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# A Study of the Expression of Functional Human Coagulation Factor IX in Keratinocytes Using a Nonviral Vector Regulated by K14 Promoter

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**Abstract** *Ex vivo* gene therapy requires a suitable bioreactor for production and delivery of the gene products into a target tissue, and keratinocyte is suitable model in this regard because of its potential for systemic release of proteins. To establish a keratinocyte-specific expression system, a mammalian-based expression plasmid equipped with a 2,240-bp fragment from the human keratin 14 (k14) gene enhancer/promoter region was constructed and used for the insertion of the human coagulation factor IX (*hFIX*)-cDNA downstream the K14-derived regulatory elements. The human epidermal keratinocytes isolated from neonatal foreskin were cultivated in keratinocyte serum-free media and transfected with the recombinant plasmid. The K14-promoter-driven expression of recombinant *hFIX* (r*hFIX*) was evaluated by performing coagulation test as well as enzyme-linked immunosorbent assay on the cultured media collected from the transfected cells at various stages. The r*hFIX* corresponding transcript and protein were confirmed by performing reverse transcription PCR as well as immunoblotting experiments, respectively. Based on the coagulation activities obtained from the conditioned media of nine isolated clones, the hFIX expression levels vary from 5% to 39% of normal human plasma. Expression levels of the *hFIX* obtained in this study are comparable to those reported for viral systems. The obtained data supported the potential of keratinocyte for the expression and secretion of biologically active r*hFIX* and underscore the importance of the examined cis sequences for enhancing gene expression in a mammalian expression system. Besides, it has provided means for

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further bioengineering strategies to improve the expression efficiency of the *hFIX* in keratinocytes and other mammalian host cells.

**Keywords** Human Factor IX · Keratinocyte · Hemophilia B · Keratin 14 gene promoter · *Ex vivo* gene therapy

## Introduction

Previous studies have documented that the epidermal keratinocyte can be used as a desirable bioreactor for production of a protein of interest to be released systemically [1, 2]. Keratinocytes are accessible targets for gene therapy and techniques for their cultivation, and genetic manipulations have already been developed [3–6]. Moreover, they are renewed throughout adult life and form the outer layer, thus making it easy to monitor the behavior of the genetically modified tissue [7]. Besides, application of a gene-switch system to induce target gene expression in the epidermis via topical administration of an inducer has also been shown [2]. The above-mentioned potentials of keratinocytes have been proven by secretion of a variety of recombinant proteins into central circulation [3, 4, 8–11].

Hemophilia B is an X-linked recessive bleeding disorder affecting about 1 in every 30,000 males and is caused by the functional deficiency or lack of FIX [12]. The *hFIX* is a plasma glycoprotein with an approximately 17% carbohydrate content and a molecular weight of 57 kDa that contains 415 amino acids [13, 14]. Currently, replacement therapy is the major treatment for the hemophilia B disease, carried out via infusion of the *hFIX* [15]. For replacement therapy, very often, plasma-derived therapeutics is used. However, in recent years, concerns over potential risks for the transmission of human blood born infectious agents such as HIV, hepatitis, and prions have motivated the use of recombinant forms of therapeutics [16, 17].

Developments in both gene- and cell-therapies of genetic diseases have also been at the centre of attentions, and hemophilia B is an attractive model in this regard [18]. For the somatic gene therapy of hemophilias, different cell types including hepatocytes, myoblasts, keratinocytes, endothelial cells, and bone marrow stromal cells have been examined [6, 19–22]. Similarly, a variety of cell lines such as Chinese hamster ovary, baby hamster kidney, and human embryonic kidney-293 have been studied for the production of recombinant *hFIX* [23–25]. Keratinocytes are well-characterized hosts and attractive bioreactors to produce factor IX, and use of viral promoters to express biologically active FIX in there have been reported by several groups [5, 12, 26, 27]. For the keratinocyte specific expression of a protein, a keratinocyte-specific regulatory system is required. Basal keratinocytes express keratin proteins such as keratin 5 (K5) and keratin 14 (K14) at high levels up to 30% of the total keratinocyte proteins [28–30]. Therefore, promoters corresponding to K5 and K14 genes might be attractive candidates for the expression of heterologous proteins in keratinocytes. Studies, carried out by other workers, showed that a 2,200-bp fragment of the human K14 gene upstream has potential to direct transgene expression in an epidermal keratinocyte [3, 31, 32]. More related to the present study is the research carried out by Page and coworkers [6, 26] that demonstrated the expression of the *hFIX* in keratinocytes under the control of CMV promoter in combination with an enhancer corresponding to K14 gene.

