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RESEARCH ARTICLE

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The leukemia associated nuclear corepressor ETO homologue genes *MTG16* and *MTGR1* are regulated differently in hematopoietic cells

Ram Ajore^{1*}, Parveen Kumar¹, Rakesh Singh Dhandra², Urban Gullberg¹ and Inge Olsson¹

Abstract

Background: *MTG16*, *MTGR1* and *ETO* are nuclear transcriptional corepressors of the human *ETO* protein family. *MTG16* is implicated in hematopoietic development and in controlling erythropoiesis/megakaryopoiesis. Furthermore, *ETO* homologue genes are 3' participants in leukemia fusions generated by chromosomal translocations responsible of hematopoietic dysregulation. We tried to identify structural and functional promoter elements of *MTG16* and *MTGR1* genes in order to find associations between their regulation and hematopoiesis.

Results: 5' deletion examinations and luciferase reporter gene studies indicated that a 492 bp sequence upstream of the transcription start site is essential for transcriptional activity by the *MTG16* promoter. The TATA- and CCAAT-less promoter with a GC box close to the start site showed strong reporter activity when examined in erythroid/megakaryocytic cells. Mutation of an evolutionary conserved GATA -301 consensus binding site repressed promoter function. Furthermore, results from *in vitro* antibody-enhanced electrophoretic mobility shift assay and *in vivo* chromatin immunoprecipitation indicated binding of GATA-1 to the GATA -301 site. A role of GATA-1 was also supported by transfection of small interfering RNA, which diminished *MTG16* expression. Furthermore, expression of the transcription factor *HERP2*, which represses GATA-1, produced strong inhibition of the *MTG16* promoter reporter consistent with a role of GATA-1 in transcriptional activation. The TATA-less and CCAAT-less *MTGR1* promoter retained most of the transcriptional activity within a -308 to -207 bp region with a GC-box-rich sequence containing multiple SP1 binding sites reminiscent of a housekeeping gene with constitutive expression. However, mutations of individual SP1 binding sites did not repress promoter function; multiple active SP1 binding sites may be required to safeguard constitutive *MTGR1* transcriptional activity. The observed repression of *MTG16/MTGR1* promoters by the leukemia associated *AML1-ETO* fusion gene may have a role in hematopoietic dysfunction of leukemia.

Conclusions: An evolutionary conserved GATA binding site is critical in transcriptional regulation of the *MTG16* promoter. In contrast, the *MTGR1* gene depends on a GC-box-rich sequence for transcriptional regulation and possible ubiquitous expression. Our results demonstrate that the *ETO* homologue promoters are regulated differently consistent with hematopoietic cell-type- specific expression and function.

Background

The highly conserved *ETO* (Eight-Twenty-One) corepressor gene family traced to *Drosophila* *ETO* homologue *Nervy* [1] contains the myeloid translocation gene (*MTG*) 16 (*MTG16*) (gene name *CBFA2T3*) or murine *ETO-2* (gene name *cbfa2t3*), *MTG8* (gene name *CBFA2T1*, *RUNXT1*, *ETO*) and *MTG*-related protein 1 (*MTGR1*) (gene name *CBFA2T2*). The *ETO* homologues do not

bind directly to DNA but rather function as protein scaffolds and bring about gene repression indirectly through an interplay with multiple transcriptional regulatory proteins [2,3]. *MTG16* has been shown to interact with canonical transcription factors such as *PLZF*, *BCL6*, *TAL1/SCL*, *Gfi1* and *Heb* [4-9], and *MTGR1* with *TAL1/SCL* [10]. The *ETO* homologues also recruit chromatin regulating proteins such as nuclear corepressors [3,11,12] and histone deacetylases (*HDACs*) that catalyze chromatin modifications, resulting in transcriptional repression. Importantly, *ETO* homologue genes are involved in

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chromosomal translocations of acute leukemia, *MTG16* in the generation of the *AML1-MTG16* fusion gene of t(16;21) [13] in patients with therapy-induced leukemia, *ETO* in the generation of the *AML1-ETO* fusion gene of t(8;21) [14,15] and *MTGR1* in the generation of *AML1-MTGR1* fusion gene of t(20;21) [16]. The leukemic fusion proteins so encoded disrupt normal function of transcriptional regulators, to promote dysregulation of hematopoietic cell differentiation [17].

Expression of the *MTG16* (murine *ETO-2*) corepressor is confined to early hematopoiesis whereas *MTGR1* is present throughout hematopoietic maturation [18]. Thus, *ETO-2* increases during differentiation of murine embryonic stem cells into hematopoietic cells [19] suggesting a role in the development of the blood cell compartment. This is consistent with *MTG16* being the most highly expressed *ETO* homologue gene in the stem/progenitor cell compartment [18]. Furthermore, *MTG16/ETO-2* has a role in controlling erythropoiesis and megakaryopoiesis. In this context, *MTG16/ETO-2* is incorporated in Ldb1 (LIM domain-binding protein 1) and TAL1 (T-cell acute lymphocytic leukemia protein 1) containing transcription factor complexes [5,8,20,21], mediating transcriptional suppression. As a heteromer with *MTG16*, also *MTGR1* complexes with TAL1 in erythroid cells, enhancing transcriptional repression [10]. The *ETO-2* recruitment within nuclear complexes may determine the onset of terminal erythroid/megakaryocytic differentiation [5], while the downmodulation of *MTG16/ETO-2* at late stages of erythropoiesis suggests that decreased transcriptional repression is necessary for late, terminal erythroid differentiation to occur. For example, in erythroid MEL cells, induction of differentiation is accompanied by dissociation of nuclear protein complexes concomitant with a decrease in the relative level of *ETO-2* [8]. Moreover, shedding the negative regulator *ETO-2* [21] transforms the Ldb complex into a positive regulator of final erythroid differentiation, supporting the notion that multi-protein complexes may keep erythroid target genes suppressed until corepressor *ETO-2* is eliminated at final differentiation [21]. Similarly, in megakaryopoiesis the *ETO-2* expression is restricted to immature cells consonant with a function in repression of genes that should be only terminally expressed [22]. Finally, a physiological hematopoietic role for *MTG16* has been proven by results from targeted disruption of murine *MTG16* (*ETO-2*) [23]. Although no complete block in hematopoietic differentiation was discovered by *ETO-2* knockout, a requirement was demonstrated for *ETO-2* in fate decisions, proliferation and stress-dependent hematopoiesis. As a corroboration of functional differences between *ETO* homologues, targeted disruption of *MTGR1* showed no phenotypic consequences for hematopoiesis [24].

Endogenous cell-type-specific hematopoietic expression of the *ETO* homologues [18] suggests differences in

gene regulation. The *ETO* promoter is regulated by the GATA transcription factor in erythroid/megakaryocytic cells [25]. We now examined functional promoter elements upstream of the coding sequences for *MTG16* and *MTGR1*. A critical role in transcriptional regulation was shown for an evolutionary conserved GATA binding site of the *MTG16* promoter. In contrast, *MTGR1* has a strong TATA-less promoter with multiple GC box-rich regions of possible importance for constitutive expression. Our results show that the *ETO* homologue promoters are regulated differently, consistent with cell-type-specific expression.

Results

Identification of transcriptional start site and sequence analysis of proximal promoters of *MTG16* and *MTGR1*

The *MTG16* corepressor is associated with developing hematopoiesis and control of erythropoiesis/megakaryopoiesis. However, transcriptional factors regulating the expression of the *MTG16* and *MTGR1* genes have not been identified. Therefore, we tried to discover the important *cis*-acting regions and *trans*-acting factors regulating expression in hematopoietic cells. First, to find the position of the transcriptional start site for *MTG16*, we performed 5'-rapid amplification of cDNA-ends (RACE). *MTG16*-cDNA amplified from erythropoietic HEL cell mRNA showed a complete sequence match with a region of *MTG16a* transcript (NCBI Ref. Seq: NT_010542.15). The transcription start site (TSS) was determined at -176 bp upstream of the translation start codon ATG at +1 (Figure 1A). The translation initiation in *MTG16b* is located further downstream to that of *MTG16a* as determined by alignment of mRNA sequence of *MTG16a* (NM_005187.5) and *MTG16b* (NM_175931.2). Inspection of the proximal promoter upstream of the transcription start site start (-176 bp) revealed several consensus binding elements (Figure 1A) such as potential ETS1 binding sites at -491 and at -419; SP1 binding sites at -425 and at -316; GATA binding sites at -356 and at -301; MZF1 sites at -251 and at -191; TFAP2A at -389 (Figure 1A). No canonical TATA or CCAAT boxes were detected in the region near the transcription start.

However, a conserved GC box close to the transcriptional start site (Figure 1A) may regulate transcriptional initiation. Sequence comparisons showed the GATA binding site at -301 to be conserved between eight species (Figure 1B).

