Synergistic up-regulation of the myeloid-specific promoter for the macrophage colony-stimulating factor receptor by AML1 and the t(8;21) fusion protein may contribute to leukemogenesis

KRISTINA L. RHOADES*[†], CHRISTOPHER J. HETHERINGTON*, JANET D. ROWLEY[‡], SCOTT W. HIEBERT[§], GIUSEPPINA NUCIFORA[‡], DANIEL G. TENEN*, AND DONG-ER ZHANG*[¶]

*Division of Hematology/Oncology, Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, MA 02215; [‡]Section of Hematology/Oncology, Department of Medicine, The University of Chicago, Chicago, IL 60637; and [§]Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38101

Contributed by Janet D. Rowley, August 8, 1996

ABSTRACT *AML1* is involved in the (8;21) translocation, associated with acute myelogenous leukemia (AML)-type M2, which results in the production of the AML1-ETO fusion protein: the amino-terminal 177 amino acids of AML1 and the carboxyl-terminal 575 amino acids of ETO. The mechanism by which AML1-ETO accomplishes leukemic transformation is unknown; however, AML1-ETO interferes with AML1 transactivation of such AML1 targets as the T-cell receptor β enhancer and the granulocyte-macrophage colony-stimulating factor promoter. Herein, we explored the effect of AML1-ETO on regulation of a myeloid-specific AML1 target, the macrophage colony-stimulating factor (M-CSF) receptor promoter. We found that AML1-ETO and AML1 work synergistically to transactivate the M-CSF receptor promoter, thus exhibiting a different activity than previously described. Truncation mutants within the ETO portion of AML1-ETO revealed the region of ETO necessary for the cooperativity between AML1 and AML1-ETO lies between amino acids 347 and 540. Endogenous M-CSF receptor expression was examined in Kasumi-1 cells, derived from a patient with AML-M2 t(8;21) and the promonocytic cell line U937. Kasumi-1 cells exhibited a significantly higher level of M-CSF receptor expression than U937 cells. Bone marrow from patients with AML-M2 t(8;21) also exhibited a higher level of expression of M-CSF receptor compared with normal controls. The up-regulation of M-CSF receptor expression by AML1-ETO may contribute to the development of a leukemic state in these patients.

The *AML1* gene is involved in the (8;21) translocation, a frequently occurring chromosomal abnormality associated with the French American British M2 subtype of acute myelogenous leukemia (AML) (1–5). In this translocation the 5' portion of *AML1* is fused with nearly the entire *ETO* (*MTG8*) gene (where ETO is eight twenty one), creating the AML1-ETO fusion protein 752 amino acids in length. The fusion of these two sequences is in frame. Exactly how expression of this fusion protein leads to leukemogenesis is unknown, although production of AML1-ETO may affect AML1 target genes. *AML1* is a member of a family of transcription factors that includes alternatively spliced forms of *AML1* (*pebp2ab/CBFA2*) on chromosome 21, *AML2* (*pebp2ac/CBFA3*) on chromosome 1, and *AML3* (*pebp2aa/CBFA1*) on chromosome 6 (6–11) (where PEPB is polyoma virus enhancer binding protein and CBF is core binding factor). Each of the AML family members contains a region of homology to the *Drosophila runt* protein, a pair rule gene involved in segmentation (10, 12). This *runt* homology domain (rhd) confers the ability of AML1 to bind to its consensus sequence, TGT/CGGT, and

the ability of AML1 to interact with its cofactor CBF β , which enhances the DNA binding ability (8, 9, 13). Several target genes for AML1 have been identified and include the macrophage colony-stimulating factor (M-CSF) receptor (*CSF1R*); T-cell receptor (TCR) α , β , δ , and γ ; neutrophil elastase; myeloperoxidase; granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) (9, 14–21). AML1 family members have been found to transactivate the regulatory sequences of each of these genes. AML1-ETO contains the first 177 amino acids of AML1 including the rhd and can, therefore, bind to the AML1 consensus sequence and also heterodimerize with CBF β (13).