Our main goal in the present study was to establish a keratinocyte-specific expression system, for studying secretion of transgene products. In this regard, we have constructed an expression plasmid equipped with a regulatory element derived from the human k14 gene.

Here, the results obtained from the expression analysis of the keratinocytes, transfected with the r*hFIX* expressing plasmid is presented.

## Materials and Methods

### Bacterial Strain, Media, Enzymes, Chemicals, and Kits

The DH5α strain of *Escherichia coli* (Stratagene, USA) was used for cloning steps. Luria–Bertani was used as the bacterial culture medium, and ampicillin (100 μg/mL) was added when required to maintain selection pressure. Enzymes including *Hind*III, *Bgl*II, *BamH*I, *Not*I, T4 DNA ligase and reverse transcriptase (M-MuLV), geneticin (G-418), and kits for the long-template PCR, PCR product purification, plasmid isolation, and RNA preparation in addition to FuGene-6 were purchased from Roche, Germany. The PCR product cloning kit (InsT/Aclone) was obtained from Fermentas, Canada. Keratinocyte serum-free medium (K-SFM) and dispase were obtained from Invitrogen, USA. Hanks' balanced salt solution (HBSS) was obtained from Baharafshan, Iran. Gentamicin was obtained from Alborzdarou, Iran. Citrated normal pooled plasma (provided by the control unit of the Iranian blood transfusion organization) was used as standard in coagulation tests. Enzyme-linked immunosorbent assay (ELISA) kit for measuring the *hFIX* antigen, *hFIX*-deficient plasma, and Owren Koller buffer were purchased from Diagnostica Stago, France. Polyclonal rabbit antibody anti-*hFIX* (ICN-USA) was used for immunoblotting experiments. Immunoreactive material was detected using peroxidase-conjugated goat antirabbit antibody (Tebsan, Iran).

### DNA Manipulations

All DNA manipulations were carried out based on standard cloning procedures [33]. The human chromosomal DNA, extracted from blood, was used as template for the amplification of a 2,240-bp fragment containing the promoter/enhancer domain of the human K14 gene (phk14). Primers, hk14-$F_1$ (5′-GGAAGATCTGCTAGGGTTCTGGTGTTGGTGCG-3′) with the *Bgl*II site (underlined) in forward direction and hk14-$R_1$ (5′-CGTCCAAGCTTGAG GAGGGAGGTGAGCGAGCGA-3′) with the *Hind*III site (underlined) in reverse direction, were designed based on the human K14 gene promoter sequence (Acc. No. U11076) [34]. The *hFIX*-cDNA was isolated from a human liver cDNA library (purchased from MRC-clinical science center at the Imperial College School of Medicine, London, UK) based on a long-range PCR procedure. For the isolation and amplification of the *hFIX*-cDNA, two primers, hIX-$F_1$ (5′-GGATCCGTTATGCAGCGCGTGAACATGA-3′) in forward direction to generate a *BamH*I site (underlined) and hIX-$R_1$ (5′-GCGGCCGCAGTGAT TAGTTAGTGAGAGGCCC-3′) in reverse direction to generate a *Not*I site (underlined), were designed based on the *hFIX* mRNA sequences (Acc. No. MN000133). Plasmids constructed in this work, including the phPK14H and pK14*hFIX* were derived from pcDNA3 plasmid (Invitrogen, USA), a mammalian expression vector that contains a neomycin-resistance gene (neo) as a selectable marker. In each step, the constructed plasmid was transferred into an *E. coli* host and subjected for amplification, restriction analysis.

The inserted fragments in the *hFIX* expressing plasmid were fully sequenced from both strands, using ABI 373A automated sequencer (MWG, Germany). Comparison of the obtained sequences (Acc. No. DQ886588, DQ898181) against the GeneBank data base was performed using Blast program [35].

