Methylation-Independent Silencing of the Tumor Suppressor *INK4b* (p15) by CBFβ-SMMHC in Acute Myelogenous Leukemia with inv(16)

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Abstract

The tumor suppressor gene *INK4b* (p15) is silenced by CpG island hypermethylation in most acute myelogenous leukemias (AML), and this epigenetic phenomenon can be reversed by treatment with hypomethylating agents. Thus far, it was not investigated whether *INK4b* is hypermethylated in all cytogenetic subtypes of AML. A comparison of levels of *INK4b* methylation in AML with the three most common cytogenetic alterations, inv(16), t(8;21), and t(15;17), revealed a strikingly low level of methylation in all leukemias with inv(16) compared with the other types. Surprisingly, the expression level of *INK4b* in inv(16)+ AML samples was low and comparable with that of the other subtypes. An investigation into an alternative mechanism of *INK4b* silencing determined that the loss of *INK4b* expression was caused by inv(16)-encoded core binding factor β-smooth muscle myosin heavy chain (CBFβ-SMMHC). The silencing was manifested in an inability to activate the normal expression of *INK4b* RNA as shown in vitamin D3–treated U937 cells expressing CBFβ-SMMHC. CBFβ-SMMHC was shown to displace RUNX1 from a newly determined CBF site in the promoter of *INK4b*. Importantly, this study (*a*) establishes that the gene encoding the tumor suppressor $p15^{INK4b}$ is a target of CBFβ-SMMHC, a finding relevant to the leukemogenesis process, and (*b*) indicates that, in patients with inv(16)-containing AML, reexpression from the *INK4b* locus in the leukemia would not be predicted to occur using hypomethylating drugs. [Cancer Res 2007; 67(3):992–1000]

Introduction

The *INK4b* (*CDKN2B*) gene encodes a 15-kDa protein that is a cyclin-dependent kinase inhibitor (CDKI; ref. 1). A role for $p15^{INK4b}$ as a tumor suppressor in acute myelogenous leukemia (AML) has become established from multiple studies. Hypermethylation of the CpG island that includes the promoter region, exon 1, and part of intron 1 has been shown to occur in up to 80% of human AML, and this epigenetic state is associated with reduced expression (2–4). In acute promyelocytic leukemia, detection of hypermethylation is associated with poor prognosis (5). This epigenetic alteration of *INK4b* is also observed in a high percentage of cases of myelodysplastic syndrome (MDS) and provides a marker for subsequent transformation in patients that progress to leukemia (6–8). Support for a role of $p15^{INK4b}$ as a tumor suppressor also comes from studies carried out in mice. The gene was hypermethylated in experimentally induced AML, and $p15^{INK4b}$ deficiency was shown to increase susceptibility to myeloid leukemia (9). Interestingly, mice that were heterozygous for loss of *INK4b* were more susceptible than wild-type mice and the second allele was hypermethylated in most cases of the leukemia (9).

It is not clear why *INK4b* is highly likely to be inactivated in human AML whereas genes encoding other CDKIs, such as $p16^{INK4A}$ and $p21^{WafCip}$, are not (3). Furthermore, it is not known why the mechanism of inactivation is hypermethylation rather than deletion. Its inactivation in leukemia is probably related to what seems to be a critical role for this CDKI in differentiation and growth arrest in hematopoietic cells. $p15^{INK4b}$ is expressed in the monocyte lineage in association with differentiation, and its expression is increased in hematopoietic cells in response to growth-arresting cytokines, such as interleukin-6, IFNs, and transforming growth factor-β (9–15). Ectopic expression of the gene induces growth arrest in myeloid progenitor cells (16). It could be presumed that preleukemic cells escape from growth arrest, at least in part, by inactivating *INK4b* and that this event is associated with progression to a more aggressive phenotype.

Inhibitors of DNA methylation are promising options for the treatment of AML and MDS. This is in part because hypermethylation of *INK4b* is so prevalent in these diseases and is reversible (reviewed in refs. 17, 18). Two agents, 5-azacytidine (azacitidine) and 5-aza-2′-deoxycytidine (decitabine), which act as DNA methyltransferase inhibitors, are effective in inhibiting methylation and reactivating a variety of genes, including *INK4b*, both *in vitro* and *in vivo* (17, 19).

