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# Estrogen modulates plasminogen promoter activity

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ABSTRACT

Postmenopausal women treated with estrogen hormone replacement therapy and female patients with hypoplasminogenemia receiving oral contraceptives show increasing plasminogen (PLG) concentrations. The elevated PLG levels are in contrast to the estrogen dependent decline of lipoptrotein(a) [Lp(a)], whose main protein component apolipoprotein(a) [APO(a)] is highly homologous to PLG in protein and gene structure and is also located in its immediate vicinity on chromosome 6q26. The intergenic region between both genes comprises several transcription-regulatory regions with enhancer sequences that increase the basal activity of the PLG core promoter. Using luciferase reporter assays we demonstrate that the minimal PLG promoter is insensitive to estrogen. However, an estrogen response element located 11.5 kb upstream of the PLG transcription start site is able to convey a dramatic estrogen-dependent elevation of PLG-minimal promoter driven reporter gene expression. In contrast, the activating effect of two additional enhancer elements, among them an DNase I hypersensitivity region that has been shown to regulate the APO(a) minimal promoter activity, is abrogated by estrogen. Thus, the identified estrogen-responsive elements provide a gene and tissue specific framework by which PLG expression is regulated and whose activity is orchestrated by yet unknown accessory factors.



## 1. Introduction

Plasminogen (PLG, MIM 173350) is the zymogen of plasmin, the enzyme of fibrin clot degradation. Apart from the involvement in fibrinolysis PLG plays a major role in wound healing, cell migration, tissue remodeling, angiogenesis and embryogenesis and is primarily synthesized by liver tissue [1,2].

The *PLG* gene is located on chromosome 6q26-q27 [3] and resides in a head-to-head configuration with the *APO(a)* gene (*LPA*, MIM 1 52200), from which it is separated by a ~40 kb intergenic region that contains various regulatory elements described to modulate *APO(a)* promoter activity in reporter gene assays (Fig. 1A) [4–6]. APO(a) is a protein component of the plasma lipoprotein Lp(a) and bears a strong homology to PLG [7,8]. Elevated plasma Lp(a) levels have been identified as an independent risk factor for the development of cardiovascular disease [9].

*PLG* gene expression is regulated by glucocorticoids, interleukin 6, nerve growth factor, and retinoic acid-related orphan receptor alpha [10–13]. Furthermore, epidemiological studies revealed an increase of PLG plasma levels in women taking oral contraceptives and in postmenopausal women receiving hormone replacement therapy, containing estrogen alone or in combination with gestagens [14,15].

Estrogen (E2, 17β-estradiol) acts primarily by binding to the estrogen receptor (ERα, *ESR1*; ERβ, *ESR2*). The activated ER is a transcription factor that binds in dimeric form to estrogen response elements (EREs) in regulatory regions of target genes. Only few genes with canonical EREs are known. Most E2 target genes do not contain a perfect ERE palindrome but have non-palindromic, imperfect EREs through which modulation of promoter activity has been demonstrated [16,17].

In this study we addressed the influence of E2 on the *PLG* promoter and adjacent regulatory elements. In PLG expressing HepG2 cells we analyzed *cis*-acting sequences and elements previously shown to play a role in *APO(a)* gene expression. Moreover, we identified two EREs that influence *PLG* promoter activity to various extent *in vitro* suggesting that the plasminogen gene is a target for estrogen action.

## 2. Material and methods

### 2.1. Plasmids

All PLG promoter and enhancer constructs were cloned into the pGL4.10 reporter vector (Promega, Madison, WI, USA) encoding the *Photinus pyralis* (firefly) luciferase. Positional information within

*Abbreviations:* APO(a), apolipoprotein(a); DH, DNase hypersensitivity; ERE, estrogen-response element; Lp(a), lipoptrotein(a); PLG, plasminogen; TSS, transcription start site.

