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Cell Type Dependent Regulation of Multidrug Resistance-1 Gene Expression by AML1-ETO

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Abstract

The AML1-ETO fusion protein is generated from the 8;21 chromosome translocation that is commonly identified in acute myeloid leukemia. AML1-ETO is a DNA binding transcription factor and has been demonstrated to play a critical role in promoting leukemogenesis. Therefore, it is important to define the molecular mechanism of AML1-ETO in the regulation of gene expression. Here, we report that the effect of AML1-ETO on the promoter of multidrug resistance-1 (MDR1) gene, a known AML1-ETO target, is highly cell type specific. Besides observing repression of the MDR1 promoter in C33A and CV-1 cells as reported previously, AML1-ETO strongly activated the promoter in K562 and B210 cells. More importantly, this activation required both the AML1 and ETO portions of the fusion protein, but did not depend on the AML1 binding site in MDR1 promoter. Furthermore, results from promoter deletion analysis and chromatin immunoprecipitation assays suggested that this activation effect was likely through the influence of the general transcription machinery rather than promoter-specific factors. Based on these data, we propose that AML1-ETO may have opposing effects on gene expression depending on the various conditions of the cellular environment.

Introduction

Human acute leukemias are often associated with translocations involving transcription factors which necessarily disrupt the normal transcriptional activities of these proteins [1]. t(8;21) is one of the most common translocations found in the M2 French-American-British subtype of acute myeloid leukemia [2;3]. This translocation involves the AML1 gene (also known as RUNX1, PEBP2A2, and CBFA2) on chromosome 21 and the ETO gene (also known as MTG8 and RUNXT1) on chromosome 8 [4;5].

The AML1 protein binds to the core enhancer motif TGT^T/C GGT along with its heterodimeric, non-DNA binding partner CBF β [6-8]. The normal functions of both AML1 and CBF β are critical for proper hematopoietic development in that knockout mice for either protein lack definitive hematopoiesis [9-11]. ETO is a zinc finger protein and is not believed to bind DNA itself. The AML1-ETO fusion protein has the AML1 DNA binding domain fused to almost the entire ETO protein. It also binds to the same DNA sequence as AML1 [12] and is involved in transcriptional regulation. AML1-ETO is thought to lead to the progression of leukemia by blocking wild-type AML1 function in a dominant negative manner. Heterozygous AML1-ETO knock-in mice died during embryonic development with a similar phenotype as homozygous

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AML1 knockout mice [13;14], supporting the dominant negative theory. Furthermore, reporter-gene assays and protein-protein interaction data provide a further molecular mechanism for this effect. AML1-ETO binds to transcription repressor complexes, such as N-CoR, Sin3A, SMRT, and class 1 histone deacetylases (HDACs), which actively shut down transcription from AML1 responsive promoters [15-20]. However, AML1-ETO does not always function as a transcriptional repressor. For example, expression of AML1-ETO has been shown to transactivate the BCL-2 and AP-1 promoters in reporter gene assays [21]. Additionally, AML1-ETO up-regulates C/EBP ϵ [22] and synergistically activates the M-CSF receptor promoter in combination with AML1 [23]. Therefore, the actual mechanism by which AML1-ETO functions is likely more complex. For example, in mouse models, until recently AML1-ETO had been unable to promote leukemia by itself; however, when the Nervy homology regions 3 and 4 (NHR3 and NHR4) at the C-terminus of AML1-ETO were removed, AML1-ETO became strongly leukemogenic on its own [24;25]. The elimination of these c-terminal domains removed regions of the fusion protein shown to be strongly involved in co-repressor interactions [18;19;26], suggesting that more studies are necessary for understanding the molecular mechanisms of AML1-ETO induced leukemogenesis.

Using the MDR1 promoter as a model, we tested the transcriptional properties of the AML1-ETO fusion protein in various cell lines and found that AML1-ETO had diverse and opposing transcriptional properties depending on the cell types in which the experiments were carried out. Indeed, in the fibroblast CV-1 and epithelial C33A cell lines, AML1-ETO acted to repress transcription from the MDR1 promoter in a manner similar to what has been reported previously [26]. In contrast, when the hematopoietic cells lines K562 and B210 were used, AML1-ETO functioned as a strong activator of transcription. This effect required both the intact DNA binding domain from AML1 as well as the ETO portion. However, an intact consensus AML1 binding site within the promoter was not required for this effect, indicating a possible unique mechanism by which AML1-ETO may regulate transcription in a cell type specific manner.