As treatments for reversing aberrant methylation become more effective, it is increasingly important to be able to identify those subgroups of AML that would be more likely to respond to such treatment. A previous study evaluated *INK4b* methylation based on AML morphology and found that hypermethylation was predominantly associated with M2 and M4 subtypes (20). Despite categorization of AML based on cytogenetic abnormalities, there has been no attempt to correlate epigenetic inactivation of *INK4b* with known cytogenetic abnormalities. We have taken three groups of leukemia with the common translocations, t(8;21), inv(16), and t(15;17), which contain the fusion genes *AML1-ETO, CBFB-MYH11*, and *PML-RARA*, respectively, and have made correlations with the

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epigenetic inactivation of *INK4b*. Altogether, these translocations make up ~31% of all cases of AML.

As a result of this study, it was discovered that AML with inv(16) was the only one that did not show hypermethylation of the gene. We present evidence that, in this leukemia subset, the core binding factor β-smooth muscle myosin heavy chain (CBFβ-SMMHC) fusion protein encoded by *CBFB-MYH11* causes transcriptional suppression of *INK4b*. Presumably, this type of silencing is sufficient to reduce expression in the absence of hypermethylation. Our data identify an important target gene of the chimeric CBFβ-SMMHC protein, which is critical to the leukemogenesis process.

Materials and Methods

Patient samples and cell lines. Patient blood and bone marrow samples were from Rush Cancer Institute and the University of Chicago (Chicago, IL; Supplementary Table S1). Cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Hyclone, Logan, UT).

Plasmids and virus infection. A retrovirus expressing CBFβ-SMMHC (MiG/inv16) was prepared by methods previously described (21) and used to infect U937 cells (ATCC 1593-CRL; American Type Culture Collection, Manassas, VA). Green fluorescent protein was used to sort virus-containing cells. The human *INK4b* promoter sequence was amplified from the bacterial artificial chromosome clone RP-11 145e5 (Invitrogen) using primers P1 and P2 (Supplementary Table S2). The fragment was cloned into *Kpn*I/*Xho*I-digested pGL4.10 vector (Promega, Madison, WI). A mutation within the CBF-binding site was introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene, Lajolla, CA) and primers CBFm1 and CBFm2 (Supplementary Table S2). Vectors encoding RUNX1 and PU.1 were described previously (12, 22).

Methylation-specific PCR and bisulfite genomic sequencing. Genomic DNA (2 μg) was treated with bisulfite as described (23). For methylation-specific PCR (MSP), the CpG Wiz p15 Amplification kit (Chemicon International, Temecula, CA) was used, which included primers and positive and negative methylation controls. The reaction, which consisted of 12.5 μL 2× TAP Master Mix (Continental Lab Products, San Diego, CA), 1 μL primer mix, and 2 μL bisulfite-treated DNA in a final volume of 25 μL, was carried out under the following conditions: 95°C for 15 min followed by 35 cycles of 94°C for 1 min, 60.5°C for 30 s, and 72°C for 1 min and terminated at 72°C for 10 min.

The bisulfite genomic sequencing protocol was carried out as previously described (11) with several modifications. Following bisulfite treatment, DNA was amplified using primers BS1 and BS2 (Supplementary Table S2) and TAP Master Mix. PCR products (811 bp) were cloned into pCR4-TOPO and sequenced using the M13 reverse primer and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The differences among means of total percentage methylation were assessed by robust parametric tests (Welch's one-way ANOVA and the Games-Howell test) as well as nonparametric tests [Kruskal-Wallis and pairwise Mann-Whitney (Wilcoxon) tests].

Real-time reverse transcription-PCR. Total RNA from patient samples was isolated using the QIAamp RNA Blood Mini kit (Qiagen, Valencia, CA) or by guanidine isothiocyanate/phenol extraction and analyzed for *INK4b* expression. Quantitative reverse transcription-PCR (RT-PCR) was carried out in triplicate using the Taqman One-Step RT-PCR Master Mix Applied Biosystems protocol, 150 ng total RNA template, primers RT1 and RT2 (250 nm each; Supplementary Table S2), and 250 nm *INK4b* probe (Supplementary Table S2). For an 18S RNA control, a kit from Applied Biosystems was used. Real-time RT-PCR was done in a 96-well plate in an ABI PRISM 7700 (Applied Biosystems). Relative quantitation was carried out by the comparative C_t method. Expression of *CBFB-MYH11* RNA was analyzed by real-time RT-PCR using the SYBR Green PCR Master Mix (Applied Biosystems) and the primers CS1 and CS2 (Supplementary Table S2). The data were normalized according to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression obtained using primers GAPDH1 and GAPDH2 (Supplementary Table S2).

