electrophoresis on 0.8% agarose-gel. Restriction analysis also was employed to confirm the recombinant plasmid.

Cell Culture and Transfection

The mammalian cells were grown in 5% CO_2 atmosphere at 37°C. One day before transfection, cells were subcultured at a density of 2×10^5 cells in 2 ml of medium in a 35 mm (8 cm^2) culture dishes (or 6-well plates). The cells were then transfected with approximately 4 μg plasmid DNA by lipofection-mediated method.

Measurement of FVIII Coagulation Activity

The activity of the secreted FVIII was measured based on one-stage coagulation assay (with a sensitivity limit of 0.01 U/ml) using FVIII-deficient plasma and activated partial thromboplastin (aPTT) reagent, according to the instructions described by manufacturer (*Diagnostica stago-France*): 100 µl of sample or standard (citrated pooled-plasma) was added to 100 µl of FVIII-deficient plasma, following of which 100 µl of the aPTT reagent was also added and incubate for a 3-minute period at 37°C. After incubation 100 µl of 25 mM $CaCl_2$ was added to initiate the reaction, and the time required to develop fibrin clot was measured. FVIII activity was determined based on a log-log standard curve. The standard curve was constructed by making five dilutions of normal citrated plasma (1/10, 1/20, 1/40, 1/80 and 1/160) and by plotting log clotting time versus log plasma FVIII activity.

Measurement of FVIII Antigen

The rhFVIII antigen in the conditioned cultured media was assayed by sandwich enzyme-linked immunosorbent assay (ELISA) on micro-plate, coated with a specific mouse anti-hFVIII monoclonal antibody, provided in the ELISA- kit (*Diagnostica Stago-France*). The bound FVIII to the first antibody was revealed by the use of a second mouse anti-FVIII monoclonal antibody, labeled with horseradish peroxidase that binds to another antigenic determinant of the FVIII. The enzymatic activity was then demonstrated by its oxidative action on the substrate ortho-phenylendiamine (OPD) in the presence of urea peroxide. After the reaction was stopped by the addition of sulphoric acid, the obtained coloration was measured at 492 nm. The observed optical density was directly proportional to the concentration of hFVIII. The detection limit for the antigen assay was 0.1 ng/ml. cultured media collected from untrasfected cell-lines (COS7 & CHO) were used in parallel as negative control for the analysis.

Reverse Transcription PCR (RT-PCR)

Total cellular RNA was extracted from CHO cells according to the manufacturer's instruction. cDNA was synthesized from the isolated RNA, pre-treated with RNase-free DNase, by AMV-reverse transcriptase. The synthesized FVIIISQ-cDNA was subsequently analyzed by amplification of two fragments, using two specific primer pairs, namely A1-8F (5′-agt cct gaa gct aga tct ctc tcc-3′), A1-8R (5′-ata aga atg cgg ccg caa tat gga gag aga tct agc-3′), C8F (5′-att tgg cgg gag gaa tgc ctt att ggc g-3′) and C8R (5′-aca cct cga gtc agt aga ggt cct gtg cct cg-3′) from hFVIIISQ coding region.

Southern Blotting

The DIG-labeled DNA-probe was synthesized from a restriction fragment covering the hFVIIISQ cDNA. Chromosomal DNA from mammalian cells was prepared by salting-out methods [16]. The genomic DNA of transfected and untransfected (negative control) cells were digested with *Bgl*II endonuclease and separated on 1% agarose gel along with their undigested high molecular weight chromosomal DNA and a super-coil pattern of the pGM3-hFVIII plasmid. The electrophoresed DNAs were then transferred onto positive-charged nylon membrane by capillary transfer method, followed by alkaline-denaturation of DNA [15]. The blot was then subjected to hybridization procedure, using the specific DNA probe followed by detection steps based on the protocols provided by manufacturer.

