Regulation of the human thromboxane A\textsubscript{2} receptor gene by Sp1, Egr1, NF-E2, GATA-1, and Ets-1 in megakaryocytes

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Abstract The \(\alpha\) and \(\beta\) isoforms of the human thromboxane A\textsubscript{2} (TXA\textsubscript{2}) receptor (TP) are encoded by a single gene but are transcriptionally regulated by distinct promoters, termed promoter 1 (Prm1) and Prm3, respectively. Herein, it was sought to identify factors regulating Prm1 within the megakaryocytic human erythroleukemia 92.1.7 cell line. Through gene deletion and reporter assays, the core Prm1 was localized to between nucleotides \(-6,320\) and \(-5,895\), proximal to the transcription initiation site. Furthermore, two upstream repressor and two upstream activator regions were identified. Site-directed mutagenesis of GATA and Ets elements disrupted the upstream activator sequence located between \(-7,962\) and \(-7,717\), significantly impairing Prm1 activity. Electrophoretic mobility shift assays and chromatin immunoprecipitations confirmed that Sp1, Egr1, and NF-E2 bind to elements within the proximal region substantially reduced Prm1 activity. Deletion/mutation of GATA and Ets elements disrupted the upstream activator sequence located between \(-7,962\) and \(-7,717\), significantly impairing Prm1 activity. The prostanoid thromboxane A\textsubscript{2} receptor superfamily, TP\textsubscript{\alpha} and TP\textsubscript{\beta}, that are encoded by a single gene on chromosome 19p13.3 and arise by differential splicing (5). TP\textsubscript{\alpha} and TP\textsubscript{\beta} are identical in their N-terminal 328-amino-acid residues but differ exclusively in their C-tail domains (5, 6). As members of the G-protein-coupled receptor superfamily, TP\textsubscript{\alpha} and TP\textsubscript{\beta} show identical coupling to G\(_{\alpha}\)-mediated phospholipase C\(_{\beta}\) activation (7), but differentially couple to other secondary effectors, including adenylyl cyclase and tissue transglutaminase (8, 9). TP\textsubscript{\alpha} and TP\textsubscript{\beta} undergo entirely distinct mechanisms of regulation, such as through agonist-induced homologous (10, 11) and heterologous desensitization (12, 13). For example, TP\textsubscript{\beta}, but not TP\textsubscript{\alpha}, undergoes tonic and agonist-induced internalization and desensitization through \(\beta\)-arrestin-dependent mechanisms (10, 14). Conversely, TP\textsubscript{\alpha}, but not TP\textsubscript{\beta}, undergoes desensitization in response to the anti-aggregatory/vasodilatory agents prostacyclin and nitric oxide involving direct phosphorylation of TP\textsubscript{\alpha} by cAMP- and cGMP-dependent protein kinase, respectively, within its unique C-tail domain (12, 13). The implication from the latter studies is that TP\textsubscript{\alpha} is the main isoform involved

Supplementary key words promoter • transcription • prostanoid • platelet

The prostanoid thromboxane A\textsubscript{2} (TXA\textsubscript{2}) plays a central role in hemostasis, acting as a potent mediator of platelet aggregation and vasoconstriction (1). Alterations in the levels of the cyclooxygenase 1-derived TXA\textsubscript{2} or of TXA\textsubscript{2} synthase or the TXA\textsubscript{2} receptor (TP) are associated with a variety of vascular disorders, including thrombosis, unstable coronary artery disease, ischemic heart disease, and congestive heart failure (2, 3). Moreover, patients with recent episodes of myocardial infarction or pregnancy-induced hypertension have elevated TP numbers in platelets, suggesting that increased expression of the TP gene in megakaryocytic precursors may underlie the increased tendency toward aggregation or may predispose such individuals to thrombosis (2, 4). Hence, identifying the mechanisms regulating TP gene expression should lead to a greater understanding of its involvement in hemostasis and vascular disease and may provide an increased rationale for intervention approaches.

In humans, TXA\textsubscript{2} actually signals through two TP isoforms, termed TP\textsubscript{\alpha} and TP\textsubscript{\beta}, that are encoded by a single gene on chromosome 19p13.3 and arise by differential splicing (5). TP\textsubscript{\alpha} and TP\textsubscript{\beta} are identical in their N-terminal 328-amino-acid residues but differ exclusively in their C-tail domains (5, 6). As members of the G-protein-coupled receptor superfamily, TP\textsubscript{\alpha} and TP\textsubscript{\beta} show identical coupling to G\(_{\alpha}\)-mediated phospholipase C\(_{\beta}\) activation (7), but differentially couple to other secondary effectors, including adenylyl cyclase and tissue transglutaminase (8, 9). TP\textsubscript{\alpha} and TP\textsubscript{\beta} undergo entirely distinct mechanisms of regulation, such as through agonist-induced homologous (10, 11) and heterologous desensitization (12, 13). For example, TP\textsubscript{\beta}, but not TP\textsubscript{\alpha}, undergoes tonic and agonist-induced internalization and desensitization through \(\beta\)-arrestin-dependent mechanisms (10, 14). Conversely, TP\textsubscript{\alpha}, but not TP\textsubscript{\beta}, undergoes desensitization in response to the anti-aggregatory/vasodilatory agents prostacyclin and nitric oxide involving direct phosphorylation of TP\textsubscript{\alpha} by cAMP- and cGMP-dependent protein kinase, respectively, within its unique C-tail domain (12, 13). The implication from the latter studies is that TP\textsubscript{\alpha} is the main isoform involved

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in hemostasis. Consistent with that hypothesis, although TPA and TPβ also show distinct patterns of mRNA and protein expression in a range of cell and tissue types of vascular origin (15), platelets exclusively express TPA (16).

Hence, although the significance of two receptors for TXA₂ in humans but not in other species is currently unknown, there is abundant and increasing evidence that they have distinct (patho)physiologic roles displaying differences in their signaling, modes of regulation, and patterns of expression. Consistent with this, the TP isoforms are under the transcriptional control of distinct promoters within the single TP gene. Whereas the originally identified promoter (Prm1) regulates TPA expression (17), a novel promoter termed Prm3 directs TPβ expression (18). Detailed characterization of Prm3 revealed a central role for both Oct-1/2 and activator protein (AP)-1 in its basal regulation (19).