ETO has not been extensively studied although it is known to contain regions conserved in transcription factors, such as three proline/serine/threonine-rich regions, a leucine-rich region, and two zinc finger domains as well as regions of homology to RP8, a rat apoptosis gene, *Drosophila nervy*, a protein important in the development of nervous tissue, and TAF110, a subunit of TFIID (22–24). Wild-type ETO is expressed at high levels only in the brain and at low levels in lung, testis, and ovaries (4, 23). Expression of ETO in hematopoietic tissues is controversial (4, 25). The function of wild-type ETO is unknown. Because AML1-ETO is expressed concurrently with AML1 in patients with AML t(8;21) and because AML1-ETO can also bind to the same DNA sequence as AML1, its effect on AML1 transactivation of target genes is of interest. AML1-ETO has been shown to interfere with AML1 transactivation of the TCR β enhancer and the GM-CSF promoter (11, 26). In this study we investigated the effect of AML1-ETO expression on AML1 transactivation of the M-CSF receptor promoter, which we previously identified as a target for AML1 (27). We report herein a synergistic transactivation by AML1 and AML1-ETO of the M-CSF receptor promoter.

The M-CSF receptor is a 150-kDa integral membrane glycoprotein with tyrosine kinase activity that is responsible for transducing the signal received from M-CSF binding that promotes the development of the monocytic/macrophage lineage (28–32). Normally, expression of the M-CSF receptor is very tightly regulated and restricted to developing and mature monocytes and placental trophoblasts (33, 34), where it is regulated by two different promoters in these two cell types (35, 36). Normal myeloid-specific M-CSF receptor expression

Abbreviations: M-CSF, macrophage colony-stimulating factor; AML, acute myelogenous leukemia; ETO, eight twenty one; CBF, core binding factor; PEBP, polyoma virus enhancer binding protein; TCR, T-cell receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; RT-PCR, reverse transcription-coupled PCR.

[†]Present address: Whitehead Institute, Nine Cambridge Center, Cambridge, MA 02142.

[¶]To whom reprint requests should be addressed at: Harvard Institutes of Medicine, Beth Israel Hospital, Room 953, 77 Avenue Louis Pasteur, Boston, MA 02115.

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is gradually up-regulated as the cells differentiate from stem cells, where there is very low level expression, to mature monocytes, where the expression is high (37–39). We have previously reported the significance of several factors important for the normal myeloid specific expression of the M-CSF receptor, including PU.1, C/EBP, and AML1 (27, 40, 41). PU.1 is crucial in TATAA-less promoters, such as M-CSF receptor, for the initiation of transcription in a myeloid-specific fashion (40). C/EBP and AML1 act synergistically to greatly up-regulate expression of the M-CSF receptor promoter (41). The presence and appropriate balance of these factors are necessary for appropriate myeloid-specific expression of the M-CSF receptor and a disturbance in the function of one of these factors, as occurs in the (8;21) translocation, might lead to aberrant expression.

Signaling through the M-CSF receptor promotes cell proliferation by inducing the G₁-S phase transition because of the effect on cyclins D and E (42, 43). This effect on proliferation can be tied in with the oncogenic nature of the M-CSF receptor, which was first discovered as the cellular counterpart to the transforming oncogene, *v-fms* (44). Overexpression of the M-CSF receptor causes transformation of NIH 3T3 cells and in mice leads to a myeloblastic leukemia (45, 46). Therefore, it is important to study the possible role of M-CSF receptor in human myeloid leukemia, especially in AML-M2 t(8;21) in which the *AML1* gene is affected. In this report we also investigate the effect of AML1 and AML1-ETO concurrent expression on M-CSF receptor expression in the bone marrow of patients with AML-M2. Our findings indicate that a premature up-regulation of M-CSF receptor expression could be one of the factors contributing to leukemogenesis in acute myelogenous leukemia.

MATERIALS AND METHODS

Cell Culture and Patient Samples. Human promonocytic U937 cells (CRL 1593; American Type Culture Collection, ATCC) were maintained in RPMI 1640 medium (GIBCO/BRL) supplemented with 10% fetal bovine serum (HyClone) and 2 mM L-glutamine (GIBCO/BRL). African green monkey kidney fibroblast-like cells (ATCC CCL 70) were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) with 10% calf serum (HyClone) and 2 mM L-glutamine (GIBCO/BRL). Bone marrow aspirates from patients diagnosed with AML-M2 were obtained with informed consent. Cytogenetics studies were performed to confirm the presence or absence of t(8;21) (University of Chicago).