## Primary Human Keratinocyte Culture and Transfection

The human normal epidermal keratinocytes were isolated from neonatal foreskins (kindly provided by Dr. M. Mohammadzadeh M.D.) and grown in K-SFM [36]; briefly, foreskin specimens were placed into complete K-SFM containing gentamicin (50 μg/mL). A basal K-SFM containing 25–30 μg/mL of bovine pituitary extract, and 0.1–0.2 ng/mL of recombinant epidermal growth factor is considered as complete K-SFM. The samples were rinsed for several minutes in $Ca^{2+}$- and $Mg^{2+}$-free Dulbecco's phosphate-buffered saline (D-PBS), containing 20 μg/mL gentamicin, and connective tissues were discarded and incubated in HBSS, containing 25 U/mL dispase, for 18 h at 4 °C, in order to separate epidermis from dermis. The epidermal layer was lifted from the dermis and treated with a solution containing 0.25% trypsin and 1 mM EDTA, for 10 min at 37 °C. During incubation, it was aspirated every 2–3 min to dissociate the cells. Trypsin was neutralized with 10% FBS in the final trypsinization solution, and cells were pelleted at 1,000 rpm for 10 min at 4 °C. The pellet was gently resuspended in approximately 2–5 mL of complete K-SFM. The primary keratinocytes were seeded into complete K-SFM at $4 \times 10^4$ cells/cm$^2$ of tissue culture plate. The cells were grown at 37 °C in a 5% (*v*/*v*) $CO_2$ atmosphere. The medium was refreshed every 2–3 days. Upon reaching 60–75% confluency, the medium was removed, and the cells were washed once with $Ca^{2+}$ and $Mg^{2+}$-free D-PBS. The keratinocytes obtained from the first or second passages were transfected with fugene-6. Briefly; keratinocytes were seeded into complete K-SFM at the density of $1.5–1.8 \times 10^5$ cells per well and incubated at 37 °C for 21 ± 3 h before transfection. Fugene-6 (3 μL) was added to 97 μl of basal K-SFM and incubated at 25 °C for 5 min. Subsequently, 2 μg of (~5–10 μL) the DNA construct (either phk14H or pK14FIX) was added to the mixture, and incubation continued for 30 min. The final transfection mix was added directly to 2 mL of keratinocyte-containing basal medium, provided from the previous step. The transfection reaction continued for up to 21 h. Following the transfection step, the basal K-SFM was harvested, and the complete K-SFM was added to the cells. On the third day of posttransfection, the cultured media were collected for analysis, and fresh media were added. The transfected keratinocytes were selected in media containing 250–300 μg/mL geneticin (after 3 weeks in selection). Using paraffin rings, the single colonies were isolated and expanded individually [37]. The expanded clones were then treated with media containing 500–600 μg/mL of geniticin.

## Measurement of *hFIX* Coagulation Activity

Biological activity of the expressed *hFIX* was determined using immunodepleted plasma for *hFIX* and activated partial thromboplastin (aPTT) reagent, according to the instructions provided by the manufacturer (Diagnostica Stago, France). The standard curve for the coagulation activity was constructed based on clotting activities of five serial dilutions of normal citrated pool plasma (1/10, 1/20, 1/40, 1/80, and 1/160) in Owren Koller buffer and plotting the log clotting time against the log plasma FIX activity. Accordingly, the conditioned cultured media (1:10 in Owren-Koller) were then used for the activity assay of the expressed *hFIX* determined by the standard curve. A 100 μl volume of 1:10 dilutions of samples were mixed with 100 μl of *hFIX*-deficient plasma and 100 μl of aPTT (cephalite) reagent. After 3 min of incubation at 37 °C, 100 μL of a prewarmed $CaCl_2$ solution (25 mM) was added to the mixture and clotting time was measured.

### Measurement of r*hFIX* Antigen

The FIX antigen in the conditioned cultured media was assayed, by the sandwich ELISA on microtiter plate, which was coated with a specific rabbit anti-*hFIX* antibody. All the experiments were carried out twice to exclude any false signals. The bound *hFIX* to the first antibody was revealed by the use of a second rabbit anti-*hFIX* antibody, labeled with horseradish peroxidase that binds to another antigenic determinant of the *hFIX*. 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 adding 100 μL of HCl (1 M), and the absorbance of the colored product was measured at 492 nm. The observed optical density is directly proportional to the concentration of FIX. 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 the untransfected keratinocytes (N) and the keratinocytes transfected with the phPK14H plasmid (NC) were used in parallel as the negative controls.

### SDS-PAGE and Western Blotting

SDS-PAGE was carried out according to a modified method explained by Laemmli [38] with slight modifications. The prepared protein samples were subjected to electrophoresis on a 13% polyacrylamide, and gels were stained with Comassie Brilliant Blue. Electroblotting of proteins onto PVDF membrane (Amersham-Pharmacia Biotech, Germany) was performed using semidry procedure in a transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) at 86 mA for overnight. Presence of r*hFIX* in the cultured media was confirmed by western blotting experiment, during which the electroblotted proteins were probed with a polyclonal antiserum (ICN, USA) prepared against the *hFIX*. The *hFIX*-antibody complex was then treated with horseradish peroxidase-conjugated antirabbit immunoglobulin and visualized using a solution of 4-chloronaphtol with hydrogen peroxidase as enzyme substrate.

