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Designing a vector for specific expression of recombinant proteins in epidermal keratinocytes, Using human factor IX cDNA as a model for expression analysis

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Abstract: We have designed a keratinocyte-specific expression plasmid equipped with the K14-promoter by inserting a 2240bp *Bgl*II/*Hind*III fragment, containing the upstream regulatory elements of the K14 gene, in a digested pcDNA3 plasmid, from which the CMV promoter had been deleted. Subsequently human epidermal keratinocyte, isolated from neonatal foreskin, was transfected with the recombinant plasmid, based on liposome mediated transfection technique. The transfection step was continued for either 6 or 21 hours. The transfected cells were then subjected for the expression analysis. The K14-promoter-deriven expression of rhFIX in the transfected cells was examined by performing one-stage clotting assay, using hFIX immuno-depleted plasma on the cultured media on the day 3 of post-transfection. The pro-coagulation activity of the cultured media collected from the transfected keratinocytes indicates in the expression of hFIX by the transfected cells. The expression of recombinant hFIX in the transfected cells was also confirmed by RT-PCR.

Keywords; *human Factor IX، keratinocyte ، haemophilia B، keratin 14 gene promoter، ex vivo gene therapy*

Introduction; *Ex vivo* gene therapy requires a desirable bioreactor for production and delivery of gene product into target tissues. Human skin epidermis is an attractive vehicle for gene therapy, and can be used as a bioreactor to produce proteins for systemic genetic disorder (2). The potentials of keratinocytes have been demonstrated by the secretion of variety of recombinant proteins into central circulation (2). Application of keratinocytes for the treatment of systemic disease, such as hemophilia B, has been suggested. A defect or absence of the Factor IX results in hemophilia B, an X-linked hereditary bleeding disorder. Previous studies has documented that recombinant factor IX (rFXI), which is secreted by human keratinocytes is biologically active (3). Transfer of hFIX-cDNA into cultured human keratinocyte has been demonstrated by utilizing a viral of promoters to drive the expression of hFIX-cDNA (4,9). Basal keratinocytes expresses keratin 5 and keratin 14 protein at high levels (5). Therefore these promoters are especially attractive candidates for use in keratinocyte-mediated gene expression (8). The expression of hFIX-cDNA under the regulation of keratinocyte-specific promoter has not reported. However the hFIX cDNA expression under the control of CMV promoter/enhancer in combination with keratin14 enhancer in keratinocyte was shown by Page and his co-workers (6). The goal of this study is to use human keratin14 gene promoter to express hFIX-cDNA in cultured keratinocytes.

Materials and Methods; DH5α (*stratgene-USA*) strain of *E. coli* was used for cloning steps. All the enzymes, Geneticin (G-418), high pure PCR product purification kit, high pure plasmid isolation kit and transfection reagent, FuGene6 were from *Roche-Germany*. Keratinocyte Serum Free Medium (K-SFM) and dispase were from *invitrogen-USA*. Hanks, balanced salt solution was from *Baharafshan-IRAN*. *BamHI* and PCR product cloning kit (InsT/Aclone) were from *Fermentas*. Gentamicin was from *Alborzdarou-IRAN.* Reverse transcriptase (M-MuLv) and RNA preparation kit were from (*Roche-Germany*). All DNA manipulations were based on standard cloning procedures (7). Human chromosomal DNA was extracted from Blood and a human liver cDNA library (MRC-*UK*), was used as templates for the amplifications of human gene. The constructed expression plasmid was derived from pcDNA3 (*Invitrogen-USA*). Primers, hk14-F_1 (5′-GGAAGATCTGCTAGGGTTCTGGTGTTGGTGCG-3′) and hk14-R_1 (5′-CGTCCAAGCTTGAGGAGGGAGGTGAGCGAGCGA-3′) were designed based on the human keratin14 gene promoter. For human factor IX cDNA amplification, two primers, hIX-F_1

