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Additions and Corrections

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Ets-dependent regulation of target gene expression during megakaryopoiesis.

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One of the cell lines studied was CMK11-5, a derivative of CMK. We used the GATA-1 (N6) antibody in some of the ChIP studies (Fig. 3). Other investigators have brought to our attention the fact that the GATA-1 gene in CMK cells is mutated, resulting in a shorter protein lacking the N6 epitope. We therefore sequenced the GATA-1 cDNA from CMK11-5 cells and found the same mutation as in CMK cells. Next we compared the Western blots probed with either the GATA-1 (N6) or the GATA-1 (C20) antibody. For reasons unknown to us, the N6 lot used in the studies recognized the same GATA-1 protein we now detect using the C20 antibody. We have repeated the ChIP experiments with the GATA-1 (C20) antibody and confirmed the published data.

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Ets-dependent Regulation of Target Gene Expression during Megakaryopoiesis*

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Pascale Jackers‡, Gabor Szalai‡, Omar Moussa, and Dennis K. Watson§

From the Department of Pathology and Laboratory Medicine and Department of Biochemistry and Molecular Biology, Hollings Cancer Center, Medical University of South Carolina, Charleston, South Carolina 29403

Megakaryopoiesis is the process by which hematopoietic stem cells in the bone marrow differentiate into mature megakaryocytes. The expression of megakaryocytic genes during megakaryopoiesis is controlled by specific transcription factors. Fli-1 and GATA-1 transcription factors are required for development of megakaryocytes and promoter analysis has defined *in vitro* functional binding sites for these factors in several megakaryocytic genes, including *GPIIb*, *GPIX*, and *C-MPL*. Herein, we utilize chromatin immunoprecipitation to examine the presence of Ets-1, Fli-1, and GATA-1 on these promoters *in vivo*. Fli-1 and Ets-1 occupy the promoters of *GPIIb*, *GPIX*, and *C-MPL* genes in both Meg-01 and CMK11-5 cells. Whereas *GPIIb* is expressed in both Meg-01 and CMK11-5 cells, *GPIX* and *C-MPL* are only expressed in the more differentiated CMK11–5 cells. Thus, *in vivo* occupancy by an Ets factor is not sufficient to promote transcription of some megakaryocytic genes. GATA-1 and Fli-1 are both expressed in CMK11-5 cells and co-occupy the *GPIX* and *C-MPL* promoters. Transcription of all three megakaryocytic genes is correlated with the presence of acetylated histone H3 and phosphorylated RNA polymerase II on their promoters. We also show that exogenous expression of GATA-1 in Meg-01 cells leads to the expression of endogenous c-mpl and gpIX mRNA. Whereas *GPIIb*, *GPIX*, and *C-MPL* are direct target genes for Fli-1, both Fli-1 and GATA-1 are required for formation of an active transcriptional complex on the *C-MPL* and *GPIX* promoters *in vivo*. In contrast, *GPIIb* expression appears to be independent of GATA-1 in Meg-01 cells.

Ets family members each contain a conserved winged helix-loop-helix DNA binding (ETS) domain that allows recognition of purine-rich DNA sequences with a core GGA(A/T) consensus, designated EBS[1] (Ets binding sequence) (1–3). These transcription factors have critical roles in the transcriptional control of genes important in development, morphogenesis, proliferation, and angiogenesis. Ets factors can function as either positive or negative transcriptional regulators (4, 5) and additional binding of other transcription factors to cis-elements located near EBSs contributes to regulated transcription of specific target genes. Thus, in addition to binding to DNA, Ets transcription factors participate in protein interactions that affect their functions (6, 7).

Fli-1, a member of the Ets gene family of transcription factors, performs functions critical for normal development and oncogenesis (for a review, see Ref. 8). Fli-1 is preferentially expressed in cells of hematopoietic lineages and vascular endothelial cells, and has been shown to transcriptionally activate genes, including the stem cell leukemia gene (9), tenascin (10), the stress response gene GADD153 (11), the anti-apoptotic gene Bcl-2 (12) and several megakaryocytic specific genes (see below). Fli-1 also forms ternary complexes through interaction with SRF to bind to SRE elements of *fos* and *Egr-1* promoters (13, 14). Fli-1 protein interaction with other regulatory proteins modulates their activities (15, 16) and the Ets family member TEL has been shown to inhibit Fli-1 transcriptional and biological activity (17–19). In addition, we (20, 21) and others (22) have shown that Fli-1 binding can result in transcriptional repression, dependent on promoter and cell context.

Overexpression of Fli-1 in transgenic mice results in death from progressive immunological renal disease associated with an increased number of autoreactive T- and B-lymphocytes (23). These mice also have an increased number of mature B-cells, which have a reduced activation-induced apoptotic response compared with B-cells from wild type animals (23). The possible role of Fli-1 in autoimmunity is further supported by our observation of elevated expression of Fli-1 mRNA in lymphocytes from patients with systemic lupus erythematosus (24). In addition, it has been demonstrated that Fli-1 expression promotes a megakaryocytic phenotype in K562 cells and increases the expression of megakaryocytic genes (25–27). Viral integration and insertional activation of Fli-1 is associated with hematological cancers, including erythroleukemia by Friend-MuLV (28), granulocytic leukemia induced by the Graffi virus (29), primitive stem cell tumors by the 10A1 isolate of MuLV (30), and non-T, non-B lymphomas by the Cas-Br virus (31). Taken together, these reports suggest that Fli-1 plays a crucial role in normal hematopoietic differentiation and lineage selection. To clarify the physiological role of Fli-1 in hematopoiesis, we (32) and others (33) generated mice with the targeted disruption of *Fli-1*. The *Fli-1* homozygous mutant (*Fli-1−/−*) embryos showed hemorrhage from the dorsal aorta into the lumen of the neural tube and the ventricles of the brain beginning on embryonic day 11.0 (E11.0) and were dead on or before day E12.0. In addition, severe dysmegakaryopoiesis (33, 34) and vascular defects (33) were found. Analysis of cultured cells from day 10.0 embryos demonstrated absence of megakaryocytes and aberrant red blood cell development (34).

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‡ Both authors contributed equally to this work.
§ To whom correspondence should be addressed: Dept. of Pathology and Laboratory Medicine, Medical University of South Carolina, 165 Ashley Ave., Charleston, SC 29403. Tel.: 843-792-3962; E-mail: watsondk@musc.edu.

[1] The abbreviations used are: EBS, Ets binding sequence; gp, glycoprotein; ChIP, chromatin immunoprecipitation; RT, reverse transcriptase; PIPES, 1,4-piperazinediethanesulfonic acid.