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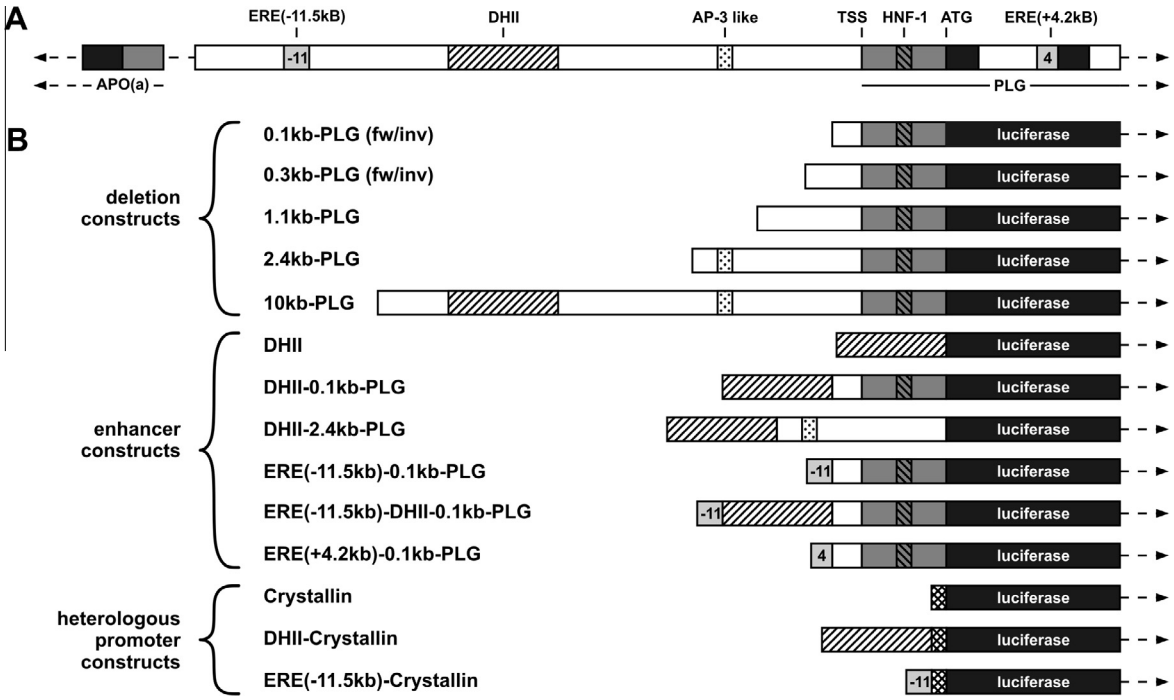


**Fig. 1.** Organization of the APO(a)–PLG chromosomal locus and plasmid constructs used in this study. (A) Genomic organization of APO(a)–PLG intergenic region on chromosome 6q26 (not drawn to scale). Black bars, exons; shaded bars, 5′-untranslated region (UTR); white bars, introns and intergenic region; hatched and dotted boxes, regulatory elements; light-gray boxes, estrogen response elements (ERE); cross-hatched box, crystallin minimal promoter; TSS, transcription start site according to Malgaretti et al. 1990 [18]; ATG, translation start site; DHII, DNase I hypersensitivity region II [22]; AP3-like, activator protein 3-like; HNF-1, hepatocyte nuclear factor 1; ERE, estrogen response elements. (B) Overview on all promoter and enhancer constructs in the pGL4.10 luciferase reporter vector backbone.

plasmid names refer to the transcription start site (TSS) as defined by Malgaretti et al. [18]. An overview on all used constructs is given in Fig. 1. Detailed cloning strategies for the specific plasmids are described in the Supplementary Material.

For *in silico* analysis to identify potential EREs within the *APO(a)-PLG* locus the publicly accessible version of MatInspector (Genomatix, Munich, Germany) was used. Double stranded oligonucleotides consisting of synthetic 5′-phosphorylated oligonucleotides corresponding to 25 base pairs around the SmaI restriction site at positions −11502 [ERE(−11.5 kB)] and around the intron 1/exon 2 boundary at position +4263 [ERE(+4.2 kB)] and their corresponding complementary counterparts were generated and cloned into pGL4.10 and specific PLG promoter and enhancer derivatives. Oligonucleotide sequences and cloning strategies are given in the Supplementary Material.

