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RESEARCH ARTICLE

A systematic study of the function of the human β-globin introns on the expression of the human coagulation factor IX in cultured Chinese hamster ovary cells

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Abstract

Background Intronic sequences have the potential to improve gene expression in eukaryotes by a variety of mechanisms. In this context, human β-globin (*hBG*) introns were inserted into the human factor *IX* (*hFIX*) cDNA in cytomegalovirus (CMV)-regulated plasmids. The resulting construct was then used for further expression analysis *in vitro*.

Methods Seven *hFIX*-expressing plasmids with different combinations of the two *hBG* introns and the Kozak element were constructed and used for a systematic expression analysis in cultured Chinese hamster ovary (CHO) cells. In parallel, the *hBG* intronic sequences were analysed for the presence of possible regulatory elements.

Results All the constructed plasmids resulted in transient expression of the *hFIX*. However, the coagulation activities varied according to the particular constructs used. Based on the *hFIX* antigenic assay, a wide range of variation was observed during persistent expression. The second *hBG* intron appears to be more effective than the first one. The expression level was further increased upon the inclusion of the Kozak element. Sequence analysis has detected several transcription factor binding (TFB) motifs in both of the introns, but with a higher frequency in the second one.

Conclusions Potentials of *hBG* introns as enhancer-like elements for the expression of the *hFIX* in cultured CHO cells and a higher activity with respect to the second *hBG* intron compared to the first one were demonstrated. The larger number of TFBs in the second *hBG* intron reflects its stronger effect. The results obtained suggest possible synergistic functions of the *hBG* introns and Kozak on the expression level of *hFIX in vitro*. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords human β-globin (hBG) intron; recombinant human coagulation factor IX (rhFIX); transcription factor binding (TFB) motifs

Introduction

Two major approaches for the treatment of genetic diseases are replacement therapy with recombinant proteins and gene therapy. In order to produce a functional protein, an efficient expression vector with suitable regulatory elements is required in either technique. Because of limitations in vector size, the inclusion of all corresponding regulatory elements of a gene in

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the expression plasmid presents a hurdle. Therefore, in many cases, the expression efficiencies of heterologous proteins in such systems are not satisfactory. Gene regulation takes place at several levels and is usually controlled by regulatory elements in noncoding regions such as promoters and upstream enhancers and terminator and polyadenylation elements in downstream regions of the genes [1]. Introns, as a major part of noncoding sequences, have the potential to improve gene expression in a broad range of organisms, including nematodes, insects and mammals [2]. The importance of introns for eukaryotic cells is explained by eukaryotic cells spending enormous amounts of energy to amplify and maintain such sequences during evolutionary periods [3]. Introns also appear to play critical roles in molecular evolution through their repeated elements, which function as recombinogenes [4]. In the human genome, while the coding sequences comprise less than 5%, the repeated sequences, which are mostly derived from retrotransposons, cover more than 50% of the genome [5]. The nonrandom distribution of the repeated elements throughout the introns in eukaryotic genomes, supports their importance as regulatory sequences [6].

Introns and their removal by spliceosomes regulate the expression of genes in different levels, including transcription, polyadenylation, nuclear mRNA export, translational efficiency and mRNA decay. Further evidence for the involvement of introns in gene regulations is provided by studies demonstrating that the nuclear export of mRNA and its stability and localization are affected by the components of exon junction complex [7–10]. Evidence from plants also indicates that introns promote the accumulation of mRNAs, apparently by facilitating their maturation or enhancing the stability of the emerging transcripts [11,12]. The intron-dependent effects may cause more than 400-fold increase in mRNA levels [13]. Moreover, the intron activity is believed to be affected by intron position within the gene and exon sequence context. There are reports indicating that introns in their native positions and in different positions could cause opposite outcomes of gene expression [14]. Promoter proximal introns can increase pre-mRNA synthesis by enhancing both transcription initiation and RNA polymerase II processivity [1]. Experiments in cell-free systems and transient transfection assays also imply that the 3′-splice acceptor intronic region is required for efficient 3′-end cleavage and polyadenylation [15,16]. In a number of genes, some elements within the 3′-end intron interact with polyadenylation processing and increase the poly A tail length, leading to longer half life for mRNA and more efficient translation [1]. Indirect support for their post-transcriptional roles could be concluded from the fact that introns must be contained within the transcribed sequences and in the proper orientation to elevate gene expression, unlike the transcriptional enhancers, which are usually position and orientation independent [12]. Introns and the 3′-untranslated region of the transcript may have synergistic effects on gene expression *in vivo* [17]. Furthermore, synergistic interactions between the splicing and polyadenylation machineries contribute to more efficient 3′-end processing in intron containing transcripts [1].

The presence of some introns is considered to be crucial for the accumulation of mRNA in certain genes, such as the human growth hormone [18] and purine nucleoside phosphorylase [19]. In other cases, such as the genes encoding triosephosphate isomerase [20] and β-globin [21], the last introns are implicated in 3′-end formation of their corresponding transcripts. Experimental analysis confirms that the presence of either partial or full length of intron(s) in the vicinity of a gene improves the expression of the corresponding gene [22]. This increase may approach up to 500-fold [1].

Experimental evidence reveals that the *hBG* introns play critical roles in the expression of the corresponding gene [21]. For example, the export of the *hBG* transcripts is highly dependent on the presence of its introns, especially the second intron, which is essential for the accumulation of stable cytoplasmic mRNA. It has also been shown that the inclusion of either intron I or intron II can restore the expression of an intronless rabbit β-globin gene in HeLa cells [23]. The enhancer-like activities of the β-globin intronic sequences on the expressions of different transgenes have also been shown. Noe *et al.* [24] showed that, insertion of the *hBG* intron I into the corresponding location in dehydofolate reductase (*Dhfr*) cDNA improved production of *Dhfr* protein even more than the natural intron I of the DHFR gene *in vitro*. In similar studies, introductions of the first intron and second introns from the rabbit β-globin into vectors containing, respectively, the human factor VIII and ceruloplasmin cDNAs successfully improved the productions of their corresponding proteins [25,26].