### Reverse Transcription PCR

Total cellular RNA was extracted from transfected keratinocyte using RNA preparation kit according to the manufacturer's instruction (Roche, Germany) and pretreated with RNase-free DNase. Using the isolated RNA, cDNA was synthesized by reverse transcriptase (M-MuLV), which was subsequently analyzed by the amplification of a piece of *hFIX* coding region, using two specific primers, namely *hFIX*-F2 (5′-GCCATGGCCCCCTTTGGATTTGAAGGAAA GAAC-3′) and *hFIX*-R2 (5′-GAAGCTTTCTCCCTTTGTGGAAGACTCTTCCC-3′).

## Results

### Construction of Recombinant Plasmids

Construction of the plasmid pK14FIX (8,223 bp) for specific expression of the *hFIX* in epidermal keratinocyte involved two steps. First, a 2,240-bp fragment from the human K14 gene upstream was amplified, *Bgl*II/*Hind*III digested, and inserted into a pcDNA3 plasmid. The resulted recombinant plasmid, in which the CMV enhancer/promoter was replaced by the hPK14 promoter, was named phPK14H (Fig. 1A). In the second step, a *Not*I/*Bam*HI fragment containing the *hFIX*-cDNA was PCR-amplified and cloned into the phPK14H

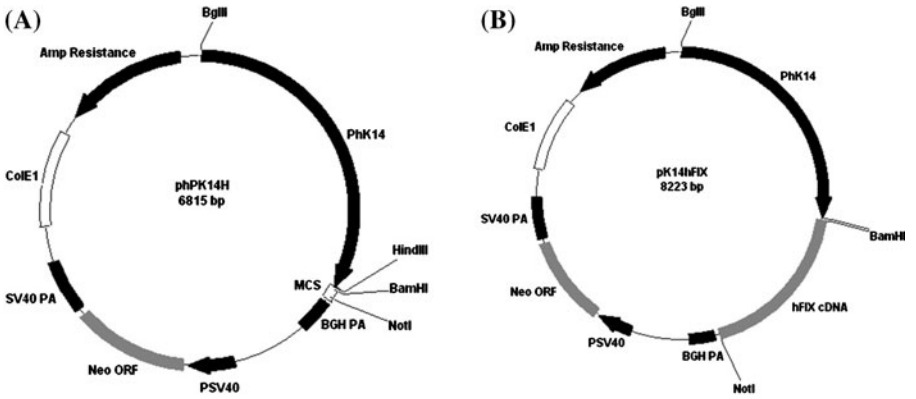


**Fig. 1** The physical map of the constructed recombinant plasmids. (**A**) phPK14H, (**B**) PK14*hFIX*

plasmid after a first round of cloning in a T/A cloning vector. The newly made recombinant plasmid was named PK14*hFIX* (Fig. 1B).

Based on the restriction analysis, outlined in Fig. 2A, the expected 6,815-bp size of the linearized phPk14H plasmid was confirmed by single digestions with either *Hind*III or *Bgl*II enzymes separately, which were supposed to have unique sites in that plasmid. A double *Hind*III/*Bgl*II digestion of the phk14H plasmid created two fragments of 2,240 and 4,575 bp, corresponding to the phK14 promoter and the pcDNA3-related fragment, respectively. The 8,223-bp size of the linearized PK14*hFIX* plasmid was also confirmed separately by single digestions with both *Not*I and *BamH*I (Fig. 2B). A double, *Not*I/*BamH*I digestion of the PK14*hFIX* plasmid created two fragments of 6,764 and 1,459 bp, corresponding to the linearized phpK14H plasmid and the *hFIX*-cDNA fragment, respectively.