TABLE I
Primers used for PCR experiments

Region	Application	Primer sequences	Amplicon size	Cycle number	Annealing temperature
			bp		*°C*
C-MPL	ChIP	5′-TAGCACAGATACAGAGGCTGAGTT-3′	230	36	56
−108 to +122	ChIP	5′-CATCCTCCCTTCAGGAAGCTG-3′			
C-MPL	ChIP	5′-GTGCTTAACACACGGCAGC-3′	236	40	58
−328 to −92	ChIP	5′-CCATCCAGACCACACTGG-3′			
C-MPL	ChIP	5′-TGCTGTTGCTCCTGGTCTACA-3′	209	36	55
−714 to −505	ChIP	5′-CAGCCATGCTGTCGTATGCTC-3′			
GPIIb	ChIP	5′-GAACCAATAGGACATGG-3′	272	35	50
−237 to +35	ChIP	5′-CATCTTCCTTCTTCCAC-3′			
GPIIb	ChIP	5′-GCACTGACTGCACTGCTG-3′	221	35	58
−625 to −404	ChIP	5′-GCTGTGGTGCTTTGTGAC-3′			
GPIIb	ChIP	5′-GCACTCCTCCTCTTCCGCTTAC-3′	182	36	55
−1133 to −951	ChIP	5′-TGCTACAGCCCTAGAGACTACC-3′			
GPIX	ChIP	5′-GAGATGTGGTTCTGTGCC-3′	245	35	55
−147 to +98	ChIP	5′-TGCTGGGTAATACGGAGG-3′			
GPIX	ChIP	5′-ACTTGGTAGAGGCTCTGCTT-3′	204	36	55
−566 to −362	ChIP	5′-CTGGCTGAGCACACCATGGT-3′			
S26 cDNA	RT-PCR	5′-GCGAGCGTCTTCGATGC-3′	406	18	55
	PCR	5′-CTCAGCTCCTTACATGGGC-3′		32	
c-mpl	RT-PCR	5′-CCAGCCAGGGGAACTTC-3′	233	18	52
Exon 4	PCR	5′-GCTTTGGTCCATCTTGCC-3′		32	
GpIIb	RT-PCR	5′-AGGCATGATCTGCTGGTGGGCGCT-3′	209	18	60
Exon 12	PCR	5′-ATTGTAGCCATCCCGGTCGAGGT-3′		32	
GpIX	RT-PCR	5′-AGCTGCAGACCCTCGATGTGACGC-3′	238	18	60
Exon 3	PCR	5′-GACCAGCGCCACGTCCCACAAG-3′		32	
GATA-1	RT-PCR	5′-TAAGGTGGCTGAATCCTCTGCATC-3′	445	18	58
Partial open reading frame		5′-CCGTCTTCAAGGTGTCCAAGAACGT-3′			
Fli-1 open reading frame	PCR	5′-GGTTTACCGATGGACGGGACTATTAAG-3′ 5′-CCTGATCAAAGAAGCTTCTAGTAGTAG-3′	1359	30	52

Significantly, multiple megakaryocytic genes contain Ets and GATA-1 DNA binding sites that are necessary for transcription *in vitro*, including glycoprotein Ibα (gpIbα) (27, 35), gpIIb (36, 37), gpV (38, 39), gpVI (26), gpIX (27, 40–42), thrombopoietin receptor (c-mpl) (43, 44), and PF4 (45). GATA-1 is a Cys2/Cys2 zinc finger DNA-binding protein that recognizes and binds the sequence (A/T)GATA(A/G) in the cis-regulatory elements of many lineage-restricted genes (46). GATA-1 is expressed in erythrocytes, megakaryocytes, eosinophils, and mast cells and has been shown to be essential for maturation of erythroid and megakaryocytic cells (46, 47). Absence of GATA-1 expression is associated with the accumulation of early megakaryocytic progenitors that fail to mature (46).

Over 200 Ets target genes have been identified by the presence of functional EBS (5), based upon transient transfection studies. However, few of these have been characterized on chromatin templates *in vivo*. To better understand how Ets and GATA-1 proteins regulate the transcription of megakaryocytic target genes, we examined the *in vivo* binding of Fli-1, Ets-1, and GATA-1 proteins to the endogenous *GPIIb*, *GPIX*, and *C-MPL* promoters. The differential expression of these genes in megakaryocytic cell lines allowed us to correlate Ets factor occupancy, histone acetylation status, and/or presence of phosphorylated RNA polymerase II with transcription from these promoters. Co-expression of GATA-1 and Fli-1 is critical for expression of endogenous *C-MPL* and *GPIX* genes. We also demonstrate for the first time that simultaneous *in vivo* promoter occupancy by these factors is directly correlated with the transcriptional status of specific megakaryocytic genes.

EXPERIMENTAL PROCEDURES

Megakaryocytic Cell Lines—Meg-01 cells (48) were obtained from Dr. Steven Lentz (University of Iowa, Iowa). CMK11-5 cells (49, 50) were provided by Dr. Nicholas Greco (The American Red Cross, Rockville, MD) with permission from Dr. T. Sato (Chiba University, Chiba, Japan), and Dr. Masao Kobayashi (Mochida Pharmaceutical Co., Tokyo, Japan). These two cell lines were maintained in RPMI 1640 media plus 10% fetal bovine serum, according to the ATCC protocol for Meg-01 cells. Briefly, cells were subcultured by dilution into fresh media at a split ratio of 1:2 to 1:3, 2 to 3 times weekly and maintained between 1×10^5 and 5×10^5 cells/ml. For Meg-01 cells, adherent cells (less than 10% of the population) were scraped into the media prior to subculture. All cell lines were propagated at 37 °C in an atmosphere containing 5% CO_2.

Antibodies—Anti-Fli-1 (C19), anti-Ets-1 (C20), and anti-GATA-1 (N6) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-acetylated H3 (06–599) and anti-phosphorylated RNA Polymerase II (clone CTD4H8) antibodies were obtained from Upstate (Lake Placid, NY). β-Actin antibody was obtained from Sigma. The specificity of the Ets-1, Fli-1, and GATA-1 antibodies was confirmed by the absence of nonspecific bands on Western blots of cells extracts. The antibodies selected also have been shown to be suitable for chromatin immunoprecipitation (ChIP) analyses (37, 51–53).