Despite the recognized importance of TPA, such as in platelets and hemostasis, the factors regulating Prm1 remain largely undefined. Whereas Prm1 is known to lack consensus TATA or CAAT elements (20), transcription initiation is thought to occur at multiple sites within exon 1 of the TP gene (20, 21), and it is suggested that the core promoter may be located within a 500 bp region proximal to the TP gene (20, 21), and it is suggested that the core promoter may be located within a 500 bp region proximal to the proposed transcription initiation site(s) (22). Considering the central role of TXA₂ in hemostasis and vascular disease, coupled with the lack of detailed knowledge of the factors regulating Prm1 and TPA expression, the central aim of the current study was to characterize Prm1, identifying the cis-acting elements and trans-acting factors that define both the proximal core promoter and upstream regulatory elements in the platelet progenitor megakaryocytic human erythroleukemia (HEL) 92.1.7 cell line. The data herein categorize Prm1, as distinct from Prm3, as a megakaryocytic promoter under the regulation of both general, including Sp1 and Egr1, and more-specific transcription factors, including NF-E2, GATA-1, and Ets-1.

EXPERIMENTAL PROCEDURES

Materials

pGL3basic and pRL-thymidine kinase (pRL-TK) were obtained from Promega Corporation, DMRIE-C®, RPMI 1640 culture media, and FBS were from Invitrogen Life Technologies. Anti-NF-E2 (sc-2913), anti-Sp1 (sc-593), anti-Egr1 (sc-1103), anti-WT-1 (sc-192), anti-cJun (sc-45), anti-GATA-1 (sc-13053), anti-Ets-1 (sc-350), and rabbit IgG (sc-2027) were obtained from Santa Cruz Biotechnology. All antibodies used for chromatin immunoprecipitation (ChIP) analysis were ChIP-validated by the supplier (Santa Cruz Biotechnology) and have been widely used in the literature for such analyses (23–30). The plasmid pCMV-Egr1 was kindly provided by Dr. Gerald Thiel, University of Saarland Medical Centre, Homburg, Germany (31). Bioinformatic analyses to identify putative transcription factor binding sites within Prm1 were carried out using the MatInspector™ program (32).

Construction of luciferase-based reporter gene plasmids

Prm1 is defined as nucleotides −8,500 to −5,895, located 5′ of the translational ATG initiation codon, designated +1. The plasmid pGL3bvPrm1, containing the Prm1 sequence in the pGL3Basic genetic reporter vector, has been previously described (18). To identify elements required for Prm1 activity, a series of 5′- and 3′-deletion subfragments were subcloned into pGL3Basic. The recombinant plasmids generated, as well as the identities, sequence, and corresponding nucleotides of the specific primers used for each fragment are listed in the expanded Materials and Methods section in the online data supplement at http://www.jlr.org/. The identity and fidelity of all recombinant plasmids were verified by DNA sequence analysis.

Site-directed mutagenesis

Site-directed mutagenesis was carried out using the Quick-Change™ method (Stratagene). The identities of the Prm1 elements subjected to site-directed mutagenesis and the corresponding plasmids generated, as well as the identity, sequence, and corresponding nucleotides of the specific primers used are listed in the expanded Materials and Methods section in the online data supplement at http://www.jlr.org/.

Cell culture

HEL 92.1.7 cells, obtained from the American Type Culture Collection, were cultured in RPMI 1640, 10% FBS, at 37°C in a humid environment with 5% CO₂.

Assay of luciferase activity

HEL cells were cotransfected with the various pGL3Basic-recombinant plasmids, encoding firefly luciferase, along with pRL-TK, encoding renilla luciferase, using DMRIE-C® transfection reagent and assayed for firefly and renilla luciferase 48 h later using the Dual-Luciferase Reporter Assay System™ as described (19). Relative firefly to renilla luciferase activities (arbitrary units) were calculated as a ratio and were expressed in relative luciferase units (RLUs).

Western blot analysis

The expression of Sp1, Egr1, NF-E2, GATA-1, and Ets-1 proteins in HEL cells was confirmed by Western blot analysis. Briefly, whole-cell protein was resolved by SDS-PAGE (10% acrylamide gels) and transferred to polyvinylidene difluoride membranes according to standard methodology. Membranes were screened using anti-NF-E2, anti-Sp1, anti-Egr1, anti-GATA-1, or anti-Ets-1 sera in 5% non-fat dried milk in 1× TBS (0.01 M Tris-HCl, 0.1 M NaCl) for 2 h at room temperature, followed by washing and screening using goat anti-rabbit HRP (sc-2204), followed by chemiluminescence detection (13).

Electrophoretic mobility shift and supershift assays

Nuclear extract was prepared from HEL cells as previously described (19). Oligonucleotides corresponding to the sense (5′-end-labeled with biotin) and antisense strands of each probe (90 μM) were annealed by heating at 95°C for 2 min, followed by slow cooling to room temperature. The identities and sequences of the biotin-labeled oligonucleotide probes and the non-labeled competitor/non-competitor oligonucleotides are listed in the expanded Materials and Methods section in the online data supplement at http://www.jlr.org/.

Initially, serial dilutions of each probe were incubated with nuclear extract (2.5 μg total protein) for 20 min at room temperature in 1× binding buffer [20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA (pH 8.0), 250 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 0.25 mg ml⁻¹ poly(dI-dC; Sigma)]. Protein-DNA complexes were subjected to electrophoresis through 6% DNA retardation gels (Invitrogen) in Tris borate EDTA buffer for 1–2 h at room temper-
perature and then transferred to Bio-Rad® B positively charged nylon membrane (Pall). Thereafter, detection was carried out using the Chemiluminescence Nucleic Acid Detection Module (33), as described by the manufacturer. Once the optimal concentration of each probe was determined, binding reactions were set up by incubating nuclear extract (2.5 μg total protein) with/without 300-fold molar excesses of nonlabeled double-stranded competitors/non-competitors in 1× binding buffer for 20 min at room temperature. The appropriate concentration of biotin-labeled probe was then added, and mixtures were incubated for 20 min at room temperature, after which electrophoresis, transfer, and detection were carried out, as before.

For supershift assays, nuclear extract (2.5 μg total protein) was preincubated with 3 μg of anti-NF-E2, anti-Sp1, anti-Egr1, anti-WT-1, or anti-c-Jun sera for 2 h at 4°C. Thereafter, the nuclear extract/antibody mixtures were incubated for 20 min at room temperature, after which electrophoresis, transfer, and detection were carried out, as before.