Plasmid Construction. The wild-type M-CSF receptor promoter luciferase reporter construct, pM-CSF-R-luc, containing positions -416 to +71 of the human M-CSF receptor promoter was described (40). The AML1B, AML1-ETO, and CBF β cDNAs were subcloned into the pCMV5 as described (11). The 3' deletions of AML1-ETO were made as described (47).

Transient Transfection. CV-1 cells and U937 cells were transfected as described (41, 48).

Reverse Transcription-Coupled PCR (RT-PCR) Analysis. RT-PCR analysis of RNA from patient samples was performed with 0.5 μ g of RNA from each bone marrow sample. Reverse transcription reactions were performed in the presence of all four dNTPs (each at 1.25 mM), 100 pM pdN6 (Pharmacia), 4 μ l of 5 $\times$ reaction buffer, 200 units of Moloney murine leukemia virus (MMLV) reverse transcriptase, 20 units of RNasin in a total volume of 20 μ l and incubated for 10 min at 65°C with just random hexamers and then for 1 h at 42°C with the other components. The volume of the RT reaction was adjusted to 50 μ l for each sample and 4 μ l was used for the PCRs. For *AML1-ETO*-nested PCR the RT reaction was performed under the same conditions with the exception that 50 ng of the external nested primer (ETOn) was used for first-strand synthesis. The PCR conditions for β_2 -microglobu-

lin, *AML1B*, *MCSF* receptor, and *AML1-ETO* within the linear range are as follows: 10 mM Tris-HCl, pH 8.3/50 mM KCl/0.5 mM MgCl₂/all four dNTPs (each at 0.1 mM)/5 pM of each primer. Amplifications were performed as follows: denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. This cycle was repeated 30 times and then extended at 72°C for 5 min. Each PCR was performed with one of the primers labeled with ³²P and the product was separated on a 4% polyacrylamide gel that was dried and then exposed to XAR film.

Oligonucleotide Primers. The sequences of the oligonucleotides are as follows: external nested ETO, 5'-GCTGTAGGAGAATGGCT-3'; sense ETO, 5'-AGCTTCAC-TCTGACCATC-3'; antisense ETO, 5'-TGAAGTGGTTCT-TGGAGC-3'; sense β_2 -microglobulin, 5'-AACCACGTG-ACCTTGTCTACAGC-3'; antisense β_2 -microglobulin, 5'-CTGCTCAGATACATCAAACATG-3'; sense AML1, 5'-TCGCTTTCAAGGTGGTG-3'; antisense AML1, 5'-GCCT-TGTATCCTGCATCTGA-3'; sense M-CSF receptor, 5'-AATTTAATACGACTCACTATAGGGAGGAATCCCAG-TGATAGAG-3'; antisense M-CSF receptor, 5'-CAGACAG-GGCAGTAGTGCG-3', sense.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from cell lines and patient samples by guanidinium isothiocyanate lysis and centrifugation through cesium chloride. A 10- μ g sample of total RNA was subjected to gel electrophoresis on a 1.0% agarose/2.2 M formaldehyde gel and subsequently transferred to Biotrans nylon membrane (Bio-Rad). The membrane was probed first with ³²P random primed labeled M-CSF receptor probe in Church-Gilbert hybridization buffer. The membrane was washed in 0.2 $\times$ standard saline citrate (SSC)/0.1% SDS and exposed to XAR film. The membrane was then probed with a ³²P-labeled oligonucleotide specific for 18S rRNA in 50% formamide/5 $\times$ SSC/5 $\times$ Denhardt's solution/0.1% SDS/0.5 M Na₂PO₄/salmon sperm DNA (250 μ g/ml). The membrane was then washed with 6 $\times$ SSC/0.1% SDS and exposed to XAR film. The autoradiographs were converted to TIFF files using a Hewlett-Packard ScanJet II CX scanner. RNA levels in each lane were quantitated using the IMAGEQUANT program, which quantitates the intensity of the band at each position. The amount of RNA in each lane was based upon the amount of 18S rRNA present in each lane.