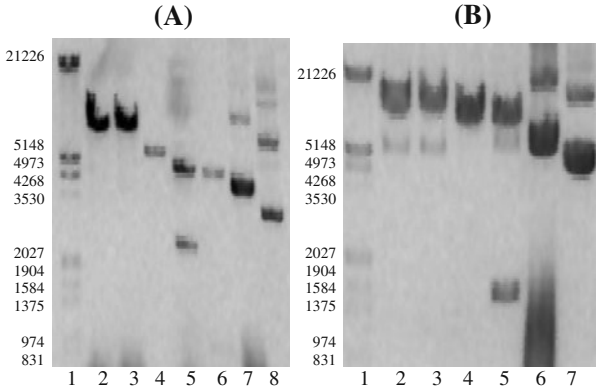


**Fig. 2** Restriction analysis of the recombinant plasmids. (**A**) phPK14H plasmid; *Lane 1*, DNA size marker. *Lanes 2 and 3*, linearized forms of phPK14H after digestion with either *Bgl*II or *Hind*III, respectively. *Lane 4*, linearized form of pcDNA3 after digestion with *Hind*III. *Lane 5*, *Bgl*II/*Hind*III double digestion pattern of phPK14H. *Lane 6*, *Bgl*II/*Hind*III double digestion pattern of pcDNA3. *Lanes 7 and 8*, supercoil patterns of phPK14H and pcDNA3, respectively. (**B**) pK14FIX plasmid; *Lane 1*, DNA size marker. *Lane 2*, pK14FIX plasmid digested with *BamH*I (linearized). *Lane 3*, pK14FIX plasmid linearized with *Not*I. *Lane 4*, phPK14H plasmid linearized with *Hind*III**.** *Lane 5*, pK14FIX plasmid digested with *Bam*HI/*Not*I. *Lane 6*, undigested pK14FIX plasmid. *Lane 7*, undigested phK14H plasmid

## Transfection and Transient Expression Analysis

The prepared primary human normal keratinocytes were transfected with the pK14*hFIX* plasmid. By performing a one-stage clotting assay on the conditioned media collected from the transfected cells on the third day of posttransfection, the possible expression of the *hFIX* was examined. The clotting times of samples obtained from ten different transfection lines were compared with the clotting time of the samples from the untransfected cells and those transfected with the phPK14H plasmid as negative controls (Fig. 3). The expression levels that are demonstrated by the r*hFIX* coagulation activity (percent) in the transfection lines vary from 16% to 42%. A unit of *hFIX* is defined as the amount that is present in 1 mL of citrated normal plasma and considered as 100% activity [39].

Taking advantage of an immunoblotting experiment, using a polyclonal anti-*hFIX* antibody, expression of a protein of 57 KDa, compatible with the size of the *hFIX*, was observable among the proteins of the cultured media corresponding to the cells transfected with the recombinant plasmids after separation by SDS-PAGE (Fig. 4).

## Activity Measurements of the hFIX Secreted by the Selected Clones

Approximately 72 h after transfection, the keratinocytes were transferred into selective media containing geniticin and incubation continued until colony appearance. Among ten transfection experiments, nine colonies were obtained. The isolated colonies, namely F1-C1, F1-C2, F1-C3, F2-C3, F2-C4, F3-C1, F3-C2, and F3-C3, were cultured separately in selective media and subsequently analyzed, based on procoagulation activity of the secreted r*hFIX*. Expression of r*hFIX* for all the examined colonies was measured before achieving a complete confluency. Accordingly, persistent expression and successful secretions of the biologically active r*hFIX* from the nine isolated clones were documented (Fig. 5). The expression levels that are demonstrated by the r*hFIX* coagulation activities (percent) among the nine clones vary from 5% to 39% of normal human plasma.

Three out of the nine isolated clones, namely F1C1, F3C1, and F3C2, survived during prolonged incubation until reaching a complete confluency. Cultured media from the survived clones were collected every day during 72 h of incubation and used for expression

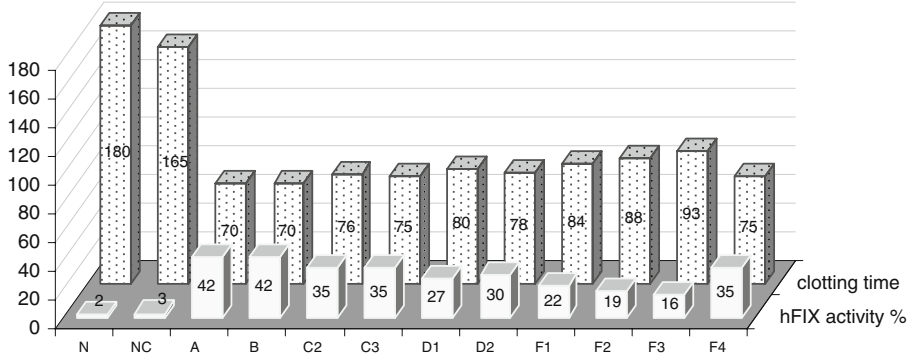


**Fig. 3** *hFIX* clotting activity obtained from the keratinocyte-conditioned cultured media before G418 treatment (3 days after transfection). The test samples obtained from ten transfection experiments are indicated by *A*, *B*, *C2*, *C3*, *D1*, *D2*, *F1*, *F2*, *F3*, and *F4*. Cultured media obtained from either untransfected cells or the cells transfected with the phPK14H plasmid were considered as negative controls and indicated by N and NC, respectively. Clotting times are indicated in seconds

