Regulation of the Human Thromboxane A₂ Receptor Gene by Sp1, Egr1, NF-E2, GATA-1 and Ets-1 in Megakaryocytes.

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Abstract

The α and β isoforms of the human thromboxane A₂ receptor (TP) are encoded by a single gene but are transcriptionally regulated by distinct promoters, termed Prm1 and Prm3, respectively. Herein, it was sought to identify factors regulating Prm1 within the megakaryocytic human erythroleukemia (HEL) 92.1.7 cell line. Through gene deletion and reporter assays, the core Prm1 was localized to between -6320 and -5895, proximal to the transcription initiation site. Furthermore, two upstream repressor and two upstream activator regions were identified. Site-directed mutagenesis of four overlapping Sp1/Egr1 elements and an NF-E2/AP1 element within the proximal region substantially reduced Prm1 activity. Deletion/mutation of GATA and Ets elements disrupted the upstream activator sequence located between -7962 and -7717, significantly impairing Prm1 activity. Electrophoretic mobility shift assays and chromatin immunoprecipitations confirmed that Sp1, Egr1 and NF-E2 bind to elements within the core promoter while GATA-1 and Ets-1 factors bind to the upstream activator sequence (between -7962 and -7717). Collectively these data establish that Sp1, Egr1 and NF-E2 regulate core Prm1 activity in the megakaryocytic-platelet progenitor cells while GATA-1 and Ets-1 act as critical upstream activators, hence providing the first genetic basis for the expression of the human TXA₂ receptor (TP) within the vasculature.
INTRODUCTION

The prostanoid thromboxane (TX) A₂ plays a central role in haemostasis, acting as a potent mediator of platelet aggregation and vasoconstriction (1). Alterations in the levels of the cyclooxygenase (COX) 1-derived TXA₂ or of TXA₂ synthase or the TXA₂ receptor (TP) are associated with a variety of vascular disorders including thrombosis, unstable coronary artery disease, ischaemic 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 haemostasis and vascular disease and may provide an increased rationale for intervention approaches.

In humans, TXA₂ actually signals through two TP isoforms, termed TPα and TPβ, that are encoded by a single gene on chromosome 19p13.3 and which arise by differential splicing (5). TPα and TPβ are identical for 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 (GPCR) superfamily, TPα and TPβ show identical coupling to Go₆-q-mediated phospholipase Cβ activation (7), but differentially couple to other secondary effectors including adenylyl cyclase and tissue transglutaminase (8,9). TPα and TPβ undergo entirely distinct mechanisms of regulation such as through agonist-induced homologous (10,11) and heterologous desensitization (12,13). For example, TPβ, but not TPα, undergoes tonic and agonist-induced internalization and desensitization through β-arrestin dependent mechanisms (10,14). Conversely, TPα, but not TPβ, undergoes desensitization in response to the anti-aggregatory/vasodilatory agents prostacyclin and nitric oxide involving direct phosphorylation of TPα by cAMP and cGMP-dependent protein kinase, respectively, within its unique C-tail domain (12,13). The implication from the latter studies is that TPα is the main isoform involved in haemostasis. Consistent with that hypothesis, while TPα and TPβ also show distinct patterns of mRNA and protein expression in a range of cell and tissues types of vascular origin (15), platelets exclusively express TPα (16).

Hence, whilst 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 signalling, 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. Whilst the originally identified promoter (Prm) 1 regulates TPα 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 TPα, such as in platelets and haemostasis, the factors regulating Prm1 remain largely undefined. While Prm1 is known to lack consensus TATA or CAAT elements (20), transcription initiation is thought to occur at multiple sites within exon (E)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 proposed transcription initiation site(s) (22). Considering the central role of TXA₂ in haemostasis and vascular disease, coupled with the lack of detailed knowledge of the factors regulating Prm1 and TPα 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 HEL 92.1.7 cell line. The data herein categorize Prm1, as distinct from Prm3, as a megakaryocytic promoter being 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 fetal bovine serum (FBS) were from Invitrogen Life Technologies. Anti-NF-E2 (sc-291X), anti-Sp1 (sc-59X), anti-Egr1 (sc-110X), anti-WT-1 (sc-192X), anti-cJun (sc-45X), anti-GATA-1 (sc-13053X), anti-Ets-1 (sc-350X) and rabbit IgG (sc-2027) were obtained from Santa Cruz Biotechnology. All antibodies used for ChIP analysis were ChIP-validated by the supplier (Santa Cruz) 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™ programme (32).

Construction of Luciferase-based Genetic Reporter Plasmids

Promoter (Prm)1 is defined as nucleotides -8500 to -5895, located 5’ of the translational ATG initiation codon, designated +1. The plasmid pGL3b:Prm1, 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 was verified by DNA sequence analysis.

Site-directed Mutagenesis

Site-directed mutagenesis was carried out using the Quik-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
Human erythroleukemic (HEL) 92.1.7 cells, obtained from the American Type Culture Collection, were cultured in RPMI 1640, 10 % fetal bovine serum (FBS) at 37 °C in a humid environment with 5 % CO₂.

**Assay of Luciferase Activity**

HEL cells were co-transfected 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 hr 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 (RLU).