The coagulation FIX is an essential vitamin K-dependent protein that participates in the intrinsic pathway of blood coagulation [27]. Mature *hFIX* is one of the serine proteases from peptidase family S1. It circulates as an inactive precursor before activation with either factor XIa and calcium ions or tissue factor/factor VIIa and calcium ions during blood coagulation [28]. Hemophilia B, an X-linked recessive bleeding disorder, is caused by the functional deficiency or lack of the *hFIX* [29]. Gene therapy is an alternative approach for treatment of hemophilia B patients [30]. Currently, replacement therapy is the major treatment for this disease, carried out via the infusion of normal *hFIX*, produced either from human plasma or recombinant expression systems [31,32]. Application of intron(s) to achieve higher expression levels of *hFIX* has been demonstrated previously by Kurachi *et al.* [33]. These authors demonstrated that sub-regions of the first *hFIX* intron inserted immediately upstream of the *hFIX* promoter exerted only marginal enhancing or even weakly negative regulatory activities on factor IX gene expression. However, mini-gene constructs containing further truncated first intron sequences (1.4 and 0.27 kb, respectively) and legitimate splicing sequences (donor,

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acceptor and branch sites) increase the *hFIX* gene expression by seven- to nine-fold compared to constructs that lack intronic sequences in cultured hepatoma cells. In transgenic mice, inclusion of the full-length 6.2-kb or a truncated 1.4-kb fragment of the first intron of the *hFIX* gene increases gene expression by 40–200-fold [34].

In most of the studies mentioned above, the intronic sequences have been placed in the 5′-untranslated reagions (UTRs) with rather ignoring the potentials of introducing the intronic sequences inside the transgene open reading frame. Introduction of introns into coding regions may have distinct advantages over their insertion into the UTRs. It is reasonable to assume that the *hBG* introns function more effectively in locations similar to their natural positions. This approach would create a gene structure reminiscent of typical mammalian genes to provide a near-natural substrate for gene expression [35]. In this regard, a set of recombinant *hFIX* expressing plasmids were constructed, which carry various combinations of introns I and II of the *hBG* gene, respectively, at the first and second intronic positions in *hFIX* cDNA. Because the complete *hBG* intronic sequences were inserted in the *hFIX* exon junction sites in six out of seven constructed plasmids, the corresponding mini-genes were expected to contain requirements for proper splicing. A direct correlation between the number of introns and the expression level of protein in a mammalian expression system has been reported previously [19]. Introns can act cooperatively to enhance the level of mRNA, an effect that would require the presence of at least two introns [36]. In the present study, the cooperative functions of the two *hBG* introns, introduced inside the *hFIX* cDNA, were also examined.

In addition to the well-known regulatory elements, the start codon context also affects expression at the translation level as suggested by Kozak [37]. In this context, a consensus hexa-nucleotide sequence (GCCACC) prior to the start-codon is believed to affect translation efficiency of the corresponding transcript. Therefore, a Kozak element was introduced into the second generation of each of the plasmids mentioned above. The present study reports the results obtained from the systematic expression analysis of the recombinant CHO cells which carry the *hFIX* mini-genes and discusses various aspects of the function of heterologous introns in this context.

Materials and methods

Bacterial strain, mammalian cell line, plasmids and primers

The DH5α strain of *Escherichia coli* (Strategene, La Jolla, CA, USA) was used as host for various cloning and subcloning steps. The CHO cell line was used as expression host. Plasmid pK14hFIX [38] was used as source of the *hFIX* cDNA. Plasmids $pBC_{(SK)}$ (Strategene) and pET26+ (Novagen, Damstatdt, Germany) were used as cloning and subcloning vectors. Plasmid pcDNA3 (Invitrogen, Carlsbad, CA, USA) was used for the construction of the *hFIX*-expressing plasmids. The oligonucleotides for polymerase chain reactions (PCR) (Table 1), were synthesized by MWG Biotech (Ebersberg, Germany). To facilitate the cloning steps, restriction sites were considered at the 5′-ends of the designed oligonucleotides. A Kozak sequence was included in primer hF9KozF next to the *BamH*I restriction site and before the *hFIX* start codon.

Media, enzymes, chemicals and kits

Luria–Bertani medium was used for bacterial growth and either ampicillin (100 µg/ml) or kanamaycin (30 µg/ml)

Table 1. List of the oligonucleotides used for the construction of *hFIX*-mini-genes