RESULTS

AML1B and AML1-ETO Act Synergistically to Transactivate the M-CSF Receptor Promoter. Previously, we have reported that AML1B and C/EBP synergistically direct myeloid-specific M-CSF receptor promoter activity (41). If the function of one of these factors is altered, then one might expect that M-CSF receptor expression would change in response. The involvement of AML1B in the (8;21) translocation leads to expression of AML1B and the AML1-ETO fusion protein in the same blast cell (2, 4). AML1B and AML1-ETO both contain the AML1 DNA binding and heterodimerization *runt* homology domain (13). Others have reported the ability of the AML1-ETO fusion protein to inhibit or repress AML1B transactivation of the TCR β enhancer and GM-CSF promoter through the AML1B binding sequence (11, 26).

To analyze the effect of the AML1-ETO fusion protein on expression of the M-CSF receptor promoter, we performed cotransactivation experiments in CV-1 cells as shown in Fig. 1A. When cotransfected with CBF β and C/EBP α , AML1-ETO has no significant effect on M-CSF receptor promoter activity, whereas AML1B cotransfected with CBF β and C/EBP α causes a 39-fold transactivation. When cotransfected with AML1B, CBF β , and C/EBP α , AML1-ETO caused a synergistic transactivation of the M-CSF receptor promoter to

FIG. 1. (A) CV-1 cells were transfected with the M-CSF receptor promoter luciferase construct pM-CSF-R-luc in the presence or absence of different amounts of AML1B and AML1-ETO expression constructs, pCMV5-AML1B and pCMV5-AML1-ETO, respectively. Each transfection includes C/EBP α and C/EBP β expression constructs, pCMV5-C/EBP β (1 μ g) and pMSV-C/EBP α (1 μ g), respectively. The total amount of DNA in each transfection was 20 μ g, which was standardized with empty expression vector and carrier DNA. The results are expressed as fold increase over activity of the M-CSF promoter luciferase construct alone. The data are the averages of three sets of experiments. Error bars indicate the standard deviation. (B) CV-1 cells were transfected as in A, except in the absence of C/EBP α . (C) RT-PCR of AML1 and AML1-ETO from Kasumi-1 and U937 cells. The primers used for amplification of AML1-ETO are as follows: sense AML1, 5'-TCGCTTTCAAGGTGGTG-3'; antisense ETO, 5'-AGGCTGTAGGAGAATGG-3'.

90-fold. We observed an increasing level of cooperativity between AML1B and AML1-ETO to transactivate the M-CSF receptor promoter when the amount of AML1B was held constant at 1 μ g in each transfection and the amount of AML1-ETO was increased from 0.1 μ g to 1 μ g.

The transfections above have all contained C/EBP β and C/EBP α . To determine if the presence of C/EBP α was necessary for the synergistic effect between AML1B and AML1-ETO, we performed the same transfections in the absence of C/EBP α . This same synergistic transactivation was seen also when C/EBP α was not cotransfected with AML1B, AML1-ETO, and C/EBP β , as shown in Fig. 1B. In this case, AML1B and C/EBP β alone caused a 6-fold transactivation while AML1-ETO and C/EBP β alone caused a 2- to 3-fold transactivation. Together, AML1, AML1-ETO, and C/EBP β caused a 28-fold transactivation of the M-CSF receptor promoter. Thus, it appears that C/EBP α is not necessary for the synergistic interaction between AML1B and AML1-ETO, since CV-1 cells do not contain detectable levels of endogenous C/EBP α (41). A titration of the amount of AML1-ETO expression plasmid in the cotransfection with AML1B and C/EBP β showed an increasing cooperative transactivation until both AML1B and AML1-ETO were present at equal levels (Fig. 1B). The amount of transactivation decreased when the amount of AML1-ETO was increased from 1 μ g to 5 μ g while

the amount of AML1 was held constant at 1 μ g, which is consistent with the finding that AML1-ETO does not transactivate the promoter alone. Higher amounts of AML1-ETO alone had no effect on promoter activity. Thus, a 1:1 ratio of AML1 and AML1-ETO resulted in the optimal synergism to transactivate the M-CSF receptor promoter. RT-PCR analysis of AML1 and AML1-ETO expression in Kasumi-1 cells revealed that they are expressed at a 1:1 ratio, as shown in Fig. 1C, and it has recently been reported that the half-life of these proteins is comparable (26).