Materials and methods

Cell lines

Monkey kidney fibroblast cell line CV-1 and human cervical carcinoma cell line C33A were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine. Human myeloid leukemia K562 and U937 cells were cultured in RPMI supplemented with 10% FBS and 1% L-glutamine. Ba/F3-p210 (B210) cells were kindly provided by Dr. George Daley (Boston, MA), which were generated by retrovirally expressing p210-BCR-ABL fusion protein in IL-3 dependent murine B lymphoid Ba/F3 cells [27]. B210 cells were cultured in RPMI supplemented with 10% FBS, 1% L-glutamine, 10% WEHI-3B conditioned medium as a source of IL-3, followed by selection with 1 mg/ml G418 (Life Technologies, Inc.) for two weeks.

Transient transfection and luciferase assay

CV-1 and C33A cells were transfected using the $\text{Ca}_3(\text{PO}_4)_2$ precipitation method of transfection as previously described [28]. Ten μg of promoter-firefly luciferase reporter plasmid and the indicated amount of other DNA constructs with herring sperm DNA as a carrier to a total 20 μg DNA were used in each transfection. 2.5×10^6 K562 cells were electroporated with 20 μg total DNA in a volume of 180 μl serum free RPMI at 152 volts and 950 μF . Cells were grown overnight in 3 ml RPMI supplemented with 10% FBS and 1% L-glutamine. 2.5×10^6 B210 cells were electroporated with 20 μg total DNA (10 μg luciferase construct and the indicated amount of additional plasmid DNA using herring sperm DNA as a carrier) in a volume of 180 μl serum free RPMI at 200 volts and 950 μF . Cells were grown overnight in 3 ml RPMI

supplemented with 10% FCS and 1% L-glutamine. For some of the experiments as indicated in the result section, 1 μ M imatinib (STI571, Novartis) was added to the culture medium after transfection. Promoterless Renilla luciferase expression plasmid pRL-Null was added to each transfection for the measure of transfection efficiency. Cell lysates were prepared 18-24 hours after transfection. Luciferase assays were done using the Promega dual luciferase assay kit following manufacturer's instructions.

Plasmid construction

Human MDR1 promoter-firefly luciferase reporter construct pGL2-MDR1 (MDR1 bp - 137 to +30 in Bgl II site of Promega pGL2-basic vector) has been previously described and was kindly provided by Drs. Scott Hiebert (Nashville, TN) and John Schuetz (Memphis, TN) [29]. The AML1 point mutation within the MDR1 promoter-luciferase construct was made using the Stratagene Quickchange kit following the manufacturer's protocol with the following oligonucleotides to mutate the consensus AML1 binding site within the MDR1 promoter from 5'-TGTGGT-3' to 5'-TGTTAG-3' [30]: 5'-CGGGAGCAGTCATCTAGGAGGCAGATTGGCTGGG-3' and 5'-CCCAGCCAATCAGCCTCCTAACAGATGACTGCTCCCG-3'. The ETS point mutations in the MDR1 promoter luciferase construct were also made using the Stratagene Quickchange kit with the following oligonucleotides: 5'-CATTGAGTAGCGGCTCTTGCTAGCTCAAAAGATCTAAGT-3', 5'-ACTTAGATCTTTTGAGCTAGCAAGAGCCGCTACTCGAATG-3', 5'-CTTCGCTCTCTTTGCCACCTCGAGCCTGAGCTCATTGAG-3', 5'-CTCGAATGAGCTCAGGCTCGAGGTGGCAAAGAGAGCGAAG-3', 5'-CCCCGGCGCTGTTAATGCCAGCCAATCAGC-3', 5'-GCTGATTGGCTGGGCATTAACAGCGCCGGG-3'.

The pGLX2 promoterless luciferase construct (pGLX2-basic) was made using the Stratagene Quickchange-multi kit with the following 5' phosphorylated oligonucleotides to mutate six consensus AML1 binding sites within the pGL2-basic luciferase construct (Promega) from 5'-TGTGGT-3' to 5'-TGTTAG-3': 5'-AGCGCGGCGGGTGTAGGGTTACGCGCAGCG-3', 5'-GCATTTTTTTTACAGCATTCTAGTACTAGTTTGTCCAAACTCATCAATGTATC-3', 5'-TTGACTAGAGATCATAATCAGCCATCTAACATTTGTAGAGGTTTTAC-3', 5'-TCTTGCTTGCTTTGCTATTTACCTAACAAAGGAAAAAGCTGCAC-3' and 5'-TGTGAAACCTTACTTCTGTTAGGTGACATAATTGGACAAAC-3'. MDR1 promoter (bp -137 to +30) was inserted into Bgl II site of pGLX2-basic to form pGLX2-MDR1.