**RESULTS**

Functional analysis of Prm1 of the human TXA2 receptor gene

The aim of this investigation was to characterize Prm1 of the human TXA2 receptor (TP) gene within the megakaryocytic HEL 92.1.7 cell line, seeking to identify the key factors regulating TPa expression in platelets and related cell types. Prm1 is defined as nucleotides −8,500 to −5,995 upstream of the translational initiation codon (18). A series of 5′ deletions was generated, where the 5′ nucleotide of each subfragment is indicated in brackets throughout. Through genetic reporter assays, the recombinant plasmid pGL3b: Prm1 directed 7.83 ± 0.70 RLU in HEL cells (Fig. 1A), compared with 23.9 ± 1.1 RLU directed by an SV40 promoter in the pGL3control vector, which acted as a reference. Deletion of Prm1 (−8,500) to Prm1B (−7,962) yielded a 2.8-fold increase in luciferase activity (P < 0.0001). Further 5′ deletion to generate Prm1BΔ (−7,717) resulted in a 2.4-fold decrease in luciferase expression (P < 0.0001). Moreover, progressive 5′ deletion to generate Prm1C (−7,504) yielded a further 1.8-fold reduction (P = 0.0014), whereas deletion of nucleotides from Prm1D (−6,848) to generate Prm1E (−6,648) resulted in a 1.3-fold increase (P = 0.0242) in luciferase expression. Hence, 5′ deletion analysis revealed two upstream repressor sequences (URSs) between −8,500 to −7,962 and −6,848 to −6,648 and two upstream activator sequences (UASs) between −7,962 to −7,717 and −7,717 to −7,504 within Prm1. The Prm1E (−6,648) subfragment directed luciferase expression comparable to that of the full-length Prm1, indicating that Prm1E contains core elements required to direct minimal Prm1 activity. Consistent with this, 3′ deletion of nucleotides −6,437 to −5,895 from Prm1BΔ, Prm1C, and Prm1E significantly reduced luciferase expression (P < 0.0001 in each case; Fig. 1B), to levels that were not substantially greater than that of pGL3Basic, such as in the case of Prm1E 3′ deletion. These data further suggest that the proximal Prm1E (−6,648) contains the core elements required to direct minimal Prm1 activity.

Identification of functional NF-E2 and overlapping Sp1/Egr1 elements in Prm1

Successive 5′ deletions of Prm1E (−6,648) further localized the positive regulatory elements(s) between −6,648 and −5,895 (Fig. 2A). Deletion of nucleotides from −6,648 to generate Prm1HΔ (−6,320) did not affect luciferase expression, but generation of Prm1K (−6,080) led to 1.3-fold (P = 0.0003) and 1.8-fold (P < 0.0001) reductions, respectively.

Further 5′ deletions, to generate Prm1F–I (data not shown), in combination with bioinformatic analysis to identify elements within the −6,320 to −5,895 region revealed five putative overlapping sites for Sp1/Egr1 and a putative NF-E2/API site (Fig. 2). Hence, site-directed mutagenesis was used to disrupt those putative Sp1/Egr1 and NF-E2/API sites within either Prm1HΔ (−6,320) or Prm1L (−6,080) to generate Prm1HΔ (−6,320) and Prm1L (−6,010) to generate Prm1HΔ (−6,320) and Prm1L (−6,010). Mutation of the Sp1/Egr1−6,907 site within Prm1L significantly reduced but did not abolish luciferase expression (Fig. 2A, P = 0.0135). Mutation of four of the five Sp1/Egr1 sites, specifically Sp1/Egr1−6,284, Sp1/Egr1−6,278, Sp1/Egr1−6,022, and Sp1/Egr1−6,007, but not Sp1/Egr1−6,098, each reduced luciferase activity directed by Prm1HΔ (P = 0.0096, P = 0.0005, P < 0.0001, and P < 0.0001, respectively; Fig. 2B). Furthermore, disruption of the putative NF-E2/API−6,080 site also reduced luciferase activity directed by Prm1HΔ (P < 0.0001).

Thereafter, to investigate possible cooperative actions of the latter, the effect of mutating combinations of the Sp1/Egr1 and NF-E2/API elements within Prm1HΔ was exam-
As stated, disruption of Sp1/Egr1\(^{-6,294}\) and disruption of Sp1/Egr1\(^{-6,022}\) both decreased luciferase expression directed by Prm1H\(^D\), where disruption of Sp1/Egr1\(^{-6,022}\) caused a more pronounced decrease (1.8-fold; \(P, 0.0001\)) than mutation of Sp1/Egr1\(^{-6,294}\) (1.3-fold; \(P, 0.0096\)). Mutation of both elements together, generating Prm1H\(^D\)Sp1/Egr1\(^{-6,294},-6,022\)*, also decreased luciferase expression compared with that of Prm1H\(^D\) (\(P, 0.0001\)). However, the magnitude of this decrease (1.8-fold) was not greater than that of Sp1/Egr1\(^{-6,022}\) alone. Furthermore, the luciferase activity directed by Prm1H\(^D\)Sp1/Egr1\(^{-6,294},-6,022\)* was not significantly different from that of Prm1H\(^A\)Sp1/Egr1\(^{-6,022}\)* (\(P, 0.1333\)) or Prm1H\(^A\)Sp1/Egr1\(^{-6,007}\)* (\(P, 0.7571\)). Hence, collectively, these and other combinations of mutations (data not shown) indicate that the Sp1/Egr1 and/or NF-E2/AP1 elements within the \(-6,320\) to \(-5,895\) region act interdependently and functionally cooperate to regulate Prm1.