The transcription start site for the *MTGR1* gene (NCBI Ref. Seq: NM_001032999.2) is present at -146 bp upstream of the translation start codon [16]. As for *MTG16*, the proximal promoter of *MTGR1* lacks canonical TATA and CCAAT boxes. However, the region between -285 to -170 bp contains multiple GC motifs showing strong homology with the classical GC box,

A

-668 AAGGGGTGCTGGGGAAAGCTCGGCTGACTGAGGGCTAGCGGAGAGGGAGGA
 -617 CACCCCAGATACCCAGGCTGAAGGAAGGGCCAAGCCCTGGGGGAAGAT
 -566 GGGTGGGCGGGACAGGCCAAGGGCTCTGGGCCAGGATGGAGCTGCCCTCAG
 -515 GCCTGCACACCTGCCCTGGGGCCG**AGGAAA**CACATGCCCAGTGGCCACGCT
 -464 GTAGGGCTCAGGGTGGGTGGGACGTCACCAGAGCTGCCT**GGGAGGAGGAAG**
 -413 TTGTTGGAAGGTCAAACCGGAATG**GCCCGAGG**GAAGGCCGCGCAGGGCAGG
 -362 GCCCC**AGATG**GTTCCTGTCAGGGAAGTGGCGGGCGCAGCTGCAGGC**CTCCG**
 -311 **GCCCC**GGCAT**TATCA**CGGGGACACAGCTGGCTGCCTCACCCGCAGGCTGCA
 -260 GGGAGACCT**TCCCC**AGCCTGCAGCCCCAG**GCCCGCCCCGCG**TCACATGAGC
 -209 CCCAGGGCTCCACCCCC**TCCCC**AGGGCAGAG**G**ACACCCAGTTGGTGGCCG
 -158 GGAGGGCCTCGGCTTTCCAGGGACAGAGGCCCAACTCCAGGACGCCCCAGC
 -107 TGGCCAGCCCCTCCTCTTTCCCTCAAGGCTGCAGGAGGTCGGGAAAGGCA
 -56 GTCCTGGTAGAGGCCTGTCCTGGGCTCCAGGTTGGCCCTGAGGGTGGCCC
 -5 TCCT**CATG**CCGG
 +1

B

	GATA	
Human	CAGGCTCC--GGCCCC---GGCAT	TATCACGCGGGACACAGCTGGCTGCCTCACCC--GC
Chimpanzee	CAGGCTCC--GGCCCC---GGCAT	TATCACGCGGGACACAGCTGGCTGCCTCACCC--GC
Orangutan	CAGGCTCC--GGCCCT---GGCAT	TATCACGCGGGACACAGCTGGCTGCCTCACCC--GC
Gorilla	CAGGCTCC--GGCCCC---GGCAT	TATCACAGGGACACAGCTGGCTGCCTCACCC--GC
Rhesus	CAGGCTCC--GGCCC-----AT	TATCACAGGGACACAGCTGGCTGCCTCACCC--GC
marmoset	CAGGCTCG--GGCC-----GGCAT	TATCACGCGGGACACAGCTGGCTGCCTCGCC--AC
Mouse	CAGGCCCC--GACCCCCACCGCAT	TATCACTGTGACACAGCTGGCTGCCTCACCCCTGA
Rat	CAGGCCCCCGACCCCCACCGCAT	TATCACGGCGACACAGCTGGCTGCCTCACCCCTGA
	***** * * **	***** * *****

Figure 1 Localization and structure of MTG16 promoter. (A) Nucleotide sequence of the MTG16 promoter. Identification of transcription start site (TSS) and amplification of the promoter was performed as described in Methods. Nucleotide +1 is the translational start site (ATG). Putative consensus binding sites for transcription factors identified with MatInspector <http://www.genomatix.de//matinspector.html> and <http://jaspar.genereg.net/> are marked. The promoter lacks TATA and CCAAT boxes, but a GC box (underlined) close to the TSS may be important for start site utilization. (B) Homology search was performed for a stretch of sequence from -251 to -499 human sequence with NCBI nucleotide blast (Blastn) programme in database for "whole-genome shotgun reads" (wgs) and an evolutionary conserved GATA site was identified (marked between straight lines). The following accession numbers appeared containing homologous sequences, Chimpanzee, b| AACZ03106631.1; Orangutan gb| ABGA01087101.1; Gorilla, emb| CABD02143420.1; Rhesus Monkey, gb|AANU01214553.1; Marmoset, gb|ACFV01163109.1; Mouse, ref|NT_078575.6| Mm8_78640_37; Rat, ref|NW_047536.2|Rn19_WGA2058_4. The homology sequences were also found at <http://genome.ucsc.edu/cgi-bin/hgBlat>.

5GGGGCGGGG3' (Figure 2). These boxes are predicted to compose multiple binding sites for SP1. In addition, ETS1 sites are noted at -335 and -276 as well as a YY1 potential binding sites at -301 bp. The different composition of the proximal promoters of *MTG16* and *MTGR1* suggests that they are under distinct regulatory mechanisms. While the promoters of both genes lack classical TATA or CCAAT boxes, the presence in the *MTGR1* promoter of multiple SP1 sites and a CpG island from -450 to -1 bp covering the transcriptional start site (data not shown) indicate characteristics of a housekeeping gene.

Functional evaluation of *MTG16* promoter

We cloned a -820 to -57 bp region (translational start codon at +1) of the sequence upstream of the transcription start of the *MTG16* for determination of transcriptional activity. The cloned region was inserted upstream of the luciferase reporter gene in promoterless pGL3/Basic vector (Promega) to give the pGL3/-820 to -57 plasmid, which was transfected into hematopoietic cell lines and luciferase activity was determined and normalized as described in Methods. pGL3/-820 to -57 showed an approximately 4-fold increased reporter signal as compared to pGL3/SV40-promoter in erythroid HEL cells

(Figure 3) indicating strong transcriptional activity in cells expressing the endogenous *MTG16* gene. The *MTG16* luciferase reporter also showed a strong signal in other erythroid and in megakaryocytic cell lines investigated (Figure 3). However, no direct correlation was observed between luciferase activity and *MTG16* transcripts in erythroid and megakaryocytic cell lines. Differences in cellular environment and regulatory elements outside the cloned promoter that modulate endogenous gene expression could explain the lack of direct correlation between promoter activity and gene expression. Promyelocytic HL-60 and acute myeloid leukemia Kasumi-1 cells showed only low luciferase activity; lymphoblastoid Raji cells and monkey kidney COS cells lacked reporter activity. The latter results correlate with low *MTG16* transcript levels.

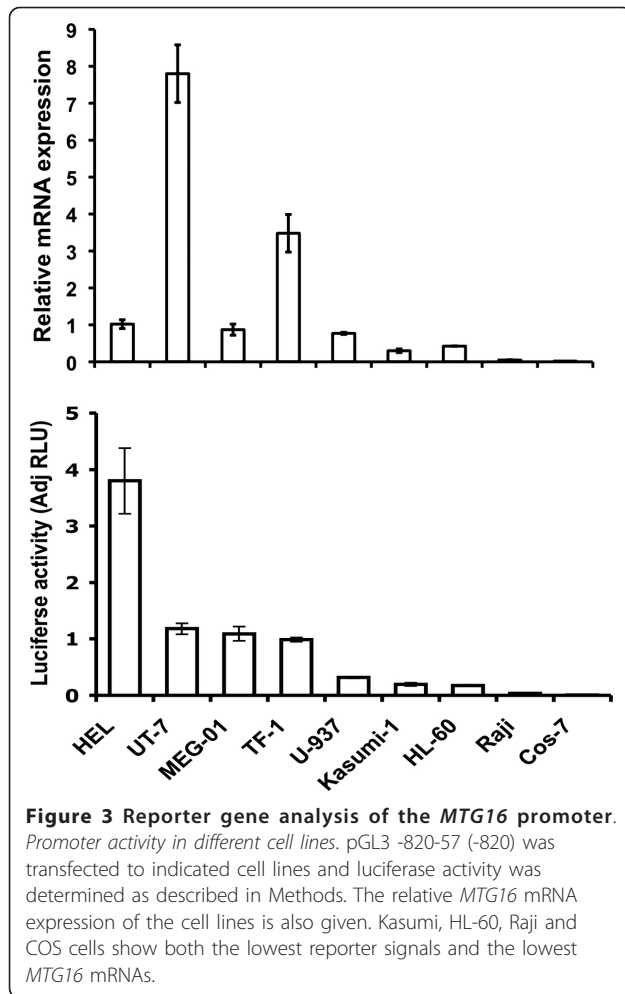
A GATA consensus binding site is critical for *MTG16* promoter function in erythroid/megakaryocytic cells

In order to identify important *cis* regulatory elements, a series of constructs was produced by sequential deletions from the 5' end of the -820 to -57 bp region shown in Figure 4. The deletion constructs were incorporated upstream of the luciferase reporter gene in promoterless pGL3/Basic resulting in the pGL3 -820-57, pGL3 -668-57,