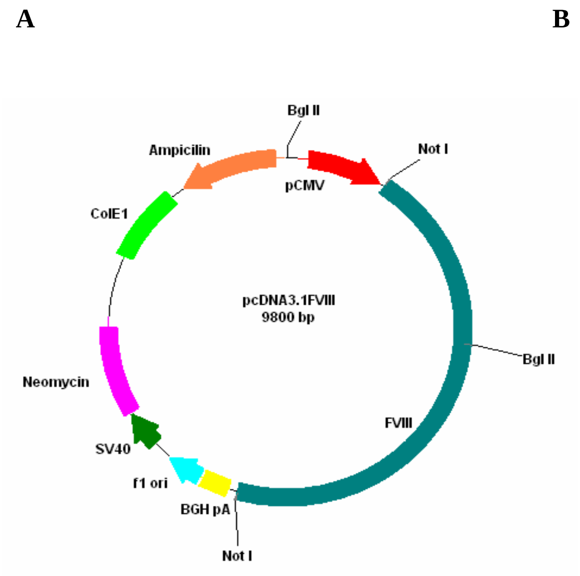
Purification

A two-step ion exchange chromatography procedure was applied for the purification of rhFVIII. The conditioned medium was clarified and then concentrated by ultra filtration. The solution was loaded onto a SP-sepharose fast-flow column, equilibrated in 0.1 M NaCl, 20 mM HEPES, 5 mM $CaCl_2$, 0.01% tween, pH 7.4. The rhFVIII was eluted with a linear 0.1-0.65 M NaCl gradient in the same buffer. The collected fractions containing FVIII were pooled. The pooled fractions were diluted with 0.2 M NaCl in the same buffer, applied to a Q-sepharose column and eluted with a linear 0.2-1 M NaCl gradient. Fractions were then assayed by coagulation assay and ELISA [17,18].

Results

Construction of the Plasmid pcDNA3-FVIII

After the insertion of a *Not*I fragment containing complete coding sequence of the B-domain-less FVIIISQ in front of CMV promoter in the pcDNA3 plasmid, amplification of the recombinant plasmid in bacterial host was carried out. Taking advantage of the only *Bgl*II site in the insert and considering the unique *Bgl*II site in the vector DNA (Fig. 2A), by performing *Bgl*II restriction analysis among the isolated clones, we selected a number of clones carrying recombinant plasmids with proper orientation of the FVIII coding fragment. As it is shown in Figure 2B, two *Bgl*II fragments with about 7200 bp and 2600 bp were generated after *Bgl*II digestion of the recombinant plasmid, which was expected from the clones with correct insert orientation. The size of the insert in the



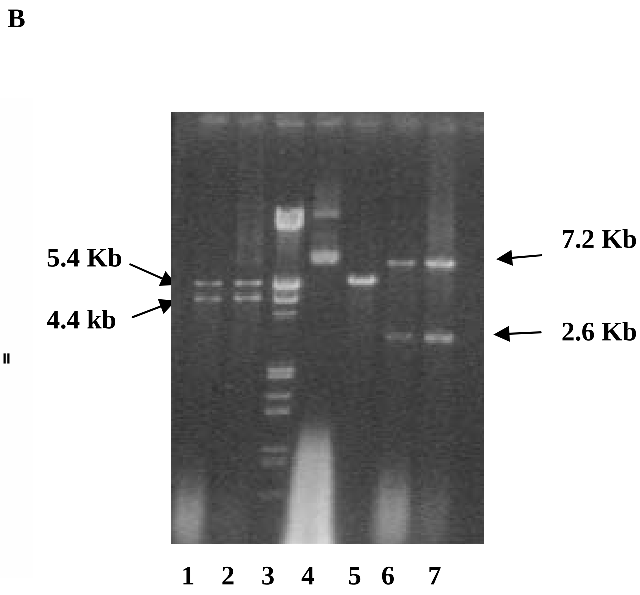


Figure 2. *Panel A.* Physical map of the recombinant pcDNA3-hFVIIISQ plasmid. *Panel B.* Restriction pattern of the recombinant plasmid. Lanes 1 and 2: *Not*I digestion of the recombinant plasmid. Lane 3: DNA size marker (λ-phage DNA digested with *Hind*III/*Eco*RI). Lane 4: Undigested recombinant plasmid. Lane 5: Linearized pcDNA3 plasmid. Lanes 6 and 7: *Bgl*II digestion. The bands corresponding to *Bgl*II and *Not*I digestion of the recombinant plasmid are indicated by their sizes.

selected clone, corresponding to the size of FVIIISQ cDNA (about 4.4 Kb), was also confirmed by the *Not*I digestion of the recombinant plasmid (Fig. 2B). The selected plasmid was considered for further expression analysis.