**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 (PVDF) membrane 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 1x 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 horseradish peroxidase (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-labelled 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-labelled oligonucleotide probes and the non-labelled competitor/non-competitor oligonucleotides are listed in the expanded Materials and Methods section in the online data supplement at [http://www.jlr.org/](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 1x 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 (dT-dC; Sigma)]. Protein-DNA complexes were subjected to electrophoresis through 6 % DNA
retardation gels (Invitrogen) in Tris borate, EDTA (TBE) buffer for 1 – 2 h at room temperature and then transferred to Biodyne® 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 non-labelled double-stranded competitors/non-competitors in 1x Binding Buffer for 20 min at room temperature. The appropriate concentration of biotin-labelled 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 pre-incubated with 3 µg of anti-NF-E2, anti-Sp1, anti-Egr1, anti-WT-1 or anti-cJun sera for 2 h at 4 °C. Thereafter, the nuclear extract–antibody mixtures were incubated for 20 min at room temperature with the appropriate biotin-labelled double-stranded probe, as described in the expanded Materials and Methods section in the online data supplement at http://www.jlr.org/.

Chromatin Immunoprecipitation (ChIP) Assays

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described (34). Specifically, HEL cells (1 x 10⁸) were pelleted, washed in ice-cold PBS and resuspended in serum-free RPMI 1640. Formaldehyde-cross linked chromatin was sonicated, as described (34), to generate fragments 500 bp to 1000 bp in length. Prior to immunoprecipitation (IP), chromatin was incubated with 60 µg normal rabbit IgG overnight at 4 °C on a rotisserie, after which 250 µl of salmon sperm DNA/protein A agarose beads (Millipore) were added and chromatin was precleared for 3 h at 4 °C with rotation. Thereafter, anti-NF-E2, anti-Sp1, anti-Egr1, anti-GATA-1, anti-Ets-1 (10 µg aliquots) or normal rabbit IgG (10 µg) were used for immunoprecipitation. Following elution, cross-links were reversed by incubation at 65 °C overnight followed by protease digestion with proteinase K (Sigma; 9 µl of 10 mg/ml) at 45 °C for 7 h. After precipitation, samples were resuspended in 50 µl dH₂O. PCR analysis was carried out using 2-3 µl of ChIP sample as template or, as a positive control, with an equivalent volume of a 1:20 dilution of the input chromatin DNA. The identities of the primers used for the ChIP PCR reactions, as well as their sequences and corresponding nucleotides within Prm1 are listed in the expanded Materials and Methods section in the online data supplement at http://www.jlr.org/.
Statistical analysis

Statistical analysis of differences were routinely analysed using the two-tailed Student’s unpaired $t$-test. All values are expressed as mean ± standard error of the mean (SEM). $P$-values < 0.05 were considered to indicate statistically significant differences and *, **, *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.
RESULTS

Functional Analysis of Promoter 1 of the human TXA₂ Receptor Gene

The aim of this investigation was to characterize promoter (Prm)1 of the human thromboxane (TX) A₂ receptor (TP) gene within the megakaryocytic human erythroleukemia (HEL) 92.1.7 cell line, seeking to identify the key factors regulating TPα expression in platelets and related cell types. Prm1 is defined as nucleotides -8500 to -5895 upstream of the translational initiation codon (18). A series of 5’ deletions was generated, where the 5’ nucleotide of each sub-fragment is indicated in brackets throughout. Through genetic reporter assays, the recombinant plasmid pGL3b:Prm1 directed 7.83 ± 0.70 RLU in HEL cells (Figure 1A), compared to 23.9 ± 1.1 RLU directed by an SV40 promoter in the pGL3control vector, which acted as a reference. Deletion of Prm1 (-8500) to Prm1B (-7962) yielded a 2.8-fold increase in luciferase activity (p < 0.0001). Further 5’ deletion to generate Prm1BΔ (-7717) resulted in a 2.4-fold decrease in luciferase expression (p < 0.0001). Moreover, progressive 5’ deletion to generate Prm1C (-7504) yielded a further 1.8-fold reduction (p = 0.0014), whilst deletion of nucleotides from Prm1D (-6848) to generate Prm1E (-6648) resulted in a 1.3-fold increase (p = 0.0242) in luciferase expression. Hence, 5’ deletion analysis revealed two upstream repressor sequences (URS; between -8500 to -7962; -6848 to -6648) and two upstream activator sequences (UAS; between -7962 to -7717; -7717 to -7504) within Prm1. The Prm1E (-6648) sub-fragment 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 -6437 to -5895 from Prm1BΔ, Prm1C and Prm1E significantly reduced luciferase expression (p < 0.0001 in each case; Figure 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 (-6648) 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 (-6648) further localized the positive regulatory element(s) between -6648 and -5895 (Figure 2A). Deletion of nucleotides from -6648 to generate Prm1HΔ (-6320) did not affect luciferase expression, but generation of Prm1K (-6067)
and Prm1L (-6010) led to 1.3-fold \((p = 0.0003)\) and 1.8-fold \((p < 0.0001)\) reductions, respectively.

Further 5’ deletions, to generate Prm1F-1J (data not shown), in combination with bioinformatic analysis to identify elements within the -6320 to -5895 region revealed five putative overlapping sites for Sp1/Egr1 and a putative NF-E2/AP1 site (Figure 2). Hence, site-directed mutagenesis was used to disrupt those putative Sp1/Egr1 and NF-E2/AP1 sites within either Prm1H\(\Delta\) (-6320) or Prm1L (-6010). Mutation of the Sp1/Egr1\(-6007\) site within Prm1L significantly reduced, but did not abolish, luciferase expression (Figure 2A, \(p = 0.0135\)). Mutation of four of the five Sp1/Egr1 sites, specifically Sp1/Egr1\(-6294\), Sp1/Egr1\(-6278\), Sp1/Egr1\(-6022\) and Sp1/Egr1\(-6007\), but not Sp1/Egr1\(-6098\), each reduced luciferase activity directed by Prm1H\(\Delta\) \((p = 0.0096, p = 0.0005, p < 0.0001, p < 0.0001\), respectively; Figure 2B). Furthermore, disruption of the putative NF-E2/AP1\(-6080\) site also reduced luciferase activity directed by Prm1H\(\Delta\) \((p < 0.0001)\).