Thereafter, electrophoretic mobility shift assays (EMSAs) were carried out to investigate the presence and identity of nuclear factors capable of binding to the NF-E2/AP1\(^{-6,080}\) element in vitro (Fig. 3A). Expression of NF-E2 (Fig. 3C) and the AP1 component cJun (data not shown) in HEL 92.1.7 cells was confirmed by immunoblot analysis. Incubation of a biotin-labeled NF-E2/AP1 probe with nuclear extract from HEL cells resulted in the appearance of a main protein-DNA complex, C1, as well as one or more faster migrating complexes (Fig. 3A, lane 2). The main C1 complex was competed by specific NF-E2/AP1\(^{-6,080}\) or consensus NF-E2 sequences but not by a consensus AP1 sequence (Fig. 3A, lanes 3–5). It appears that the faster migrating complexes were competed in a similar manner to C1. Following prolonged exposure of the chromatogram shown in Fig. 3A, a further slower migrating complex, designated

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**Fig. 1.** Effect of 5′ and 3′ deletions on Prm1-directed gene expression. Schematic of the human TP gene spanning nucleotides \(-8,500\) to \(+786\), encoding Prm1 \((-8,500\) to \(-5,895\)), Prm3, exon (E)1, intron (I)1 and E2, where nucleotide +1 represents the translational start site (ATG). Plasmids (2 μg) encoding (A) pGL3control [positive control; 23.9 ± 1.1 relative luciferase units (RLUs)], Prm1, Prm1B, Prm1AΔ, Prm1C, Prm1D, and Prm1E; (B) Prm1HΔ, Prm1HΔ 3′ deletion, Prm1C, Prm1C 3′ deletion, Prm1E, Prm1E 3′ deletion, or, as a negative control, pGL3Basic (A, B) were cotransfected with pRL-TK into HEL 92.1.7 cells. Mean firefly relative to renilla luciferase activity was expressed in arbitrary relative luciferase units (RLU ± SEM; \(n = 5\)).
Fig. 2. Identification of NF-E2/AP1 and Sp1/Egr1 elements within Prm1. Putative Sp1/Egr1 and NF-E2/API elements within Prm1, where the 5′ nucleotide is indicated and the star symbol signifies mutated elements. pGL3Basic plasmids (2 μg) encoding (A) Prm1E, Prm1HΔ, Prm1K, Prm1L, Prm1LSp1/Egr1(−6007)* and, as a control, pGL3Basic; (B) Prm1HΔ, Prm1HΔSp1/Egr1(−6026, 26,022)*, Prm1HΔSp1/Egr1(26,278)*, Prm1HΔNF-E2/API(−6020)*, Prm1HΔSp1/Egr1(−6022)*, and Prm1HΔSp1/Egr1(−6007)*; or (C) Prm1HΔ, Prm1HΔSp1/Egr1(−6026, 26,022)*, Prm1HΔSp1/Egr1(−6022)*, and Prm1HΔSp1/Egr1(−6007)* were cotransfected with pRL-TK into HEL 92.1.7 cells. Luciferase activity was expressed as mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 5).
C2, and equivalent to C2 shown in Fig. 3B, was observed and, like C1, was competed by NF-E2/AP1 26,080 and consensus NF-E2 sequences but not by the consensus AP1 sequence (data not shown). Thereafter, preincubation of nuclear extract with an anti-NF-E2 antibody resulted in a supershifted complex (Fig. 3B). However, it appeared that C1 was not significantly reduced following formation of this supershift, suggesting that the supershifted NF-E2 may have originated from a complex other than C1. Although no supershift was observed with an anti-cJun antibody, it appeared that addition of this antibody reduced both C1 and C2, suggesting a possible role for c-Jun binding to the NF-E2/AP1 probe (Fig. 3B, lane 4). To investigate whether NF-E2 can directly bind to Prm1 in vivo, ChIP assays were carried out on chromatin extracted from HEL cells (Fig. 3D). PCR analysis using primers specific to the proximal Prm1 region (−6,368 to −5,895; (D)) from input chromatin or anti-NF-E2 or, as a control, normal rabbit IgG immunoprecipitates of cross-linked chromatin from HEL cells. Primers to detect an upstream region of Prm1 (−8,460 to −8,006; (D)) from input chromatin, anti-NF-E2, or normal rabbit IgG precipitates were used as a negative control. Images are representative of three independent experiments.

Fig. 3. NF-E2 binding to the proximal Prm1. Electrophoretic mobility shift assays (EMSAs) (A) or supershift assays (B) using nuclear extract from HEL cells and a biotin-labeled double-stranded NF-E2/AP1 probe (Probe) spanning −6,087 to −6,049 of the TP gene, as indicated by the horizontal bar. A: Nuclear extract was preincubated with the vehicle (−) or with excess nonlabeled competitor oligonucleotides (+) prior to addition of the NF-E2/AP1 probe. One main complex, C1, as well as one or more faster migrating complexes, was observed; prolonged exposure revealed a slower migrating C2 complex (not shown). B: Nuclear extract was preincubated with vehicle (−), anti-NF-E2 (+), or anti-cJun (+) sera before addition of the biotinylated NF-E2 probe. Two main complexes, C1 and C2, were observed. The arrow to the right indicates the supershifted transcription factor:DNA complex detected with the anti-NF-E2 serum (lane 3). C: Immunoblot analysis of NF-E2 expression in HEL cells (60 μg whole-cell protein). D, E: Chromatin immunoprecipitation (ChIP) analysis of Prm1. Schematic of Prm1 and primers (arrows) used in the PCR to detect the proximal Prm1 region (−6,368 to −5,895; (D)) from input chromatin or anti-NF-E2 or, as a control, normal rabbit IgG immunoprecipitates of cross-linked chromatin from HEL cells. Primers to detect an upstream region of Prm1 (−8,460 to −8,006; (D)) from input chromatin, anti-NF-E2, or normal rabbit IgG precipitates were used as a negative control. Images are representative of three independent experiments.
shifts demonstrate that NF-E2 specifically binds to the NF-E2/API probe in vitro, whereas ChIP assays establish that NF-E2 occupies element(s) within the −6,368 to −5,895 region of Prm1 in vivo.

EMSAs also investigated nuclear factor binding to the Sp1/Egr1 and Sp1/Egr1 elements in vitro. Immunoblot analysis confirmed abundant expression of Sp1 and Egr1 in HEL cells (Fig. 4A, B). Incubation of the Sp1/Egr1 probe with nuclear extract generated two DNA-protein complexes, C1 and C2 (Fig. 4C, lane 2). Both C1 and C2 were efficiently competed by the Sp1/Egr1 and consensus Egr1 sequences (Fig. 4C, lanes 3 and 5, respectively), and to a lesser extent by consensus Sp1 and WT-1 sequences (Fig. 4C, lanes 4 and 6, respectively). Neither C1 nor C2 were competed by a nonspecific randomized sequence based on the TP gene (Fig. 4C, lane 7). Moreover, addition of an anti-Egr1 antibody resulted in generation of a supershift complex, as well as reducing both C1 and C2 (Fig. 4D, lane 4). Although no supershift was observed with an anti-Sp1 antibody, both C1 and C2 were substantially reduced following its addition (Fig. 4D, lane 3), indicating a possible role for Sp1 binding to the probe. Addition of an anti-WT-1 antibody or an anti-cJun antibody, used as a control, had no substantial effects on binding patterns to the probe (Fig. 4D, lanes 5 and 6, respectively). Collectively, these data indicate that complexes of Sp1 and Egr1 from HEL cell nuclear extract can bind to the Sp1/Egr1 element within Prm1 in vitro.