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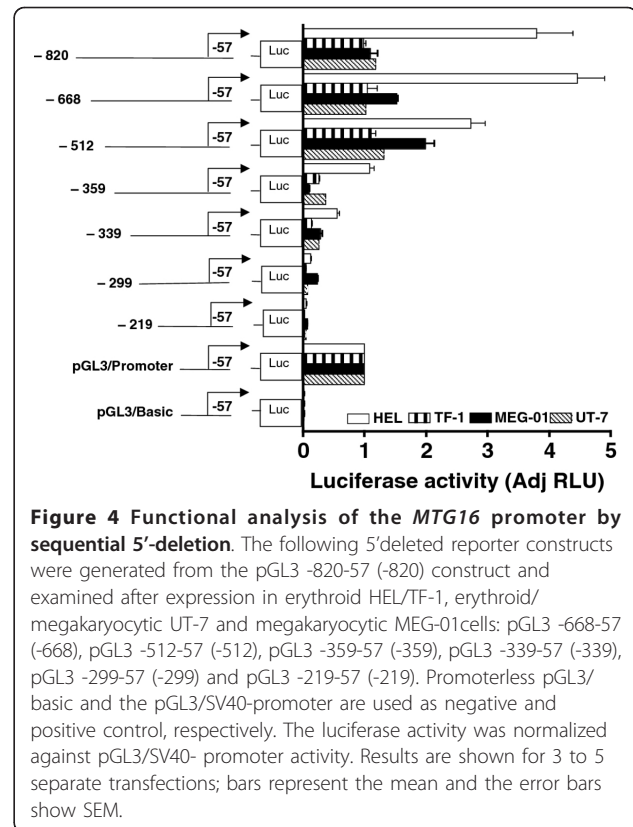
-498 TGTAATCCCTCCCCCTAGCACTCGGCCCCGAGCCTGGGCCACTGCCCTGG
-447 CTACACCGGGAAGTCTGGAGCTAGAAAGTGGGCCGTGCGCGTGACGGGCGC
-396 CGTGACGCCGAGGGGGCAGGTCTGAGAAGCAGGCTGCGCCGGGATCGGCGG
      ETS-335                               YY1-301
-345 TCGGTTCCCGCGGAAGGGGCGGGTCAAGTTAGGGGGCGGTGCTTAATGGGA
      ETS-276
      SP1-285
-294 GCTGGTCTTGGGGCCGGCTTCCGCGTGCCTTGGCCTGGCGGCGAGAGCGC
      SP1-243           SP1-225           SP1-
-243 GCGGCGGGGGCGTGCCCGGCGGGCGGGCGGGGCCAGCCGGGGCGGGGGCG
      -206           SP1-170
      SP1-192           SP1-178           TSS
-192 GGGCGCGGCCTAACGGCGGCGGCGGCGGCGGCGACGGCGACAGCAG|C|GGT
-142 GGTGGTGTCTGGTTAGCTCGGCGGCTGCAGATCTCGCGGCGACGCCTGCGA
-91  GGGACCCGGGCGCGGGTTCGAGGCGGGCGGCGCCTGCGAGGGACCCGTGTC
-40  GCGGGTAGAGGCGGGCGGCGCGGCGGCGGCGGCGCTCGGCGATGGTAGGCGT
      +1
+11  CCCTGGAGCGGCCCTTCCAGCGTAAGTGGGGCGTGAATGC
  
```

Figure 2 Localization and structure of *MTGR1* promoter. Nucleotide sequence of the *MTGR1* promoter. The promoter of *MTGR1* was amplified by PCR as described in Methods. Nucleotide +1 is the translational start site (ATG) and transcription start site at -176 is marked (TSS). The *MTGR1* promoter lacks TATA and CCAAT boxes but is GC-rich with multiple SP1 binding sites. Putative consensus binding sites for transcription factors identified with MatInspector <http://www.genomatix.de/matinspector.html> and with <http://jaspar.genereg.net/> are marked.



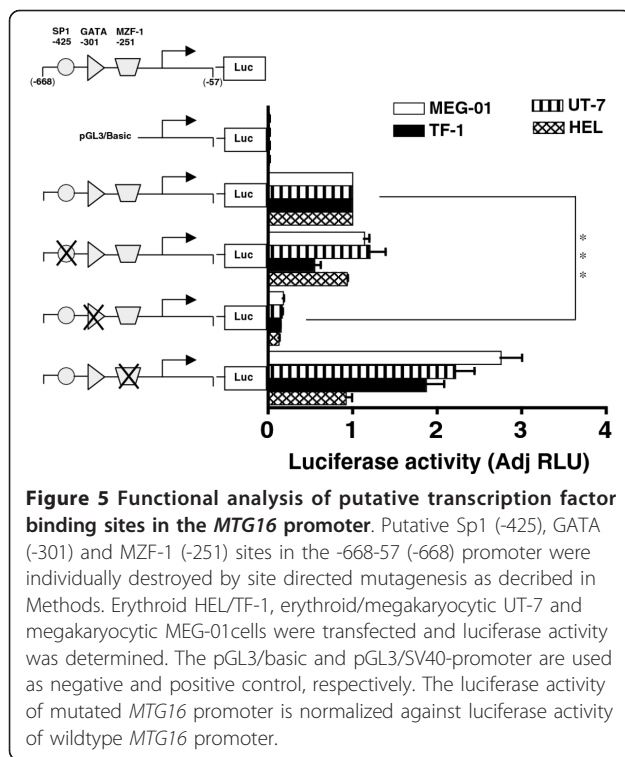
pGL3 -512-57, pGL3 -359-57, pGL3 -339-57, pGL3 -299-57, and pGL3 -219-57 reporter constructs, which were transfected into erythroid HEL/TF-1, erythroid/megakaryocytic UT-7 and megakaryocytic MEG-01 cells. Deletion of the region from -820 to -668 did not reduce the transcriptional activity of the promoter, while extended 5'-deletions caused a prominent decrease in luciferase activity (Figure 4). We conclude that the region between -668 and -57 bp is likely to contain the major regulatory *cis*-elements of the proximal *MTG16* promoter, as it was the smallest fragment created with retention of full transcriptional activity (Figure 4).

As noted in Figure 1A potential ETS1, SP1, GATA and MZF1 binding sites are detected in the proximal *MTG16* promoter. We examined whether these binding sites contribute to promoter transactivation using site-directed mutagenesis. Most notably, disruption of the GATA-301 site led to a 4 to 10-fold reduction in luciferase reporter gene activity in either HEL, TF1, UT-7 or MEG-01 cells relative to intact *MTG16* promoter ($P < 0.0001$) (Figure 5), indicating this GATA site as an important *cis*-factor for



MTG16 gene expression. Furthermore, the GATA -301 site is highly conserved among species (Figure 1B) supporting its functional importance. Moreover, GATA factors are known to play an important role in erythroid/megakaryocytic differentiation [26-28]. Our results suggest that the GATA -301 site may be involved in transcriptional activation in erythroid/megakaryocytic cells.

The GATA-301 site is necessary but not sufficient for transactivation of the *MTG16* promoter as some deletion constructs (-359, -339) that retain the GATA -301 site do not retain transcriptional activity (Figure 4). However, all mutation experiments were done with the longer -668 construct and its promoter activity was blocked completely by mutation of the GATA-301 site. An element upstream of the GATA-301 site that cooperates with GATA bound to -301 might explain our results. However, no such element was identified as disruption of all upstream potential binding sites that were identified e. g. the SP1 -425, ETS1 -491, ETS1 -419, SP1 -316, and GATA -356 sites did not affect the reporter signal (data not shown). Disruption of the MZF1 -191 sites downstream of the GATA-301 site did not affect the luciferase signal either (data not shown). Mutation of the downstream MZF1 -251 increased the signal except in HEL cells (data not shown), suggesting that this site possibly mediates binding to some transcriptional repressor.



GATA-1 binds *in vitro/in vivo* to the -301 consensus site in the *MTG16* promoter

The finding that destruction of the conserved GATA (-301) site reduced gene promoter activity (Figure 5) suggested binding of trans-acting factors. Therefore, Electrophoretic Mobility Shift Assays (EMSA) and antibody supershift assays were performed to examine whether the -301 site binds GATA protein *in vitro*. A double-stranded biotin-labeled oligonucleotide probe (see Methods) was prepared for the core consensus GATA -301 site, the sequence and location of which is shown in Figure 6. The oligonucleotide probe was incubated with nuclear extracts prepared from HEL, TF-1, UT-7 or MEG-01 cells to examine interactions between probe and proteins *in vitro*. Nuclear extract proteins from all cell types showed a shift, indicating binding of protein to the biotinylated probe (Figure 6). Binding was specific, as shown by absence of shift in the presence of excess unlabeled competitor probe. Protein bound to the GATA-301 probe was “super-shifted” by anti-GATA-1 antibody, but not with control anti-CD63 antibody (Figure 6). Thus, our results demonstrate that GATA-1 can bind to the consensus GATA-binding site (-301) within the *MTG16* promoter. No supershift was observed by anti-GATA-2 antibodies (Figure 6) despite testing of several different antibodies (data not shown), suggesting lack of strong binding of GATA-2 to the consensus site. This finding is similar to previous results from the characterization of

the ETO promoter, in which a GATA-site binds GATA-1 but not GATA-2 [25].

In addition to the specific shift, another band is clearly visible in the EMSA (Figure 6). This band-shift is unspecific since it is unaffected or only weakly affected by the competitor probe. However, it obviously contains GATA-1 protein as it is lost by supershifting with anti GATA-1 (Figure 6).

The SP1 -425 and the MZF1 -251 binding sites of the *MTG16* promoter were also examined by EMSA. No supershifting was observed by appropriate antibodies suggesting lack of binding of these transcription factors to the respective consensus binding sites (data not shown).