Plasmid Constructs—The pXM GATA-1 expression vector was kindly provided by Dr. S. Orkin (Harvard Medical School, Boston, MA) (54, 55). Mouse GATA-1 cDNA was excised by XhoI digestion of pXM GATA-1 and cloned into the eukaryotic expression vector, pSGNeoKS (modification of pSG5 (Stratagene, La Jolla, CA) to contain a neomycin/G418-resistance cassette and the multiple cloning site from pBluescript II KS vector) at the XhoI restriction site to generate pSGNeo GATA-1. Orientation and sequence were verified by analysis on an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA).

RNA Preparation and Northern Blot Analysis—Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Gel electrophoresis of RNA and transfer to nylon membrane (Duralon-UV membrane, Stratagene) was performed according to Sambrook *et al.* (56). Prehybridization and hybridization were performed at 65 °C with QuikHyb Hybridization Solution (Stratagene) according to the manufacturer's specifications. Membranes were washed once at room temperature in 2× SSPE, 0.2% SDS for 15 min, once at room temperature in 1× SSPE, 0.2% SDS for 15 min, and then twice at 55 °C in 0.2× SSPE, 0.2% SDS for 30 min.

Probes for Northern and Southern Blots—Each probe was labeled with [α-^{32}P]dCTP using Rediprime II (Amersham Biosciences). Ets-1 cDNA was excised by ApaI-SmaI digestion of pSG5-Ets-1 (10). GATA-1 cDNA was excised by XhoI digestion of pXM GATA-1 (54, 55). Fli-1 was prepared by PCR using the previously described human Fli-1 cDNA vector (57) as a template with the primers and conditions provided in Table I. For the c-mpl, gpIIb, and gpIX probes, a first strand cDNA generated from CMK11-5 mRNA was used in PCR utilizing the conditions provided in Table I. The resultant PCR products were cloned into the pCR2.1-TOPO vector and sequence-verified clones served as templates in PCR to generate the DNA for labeling.

Protein Preparation and Western Blot Analysis—Cells were washed in

ice-cold phosphate-buffered saline and lysed (100 μl per 1.5×10^6 cells) in cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.1% SDS, 1% deoxycholate and protease inhibitor mixture (Sigma, catalog P-8340)) for 15 min on ice. Extracts were clarified by centrifugation at $13,000 \times g$ at 4 °C for 15 min and quantitated using the BCA protein assay (Pierce). For Western analysis, protein extracts (20 μg) were separated on a 12.5% denaturing polyacrylamide gel (SDS-PAGE) and transferred onto 0.45-μm polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat dry milk in TBST (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Subsequently, membranes were incubated overnight at 4 °C with the following primary antibodies: anti-Fli-1 (C19) and Ets-1 (C20) (Santa Cruz Biotechnology) using a 1:1000 dilution for both antibodies, anti-GATA-1 (N6) (Santa Cruz Biotechnology) using a 1:2000 dilution. After several washes with TBST, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Caltag Laboratories, Burlingame, CA) using a 1:2000 dilution. Immunocomplexes were visualized with SuperSignal West Pico kit (Pierce). Membranes were stripped (0.2 N NaOH) at room temperature for 5 min and re-probed with a β-actin antibody (Sigma; 1:10,000 dilution) to assess equivalent loading.

ChIP Assays—ChIP assays were adapted from Boyd and Farnham (58) with modifications. Cells (10^7) were resuspended in phosphate-buffered saline and cross-linked with 1% formaldehyde at room temperature for 15 min and the reaction was stopped by the addition of glycine to a final concentration of 200 mM. Cells were then washed with ice-cold phosphate-buffered saline and lysed in 1 ml of ChIP cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, 25 μl protease inhibitor mixture, Sigma). Nuclei were obtained by centrifugation at $3,500 \times g$ and lysed in ChIP nuclei lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 50 μl of protease inhibitor mixture, Sigma). DNA was sheared by sonication to yield an average fragment size of 500 bp and centrifuged for 15 min at $14,000 \times g$. The supernatants were stored at −70 °C. For immunoprecipitation, supernatants were diluted 5-fold in IP buffer (16.7 mM Tris-HCl, pH 8.1, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, protease inhibitors) and pre-cleared with 80 μl of a 50% protein A-Sepharose slurry (equilibrated in 50 mM Tris-HCl, pH 7.0, 20 μg/ml salmon sperm DNA, 0.5 mg/ml bovine serum albumin, 100 μg/ml preimmune IgG) for 2 h at 4 °C. After centrifugation at $14,000 \times g$ for 2 min, specific antibodies (10 μg) were added to the supernatants. Immunocomplexes were formed overnight at 4 °C and collected with 60 μl of 50% protein A-Sepharose (equilibrated as above, except no IgG was added) for 1 h at 4 °C. Beads were washed for 5 min on ice in buffer A (20 mM Tris-HCl, pH 8.1, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 0.1% SDS), buffer B (buffer A with 500 mM NaCl), buffer C (10 mM Tris-HCl, pH 8.1, 0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA), buffer D (buffer C with 500 mM LiCl) and twice in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). Immunocomplexes were eluted off the beads with 150 μl of 1% SDS, 0.1 M $NaHCO_3$ and cross-links were reversed by incubation for 4 h at 65 °C after addition of NaCl to the final concentration of 0.3 M. Proteins were digested with proteinase K (40 μg/ml) for 1 h at 50 °C. DNA was purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Of the total yield of 30 μl of PCR template, 4 μl was used in each PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems). Gene-specific primer sequences and predicted amplicon sizes are summarized in Table I. In all experiments, the specificity of the ChIP reaction was monitored by PCR utilizing primers derived from the coding region of the *C-MPL* gene.

Sequential ChIP Assays—After primary immunoprecipitation, the cross-linked immunocomplexes were eluted from the beads by incubation in elution buffer at room temperature, and the eluate then diluted 1:10 in IP buffer, followed by re-immunoprecipitation with the second antibody. All the subsequent steps were carried out as described above.

DNA Transfection and Selection of Stable Clones—Meg-01 cells were transfected with pSGNeo GATA-1 or pSGNeo control vector using the FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. After transfection, cells were allowed to recover for 16 h, and G418 (Calbiochem, San Diego, CA) was added to a final concentration of 0.8 mM to allow selection of clones with stable expression. After 3 weeks, pools of selected cells were used for RNA isolation.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—Total RNA was isolated from pools of transfected Meg-01 cells using TRIzol reagent (Invitrogen). RNA (1 μg) was reverse transcribed in first strand synthesis buffer according to the manufacturer's recommendations (Invitrogen). 14 ng of the cDNA products were used as template in subsequent PCR using the primers and conditions listed in Table I. The products were electrophoretically separated on a 1.5% agarose gel. After ethidium bromide staining of the molecular weight marker, the gel was used for Southern blotting and hybridization.