Thereafter, to investigate possible cooperative actions of the latter, the effect of mutating combinations of the Sp1/Egr1 and NF-E2/AP1 elements within Prm1H\(\Delta\) was examined (Figure 2C & data not shown). As stated, disruption of Sp1/Egr1\(-6294\) and Sp1/Egr1\(-6022\) both decreased luciferase expression directed by Prm1H\(\Delta\), where disruption of Sp1/Egr1\(-6022\) caused a more pronounced decrease (1.8-fold; \(p < 0.0001\)) than mutation of Sp1/Egr1\(-6294\) (1.3-fold; \(p = 0.0096\)). Mutation of both elements together, generating Prm1H\(\Delta\)\(^{Sp1/Egr1\(-6294,-6022)\}}\), also decreased luciferase expression compared to that of Prm1H\(\Delta\) \((p < 0.0001)\). However, the magnitude of this decrease (1.8-fold) was not greater than of Sp1/Egr1\(-6007\) alone. Furthermore, the activity directed by Prm1H\(\Delta\)\(^{Sp1/Egr1\(-6294,-6022)\}}\) was not significantly different from that of Prm1H\(\Delta\)\(^{Sp1/Egr1\(-6022)\}}\) \((p = 0.5033)\). Similarly, mutation of Sp1/Egr1\(-6022\) and Sp1/Egr1\(-6007\) both led to decreased luciferase expression directed by Prm1H\(\Delta\) (1.8-fold; \(p < 0.0001\) and 1.5-fold; \(p < 0.0001\), respectively). Disruption of both elements, generating Prm1H\(\Delta\)\(^{Sp1/Egr1\(-6022,-6007)\}}\), reduced luciferase expression relative to that of Prm1H\(\Delta\) \((p = 0.0001)\). The extent of this decrease (1.5-fold) was of the same order as that caused by Sp1/Egr1\(-6007\). Moreover, the luciferase activity directed by Prm1H\(\Delta\)\(^{Sp1/Egr1\(-6022,-6007)\}}\) was not significantly different from that of Prm1H\(\Delta\)\(^{Sp1/Egr1\(-6022)\}}\) \((p = 0.1333)\) or Prm1H\(\Delta\)\(^{Sp1/Egr1\(-6007)\}}\) \((p = 0.7571)\). Hence, collectively, these and other combinations of mutations (data not shown) indicate that the Sp1/Egr1 and/or NF-E2/AP1 elements within the -6320 to -5895 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\(^{−6080}\) element \textit{in vitro} (Figure 3A). Expression of NF-E2 (Figure 3C) and the AP1 component cJun (\textit{data not shown}) in HEL 92.1.7 cells was confirmed by immunoblot analysis. Incubation of a biotin-labelled 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 (Figure 3A, lane 2). The main C1 complex was competed by specific NF-E2/AP1\(^{−6080}\) or consensus NF-E2 sequences but not by a consensus AP1 sequence (Figure 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 in Figure 3A, a further slower-migrating complex, designated C2, and equivalent to C2 in Figure 3B, was observed and, like C1, was competed by NF-E2/AP1\(^{−6080}\) and consensus NF-E2 sequences but not by the consensus AP1 sequence (\textit{data not shown}).

Thereafter, pre-incubation of nuclear extract with an \textit{anti}-NF-E2 antibody resulted in a supershifted complex (Figure 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. While no supershift was observed with an \textit{anti}-cJun antibody, it appeared that addition of this antibody reduced both C1 and C2, suggesting a possible role for cJun binding to the NF-E2/AP1 probe (Figure 3B, lane 4). To investigate whether NF-E2 can directly bind to Prm1 \textit{in vivo}, chromatin immunoprecipitation (ChIP) assays were carried out on chromatin extracted from HEL cells (Figure 3D). PCR analysis using primers specific to the 3’ Prm1 region (-6368 to -5895) generated amplicons from both the input chromatin and from an \textit{anti}-NF-E2, but not from a control IgG, immunoprecipitate (Figure 3D). PCR analysis using primers specific to the -8460 to -8006 region of Prm1, which does not contain any predicted NF-E2 elements, resulted in generation of an amplicon from input chromatin, but not from \textit{anti}-NF-E2 nor IgG precipitates (Figure 3E). Taken together, EMSA and supershifts demonstrate that NF-E2 specifically binds to the NF-E2/AP1\(^{−6080}\) probe \textit{in vitro}, while ChIP assays establish that NF-E2 occupies element(s) within the –6368 to –5895 region of Prm1 \textit{in vivo}.

EMSAs also investigated nuclear factor binding to the Sp1/Egr1\(^{−6294}\) and Sp1/Egr1\(^{−6278}\) elements \textit{in vitro}. Immunoblot analysis confirmed abundant expression of Sp1 and Egr1 in HEL cells (Figure 4A & 4B). Incubation of the Sp1/Egr1\(^{−6294}\) probe with nuclear extract generated
two DNA-protein complexes, C1 and C2 (Figure 4C, lane 2). Both C1 and C2 were efficiently competed by the Sp1/Egr1-6294 and consensus Egr1 sequences (Figure 4C, lanes 3 & 5, respectively), and to a lesser extent by consensus Sp1 and WT-1 sequences (Figure 4C, lanes 4 & 6, respectively). Neither C1 nor C2 were competed by a non-specific randomized sequence based on the TP gene (Figure 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 (Figure 4D, lane 4). While no supershift was observed with an anti-Sp1 antibody, both C1 and C2 were substantially reduced following its addition (Figure 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 (Figure 4D, lanes 5 & 6, respectively). Collectively, these data indicate that complexes of Sp1 and Egr1 from HEL cell nuclear extract can bind to the Sp1/Egr1-6294 element within Prm1 in vitro.

