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ORIGINAL RESEARCH PAPER

Enhancement of the human factor IX expression, mediated by an intron derived fragment from the *rat aldolase B* gene in cultured hepatoma cells

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Abstract Combinations of a liver-specific rat aldolase B intronic enhancer (*rAB*E) with either of the hepatocyte-specific human α1-antitrypsin promoter (hAATp) and cytomegalovirus enhancer/promoter (CMVp) were used to construct a number of plasmids expressing non-viral human factor IX (hFIX). The efficacies of the plasmids were evaluated in a hepatocyte cell line (HepG2). Potential of the *rAB*E was evidenced, by 300%—and 800% increase of the hFIX expression levels when it was combined with the CMVp and hAATp, respectively. The highest hFIX expression level was obtained when the *r*ABE was combined with the CMVp for which the maximum intracellular accumulation of hFIX was also evidenced. Therefore, the *rAB*E is suggested as a suitable *cis*-acting element for protein expression in hepatocytes. Considering the potential of introns during post-transcriptional processes, the function of the human *β*-globin (*hBG*) intron-II, within the hFIX coding region, in the second generations of the hFIX expressing plasmids was also examined, which leaded to reduction of the hFIX expression level, probably due to improper splicing of the *hBG* intron-II.

Keywords Aldolase B intronic enhancer · Cytomegalovirus promoter · Human α1-antitrypsin promoter · Human factor IX · Non viral plasmid

Introduction

Current treatment of hemophilia B patients is replacement therapy with either pooled plasma concentrates or recombinant hFIX (r-hFIX) (Roth et al. 2001). A variety of cell types, such as Chinese hamster ovary (CHO), baby hamster kidney (BHK) and hepatocytes, have been examined for the production of recombinant hFIX (Berkner 1993; Wajih et al. 2006; Armentano et al. 1990). Among these, hepatocytes are the most suitable host for the r-hFIX expression. The HepG2 cell, which has maintained

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several features of a differentiated hepatocyte, does not express hFIX (Fair and Bahnak 1984) and might be a suitable host for evaluation of the hFIX transgene expression.

In addition to the choice of host cell, an improved expression cassette in the context of a plasmid vector might facilitate efforts towards gene expressions at therapeutic levels. Among the challenges in this regard are the low expression level of the hFIX and immunological complications after using viral vectors (High 2000). Moreover, viral promoters appeared to be down regulated when shifted to in vivo conditions (Miao et al. 2000) which has been partly overcome by the use of non-viral tissue specific promoters (Miao et al. 2000). Combinations of suitable *cis*-acting elements such as strong liver specific promoters and efficient enhancers are thought to be useful to overcome the limitations mentioned above.

The hAATp and *rAB*E (from the first intron of the *rat aldolase B* gene) which exhibit high degrees of liver specificities and activities (Sifers et al. 1987; Gregori et al. 1998) are attractive candidates for hepatocyte-specific over-expressions of proteins. Having this in mind, in the first step of this work four hFIX expression plasmids regulated by the hAAT and CMV promoters with and without the *rAB*E, were generated.

Introns, as the major parts of the eukaryotic genome, have crucial roles in gene regulation through various pathways (Le Hir et al. 2003). It was previously shown that the hFIX intron-I could enhance expression of FIX transgene in vitro (Kurachi et al. 1995). In our previous study, we introduced *hBG* intron-II in the second intronic position of the *hFIX*-cDNA and observed 500% increase of expression level relative to that of the intron-less *hFIX*-cDNA in vitro (Haddad-Mashadrizeh et al. 2009). Improvements of transgene expressions by application of the *hBG* intron-II in a number of expression systems have also been shown (Harding et al. 2004; Palmiter et al. 1991). Therefore, we assumed that this intron would improve the hFIX expression in a HepG2 expression system. To examine this assumption, in the second generations of the above mentioned plasmids, the *hFIX*-cDNAs were interrupted with the *hBG* intron-II.

This paper presents results obtained from a systematic expression analysis of the HepG2 cells carrying the hFIX expressing plasmids and discusses various aspects of the functions of the applied regulatory elements on the expression of the hFIX in vitro.