Image Analysis—The Kodak Digital Science 1D version 2.0.2 software running on a PowerMac G3 machine was used to calculate the mean intensity of each ethidium bromide or SYBR Green-stained PCR band or the sum intensity of bands on autoradiograms.

Computational Analysis—For prediction of putative transcription factor binding sites the MatInspector professional software (Genomatix) was used as described in Quandt *et al.* (59). Sequences were retrieved using the following accession numbers: GI:183505 (*GPIIb*), GI:1546803 (*C-MPL*), and GI:1772612 (*GPIX*).

RESULTS

Expression of Transcription Factors and Megakaryocytic Marker Genes—The Meg-01 and CMK11-5 cell lines were selected as model systems representing early and late stages of megakaryocytic differentiation, respectively. Meg-01 is a human megakaryoblastic leukemia cell line derived from bone marrow cells of a patient in megakaryoblastic crisis of chronic myelogeneous leukemia. Meg-01 is characterized by a mixed population of adherent and floating cells, does not display B or T lymphocyte markers, whereas platelet glycoprotein gpIIb/IIIa is present on their surface (48). CMK is a cell line of the megakaryocyte/platelet lineage (49, 60) and CMK11-5 is its more differentiated subclone. CMK11-5 cells are larger than CMK cells, and contain multinucleated giant cells. CMK11-5 cells express the platelet antigens gpIb, gpIIb/IIIa, and gpIV and shed platelet-like particles (49, 50).

To characterize molecular events during megakaryopoiesis, we first determined the expression level of two Ets transcription factors, Ets-1 and Fli-1, in these cells. Fli-1 mRNA and protein levels were not significantly lower in Meg-01 cells, compared with CMK11-5 (91 and 83%, respectively). Whereas Ets-1 mRNA in Meg-01 cells is 15% of that detected in CMK11-5 cells, Ets-1 protein levels are more similar (Fig. 1). Next we analyzed the GATA-1 expression in the two cell lines. CMK11-5 cells contain six times more GATA-1 mRNA than detected in Meg-01 cells; more importantly, no GATA-1 protein is detected in Meg-01 cells, even after prolonged film exposure (data not shown).

We next assessed the expression of three megakaryocytic genes, *GPIIb*, *GPIX*, and *C-MPL*, in Meg-01 and CMK11-5 cells. As shown in Fig. 1*A*, Northern blot analysis of RNA prepared from Meg-01 and CMK11-5 cells demonstrates that gpIIb is expressed in both cell lines at essentially equivalent levels. In contrast, whereas gpIX and c-mpl mRNA are each expressed in both cells, the mRNA level found in Meg-01 cells is at least 1 order of magnitude less than that detected in CK11-5 cells. This expression pattern is consistent with the model that CMK11-5 cells are further differentiated along the megakaryocytic lineage than Meg-01 Cells.

Fli-1 and Ets-1 Bind to Endogenous Megakaryocytic Promoters in Meg-01 and CMK11-5 Cell Lines—We next examined whether Fli-1 and/or Ets-1 occupies the *GPIIb*, *GPIX*, and *C-MPL* promoters *in vivo*, using Meg-01 and CMK11-5 cells for ChIP assays. After immunoprecipitation with antibodies against Fli-1 or Ets-1, enrichment of the endogenous promoter fragments in each sample was monitored by PCR amplification using primers specific for each promoter (Fig. 2). As a positive control, 5% of the input chromatin was used in a PCR (Fig. 2, *input*). As a negative control, a reaction lacking precipitating antibody was also performed (Fig. 2, *no Ab*). Both Ets-1 and Fli-1 occupy the −237 to +35 and −1133 to −951 regions of the *GPIIb* promoter in both cell lines. However, only Fli-1 was bound to the −625 to −404 region and this was observed only in CMK11-5 cells. With the *C-MPL* promoter, both Ets-1 and Fli-1 occupy the −108 to +122 and the −715 to −505 region and no difference was observed between the two cell lines. At

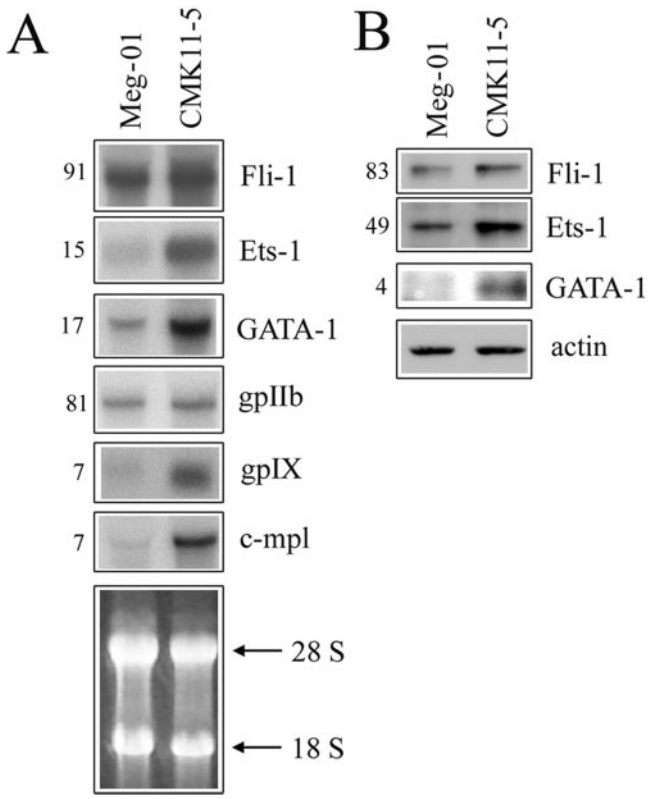

FIG. 1. **Expression of Ets factors, GATA-1, and megakaryocytic genes in Meg-01 and CMK11-5 cell lines.** *A*, *top panels*, Northern blot analysis of RNA transcript levels of Ets-1, Fli-1, and GATA-1 transcription factors and their putative target genes (gpIIb, gpIX, and c-mpl) in Meg-01 and CMK11-5 cells. *Numbers* on the *left* indicate percent relative intensity of RNA in Meg-01 compared with that in CMK11-5 cells after normalization to 28 S rRNA levels. *Bottom panel*, ethidium bromide staining of 28 S and 18 S rRNA as a loading control. *B*, Western blot analysis of Fli-1, Ets-1, and GATA-1 in the indicated megakaryocytic cell lines. *Bottom panel*, the membrane was re-probed with anti-β-actin antibody as a loading control. *Numbers* on the *left* indicate percent relative intensity compared with the protein level in CMK11-5 cells after normalization to β-actin levels.