Table 1. Summary of methylation results

Translocation	Sample no.	No. clones (complete)*	% Methylation †	MSP ‡
t(8;21)	1	9 (9)	6	No
	2	5 (5)	43	No
	3	7 (7)	13	No
	4	8 (5)	4	No
	5	7 (7)	18	Yes
	6	7 (7)	25	Yes
	7	8 (6)	5	No
inv(16)	8	6 (1)	2	No
	9	ND	ND	No
	10	ND	ND	No
	11	7 (4)	11	No
	12	5 (5)	2	No
	13	2 (0)	7	No
	14	8 (8)	2	No
	15	9 (9)	2	No
	16	5 (5)	2	No
	17	8 (7)	3	No
	18	8 (6)	8	No
	19	8 (7)	7	No
t(15;17)	20	6 (6)	19	Yes
	21	ND	ND	No
	22	10 (8)	39	No
	23	8 (8)	10	No
	24	7 (7)	4	No
	25	8 (8)	11	No
	26	8 (8)	19	Yes
	27	8 (8)	11	Yes
	28	8 (8)	22	Yes
	29	ND	ND	No
	30	ND	ND	Weak

Abbreviation: ND, not determined.
*Number of individual clones that were sequenced for each sample. In parentheses are the numbers of sequences that were complete and included all 54 CpGs.
† For each sample, the total percentage of methylation was the sum of methylated CpGs in all sequences divided by the total number of CpGs in all clones.
‡ "Yes" means that a band was observed on a gel following amplification.

Western blot. Western blot analyses were carried out as previously described (11) using the anti-CBFβ monoclonal antibody (mAb; ref. 24), anti-actin mAb I-19 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-RUNX1 mAb (MAB2399, R&D Systems, Minneapolis, MN).

Electrophoretic mobility shift assay. Screening of *INK4b* genomic sequence for the transcription factor binding sites was done using Genomatix MatInspector software.[5] Electrophoretic mobility shift assays (EMSA) were carried out as described (12) using probes complementary to two regions of *INK4b* promoter: −629 to −602 (WT1, WT2, MUT1, and MUT2) and +357 to +384 (WT3, WT4, MUT3, and MUT4). The numbering refers to the distance from the translation start site. Where indicated, 5-, 10-, or 50-fold excess of unlabeled probe or 1 μg of anti-RUNX1 serum (PC284, Calbiochem, San Diego, CA) was added to the reaction.

[5] http://www.genomatix.de.

Transient transfection and luciferase assay. Cells were plated in a 24-well plate at a density of 4×10^5 per well and transfected with 1 μL of Fugene 6 reagent (Roche, Indianapolis, IN), 100 to 400 ng of luciferase reporter construct, indicated amounts of RUNX1 and PU.1 expression vectors, and 50 ng of the pCMV-RL plasmid to normalize for transfection efficiency. The total amount of DNA was adjusted to 1 μg using pcDNA3.1 plasmid. Cells were harvested 48 h after transfection and lysed in 35 μL of passive lysis buffer (Promega). Lysate (20 μL) was used to measure the luciferase activity using the Dual-Luciferase Assay System (Promega) and the Turner TD-20 luminometer. All experiments were done at least twice in triplicate.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was done as described previously (12) with several modifications as follows. Lysates were sonicated four times for 30 s on ice. Immunoprecipitation was done overnight using 8 μg of anti-RUNX1 (PC284) or anti-HA (Y-11, Santa Cruz Biotechnology) antibodies. The presence of *INK4b* sequence was determined by PCR using primers ChIP1 and ChIP2 (amplifying area, +125 to +239) or primers ChIP3 and ChIP4 (−1,405 to 1,225; Supplementary Table S2).

Results

Comparison of methylation frequencies in AML subgroups. AML with the most commonly found rearrangements inv(16), t(15;17), and t(8;21) were examined for methylation of the CpG island at the 5′-end of the *INK4b* gene. The first method for evaluating these samples was MSP (25). An example of the results obtained is presented in Supplementary Fig. S1, and a summary of all the data is presented in Table 1. Interestingly, no samples (0 of 12) with inv(16) were positive, whereas 4 of 11 (36%) samples with t(15;17) and 2 of 7 (29%) samples with t(8;21) were positive for methylation.