(5′ GGATCCGTTATGCAGCGCGTGAACATGA-3′) and hIX-R_1 (5′-GCGGCCGCAGTGATTAGTTAGTGAGAGGCCC-3′) were designed based on the human FIX mRNA sequences. The inserted fragments in the recombinant hFXI-expressing, pK14hFIX (including hPK14 promoter and hFIX cDNA) were fully sequence from both strands (*MWG-Germany*). Comparison of the obtained sequences against the *Gene-Bank* was performed using *Blast* program (1). Primary human normal epidermal keratinocytes were isolated from neonatal foreskins, [kindly provided by Dr. M. Mohammadzadeh (M.D.)] by over night dispase, followed by trypcinization of the separated epidermis and seeded in complete K-SFM (Figure 1). The cells were grown at 37^{o}C. The medium was changed with fresh KSFM every 2-3 day. Upon reaching 60-75% confluency the medium is removed and the cells were washed once with Ca and Mg-free D-PBS and passaged. The 1st and 2nd passaged keratinocytes were transfected with fugene-6 transfection reagent according protocol provided by manufacturer, in sex-well plates. The biological activity of the expressed hFIX was determined by performing clotting test, according to the instructions described by manufacturer. Reverse transcription was performed to detect the presence of recombinant hFIX transcript in the transfected cells. Total cellular RNA was extracted from transfected keratinocytes and used for the sythesis of cDNA. The synthesized FIX-cDNA was subsequently amplified by using two specific primer pairs, namely hFIX-F2 (5'-GCCATGGCCCCCTTTGGATTTGAAGGAAAGAAC-3'), and hFIX-R2 (5'-GAAGCTTTCTCCCTTTGTGGAAGACTCTTCCC-3').

Results:

Construction of Recombinant plasmids; The construction of the 8223 bp plasmid (pk14hFIX) for specific expression of recombinant proteins was carried out in two steps (figure2). In the first step, a ~2240 bp fragment containing hPK14 promoter was amplified (PCR1), *Bgl*II/*Hind*III digested and inserted in the pcDNA3 plasmid, which was digested similarly. The recombinant plasmid was named phPK14H, in which the CMV promoter was replaced by the hPK14 promoter. The recombinant plasmid was transferred to *E. coli* host and subjected for restriction analysis (figure 3A). The 6815 bp size of the linearized phk14H plasmid was confirmed separately by single digestion with either *Hind*III or *Bgl*II which were supposed to have unique sites in the recombinant phPK14H plasmid. A double *Hind*III/*Bgl*II digestion of the plasmid created to fragments of 2240 bp and 4575 bp, corresponding to the phK14 promoter and the pcDNA-related fragment, respectively. In the second step hFIX-cDNA was PCR-amplified (PCR2) and cloned in the phpk14H after a first round of cloning in T/A cloning vector (Figure 2). The newly made recombinant plasmid was named PK14hFIX and confirmed by restriction analysis (figure 3B) and nucleotide sequencing.

Expression analysis; **T**he primary human normal keratinocytes were subjected for transfection with the PK14hFIX. The transfection was carried out with various ratios of fugene6 and DNA (as the manufacturer recommended) and incubations were continued till confluency. The possible expression of hFIX at this stage was examined by performing clotting test on the media collected from the transfected cells. The clotting times obtained from the samples of different transfection lines were compared to the clotting time of the samples from negative controls (Table 1). The results indicate in the presence of coagulation activity in the cultured media collected from tranasfected cells that shows the presence of rhFIX, expressed by the transfected keratinocytes. About 72 hours after transfection, the cells were transferred into selective media containing geniticin and incubation continued until appearance of 9 isolated colonies which were cultured separately in selective media. The expression of rhFIX in the media collected from each colony was measured before achieving a complete confluency, that indicates in the presence of coagulation activity in the cultured media (Table 2). The expression of hFIX-cDNA

in the transfected cells was also confirmed by performing RT-PCR. The RT-PCR experiment resulted in the production of a PCR product of about 735 Bp that is in accordance with the expected size of hFIX cDNA(Figure 4). In conclusion, our preliminary results support the idea that human keratinocyte has potential for the production of biologically active FIX. In besides, the expression plasmid constructed in this work, has provided useful means for the expression of different protein of medical importance in keratinocyte for further biochemical and cellular studies.

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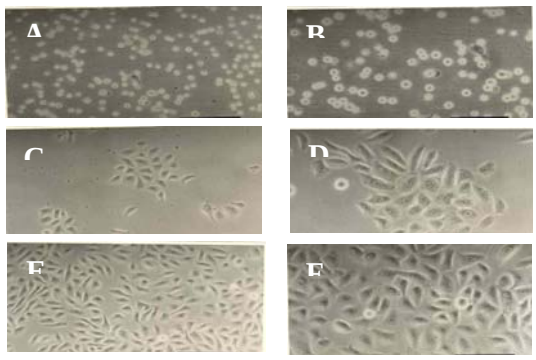

Figure 1: Human keratinocytes, immediately after being transferred to flask (A x200, B x320), after colony formation (C x200, D x320) and after confluency (E x200, F x320)