Restriction site	Nucleotide sequence	Name
*Bam*HI	5′-GGATCCGTTATGCAGCGCGTGAACATGATC-3′	hFIXE1-F
	5′-AACCTTGATACCAACCTGTACATTCAGCACTGAGTAGATATCCTA-3′	hFIXE1-R
	5′-AGTGCTGAATGTACAGGTTGGTATCAAGGTTACAAGACAGG-3′	hBI1-F
	5′-ATGATCAAGAAAAACTAAGGGTGGGAAAATAGACCAATAG-3′	hBI1-R
	5′-TTTTCCCACCCTTAGTTTTTCTTGATCATGAAAACGCCAAC-3′	hFIXE2-F
*Dra*I	5′-CCTTGCAACTGCCGCCATTTAAAC-3′	hFIXE4-R
*Not*I	5′-GCGGCCGCAGTGATTAGTTAGTGAGAGGCCC-3′	hIX-R1
*Nco*I	5′-GCCATGGCCCCCTTTGGATTTGAAGGAAAGAACT-3′	hFIX-F2
*Hind*III	5′-GAAGCTTTCTCCCTTTGTGGAAGACTCTTCCC-3′	hFIX-R2
*Bam*HI	5′-GGATCCGCCACCATGCAGCGCGTGAACATGAT-3′	hF9KozF
*Xho*I	5′-CCGCTCGAGCTTCTCCAAAACTACACTTTTC-3′	hFIXE2-R
*Eco*RI	5′-CCGAATTCTCAAGAAAAACTGAAATGTAAAAGA-3′	RI2 : EcoRI site
*Eco*RI	5′-CGGGAATTCTGGAAGCAGTATGTTGA-3′	EcoRI-E39FIX-F
	5′-GTGAGTCTATGGGACGCTTG-3′	SIN2-BGLOBIN-F
*Eco*RI	5′-CGGGAATTCAGTCTGTGGGAGGAAGATAAGAG-3′	EcoRI-E3FIXI2BG-R
*Xho*I	5′-CCGCTCGAGCTTCTCCAAAACTACACTTTTC-3′	XhoI-E2FIX-R
*Xho*I	5′-CCGCTCGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTCTATGGGACGCTTGAT-3′	XhoI-E2FIXIN2BG-F
*Not*I	5′-GCGGCCGCAGTGATTAGTTAGTGAGAGGCCC-3′	NotI-FIX-R
*Bam*HI	5′-GGCGGTACCGGATCCGTTATGCAGCGCGTGAACATGA-3′	KpnI/BamHI-FIX-F

Restriction sites are underlined and indicated in front of the corresponding oligonucleotide. The Kozak sequence is shown in grey.

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was added when required, to maintain selection pressure. The CHO cells were grown in Dulbeco's modified Eagle's medium and Hams-F12 (Gibco-BRL Life Technology, Karlsrahe, Germany) at a 1 : 1 ratio supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco-BRL Life Technology) plus 100 U/ml of penicillin G and 100 μg/ml of streptomycin (Sigma-Aldrich, Munich, Germany). All the enzymes used for molecular techniques in addition to kits for PCR product purification, plasmid isolation and RNA preparation and other chemicals such as FuGene-6 and geneticin (G-418) were purchased from Roche (Mannheim, Germany). Alkaline lysis method was also applied for plasmid DNA preparations [39]. The kit for the cloning of PCR products (InsT/Aclone) was obtained from Fermentas (Burlington, Ontario, Canada). The enzyme-linked immunosorbent assay (ELISA) kit for the measurement of the *hFIX* antigen (Asserachrom hFIX::Ag) and FIX-deficient plasma were purchased from Diagnostica Stago (Asnières sur Seine, France). Citrated normal pooled plasma (kindly provided by Dr Amirizadeh at the Quality Control Unit of the Iranian Blood Transfusion Organization) was used as a standard sample in the coagulation test. RNX™ (plus) kit (Cinnagen, Tehran, Iran) was used for isolation of total cellular RNA.

Construction of rhFIX expressing plasmids

All DNA manipulations were carried out based on standard cloning procedures [39]. Human genomic DNA, extracted from blood, was used as template for amplifications of the *hBG* introns. PCR-mediated procedures were carried out to generate different *hFIX* mini-genes (Figure 1). Seven recombinant CMV-regulated *hFIX* expressing plasmids were constructed, as described below. Three of the plasmids, namely phFIX-I, phFIX-II and phFIX-I-II, carry intron I, intron II and inrons-I/-II of the *hBG* in the *hFIX* cDNA, respectively. In the three other plasmids, namely pkhFIX-I, pkhFIX-II and pkhFIX-I-II representing the second generations of the above mentioned plasmids, a Kozak sequence was engineered prior to the *hFIX* start codon.

The first plasmid, phFIX carrying an intron-less *hFIX* cDNA was constructed: a *BamH*I/*Not*I restriction fragment, originating from the pK14hFIX plasmid [38] was subcloned between *Not*I and *BamH*I sites of the pcDNA3 plasmid. The resulting phFIX plasmid was then used both as the parental *hFIX* expressing plasmid for the construction of other intron-containing plasmids and as a control (parental) *hFIX* expressing plasmid.

The second plasmid, phFIX-I carriying intron I of *hBG*, which is 130 bp and located between exons 1 and 2 of the *hFIX* gene. In order to insert the *hBG* intron I into the *hFIX* cDNA, a *BamH*I/*Not*I restriction fragment containing the *hFIX*-cDNA (from the previous step) was inserted into a pET26+ plasmid to generate the pET26-hFIX plasmid. To insert the *hBG* intron I into the acceptor-donor site between the *hFIX* exons 1 and 2, a PCR-mediated method, known as splice overlap extension-PCR (SOE-PCR), was performed. For this purpose, three pairs of primers, namely [$hFIXE_1$-F/$hFIXE_1$-R], [$hFIXE_1$-F/hBI_1-R] and