The human ER α (hERα) cDNA pSG5 plasmid was kindly provided by M. Marino (University of Rome, Italy). For transfection assays hERα cDNA was subcloned into the EcoRI sites of the eukaryotic expression vector pcDNA3.1(+) (pcDNA3.1-hERα). Expression of hERα in transiently transfected HepG2 cells was verified by immunoblot analysis (mouse-anti-hERα antibody, sc-8005, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### 2.2. Cell culture, transfection and Dual Luciferase Assays

The human hepatoma cell line HepG2 and breast cancer cell line MCF-7 were maintained under standard conditions in RPMI 1640 and DMEM-F12, respectively.

For transfection assays $5 \times 10^5$ HepG2 cells/well or $2.5 \times 10^5$ MCF-7 cells/well, were seeded in 6-well plates in medium without phenol red, supplemented with 5% charcoal/dextran treated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 2.5 μg of reporter constructs were co-transfected with 0.25 μg pGL4.70 (encoding the Renilla-Luciferase cDNA as an internal control; Promega), and 0.2 μg of expression plasmid pcDNA3.1-hERα using Lipofectamin 2000 (Life Technologies, Paisley, UK). Stimulation experiments were performed in fresh medium containing either 100 nM 17β-estradiol (Sigma–Aldrich, Munich, Germany) in ethanol or ethanol alone as vehicle control at a final concentration of 0.01%. After 24 h of incubation, lysates were assayed for luciferase activity using the Dual Luciferase Reporter Assay (Promega) following the manufacturers protocol. In order to normalize for transfection efficiency, luciferase activities are reported as the ratio of the reporter plasmid (pGL4.10) to control plasmid (pGL4.70) activity. In addition, activity of PLG promoter constructs is further normalized to promoter-less pGL4.10 activity.

Primary human hepatocytes were isolated from liver biopsies (3 patients with gastrointestinal malignancies with liver metastases), kindly provided by Prof. R. Gebhardt (Institute for Biochemistry, Medical Faculty, University of Leipzig, Germany) through the "HepatoSys" program according to the guidelines of the "Charitable state controlled foundation HTCR (Human Tissue and Cell Research)" with written informed consent of patients [19]. Culture, stimulation and real time PCR protocols are described in detail in the Supplementary Material.

### 2.3. Statistical analysis

All experiments were performed in triplicates. Statistical analyses were performed using students *t*-test. Data are shown as means ± SEM.

## 3. Results and discussion

### 3.1. Effect of E2 on PLG minimal promoters

To verify and compare transcriptional activities under conditions employed in the present study, three minimal promoters

of differing length driving PLG expression [0.1 kb-PLG, 0.3 kb-PLG, and 1.1 kb-PLG (Fig. 1)] described previously were tested. After co-transfection with hERα into HepG2 cells, all core promoters in their native orientation were able to drive luciferase activity 2-3-fold above the promoterless plasmid, whereas 0.1 kb-PLG and 0.3 kb-PLG minimal promoters in antisense direction were not effective (Fig. 2A). These results confirm previous studies, that demonstrated constitutive expression of PLG depending on the presence and orientation of a hepatocyte nuclear factor (HNF) 1α (HNF1A) binding site within the PLG 5′-UTR. HNF1α is a liver enriched transcription factor that binds to DNA sequences near the TSS (between +48 and +61 relative to PLG TSS) [20], a region that is included in three minimal promoter constructs.

When 17β-estradiol (E2, 100 nM) was administered to transfected HepG2 cells none of the minimal promoter constructs showed a significant modulation of transcriptional activity (Fig. 2A). For further analysis of PLG regulatory elements the 0.1-PLG minimal promoter was used because it was sufficient to mediate basal PLG transcription.