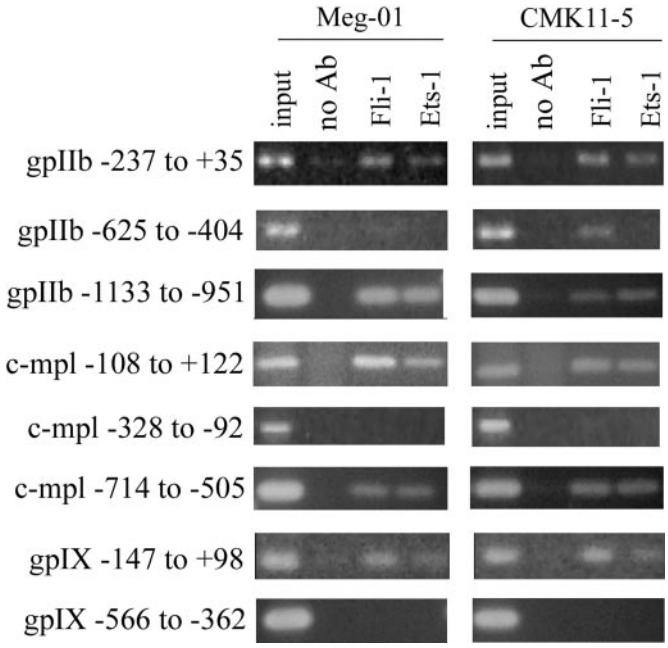

FIG. 2. **Both Fli-1 and Ets-1 bind to the promoters of the megakaryocytic target genes *in vivo*.** PCR was performed on chromatin fragments enriched by immunoprecipitation with or without the indicated antibodies. The antibodies used in these experiments are shown *above* each lane. *Input* represents 5% total cross-linked, reversed chromatin before immunoprecipitation. To show the efficiency of washing, no antibody was added to one sample, marked as *no Ab*. Primers specific for the promoter regions of the genes indicated on the *left* were used in PCR.

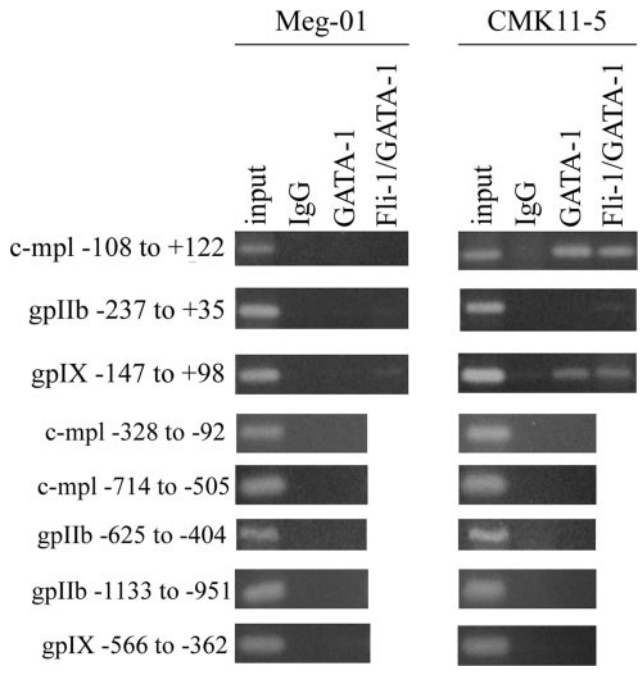

FIG. 3. **Fli-1 and GATA-1 co-occupy the *C-MPL* and *GPIX* promoters *in vivo* in CMK11-5 cells.** ChIP experiments were carried out to investigate whether Fli-1 and GATA-1 bind to the *C-MPL, GPIIb*, or *GPIX* promoter regions in the indicated cell lines. *Input* represents 5% total cross-linked, reversed chromatin before immunoprecipitation. In lanes marked *Fli-1/GATA-1*, anti-Fli-1 antibody was used in the first round and anti-GATA-1 antibody was used in the second round of immunoprecipitation. As a negative control, isotype matched pre-immune IgG was added to one sample, marked as *IgG*.

the *GPIX* promoter, Ets-1 and Fli-1 were found only at the −147 to +98 region in both cell lines. In summary, we found that both Fli-1 and Ets-1 bind, simultaneously or individually, to EBSs present in these megakaryocytic promoters in both Meg-01 and CMK11-5 cells (Fig. 6).

Fli-1 and GATA-1 Proteins Are Present Simultaneously on the C-MPL and GPIX Promoters in CMK11–5 Cells—Based on previous studies demonstrating that Ets and GATA-1 proteins bind and transcriptionally activate the *GPIIb* (36, 37), *GPIX* (27, 40–42), and *C-MPL* (43, 44) promoters *in vitro* and the observation that Fli-1 binds to the *GPIIb*, *GPIX*, and *C-MPL* promoters *in vivo* (Fig. 2), we next determined whether GATA-1 is bound to these promoters *in vivo*. ChIP analysis was performed using Meg-01 and CMK11-5 cell lines. These experiments revealed that GATA-1 is bound to the *C-MPL* and *GPIX* promoters (−108 to +122 and −147 to +98 regions, respectively) in CMK11-5 cells (Fig. 3). Consistent with low GATA-1 protein level, these regions were not enriched in ChIP fragments from Meg-01 cells. Furthermore, we did not detect GATA-1 at the *GPIIb* promoter in either cell line. Other regions of the analyzed promoters were not detected in the GATA-1 ChIP fragments from either cell line.