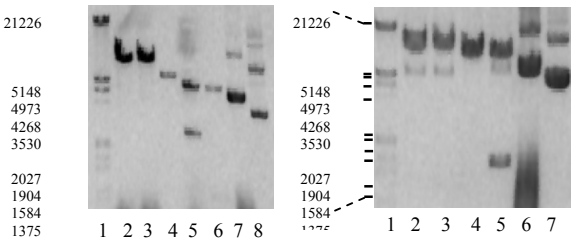

Figure 3: Restriction -analysis of the recombinant plasmids.
Panel A: phPk14H plasmid: Lane 1: DNA size marker. Lanes 2 & 3: linearized forms of phPk14H with either *Bgl*II or *Hind*III respectively. Lanes 4: linearized form of pcDNA3 with *Hind*III. Lanes 5: *Bgl*II/*Hind*III double digestion pattern of phPk14H. Lane 6: *Bgl*II/*Hind*III double digestion of pcDNA3. Lanes 7 & 8: Super-coil patterns of phPk14H and pcDNA, respectively.
Panel B : phK14FIX plasmid: Lane1: DNA size marker, Lane2: phK14FIX plasmid Digested with BamHI (linearized). Lane3: phK14FIX plasmid digested with *Not* . Lane4: linearized phPk14H plasmid. Lanes5: phK14FIX plasmid digested with *Bam*HI/*Not*I. Lane 6: undigested phK14FIX plasmid. Lane7:undigested phK14H plasmid.

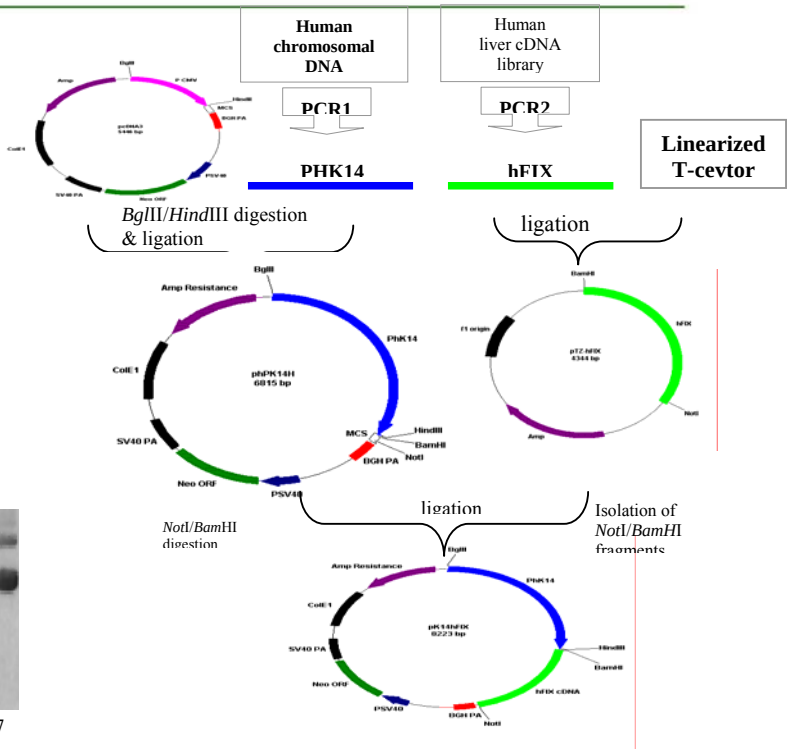

Figure2: Stepwise view of the constructions of recombinant plasmids

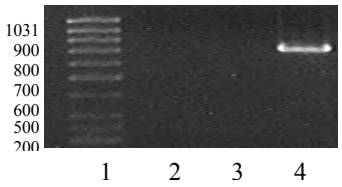

Figure 4: PCR product amplified from the reverse transcribed total RNA. In each case, the prepared RNA was treated with RNase-free DNase. **Lane 1:** DNA size marker. **Lane 2:** Transfected keratinocytes, using total RNA pre-treated with RNase-free DNase as template. **Lane 3:** Negative control. **Lane 4:** Transfected keratinocytes, using Reverse transcribed product cDNA as template.

Table1; Human FIX clotting activity in conditional cultured media before G418 treatment (day 3 after transfection reaction)

Row	Sample	Clotting time (second)
1	Negative control	180
2	Negative control (PhpK14H)	165
3	A (pK14hFIX)	70
4	B (pK14hFIX)	70
5	C2 (pK14hFIX)	76
6	C3 (pK14hFIX)	75
7	D1 (pK14hFIX)	80
8	D2 (pK14hFIX)	78
9	F2 (pK14hFIX)	75
10	F1 (pK14hFIX)	84
11	F2 (pK14hFIX)	88
12	F3 (pK14hFIX)	93

Table 2; Human FIX clotting activity in conditinal cultured madia after clony selection following G418 treatment(during 18±3 hr).all media were diluted

Row	Clones	Clotting time (second)
1	F1-C1	100
2	F1-C2	100
3	F1-C3	100
4	F1-C4	100
5	F2-C3	133
6	F2-C4	135
7	F3-C1	87
8	F3-C2	70
9	F3-c3	93

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