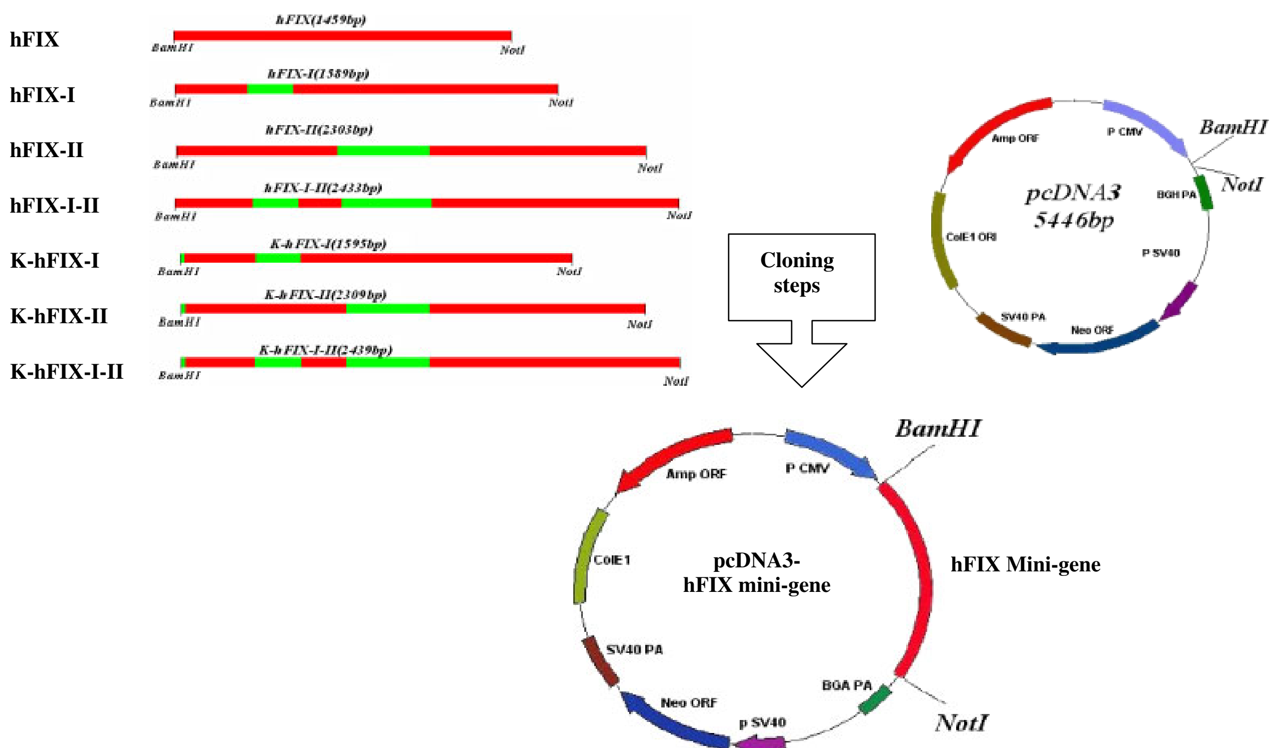

Figure 1. Schematic view of the constructed *hFIX* cDNA and six different *hFIX* mini-genes, subcloned in the pcDNA3 plasmid. The names of the corresponding *hFIX* mini-genes are indicated on the left

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[hFIXE$_1$F/hFIXE$_{2-4}$R], were used for amplification of three overlapping DNA fragments, respectively; exon 1 of the *hFIX* (E1), intron I of the *hBG* (I) and exons 2–4 of the *hFIX* (E$_{2-4}$). Products of the first and second PCRs were joined by performing a third PCR to generate the E1-I fragment. The E$_1$-I and the E$_{2-4}$ were used subsequently in a second round of SOE-PCR to create a new product, named E$_1$-I-E$_{2-4}$. The chimeric E1-I-E$_{2-4}$ fragment carrying *BamH*I and *Dra*I sites on its 5′- and 3′-ends, respectively, was used to substitute a piece of DNA covering exons 1–4 of the *hFIX* cDNA in the pET26-hFIX plasmid. The constructed plasmid was designated as pET26-hFIX-I. The intron I containing *hFIX*-cDNA was subcloned between the *BamH*I and *Not*I sites in a pcDNA3 plasmid, leading to the generation of the phFIX-I plasmid.

The third plasmid, phFIX-II carries the *hBG* intron II, which is 850 bp in length, and is located between exons 2 and 3 of hFIX. In order to insert the *hBG* intron II within the acceptor-donor site between the exons 2 and 3 of hFIX-cDNA in the expression plasmid, the following steps were carried out by using the two oligonucleotides EcoRI-E39FIX-F and NotI-FIX-R, a PCR was performed to generate a DNA fragment covering exons 3–9 of hFIX, which was subsequently cloned between the *EcoRI* and *Not*I sites of pBC$_{(SK)}$ to generate pBC-E39-FIX. Using human genomic DNA as template and two oligonucleotides, EcoRI-E3FIX-I2BG-R and XhoI-E2FIX-IN2BG-F, a PCR was carried out to amplify the *hBG* intron II carrying parts of either exons 2 and 3 of the hFIX gene at its 5′- (with *Xho*I) and 3′- (with *EcoRI*) ends, respectively. After *Xho*I/*Eco*RI digestion, the amplified fragment was inserted into the pBC-E39-FIX plasmid prior to the hFIX exons 3–9, leading to the generation of the pBC-II-E39-FIX plasmid. By using two oligonucleotides, KpnI/BamHI-FIX-F and Xho1-E2FIX-R, a third round of PCR was performed to generate a fragment covering exons 1 and 2 of hFIX, which was then used for cloning between the *Xho*I/*Kpn*I sites prior to the *hBG* intron II of the pBC-II-E39-FIX plasmid. The hFIX mini-gene containing the *hBG* intron II was isolated from pBC-II-E39-FIX plasmid after *BmaHI/Not*I digestion and subcloned in to a pcDNA3 plasmid digested similarly to generate a plasmid named phFIX-II.