MDR1 promoter deletion constructs were made using PCR mutagenesis. Various upstream oligonucleotides containing an Nhe I site that were complimentary to different sequences within the MDR1 promoter were used in combination with a single antisense oligonucleotide complimentary to the start of the luciferase open reading frame. Upstream oligonucleotide sequences were 5'-AGGGAGCTAGCATCTGTGGTGAGGC-3', 5'-AGTGGGCTAGCTGATTGGCTGGGCAG-3', 5'-AGATTGCTAGCGCAGGAACAGCGCC-3', 5'-ACTGGGCTAGCACAGCGCCGGGG-3', 5'-AAGGGGCTAGCACAGCCGCTTCGC-3' while the downstream oligonucleotide sequence was 5'-CTTTATGTTTTTGGCGTCTTCCA-3'. PCR fragments were cloned into the pGLX2 vector using Nhe I and Hind III.

The luciferase reporter plasmid for the p21^{waf1} promoter in pGL2-basic (p21P) was provided by Dr. Xiao-Fan Wang (Durham, NC) [31]. The Renilla luciferase pRL-null reporter is from Promega.

pCMV5-AML1-ETO, pCMV5-AML1-ETO-L148D, pCMV5-CBF β , and pCMV5 were received from Dr. S. Hiebert (Vanderbilt University, Vanderbilt, TN) [32]. pCMV5-AML1 (1-213) was described previously [33]. pCMV5-AML1-ETOTr was created by replacing pCMV5-AML1-ETO C-terminal BamHI fragment with AML1-ETOTr cDNA BamHI fragment from MigR1-HA-AETr [24]. pCMV5-ETO was generated by inserting ETO cDNA into KpnI/XbaI sites of pCMV5 vector.

Northern blot analysis

RNA was isolated using the RNeasy Mini kit (Qiagen). Ten μ g of RNA were electrophoresed on a 1% formaldehyde gel and probed with an approximately 500 bp radiolabeled Xba I/EcoR I digested luciferase cDNA fragment.

Chromatin immunoprecipitation assay

2×10^6 K562 cells were transfected by electroporation with 1 μ g of pGLX2-basic or pGLX2-MDR1, and with 10 μ g of pFLAG-CMV2 control vector or pFLAG-AML1-ETO. Duplicate electroporations were performed for each luciferase construct with each transcription factor. Approximately 40 hours following electroporation, cells were fixed in 1.1% formaldehyde, nuclei were isolated and DNA was sonicated to obtain chromatin of 0.5 kb using a Branson 450 Sonifier: power setting 2, 3 pulses of 30 seconds as previously described [34]. Soluble chromatin was precleared in sepharose 4G (GE Healthcare) and was diluted to obtain chromatin in 0.1% Triton X-100 and 0.1% sodium deoxycholate. Immunoprecipitations were performed with 2.5 μ g anti-Flag M2 antibody (Sigma) overnight at 4°C with gentle rotation. Immune complexes were precipitated using protein G sepharose (GE Healthcare) containing 1 μ g/ μ l herring sperm DNA and 1 μ g/ μ l BSA. Complexes were washed eight times with RIPA buffer (50 mM Hepes pH 7.6, 1mM EDTA, 0.7% sodium deoxycholate, 1% NP-40, 0.5 M LiCl and 1 mM PMSF) and once with TE (10 mM Tris pH 8.0 and 1 mM EDTA pH 8.0). Protein-DNA crosslinks were reversed by incubation at 65°C overnight. Proteinase K and RNase treatment were performed and purified DNA was obtained. DNA was further purified using a Qiaquick PCR column (Qiagen).

Approximately 2% of the eluted purified DNA was used in real-time PCR reactions with Platinum SYBR green QPCR Supermix (Invitrogen) and 0.5 μ M of forward and reverse primers. Reactions were analyzed using the iCycler iQ (Bio-Rad Laboratories) with melt curve analysis. Products were analyzed by agarose gel electrophoresis to check for the absence of primer dimers and to check for specificity of amplification. Forward primer used was 5'-CGGGAGCAGTCATCTAGGAGGCAGATTGGCTGGG-3' (bp -104 to -67 of MDR1 promoter) and reverse primer was 5'-CTTTATGTTTTTGGCGTCTTCCA-3', which binds in the luciferase gene within the pGL2 vector (Promega). Threshold cycle (Ct) values were obtained for duplicate PCR reactions from two independent electroporations and independent chromatin immunoprecipitations. Ct values were corrected between input Ct values for each electroporation. Relative binding was determined by calculating the fold differences between vector control and AML1-ETO wild type after normalizing the amount of genomic input in each chromatin IP.