Chromatin immunoprecipitation (ChIP) was used to determine whether GATA-1-2 bound to the putative *MTG16* gene promoter *in vivo*. The assays were carried out with chromatin isolated from HEL, TF-1, UT-7 or MEG-01 cells and antibody towards chromatin-bound GATA-1 or GATA-2. Precipitated DNA was identified by PCR amplification of the *MTG16* promoter fragment with gene specific oligonucleotides. By the use of primers specific for the -358 to -252 region, which flank the conserved GATA (-301) site, a 107 bp PCR product was generated using precipitation with either anti-GATA-1 or anti-GATA-2 (but not with anti-actin antibody or with no antibody, serving as negative controls) of all the cell lines examined (Figure 7). Control precipitation with anti-GATA-1 or anti-GATA-2 and amplification of a downstream control region was negative. Thus, specific amplification was achieved after precipitation with both anti-GATA-1 or anti-GATA-2.

Taken together, the results from EMSA/supershift assays demonstrate GATA-1 binding *in vitro* to the GATA -301 binding site and results from ChIP assays demonstrate binding *in vivo* of both GATA-1 and GATA-2 to the putative *MTG16* promoter. This result further supports a functional role at least for GATA-1 in activation of the *MTG16* promoter, consistent with the results of the mutagenesis studies shown in Figure 5.

Overexpression of GATA-1

GATA-1 was transiently overexpressed to examine its effect on co-transfected *MTG16* promoter activity in erythroid/megakaryocytic cells. The reporter signal was increased by overexpression in megakaryocytic MEG-01 cells ($p < 0.05$) (Figure 8). The specificity of this result is supported by lack of impact by GATA-1 overexpression on the Renilla control (data not shown).

Repression of GATA-1 by siRNA and inhibition of GATA-1 transcriptional activity with HERP2 leads to decreased *MTG16* promoter activity

To verify a role of GATA-1 in *MTG16* expression, siRNA was transfected into HEL cells as described in Methods.

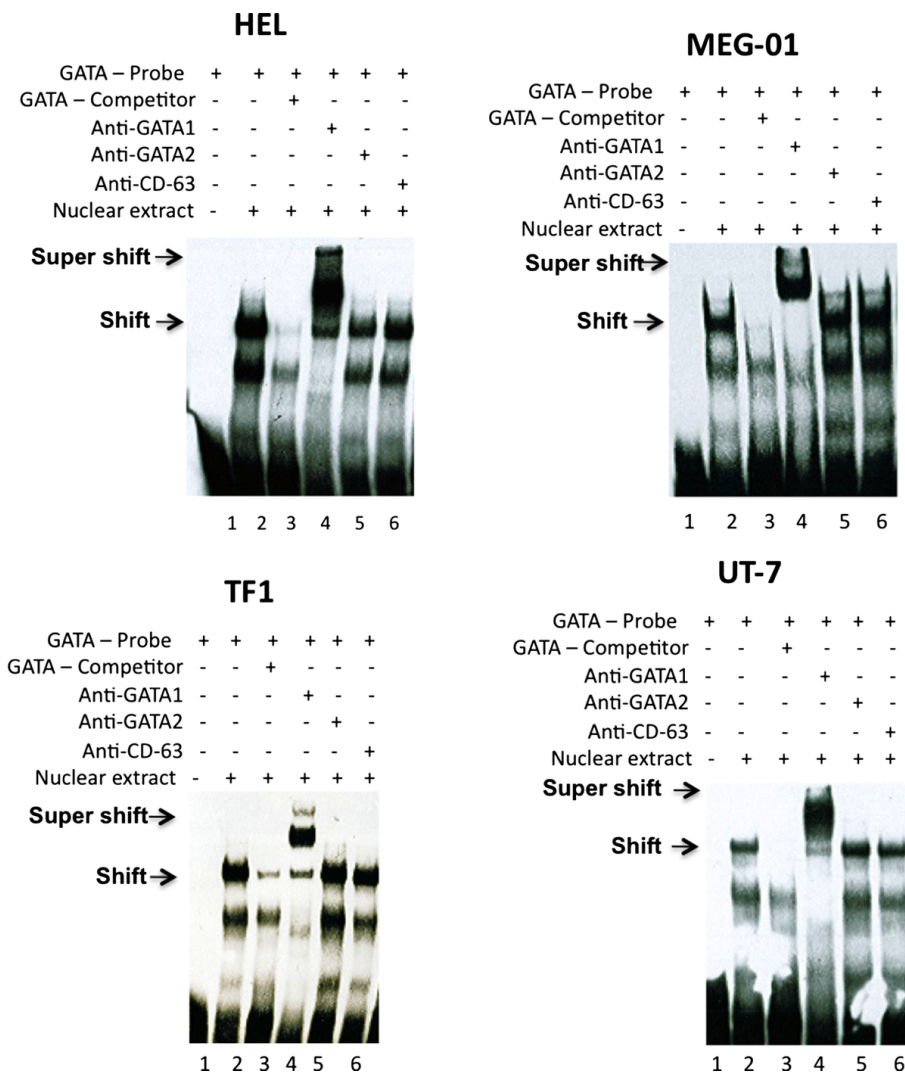


Figure 6 Detection of DNA-protein interactions using electrophoretic mobility shift/supershift assays *in vitro* of consensus binding sequences in the 5'promoter of *MTG16* and nuclear extracts. The following sequence of the oligonucleotide probe was used in EMSA for the conserved GATA (-301) core consensus site (red): **GATA-301 probe 5'- CCCGGCATTATCACGGGGACAC**. Results are given for HEL/MEG-01/TF-1/UT-7 cells. Primary DNA-nuclear protein interactions are shown by arrows marked shift; DNA- nuclear protein-antibody interactions are shown by arrows marked supershift. All cell lines tested showed identical. A shift is shown for the GATA -301 probe that is competed for by excess unlabelled probe (competitor) (3) indicating binding of nuclear extract protein to the biotinylated probe that containing the the GATA -301 sequence. Proteins bound to the probe were "super-shifted" by antibody to GATA-1 (4) but not with antibody to GATA-2 (5). All experiments were repeated at least twice.

Introduction of GATA-1 siRNA led to a decrease by approximately 50% of both GATA-1 and *MTG16* mRNA levels and the results were confirmed on the protein levels (Figure 9), indicating a role of GATA-1 in *MTG16* transcriptional regulation. Three different siRNA constructs, all bioinformatically (Rosetta algorithm, SIGMA) designed to minimize off-target effects were used. However, only one resulted in a robust suppression of GATA-1 protein. Off-target effects can therefore not be excluded. However, in combination with our results from reporter experiments, EMSA, ChIP, and overexpression,

we believe that we can draw the conclusion that GATA-1 is a positive trans-acting factor on the promoter. Additional support for a role of GATA-1 was obtained by use of the basic helix-loop-helix transcription factor HERP2, which shows physical interaction with GATA-1 and represses GATA-1-mediated transcriptional activation [29]. Both erythroid and megakaryocytic cell lines cotransfected with HERP2 showed strong inhibition of the *MTG16* promoter reporter (Figure 10) consistent with a role of GATA-1 in transcriptional activation of *MTG16*.

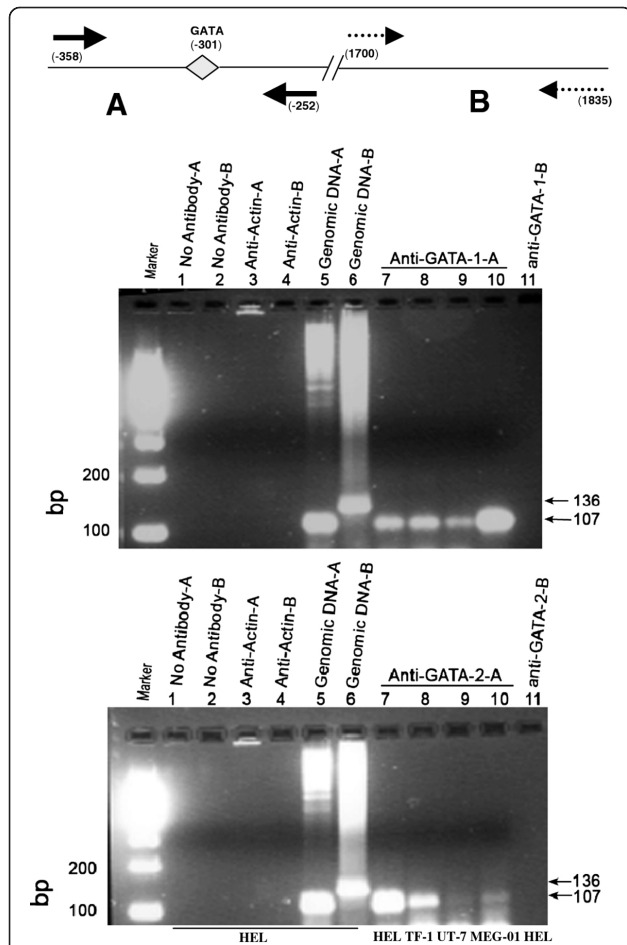


Figure 7 Chromatin immunoprecipitation (ChIP) assay for examining interactions in vivo of consensus binding sequences in the 5' promoter of *MTG16*. The forward and reverse primers used to amplify the proximal promoter region from -358 to -252 (primers **A**, solid arrows) and forward and reverse primers for a downstream region from 1700 to 1835 as control (primers **B**, dashed arrows) are shown. ChIP assays were carried out as described in Methods using chromatin isolated from erythroid HEL/TF-1, erythroid/megakaryocytic UT-7 and megakaryocytic MEG-01 cells. PCR products were separated on a 2% gel and representative results are shown. Lane 1-2, no antibody and primers A or B; lanes 3-4, actin antibody and primers A or B; lanes 5-6, genomic DNA and primers A or B; lanes 7-11, GATA-1 (top) or GATA-2 (bottom) antibody and primers A or B (lane 11). By use of the specific primers, a PCR product is generated both from the anti-GATA-1 and the anti-GATA-2 immunoprecipitated chromatin. No amplification is seen without antibody or in the presence of anti-actin. All experiments were repeated at least twice.

Functional evaluation of the *MTGR1* promoter

We cloned a -989 to +52 bp region (translational start codon at +1) for examination of transcriptional activity. The cloned region was inserted upstream of the luciferase reporter gene in promoterless pGL3/Basic vector to give the pGL3/-989 to +57 plasmid, which was transfected into hematopoietic cell lines and luciferase

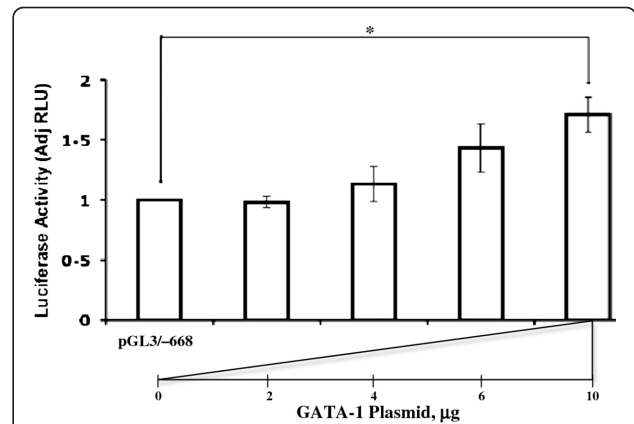


Figure 8 Effects on the *MTG16* promoter activity of GATA-1 overexpression. MEG-01 cells were co-transfected with 15 µg *MTG16* -668 to -57 bp promoter plasmid and increasing amounts of GATA-1 plasmid. The luciferase activity is normalized against Renilla and the activity of the *MTG16* -668 to -57 bp promoter. The *MTG16* promoter is activated by GATA-1 overexpression. The data are from 3 separate experiments. Bars represent the mean and the error bars show SEM. Statistically significant differences are marked by star.

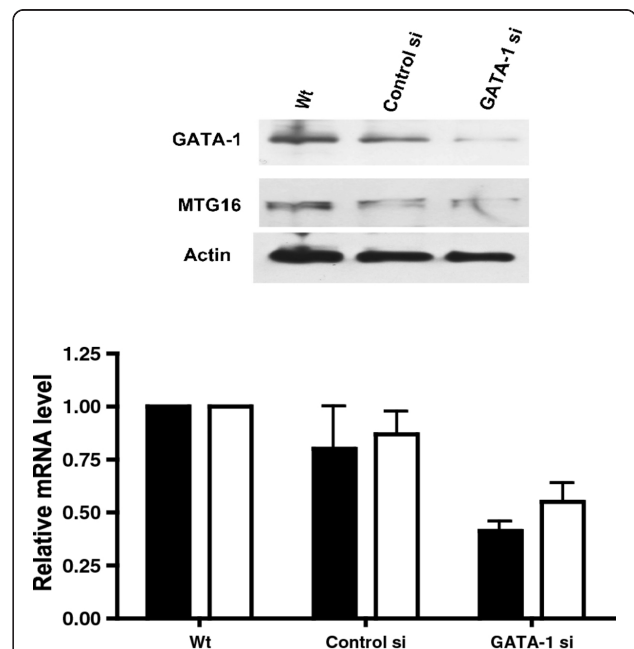
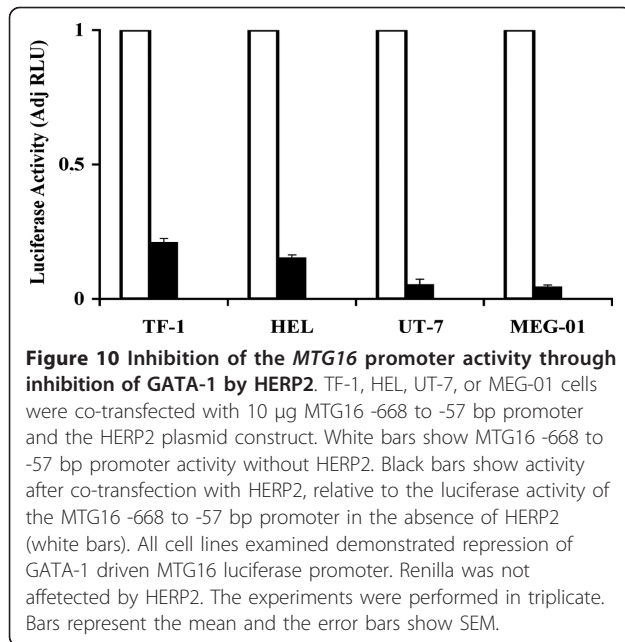


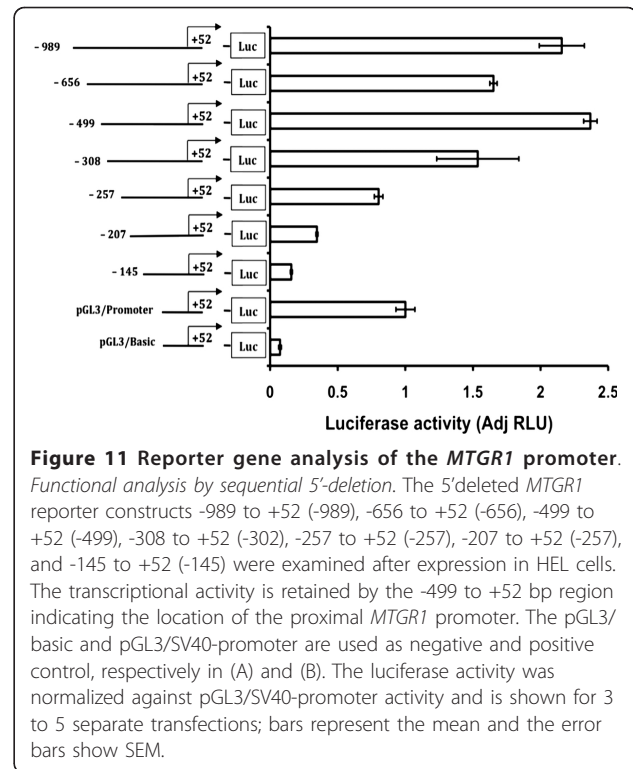