The fourth plasmid, phFIX-I-II carries both of the *hBG* introns at their corresponding positions in the hFIX cDNA. To construct the phFIX-I-II plasmid, a 387-bp DNA fragment, representing the *hBG* intron I was PCR-amplified (using primers hBI1-F and hBI1-R) and inserted into the splice site between the first and second exons of the hFIX, by using the *BamH*I/*Xho*I restriction sites in pBC-II-E39-FIX, after a first round of cloning in the pTZ57R/T vector (InsT/Aclone). Finally, the whole chimeric hFIX-I-II cDNA was inserted into the pcDNA3 plasmid using *Bam*HI/*Not*I restriction sites in order to create the phFIX-I-II plasmid.

The last three plasmids, namely pKhFIX-I, pKhFIX-II and pKhFIX-I-II, are second generations of the plasmids, described above. However, they carry a Kozak sequence upstream the hFIX-cDNA. To construct the Kozak containing mini-genes, an oligonucleotide (hF9KozF), carrying the Kozak sequence together with either hFIXE2-R or hFIXE4-R oligonucleotides were used as the forward and reverse primers, respectively, to amplify the hFIX mini-genes with Kozak sequences prior to their start codons. Each of the Kozak containing PCR products were then used to substitute their corresponding fragments in the plasmids phFIX-I, phFIX-II and phFIX-I-II.

The recombinant plasmids generated in different cloning and subcloning steps were transferred into the DH5α strain of *E. coli* and the resulting transformants were isolated on selective media and verified through restriction analysis, as well as complete sequencing of both strands of the cloned fragments by using the ABI 373A automated sequencer (MWG Biotech).

Online sequence analysis

The hFIX and *hBG* nucleotide sequences were retrieved from GeneBank at National Center for Biotechnology Information with the accession numbers of AY769950 and L48217, respectively [40]. Presence of regulatory motifs in the examined sequences was investigated by the PromoterPlot software [41] and NSITE program [42]. The Repeat Masker program (A. F. A. Smith, R. Hubley and P. Green; http://repeatmasker.org) was used to detect the repeated sequences. Comparison of the obtained sequences against the GeneBank database was performed using the BLAST program [43].

Cell culture and transfection

The CHO cells were grown in a 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 six-well plates. The cells were transfected with 2 μg of plasmid DNA, using FuGene–6. After transfection, the medium was harvested and fresh rich medium (containing 1 μg/ml vitamin K_1 and 10% (v/v) (FBS) was added to the cells. On the second day of post-transfection, the cultured medium was collected for transient expression analysis and fresh medium was added again to the cells. For stable transfection, the transfectants were selected in media containing 450 μg/ml geneticin. After the emergence of stably-transfected cells, the colonies were expanded individually for further analysis as described by Otter-Nilson and Nilson [44]. In another approach, the expanded colonies of each mini-gene were pooled in media containing geneticin. The hFIX expression analysis in each step was performed on cultured media taken from cells after achieving approximately 70% confluency.

Measurement of hFIX coagulation activities

Biological activity of the expressed hFIX was examined using immuno-depleted plasma for FIX and activated

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partial thromboplastin (aPTT) reagent, according to the instructions provided by the manufacturer (Diagnostica Stago). A standard curve was constructed by making five serial dilutions of normal citrated pool plasma (1 : 10, 1 : 20, 1 : 40, 1 : 80 and 1 : 160) in Owren-Koller buffer (Diagnostica Stago) and plotting the log clotting time against the log plasma FIX activity. The conditioned cultured media (1 : 10 in Owren-Koller) were then used for determining the activity of the expressed hFIX, based on the standard curve. Samples (100 µl) at 1 : 10 dilutions were mixed with 100 µl of FIX-deficient plasma and 100 µl of aPTT (cephalite) reagent. After 3 min of incubation at 37 °C, 100 µl of a pre-warmed (at 37 °C) $CaCl_2$ solution (25 mM) was added to the mixture and the clotting time was measured. By definition, the normal concentration of 5 µg/ml of hFIX in human plasma is equal to 100% activity and thus a unit of hFIX is defined as the amount that is present in 1 ml of normal plasma and considered as 100% activity [45,46].

Measurement of hFIX antigen (hFIX::Ag)

The rhFIX antigen in the conditioned cultured media was assayed by the sandwich ELISA using a micro-plate, coated with a specific anti-hFIX antibody, provided by the ELISA-kit. The FIX bound to the first antibody was revealed by using a second mouse anti-FIX monoclonal antibody, labeled with horseradish peroxidase that binds to another antigenic determinant of hFIX. The enzymatic activity was then demonstrated by its oxidative action on the substrate ortho-phenylendiamine in the presence of urea-hydrogen peroxide. The reaction was then stopped by the addition of sulfuric acid and the resulting colour was measured at 492 nm. The observed optical density was directly proportional to the concentration of hFIX. The detection limit of the hFIX antigen assay is 50 ng/ml (1% of the normal hFIX content of human plasma). The cultured media collected from both untransfected cells and cells transfected with the parental pcDNA3 plasmid were used as negative controls.

Reverse transcription-PCR

Total cellular RNA was extracted from transfected cells according to the manufacturer's instructions (Cinnagen, Tehran, Iran) and pre-treated with RNase-free DNase to synthesize cDNA by reverse transcriptase (M-MuLV), which was subsequently analysed by the amplification of a section of the hFIX coding region, using two hFIX-specific primers (Table 1).