Thereafter, EMSAs were carried out to investigate the presence of nuclear factors capable of binding to the Sp1/Egr1-6278 element in vitro. Incubation of the Sp1/Egr1-6278 probe with HEL cell nuclear extract generated one main complex, designated C1 (Figure 4E, lane 2). C1 was efficiently competed by Sp1/Egr1-6278, consensus Sp1 and consensus Egr1 sequences, and to a much lesser extent by the WT-1 sequence (Figure 4E, lanes 3 – 6, respectively). C1 was not competed by a non-specific randomized sequence based on the TP gene (Figure 4E, lane 7). Moreover, addition of an anti-Egr1 antibody generated a supershift complex, as well as reducing the main complex C1. While addition of an anti-Sp1 antibody did not lead to observation of a supershift complex, it reduced C1 in a similar manner to 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/Egr1-6278 element within Prm1 in vitro.

EMSAs also confirmed the presence of nuclear factors capable of binding to the Sp1/Egr1-6022 and Sp1/Egr1-6007 elements in vitro. Incubation of the Sp1/Egr1-6022-6007 probe with HEL cell nuclear extract resulted in two main complexes, C1 and C2 (Figure 5A). Both C1 and C2 were competed by both Sp1/Egr1-6022 and Sp1/Egr1-6007 specific sequences (Figure 5A, lanes 3 – 5, respectively). The faster migrating C1 complex was efficiently competed by consensus Sp1, consensus Egr1 and WT-1 sequences (Figure 5A, lanes 6 – 8, respectively). It was notable,
however, that C2 was actually increased by consensus Sp1, Egr1 or WT-1 oligonucleotides (Figure 5A, lanes 6 – 8, respectively), suggesting that nuclear factor(s) other than Sp1, Egr1 or WT-1 may possibly bind to the Sp1/Egr1\textsuperscript{-6022,-6007} probe \textit{in vitro}, and that these factor(s) may bind more efficiently to the probe when the protein(s) involved in the formation of C1 are unavailable for binding. A non-specific randomized sequence based on the TP gene (Figure 5A, lane 9) failed to inhibit C1 or C2. These data indicate that Sp1, Egr1 and/or WT-1 proteins from HEL cells bind to the sites at -6022 and -6007 within Prm1. Moreover, \textit{anti-}Sp1 (Figure 5B left, lane 3) and \textit{anti-}Egr1 (Figure 5B right, lane 4) antibodies both resulted in supershift complexes while no supershifts were observed with either \textit{anti-WT-1} or, as a control, \textit{anti-cJun} antibodies, even following prolonged exposure of the chromatogram (Figure 5B, lanes 5 and 6, respectively). Due to the weak nature of the supershifted complexes observed following pre-incubation with either \textit{anti-}Sp1 or \textit{anti-}Egr1 antibodies, it was not clear whether the Sp1 or Egr1 in the supershifted complexes actually originated from C1.

Thereafter, in order 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 \textit{anti-}Sp1 and \textit{anti-}Egr1, but not from the control IgG, immunoprecipitates (Figure 5C). Conversely, PCR analysis using primers specific to an upstream region of Prm1 from -8460 to -8006 resulted in generation of an amplicon from input chromatin, but not from \textit{anti-}Sp1, \textit{anti-}Egr1 nor IgG precipitates (Figure 5D). Additionally, over-expression of Egr1 led to a modest, but significant, decrease in the luciferase expression directed by Prm1\textsubscript{H\textDelta} in HEL cells (Figure 5E). Hence, collectively, four overlapping Sp1/Egr1 sites and a NF-E2 site have been identified in the proximal Prm1 region. Both Sp1 and Egr1, in addition to NF-E2, bind to those elements \textit{in vitro} and \textit{in vivo} to regulate core Prm1 while over-expression 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 an UAS between -7962 (Prm1B) and -7717 (Prm1B\textDelta), deletion of which yielded a 2.8-fold reduction in luciferase expression (Figure 1A). To localise the regulatory element(s) within this region, an additional 5’ sub-fragment Prm1B\textDeltaGata/Ets (-7859) was generated. Removal of nucleotides between -7962 (Prm1B) and -7859 (Prm1B\textDeltaGata/Ets) led to a 2.3-fold reduction in luciferase activity (Figure 6A; \(p < 0.0001\))
while there was no difference in expression between Prm1BΔGata/Ets and Prm1BΔ (p = 0.261). Amongst the transcription factor elements identified between -7962 and -7859 were putative GATA and Ets elements at -7890 and -7870, respectively. Mutation of GATA\(^{-7890}\) and Ets\(^{-7870}\) elements both reduced luciferase expression directed by Prm1B (Figure 6B), where the decrease due to the Ets\(^{-7870}\) mutation (2.2-fold; \(p < 0.0001\)) was more pronounced than that caused by the GATA\(^{-7890}\) mutation (1.8-fold; \(p < 0.0001\)). Luciferase expression directed by Prm1B\(^{GATA(-7890)*,Ets(-7870)*}\), where both GATA\(^{-7890}\) and Ets\(^{-7870}\) 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\(^{-7870}\) mutation alone. Moreover, luciferase activity directed by Prm1B\(^{GATA(-7890)*,Ets(-7870)*}\) was not significantly different to that directed by Prm1B\(^{Ets(-7870)*}\) (\(p = 0.9293\); Figure 6B). Collectively, these single and combination mutations suggest that the GATA\(^{-7890}\) and Ets\(^{-7870}\) elements do not act independently but rather, cooperatively in an interdependent manner.