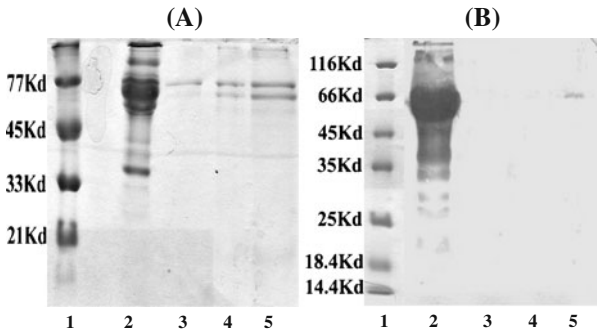


**Fig. 4** Analysis of the proteins of cultured media taken from transfected keratinocytes by SDS-PAGE (13%) (**A**) and immunoblotting using polyclonal anti-*hFIX* antibody (**B**). *Lanes 1*, protein size marker. *Lanes 2*, normal *hFIX*. *Lanes 3*, untransfected cells. *Lanes 4*, cells transfected with phPK14H. *Lanes 5*, cells transfected with pK14*hFIX*

analysis. Based on the results obtained from clotting test, secretion of the r*hFIX* was detectable during the incubation time (Fig. 6). Among the three examined clones, the F3C2 clone showed a relatively higher activity under the same condition. The secretion level of the active *hFIX* by $10^6$ cells of the clone F3C2 was highest on the first day and estimated around 600–700 ng/mL. By definition, the normal concentration of 4,500 ng/mL of *hFIX* in human plasma is equal to 100% activity [6]. Therefore, based on the coagulation activity of the cultured media, the highest level of *hFIX* expression obtained from the recombinant keratinocytes was estimated around 13–15% of the *hFIX* activity of normal human plasma.

## Measurement of the Expressed r*hFIX* Antigen

Presence of the *hFIX* in the cultured media of the F1C1, F3C1, and F3C2 clones was investigated by ELISA (Fig. 7). Indeed the antigenicity of the *hFIX* (*hFIX*:Ag) were detectable in the examined samples; among them the highest expression level of *hFIX* was obtained by the clone F3C1 on the first day that was estimated between 160 and 180 ng/mL/$10^6$ cells.

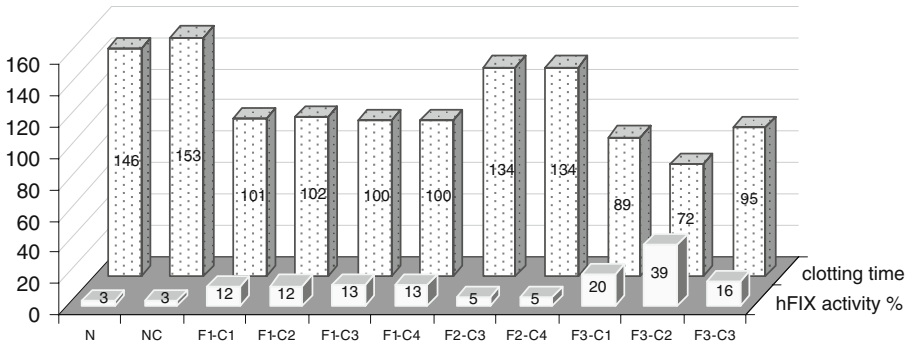


**Fig. 5** Clotting activity of the *hFIX* measured in cultured media after colony- selection in the G418-containing media. The day before the coagulation test (18±3 h) the cells were transferred into fresh media. The tested samples obtained from various transfection experiments are indicated by specific names. Cultured media obtained from either untransfected cells or the cells transfected with the phPK14H plasmid were considered as negative controls and indicated by N and NC, respectively. Clotting times are indicated in seconds