Statistical analysis

All expression analysis experiments including coagulation and ELISA, were carried out in triplicates and the generated data were presented as the mean ± SD. Analysis of variance followed by a Tukey post-hoc test was used to evaluate differences among the mini-genes. $p < 0.05$ was considered statistically significant. All statistical analyses were carried out with SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Transient expression of hFIX

For any of the transfectants, the activity of the secreted hFIX was examined by the one-stage clotting assay of cultured media 48 h after transfection. The relatively shorter clotting times of the samples from cells with the normal hFIX cDNA and other hFIX mini-genes in comparison with negative controls indicated the expression of biologically active hFIX by all the recombinant constructs. In general, the mean coagulation activities of the cultured media taken from the *hBG* intron containing cells are higher than that of parental hFIX expressing cells. The highest hFIX coagulation activity (4%) occurred for the mini-gene that carries both of the *hBG* introns in addition to the Kozak sequence (phKFIX-I-II; Figure 2). No significant variations were observed among other intron containing mini-genes. In comparison with the normal hFIX cDNA, with approximately 1.5% activity, a two-fold increase was observed in the cases of either hFIX-II or hFIX-I-II mini-genes (Figure 2).

Stable expression of hFIX

For each of the examined constructs, a number of stably-transfected colonies appeared on selective media, which were subsequently cultured separately for further analysis. Based on the data obtained from the coagulation assays, successful secretions of the biologically active rhFIX by all the isolates were documented (Table 2). However, a wide range of variation was observable among different isolates even in a same mini-gene group. At the same time, the average hFIX activities of the clones carrying the *hBG* intron II either alone or together with intron I were higher than that of the parental hFIX expressing cells. The clones with the highest coagulation activities were tracked among the hFIX-II mini-gene containing clones. Those clones that contained the hFIX-I mini-gene did not show significant differences from that of the intron less hFIX cDNA.

The main goal of the present study was to select the most efficient hFIX expressing mini-gene. Therefore, examination of the stably-transfected cells were continued by performing a comparative expression analysis, among the separate pools of expanded colonies, as described below. Based on both the clotting test and ELISA and under similar conditions, the highest level of hFIX coagulation activity among the examined cell pools, was observed in cells containing the K-hFIX-I-II mini-gene (Figure 3). The hFIX::Ag level estimated from

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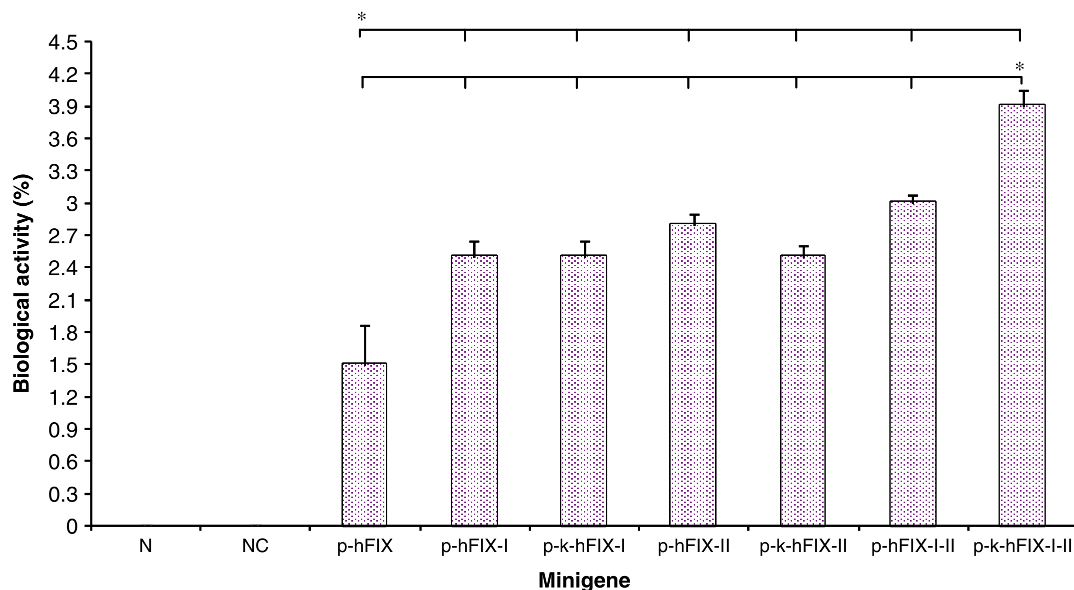

Figure 2. Coagulation activities of the cultured media taken from transfected cells 48 h after transfection. The transfected cell line is indicated by plasmid names below the corresponding columns. N, cultured media taken from untransfected cells; NC, cultured media taken from cells transfected with plasmid pcDNA3. Asterisks indicate samples that are significantly different ($p < 0.05$) compared to other samples, using analysis of variance

Table 2. Number of isolates and range of coagulation activities of *hFIX* expressed in 10^6 cells of different isolates for each *hFIX* mini-gene group after 48 h of confluency

Construct name	Number of isolated expression clones	ELISA (ng/ml)/ 10^6 cells[a]	Biological activity (%)/10^6 cells	Mean ± SD coagulation activity
HFIX	8	87.3	0.34–1.12	0.5 ± 0.27
hFIX-I	14	38.7	0.23–1.18	0.51 ± 0.25
KhFIX-I	8	ND	0.18–1.03	0.45 ± 0.31
hFIX-II	3	400	2–2.6	2.26 ± 0.3
KhFIX-II	11	ND	1.8–2.2	1.23 ± 0.71
hFIX-I-II	13	260.4	0.22–2.5	0.85 ± 0.76
KhFIX-I-II	9	2222.2	0.24–1.83	0.6 ± 0.5

The average coagulation activity for each mini-gene group is indicated.
[a]The results represent the highest hFIX::Ag obtained for each mini-gene. ND, not determined.