An AP3-like (activator protein 3) enhancer element, located −2.4 kb upstream of PLG-TSS, is involved in PLG transcriptional regulation [20]. In our study we could show that a construct comprising the entire upstream region, containing the AP3 response element (2.4 kb-PLG) was able to further elevate basal luciferase activity 1.7-fold above that of PLG minimal promoters (Fig. 2A). The observed increase corresponds well to previously published results [20] and the 2.4 kb-PLG construct represents the most active contiguous promoter fragment analysed in this study. Surprisingly, with E2 stimulation this effect decreased by 54% down to the activity level of the PLG core promoter, although no ERE could be identified within this region. The identity of an activator protein that in HepG2 cells constitutively binds to a sequence between −2.4 kb and −1.1 kb but whose activity is completely suppressed by estrogen remains elusive. The poorly defined liver enriched AP3 transcription factor might represent a candidate. To the best of our knowledge functional interference of AP3 with activated ERs has never been described.

### 3.2. Modulation of PLG promoter activity by a DNase hypersensitivity region

Within the *PLG-APO(a)* intergenic region, four liver specific DNase hypersensitivity regions (designated as DHI-IV) were identified [21]. DHII (−8767 to −9506 bp) was described to enhance *APO(a)* gene expression [4–6,22]. An ERU (minimal estrogen responsive unit) was identified within DHII containing parts of an ERE, responsible for E2 dependent inhibition of *APO(a)* expression [4,5]. When DHII was cloned in the same orientation as the *PLG* gene (i.e., as organized at the chromosomal locus) in front of 0.1-PLG minimal promoter, reporter activity increased 3-fold above the 0.1-PLG minimal promoter without DHII. Following E2 treatment a decrease in activity of 49% was detected (Fig. 2B). Since HepG2 cells do not express detectable levels of endogenous ERα, the E2 responsiveness of the DHII region in front of the PLG minimal promoter was dependent on the presence of co-transfected hERα (data not shown). This mode of DHII action is almost identical to the findings reported for the DHII-estrogen interaction within the APO(a) promoter [4,6].

Neither basal nor E2 dependent activation of the larger 2.4 kb-PLG promoter were affected by the presence of the DHII enhancer (Fig. 2B) suggesting a dominant impact of the putative AP3-like factor binding enhancer element centered at nucleotide −2200 bp (e.g., by blocking access of the DHII bound protein to another accessory *trans*-acting factor) [20].

In order to analyse the estrogen response of the DHII and −2.4 kB regulatory elements in a native context, a fragment containing 10 kB of the *PLG-APO(a)* intergenic region was cloned into pGL4.10. Under treatment with E2 a significant reduction (52%) of luciferase expression was detected, which points to an inhibitory E2 dependent net effect in the 10 kb upstream promoter region on PLG expression (Fig. 2B). This estrogen dependent decrease parallels the decline observed for the 2.4 kb- and DHII-fragments alone or in combination. Overall activity did not reach pGL4.10 level, which is probably caused by the extensive size of this plasmid supposed to result in a low transfection efficiency.

### 3.3. Identification and activity of distal estrogen responsive elements (EREs)

To evaluate whether additional putative EREs are located within the 40 kb *APO(a)-PLG* intergenic region as well as in the region downstream of the PLG transcription initiation site comprising the 5′-untranslated region and the first two exons/introns, *in silico* analysis was performed. Within the upstream intergenic region five out of six identified EREs were excluded from further analyses due to more than one mismatch compared to the canonical 13 bp ERE palindrome (5′-GGTCAnnnTGACC-3′) or a spacer length between the ERE half-sites other than the obligatory three nucleo-