Results

AML1-ETO has opposing transcriptional effect in different cells lines

To examine the transcriptional properties of AML1-ETO in various cell-types, the fusion protein was co-transfected into C33A and CV-1 cells along with the MDR1 promoter luciferase construct (Fig. 1A & B). In these cells lines, AML1-ETO was shown to repress basal transcription from this promoter. This data was in agreement with previously published work demonstrating the dominant negative activity of AML1-ETO [15;26]. AML1-ETO had very

little effect on MDR1 promoter activity when these studies were conducted in U937 cells (data not shown). Interestingly, when these same assays were carried out in K562 cells, AML1-ETO no longer repressed transcription from this promoter, but instead increased the luciferase activity from the same promoter construct (Fig. 1C). This result suggests that in K562 cells AML1-ETO functions as a transcriptional activator instead of a repressor in controlling MDR1 promoter activity. Additionally, it suggests that AML1-ETO may function in cooperation with factors present in K562 cells that may be absent in the other cell lines where it behaves as a repressor.

Transcriptional activation by AML1-ETO is not the direct result of BCR-ABL expression in K562 cells

The K562 cell line was established from a Philadelphia chromosome-positive chronic myeloid leukemia patient at blast crisis and expresses the BCR-ABL fusion protein. Despite its malignant origin, K562 cells retain some capacity for differentiation into multiple cell types, a characteristic of the normal multipotent hematopoietic progenitor cell [35-38]. Because these cells express BCR-ABL, we examined whether this was the reason for the activation of the MDR1 promoter observed in the presence of AML1-ETO. As shown in figure 2A, AML1-ETO was still able to activate the promoter in the presence of 1 μ M imatinib, which blocks the function of BCR-ABL [39]. To confirm this result, we also checked the effect of AML1-ETO on the MDR1 promoter using B210 cells, which is a B-cell line expressing BCR-ABL. In B210 cells, AML1-ETO also activated the MDR1 promoter either in the presence or absence of 1 μ M imatinib (Fig. 2B). Additionally, we also examined the possible role of BCR-ABL on AML1-ETO transactivation using U937 cells. As mentioned in the above section, AML1-ETO alone had very little effect on MDR1 promoter activity. When a BCR-ABL expression plasmid was co-transfected in these assays, again, there was almost no change in promoter activity in the presence of AML1-ETO (data not shown). Although these latter experiments cannot rule out any indirect effects resulting from the chronic expression of BCR-ABL, they do suggest that BCR-ABL most likely does not directly transform AML1-ETO from a transcriptional repressor into an activator.

Promoter activation by AML1-ETO is independent of the AML1 binding site

The MDR1 promoter construct contains a single consensus AML1 binding site with the sequence 5'-TGTGGT-3' [26]. To examine whether this AML1 binding site was critical for AML1-ETO activation of the MDR1 promoter, we mutated this site to 5'-TGTTAG-3'. Using a transient transfection assay, we then compared the effect of AML1-ETO between the wild-type promoter and the AML1-site mutant promoter in a luciferase assay (Fig. 3A). Interestingly, the AML1-site mutant promoter was activated more strongly by AML1-ETO than was the wild-type promoter suggesting that the AML1 site somehow titrates away, or reduces, the positive transcriptional potential of AML1-ETO on the MDR1 promoter in K562 cells. A single amino acid substitution in the DNA-binding portion of AML1-ETO (termed AML1-ETO L148D), which has been reported to eliminate DNA binding and CBF β interaction [32], was completely lacking in its ability to alter promoter activity. Taken together, these data suggest a couple possibilities. Either DNA binding to a non-consensus AML1 site is occurring through the runt homology domain to drive the transcriptional activation of this promoter or an interaction with another protein through this domain is occurring for the observed effect in K562 cells. What does not appear to be necessary for the observed results is the binding of AML1-ETO to the consensus AML1 site within the MDR1 promoter luciferase construct.