A more detailed analysis of the methylation pattern is provided by the bisulfite genomic sequencing. For each leukemia sample, five to nine clones were sequenced (only two clones for sample 13 were sequenced). A major finding, consistent with the MSP results, was that leukemias with inv(16) had a significantly lower

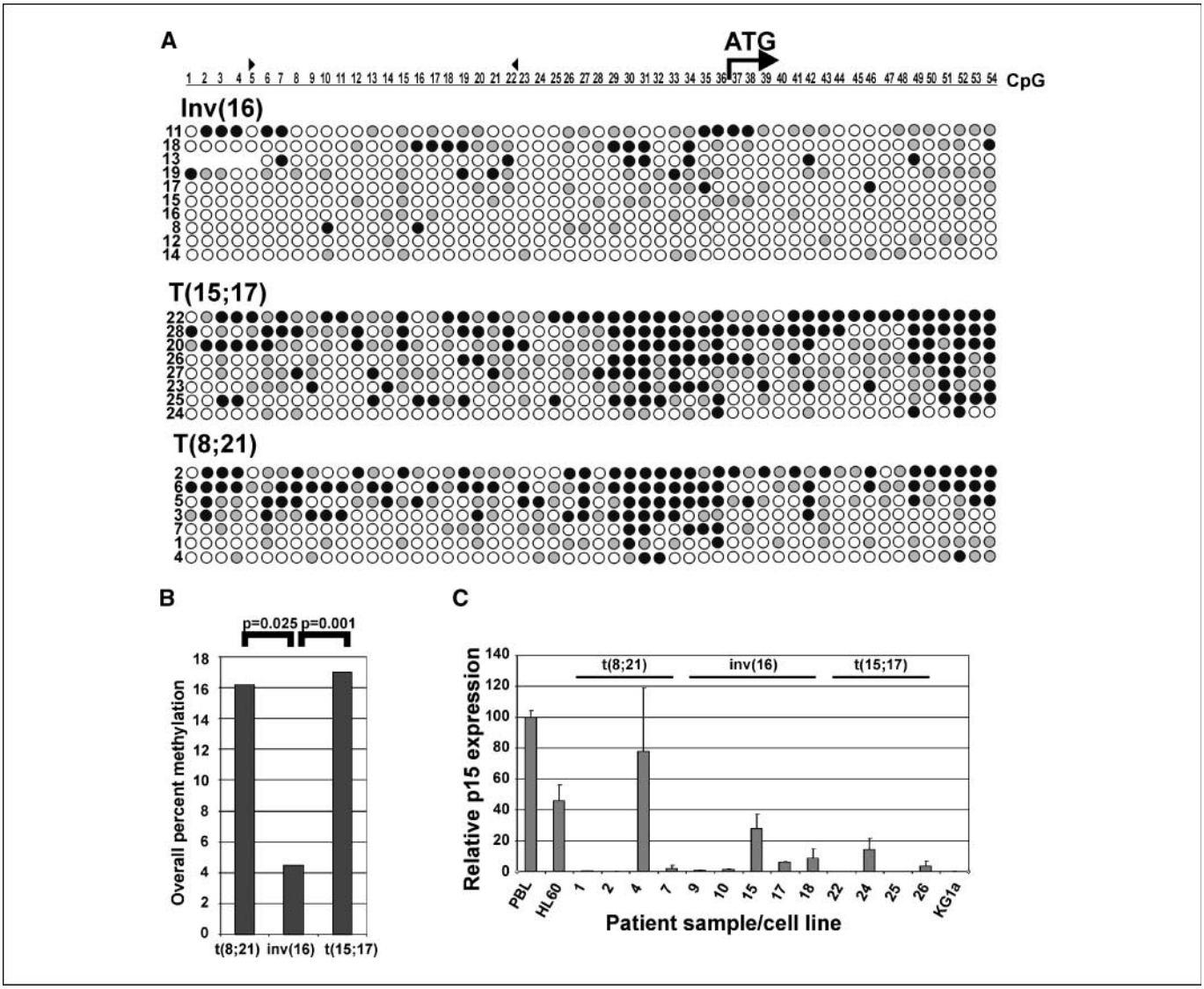

Figure 1. Frequency of methylation for each patient sample. *A, line,* single patient sample; *circle,* CpG dinucleotide (potential target of methylation). Shading of the circle corresponds to the level of methylation detected on a given CpG: *open circle,* no methylation; *gray circle,* 1% to 24% methylation; *closed circle,* ≥25% methylation. *Arrowheads,* beginning and end of the area amplified in MSP assay. *B,* average overall methylation of the *INK4b* promoter in patients carrying particular translocations. *C,* a quantitative real-time RT-PCR assay was used to compare the levels of $p15^{INK4b}$ expression in patient samples, PBLs from healthy individuals, and the HL60 and KG1a cell lines. For each sample, the relative expression was calculated using the comparative C_t method and plotted on the *Y axis. INK4b* real-time data are depicted relative to that expressed in PBLs from healthy individuals. *Bars*, SD.