The previous transfections were done in the monkey kidney CV-1 cell line. However, the (8;21) translocation occurs in a myeloid precursor that then expresses both AML1B and AML1-ETO. To assess whether AML1-ETO would show the same cooperative effect with AML1B in a myeloid cell line, we performed cotransfection of the M-CSF receptor promoter with AML1B and AML1-ETO in the U937 promonocytic cell line. The same synergistic transactivation of the M-CSF receptor promoter by AML1B and AML1-ETO was also observed in the U937 promonocytic cell line as shown in Fig. 2. The fold transactivation by AML1B was approximately 5.5-fold over basal promoter activity in the presence of only C/EBP β . Cotransfection of AML1-ETO again caused a synergistic transactivation raising the fold transactivation in the presence of C/EBP β from 5.5- to 27-fold. Thus the cooperative transactivation of the M-CSF receptor by AML1B and AML1-ETO can also occur in a myeloid cell line that contains endogenous myeloid-specific factors.

Identification of Regions of AML1-ETO Required for Cooperative Transactivation with AML1B of the M-CSF Receptor Promoter. AML1-ETO contains amino acids 1-177 of AML1B, including the rhf, and the carboxyl-terminal 575 amino acids of ETO (4). To determine which regions of AML1-ETO are required for the cooperative transactivation of M-CSF receptor promoter with AML1B, we performed cotransfection experiments in CV-1 cells with AML1B and wild-type AML1-ETO or truncation mutants of AML1-ETO, as shown in Fig. 3. Truncation to amino acid 592, 579, or 540 did not significantly reduce the ability of AML1-ETO to synergize with AML1B. These truncations delete the two zinc finger domains and one of the proline/serine/threonine-rich regions. Truncation to amino acid 469 lowers the ability of AML1-ETO to transactivate to $\approx$ 50%. Further truncation into the leucine-rich region to amino acid 347 completely eliminates the ability of AML1-ETO to transactivate the M-CSF receptor promoter cooperatively with AML1B. The truncation to amino acid 347 also deletes a portion of the region of homology to TAF110 and *Drosophila* nery, which runs from amino acids 242-409. Thus it appears that the ETO

FIG. 2. U937 cells were transfected with the M-CSF receptor promoter luciferase construct pM-CSF-R-luc in the presence or absence of 1 μ g of AML1B expression constructs pCMV5-AML1B and different amounts of the AML1-ETO expression construct pCMV5-AML1-ETO. Each transfection includes 1 μ g of the C/EBP β expression construct pCMV5-C/EBP β . The results are expressed as fold increase over activity of the M-CSF promoter luciferase construct alone. The data are the averages of three sets of experiments. Error bars indicate the standard deviation.

FIG. 3. CV-1 cells were transfected with the M-CSF receptor promoter luciferase construct pM-CSF-R-luc and 1 μ g of the wild-type (WT) AML1-ETO expression construct pCMV5-AML/ETO or 1 μ g of an expression construct with a truncated AML1-ETO. These constructs express AML1-ETO proteins truncated at amino acid 592, amino acid 579, amino acid 540, amino acid 469, amino acid 347, and amino acid 302 of the ETO protein, as indicated. Each transfection includes the AML1B and the CBF β expression constructs, pCMV5-AML1B (1 μ g) and pCMV5-CBF β (1 μ g), respectively. The results are expressed as fold increase over AML1B transactivation of the M-CSF promoter luciferase construct alone. The data are the averages of four sets of experiments. Error bars indicate the standard deviation.

portion of AML1-ETO necessary for the synergistic transactivation with AML1B lies between amino acids 347 and 540.