In an effort to determine whether the ETO portion of this fusion protein was required for the effect, a construct containing only the DNA binding runt homology domain of AML1 [AML1B (1-213), which C-terminal amino acid is equivalent to 177 in AML1b] was tested in a luciferase assay. This portion of AML1 was incapable of activating transcription (Fig. 3A). Furthermore,

expression of AML1-ETO_{tr}, lacking the C-terminal NHR3 and NHR4 domains of the fusion protein, was also used in the assay [24]. The NHR4 domain has been shown to interact with the transcriptional repressors NCoR and SMRT and is important for the reported dominant negative effect of the AML1-ETO fusion protein [18;19;26]. AML1-ETO_{tr} was a less potent inducer of transcription as compared with full-length AML1-ETO; however, it was still able to increase transcription from this promoter unlike L148D or the AML1(1-213) constructs. ETO by itself was also unable to increase promoter activity (Fig. 3B). Indeed, ETO was found to slightly decrease promoter activity in K562 cells.

The MDR1 promoter-luciferase construct used in these assays had been cloned into the pGL2-basic luciferase vector. The vector sequence itself contains six consensus AML1 binding sites located outside of the ampicillin resistance gene and luciferase open reading frames. These sites could potentially bind AML1 or AML1-ETO and give questionable results. To rule out that possibility, point mutations were made in all of the six potential AML1 binding sites within the pGL2-basic luciferase vector. This new vector was named pGLX2-basic. The MDR1 promoter was then cloned into pGLX2-basic and used to examine the activation effect observed by AML1-ETO in K562 cells. As shown in figure 4A, AML1-ETO was still able to activate the MDR1 promoter using this modified vector. Additionally, when the AML1 binding site within the promoter was also mutated, a similar increase in promoter activity was again observed. This was consistent with what was seen previously using the pGL2 series of constructs. These data therefore suggest that activation by AML1-ETO was not through the consensus AML1 sites either within the vector or the promoter.

Because AML1-ETO was not increasing promoter activity directly through the consensus AML1 binding sites it was still possible that it may be interacting with other transcription factors through protein-protein interactions within the promoter. The MDR1 construct used in these experiments contained three ETS core binding motifs (Fig. 4B). AML1 has been shown to interact with ETS family members via its runt homology domain [33;40;41], which remains in AML1-ETO and is required for the transcriptional activation observed in K562 cells. It was therefore possible that AML1-ETO activated transcription through an interaction with ETS factors binding to the MDR1 promoter. The wild-type promoter was therefore compared with promoter constructs containing a point mutation in one, two, or all three of the core ETS motifs (Fig. 4B). When mutations were placed in the 5' and middle ETS binding site, there was no decrease in the level of activation caused by the addition of AML1-ETO, instead there was an increase. This increase in promoter activation was similar to the increase observed when the consensus AML1 site was mutated, suggesting that interaction of AML1-ETO with upstream ETS factors titrates away its activation potential. More importantly, however, no difference in the activation was detected between the wild type promoter and the MDR1 promoter containing mutations in all three ETS binding sites (Fig. 4B). These data indicate that the ETS sites are not responsible for promoter activation in the presence of AML1-ETO.

Multiple regulators of transcription are known to interact with the MDR1 promoter [42;43]. In an effort to locate the site(s) within the promoter that allow AML1-ETO to stimulate promoter activity, a series of deletion mutants were made in the MDR1 promoter (Fig. 4C). The expected result for this set of experiments was that AML1-ETO would activate the promoter until a critical factor was deleted, after which it would have little or no effect on promoter activation. As expected, deletions upstream of the AML1 site had little effect. When the AML1 site itself was deleted, activation of the promoter in response to AML1-ETO was increased. This increase in AML1-ETO induced promoter activity with the loss of the AML1 binding site was consistent with the mutational studies shown earlier (Figs. 3A and 4A). With continued deletions, however, there was a gradual decrease in the transactivation by AML1-ETO, but not an expected sharp drop. Although there appears to be a slight increase in luciferase activity with the addition of AML1-ETO to the empty vector pGLX2, the basal level of the

empty vector was at or below background levels so any subtle changes in either firefly or Renilla luciferase activity became amplified as a result, but are likely not significant. This implied that there was no single critical factor within the promoter that cooperated with AML1-ETO to stimulate promoter activity.

The increase in luciferase activity in the presence of AML1-ETO is a general effect on transcription

If the activation of transcription observed with AML1-ETO was a general effect on transcription as a whole and not specific to the MDR1 promoter, then other promoters should also be increased by the addition of AML1-ETO. As shown in figure 5A this is indeed the case. In a side-by-side comparison with the MDR1 promoter, AML1-ETO was able to increase expression of the p21 promoter luciferase construct. Additionally, AML1-ETO was able to increase luciferase activity in other constructs including the TCR β enhancer and myeloperoxidase promoters (Data not shown).