overall level of methylation than those with t(8;12) or t(15;17) (Fig. 1*A*; Table 1). AML samples with inv(16) only had an overall average methylation level of 4.6% with a range of 2% to 11%, whereas the methylation ranged from 11% to 39% in 75% of t(15;17)-associated AML and from 13% to 43% in 57% of t(8;21)-associated AML. Significant differences were found in the total percentage methylation between inv(16) and both t(8;21) and t(15;17) samples (P = 0.025 and 0.001, respectively; Fig. 1*B*). No evidence was found for average differences between t(8;21) and t(15;17) (P = 0.779).

Although there is not complete concordance between the MSP data and the sequence data for each leukemia, overall they show the same trend, a low frequency of methylation in leukemias with inv(16) in contrast to a relatively high frequency of methylation in leukemias with t(15;17) and t(8;21). Only two cases of AML (nos. 2 and 22) showed a marked difference in outcome between the two methods of analysis (see Table 1). The sequencing protocol detected levels of methylation of 43% and 39% in these two samples, respectively, which were negative in the MSP assay. This is probably due to the fact that MSP evaluates only a limited number of CpG sites where the primers bind. Furthermore, the primers for the MSP assay are not located in the regions where we observed peaks in the methylation.

Expression of *INK4b* RNA in patient samples. Density of methylation is a critical factor for transcriptional silencing (26). We therefore wanted to determine if there was an inverse correlation between the level of methylation and the level of *INK4b* RNA expression in the AML cases. In the *INK4b* RNA expression analysis of AML samples, RNA from the peripheral blood lymphocytes (PBL) of healthy individuals and the human cell line HL60 provided positive controls and the Kg1a cell line provided a negative control (27). In Fig. 1*C*, *INK4b* real-time data are depicted relative to the amount of *INK4b* RNA expressed in PBLs from healthy individuals. Surprisingly, in most samples from inv(16) AMLs, the level of *INK4b* RNA expression was low, comparable with that found in leukemias with t(15;17) or t(8;21) (Fig. 1*C*). The low level of *INK4b* RNA in inv(16) AML in the absence of high levels of methylation suggests that there is an alternative mechanism to account for *INK4b* transcriptional repression in these patient samples.

CBFβ-SMMHC inhibits the expression of *INK4b* RNA in human myeloid cells. Previously, it was postulated that CBFβ-SMMHC functions as a dominant repressor of the target genes of the CBF, an important regulator of myeloid lineage genes (reviewed in ref. 28). Therefore, we tested whether ectopic expression of inv(16)-associated CBFβ-SMMHC is sufficient to inhibit the normal up-regulation of *INK4b* transcription during differentiation. U937 cells were infected with a retrovirus carrying *CBFB-MYH11* (U937/inv16) and analyzed for the expression of *CBFB-MYH11* RNA and CBFβ-SMMHC protein (Fig. 2*A* and *B*). To prevent indirect effects of long-term CBFβ-SMMHC overexpression, experiments with these cells were done within 2 to 3 weeks after infection. To induce *INK4b* RNA, the cells were treated with 200 nmol/L 1,25-dihydroxyvitamin D3. The results of a quantitative real-time RT-PCR analysis of *INK4b* RNA expression in the cells at 24-h intervals are depicted in Fig. 2*C*. As expected, in U937/MiG cells, *INK4b* RNA was induced with maximum expression 72 h after treatment. In contrast, in the U937/inv16 cells, expression of the *INK4b* gene was maintained at a low level. Even after 72 h of vitamin D3 treatment, *INK4b* RNA in the CBFβ-SMMHC-expressing cells was at a level no higher than that in untreated U937/MiG cells (Fig. 2*C*).