ELISA for the K-hFIX-I-II containing cells (10^6) was approximately 2.75×10^3 ng/ml, with more than a 25-fold increase relative to the intron-less hFIX expressing clone (Figure 3). Based on the same result, the second highest level of hFIX::Ag belongs to the hFIX-II mini-gene, estimated to be approximately 0.5×10^3 ng/ml for 10^6 cells, which was five-fold more than that of the intron-less hFIX cDNA. The results obtained from the coagulation activity assays of the expressed hFIX also showed that the highest expression level of rhFIX belongs to K-hFIX-I-II mini-gene. The presence of an antigenic determinant may not be accompanied completely by the biological activity of the corresponding protein. Therefore, as expected, the amount of the hFIX::Ag detected by ELISA was higher than that of the FIX activity detected by the clotting test, which indicates that a major section of the expressed hFIX might not be biologically active.

Further analysis of the cultured media from selected clones of each hFIX-mini-gene showed that the three highest levels of hFIX::Ag belong to selected clones of the K-hFIX-I-II, hFIX-II and hFIX-I-II mini-genes. Surprisingly, at this stage, no significant hFIX::Ag activity was detected in the rest of the representative clones and, in agreement with the results obtained during transient expression analyses, higher levels of rhFIX expression occurred whenever the *hBG* intron II was present in a mini-gene.

Analysis of the hFIX transcript

Presence of the properly spliced hFIX transcripts in the representative clones of different hFIX mini-genes was confirmed by PCR-amplification of a 1.5 kb FIX cDNA, reversely transcribed from mRNAs of the stably-transfected cells, using a primer pair from the first and fourth hFIX exons (data not shown).

Analysis of hBG intronic sequences

Considering the positive effects of *hBG* introns on the expression of the *hFIX* in the present study, the intronic

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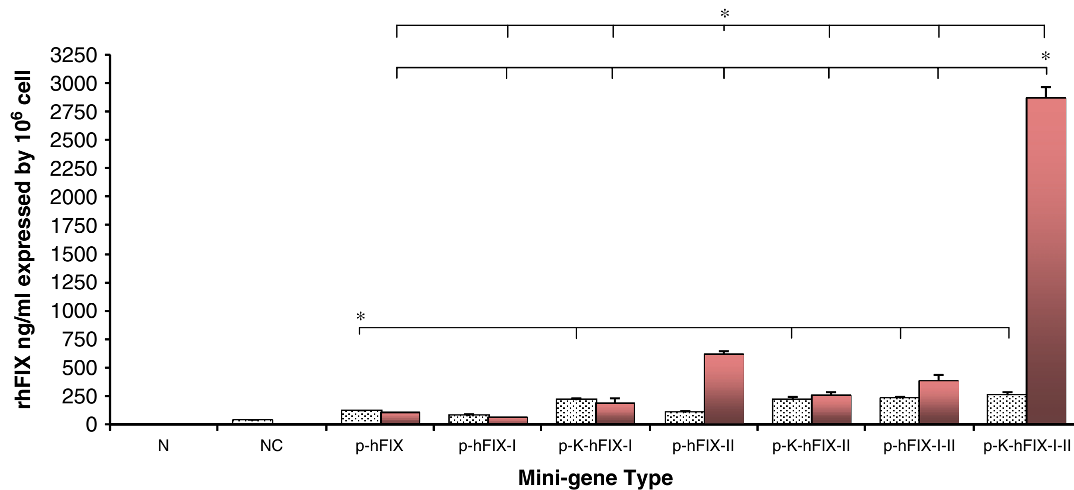

Figure 3. ***hFIX*** **(ng/ml) expressed in 10^6 cells of pooled permanent clones (48 h after confluency) based on ELISA ▪ and coagulation activity ▫. In the case of the hFIX-II mini-gene, and because of loss of the two clones, the pooled cells were derived from a single clone that survived during prolonged incubation. Asterisks indicate samples that are significantly different ($p < 0.05$) compared to other samples, using analysis of variance**

sequences of *hBG* gene were screened for the possible presence of regulatory motifs. Sequence analysis detected several motifs in both of the *hBG* introns, distributed throughout both strands of the *hBG* introns. Based on the results from the PromoterPlot program, in total, 42 and 98 TFB sites were detected within the *hBG* introns I and II, respectively. Analysis of the two intronic sequences by the NSITE program confirmed the presence of TFB sites, but with a higher frequency in the *hBG* intron II. Analysis of the *hBG* intronic sequences detected a 96-bp sequence within intron II of the *hBG* sequence, which belongs to the L1 family of non-LTR retrotransposons (L1MA6). These findings may provide an explanation for the relatively stronger effect of the *hBG* intron II on the expression of *hFIX*, as demonstrated by the experimental data described above.

Discussion

The results obtained in the present study provide evidence that both of the *hBG*-derived introns are potent enhancer-like elements, which can act positively with respect to the expression of *hFIX* in a mammalian expression system *in vitro*. It has been shown that intronic sequences of autologous, heterologous or synthetic origins potentiate gene expression *in vitro* or *in vivo* by various mechanisms [13,18,24,35]. More related to this work, is the result obtained by Harding *et al*. [47] who used the second *hBG* intron located between a liver-specific promoter and the hFIX cDNA. They showed that the addition of the *hBG* second intron enhances the *hFIX* expression level by approximately 85-fold. In another study, Palmiter *et al*. [18] inserted the *hBG* intron II between the mouse metallothionein I promoter and the rat growth hormone cDNA and observed a substantial increase at the mRNA level *in vivo*. Improvement of transgene expression by applying the *hBG*-derived introns has also been shown in other studies [25,26]. The results of the present study, in particular the parts related to the function of the second *hBG* intron, are in principle in agreement with the findings of the abovementioned studies. However, there is a major difference between our work and those previous studies. In the present study, an *in vitro* system was used, whereas, in the other studies, the major results were obtained from *in vivo* analysis, to demonstrate the intron functions. The results obtained from *in vitro* experiments (cell culture) usually diverge from those of the *in vivo* studies [47]. Brinster *et al*. [48] have also reported that introns increase the transcription rates of genes in transgenic mice. However, no effect of introns was observed when the same constructs were examined in cultured animal cells, which is in direct contrast to the results of the present study. Thus, the basis for the results obtained in the present study versus those of Brinster *et al*. [48] may be different. Therefore, a comparison of the results obtained from an *in vitro* system with that of *in vivo* system may not lead to a proper interpretation.