Materials and methods

Amplification of the *rAB*E and hAATp

Genomic DNA extracted from the adult *Rattus Norvegicus* peripheral blood (Sambrook 2001) was used as the template for PCR-amplification of a 417 bp fragment containing the liver-specific enhancer sequence (+1,912 nt to +2,329 nt) of the *rat aldolase B* (*rAB*) gene using oligonucleotides *rAB*E-1912F and *rAB*E-2329R as forward and reverse primers, respectively (Table 1). Genomic DNA extracted from the human peripheral blood (Sambrook 2001) was used as the template for PCR-amplification of a 320 bp fragment (−266 nt to +54 nt) representing a minimal length of the hAATp. Oligonucleotides, AAT-266F and AAT-54R, were used as forward and reverse primers, respectively for amplification of the hAATp (Table 1). To facilitate cloning of the generated PCR products, restriction sites were considered at the 5′-ends of the forward and reverse primers.

Construction of the hFIX expressing plasmids

For detailed description of the construction of vectors, see Supplementary data. Schematic views of the constructed hFIX expression cassettes are outlined in Fig. 1.

Cell culture and transfection

The HepG2 cells (National Cell Bank of Iran) were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 1% (w/v) sodium pyruvate, 1% non-essential amino acids in addition to 100 U penicillin/ml and 100 μg streptomycin/ml at 37°C with 5% CO_2. Cells were transfected by 2 μg *Pvu*I-linearised plasmids mixed with 6 μl FuGene-6 (Roche) according to the manufacturer's instruction. Six hours after transfection, conditioned medium was replaced by fresh medium containing 1 μg vitamin K1/ml 48 h later the conditioned medium was replaced with fresh medium containing 0.6 mg G418/ml. After

Table 1 List of the oligonucleotides, used for the construction of the hFIX expression cassettes

Primer name	Nucleotide sequence (5′–3′)	Restriction site
rABE-1912F	GAAGATCTAGGCCTCCACACTCTATTGCA	*Bgl*II
rABE-2329R	AGCAATTGGTCAAGTCAAACTCGTCCTGT	*Mun*I
AAT-266F	AGTCGCGATGACTCACGCCACCCCCT	*Nru*I
AAT-54R	TGAAGCTTATACTTACGATTCACTGTCCC	*Hind*III
hKozF9-F	GGATCC**GCCACC**ATGCAGCGCGTGAACATGAT	*Bam*HI
hFIXE4-R	CCTTGCAACTGCCGCCATTTAAAC	*Dra*I
hFIX-R2	GAAGCTTTCTCCCTTTGTGGAAGACTCTTCCC	*Hind*III
β actin-F	GAGACCTTCAACACCCCAGCC	–
β actin-R	AGACGCAGGATGGCATGGG	–

Restriction sites are *underlined*. The Kozak sequence is emphasized in *bold*

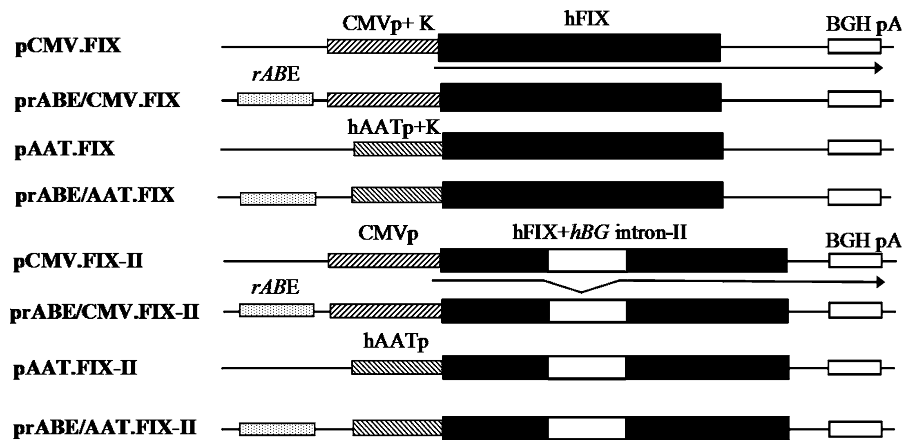

Fig. 1 Schematic view of the constructed hFIX expression cassettes in pcDNA3.1 plasmid. Names of the recombinant plasmids carrying the expression cassettes are indicated on the left. *CMVp* cytomegalovirus enhancer/promoter, *K* Kozak sequence, *rAB*E rat aldolase B intronic enhancer, *hAATp* human α1-antitrypsin promoter, *hFIX + hBG intron-II* human factor IX + human β-globin intron-II, *BGH pA* bovine growth hormone polyadenylation signal

3–4 weeks cultivation, G418 resistant cells were pooled and seeded in duplicates at 2×10^5 cells in 1 ml of vitamin K1-supplemented medium in the absence of G418. The culture supernatants were collected at various time intervals and used to assay the expressed hFIX.