This result opened the possibility that the increase in luciferase activity may be due to reasons other than promoter activation, such as a non-specific alteration in the protein translation or stability of luciferase mRNA. For this reason, a northern blot was conducted to study whether the increase in luciferase activity correlated with an increase at RNA level. We detected a thirteen-fold increase in luciferase activity with the addition of AML1-ETO in an MDR1 promoter-reporter gene assay (Fig. 5B). This increase in luciferase activity correlated well with the northern blot result, which showed a similar increase in luciferase mRNA in the presence of AML1-ETO (Fig. 5C). Taken together, these data imply that the activation observed in the presence of AML1-ETO is a general effect on transcription and not on luciferase protein stability.

The transactivation effect of AML1-ETO is not via its involvement in MDR1 promoter complex

The above results indicate that AML1-ETO strongly activated the MDR1 promoter independently of an AML1 binding site. Furthermore, no specific region upstream of the transcription initiation site within the MDR1 promoter was identified that could be responsible for the effect seen in the presence of AML1-ETO through protein-protein interactions. To examine whether the transactivation effect of AML1-ETO required interaction with the MDR1 promoter, we performed chromatin immunoprecipitation assays with cells cotransfected with either the wild type MDR1 promoter or a promoter construct containing a mutation in the AML1 binding site. To this, either an AML1-ETO expression plasmid or the vector alone was cotransfected as a control. AML1-ETO was only detected in the promoter complex when the AML1 binding site was present in the promoter (Fig. 6). Since the AML1 binding site was not needed for the transactivation effect, this result indicated that transactivation by AML1-ETO was not through its direct binding to the promoter.

Discussion

Previous studies by many groups have indicated that t(8;21) fusion transcription factor AML1-ETO affects hematopoietic cell proliferation, differentiation, and survival. Some of these effects favor and others oppose leukemogenesis [44]. Analyses of various mouse models of AML1-ETO expression also demonstrate that AML1-ETO plays a critical role in leukemogenesis. By itself however, it is not sufficient for disease development [45-49]. Since AML1-ETO has the AML1 DNA binding domain and binds to the same DNA sequence as AML1, it has been believed that the function of AML1-ETO is through the regulation of gene expression. Indeed, AML1-ETO has been shown to act as a dominant negative inhibitor of AML1 [50]. Multiple repressors of transcription, such as Sin3A, HDAC, N-CoR, and SMRT, interact with the ETO portion of the fusion protein [17-20], which provides the molecular

mechanisms of repression. AML1-ETO has been reported to repress the MDR1 promoter in hematopoietic HEL cells and non-hematopoietic C33A cells [26]. By using the MDR1 promoter as a model to study AML1-ETO, we discovered that AML1-ETO activated the MDR1 promoter in two hematopoietic cell lines (K562 and B210), repressed the promoter in two non-hematopoietic cell lines (CV-1 and C33A), and had no obvious influence to the promoter in myeloid U937 cells. Thus, these studies indicate that AML1-ETO can activate, repress, or have no effect on the expression of the same target gene depending on the cellular conditions.

MDR1 is a large transmembrane protein and mediates energy dependent efflux of a variety of chemotherapeutic agents [43;51]. Therefore, its over expression is directly related to the development of multidrug resistance in leukemia and other cancer patients. Investigations regarding the regulation of MDR1 expression have great clinical value in treating these patients. Both previous reports in combination with our current study indicate that the AML1 binding site in the MDR1 promoter mediates the repression of promoter activity by AML1-ETO in some non-myeloid cell types. However, this study demonstrates that in some hematopoietic cell lines, AML1-ETO can behave as a transcriptional activator. Additionally, as shown in figures 3 and 4, mutation or deletion of the AML1 binding site within the MDR1 promoter further enhances the observed activation. We hypothesize that the initial effect of AML1-ETO on MDR1 is the repression of its expression via this AML1 binding site in the promoter. With leukemia progression, which triggers additional genetic changes and the alteration of currently unidentified gene expression, AML1-ETO becomes an activator of MDR1 expression (and possibly other genes as well) in a manner independent of the AML1 binding site in the MDR1 promoter. This model is well supported by clinical studies. First of all, t(8;21) patients had a high response to initial chemotherapy [52;53]. Second, MDR1 expression was more frequently detected in adult M1 and M2 AML patients than in M3, M4, and M5 [51] and about 40% of M2 patients are t(8;21). Furthermore, relatively high levels of MDR1 expression have been reported in pediatric t(8;21) AML patients and uneven expression has been reported in adult t(8;21) AML patients [54]. It will be informative to compare the relative levels of MDR1 expression on t(8;21) positive AML cells during the initial treatment and after disease progression.