Figure 9 Effects on the *MTG16* promoter activity of GATA-1 siRNA. Transfection was as described in Methods. The relative mRNA expression of GATA-1 (black bar) and *MTG16* (white bar) is given for HEL cells (wt), HEL cells transfected with non silencing scrambled control siRNA (control si) and HEL cells transfected with siRNA for GATA-1 (GATA-1 si). The protein expression of GATA-1 and *MTG16* are shown with GAPDH as a control of equal loading. The experiments were performed in triplicate. Bars represent the mean and the error bars show SEM.



activity was determined and normalized as described in Methods. An approximately 2-fold luciferase signal in HEL cells, as compared to the positive control pGL3/promoter, indicated strong transcriptional activity of the cloned region (data not shown). Upon transfection of the shortest luciferase construct with full promoter activity (pGL3 -499 to +52 bp) (see Figure 11 below) to a panel of hematopoietic cell lines, different activities were revealed (Figure 12). As with the *MTG16* promoter, the strongest activity was found in HEL cells, but the activity patterns between the two promoters were not uniform (compare Figure 3 to Figure 12). The *MTGR1* promoter construct produced a signal in all cell lines examined except for COS cells. All cell lines examined expressed endogenous *MTGR1* mRNA supporting ubiquitous expression (Figure 12).

To define the promoter in more detail, a series of constructs was generated by sequential deletions from the 5' end of the -989 +52 bp region after which transcriptional activity was analyzed in HEL cells (Figure 11). Deletion to -499 did not affect transcriptional activity, but further deletion reduced the luciferase reporter expression. Thus the sequence from -499 to +52 bp retained full transcriptional activity and therefore is likely to contain the core proximal *MTGR1* promoter. Further sequential 5' deletions of the -499 to +52 bp region resulted in a step by step decreasing luciferase reporter signal (Figure 11) suggesting cooperation between factors binding to different regions of the promoter.

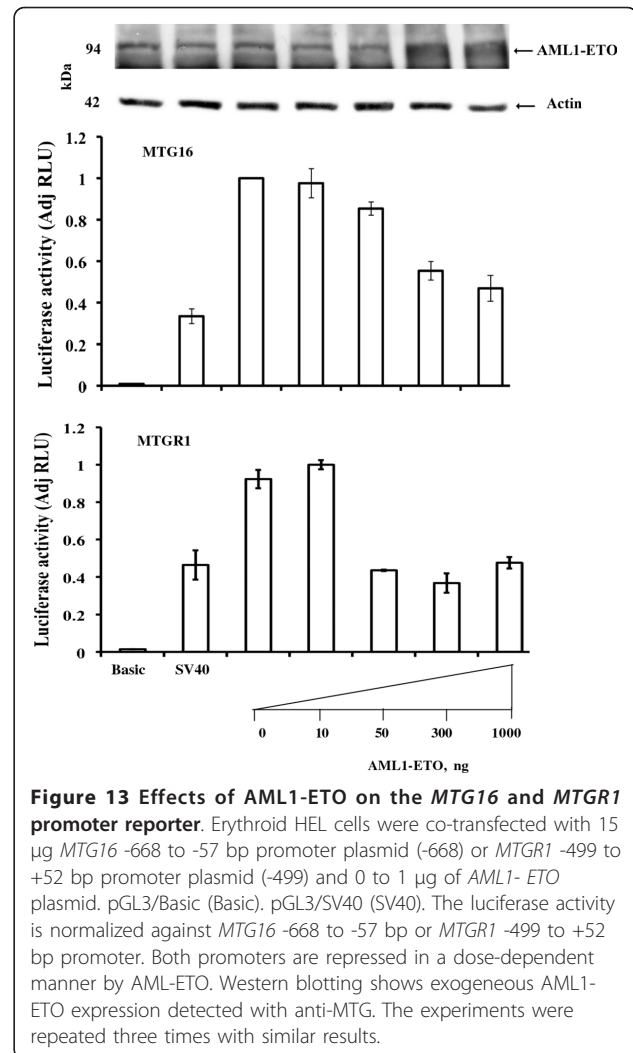
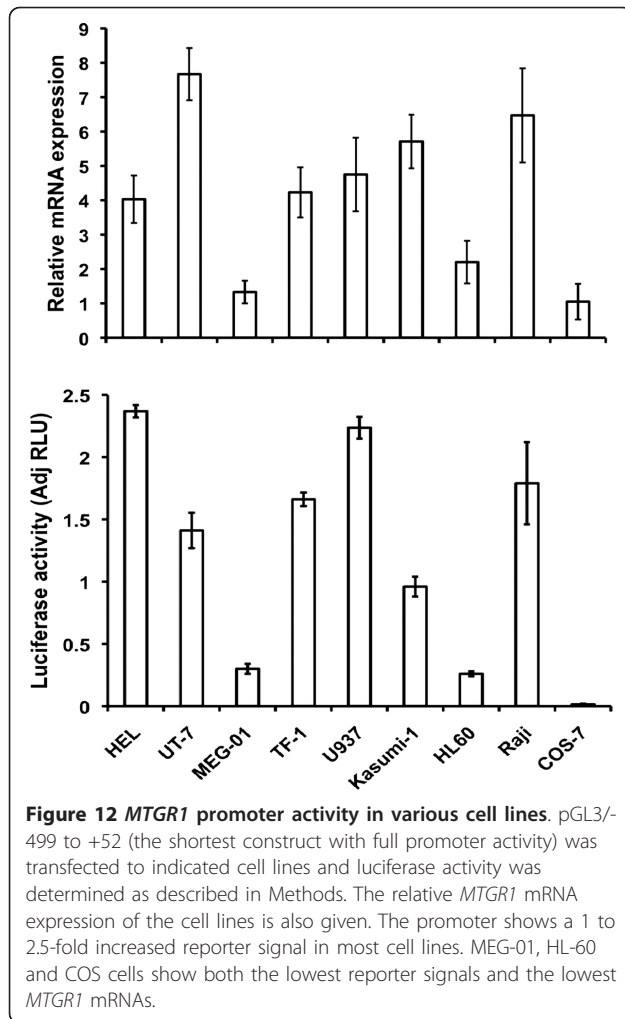
Site-directed mutagenesis was used to examine whether potential transcription factor binding sites contributed to promoter transactivation. The -308 to +52 bp region



retains approximately two thirds of the transcriptional activity of the fully active promoter as was shown in Figure 11. This region is GC box-rich with 9 SP1 consensus binding sites (Figure 2) located immediately upstream of the transcription start site at -146 bp consistent with the housekeeping gene character of *MTGR1* with ubiquitous expression. However, disruption of individual SP1 sites; SP1-285, SP1-243, SP1-229, SP1-225, SP1-213, SP1-206, SP1-192, SP1-178, SP1-170; did not affect reporter gene activity relative to intact *MTGR1* promoter (data not shown), suggesting that SP1 is not important for promoter activity. However, it is possible that multiple active SP1 binding creates a redundancy in SP1 binding and elimination of several SP1 binding sites is needed to reveal their functional relevance. Disruption of the -335 ETS1, the -276 ETS1 and the -301 YY1 sites did also not affect promoter activity (data not shown). Overall, our results indicate that a GC-box-rich sequence with multiple SP1 sites is important for transcriptional regulation of *MTGR1* promoter.

AML1-ETO represses *MTG16* and *MTGR1* gene reporters

The AML1-ETO fusion protein, which is a gene product of t(8;21) of acute leukemia [14,15] binds the promoter region of many genes causing transcriptional suppression [30]. AML1- ETO has been shown to suppress an *ETO* promoter reporter in erythroid/megakaryocytic cells [25]. Therefore, AML1-ETO was transiently expressed in HEL



cells to examine also the effect on co-transfected *MTG16* and *MTGR1* promoter reporters. In both cases the reporter was strongly repressed in a dose-dependent manner by AML1-ETO (Figure 13). This finding is not directly consistent with the inhibition observed *in vivo* in AML1-ETO-positive Kasumi cells of only *MTG16* (Figure 3) but not *MTGR1* transcripts (Figure 12) suggesting influence of cell-specific contexts.

Discussion

The negative transcriptional regulator ETO was originally discovered as the 3' participant in the leukemia chromosomal translocation t(8;21) [31]. Now, ETO and its homologues *MTG16* and *MTGR1* are known to be involved in transcriptional regulation of genome-wide targets. To explain cell-type-specificity of ETO homologue expression and function [18] exploration of promoter regulation is warranted. The human *ETO* proximal promoter is regulated by the GATA-1 transcription factor in an erythroid/megakaryocytic-specific manner [25]. Here, we continued

to identify essential *cis*-acting elements and *trans*-acting factors also of the promoters of *MTG16* and *MTGR1* within human hematopoietic cells.

MTG16 promoter regulation

TATA and CAAT-like promoter motifs facilitate interactions with general transcription factors and are thus involved in initiation of transcription. The proximal *MTG16* promoter investigated lacks these motifs. GC-boxes are known to regulate the utilization of start sites in TATA-less promoters [32-35]. Therefore, the conserved GC-box situated close to start site (Figure 1A) may be involved in transcriptional initiation. Among hematopoietic cell lines, the promoter activity was strongest in the erythroid HEL/TF-1, erythroid/megakaryocytic UT-7 and megakaryocytic MEG-01 cells consistent with a role of *MTG16* in erythropoiesis/megakaryopoiesis [5,8,20,21]. Deletion analysis of the proximal *MTG16* promoter defined a 492 bp region upstream of the transcription start

to be required for maximal promoter activity. This region includes a potential GATA binding site (-301), which is strongly conserved between species. Disruption of the GATA -301 binding site, repressed transactivation of the *MTG16* promoter. The importance of this *cis*-acting binding site was confirmed by results from examination *in vitro* with EMSA, which revealed specific GATA-1 binding. Further, *in vivo* binding of both GATA-1 and GATA-2 to this region of the promoter was confirmed by ChIP/PCR analysis. Taken together these results demonstrate binding of the GATA-1 transcription factor to the *MTG16* promoter and strongly support a role for GATA-1 in transcriptional regulation of the *MTG16* gene in hematopoietic cells.

MTGR1 promoter regulation