To investigate whether both Fli-1 and GATA-1 co-occupy these promoters *in vivo*, we performed sequential ChIP analysis. We first used Meg-01 cells and anti-Fli-1 antibody in both the first and second round of immunoprecipitation. The *C-MPL* promoter region was successfully detected in the ChIP-enriched DNA fragments using PCR (data not shown). Next, we used chromatin prepared from both Meg-01 and CMK11-5 cells

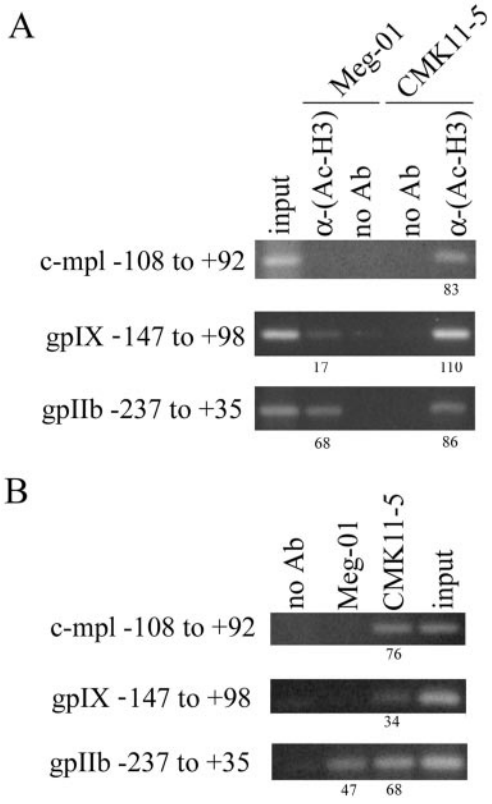

FIG. 4. **ChIP analysis of histone modification status and occupancy by phosphorylated RNA polymerase II on Fli-1 occupied megakaryocytic promoters.** *A*, histone H3 is acetylated at the Fli-1 occupied transcriptionally active promoters. Anti-Fli-1 and anti-acetylated-H3 antibodies were used in sequential ChIP assays to precipitate fragments representing promoters of Fli-1-regulated active genes. Primers specific for *C-MPL*, *GPIIb*, and *GPIX* promoter regions were used in PCR. *Input* and *No Ab* controls were performed as described above (Fig. 2). *Numbers below* bands indicate percent intensity relative to input. *B*, phosphorylated RNA polymerase II is present on the Fli-1-occupied active promoters. Anti-Fli-1 and anti-phosphorylated RNA polymerase II antibodies were used in sequential ChIP experiments. *input* and *no Ab* controls were as described above.

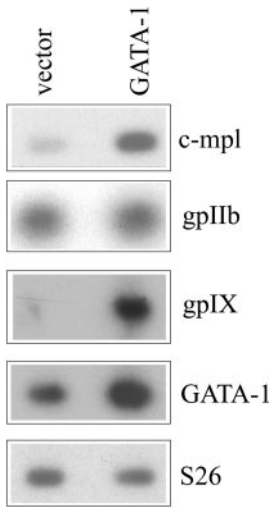

FIG. 5. **Exogenous expression of GATA-1 induces expression of the endogenous *C-MPL* and *GPIX* genes.** Total RNA was harvested from either pSG5Neo GATA-1 or pSG5Neo transfected Meg-01 cells and used for semiquantitative RT-PCR. Reverse transcribed cDNA products were amplified with primers for GATA-1, gpIIb, gpIX, c-mpl, and S26. Southern blot detection of the amplified cDNAs from stable GATA-1-transfected cells (*GATA-1*) showed a marked increase in GATA-1, gpIX, and c-mpl, when compared with *vector only* transfected cells. Analysis of S26 mRNA served as loading control.

in the sequential ChIP assays, employing anti-Fli-1 antibody in the first round and anti-GATA-1 antibody in the second round of immunoprecipitation. The −108 to +122 *C-MPL* promoter region was detected in the ChIP-enriched fragments from CMK11-5 cells, but not from Meg-01 cells (Fig. 3). This is consistent with our results that GATA-1 is not bound to the *C-MPL* promoter in Meg-01 cells (Fig. 3). Moreover, these results show that Fli-1 and GATA-1 proteins are simultaneously present on this *C-MPL* promoter region in CMK11-5 cells *in vivo*. Similarly, sequential ChIP analysis demonstrated simultaneous occupancy by Fli-1 and GATA-1 proteins on the *GPIX* promoter (−147 to +98 region) *in vivo* in CMK11-5 cells. In contrast, we were not able to detect *GPIIb* promoter fragments in ChIP-enriched chromatin from either cell line.

Histone Acetylation and RNA Polymerase II Phosphorylation on Fli-1 Target Gene Promoters—We have shown above that both Ets-1 and Fli-1 occupy the *GPIIb*, *GPIX*, and *C-MPL* promoters in Meg-01 cells. However, in these cells the *C-MPL* and *GPIX* mRNA levels are less than 10% of that detected in CMK11-5 cells. Additional criteria for transcriptionally active Fli-1 target genes, independent of GATA-1 status, were investigated. One hallmark of many transcriptionally active genes is the presence of acetylated histones at the respective promoters (61). Therefore, we analyzed the acetylation status of histone H3 at Fli-1 occupied megakaryocytic promoters (*C-MPL*, *GPIIb*, and *GPIX*) by performing sequential ChIP assays using anti-Fli-1 and anti-acetylated-H3 antibodies. ChIP enriched *C-MPL*-, *GPIIb*-, and *GPIX*-derived fragments were detected in chromatin prepared from CMK11-5 cells, indicating that Fli-1 and acetylated histone H3 are both present on these transcribed promoters (Fig. 4*A*). Similarly, ChIP demonstrated that Fli-1 and acetylated H3 are both present on the *GPIIb* promoter in Meg-01 cells. The ratio of band intensity of the *GPIIb* ChIP fragments for Meg-01 and CMK11-5 was 0.78, which correlates with the relative mRNA expression between the two cell lines (81%, Fig. 1). Consistent with reduced *GPIX* and *C-MPL* expression in Meg-01 cells, ChIP analyses demonstrated that acetylated H3 on these Fli-1 occupied promoters in Meg-01 cells is significantly reduced (17% of control compared with 110% of control) or absent (0%), respectively (Fig. 4*A*).

The presence of phosphorylated RNA polymerase II is another property of most actively transcribed genes (62). We therefore analyzed the same megakaryocytic promoters using sequential ChIP experiments utilizing anti-Fli-1 and anti-phosphorylated RNA polymerase II antibodies. Our results show that the *C-MPL* and *GPIX* promoters were detected in the ChIP-enriched fragments from CMK11-5, but not from Meg-01 cells (Fig. 4*B*). In contrast, the *GPIIb* promoter fragments were found to be enriched by ChIP using chromatin prepared from both Meg-01 or CMK11-5 cells. Moreover, the relative band intensity for *GPIIb* ChIP fragments for Meg-01 and CMK11-5 (0.69) correlated with the observed mRNA ratio (Fig. 1). Collectively, these ChIP results are consistent with the differential expression of these three genes and demonstrate the utility of sequential ChIP for identification of transcriptionally active Fli-1 target genes.