Mutation and deletion analysis of AML1-ETO in K562 cells demonstrated that both the AML1 portion and the ETO portion of the fusion protein were important for its activity and neither was capable of activating the promoter by themselves. The AML1 portion contains the runt homology domain. This domain is responsible for binding DNA, interacting with the AML1 heterodimeric partner CBF β , and participating in protein-protein interactions with at least nine other transcription factors [44]. AML1 binds to gene upstream regulatory elements with its heterodimeric partner CBF β and affects transcription. With the AML1-ETO fusion protein, almost all of ETO is attached to the AML1 runt homology domain. ETO is a zinc finger nuclear protein and no DNA binding has been detected for it. Instead, ETO contains regions highly homologous to the Drosophila Nrvy protein and has been found to interact with transcription factors and negative regulators of transcription [3;44;55]. The current model for the function of AML1 ETO is that it binds to AML1 sites within the promoters of various AML1 target genes through the runt homology domain of the fusion protein. The ETO portion of AML1-ETO then recruits certain repressors of transcription causing a reduction in the expression of these target genes. This study agrees with these models in some non-myeloid cell types (Fig. 1A and B). However, in the various hematopoietic cell lines used in the current study, AML1-ETO was found to consistently activate these same target genes. This effect required both the AML1 runt homology domain and the ETO portion suggesting that a possible interaction between AML1-ETO and a cellular factor found in these hematopoietic cells is occurring. Interestingly, when the AML1 binding site was either mutated or deleted, the activation observed by AML1-ETO was increased. This effect could be explained if the cellular factor

that is recruited by AML1-ETO was able to interact directly with the general transcription machinery.

There are two models, one direct and one indirect, that could explain the data presented in this paper (Fig. 7). Assuming the regulation involves transcription initiation, the direct model is that AML1-ETO binds to a cellular factor present in K562 cells creating a transcriptional activating complex. When an AML1 binding site is present in the promoter, this complex binds to the DNA via the runt homology domain. In this situation, the complex is able to increase promoter activity by a modest extent. In the absence of the AML1 binding site, the activating complex is able to interact directly with the general transcription factor machinery in a positive way resulting in an even greater increase of promoter activity. If the cellular factor is present in K562 cells and absent in CV-1 and C33A cells, this model would explain why AML1-ETO behaves as a transcriptional activator in one cell type and a repressor in the other (Fig. 1). Additionally, this model helps explain the surprising observation that the transcriptional activation induced by AML1-ETO is stronger when the AML1 binding site was either mutated or deleted (Fig. 4). Finally, this model also addresses that this appears to be a general effect that occurs at multiple promoters and does not seem to be specific only to MDR1 (Fig. 5A and data not shown).

A second model that is not ruled out by our data is that AML1-ETO binds to the regulatory element of another gene and changes its expression. In this indirect model, the change in the other gene is responsible for the activation of the MDR1 promoter. Similar types of modulation by p53 family members have been reported for the MDR1 promoter [56]. This mechanism is also similar to the way that AML1-ETO induces expression of the G-CSF receptor through stimulation of C/EBP ϵ in L-G cells [22]. Furthermore, since the site directed mutations or deletions of the upstream regions of the MDR1 promoter did not dramatically diminish the activation by AML1-ETO (Fig. 4C), it is likely that the AML1-ETO activation effect is through the enhancement of the transcription machinery close to transcription initiator.

In summary, we reported here that AML1-ETO can have opposing effect on the expression of the same gene in different cell types. Such different cellular environments may be directly related to the availability of specific cofactors of AML1-ETO or differential gene expression pattern in these cells. These differential effects of AML1-ETO on gene expression may play important roles in the outcome of specific clinical treatment of AML1-ETO related leukemia and may also explain the different stages of leukemia development and their responses to the treatment. The MDR1 promoter provides an excellent model to extend the study in the future.

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Figure 1. A comparison of the differential effect exhibited by AML1-ETO in three different cells lines

pGL2-MDR1 was transfected into C33A cells (A), CV-1 cells (B), and K562 cells (C) in the absence (-) of presence (+) of AML1-ETO expression plasmid. AML1-ETO functions as a transcriptional repressor in both C33A and CV-1 cells (panels A and B) and as a transcriptional activator in K562 cells (panel C). Assays were done in duplicate and repeated two or more times with similar results, data shown are a single representative experiment.