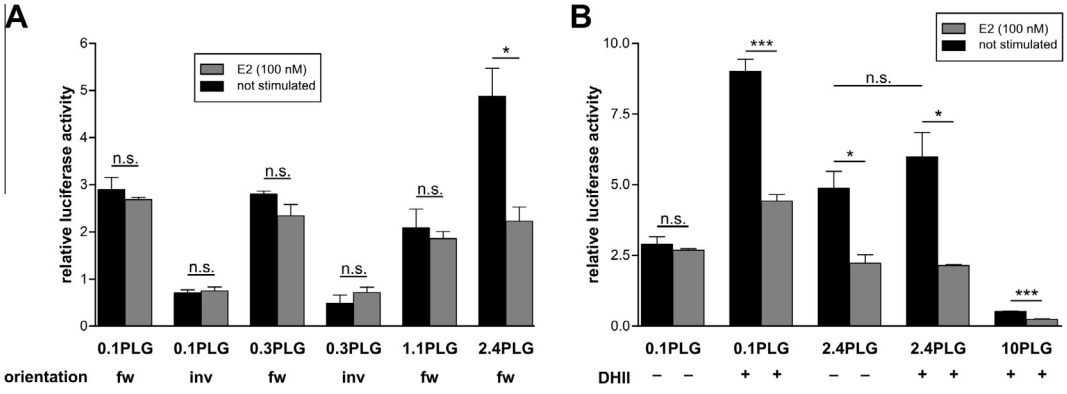


**Fig. 2.** Basal activity and E2 response of PLG promoter and enhancer constructs. HepG2 cells were transfected and stimulated as described. Plasmids as shown in Fig. 1; 'inv', inverse; 'fw', forward (native) orientation of the indicated core promoters. Transcriptional activities were normalized to pGL4.70 Renilla luciferase activity and expressed relative to pGL4.10 vector only control. All experiments were performed in triplicates and are shown as means ± SEM. *P* values $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

tides [16,17]. The remaining putative ERE [ERE(−11.5 kB)] resides approximately 11.5 kb upstream of the PLG transcription start site. It corresponds to the canonical 13 bp palindrome apart from one deviation at the third position within the left half-site of the palindrome (Fig. 3A). Interestingly, the adjacent three residues at either half-site extend the (imperfect) palindrome to 19 bp. In addition, 4 nucleotides upstream of the palindrome resides a second ERE half-site forming a perfect direct repeat with the right half-site of the palindrome separated by a 12 bp spacer (Fig. 3A). Extension of the ERE palindrome has been shown to increase ERα affinity; direct repeats of an ERE half-site may provide an additional ER binding target (reviewed in [17]). The ERE(−11.5 kb) element maps to a cluster of putative *cis*-regulatory sequences encompassing approximately 1600 bp within the APO(a)-PLG intergenic region that was previously identified by a modified PCR-electromobility shift assay (EMSA) multistep selection technique [23]. The location of the ERE within a region of increased regulatory activity provides an indication for the functional relevance of the identified sequence.

Within the region downstream the transcription start site only one putative ERE [ERE(+4.2 kb)] was identified and included in the *in vitro* analysis. ERE(+4.2 kb) locates about 4.200 bp downstream the TSS at the intron 1/exon 2 boundary and represents an imperfect palindrome because of two aberrations from the consensus sequence within the right half-site (Fig. 3A).

Both EREs in front of 0.1 kb-PLG in sense and antisense orientation were not able to modify basal luciferase expression. In contrast, under the influence of E2 ERE(−11.5 kb)–0.1 kb-PLG showed a massive 170-fold increase of luciferase activity in forward orientation (Fig. 3B). According to a comprehensive overview given by C.M. Klinge the observed estrogen caused increase in reporter activity is the most dramatic ever reported (note: assays using 10 nM E2 are mainly compared in this review) [17]. It is tempting to assume that the magnitude of the activation results from the remarkable structure of ERE(−11.5 kb); however, if the exact mechanism relies on high affinity, the synergistic action of more than one ERα molecules bound to the expanded response element, or a combination of both mechanisms remains to be clarified in detail. A weaker effect was observed using an inverse construct (43.1-fold increase of activity) (Fig. 3B).