Exogenous Expression of GATA-1 in Meg-01 Cells Leads to Increased Expression of the C-MPL and GPIX Genes—Because GATA-1 has been shown to be a key regulator of megakaryocytic gene expression, we next investigated whether expression of GATA-1 in Meg-01 cells would be sufficient to promote *C-MPL* and *GPIX* expression. We transfected Meg-01 cells with the GATA-1 expression plasmid, enriched for stable GATA-1 transfected Meg-01 cells and analyzed the c-mpl, gpIX, gpIIb, and GATA-1 mRNA levels by RT-PCR. Total RNA was prepared from GATA-1 or pSG5/neo-transfected cells and used for

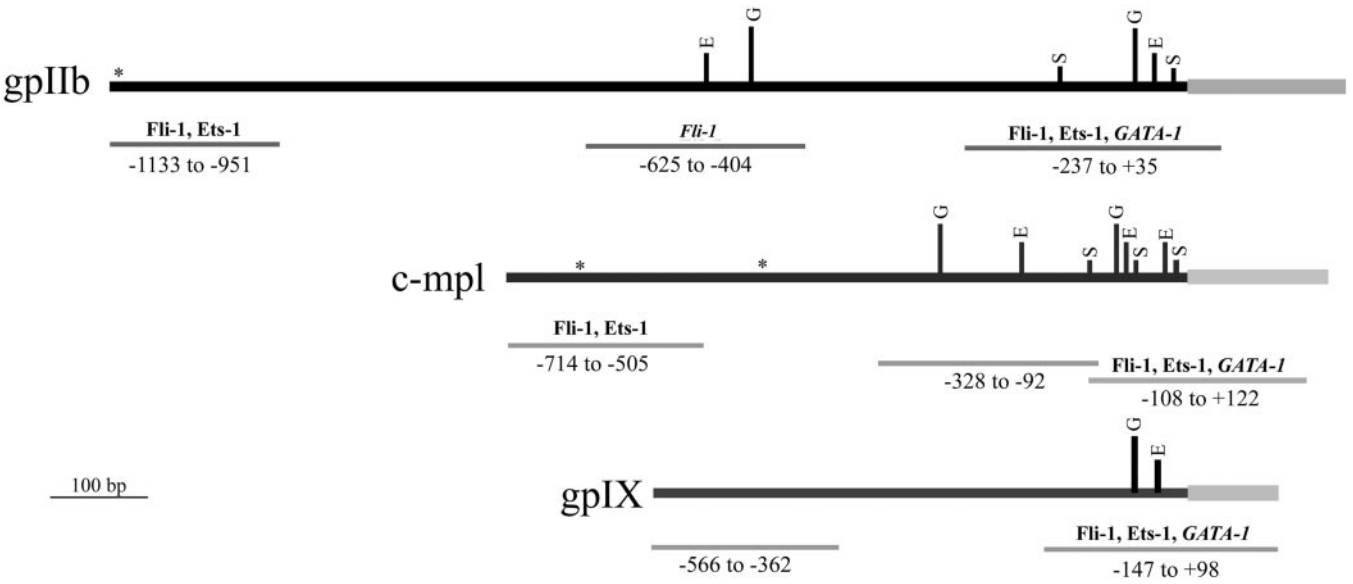

FIG. 6. **Schematic representation of the promoters analyzed.** The *gray line* represents the 5′ transcribed region of the respective genes. The *vertical bars* indicate binding sites previously characterized *in vitro*. *G*, GATA-1; *E*, Ets binding site; *S*, Sp1. *Asterisks* indicate computer predicted Ets binding sites. *Dark gray lines* indicate PCR fragments, *numbers below* show relative boundaries to the transcriptional start. *Text above* indicates transcription factor binding determined by ChIP analysis. *Italicized letters* indicate transcription factor binding observed only in CMK11-5 cells and not in Meg-01 cells.

the preparation of cDNA. PCR was performed using specific primers for c-mpl, gpIX, gpIIb, GATA-1, and S26. Relative to S26, significantly higher expression of c-mpl and gpIX mRNA was detected in the cells with exogenous expression of GATA-1 (Fig. 5). Thus, exogenous expression of GATA-1 mRNA (over 3-fold) led to a significant increase of c-mpl and gpIX mRNA transcripts in the transfected cells, consistent with a direct role for regulation of these promoters by this transcription factor. In contrast, GATA-1 expression did not significantly alter gpIIb expression.

DISCUSSION

Specific combinations of cell-restricted and ubiquitous transcription factors control the maturation of pluripotent hematopoietic stem cells into multiple differentiated cell lineages. During megakaryopoiesis, the expression of megakaryocytic genes, such as *GPIIb*, *GPIX*, and *C-MPL*, is strictly regulated. An early marker of the megakaryocytic lineage, gpIIb (CD41) (63, 64), combines with the more broadly expressed β_3 (gpIIIa, CD61) subunit to form the gpIIb/IIIa heterodimer receptor that regulates cell adhesion and functions in aggregation of activated platelets by binding fibrinogen, fibronectin, vitronectin, collagen, and von Willebrand factor (63, 64). gpIX is required for assembly of the gpIb·V·IX complex (65) that provides the binding site for the von Willebrand receptor and is critical for initial adhesion of platelets to damaged blood vessels. The thrombopoietin receptor, c-mpl, is required for megakaryocytic differentiation and mice lacking either the c-mpl receptor or its ligand, thrombopoietin, have reduced megakaryocytes and are severely thrombocytopenic (66, 67). Consistent with the importance of these genes in megakaryopoiesis and the dysmegakaryopoiesis observed in Fli-1 mutant mice, c-mpl (34) and gpIX (33) mRNA levels are significantly reduced in Fli-1 mutant embryos. Ets factors and GATA-1 functionally regulate the transcription of these (27, 36, 37, 40–44) and other (26, 27, 35, 38, 39, 45) megakaryocytic genes *in vitro*.

To better understand the molecular basis for Ets-1, Fli-1, and GATA-1 target gene regulation, we utilized Meg-01 and CMK11-5 cells. Whereas Ets-1 and Fli-1 proteins were detected in both cells, GATA-1 protein was restricted to the more differentiated CMK11-5 cell line (Fig. 1). Although Northern blot analysis revealed no significant difference in gpIIb expression level between these two cell lines, gpIX and c-mpl were expressed at a significantly lower level (7%) in Meg-01 cells.