M-CSF Receptor Expression in Cell Lines and AML-M2 Patient Samples. The data from transient transfections indicate that M-CSF receptor expression is up-regulated by AML1-ETO in combination with AML1B. In light of this evidence, we examined the effect of AML1-ETO expression on endogenous M-CSF receptor expression. Using RT-PCR we examined the levels of M-CSF receptor expression in U937 cells, and also in a leukemic cell line, Kasumi-1, derived from a patient with AML-M2 t(8;21). In this analysis, Kasumi-1 cells express significantly higher levels of M-CSF receptor RNA than do the U937 cells (10-fold), as shown in Fig. 4. β_2 -Microglobulin was used as an internal standard for the amount of RNA in these samples. The data indicate that expression of the AML1-ETO fusion protein in myeloid cells correlates with the level of M-CSF receptor expression.

The data from analysis of the cell lines led us then to question what happens to the levels of M-CSF receptor expression in patients with AML-M2 t(8;21). To address this question, we examined RNA extracted from the bone marrow of patients with AML-M2 t(8;21) by Northern blot analysis. On this same gel, we included samples from a normal donor and also from patients with AML-M2 with a non-t(8;21) karyotype. Also included are RNA samples from HeLa cells, vitamin D₃-treated THP-1 cells, and human monocytes. The Northern blot analysis is shown in Fig. 5. M-CSF receptor expression was assessed and quantitated. The level of expression from each sample was compared with the amount of total RNA from that sample as quantitated by the amount of 18S RNA present on the gel. The amount of M-CSF receptor RNA is then expressed as percent of 18S RNA expression in Fig. 5. M-CSF receptor expression in both patients with AML-M2 t(8;21) is high as compared M-CSF expression in bone marrow from a normal donor and is as abundant as expression in mature monocytes, where the level of expression is known to be high.

FIG. 4. Ethidium bromide-stained RT-PCR fragments of AML1, AML1-ETO, M-CSF receptor, and β_2 -microglobulin, as an internal control, from Kasumi-1 cells (lanes K) and U937 cells (lanes U). Lanes - contain RT-PCR products in the absence of an RNA template as a negative control. Lanes M contain the molecular weight markers. Nested PCR was used for AML1-ETO amplification with the primers described in text. The external nested primer was used for the RT reaction rather than random hexamers.

Not all of the patient samples available yielded enough RNA for Northern blot analysis. We, therefore, used RT-PCR to examine the levels of M-CSF receptor expression in the patient samples that had smaller amounts RNA. For these studies we used β_2 -microglobulin as a standard for the amount of RNA in each sample. We examined M-CSF receptor, AML1B and AML1-ETO expression for each of the samples, as shown in Fig. 6. The levels of expression of M-CSF receptor was then compared with the amount of RNA in each sample and expressed as percent β_2 -microglobulin. As in the Northern blot analysis, these data show that high levels of M-CSF receptor expression can be detected in three of the four patients with AML-M2 t(8;21). M-CSF receptor expression is also higher in patients with AML M2 non-t(8;21) compared with normal controls and may be up-regulated in response to altered expression of other factors involved in the regulation of M-CSF receptor expression, such as C/EBP α or PU.1. AML1B expression is fairly comparable in each of the patient samples and each of the cell lines, with the exception of HeLa cells and the normal control which express significantly less AML1B. t(8;21) cells were positive for expression of AML1-ETO (data not shown).

DISCUSSION

AML1 was first identified because of its involvement in AML-M2 with t(8;21) and has since been discovered to also be involved in other translocations, such as the t(3;21) in therapy-related acute myeloid leukemia/myelodysplasia or chronic myelogenous leukemia in blast crisis and also the t(12;21) in childhood B-cell acute lymphoblastic leukemias (4, 49, 50). AML1 is normally expressed in hematopoietic tissue and during myeloid differentiation (6). Knockout studies of AML1

FIG. 5. (A) Northern blot analysis of 10 μ g of total RNA from U937 cells, Kasumi-1 cells, THP-1 cells treated for 6 h with vitamin D₃, and HeLa cells for M-CSF receptor expression. (B) Northern blot analysis of 10 μ g of total RNA from bone marrow of patients with AML-M2 t(8;21), patients with AML-M2 with a normal karyotype, a normal donor (N), and RNA from THP-1 cells treated for 6 h with vitamin D₃, HeLa cells, and human monocytes. These autoradiographs were used to quantitate expression of M-CSF receptor and 18S RNA for lanes that are light on the exposure shown.