Although, it was already established (Figure 6B) that there was no difference in luciferase expression directed by Prm1BΔGata/Ets (-7859) and Prm1BΔ (-7717), a second putative Ets site, namely Ets\(^{-7805}\), was identified adjacent to the aforementioned GATA\(^{-7890}\) and Ets\(^{-7870}\) sites. However, site-directed mutagenesis of the latter Ets\(^{-7805}\) element did not significantly affect the level of luciferase activity (Figure 6B; \(p = 0.4287\)). Hence, collectively these data suggest that GATA\(^{-7890}\) and Ets\(^{-7870}\) elements act as upstream activators of Prm1 and may functionally cooperate to positively regulate basal Prm1 in HEL cells, while the putative Ets\(^{-7805}\) element does not appear to be functional.

To investigate the ability of the UAS encoding GATA\(^{-7890}\) and Ets\(^{-7870}\) elements to regulate general gene expression in HEL cells, a Prm1 sub-fragment spanning -7962 to -7718 was placed upstream of the heterologous SV40 promoter in the plasmid pGL3Control. The Prm1GATA/Ets sub-fragment resulted in a 4.1-fold increase in luciferase expression relative to that of the SV40 promoter alone (Figure 6C; \(p = 0.0003\)). Moreover, the level of luciferase expression directed by the Prm1GATA/Ets variant, in which both the GATA\(^{-7890}\) and Ets\(^{-7870}\) elements were mutated, was significantly impaired (\(p = 0.0012\)), resulting in only a 1.8-fold increase in SV40-directed luciferase activity (Figure 6C, \(p = 0.0003\)). These data indicate that the Prm1 region from -7962 to -7718 acts as an UAS, greatly increasing the activity of the SV40 promoter in HEL cells, an effect mainly attributable to the GATA\(^{-7890}\) and Ets\(^{-7870}\) cis-acting elements.
Thereafter, EMSAs explored the presence of nuclear factors capable of binding to the GATA\textsuperscript{-7890} and Ets\textsuperscript{-7870} elements \textit{in vitro}. Immunoblot analysis confirmed expression of both GATA-1 and Ets-1 in HEL cells (Figure 7B & 7C). Incubation of a GATA/Ets probe with nuclear extract prepared from HEL cells generated four DNA-protein complexes, C1-C4 (Figure 7A, lane 2). C2 was competed by either the Prm1 GATA\textsuperscript{-7890} or Ets\textsuperscript{-7870} sequences, as well as by a consensus Ets-1, but was not competed by a consensus GATA-1 sequence (Figure 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\textsuperscript{-7890} and consensus GATA-1, but not by Ets\textsuperscript{-7870} or consensus Ets-1 sequences (Figure 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\textsuperscript{-7890} or consensus GATA-1 sequences, as well as by Ets\textsuperscript{-7870} and consensus Ets-1 sequences (Figure 7A, lanes 3-6, respectively). The non-specific competitor, based on a randomized TP gene failed to inhibit any of the C1-C4 complexes (Figure 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\textsuperscript{-7890} and Ets\textsuperscript{-7870} elements \textit{in vitro}. Moreover, ChIP assays confirmed the specific amplification of the Prm1 proximal region from \textit{anti}-GATA-1 and \textit{anti}-Ets-1 immunoprecipitates, but not from the control IgG precipitate (Figure 7D), confirming that both GATA-1 and Ets-1 occupy element(s) within the –7978 to –7607 region of Prm1 \textit{in vivo}. Conversely, primers specific to the proximal region of Prm1 from -6368 to -5895 resulted in generation of an amplicon from the input chromatin, but not from the GATA-1, Ets-1 or control IgG precipitates (Figure 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. As 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, AP1 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 from those studies is that the TZD PPAR$_{\gamma}$ ligands may have combined therapeutic benefits in the treatment of type II diabetes and of 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 TFII D (36). Adjacent Sp1 sites may activate transcription independently from one another, or synergistically through formation of homomultimeric complexes (37). Early growth response protein (Egr)1, 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, while 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, over-expression 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 over-expression 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 suggest that it is likely that Sp1 activates transcription from the TATA-less Prm1 of the TP gene, and 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/AP1 element by mutational analysis of the proximal Prm1. NF-E2 is a heterodimeric transcription factor that binds to the consensus sequence (T/C)GCTGA(G/C)TCA(T/C), with a core AP1 motif (in italics). While data from EMSA, supershift and ChIP assays confirmed that endogenous NF-E2 specifically bound to the NF-E2/AP1 element within Prm1 both in vitro and to chromatin in vivo in HEL cells, further studies are necessary to comprehensively investigate the possible binding of AP1 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 haematopoietic 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 due 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 haemorrhage (42). Notable amongst its megakaryocytic targets, NF-E2 regulates expression of the human (43) and rodent thromboxane synthases (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 TPα during megakaryocytic differentiation and in platelets in humans. Collectively, whilst 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 elements/factors in this region.