ERE(+4.2 kb) in forward orientation in front of 0.1 kb-PLG displayed a small but significant E2 response (2.19-fold). In inverse orientation it was not able to increase luciferase activity under E2 treatment compared to the unstimulated control (Fig. 3B).

### 3.4. Interaction of estrogen responsive PLG enhancer elements

We studied the regulatory interplay of both estrogen responsive units, DHII region and ERE(−11.5 kb), by inserting ERE(−11.5 kb) in front of DHII-0.1 kb-PLG. The presence of the ERE(−11.5 kb) decreased the basal, unstimulated luciferase activity compared to DHII-0.1 kb-PLG alone by approximately two-thirds (Fig. 3C) overriding the activating effect of the DHII unit on the PLG minimal promoter. Whereas stimulation of the ERE(−11.5 kb)–0.1 kb-PLG construct with E2 lead to a massive increase in reporter activity the stimulatory effect of ERE(−11.5 kb) was completely abrogated in combination with DHII (Fig. 3C). This mutual suppressive action of the combination of the two response units (separated by about 2000 bp on gene level or 100 bp in our construct) is opposed to the transcriptional synergism that was reported for two to four ERE tandem copies (reviewed in [17]). The functional implications and underlying mechanisms of the ERU/ERα/estrogen interaction conferred by the DHII region have been intensely studied but results are partly conflicting [4,5]. These investigations indicated that the *modus operandi* is determined by additional nuclear receptors with the peroxisome proliferator-activated receptor (PPAR) α probably playing a role as described in previous studies with *APO(a)* gene enhancers [5]. Our results obtained from analyses of the DHII-ERE(−11.5 kb) and DHII−2.4 kb interrelationship corroborate the assumption of a contribution of accessory molecules such as coactivators and/or corepressors that mediate a tightly controlled E2 response.

### 3.5. Enhancer elements are PLG promoter and liver cell specific

As described above the exact mechanism mediating the dramatic estrogen response of the ERE(−11.5 kb) sequence is unknown. Assays shown in Fig. 4A indicate that interaction of the distal ERE element with factors bound to the PLG minimal promoter mediate a large fraction of the E2 dependent increase, since replacement of the *PLG* gene specific minimal promoter by the heterologous chicken δ-crystallin promoter results in a about 20-fold lower response to estrogen (170-fold versus 8.4-fold, compare Fig. 3b and Fig. 4a). As a proof of functionality the crystallin minimal promoter induced reporter expression about fourfold in HepG2 cells, an effect that was not modified by estrogen (Fig. 4A). Thus, the enormous enhancer potential of ERE(−11.5 kb) results from its exceptional structure and interaction with PLG-specific *trans*-acting factors.