Transfection of the Mammalian Cells with the Recombinant Plasmid

In order to study the expression of hFVIIISQ, two mammalian cell lines, COS7 and CHO, were separately transfected by the recombinant plasmid that contains neomycin-resistance (neo) selectable marker, allowing selection of stable transfectants among mammalian host cells. For any of the examined cell lines, after transfection, the activity of the secreted FVIII was determined by one-stage clotting assay as well as enzyme-linked immunnosorbent assay (ELISA) at different post-transfection intervals (see below). After demonstration of a transient expression of rhFVIII by the transfected COS7 cells, our studies focused on the expression of rhFVIII by the transfected CHO cells to obtain a stable expression cell line. Accordingly, the transfected CHO cells were selected in geniticin (G-418) containing media (500 μg/ml), and G-418-resistant colonies appeared approximately 14 days after transfection. Five stable clones of the transfected CHO cells were then isolated after several attempts of reselection and were stored for further use.

Expression Analysis

Transient Expression of hFVIIISQ

In the case of the transfected COS7 cells, transient expression of hFVIII was confirmed with clotting test (Fig. 3A). The activity of the rhFVIII was also measured by performing ELISA on the cultured media at various post-transfection time periods from day 1 to day 5 (Fig. 3B) that was comparable with the results obtained from clotting test. According to the obtained results, the highest expression level of rhFVIII is achieved on day 4 following transfection that was estimated to be around 0.1 U/ml of the cultured media. A unit of FVIII is defined as the amount that is present in 1 ml of citrated normal plasma [19].

Stable Expression of hFVIIISQ

Following the isolation of the five stable transfected CHO cells, the expression analysis was continued on the cultured media collected at different passages during 4 weeks following selection (Fig. 4). Accordingly, a successful and stable secretion of the biologically active rhFVIII in the five isolated clones was confirmed, while no activity was observed in the cultured media collected from untransfected cells. As the data indicated, the expression levels in the five clones were apparently similar with slight variations during first four weeks after selection. As it is shown in Figure 4, clone number 2 among others shows less variation in expression level and higher activity of the media collected during several passages. Therefore, it was considered for further expression analysis.

To examine the state of the rhFVIII expressing plasmid in the stably transfected CHO cells, we performed Southern blotting analysis on both high molecular weight and *Bgl*II digested genomic DNA of the transfected cells in comparison with that of untransfected CHO cells, using a *Not*I restriction fragment containing the BDDhFVIII cDNA as probe. The Southern blotting results are shown in Figure 5. No signal was detected in negative controls. A high molecular band (lane 6) was detected, corresponding to the undigested chromosomal DNA of transfected cells. At least three different bands were also appeared in the samples corresponding to the *Bgl*II digested chromosomal DNA from the transfected cells that are probably corresponded to the plasmid DNA. The signal detected in the high molecular weigh chromosomal DNA as well as the digested DNA, obtained from the Southern blotting analysis, suggest for a stable presence of the rhFVIII expressing plasmid in the transfected CHO cells.

Analysis of the B-domain-less hFVIII Transcript

The presence of the FVIIISQ transcript in the selected stably transfected CHO cells were shown by the PCR-amplification of two fragments from FVIII cDNA, reversely transcribed from stably transfected cells' mRNA. As the electrophoresis pattern of the RT-PCR products indicates, bands of the appropriate size support the transcription of hFVIIISQ in the CHO cells, transfected with the recombinant plasmid (Fig. 6).