Immunoassay of the hFIX antigen in cultured media

The hFIX antigen was assayed using an ELISA kit. A standard curve was provided from two-fold serial dilutions of lyophilized normal human pooled plasma, containing 5 μg hFIX. Subsequently, concentrations of the hFIX in the cultured media were calculated based on the standard curve and stated in ng/ml.

Analysis of intracellular accumulation of the expressed hFIX

The stably-transfected HepG2 cells were lysed in ice-cold lysis buffer containing protease inhibitors (Roche) for 10 min followed by centrifugation at $12{,}000\times g$ for 15 min at 4°C. Total protein concentration was determined by the Bradford method. The hFIX antigen in the cell lysate was measured by ELISA and normalized to the total protein of cell lysate and finally stated in ng protein/mg.

Reverse transcription-PCR

Total RNA, isolated from 5 to 10×10^6 cells using the Tripure kit (Roche), was used as the template to

perform RT-PCR using a one-step RT-PCR kit to generate a first cDNA strand according to the manufacturer instruction. The generated fragments were subsequently used as the template for the PCR-amplification of the double stranded cDNA corresponding to a section of the hFIX coding sequence, using oligonucleotides hKozF9-F and hFIX-R2 as forward and reverse primers, respectively (Table 1). In parallel, for internal control and normalization, the generated cDNA was used as template for the PCR-amplification of a section of the human β-actin coding sequence, using oligonucleotides β actin-F and β actin-R as forward and reverse primers, respectively (Table 1).

Statistical analysis

Analysis of variance followed by a Tukey post-hoc test was performed to evaluate differences among the constructs. $P < 0.05$ was considered statistically significant. All statistical analyses were carried out with SPSS 11.5.

Results

Stable expression of hFIX

Expression analysis of the transfected cells were carried out in two separate experimental groups. In the first group, the cells were transfected with the intron-less constructs namely; pCMV.FIX, p*rAB*E/CMV.FIX, pAAT.FIX and p*rAB*E/AAT.FIX, and in the second group, the cells were transfected with the *hBG* intron-II containing constructs including; pCMV.FIX-II, p*rAB*E/CMV.FIX-II, pAAT.FIX-II and p*rAB*E/AAT.FIX-II, prior to expression analysis.

Within the first experimental group, the hFIX expression level of the CMV-regulated construct was higher than that of the hAATp-regulated one under similar conditions (Fig. 2a). When the cell numbers on the 4th day were adjusted, the hFIX expression level of the cells containing CMV-regulated construct was (22.9 ng/ml per 10^6 cells) with 440% increase when compared with that of the hAATp-regulated construct (5.2 ng/ml per 10^6 cells).

The *rAB*E augmented the hFIX expression level when it was combined with either of the CMV or hAAT promoters (Fig. 2a). The highest hFIX expression level occurred for the *rAB*E/CMV-regulated construct (71.4 ng/ml per 10^6 cells) with 300% increase when compared with that of the CMV-regulated one. In the case of the *rAB*E/hAATp-regulated construct, the hFIX expression level reached 39.5 ng/ml per 10^6 cells, thus representing an 800% increase in comparison with that of the hAATp-regulated one, when the cell numbers were adjusted on the 4th day (Table 2a).

In the second group, a general reduction in the hFIX expression levels for all the examined minigenes in comparison with their intron-less counterparts was observed (Fig. 2b). These data have also provided supporting evidences for the enhancer activity of the *rAB*E, in combinations with both hepatocyte-specific and non-specific promoters (Table 2b).