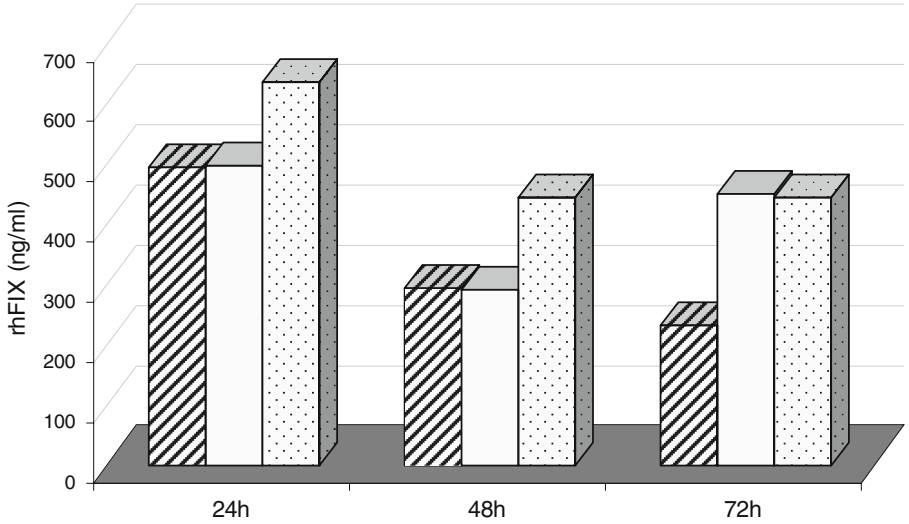


**Fig. 6** Expression assays of the three isolated clones, F1C1 (*hashed boxes*), F3C1 (*white boxes*), F3C2 (*dotted boxes*), based on clotting test of the secreted r*hFIX* in the cultured media during 3 days of subculturing. The amount of the secreted *rhFIX* was calculated for $10^6$ cells

## Analysis of the *hFIX* Transcripts

Presence of the *hFIX* transcript in the cells from the F3C2 clone was examined by PCR amplification of a 735-bp fragment of the *hFIX* coding region, reversely transcribed from the transfected cells' mRNA (reverse transcription PCR (RT-PCR)). As the electrophoresis pattern of the RT-PCR product indicates, the presence of appropriate PCR product size confirms the presence of *hFIX* transcript within the transfected cells of the analyzed clone (Fig. 8).

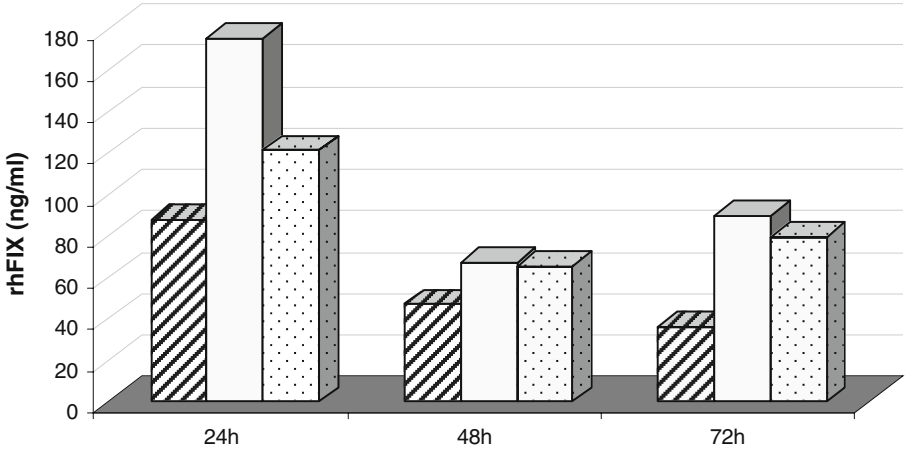


**Fig. 7** Expression assay of r*hFIX* secreted into the cultured media of the three isolated clones, F1C1 (*hashed boxes*), F3C1 (*white boxes*), F3C2 (*dotted boxes*), based on ELISA during 3 days of subculturing. The amount of the secreted r*hFIX* was calculated for $10^6$ cells

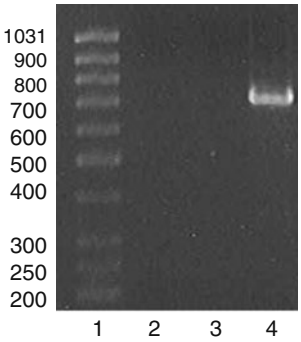


**Fig. 8** PCR product amplified from the reverse transcribed total RNA. *Lane 1*, DNA size marker. *Lane 2*, transfected keratinocytes, using total RNA as template. *Lane 3*, negative control. *Lane 4*, transfected keratinocytes, using the reverse transcribed product cDNA as template. For each test, the prepared RNA was treated with RNase-free DNase

## Discussion

Hemophilia B is a genetic disease widely prevalent throughout the world, and *ex vivo* gene therapy could be an alternative approach to challenge such genetically caused systemic diseases [40]. There are a number of evidences that support the application of epidermal keratinocytes as bioreactors for the production and systemic release of proteins [2, 4]. Therefore, keratinocytes have been suggested as attractive target cells to treat a diverse range of diseases by delivering proteins such as clotting factor [5, 6, 41], growth hormone [9, 10], and alpha-1 antitrypsin [42] systemically. The system developed in this work demonstrated a transient expression of the recombinant proteins. As it is documented here, a keratinocyte expression system has potential to produce biologically active *hFIX*, taking advantage of two key important elements: the well characterized primary keratinocyte as host that has been known as a suitable choice for *ex vivo* gene therapy and the keratinocyte-specific nonviral regulatory element that is a 2,240-bp DNA containing the hK14 gene promoter/enhancer elements, which is longer than the sequence taken as the k14 gene promoter in previous investigations [3, 31, 32].