Thereafter, EMSAs were carried out to investigate the presence of nuclear factors capable of binding to the Sp1/Egr1−6,278 element in vitro. Incubation of the Sp1/Egr1−6,278 probe with HEL cell nuclear extract generated one main complex, designated C1 (Fig. 4E, lane 2). C1 was efficiently competed by Sp1/Egr1−6,278, consensus Sp1, and consensus Egr1 sequences, and to a much lesser extent, by the WT-1 sequence (Fig. 4E, lanes 3–6, respectively). C1 was not competed by a nonspecific randomized sequence based on the TP gene (Fig. 4E, lane 7). Moreover, addition of an anti-Egr1 antibody generated a supershift complex, as well as reducing the main complex, C1 (Fig. 4F, lane 4). Although addition of an anti-Sp1 antibody did not lead to the observation of a supershift complex, it reduced C1 in a manner similar to that of the anti-Egr1 antibody, indicating a possible role for Sp1 binding. Addition of an anti-WT-1 antibody or an anti-cJun antibody, used as a control, did not have any substantial effects on binding patterns to the probe. Collectively, these data indicate that a complex of Sp1 and Egr1 from HEL cell nuclear extract can bind to the Sp1/Egr1 element within Prm1 in vitro.

EMSAs also confirmed the presence of nuclear factors capable of binding to the Sp1/Egr1−6,007 and Sp1/Egr1−6,007 elements in vitro. Incubation of the Sp1/Egr1−6,007 probe with HEL cell nuclear extract resulted in two main complexes, C1 and C2 (Fig. 5A). Both C1 and C2 were competed by both Sp1/Egr1−6,022, and Sp1/Egr1−6,007-specific sequences (Fig. 5A, lanes 3–5, respectively). The faster migrating C1 complex was efficiently competed by consensus Sp1, consensus Egr1, and WT-1 sequences (Fig. 5A, lanes 6–8, respectively). It was notable, however, that C2 was actually increased by consensus Sp1, Egr1, or WT-1 oligonucleotides (Fig. 5A, lanes 6–8, respectively), suggesting that nuclear factors other than Sp1, Egr1, or WT-1 may possibly bind to the Sp1/Egr1−6,022,−6,007 probe in vitro, and that these factors may bind more efficiently to the probe when the proteins involved in the formation of C1 are unavailable for binding. A nonspecific randomized sequence based on the TP gene (Fig. 5A, lane 9) failed to inhibit C1 or C2. These data indicate that Sp1, Egr1, and/or WT-1 proteins from HEL cell nuclei bind to the sites at −6,022 and −6,007 within Prm1. Moreover, anti-Sp1 (Fig. 5B left, lane 3) and anti-Egr1 (Fig. 5B right, lane 4) antibodies both resulted in supershift complexes, whereas no supershifts were observed with either anti-WT-1 or, as a control, anti-cJun antibodies, even following prolonged exposure of the chromatogram (Fig. 5B, lanes 5 and 6, respectively). Due to the weak nature of the supershifted complexes observed following pre-incubation with either anti-Sp1 or anti-Egr1 antibodies, it was not clear whether the Sp1 or Egr1 in the supershifted complexes originated from C1.

Thereafter, to further investigate the possible binding of Sp1 and Egr1 to the proximal Prm1, ChIP analysis was carried out. Primers based on the proximal Prm1 region generated amplicons from both anti-Sp1 and anti-Egr1, but not from the control IgG immunoprecipitates (Fig. 5C). Conversely, PCR analysis using primers specific to an upstream region of Prm1 from −8,460 to −8,006 resulted in generation of an amplicon from input chromatin, but not from anti-Sp1, anti-Egr1, or IgG precipitates (Fig. 5D). Additionally, overexpression of Egr1 led to a modest but significant decrease in the luciferase expression directed by Prm1Δ in HEL cells (Fig. 5E). Hence, collectively, four overlapping Sp1/Egr1 sites and an NF-E2 site have been identified in the proximal Prm1 region. Both Sp1 and Egr1, in addition to NF-E2, bind to those elements in vitro and vivo to regulate core Prm1, whereas overexpression of Egr1 appears to negatively regulate that transcriptional activity.