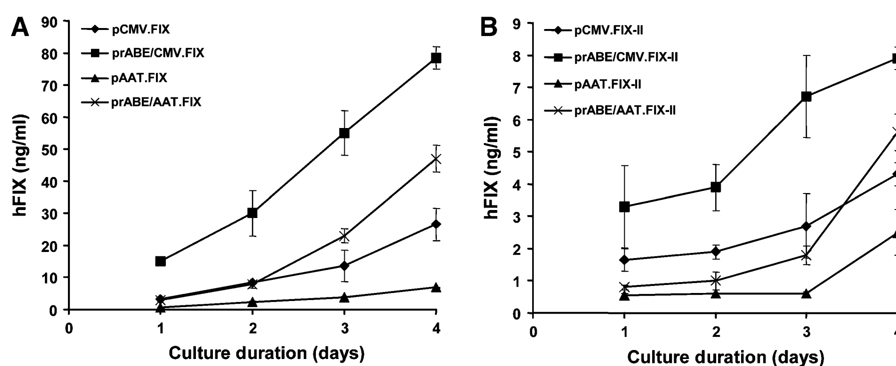

Fig. 2 Determination of the hFIX concentration in the cultured media of the cells with intron-less constructs (**a**) and with β-globin intron-II-containing constructs (**b**), at various culture durations, based on ELISA. The results represent the mean hFIX production of two independent cell-pools. Detectable level of hFIX was not identified in the culture medium of non-transfected HepG2 cells (data not shown)

Table 2 Comparison of the recombinant plasmids for expression of the hFIX in the cultured media after 4 days of cultivation

	Fold (hFIX expression level)	*P* value
(a) Intron-less plasmids		
prABE/CMV.FIX vs.		
prABE/AAT.FIX	1.8	0.002
pCMV.FIX	3	0.000
pAAT.FIX	137	0.000
prABE/AAT.FIX vs.		
pCMV.FIX	1.7	0.03
pAAT.FIX	8	0.002
pCMV.FIX vs.		
pAAT.FIX	4.4	0.02
(b) β globin intron-II containing plasmids		
prABE/CMV.FIX-II vs.		
prABE/AAT.FIX-II	1.4	0.03
pCMV.FIX-II	2	0.005
pAAT.FIX-II	3.3	0.001
prABE/AAT.FIX-II vs.		
pCMV.FIX-II	1.4	0.02
pAAT.FIX-II	2.3	0.01
pCMV.FIX-II vs.		
pAAT.FIX-II	1.7	0.02

Measurement of the hFIX level expressed by isolated clones

The lower expression levels of the hFIX in the cases of the cells, carrying the intron-containing hFIX minigenes, were thought to be correlated to a heterogenous population of cells (pooled-cells), composed of both expressing and non-expressing cells. In order to examine this assumption, a number of individual isolates (4–6 resistant clones) for each minigene were taken for expression analysis. Accordingly, the low expression levels of the cell-pools from the intron-containing cells were still observable in the results obtained from the expression analysis of the various isolates of each construct (Table 3).

Evaluation of the intracellular hFIX

The possible accumulation of the expressed hFIX inside the recombinant cells was investigated by measurement of the hFIX:Ag in cellular lysates of the cell-pools of different transfectants. The highest and lowest amounts of intracellular hFIX were found among the groups, carrying the intron-less and intron-containing plasmids, respectively. The maximum accumulation of intracellular FIX occurred for the p*rAB*E/CMV.FIX containing cells, which was approximately 100 times higher than the lowest amount of intracellular hFIX, observed from the pAAT.FIX-II containing cells (Fig. 3).

Analysis of the hFIX transcript

In the cases of the intron-containing minigenes which displayed lower expression levels of the hFIX, evaluation of the intron removal was necessary. With this aim, the recombinant HepG2 cells carrying either the p*rAB*E/CMV.FIX-II or p*rAB*E/CMV.FIX plasmids, as the representatives of the intron-containing constructs or the intron-less constructs, respectively, were subjected to RT-PCR analysis. Electrophoresed patterns of the two RT-PCR products, obtained from the intron-containing clones in comparison with and intron-less ones, detected aberrant splicing of the pre-mRNAs in samples taken from the intron-containing minigenes (Fig. 4).