A 499 bp region upstream of the translation start site of *MTGR1* was defined as sufficient for maximal activity in erythroid HEL cells. Upon analysis using different cell lines, we found that the promoter is active in most of them, which is in agreement with ubiquitous presence of native *MTGR1* gene product. Furthermore, the lack of classical TATA or CCAAT boxes, the presence of multiple SP1 sites and a CpG island covering the transcriptional initiation site of the *MTGR1* promoter (Figure 2) are similar to characteristics of a housekeeping gene [36], expected to be generally active in a non-tissue specific manner. Few potential binding sites for known transcription factors were found in the -499 promoter, rather a GC-box-rich sequence stretch with multiple SP1 binding sites is a prominent part of the promoter. Importantly, the GC-box-rich region was responsible for most of the transcriptional activity. However, we were not able to inhibit promoter activity by the elimination of individual SP1 binding sites. Possibly, individual SP1 binding sites may be equally involved in guaranteeing constitutive *MTGR1* expression. Further 5' deletion of the -499 promoter region showed gradually decreasing promoter activity observed upon successive deletions, indicating transcriptional cooperation between promoter sequences consistent with cooperating SP1- binding sites.

ETO homologues in hematopoiesis

The regulation of both the *ETO* [25] and the *MTG16* promoter (this work) by GATA-1 is consistent with a role of human ETO/*MTG16*-mediated gene suppression at a phase of erythropoiesis/megakaryopoiesis when GATA-1 levels are high [37,38]. However, despite shared *trans*-activation, *MTG16* and *ETO* are differently expressed in human hematopoiesis [18]; *MTG16* is downmodulated at late stages of erythropoiesis whereas *ETO* is expressed transiently in (human) erythropoiesis. Therefore, cell-type-specific expression of these ETO homologues may be governed also by *trans*-activating

factors other than GATA. Although both *MTG16* and *ETO* are expressed in human erythroid cells [18] yet the role of *ETO* in murine erythropoiesis is not yet supported [10].

GATA-1 and GATA-2 are expressed reciprocally during development of the erythropoietic lineage, GATA-2 decrease is followed by GATA-1 increase [39]. GATA-2 seems to be essential for the development of early and definitive erythropoiesis/megakaryopoiesis and GATA-1 for terminal erythroid/megakaryocytic maturation [26-28]. The present results are consistent with upregulation of *MTG16* as a result of rising GATA-1 levels. Furthermore, results from genome-wide analyses [40] are consistent with early GATA-2 induction of murine *ETO-2* during erythropoiesis. These analyses also showed that most occupied sites are located away from promoters indicating that GATA factors may be involved in long- range control by occupancy at non-promoter regions.

Our finding of *in vitro* binding of GATA-1 but lack of GATA-2 binding to the GATA -301 binding site raises questions about the relative importance of GATA-1/GATA-2 in *MTG16* promoter regulation. GATA-2 is reported to contribute in hematopoietic stem cell and progenitor survival [41] and GATA-1 in erythroid cell survival [42,43]. Furthermore, genetic complementation studies show that GATA-1 can replace GATA-2 [44,45]. A lack of binding in EMSA experiments suggests that GATA-2 may not be a primary transcription factor to initiate nucleation of the transcription complex at the *MTG16* promoter. Possibly, GATA-2 has a weaker binding affinity than GATA-1. However, our finding in ChIP assay of the binding of both GATA-1 and GATA-2 to *MTG16* promoter suggests that GATA-1 and GATA-2 cooperate. This is supported by results from *in vivo* studies by ChIP-seq with human K562 erythroleukemia cells [40] showing chromatin occupancy by both GATA-1 and GATA-2 at the conserved GATA motif corresponding to the -301 site of the *MTG16* promoter. In addition, a role of GATA-2 in stimulating murine *ETO-2* expression was also suggested [40]. Thus, GATA-2 was found to occupy a conserved GATA binding site in the murine erythroid G1E cell, which lack GATA-1 expression; the occupied murine site corresponds to the human -301 GATA binding site of *MTG16* promoter. Overall, available data suggest a role of both GATA-1 and GATA-2 in *MTG16* promoter activation.

Murine *MTG16* (*ETO2*) is involved in erythroid progenitor expansion [5], which is coupled to inhibition of differentiation. This is also the case in megakaryopoiesis where *ETO2* represses inappropriate early expression of terminal genes [22] coupled to restrained differentiation of immature megakaryocytes. *MTG16* and *ETO* show reciprocal expression during *in vitro*-induced human erythropoiesis; *MTG16* decreases and *ETO* increases during the

peak of erythropoiesis [18]. As both promoters are regulated by GATA-1, specific negative autoregulation of *MTG16* [40] may explain the difference. This is consonant with a role for *MTG16* in repressing genes associated with terminal erythroid differentiation thereby inhibiting differentiation and a role for *ETO* in repressing genes associated with self-renewal and proliferation thereby supporting erythroid differentiation.

Suppression of *MTG16/MTGR1* promoter by AML1-ETO

The leukemic AML1-ETO fusion protein [14,15] binds to the promoter region of a number of genes resulting in transcriptional suppression [30]. However, some genes are affected by AML1-ETO in an indirect manner and do not bind the fusion protein directly [30,46]. The observed suppression of the *MTG16* and the *MTGR1* promoter by AML1-ETO is likely to be indirect as binding sites for AML1 were not detectable on the promoters. Furthermore, a potential mechanism for the *MTG16* promoter inhibition by AML1-ETO would be suppression of *GATA-1* through impaired acetylation [47]. Suppression of *MTG16* is consistent with the low *MTG16* promoter reporter signal observed in AML1-ETO-positive Kasumi cells, which showed very low *MTG16* transcripts (Figure 3), whereas *MTGR1* transcripts were high in this cell line (Figure 12). AML1-ETO-mediated suppression of the *ETO* promoter has also been reported [25] and suggested to facilitate the AML1-ETO-induced block of erythroid lineage commitment [47,48]. AML1-ETO-mediated repression of *MTG16* may have a similar effect because of blocking *MTG16/ETO2*-mediated erythroid progenitor expansion [5]. The repression of all *ETO* homologue genes by AML1-ETO may have a general role in inhibition of hematopoietic differentiation in t(8;21) leukemia.