Identification of functional GATA and Ets sites within Prm1

As stated, 5′ deletions of Prm1 revealed a UAS between −7,962 (Prm1B) and −7,717 (Prm1Δ), deletion of which yielded a 2.8-fold reduction in luciferase expression (Fig. 1A). To localize the regulatory element(s) within this region, an additional 5′ subfragment, Prm1ΔGata/Ets, (−7,859) was generated. Removal of nucleotides between −7,962 (Prm1B) and −7,859 (Prm1ΔGata/Ets) led to a 2.3-fold reduction in luciferase activity (Fig. 6A; P < 0.0001), whereas there was no difference in expression between Prm1ΔGata/Ets and Prm1Δ (P = 0.261). Among the transcription factor elements identified between −7,962 and −7,859 were putative GATA and Ets elements at −7,890 and −7,870, respectively. Mutation of GATA−7,890 and Ets−7,870 elements both reduced luciferase expression directed by Prm1B (Fig. 6B), where the decrease due to the Ets−7,870 mutation (2.2-fold; P < 0.0001) was more pronounced than that caused by the GATA−7,890 mutation (1.8-fold; P < 0.0001). Luciferase expression directed by...
Fig. 4. Nuclear factor binding to overlapping Sp1/Egr1 sites within Prm1. Immunoblot analysis of Sp1 (A) and Egr1 (B) expression in HEL cells (80 μg whole-cell protein/lane). EMSAs (C) or supershift assays (D) using nuclear extract from HEL cells and a biotinylated double-stranded Sp1/Egr1\(^{–6,278} -^{–6,294}\) probe (Probe) spanning \(-6,299\) to \(-6,276\) of the TP gene, as indicated by the horizontal bar. C: Nuclear extract was preincubated with the vehicle (−) or excess nonlabeled competitor oligonucleotides (+) before addition of the Sp1/Egr1\(^{–6,294}\) probe. Two main complexes, C1 and C2, were observed. D: Nuclear extract was preincubated with vehicle (−) or with (+) anti-Sp1, anti-Egr1, anti-WT-1, or anti-cJun sera before addition of the Sp1/Egr1\(^{–6,294}\) probe. EMSAs (E) or supershift assays (F) using nuclear extract from HEL cells and a biotinylated double-stranded Sp1/Egr1\(^{–6,283} -^{–6,258}\) probe (Probe) spanning \(-6,283\) to \(-6,258\) of the TP gene, as indicated by the horizontal bar. E: Nuclear extract was preincubated with the vehicle (−) or excess nonlabeled competitor oligonucleotides (+) before addition of the Sp1/Egr1\(^{–6,258}\) probe. One main complex, C1, was observed. F: Nuclear extract was preincubated with vehicle (−) or with (+) anti-Sp1, anti-Egr1, anti-WT-1, or anti-cJun sera before addition of the Sp1/Egr1\(^{–6,294}\) probe. Images are representative of three independent experiments.
Prm1BGATA(−7,890)*,Ets(−7,870)*, where both GATA−7,890 and Ets−7,870 were mutated, was also significantly lower than that of the wild-type, Prm1B. However, the magnitude of this decrease (2.2-fold) was not greater than that caused by the Ets−7,870 mutation alone. Moreover, luciferase activity directed by Prm1BGATA(−7,880)*,Ets(−7,870)* was not significantly different from that directed by Prm1BEts(−7,870)* (P = 0.9293; Fig. 6B). Collectively, these single and combi-
Fig. 6. Identification of functional GATA and Ets elements within Prm1. The positions of putative GATA and Ets elements within Prm1, where the 5′ nucleotide of each element is shown and the star symbol signifies mutated elements. Recombinant pGL3Basic plasmids (2 μg) encoding (A) pGL3control (positive control), Prm1B, Prm1BGata/Ets, and Prm1BΔ, or (B) Prm1B, Prm1BGATA(−7,890)*, Prm1BEts(−7,870)*, Prm1BGATA−7,890), Ets1(−7,870)*, Prm1B Gata/Ets, Prm1B Gata/Ets Ets(−7,805)*, and Prm1BΔ were cotransfected with pRL-TK into HEL cells. A 245 bp subfragment of Prm1, spanning nucleotides −7,962 to −7,718, encoding either the wild-type or mutated (⁎) GATA(−7,890) and Ets(−7,870) elements was subcloned into pGL3control vector upstream of the SV40 promoter. Resulting recombinant plasmids (0.5 μg), as well as pGL3control (0.5 μg), were cotransfected with pRL-TK into HEL cells. Luciferase activity was expressed as mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 4). Asterisks (*) indicate that the wild-type Prm1 sequence from −7,962 to −7,718 increased luciferase expression directed by pGL3control, while site-directed mutagenesis of the GATA(−7,890) and Ets(−7,870) elements led to decreased luciferase expression compared to that directed by the wild-type sequence.
nation mutations suggest that the GATA-7,890 and Ets-7,870 elements do not act independently, but rather, cooperatively, in an interdependent manner.

Although, it was already established (Fig. 6A) that there was no difference in luciferase expression directed by Prm1ΔGata/Ets (-7,859) and Prm1ΔEts (-7,717), a second putative Ets site, namely Ets-7,805, was identified adjacent to the aforementioned GATA-7,890 and Ets-7,870 sites. However, site-directed mutagenesis of the latter Ets-7,805 element did not significantly affect the level of luciferase activity (Fig. 6B; P = 0.4287). Hence, collectively these data suggest that GATA-7,890 and Ets-7,870 elements act as upstream activators of Prm1 and may functionally cooperate to positively regulate basal Prm1 in HEL cells, whereas the putative Ets-7,805 element does not appear to be functional.

To investigate the ability of the UAS encoding GATA-7,890 and Ets-7,870 elements to regulate general gene expression in HEL cells, a Prm1 subfragment spanning −7,962 to −7,718 was placed upstream of the heterologous SV40 promoter in the plasmid pGL3Control. The Prm1GATA/Ets subfragment resulted in a 4.1-fold increase in luciferase expression relative to that of the SV40 promoter alone (Fig. 6C; P = 0.0003). Moreover, the level of luciferase expression directed by the Prm1GATA/Ets variant, in which both the GATA-7,890 and Ets-7,870 elements were mutated, was significantly impaired (P = 0.0012), resulting in only a 1.8-fold increase in SV40-directed luciferase activity (Fig. 6C; P = 0.0003). These data indicate that the Prm1 region from −7,962 to −7,718 acts as a UAS, greatly increasing the activity of the SV40 promoter in HEL cells, an effect mainly attributable to the GATA-7,890 and Ets-7,870 cis-acting elements.

Thereafter, EMSAs explored the presence of nuclear factors capable of binding to the GATA-7,890 and Ets-7,870 elements in vitro. Immunoblot analysis confirmed expression of both GATA-1 and Ets-1 in HEL cells (Fig. 7B, C). Incubation of a GATA/Ets probe with nuclear extract prepared from HEL cells generated four DNA-protein complexes, C1–C4 (Fig. 7A, lane 2). C2 was competed by either the Prm1 GATA-7,890 or Ets-7,870 sequences, as well as by a consensus Ets-1, but was not competed by a consensus GATA-1 sequence (Fig. 7A, lane 3–6). These data indicate that C2 consists of Ets-1 and another factor bound to the GATA/Ets probe. C3 was competed by GATA-7,890 and consensus GATA-1, but not by Ets-7,870 or consensus Ets-1 sequences (Fig. 7A, lanes 3–6, respectively), suggesting that C3 consists of GATA-1 protein, possibly complexed...
with another factor, bound to the GATA/Ets probe. Complexes C1 and C4 were competed by either GATA\(^{–7,890}\) or consensus GATA-1 sequences, as well as by Ets\(^{–7,870}\) and consensus Ets-1 sequences (Fig. 7A, lanes 3–6, respectively). The nonspecific competitor, based on a randomized TP gene, failed to inhibit any of the C1–C4 complexes (Fig. 7A, lane 7), confirming the specificity of the GATA/Ets probe. Therefore, complexes of GATA-1 and Ets-1 proteins from HEL cell nuclear extract can bind to Prm1 GATA\(^{–7,890}\) and Ets\(^{–7,870}\) elements in vitro. Moreover, ChIP assays confirmed the specific amplification of the Prm1 proximal region from anti-GATA-1 and anti-Ets-1 immunoprecipitates, but not from the control IgG precipitate (Fig. 7D), confirming that both GATA-1 and Ets-1 occupy element(s) within the −7,978 to −7,607 region of Prm1 in vivo. Conversely, primers specific to the proximal region of Prm1 from −6,368 to −5,895 resulted in generation of an amplicon from the input chromatin, but not from the GATA-1, Ets-1, or control IgG precipitates (Fig. 7E).