Discussion

The present study aimed to establish an efficient hepatocyte specific vector for the hFIX expression in vitro. Based on the data obtained, the *rAB*E is able to improve the activities of both the liver-specific hAAT and the CMV promoters in stably transfected HepG2 cells, significantly. Although the *rAB*E as a liver specific element in combination with the hAATp ensure the specificity of the hFIX expression in hepatocytes, it did function more effectively in combination with the CMVp in vitro. This result is in agreement with those findings that showed the enhancer activity of the *rAB*E on the heterologous promoters such as the liver-specific L-type pyruvate kinase promoter and non-specific thymidine kinase promoter in the HepG2 cells, up to 18- and 15-fold, respectively, and its activity is a position and distance-independent (Gregori et al. 1998). In this study, applicability of the *rAB*E in conjunction with both liver-specific AAT and the viral CMV promoters

Table 3 Range of the hFIX concentrations in the cultured media of different isolates for each construct, as determined by ELISA after 4 days of cultivation

The hFIX expressing plasmids	Number of isolates	Range of the hFIX expression level of the isolates (ng hFIX/ml)/10^6 cells	Mean	hFIX expression of the cell-pools on the 4th day
pCMV.FIX-II	6	0.1–4.6	1.1	2.2
p*rABE*/CMV.FIX-II	4	1–2.6	1.8	4.3
pAAT.FIX-II	5	3.7–9.3	5	1.3
p*rABE*/AAT.FIX-II	6	1.8–11.3	4.6	3

The results represent the mean production of three independent experiments

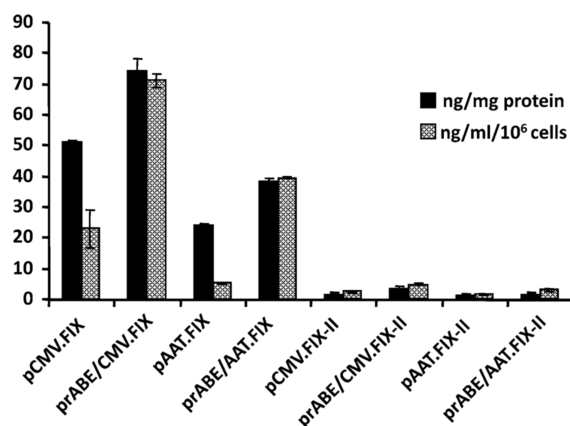

Fig. 3 Evaluation of the intracellular and extracellular hFIX using ELISA on the 4th day. Each column represents the mean values of either the intracellular or the extracellular hFIX, obtained from two independent experiments. Detectable level of hFIX was not identified in the lysate of non-transfected HepG2 cells (data not shown)

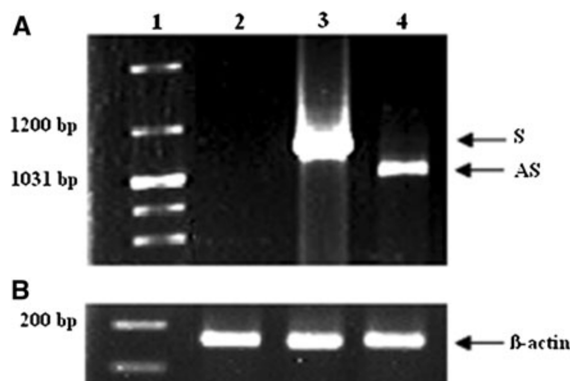

Fig. 4 PCR products amplified from the reverse transcribed total RNA. In each case, the prepared RNA was treated with RNase-free DNase. Panel A: *Lane 1*: DNA size marker (Fermentas), *Lane 2*: non-transfected HepG2 cells, *Lane 3*: cells transfected with prABE/CMV.FIX, *Lane 4*: cells transfected with prABE/CMV.FIX-II. Panel B: amplified β-actin cDNA from each sample (161 bp). *S* spliced product (1,104 bp), *AS* aberrant spliced product