Conclusions

This work shows differences in promoter regulation between *MTG16* and *MTGR1* that may explain differences in hematopoietic expression of these genes. Evolutionary conserved GATA binding sites are critical in transcriptional regulation of both *MTG16* (this work) and *ETO* [25] promoters. The *MTGR1* promoter has a GC box-rich sequence with multiple SP1 binding sites, which may secure transcriptional regulation and ubiquitous expression of this gene.

Methods

Cell Culture

Myelomonocytic U-937, erythroid HEL and TF-1, megakaryocytic MEG-01 cells were grown in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) (Gibco BRL, Life Technologies, Rockville, MD, USA); TF-1 cells also received 20 ng/ml GM-CSF (R&D Systems, MN, USA). The erythroid/

megakaryocytic UT-7 cells were grown in MEM Alpha modification medium with L-Glutamine and Nucleosides (PAA Laboratories GmbH, Austria) supplemented with 20% FBS and 30 ng/ml GM-CSF. The acute myeloid leukemia Kasumi-1, Burkitt's lymphoma Raji, and promyelocytic HL-60 cells were supplemented with 20% FBS. Monkey kidney COS cells were grown in DMEM medium with 10% FBS supplemented with high glucose (4.5 g/L) and L-glutamine.

Identification of the transcription start site of the *MTG16* gene

The 5'-end (transcription start) of the mRNA was identified by 5'-RACE carried out with mRNA prepared from HEL cells using Oligotex Direct mRNA mini Kit (Qiagen, Hilden, Germany), using the First choice RNA Ligase mediated (RLM)-RACE kit (Ambion Inc., TX, USA). Nested PCR of RACE reactions was performed with adapter primer 5'-CGCGGATCCGAACACTGCGTTTGCTGCTTTGATG-3' and nested gene specific primer 5'-GCCTTAGCTTTCCTGTCCACTGG-3'. RACE-products were cloned into pGEM-T Easy Vector system (Promega Corporation, WI, USA) and sequenced.

Amplification of *MTG16* promoter region

A 1208 bp region upstream of the translation start (from -1264 to -57 bp) was amplified from human genomic DNA by PCR. The forward and reverse primers used were 5'-TGAGGCGGTACCACCTCCAGCCATGGCATC-3' and 5'-TGCCTAGATCTACCTCCTGCAGCCTTGAGG-3', respectively. Regions corresponding to -905 to -57 and -820 to -57 bp were amplified from the 1208 bp fragment by nested PCR with forward primers 5'-TATGG-TACCGTGGGGGAGCCCTGCTGTCTCCACA-3' (KpnI restriction site underlined), 5'-AGGCGGGAGGTACCTT-GAGGACAGGTCAGG-3' (KpnI restriction site underlined), and a common reverse primer 5'-TGCCTAGA-TCTACCTCCTGCAGCCTTGAGG-3' (BglII restriction site underlined).

Sequential 5' deletions of the -820 to -57 bp promoter region were generated by PCR from the cloned genomic DNA as template to generate -668 to -57, the -512 to -57, the -359 to -57 bp, the -339 to -57, the -299 to -57 and the -219 to -57 regions. Forward primers were 5'-TAAGGTACCCTGGGGAAAGCTCGGCTGAC-3', 5'-TATGGTACCCTGCCCTGGGGCCGAGG-3', 5'-CCAGGTACCTCC TGTCAGGGAAGTGGCGG-3', 5'-TATGGTACCGGGC GCAGCTGCAGGCC

-3', 5'-TATGGTACCTCACGGGGACACAGCTGGC-3' and 5'-TATGGTACCATGAGCCCCAGGGCTCCCACCC-3' with KpnI restriction sites underlined. The common reverse primer is the same as used for amplification of the -1208 to -57 bp region. All sequences were verified by sequencing.

Amplification of *MTGR1* promoter region

A 5' flanking region of 1965 bp from -1913 to +52 bp was amplified from human genomic DNA by PCR. The forward and reverse primers used were 5'-TATTGGTACCG-CAACACCATGTCTGGCTAA -3' (KpnI restriction site underlined) and 5'-CTACAGATCTGCATTACGCCC-CACTTAC -3' (BglII restriction site underlined), respectively. Regions corresponding to -989 to +52, -656 to +52 and -499 to +52 bp were amplified from the 1965 bp fragment by nested PCR with forward primers 5'-TTGCGGTACCCCTTCAAACCTCTGACCTCGTGA -3' (KpnI restriction site underlined), 5'-TTGCGGTACCCCGGC-CAACAGTTATTTAAA -3' (KpnI restriction site underlined), 5'-TTGCGGTACCTGTAATCCCTCCCCCTAGACT -3' (KpnI restriction site underlined), and a common reverse primer 5'-CTACAGATCTGCATTACGCCC-CACTTAC -3' (BglII restriction site underlined).

Sequential 5' deletions of the -499 to +52 bp promoter region were generated by PCR from the cloned genomic DNA as template to generate -308 to +52, the -257 to +52, the -207 to +52 bp and the -145 to +52 regions. Forward primers were 5'-TTGCGGTACCGTGCT-TAATGGGAGCTGGTCT.-3', 5'-TTGCGGTACCGCGGCGAGAGCGCGCGGGGGGCG -3', 5'-TTGCGGTACCGCGGGGGGCGGGGGCGGGGCGCGGCC -3' and 5'-TTGCGGTACCGTGGTGGTGTCTGGTTAGCTC-3', with KpnI restriction sites underlined. The common reverse primer is the same as used for amplification of the -499 to +52 bp region. All sequences were verified by sequencing.

Site-directed mutagenesis of transcription factor-binding sites

Oligonucleotide primers including the desired mutations were synthesized and used in two-step spliced overhang extension PCR. The following potential *MTG16* promoter transcription factor binding sites were mutated: ETS1/PU.1 sites at positions -491, 5'-AGGAAA-3' changed to 5'-ATTCAA-3'; SP1 sites at positions -425, 5'-GGGAGG-3' changed to 5'-GTTCCG-3'; ETS1/SPI1 sites at positions -419, 5'-AGGAAGT-3' changed to 5'-ATTCAGT-3'; TFAP2A sites at positions -389, 5'-GCCCCGAGGG-3' changed to 5'-GCCTTCGGG-3'; GATA/YY1 sites at positions -356, 5'-GATGGT-3' changed to 5'-GACTTT-3'; SP1 sites at positions -316, 5'-CTCCGGCCCC-3' changed to 5'-CTCCTTACCC-3'; GATA sites at positions -301, 5'-TATCA-3' changed to 5'-TGGTA-3' and MZF1 sites at positions -251 & -191, 5'-TCCCCA-3' changed to 5'-TAAACA-3'.

The following potential *MTGR1* promoter transcription factor binding sites were mutated: ETS1 sites at positions -335, 5'-CTTCCG-3' changed to 5'-CGTATG-3'; YY1 sites at positions -301, 5'-CCCATT-3' changed to 5'-

CTTCTT-3'; SP1 sites at positions -285, 5'-CCCCGCCCCA-3' changed to 5'-CACTGTCCCA -3'; ETS1 sites at positions -276, 5'-CTTCCG-3' changed to 5'-CGTATG -3' SP1 sites at positions -243, 5'-CCCCCGCCGC-3' changed to 5'-CCCTTGCAGC -3'; SP1 sites at positions -229, 5'-CCCCGCCCCG-3' changed to 5'-CCTTGCACGC -3'; SP1 sites at positions -225, 5'-CCCCGCCCCG-3' changed to 5'-CCTTGCACCG -3'; SP1 sites at positions -213, 5'-CCCCGCCCCC-3' changed to 5'-CCTTGCACCC -3'; SP1 sites at positions -206, 5'-CCCCGCCCCG-3' changed to 5'-CCTTGCACCG -3'; SP1 sites at positions -192, 5'-CGCCGCCGCC-3' changed to 5'-CGTTGCAGCC -3'; SP1 sites at positions -178, 5'-CGCCGCCGCC-3' changed to 5'-CGTTGCAGCC -3' and SP1 sites at positions -170, 5'-CGCCGCCGCC-3' changed to 5'-CGTTGCAGCC -3'. After subcloning into promoterless pGL3/Basic reporter plasmid, all mutations were verified by sequencing.