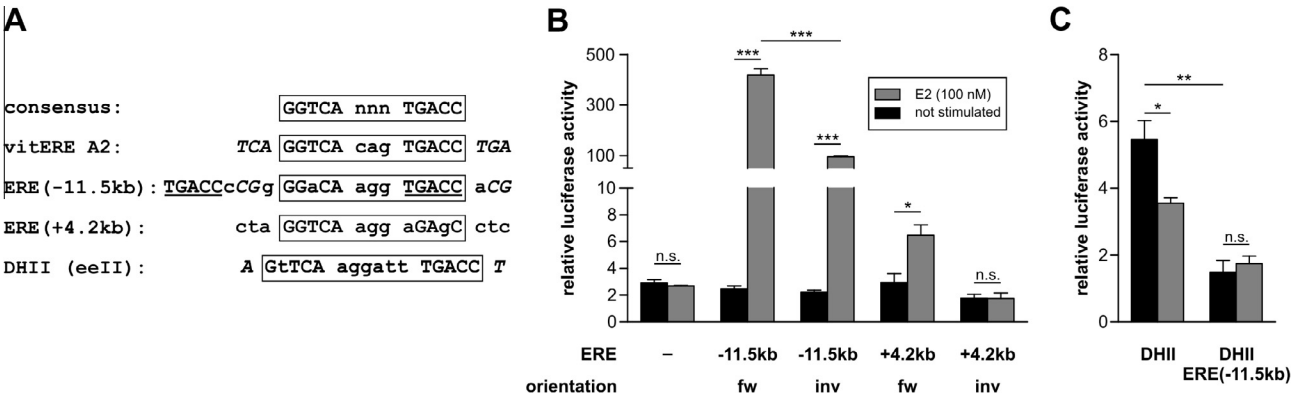


**Fig. 3.** Structure as well as basal and E2 induced activity of identified estrogen response elements. (A) Sequence of identified EREs (−11.5 kb and +4.2 kb) aligned to the minimal 13 bp ERE consensus palindrome (boxed; *n* = any nucleotide) derived from African clawed frog *Xenopus laevis* genes encoding vitellogenin isoforms exemplified by vitellogenin A2 (vitERE A2) [16]. DHII (eeII) represents the imperfect ERE palindrome suggested to be responsible for E2 dependent regulation of the APO(a) promoter [4,5]. Note the atypical 6 bp spacer between the ERE half sites. Lower-case characters within ERE half sites indicate nucleotides that deviate from the consensus sequence. Extensions of the 13 bp palindrome are shown in italic letters. Direct repeat sequences within ERE(−11.5 kb) are underlined. (B) and (C) HepG2 cells were transfected and assayed as described for Fig. 2. Estrogen dependent activation of the PLG minimal promoter (0.1 kb-PLG) driven reporter expression by the indicated ERE enhancers cloned in forward (fw) and inverse (inv) orientation (panel b) and in combination with the estrogen responsive DHII fragment (panel c).

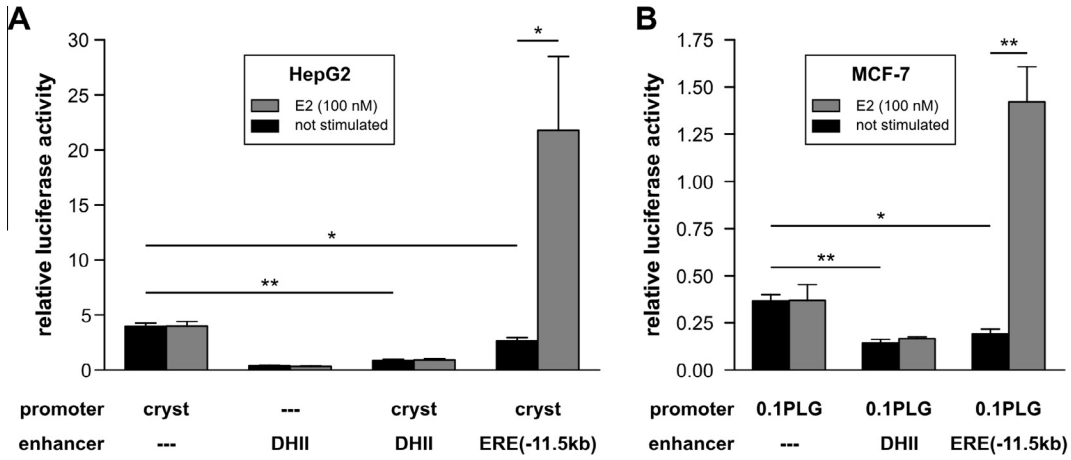


**Fig. 4.** Promoter- and cell specificity of PLG enhancer elements. HepG2 cells were transfected and assayed as described for Fig. 2. (A) Transcriptional effect of the DHII and/or ERE (−11.5 kb) enhancers on the heterologous crystallin promoter (cryst) driven luciferase activity in HepG2. (B) Estrogen dependent response of the PLG minimal promoter driven reporter expression and the influence of enhancer elements in MCF-7 cells.