**DISCUSSION**

In humans, TXA\(_2\) signals through the TP\(\alpha\) and TP\(\beta\) isoforms of its cognate G-protein-coupled receptor. Imbalances in the levels of TXA\(_2\) and TP are implicated in a range of cardiovascular disorders (1–4), but the relative extent to which TP\(\alpha\) and TP\(\beta\) contribute to such pathologies is unknown. Because TP\(\alpha\) and TP\(\beta\) are under the transcriptional control of distinct promoters (18), identification of the factors regulating Prm1 and Prm3 may lead to a greater understanding of their contributory roles in health and disease. Through studies aimed at characterizing Prm3, API and Oct-2 were identified as the key factors regulating its basal activity in HEL cells (19). Moreover, the endogenous cyclopentone 15-deoxy-\(\Delta\)12,14-prostaglandin J\(_2\) (15d-PGJ\(_2\)), a peroxisome proliferator-activated receptor (PPAR)\(\gamma\) ligand, suppressed the transcriptional activity of Prm3 but had no effect on Prm1 (21). Additionally, the synthetic thiazolidinedione (TZD) PPAR\(\gamma\) ligands rosiglitazone and troglitazone, used in the treatment of type II diabetes mellitus, selectively suppressed Prm3 activity, without affecting Prm1 (35). An implication of those studies is that the TZD PPAR\(\gamma\) ligands may have combined therapeutic benefits in the treatment of type II diabetes and the associated cardiovascular disease, partly due to their suppression of TP\(\beta\) expression (35).

Prm1 represents the main promoter within the human TP gene (17, 18), but despite this, to date the factors regulating its expression, such as within the vasculature or indeed other tissue/cell types, remain largely undefined. Herein, we sought to identify the key factors regulating basal Prm1 activity in the HEL 92.1.7 megakaryocytic cell line. Prm1 belongs to the class of promoters that lack TATA or CAAT elements. Many TATA-less promoters contain multiple GC-rich elements in their proximal promoter, from which transcription can be activated by the ubiquitously expressed zinc finger transcription factor Sp1 by its recruitment of multi-subunit complex(es) involving TF\(_{ID}\) (36). Adjacent Sp1 sites may activate transcription independently from one another, or synergistically through formation of homomultimeric complexes (37). Early growth response protein (Egr1), another zinc finger transcription factor, also has a GC-rich binding site, and because of the similarity in their consensus elements, adjacent or overlapping sites for Sp1 and Egr1 are frequently found in promoter sequences (38). By mutational analysis and EMSAs, four functional overlapping Sp1/Egr1 elements were identified within the proximal region of Prm1. EMSA and supershift analyses indicated a role for Sp1 and Egr1 binding to each of these elements in vitro, whereas ChIP analysis confirmed the in vivo binding of both endogenous Sp1 and endogenous Egr1 to the proximal Prm1 region of chromatin extracted from HEL cells. Several studies have shown that where overlapping Sp1/Egr1 sites occur in proximal promoter regions, Egr1 negatively regulates Sp1-mediated basal transcription by competitively binding to the overlapping element (38). The four functional Sp1/Egr1 elements identified herein within the proximal Prm1 were adjacent overlapping sites that, through mutational studies, were shown to cooperatively regulate Prm1. Consistent with this, herein, EMSAs confirm that Sp1 and Egr1 generally compete for the same sites within Prm1. Moreover, overexpression of recombinant Egr1 in HEL cells led to a modest but significant reduction in the level of luciferase expression directed by the proximal Prm1. It is likely that overexpression of Egr1 may have led to a more pronounced reduction in Prm1-directed gene expression if the total amount of transfected DNA herein was not limited by the luciferase-based reporter assay itself. Therefore, the combined assessment of these studies suggests that it is likely that Sp1 activates transcription from the TATA-less Prm1 of the TP gene, and that Egr1 negatively regulates this transcription by competing with Sp1 for binding at each of the four overlapping Sp1/Egr1 sites.

In addition to the Sp1/Egr1 sites, we also identified a functional NF-E2/API element by mutational analysis of the proximal Prm1. NF-E2 is a heterodimeric transcription factor that binds to the consensus sequence (T/C)GCC\(_{G/C}\)TGA\(_{G/C}\)/TCA\(_{T/C}\) (G/C), with a core API motif (in italics). Although data from EMSA, supershift, and ChIP assays confirmed that endogenous NF-E2 specifically bound to the NF-E2/API element within Prm1 both in vitro and in chromatin in vivo in HEL cells, further studies are necessary to comprehensively investigate the possible binding of API components such as Jun B, Jun D, c-Fos, FosB, Fra1, and Fra2 to the proximal Prm1. The heterodimeric NF-E2 is composed of a tissue-restricted p45 subunit associated with a ubiquitously expressed p18 member of the Maf family (39). Expression of p45 is restricted mainly to hematopoietic progenitors, as well as differentiated erythroid and megakaryocytic cells, mast cells, and granulocytes (40). Originally, it was thought that the primary role of NF-E2 was in erythroid development, owing to its regulation of the porphobilinogen deaminase (40) and globin (41) genes. However, p45-deficient mice exhibited only mild disruption to erythropoiesis but displayed severe thrombocytopenia (<5% of normal platelet count) and high mortality.
due to hemorrhage (42). Notable among its megakaryo-
cytic targets, NF-E2 regulates expression of the human
(43) and rodent TX syntheses (44, 45), platelet-specific
Rab27b, β1-tubulin, and caspase-12 (42, 43). Thus, it is
suggested that NF-E2 acts as a critical mediator of platelet
shedding, regulating a subset of genes involved in late-
stage megakaryocyte maturation. Consistent with this,
herein, we report that Prm1 of the TP gene is also a bone
fide target of NF-E2, suggesting that it plays a critical role
in regulating expression of TPs during megakaryocytic
differentiation and in platelets in humans. Collectively,
although our data have established a role for Sp1, Egr1, and
NF-E2 in regulating the core proximal Prm1, they do
not exclude the possible involvement of other regulatory ele-
ments/factors in this region.