We next examined the *in vivo* binding of Ets factors and GATA-1 to these megakaryocytic genes (Fig. 6). Our ChIP studies demonstrate that Fli-1 and Ets-1 occupy the *GPIIb*, *GPIX*, and *C-MPL* proximal promoter regions *in vivo* in both Meg-01 and CMK11-5 cells, consistent with previous *in vitro* studies (36, 40, 44). The only Ets-specific and cell-specific *in vivo* interaction was found on the *GPIIb* promoter (−625 to −404). All other cell-specific interactions were directly correlated with GATA-1 expression. Furthermore, we also observed Fli-1 and Ets-1 occupancy of the *GPIIb* promoter at the −1133 to −951 region and of the *C-MPL* promoter at the −714 to −505 region. Although factor-binding sites within these regions were not identified by previous *in vitro* studies, potential EBSs were predicted by computer analysis. In addition, we determined that potential Ets and GATA-1 sites of *C-MPL* previously defined by *in vitro* studies are not bound *in vivo* by Ets-1, Fli-1, or GATA-1. The chromatin structure around these sites may account for these disparate observations. Alternatively, other Ets or GATA family members may bind to these regions. Overall, we conclude that Fli-1 and Ets-1 may be part of the complex that mediates transcription of these genes *in vivo*. In addition, based upon the absence of GATA-1 in Meg-01 cells, our results indicate that Ets-1 and Fli-1 are able to bind to these megakaryocytic promoters independently of GATA-1.

Northern analysis demonstrated that gpIIb mRNA is present at similar levels (81% relative ratio) in Meg-01 and CMK11-5 cells, independent of GATA-1 expression. Thus, although GATA-1 and Fli-1 have been shown to synergistically activate the *GPIIb* promoter *in vitro* (37), *GPIIb* transcription appears to be independent of GATA-1 in Meg-01 cells. In contrast, both gpIX and c-mpl mRNA are highly abundant in CMK11-5 cells, with only 7% relative expression level in Meg-01 cells, suggesting that transcription of these genes requires GATA-1 and/or other factors that are differentially expressed between these two cell lines. Either *GPIIb* transcriptional regulation is cell-type specific, or it is possible that the *GPIIb* promoter has a higher affinity for the limited amount of GATA-1 protein ex-

pressed in Meg-01 cells, compared with that for the *GPIX* and *C-MPL* promoters. However, the failure to enrich for *GPIIb* promoter fragments by our ChIP experiments argues against this model. Furthermore, the level of gpIIb mRNA was not significantly affected by exogenous expression of GATA-1 in Meg-01 cells (Fig. 5). It is likely, however, that gpIIb transcription requires additional factors. Cell-type specific transcription of gpIIb is also controlled by silencer elements at −120 to −116 present near the proximal EBS in both the human (68, 69) and rat (70) promoters. Whether Fli-1 presence on the promoter modulates the binding of a silencer complex to this element remains to be directly evaluated.

We did not detect significant GATA-1 bound to the *GPIX* or *C-MPL* promoter in Meg-01 cells *in vivo*, whereas this was readily observed in CMK11-5 cells (Fig. 3). Although Ets-1 and Fli-1 bind these endogenous promoters, binding of either Ets factor is not sufficient for gene transcription in Meg-01 cells. Sequential ChIP analysis demonstrated that Fli-1 and GATA-1 co-occupy the *C-MPL* and *GPIX* promoters *in vivo*. Such co-occupancy was not observed for Ets-1 and GATA-1 (data not shown). Furthermore, although the *C-MPL* promoter contains several potential EBSs, we did not detect Ets-1 and Fli-1 co-occupancy of the *C-MPL* promoter as assessed by sequential ChIP experiments (data not shown). Transient transfection with either Ets-1 or Fli-1 with GATA-1 activates transcription of the *C-MPL* promoter in non-hematopoietic cells (43, 44). Our *in vitro* analysis confirmed these findings in HeLa cells and demonstrated that Fli-1 and GATA-1 synergistically activate *C-MPL* (data not shown). These results support the model that Fli-1 and GATA-1 interact and mediate synergistic expression of specific megakaryocyte-specific genes (27).

Promoter occupancy by Fli-1 or Ets-1 alone is not correlated with transcription of *C-MPL* or *GPIX*. The presence of acetylated histone H3 on the Fli-1 occupied *C-MPL* and *GPIX* promoters was directly correlated with their expression status. Relevant to these observations, GATA-1 has been reported to induce histone H3 acetylation at the β-globin promoter (71, 72). Furthermore, GATA-1 associates with histone acetyltransferases and CREB protein (73), supporting a role in chromatin remodeling necessary to enhance transcription. Also, GATA-1 has been associated with RNA polymerase II recruitment to the β-globin promoter (74). Consistent with the expression status of the *C-MPL* and *GPIX* genes in CMK11-5 and Meg-01 cells, we further demonstrate that phosphorylated RNA polymerase II is present on these promoters in only CMK11-5 cells. The relative mRNA expression level (81%) of *GPIIb* in Meg-01 and CMK11-5 cells is correlated with that of Fli-1/acetylated H3 occupied DNA (79%) and Fli-1/phosphorylated RNA polymerase II occupied DNA (70%).

Exogenous expression of GATA-1 in Meg-01 cells resulted in the expression of the *C-MPL* and *GPIX* genes (Fig. 5), demonstrating a critical role for GATA-1 for the expression of these genes. This result complements the previous observation that exogenous expression of Fli-1 in K562 cells, which expresses GATA-1 endogenously, results in expression of megakaryocytic genes, *GPIIb*, *GPVI*, *GPIbα*, and *GPIX* (25–27). Furthermore, gpVI mRNA was only detected in megakaryocytic cell lines expressing both Fli-1 and GATA-1 (26). Taken together, it is clear that both Fli-1 and GATA-1 are critical for the expression of many, but not all, megakaryocytic genes. However, other transcription factors, including FOG-1 (Friend of GATA-1) (37), MafB/Kreisler (75), and TEL (19) also regulate expression of these genes.

Models for Ets factor and GATA-1-mediated transcriptional control have recently been proposed (27, 37, 71). Although Fli-1 and GATA-1 expression is not limited to megakaryocytes, we show that GATA-1 is important for transcriptional activation of specific Fli-1 target genes in megakaryocytes. Our results demonstrate that both Ets-1 and Fli-1 are able to bind to target promoters independently of GATA-1. It has been shown that Fli-1 and GATA-1 proteins directly interact via their Ets and zinc finger domains, respectively, and the *in vitro* binding of GATA-1 to DNA is markedly increased in the presence of Fli-1 (27). When GATA-1 and Fli-1 are both present on a promoter, Fli-1 converts FOG-1 into a co-activator (37). GATA-1 also recruits other nuclear factor(s) with histone acetyltransferase activity (73). Histone acetylation-dependent chromatin remodeling allows other nuclear factors to bind, enhancing phosphorylated RNA polymerase II-mediated transcription. Further studies will be required to identify the precise molecular mechanisms that regulate each of these events.

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