Activity Measurement of hFVIIISQ Secreted by the Stably Transfected CHO Cells

The expression of hFVIIISQ by the stably transfected CHO cells was measured based on procoagulation as well as antigen activities of the secreted rhFVIII in conditioned culture media from the same cells (Figs. 7 and 8). As the results show, the highest procoagulant activity occurs on day 4, whereas the highest FVIII: Ag measured by ELISA was observed on day 5. Based on the cell concentrations, calculated during the same period, the amount of the hFVIIISQ secreted by 10^6 cells was also estimated. Accordingly, the amount of the secreted active hFVIISQ on day 4 was estimated to be around 0.1 U/ml of the cultured media that emanated from 0.06 units of hFVIIISQ secreted form 10^6 cells per ml of the cultured conditioned media.

A primary protein analysis of the fractions collected from the two-step purification, compared with the initial samples indicated the removal of a significant part of proteins from the conditioned media of the transfected cells after chromatography. The activity measurement of both purified fraction and concentrated medium indicated the presence of rhFVIII, expressed by the transfected cells (Table 1). The coagulation activity of concentrated samples was ten times higher than that of the initial sample, whereas the activities of the purified fractions corresponding to the step-1 and step-2 chromatography were much lower than that of the initial samples.

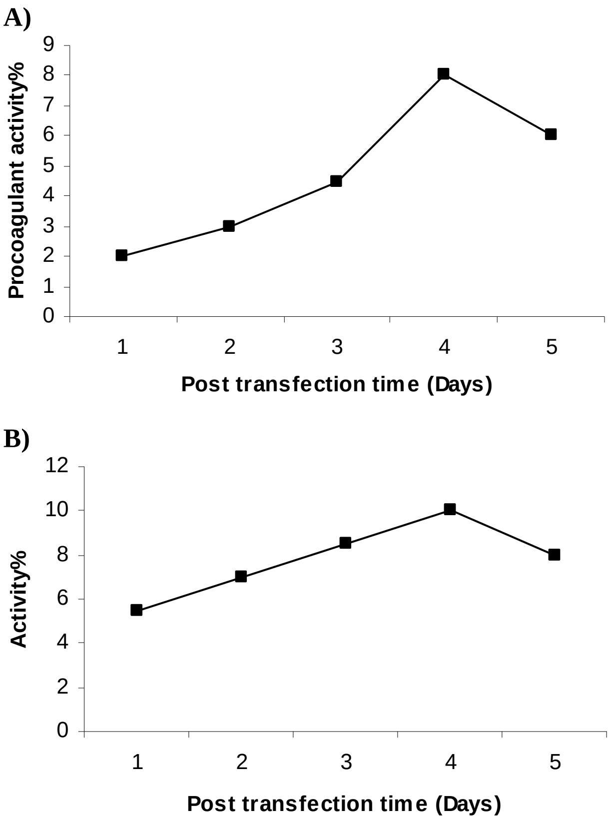


Figure 3. Transient production of rhFVIII from COS7 cells transfected with the recombinant plasmid (pcDNA3hFVIIISQ), in various post-transfection times. The activity of the rhFVIII, secreted by the transfected cells into the conditioned cultured media was measured after periods varying from 1 to 5 days. A) Procoagulant activity by one-stage clotting assay. B) Activity of hFVIII:Ag by ELISA.