Most eukaryotic promoters contain UASs andURSs. Herein, 57 deletion analyses revealed two UAS and two
URS regions within Prm1. Mutational analysis of the first
UAS region (−7,962 to −7,717) identified functional GATA
and Ets elements capable of regulating Prm1 and the het-
erologous SV40 promoter in HEL cells. EMSAs confirmed
the presence of nuclear factors in HEL cells capable of
binding to the GATA and Ets elements in vitro. Owing to
the complex binding patterns of the probe encoding the
GATA and Ets elements, supershift assays failed to provide
a clear interpretation of the identities of specific trans-
scription factors that bind to these sites in vitro. However,
supershift assays were superseded by in vivo ChIP analysis,
which confirmed binding of endogenous GATA-1 and endo-
rogenous Ets-1 to the Prm1 region of the chromatin in HEL
 cells. The GATA family of transcription factors are so called
because they bind to a consensus A/TGATAA/G DNA se-
quency. GATA-1 interacts with the coactivator Friend of
GATA (FOG)-1 to regulate several genes involved in mega-
karyocyte differentiation (46). GATA-1 is expressed in he-
matoepoietic progenitor cells, erythrocytes, megakaryocytes,
esinophils, and mast cells and is essential for normal erythro-
poiesis and megakaryopoiesis (47–49). Loss of
megakaryocytic GATA-1 expression in mice resulted in
aberrant proliferation and maturation of megakaryocyte
cells (50). The Ets family of transcription factors consists
of approximately 30 proteins that play a role in a variety of
cellular processes such as differentiation, apoptosis, and
development. Family members Ets-1, Fli-1, and PU-1
play an important role in megakaryocytic and erythroid
differentiation (51). While Ets-1 is downregulated and
exported from the nucleus during erythroid maturation, it
promotes differentiation and maturation of megakaryo-
cytes (52). ChIP assays have demonstrated that Ets-1 binds
to proximal regions in the GPIIb, GPIX, and thrombo-
poietin receptor (MPL) promoters (53). Moreover, Ets-1
and GATA-1 activate promoters for rat platelet factor 4
(54) and human thrombopoietin receptor, or MPL (55).
It is indeed notable that the promoters of these genes
are characterized by closely spaced GATA-1 and Ets
binding elements, similar to those identified herein in Prm1.
Functional cooperativity among GATA-1, FOG-1, and spe-
cific Ets family members is required for efficient ex-
pression of the megakaryocytic-specific αIIb gene (56).

Herein, we report that Prm1 of the human TP gene con-
tains an upstream activator sequence that contains func-
tional elements for GATA and Ets factors separated by
5 bp and that GATA-1 and Ets-1 functionally cooperate
by binding to these elements, thereby increasing the ex-
pression of TPs in HEL cells. In addition, the ability of a
250 bp subfragment encoding the aforementioned GATA-1
and Ets-1 sites to act as an independent UAS in HEL cells
was confirmed whereby it resulted in a 4-fold increase in
reporter gene expression directed by the heterologous
SV40 promoter.

In conclusion, several critical regulatory regions have
been identified within Prm1 of the TP gene, including two
UASs and two URSs and a proximal core Prm1 region.
Whereas the trans-acting factors regulating one of the
UASs (−7,717 to −7,504) and the two URS (−8,500 to
−7,962 and −6,848 to −6,648) are the subject of ongoing
investigations, we have identified four functional over-
lapping Sp1/Egr1 elements and a single NF-E2 element in
the proximal Prm1 region, as well as functional GATA and
Ets elements within the UAS, located between −7,962
and −7,859, that regulate basal Prm1 activity. It seems likely
that cooperative binding of Sp1 to multiple sites in the proxi-
mal Prm1 is an important step required for initiation of
transcriptional activity. A similar model has been proposed
for regulation of the β-globin gene in erythroleukemia
cells, whereby Sp1 binding to multiple sites is required to
open the nucleosomal structure, facilitating transcription
(57). Herein, it appears that overexpression of Egr1 inhib-
its the Sp1-mediated activation of Prm1, suggesting that it
is the relative balance between Sp1 and Egr1 binding that
determines its basal transcription. Additionally, the activity
of Prm1 in the HEL megakaryocyte cell line is increased
owing to a functional NF-E2 element in the proximal pro-
mar, as well as functional GATA and Ets elements in a
UAS. It has been suggested that the hematopoietic-specific
factors NF-E2 and GATA-1 stabilize the open nucleosomal
structure of the β-globin gene following Sp1 binding (57).
Additionally, interactions between Ets factors and Sp1 sta-
bilize Sp1 binding to the αIIb promoter (58). Although
further studies are required to elucidate the molecular
dynamics of binding, it is likely that the combination of
NF-E2, GATA-1, and Ets-1 proteins plays a critical role in
regulating Prm1 activity and expression of TPs during
different stages of megakaryocytic differentiation and in
platelets. Future studies involving overexpression or knock-
down of specific factors may, in principle, provide further
insight into the regulation of Prm1 and TP expression.
However, owing to the number of factors involved and
the various combinations thereof required to complete such
an assessment, those experiments are outside the scope of
the current study.

The functional characterization of Prm1 herein greatly
increases knowledge of the factors regulating expression of
the human TP gene. These data not only provide a molecu-
lar and genetic basis for understanding the role of TXA2
and its receptor TP in hemostasis and vascular disease but
also provide a rationale for understanding how altered
numbers of TPs, such as through dysregulation of signal-
ing by the trans-acting factors involved or indeed through genetic polymorphisms in Prm1 itself, contribute to such diseases. Moreover, these data also significantly increase appreciation that expression of the individual TPa and TPβ isoforms, as products of Prm1 and Prm3, respectively, are subject to entirely distinct regulatory mechanisms.

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Supplemental Material can be found at: http://www.jlr.org/content/suppl/2008/08/13/M800256-JLR20.DC1.html