were documented. The higher expression level of the hFIX under the regulation of the chimeric *rAB*E/CMVp was evidenced by the higher levels of both secreted hFIX in the cultured media of the recombinant HepG2 cells (containing p*rAB*E/CMV.FIX) and the hFIX, accumulated inside the cells. This result and those reported by others have provided convincing evidences that tissue-specific enhancers have potential to improve the transcriptional activity of the CMVp (Dai et al. 1992; Hagstrom et al. 2000). Under similar conditions, the hFIX levels produced by either of the *rAB*E/CMVp and CMVp regulated plasmids in the HepG2 cells were higher than those produced by the *rAB*E/hAATp and hAATp regulated plasmids, respectively indicating the stronger activity of the CMVp than the 320 bp hAAT-derived promoter. This finding is consistent with the results reported by Kramer et al. (2003) who showed that the activity of a similar section of the hAATp (305 bp), is about 40% of that of the CMVp in the cultured HepG2 cells. However, it is in contrast to several other reports that have shown stronger activities of tissue-specific promoters in comparison with viral promoters (Rodriguez et al. 2002; Wang et al. 1997).

Application of intron(s) to achieve higher expression levels of hFIX has been demonstrated by Kurachi et al. (1995). Here, we have examined the possible regulatory functions of intron-derived fragments on the expression of hFIX at two different locations with respect to the *hFIX*-cDNA. First, the enhancer-like activity of the 417 bp fragment, derived from the first intron of the *rAB* gene in the

hFIX-expressing cassette, provided supporting evidence for the presence of regulatory elements in this intron, which is in accordance with the results obtained by others (Gregori et al. 1998; Sabourin et al. 1996). In this regard, it has been hypothesized that the first introns of mammalian genes facilitate transcription, due to the probable presence of additional transcription factor binding sites (Majewski and Ott 2002). Enhancement of both transcription initiation and RNA polymerase II processivity is conducted by promoter-proximal introns, which boost pre-mRNA synthesis (Furger et al. 2002). Secondly, the probable function of the *hBG* intron-II within the coding region of *hFIX*-cDNA was also examined. In contrast to our previous results (Haddad-Mashadrizeh et al. 2009), application of the *hBG* intron-II in this study, in a similar position but under a different regulation system caused a dramatic reduction in the expression levels of the hFIX.

Based on the results obtained from RT-PCR, the transcript precursors of the hFIX-II minigene in the cells carrying the p*rAB*E/CMV.FIX-II plasmid, are not properly spliced, which may explain the low expression levels of the hFIX by the intron-containing hFIX minigenes in the cultured HepG2 cells. Considering the importance of the sequences surrounding the intron as well as intron identity with regard to splicing efficiency, the chimerical structure generated by the introduction of the second *hBG* intron, inside the *hFIX*-cDNA, might not favor efficient splicing. At this stage, one may explain this behavior in the context of the heterologous intron–exon function and a possible effect of the host cell type on intronic fate and functions.

In the present study, the higher hFIX secretion from the p*rAB*E/CMV.FIX containing cells was shown to coincide with the higher intracellular accumulation of the hFIX, which indeed is a major obstacle for an efficient expression of the hFIX. Accumulation of the expressed protein within the intracellular compartments can occur as a result of inefficient secretion of the over-expressed protein in such system. Tagliavacca et al. (2000) showed that, cells which express the human coagulation factor VIII (hFVIII) at high level are susceptible to intracellular accumulation of the protein. In similar experience, Plantier et al. (2001) demonstrated that vector containing the *hFVIII* minigene with higher expression levels of the hFVIII secretion are susceptible to accumulation of nearly 100 times of intracellular hFVIII. Wajih et al. (2006) used siRNA against calumenin, an inhibitor of the γ-carboxylation system, in the engineered BHK cells over-expressing vitamin K1 2,3-epoxide reductase (VKORC1) and hFIX and were thus able to increase the hFIX secretion significantly. Therefore, to improve secretion efficiency of the hFIX in an expression system such as the one used in this work, manipulation of various components involved in the γ-carboxylation system, in addition to cellular chaperones, such as calumenin would be highly desirable.

Another key element in secretion of a protein is its attached signal peptide which must be processed efficiently. Accordingly, one approach to improve the secretion efficiency of the hFIX during an over expression state would be to investigate the function of different heterologous signal peptides on the hFIX expression followed by optimization of the culture media and growth conditions.

In conclusion, the data obtained from the application of various combinations of the *cis*-acting heterologous elements in this study have provided suitable means for further steps toward the expression of the hFIX in hepatocytes. Accordingly, *rAB*E is suggested as an attractive *cis*-acting element to achieve a higher expression level of proteins in hepatocytes.

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