Most eukaryotic promoters contain UAS and URS. Herein, 5’ deletion analyses revealed two UAS and two URS regions within Prm1. Mutational analysis of the first UAS region (-7962 to -7717) identified functional GATA and Ets elements capable of regulating Prm1 and the heterologous 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. Due 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 transcription 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 endogenous 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 sequence. GATA-1 interacts with the co-activator Friend of GATA (FOG)-1 to regulate several genes involved in megakaryocyte differentiation (46). GATA-1 is expressed in haematopoietic progenitor cells, erythrocytes, megakaryocytes, eosinophils and mast cells and is essential for normal erythropoiesis 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). Whilst Ets-1 is downregulated and exported from the nucleus during erythroid maturation, it promotes differentiation and maturation of megakaryocytes (52). ChIP assays have demonstrated that Ets-1 binds to proximal regions in the GPIIb, GPIX, and thrombopoietin receptor (MPL) promoters (53). Moreover, Ets-1 and GATA-1 activate promoters for rat platelet factor (PF)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 specific Ets family members is required for efficient expression of the megakaryocytic-specific αIIb gene (56). Herein, we report that Prm1 of the human TP gene contains an upstream activator sequence that contains functional 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 expression of TPα 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 UAS and two URS and a proximal “core” Prm1 region. While the trans-acting factors regulating one of the UAS (-7717 to -7504) and the two URS (-8500 to -7962; -6848 to -6648) are the subject of on-going investigations, we have identified four functional overlapping 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 -7962 and -7859, that regulate basal Prm1 activity. It seems likely that cooperative binding of Sp1 to multiple sites in the proximal 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 over-expression of Egr1 inhibits 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 due to a functional NF-E2 element in the proximal promoter, as well as functional GATA and Ets elements in an UAS. It has been suggested that the haematopoietic-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 stabilize Sp1 binding to the alphaIIb promoter (58). Whilst 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 play a critical role in regulating Prm1 activity and expression of TPα during different stages of megakaryocytic differentiation and in platelets. Future studies involving over-expression or knock-down of specific factors may, in principle, provide further insight into the
regulation of Prm1 and TPα 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 molecular and genetic basis for understanding the role of TXA₂ and its receptor TP in haemostasis and vascular disease but also provide a rationale for understanding how altered numbers of TPs, such as through dysregulation of signaling 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 TPα and TPβ isoforms, as products of Prm1 and Prm3, respectively, are subject to entirely distinct regulatory mechanisms.

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References


Figure 1: Effect of 5' and 3' Deletions on Prm1-directed Gene Expression. Schematic of the human TP gene spanning nucleotides -8500 to +786, encoding Prm1 (-8500 to -5895), Prm3, exon (E)1, intron (I)1 and E2, where nucleotide +1 represents the translational start site (ATG). Plasmids (2 µg) encoding: Panel A: pGL3control (positive control; 23.9 ± 1.1 RLU), Prm1, Prm1B, Prm1BΔ, Prm1C, Prm1D, Prm1E; Panel B: Prm1BΔ, Prm1BΔ 3’deletion, Prm1C, Prm1C 3’deletion, Prm1E, Prm1E 3’deletion or, as a negative control, pGL3Basic (A & B) were co-transfected 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).

Figure 2: Identification of NF-E2/AP1 and Sp1/Egr1 Elements within Prm1. Putative Sp1/Egr1 and NF-E2/AP1 elements within Prm1, where the 5’ nucleotide is indicated and the star symbol signifies mutated elements. pGL3Basic plasmids (2 µg) encoding: Panel A: Prm1E, Prm1HA, Prm1K, Prm1L, Prm1L_Sp1/Egr1(-6007)* and, as a control, pGL3Basic or Panel B: Prm1HAΔ, Prm1HA_Sp1/Egr1(-6294)*, Prm1HAΔ_Sp1/Egr1(-6278)*, Prm1HA_Sp1/Egr1(-6098)*, Prm1HA_{NF-E2/AP1(-6080)*}, Prm1HA_{Sp1/Egr1(-6022)*}, Prm1HA_{Sp1/Egr1(-6007)*} or Panel C: Prm1HAΔ, Prm1HA_{Sp1/Egr1(-6294,-6022)*}, Prm1HA_{Sp1/Egr1(-6222,-6007)*} were co-transfected 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).

Figure 3: NF-E2 Binding to the Proximal Prm1. EMSAs (Panel A) or supershift assays (Panel B) using nuclear extract from HEL cells and a biotin-labelled double-stranded NF-E2/AP1 probe (Probe) spanning -6087 to -6049 of the TP gene, as indicated by the horizontal bar. Panel A: Nuclear extract was pre-incubated with the vehicle (-) or with excess non-labelled competitor oligonucleotides (+) prior to addition of the NF-E2/AP1 probe. One main complex C1, as well as one or more faster-migrating complexes, were observed; prolonged exposure revealed a slower-migrating C2 complex (not shown). Panel B: Nuclear extract was pre-incubated 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). Panel C: Immunoblot analysis of NF-E2 expression in HEL cells (60 µg whole cell protein). Panels D & E: ChIP analysis of Prm1.
Schematic of Prm1 and primers (arrows) used in the PCR to detect the proximal Prm1 region (-6368 to -5895; Panel D) from input chromatin or anti-NF-E2 or, as a control, normal rabbit IgG immunoprecipitates of crosslinked chromatin from HEL cells. Primers to detect an upstream region of Prm1 (-8460 to -8006; Panel 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.