FIG. 6. RT-PCR analysis of 0.5 μ g of total RNA from bone marrow of patients with AML-M2 t(8;21), patients with AML-M2 with a normal karyotype, and RNA from U937 cells, Kasumi-1 cells, THP-1 cells treated for 6 h with vitamin D₃, HeLa cells, and human monocytes. PCR was performed with ³²P-labeled primer, electrophoresed on 4% acrylamide gel, which was dried and exposed to film.

have determined that AML1 is necessary for normal development of all hematopoietic lineages (51, 52). Several targets have been identified for AML1 including the TCR α , β , δ , and γ , myeloperoxidase, neutrophil elastase, M-CSF receptor, GM-CSF, and interleukin 3 (9, 14–21, 26, 27). The mechanism by which AML1-ETO causes leukemogenesis has not been elucidated, although the AML1-ETO fusion protein has been shown to interfere with normal AML1 function. In regard to the regulation of the TCR β enhancer and the GM-CSF promoter, AML1 causes an increase in enhancer or promoter activity and AML1-ETO acts as a transdominant negative regulator to repress AML1 transactivation (11, 26). It has recently been reported that the t(12;21) fusion protein TEL-AML1 also causes the same interference with AML1 transactivation of the TCR β enhancer (53). Herein we have demonstrated that AML1-ETO and AML1 synergize to transactivate the M-CSF receptor promoter, an AML1 target which is crucial for myeloid differentiation and proliferation. This indicates that AML1-ETO not only can repress but also can enhance AML1 transactivation of its target genes.

Expression of the M-CSF receptor is very low in hematopoietic stem cells and increases as the cells begin to differentiate (37–39). Premature expression in early progenitors may cause aberrant proliferation. Experiments have demonstrated that signaling through the M-CSF receptor in a multilineage progenitor cell promotes only proliferation by inducing the G₁-S phase transition but does not result in differentiation at this stage (54, 55). Therefore, normal timely expression of this molecule is crucial for normal differentiation into the monocyte/macrophage lineage and expression too early could contribute to aberrant proliferation. We have previously described several factors critical to normal expression of the M-CSF receptor, including PU.1, C/EBP α , and AML1 (27, 40, 41). However, PU.1 is expressed not only in myeloid cells but also in B cells (56, 57). AML1 expression is also not restricted to myeloid cells but is also expressed in other hematopoietic cells, nervous tissue, skeletal muscle, and reproductive tissue (58). Likewise C/EBP α is expressed in myeloid cells and hepatocytes as well as adipocytes (59). Although each of these factors is expressed elsewhere, they are all concurrently expressed during myeloid hematopoiesis. Thus, it is presumably the unique balance of each of these factors in normal myeloid differentiation that accounts for the myeloid-specific expression of the M-CSF receptor.

Abnormal expression of the M-CSF receptor is also very important and has been associated with leukemic development. M-CSF receptor was first identified as the cellular counterpart to the transforming oncogene *v-fms* (44). Overexpression of the M-CSF receptor in mice leads to a murine myeloblastic leukemia, thus exhibiting its oncogenic potential (46). It is possible that abnormal expression of the M-CSF receptor might lead to development of leukemia in humans as

well. Because M-CSF receptor expression is dependent on a combination of factors, if one of the factors were not expressed normally or if its function were altered, this could lead to aberrant expression of the M-CSF receptor. Since AML1 is one of the key regulators of M-CSF receptor expression and expression of AML1-ETO may alter the effect of AML1 on its target genes, it is important to study the effect of AML1-ETO on expression of the M-CSF receptor promoter.

Our studies have demonstrated that the AML1-ETO fusion protein does not act as a transdominant repressor in this system but, rather, acts to synergistically up-regulate M-CSF receptor promoter activity with AML1B. In the transient transfection done with the M-CSF receptor promoter, CBF β , the cofactor for AML1B, was necessary for the synergistic effect that was observed. While C/EBP α did show a synergistic effect with AML1B alone, it was not necessary for the cooperative transactivation by AML1B and AML1-ETO. Thus it would be possible to see an up-regulation of M-CSF receptor expression even in low levels or absence of C/EBP α .