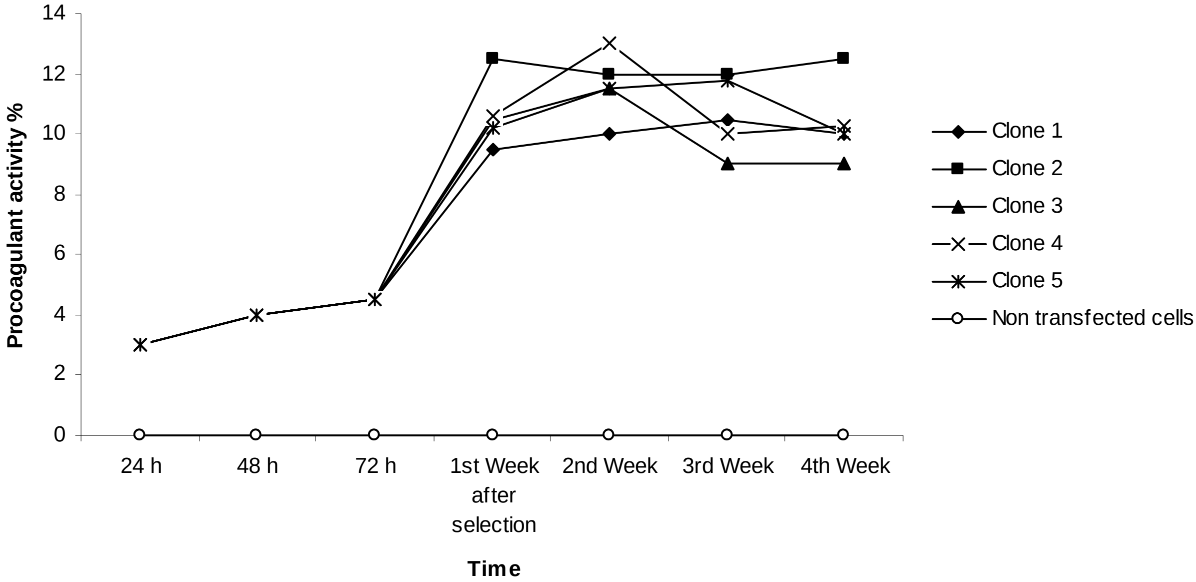


Figure 4. Production of rhFVIII during the periods before and after isolation of stably transfected CHO clones. Procoagulant activity of the rhFVIII secreted by the transfected CHO cells into the conditioned cultured media was measured based on one-stage clotting assay after periods varying from 1 to 3 days before selection and 1 to 4 weeks after selection of the 5 clones. The graph represents the mean value of three measurements of each sample.

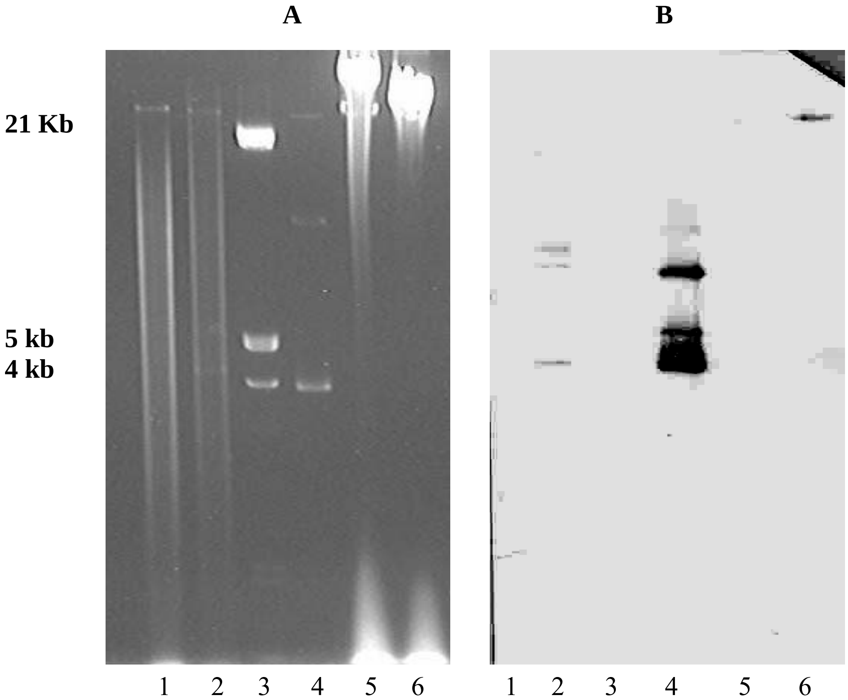


Figure 5. Southern analysis of the chromosomal DNA extracted from CHO cells. A) Pattern of the DNA separated on 1% agarose gel. B) Southern results of the DNA pattern, in Panel A. A *Not*I restriction fragment containing the B domain deleted hFVIII cDNA was used as prob. Lanes 1: Chromosomal DNA from the normal CHO cells digested with *Bgl*II (negative control). Lanes 2: Chromosomal DNA from transfected CHO cells digested with *Bgl*II. Lanes 3: DNA size marker (λ-phage DNA digested with *Hind*III/*Eco*RI). Lanes 4: pGEM3 plasmid containing FVIII cDNA as control. Lanes 5: High molecular weight chromosomal DNA from a normal (untransfected) CHO cells (Negative control). Lanes 6: High molecular weight chromosomal DNA from transfected CHO cells.