Indeed, a major deficiency of an epidermal-mediated gene expression is its inability for a prolonged gene expression *in vivo*, due to either the absence of holoclone keratinocytes among targeted cells during the transfection step or/and the down-regulation (or loosing) of episomal expression vector during progeny segregation [43]. In this regard, presence of a viral origin of replication in the expressing plasmid allows episomal plasmid replication and an increased durability of gene expression [44]. The pcDNA3-based plasmid constructed in this work takes advantage of an SV40 origin of replication.

Based on the clotting assay, the level of the active *hFIX* secreted from $10^6$ cells of each of three different isolates was estimated between 300 and 700 ng/mL (Fig. 6), whereas the values for FIX::Ag obtained by ELISA (as displayed by $10^6$ cells of the same clones in the same conditions) was estimated between 30 and 180 ng/mL (Fig. 7). The presence of an antigenic determinant may not be accompanied completely by the biological activity of the corresponding protein. Therefore, it was expected that the amount of the *hFIX*::Ag detected by ELISA to be higher than that of the FIX activity

detected by the clotting test. Unexpectedly, our results showed much less value for *hFIX*::Ag than the value estimated for the expressed *hFIX* by coagulation test. This difference could be explained by the fact that there are several proteins with antigenic similarities to *hFIX* (in Gla-domain) in human plasma which is not present in the K-SFM. Therefore, the use of human pool plasma for drawing the standard curve in addition to the application of polyclonal anti-*hFIX* to perform ELISA could underestimate the antigenicity of the *hFIX* presents in the samples taken from serum-free media. The relatively higher levels of r*hFIX*, estimated from the clotting tests, could also be correlated to a probable occurrence of an extrinsic coagulation, which might be mediated by a tissue factor released from the cultured keratinocytes, as suggested by others [45]. However, since basal coagulation activity was not detected in either of the negative controls, thus, the possibility of basal coagulation activity in the serum-free media used in this work is ruled out.

To evaluate the expression level of the *hFIX* secreted in the cultured media, if we refer to the results obtained from the ELISA, an expression level of 185–190 ng/mL of r*hFIX* after 24 h is estimated, which is still comparable to those obtained by other groups who used regulatory elements from CMV (250 ng/mL), retrovirus (830 ng/mL), or moloney murine leukemia virus (600 ng/mL) for the expression of *hFIX* [6, 13].

Although viral promoters are strong, they appeared to be downregulated when shifted to *in vivo* conditions [20, 46–48]. For example, expression of the *hFIX* under the control of moloney murine leukemia 5′ long terminal repeat promoter/enhancer was downregulated when the transfected cells were grafted beneath a skin flap in athymic mice [6]. However, in some cases, use of viral vectors to transfect keratinocyte cell lines were shown to achieve a prolonged expression, but when transplanted, the expression pattern appeared to be more transient [10, 49].

The problem of viral promoter shot-off has been partly overcome using nonviral promoters such as those belonging to phosphoglycerate kinase [50], dehydroflate reductase [51], metallothionine [52], beta-actin [53], polymerase II [54], or human alpha-1 antitrypsin [55] promoters. The native regulatory elements of both K14 and K5 genes are especially attractive candidates for keratinocyte-mediated gene expression [56–58]. However, despite the obvious success in achieving long-term expression *in vivo*, they are generally much less active than viral promoters [21, 53, 59]. In order to improve expression efficiency of a transgene in keratinocytes in a specific manner, use of hybrid elements belonging to viral/tissue-specific regulatory elements has been suggested, in which combinations of strong viral promoter with the long term effects of tissue-specific enhancer are expected [46, 60]. Page and Brownlee [6] reported an expression level of about 52 ng/mL of *hFIX* by $10^6$ keratinocytes when a hybrid promoter composed of CMV and a 240 bp of keratin 14 gene promoters was used. Considering the expression level of at least 185–190 ng/mL *hFIX* achieved in this work under the control of 2,240 bp of human k14 gene enhancer/promoter, it is assumed that a combination of the keratinocyte specific regulatory element and a viral enhancer might increase significantly the expression level of *hFIX* in a similar system. Having now identified some of the limitations in current keratinocyte-based expression systems, the recombinant *hFIX* expression system developed in this study has provided the means for further bioengineering strategies to improve the expression efficiency of *hFIX* in keratinocytes.

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