Furthermore, the ligand-independent stimulatory action of the DHII sequence unit was dependent on the PLG minimal promoter context: subcloning of DHII either alone or in front of the heterologous chicken δ-crystallin promoter into pGL4.10 had a suppressing effect (Fig. 4A) indicating that DHII does not exert promoter activity on its own and requires the PLG context to act as an enhancer. DHII alone as well as the combined DHII-Crystallin construct were not responsive to E2. Lacking or diminished enhancer activity of the DHII region has also been demonstrated for the heterologous SV40 and – partially – the low density lipoprotein (LDL) receptor [6].

The dramatic ERE(−11.5 kb) mediated E2 triggered transcription activation was not only dependent on interaction with PLG specific *cis*-regulatory promoter elements but also on liver specific *trans*-acting factors as demonstrated by transfection experiments in MCF-7 cells. In this breast carcinoma cell line the PLG minimal promoter was not active; i.e., reporter expression did not even reach the level of the empty reporter plasmid (Fig. 4B) thus confirming liver-specificity shown for the PLG and the APO(a) minimal promoters [10,24]. Neither presence of ERE(−11.5 kb) nor DHII in front of the PLG minimal promoter lead to a basal or E2-stimulated luciferase expression level in MCF-7 cells comparable to those observed in liver-derived HepG2 cells (Fig. 4B).

### 3.6. Influence of E2 on PLG mRNA expression

The opposing regulation of the PLG promoter by distinct enhancer elements in the co-transfection assays led us to the question in which way endogenous *PLG* mRNA is regulated in the HepG2 cell model. Therefore HepG2 cells were transfected with hERα and after 24 h of stimulation with E2 PLG expression was quantified by real-time (RT) PCR. RT-PCR revealed that HepG2 cells produced only low amounts of *PLG* mRNA barely above the detection limit and no regulation by E2 was detected/observed (data not shown).

As HepG2 cells did not appear to be an appropriate cell model for the analysis of endogenous *PLG* mRNA regulation, human primary hepatocyte culture was established and studied. Liver cells of three patients, suffering from gastrointestinal malignancy with liver metastases, were treated as described in Materials and methods and RT-PCR was performed. We could detect an adequate PLG production, but, again, no regulation by E2 (data not shown). Liver cells are described to express hERα [25], but as the donors of the liver cells were about 60 years old and had a history of cancer, the state of receptor expression was unknown. Using PCR for hERα only weak signals of the receptor mRNA were detected (data not shown). Western blotting employing a specific hERα antibody revealed no protein expression in the two female patients tested. ERα protein expression in samples from the male patient was not analyzed due to insufficient material. Thus, a primary cell model with proven endogenous expression of both plasminogen and ERα was not available and E2 dependent regulation of PLG expression could not be assessed.

In summary we demonstrated that estrogen regulates plasminogen promoter activity. The 5'-flanking region of the *PLG* gene contains several *cis*-acting elements that are responsive to estrogen. An enhancer located at −11.5 kb confers a dramatic estrogen response in reporter assays. Estrogen action is *PLG* gene and liver tissue specific. The estrogen mediated net effect *in vitro* strongly suggests a complex regulation *in vivo*. The regulatory network centered around the ERs depends on a plethora of interacting nuclear factors: Other members of the nuclear receptor super family, auxiliary co-activators and co-repressors that might be active in liver cells but not HepG2 cells. This indirect action of the ER is supported by the observation of PLG minimal promoter dependency, hence *bona fide* enhancers convey their stimulatory signal on any accessible promoter in their proximity. Moreover, the multifaceted extra-nuclear actions of estrogen might play a role [26], possibly by modifying PLG release from E2 responding cell populations by posttranscriptional mechanisms. As an additional level of complexity, in the physiological whole-body situation estrogen effects on PLG expression might be modulated by other hormonal factors that represent themselves targets of estrogen control. Up to now no appropriate human liver cell model is available to assess the net effect of estrogen on PLG protein expression. Such model would be essential to investigate further therapeutical approaches for patients with PLG deficiency type I, a rare autosomal recessive disorder resulting in a life threatening reduction of PLG expression and activity [27].

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.035.

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