**Figure 4: Nuclear Factor Binding to Overlapping Sp1/Egr1 Sites within Prm1.** Immunoblot analysis of Sp1 (Panel A) and Egr1 (Panel B) expression in HEL cells (80 µg whole cell protein/lane). EMSAs (Panel C) or supershift assays (Panel D) using nuclear extract from HEL cells and a biotinylated double-stranded Sp1/Egr1-6294 probe (Probe) spanning -6299 to -6276 of the TP gene, as indicated by the horizontal bar. Panel A: Nuclear extract was pre-incubated with the vehicle (-) or excess non-labelled competitor oligonucleotides (+) before addition of the Sp1/Egr1-6294 probe. Two main complexes, C1 and C2, were observed. Panel B: Nuclear extract was pre-incubated with vehicle (-) or with (+) anti-Sp1, anti-Egr1, anti-WT-1 or anti-cJun sera before addition of the Sp1/Egr1-6294 probe. EMSAs (Panel E) or supershift assays (Panel F) using nuclear extract from HEL cells and a biotinylated double-stranded Sp1/Egr1-6278 probe (Probe) spanning -6283 to -6255 of the TP gene, as indicated by the horizontal bar. Panel E: Nuclear extract was pre-incubated with the vehicle (-) or excess non-labelled competitor oligonucleotides (+) before addition of the Sp1/Egr1-6278 probe. One main complex, C1, was observed. Panel F: Nuclear extract was pre-incubated with vehicle (-) or with (+) anti-Sp1, anti-Egr1, anti-WT-1 or anti-cJun sera before addition of the Sp1/Egr1-6294 probe. Images are representative of three independent experiments.

**Figure 5: EMSA and ChIP analysis of Sp1 and Egr1 Binding to Prm1.**
EMSAs (Panel A) or supershift assays (Panel B) using nuclear extract from HEL cells and a biotinylated double-stranded Sp1/Egr1-6022-6007 probe (Probe) spanning -6027 to -5985 of the TP gene, indicated by the horizontal bar. Panel A: Nuclear extract was pre-incubated with vehicle (-) or excess non-labelled competitor oligonucleotides (+) before addition of the Sp1/Egr1-6022-6007 probe. Two main complexes, C1 and C2, were observed. Panel B: Nuclear extract was pre-incubated with vehicle (-) or with (+) anti-Sp1, anti-Egr1, anti-WT-1 or anti-cJun sera before
addition of the Sp1/Egr1-6022-6007 probe. The image on the right represents a longer exposure of the upper section of the same chromatogram on the left. The arrows to the right indicate the supershifted transcription factor:DNA complex detected with the anti-Sp1 (lane 3, left panel) and anti-Egr1 (lane 4, right panel) sera, respectively. **Panels C & D:** ChIP analysis of Prm1. Schematic of Prm1 and primers (arrows) used in the PCR to detect the proximal Prm1 region (-6368 to -5895; **Panel C**) from input chromatin or immunoprecipitated crosslinked chromatin from HEL cells using anti-Sp1, anti-Egr1 or normal rabbit IgG sera. Primers to detect an upstream region of Prm1 (-8460 to -8006; **Panel D**) from input chromatin, anti-Sp1, anti-Egr1 or normal rabbit IgG precipitates were used as a negative control. Images are representative of three independent experiments. **Panel E:** Effect of over-expression of Egr1 on Prm1-directed gene expression. HEL cells were transiently co-transfected with pGL3b:Prm1HΔ plus pRL-TK in the presence of pCMV-Egr1 (+ Egr1) or with pCMV5 (Control) and expressed as mean relative firefly to renilla luciferase activity (RLU ± SEM; n = 19).

**Figure 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: **Panel A:** pGL3control (positive control), Prm1B, Prm1BΔGata/Ets, Prm1BΔ or **Panel B:** Prm1B, Prm1B\textsuperscript{GATA(-7890)*}, Prm1B\textsuperscript{Ets(-7870)*}, Prm1B\textsuperscript{GATA(-7890)*,Ets1(-7870)*}, Prm1BΔGata/Ets, Prm1BΔGata/Ets\textsuperscript{Ets(-7805)*} and Prm1BΔ were co-transfected with pRL-TK into HEL cells. **Panel C:** A 245 bp subfragment of Prm1, spanning nucleotides -7962 to -7718, encoding either the wild type or mutated (*) GATA\textsuperscript{-7890} and Ets\textsuperscript{-7870} 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 co-transfected with pRL-TK into HEL cells. Luciferase activity was expressed as mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 4).

**Figure 7: GATA-1 and Ets-1 Binding to Prm1.**
**Panel A:** EMSAs using nuclear extract from HEL cells and a biotinylated double-stranded GATA,Ets probe (Probe) spanning -7890 to -7848 of the TP gene, indicated by the horizontal bar. Nuclear extract was pre-incubated with vehicle (-) or excess non-labelled competitor
oligonucleotides (+) before addition of the GATA,Ets probe. Four complexes, C1 –C4, were observed. The image is representative of three independent experiments. **Panels B & C:** Immunoblot analysis of GATA-1 (50 kDa) and Ets-1 (54 kDa) expression in HEL cells (100 µg per lane). **Panels D & E:** ChIP analysis of Prm1. Schematic of Prm1 and primers (arrows) used in the PCR to detect the Prm1 region from –7978 to –7607 (Panel D) from input chromatin, anti-GATA-1, anti-Ets-1, or as a control, normal rabbit IgG immunoprecipitates, as indicated. Primers to detect the proximal Prm1 (-6368 to -5895; Panel D) from input chromatin, anti-GATA-1, anti-Ets-1 or normal rabbit IgG precipitates were used as a negative control. Images are representative of three independent experiments.
Figure 1:

(A)

(B)

Luciferase Activity (RLU)