Figure 2. Activation of promoter by AML1-ETO is independent of BCR-ABL

pGL2-MDR1 was transfected into K562 cells or B210 cells either without or with AML1-ETO expression construct (A/E) in duplicate sets. BCR-ABL tyrosine kinase inhibitor Imatinib was added to one set of the cell culture medium after the transfection. Fold increases in promoter activity are relative to that of the promoter in the absence of AML1-ETO.

Figure 3. Both AML1 and ETO portions of AML1-ETO are important for the activation of MDR1 promoter

(A) Various AML1 or AML1-ETO constructs as indicated in the figure were transfected in K562 cells with wild type pGL2-MDR1 or the mutant that contains a mutation at the AML1 binding site. Fold increases in promoter activity are relative to that of each promoter in the absence of any additional transcription factors. (B) AML1 portion is required for the transactivation by AML1-ETO fusion protein. Increasing amounts of AML1-ETO or ETO expression construct (0.5 µg and 2.0 µg) were co-transfected into K562 cells along with pGL2-MDR1. AML1-ETO addition resulted in promoter activation while the addition of ETO alone resulted in a slight decrease in promoter activity.

Figure 4. Analysis of AML1-ETO transactivation of the MDR1 promoter

(A) The MDR1 promoter is activated by AML1-ETO independent of consensus AML1 binding site. pGLX2 based wild type MDR1 promoter luciferase reporter construct pGLX2-MDR1 and AML1 binding site mutant were transfected into K562 cells in the absence or presence of AML1-ETO expression plasmid. (B) MDR1 promoter is activated by AML1-ETO independent of consensus ETS binding sites. pGLX2-MDR1 or one or more consensus ETS binding sites mutated MDR1 promoter constructs as indicated in the figure were transfected into K562 cells in the absence or presence of AML1-ETO expression plasmid. (C) MDR1 promoter deletion analysis of AML1-ETO activation. pGLX2-MDR1 and a series of deletion constructs as indicated in the figure were transfected into K562 cells in the absence or presence of AML1-

ETO expression plasmid. Fold increases in promoter activity are relative to that of each promoter construct in the absence of AML1-ETO.

Figure 5. AML1-ETO activates gene expression in K562 cells

(A) Both p21 and MDR1 promoters are activated by AML1-ETO in K562 cells. p21 promoter luciferase reporter construct p21P and MDR1 promoter luciferase reporter construct pGLX2-MDR1 were transfected into K562 cells in the absence or presence of AML1-ETO expression plasmid. Fold increases in promoter activity are relative to that of each promoter construct in the absence of AML1-ETO. (B) Northern blot analysis of luciferase mRNA. pGLX2-MDR1 was transfected into K562 cells in the absence or presence of AML1-ETO expression plasmid. Twenty-four hours after the transfection, protein lysates were used in luciferase assays (Left panel) and RNA was used in northern blot for the study of luciferase mRNA level (Right panels). EtBr stained gel is shown as RNA loading control.

Figure 6. Chromatin immunoprecipitation of the MDR-1 luciferase promoter

K562 cells were co-transfected in duplicate with vector control or plasmids encoding Flag-AML1-ETO along with the wild type or AML1 binding site mutated pGLX2-MDR1. Forty hours after transfection, chromatin immunoprecipitation was performed with anti-Flag antibody. Real time PCR was performed to study the interaction of AML1-ETO with MDR1 promoter. The bar graph reflects quantitation by real-time PCR of duplicate reactions from duplicate transfections of the wild-type or mutant MDR-1 luciferase promoter bound to vector or AML1-ETO after correcting for differences in chromatin input. The average promoter DNA enrichment in the vector control sample from two independent transfections and chromatin immunoprecipitations was set to 1.

Figure 7. Two possible models by which AML1-ETO can activate gene transcription
 (A) In the direct model, AML1-ETO binds to an already existing cellular factor creating a transcription activating complex. This complex can either bind to an AML1 binding site, increasing promoter activity by a modest extent or, in the absence of an AML1 binding site, interact directly with the general transcription factors increasing promoter activity by a greater extent. Abbreviations: GTFs - general transcription factors, TGTGGT - consensus AML1 binding site, RHD - runt homology domain. (B) In the indirect model, AML1-ETO alters the expression of another gene (activation or repression). The alteration of the expression of this other gene is then able to increase the expression of the MDR1 promoter.