Recent progress in understanding of how introns and their removal by the spliceosomes can influence and enhance almost every step of mRNA metabolism has been reviewed by Le Hir *et al*. [49]. It was previously speculated that augmented gene expression with the inclusion of introns in transgenic studies was not the result of specific enhancer elements present in the intron, but rather the increased precursor mRNA stability mediated by its splicing sequences [33]. Indeed, spliceosomes assembly, in general provides a positive feedback to the RNA polymerase [1]. The correlations between splicing signal and transcription have been shown in a number of works. For example, the function of introns in regulation of transcription by controlling DNA accessibility through modulation of nucleosome position [50] and stimulation of transcription by enhancing RNA polymerase II initiation and processivity [51] have been demonstrated. Based

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on evidence obtained in yeast and mammalian cells, it is thought that the promoter-proximal introns mainly function on transcription efficiency [1]. These properties can also be the case for the *hBG* introns and it is likely that many other introns behave in similar way. Harding *et al.* [47] however, have correlated the enhancer-like effect of the second *hBG* intron to its probable ability to increase mRNA stability and transport within the host cell. Based on data obtained from the expression analysis of the *hFIX* in the present study, among the *hFIX* mini-genes with a single intron, the *hBG* intron II, with a five-fold increase of expression level relative to its intron-less counterpart, functions more effectively than the *hBG* intron I. In other words, a significant increase in the *hFIX* expression level occurs whenever the *hBG* intron II was present in a mini-gene, regardless of the presence of the first *hBG* intron. Naturally the *hBG* intron II functions as the 3′-terminal intron in its native host gene. The stronger activity of the *hBG* intron II with respect to its native gene expression has been correlated to its position in the *hBG* gene as the last intron, where it is essential for the accumulation of stable cytoplasmic mRNA and it is implicated in promoting efficient 3′-end formation [21]. Because the second *hBG* intron was not positioned as the last intron in the present study, its function with respect to the pre-mRNA 3′-end formation is unlikely.

Considering the stronger effect of the second *hBG* intron, in spite of its longer distance from the promoter in comparison with the first *hBG* intron, one may wonder about the higher frequency of factors with enhancer like activities in the second *hBG* intron. This type of transcriptional regulatory function of the intronic sequences might be caused by elements such as TFB sites. The higher number of potential TFBs in the *hBG* intron II may provide an explanation for its stronger activity compared to the *hBG* intron I. The presence of a 96-bp sequence within the second *hBG* intron, which belongs to the L1 family of non-LTR retrotransposons may also contribute to the enhancer-like activity of this intron, as already suggested by Le Hir *et al.* [49].

In the present study, the two *hBG* introns are located in the first and second intronic sites of the *hFIX* cDNA. Therefore, by considering their potential TFBs, one may assume that they might function through their promoter proximity positions. Although indirect support for a post-transcriptional role is provided by studies indicating that introns must be contained within the transcribed sequences [11,12], the results obtained from both expression analysis and the *hBG* intronic sequences in the present study support a transcriptional activity of introns and, therefore, the position-dependence of the examined introns may not be ruled out.

In a number of genes, there are elements within the 3′-end intron that interact with polyadenylation processing and increase the polyA tail-length, thereby increasing the half life of mRNA and translation rate [1]. The necessity of a functional 3′-terminal intron for an efficient 3′-end formation of transcripts is supported by studies carried out on the human glycolytic enzyme triosephosphate isomerase using cultured cells [20]. A further step in this investigation on *hFIX* expression in a heterologous mammalian system, with regard to intron position, is to study the effect of the *hBG* intron II as the 3′-terminal intron, on the expression of the *hFIX* (currently being carried out by E. Moeen and colleagues).

The highest expression level of the *hFIX* in the present study was obtained from the clone containing both of the *hBG* introns and Kozak. This result suggests a possible cooperative function between the examined elements, including the *hBG* introns and Kozak, on the expression level of the *hFIX in vitro*, even when the expression is driven by the strong CMV promoter. Synergistic effect of introns and 3′-untranslated region, which has an increased gene expression of up to 1000-fold *in vivo,* has been described by Kaleko *et al.* [17].

The recombinant plasmids, as well as the stable FIX expressing cells, have provided a baseline for further molecular studies of various important factors influencing the expression efficiency of the recombinant *hFIX*, including *cis* acting elements, in addition to studies dealing with the large-scale production of *hFIX*. Based on the systematic expression analysis carried out in the present study, all of the examined *hFIX* mini-genes have potential for the production and secretion of active rhFIX and also suggest that it is possible to confirm the construct functionality of the examined mini-genes prior to their application in both *in vitro* and *in vivo* studies. Optimization of the culture media and growth conditions for any of the clones developed in the present study is necessary to achieve an improved expression of *hFIX*. However, as a result of the experimental evidence provided under the examined conditions, the K-hFIX-I-II mini-gene can be considered as the first candidate to be used for further experiments aiming to achieve higher expression levels of *hFIX*.

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