Prm1  E1  I1  Prm3  E2

pGL3Control  SV40
Prm1  -8500  -5895
Prm1B  -5962  -5895
Prm1A  -7717  -5895
Prm1C  -7504  -5895
Prm1D  -6848  -5895
Prm1E  -6648-5895
pGL3Basic  -7717  -5895

Luciferase Activity (RLU)

Prm1  E1  I1  Prm3  E2

Prm1A  -7717  -5895
Prm1A  -7717  -6437
Prm1C  -7504  -5895
Prm1C  -7504  -6437
Prm1E  -6648  -5895
Prm1E  -6648-6437
pGL3Basic

Luciferase Activity (RLU)
Figure 2A:

(A)

- Luciferase Activity (RLU)

0 2 4 6 8 10

pGL3Basic
Prm1E
Prm1HA
Prm1K
Prm1L
Prm1L_{Sp1/Egr1(-6007)*

(luc)

(luc)

(luc)

(luc)

(luc)

0 2 4 6 8 10

Luciferase Activity (RLU)

pGL3Basic
Prm1E
Prm1HA
Prm1K
Prm1L
Prm1L_{Sp1/Egr1(-6007)*

(luc)

(luc)

(luc)

(luc)

(luc)

(A)
Figure 2B & 2C:

(B) 

(C)
Figure 4:

(A) 97 kDa — 95 kDa
66 kDa

(B) 97 kDa — 82 kDa
66 kDa

(C) Sp1/Egr1
Sp1/Egr1
Sp1/Egr1
NF-E2/
NF-E2/
NF-E2/
AP1
AP1
AP1
(-6278)
(-6294)
(-6294)
(-6022)
(-6022)
(-6022)

Free Probe

1 2 3 4 5 6 7

Nuclear Extract
Probe
Sp1/Egr1
Sp1 consensus
Egr1 consensus
WT-1 consensus
Non Specific

- + + + + + +
- + + - - -
- + + - - -
- + + - - -
- + + - - -
- + + - - -
- + + - - -

(D) Sp1/Egr1
Sp1/Egr1
Sp1/Egr1
NF-E2/
NF-E2/
NF-E2/
AP1
AP1
AP1
(-6278)
(-6294)
(-6294)
(-6022)
(-6022)
(-6022)

Free Probe

1 2 3 4 5 6

Nuclear Extract
Probe
Sp1/Egr1
Sp1 consensus
Egr1 consensus
WT-1 consensus
Non Specific

- + + + + + +
- + + - - -
- + + - - -
- + + - - -
- + + - - -
- + + - - -
- + + - - -

(E) Sp1/Egr1
Sp1/Egr1
Sp1/Egr1
NF-E2/
NF-E2/
NF-E2/
AP1
AP1
AP1
(-6278)
(-6294)
(-6294)
(-6022)
(-6022)
(-6022)

Free Probe

1 2 3 4 5 6 7

Nuclear Extract
Probe
Sp1/Egr1
Sp1 consensus
Egr1 consensus
WT-1 consensus
Non Specific

- + + + + + +
- + + - - -
- + + - - -
- + + - - -
- + + - - -
- + + - - -
- + + - - -

(F) Sp1/Egr1
Sp1/Egr1
Sp1/Egr1
NF-E2/
NF-E2/
NF-E2/
AP1
AP1
AP1
(-6278)
(-6294)
(-6294)
(-6022)
(-6022)
(-6022)

Free Probe

1 2 3 4 5 6

Nuclear Extract
Probe
Sp1/Egr1
Sp1 consensus
Egr1 consensus
WT-1 consensus
Non Specific

- + + + + + +
- + + - - -
- + + - - -
- + + - - -
- + + - - -
- + + - - -
- + + - - -

antibodies

Anti-Sp1
Anti-Egr1
Anti-WT-1
Anti-cJun

Anti-Sp1
Anti-Egr1
Anti-WT-1
Anti-cJun
Figure 5:

(A) Sp1/Egr1 (-6294)  Sp1/Egr1 (-6278)  NF-E2/Sp1 (-6007)
C1
Free Probe
Nuclear Extract
Probe
Sp1/Egr1 (-6007)
Sp1/Egr1 (-6022)
Sp1 consensus
Egr1 consensus
WT-1 consensus
Non-Specific

(B) Sp1/Egr1 (-6294)  Sp1/Egr1 (-6007)  Sp1/Egr1 (-6022)
C2
Nuclear Extract
Probe
Sp1/Egr1 (-6007)
Sp1/Egr1 (-6022)

(C) Input
Sp1/Egr1 (-6278)  Sp1/Egr1 (-6007)
NF-E2/Sp1 (-6007)
Sp1/Egr1 (-6022)
Control
IgG

(D) 1 2 3 4

(E) Luciferase Activity (RLU)
Control + Egr1

*
Figure 6A & 6B:

(A)  
\[\text{Luciferase Activity (RLU)}\]

(B)  
\[\text{Luciferase Activity (RLU)}\]
Figure 6C:

(C)
Figure 7:

(A) GATA (-7890) Ets (-7870)

Nuclear Extract: + + + + + +
Probe: + + + + + +
Prm1<sub>GATA</sub>: - - + - - -
GATA-1 consensus: - - + - - -
Prm1<sub>Ets</sub>: - - - + - -
Ets-1 consensus: - - - - + -
Non-Specific: - - - - - +

(B) 45 kDa

(C) 50 kDa

(D) 45 kDa

(E) 54 kDa

Input GATA-1

45 kDa Ets-1

IgG Control

54 kDa IgG Control

-8500 Ets (-7870) GATA (-7890)

-5895 Input GATA-1

IgG Control

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