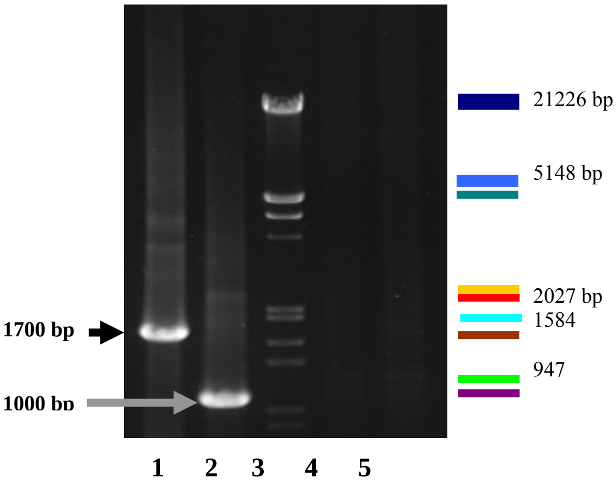


Figure 6. PCR products amplified from the reverse transcribed total RNA. In each case, the prepared RNA was treated with RNase-free DNase. Lane 1: Transfected CHO cells, using A18F/A18R primers. Lane 2: Transfected CHO cells, using C8F/C8R primers. Lane 3: DNA size marker (λ-phage DNA digested with *Hind*III/*Eco*RI). Lane 4: Transfected CHO cells, using total RNA pre-treated with RNase-free DNase as template and A1-8F/A1-8R primers Lane 5: Untransfected CHO cells, using A1-8F/A1-8R primers.

Discussion

Hemophilia A is an X-linked recessive bleeding disorder, widely prevalent throughout the world. Replacement therapy is current hemophilia A treatment, done by infusion of either human plasma derived FVIII or recombinant FVIII [26]. Both full-length and B-domain deleted forms of hFVIII have therapeutic applications that are equivalent to plasma derived ones. However, the FVIII replacement remains a very expensive therapeutic with the average patient using up to $100,000 of hFVII concentrate per year [20]. In spite of characterization of human factor VIII gene, investigations on the over-production of recombinant hFVIII are in progress in several laboratories throughout the world [14,20,21]. Here, we have reported a successful expression of biologically active rBDDhFVIII in our laboratory. The biological activities demonstrated for the expressed rhFVIII indicate a proper structure of the protein. However, the amount of activity detected by ELISA is slightly higher than that of the results shown by one-stage clotting test. This difference in activity can be attributed to the fact that the presence of the antigenic determinant may not be accompanied completely by the biological activity. As it has been reported by other researchers [22], a low expression level of rhFVIII is expected. The results of present study are similar to those reported in other studies. Two separate research groups, who used multiple rounds of selection with methotrexate for the amplification of the expressed hFVIII, reported expression levels of 0.2-0.3 U/ml hFVIII in dHFR$^-$-CHO cells [20,23].