The AML1-ETO fusion protein contains the entire rhd of AML1B. The function of this region has been extensively studied and is necessary for the ability of AML1B to bind to the consensus sequence, TGT/CGGT, and also the ability of AML1B to interact with the cofactor CBF β (13). The ETO portion of AML1-ETO has been less well characterized, although it has been found to contain several regions of similarity to other proteins. It contains two putative zinc finger DNA binding motifs similar to that of a cell-death-associated protein RP8, one resembling a type found in the glucocorticoid receptor family and one found in retroviral DNA binding proteins (23). These two zinc finger motifs are not necessary for the synergistic interaction of AML1-ETO with AML1B. ETO also contains three proline-rich regions, two of which also have a high serine and threonine content, a common characteristic of transcription factors, and a leucine-rich region, a feature characteristic of protein-protein interactions. Also within ETO are areas of similarity to *Drosophila nervy*, and TAF110, a subunit of TFIID (24). The truncation that leads to a complete loss in the ability of AML1-ETO to synergize with AML1B deletes a portion of the leucine-rich region, which also contains the region of similarity to TAF110. TAF110 is able to interact with Sp1 (60). It is possible that this region of similarity allows AML1-ETO to interact with other transcription factor such as Sp1 or perhaps with the transcriptional machinery itself. The mechanism of AML1-ETO transactivation is not understood. Preliminary studies with mutants within the rhd suggest that DNA binding function of this region is not necessary for the transactivation by AML1-ETO, whereas the protein-protein interaction is important. The mechanism of the synergy between AML1 and AML1-ETO may rely upon AML1 binding to DNA where AML1-ETO can possibly interact with it as well as other transcription factors or the transcriptional machinery. This question will require further investigation.

Examination of the patient data indicated that in AML-M2 patients with the (8;21) translocation M-CSF receptor is highly expressed compared with bone marrow from a normal donor. Early myeloid cells make very little M-CSF receptor mRNA compared with mature cells (37–39). However, blast cells in the bone marrow of these AML-M2 patients express relatively large amounts of M-CSF receptor mRNA for that stage of myeloid differentiation. This evidence is supported by the transient transfection data. In the patients with t(8;21), AML1B and AML1-ETO are both expressed, most likely at equal levels, since they are both regulated by the AML1B promoter. This coexpression, which in other systems may cause a down regulation of gene expression, particularly of TCR β and GM-CSF, may cause an over expression of M-CSF receptor, which, subsequently, may contribute to leukemogenesis in AML-M2. Leukemogenesis is a complex event that overrides

the cell's normal controls to cause a block in differentiation as well as increased proliferation. In AML-M2 t(8;21), the production of the AML1-ETO fusion protein is what causes these events to occur by causing altered expression of other genes, in particular M-CSF receptor, which are crucial for normal differentiation and proliferation. Because of its oncogenic potential, the M-CSF receptor is a good candidate target for AML1-ETO since increased levels of M-CSF receptor in early myeloid cells may lead to transformation into a leukemic state. It is also likely that the expression of other AML1 target genes that have not yet been identified would be altered due to the expression of the t(8;21) fusion protein, AML1-ETO. Further studies will be necessary to identify these target genes and also elucidate the mechanism by which AML1-ETO exerts its effects.

We thank N. Kamada for providing the Kasumi-1 cell line, D. A. Gonzalez for synthesizing oligonucleotides, and M. Petrovick and I. Oliff for helpful discussions. This work was supported by National Institutes of Health Grants CA68727-02 (K.L.R.), CA/AI59589 (D.E.Z.), CA64140 (S.W.H.), CA41456 (D.G.T.), CA67189 (G.N.), and CA42557 and Department of Energy Grant DE-FG02-86ER60408 (J.D.R.), and American Cancer Society Grants DHP-166 (D.E.Z.) and JFRA-591 (S.W.H.). G.N. is a Special Fellow of the Leukemia Society of America.

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