Luciferase reporter assays of *MTG16* and *MTGR1* promoter

MTG16 promoter was cloned into promoterless pGL3/Basic reporter plasmid using firefly luciferase as reporter to generate pGL3 -905-57, pGL3 -820-57, pGL3 -668-57, pGL3 -512-57, pGL3 -359-57, pGL3 -339-57, pGL3 -299-57 and pGL3 -219-57 reporter constructs. Mutants were cloned into the same reporter plasmid. Transient transfections of hematopoietic cell lines were performed by electroporation as previously described by [49]. The pGL3/SV40-promoter vector served as positive control and promoterless pGL3/Basic vector as negative control. Renilla luciferase, pRL/SV40 vector (Promega Corporation, WI, USA) was used as internal control for transfection efficiency. Thirtyfive µg pGL3 DNA were used for HL-60 target cells and 15 µg for HEL, TF-1, U937, UT-7, MEG-01, Raji and Kasumi-1 target cells. COS cells were transfected with 1.5 µg of DNA by use of Polyfect (Qiagen, Hilden, Germany). Twentyfour hours after transfection, cells were lysed and subjected to luciferase determination using the Dual luciferase reporter assay kit (Promega Corporation, WI, USA). Firefly and Renilla luminiscense were quantified (Run Promega Protocol, DLR-0-INJ) in a GLOMAX 20/20 Luminometer (Promega Corporation, WI, USA). Firefly was normalized to Renilla luciferase as internal control for transfection efficiency. Results are given as adjusted Relative Luciferase Units (AdjRLU) normalized to pGL3/promoter (set to 1). Three to five independent transfections were performed in each case and each sample was measured in triplicate.

Similarly, *MTGR1* pGL3 -1913 + 52, pGL3 -989 + 52, pGL3 -656 + 52, pGL3 -499 + 52, pGL3 -308 + 52, pGL3 -257 + 52, pGL3 -207 + 52 and pGL3 -145 + 52 reporters were generated, transfected into cells and assayed as described above.

Transfection of GATA-1 small interfering RNA

Twentyfour-well Falcon plates (Becton Dickinson, New Jersey 07417, USA) were coated with 1 mg/ml retronectin (Takara Bio inc, Verviers, Belgium) in PBS for two h at room temperature. Excess retronectine was removed and wells were blocked with 2% BSA in PBS for 30 min. After removal of the supernatant, 50,000 HEL cells were added per well in 600 μ l RPMI medium containing 10% FBS and 20 nM phorbol 12-myristate 13-acetate (PMA) to generate adherent cells by incubation at 37°C for 24 h. After washing with PBS transfection was carried out with 5 μ M siRNA using the N-TER transfection reagent (Sigma-Aldrich Corp. St. Louis, MO USA) in RPMI without FBS. After 4 h, 300 μ l of RPMI with 20% FBS was gently added and cells were incubated for 48 h. Eventually, gently rinsed with PBS, trypsinized and examined by Western blotting. The GATA-1 siRNA sequence was 5'-AGUUGAGG-CAGGGUAGAGC-3' (Oligo SASI_Hs02_00333089, Sigma).

Inhibition of GATA-1 transcriptional activity by HERP2

The transcription factor HERP2 represses transcriptional activation by GATA-1 [29] by physical interaction. To get support for a role of GATA-1 in gene activation of *MTG16*, HEL, TF-1, UT-7, and MEG-01 cells were co-transfected with 10 μ g *MTG16* pGL3 -668 luciferase reporter and 10 μ g HERP2 plasmid (kindly provided by Dr N Goldfarb, Charlottesville, Virginia) and the reporter signal was measured after 24 h as described above for luciferase reporter assays. The results were normalized to the luciferase activity of pGL3 -668 luciferase reporter.

Electrophoretic Mobility Shift Assay (EMSA) of *MTG16* promoter

EMSA was performed as described previously [25]. The potential GATA-1 sites at -301 was examined. The probe sequence was biotin-5-CCCGGCATTATCACGGGGA-CAC -3'. Nuclear extracts from HEL, TF-1, UT-7 and MEG-01 cells were prepared as described by Andrews and Faller [50]. One to two μ l polyclonal anti-GATA-1 (Active Motif, Carlsbad, CA, USA), monoclonal anti-GATA-2 (Santa Cruz Biotechnology Inc., CA, USA, sc-9008) or polyclonal anti-CD63 (sc-7080, Santa Cruz Biotechnology Inc., CA, USA) antibodies were added to the reaction mixtures.

Chromatin Immunoprecipitation (ChIP) assay of *MTG16* promoter

ChIP was performed as described previously [25]. For IP, 4 μ l of polyclonal anti-GATA-1 (Active Motif, Carlsbad, CA, USA) or 0.8 μ g monoclonal anti-GATA-2 antibodies (R&D Systems, MN, USA) were used. Forward and reverse primers for GATA sites were 5'-CAGATGGTTCCTGT-CAGGGAAGTGGCG-3' and 5'-AGGTCTCCCTG-CAGCCTGCGGGTGAG-3'. Control forward and reverse

primers were: 5'-TAACACAGAGTACCCAGC-CACTGTGC-3' and 5'-TGGTTGCACGGACA-GAAGCCCT-3'. Three different chromatin preparations were used for each IP.

Quantitative real-time PCR

Real-time PCR was performed as described previously [18]. Transcript levels were calculated from a standard curve based on the Ct values of the samples. Relative quantification based on the Δ Ct method [51] was used. Normalization: Δ Ct = Ct (sample) - Ct (HEL corresponding DNA concentration). Relative quantification = $2^{-\Delta$ Ct}.

Immunoprecipitation (IP) and Western blotting

IP and Western blotting were performed as described previously [52]. The following antibodies were used: polyclonal anti-GATA-1 (Active Motif, Carlsbad, CA, USA) and polyclonal anti-GATA-2 (R&D Systems, MN, USA).

Bioinformatics

The cDNA sequences were analyzed by use of the NCBI Blast program <http://www.ncbi.nlm.nih.gov/BLAST/>. Conserved regions were searched by multiple alignment to genomic sequences using ClustalW <http://www.ebi.ac.uk/Tools/clustalw2/index.html>.

Potential transcription factor binding sites were examined with the MatInspector <http://www.genomatix.de/matinspector.html> and the Jaspar database (Jaspar.genereg.net).

Statistical analysis

The statistical significance between two samples was determined by unpaired t-test in Prism Programme. Triple and single asterisk represents $P < 0.0001$ and $P < 0.05$, respectively.

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Authors' contributions

RA carried out experiments, analyzed data, supervised experimental design/data analysis and drafted the manuscript. PK carried out experiments, analyzed data and drafted the manuscript. RSD supervised experimental design/data analysis. UG supervised experimental design/data analysis. IO supervised experimental design/data analysis and drafted the manuscript. All authors critically revised and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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