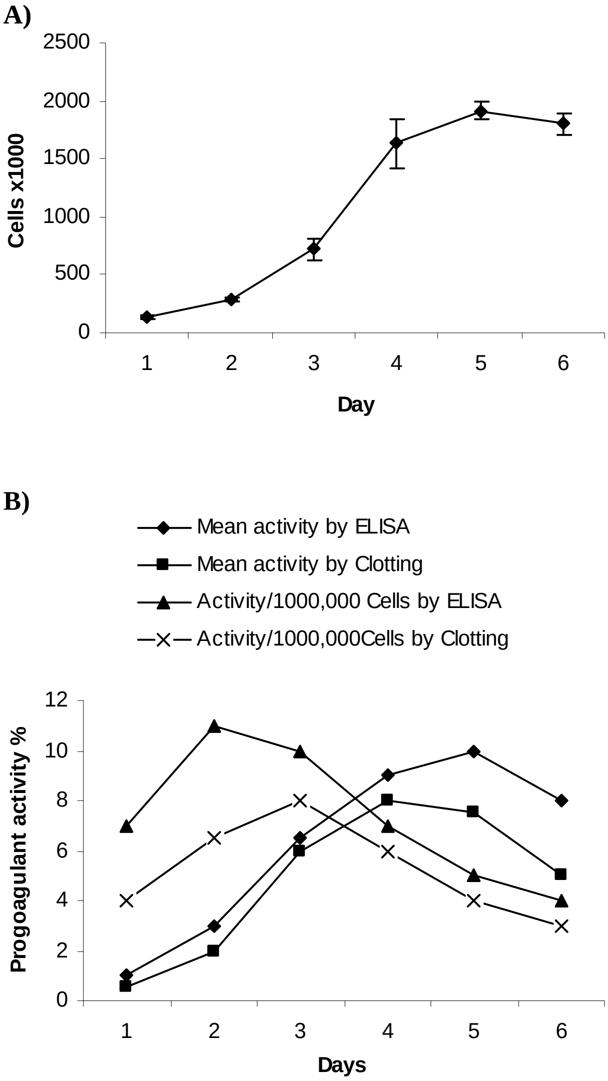


Figure 7. Expression assay of rhFVIII by the stably transfected CHO cells. A) Cell growth pattern of the stably transfected CHO cells. 1×10^5 cells were seeded in 35 mm plate with 3 ml culture media and number of cells was measured after a period varying from 1 to 6 days. B) Production of rhFVIII during 6 days of sub-culturing, based on the procoagulant and antigen activities of the rhFVIII secreted by the transfected CHO cells into the conditioned cultured media.

1 2 3 4

Figure 8. *ELISA Results*, A1: Blank, B1: 1/10 dilution of standard hFVIII. C1: 1/20 dilution of standard hFVIII. D1: 1/40 dilution of standard hFVIII. E1: 1/40 dilution of standard hFVIII. F1: 24 h after transfection. G1: 48 h after transfection. H1: 72 h after transfection. A2: 96 h after transfection. B2: 120 h after transfection. C2: 148 h after transfection. D2: Negative control. E2: 48 h after transfection. F2: 72 h after transfection. G2: 96 h after transfection. H2: 120 h after transfection. A3: 148 h after transfection. B3: Concentrated cultured medium by Amicon filter. C3: 48 h after transfection. D3: 72 h after transfection. E3: 48 h after transfection. F3: 72 h after transfection. G3: 96 h after transfection. H3: 120 h after transfection. A4: h after transfection. B4: 96 h after transfection. C4: Fraction B from step-1 chromatography. D4: Fraction A from step-2 chromatography. E4: Fraction B from step-2 chromatography. F4: Fraction C from step-2 chromatography. G4: Negative control. H4: fraction B from step-2 chromatography (concentrated).

Several mechanisms have been identified in limiting FVIII expression including inefficient expression of FVIII mRNA, inefficient folding of the primary translation product within endoplasmic reticulum (ER), the requirement for facilitated transport from the ER to the Golgi apparatus, the requirement for association and stabilization within plasma by von Willebrant Factor [24], and finally the instability of the thrombin-activated form of FVIII (FVIIIa) by proteases [10]. Many studies support that the B-domain is dispensable for hFVIII procoagulant function and therefore several independent investigations have focused on the production of B-domain-deleted forms of hFVIII [25]. However, a recent study [20] showed that there are several sites for N-linked glycosylation in B-domain that effect the secretion efficiency of the hFVIII. Therefore, the absence of the B-domain can be considered as one of the reasons for the low expression level of rhFVIII in addition to other limiting factors in this study. The primary results obtained from the chromatography-based purification, suggest that applied procedure in this study is unable to maintain the hFVIII biological activity. Therefore stabilizing factors would be required to maintain coagulation activity of this protein.

Having now identified some of the limitations in current rhFVIII technology, the recombinant hFVIII expression system developed in this study has provided means for further bioengineering strategies to enhance expression efficiency of hFVIII.

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