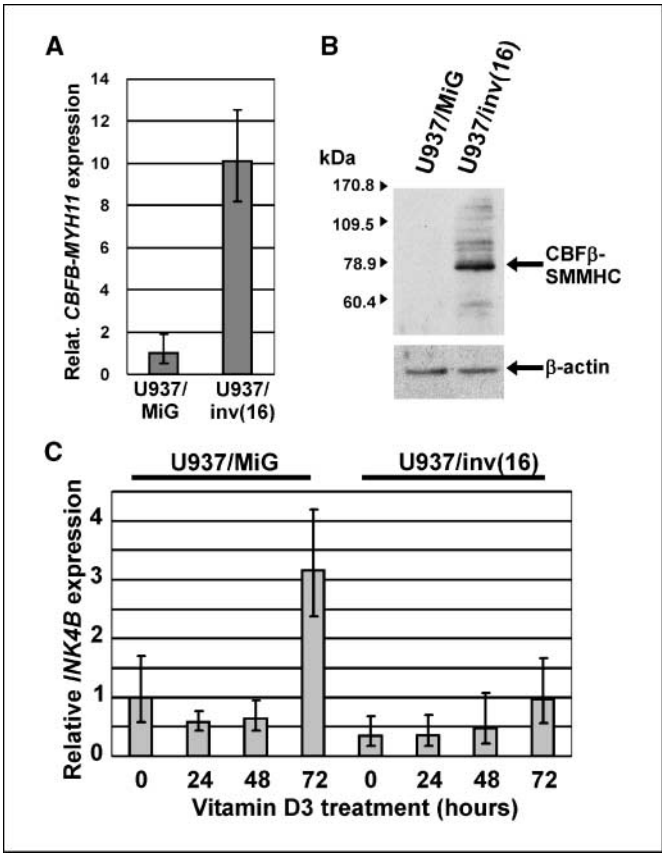

Figure 2. p15^{INK4b} RNA expression in U937 cells ectopically expressing inv(16). *A,* analysis of *CBFB-MYH11* RNA expression by real-time RT-PCR. *B,* Western blot analysis of CBFβ-SMMHC in U937/inv16 cells. *C,* real-time RT-PCR analysis of cells at indicated times following 1,25-dihydroxyvitamin D3 treatment. *Bars*, SD.

CBF-containing protein complexes bind to *INK4b* promoter sequences and are disrupted by CBFβ-SMMHC. The next objective was to determine if the inhibitory activity of CBFβ-SMMHC was through CBF-binding sites in the *INK4b* promoter and dysregulation of CBF-associated transcription. Two subunits are reported to act through CBF-binding sites in the regulation of genes; RUNX family proteins RUNX1, RUNX2, and RUNX3 (AML1, AML2, and AML3) bind to DNA, whereas the non–DNA-binding component of the complex, CBFβ, interacts with RUNX proteins through the Runt domain to enhance their binding and transcriptional activity (29).

Although CBF has not been previously shown to regulate the *INK4b* promoter, it is an excellent candidate. First, a computer analysis revealed two potential CBF-binding sites in close proximity of the *INK4b* transcription start site at positions +616 and +367 from ATG. Second, p15^{INK4b} RNA is induced in myeloid cells during differentiation and CBF is an important regulator of genes associated with myeloid cell maturation.

To determine if the CBF is capable of binding to putative CBF-binding sites found in the *INK4b* promoter, an EMSA was done. Separate probes representing the two potential binding sites were used and produced similar results. As shown in Fig. 3*A*, multiple protein complexes in a nuclear lysate from

U937/MiG cells were found to be bound to either DNA probe. Addition of anti-RUNX1 serum to the reaction resulted in a supershift of several bands (marked by arrows), suggesting that several of the observed complexes contained this DNA-binding subunit of CBF (Fig. 3*A*). There was a reduction in all the original bands, although none disappeared completely. This may be due to the reported low affinity of available antibody to endogenous RUNX1 (see data sheet PC284). However, efficient binding of most, if not all, observed complexes seems to be dependent on an intact CBF-binding motif sequence. Probes with mutations in the core of the CBF recognition motif did not bind any proteins observed on wild-type probes nor were they able to outcompete the observed complexes (Fig. 3*A* and *B*). Interestingly, complexes interacting with wild-type probes were not present in the cells overexpressing CBFβ-SMMHC (Fig. 3*C*). Instead, higher migrating bands appeared. These higher bands seemed to be CBFβ-SMMHC specific as they were also detected with a lysate from ME-1 cells (myeloid cell line derived from the patient with inv(16) (Fig. 3*C*). These results confirm that CBF is able to bind to the *INK4b* promoter sequences *in vitro* and this binding is disrupted in the presence of inv(16)-associated CBFβ-SMMHC.

CBFβ-SMMHC reverses the transcriptional activation of the *INK4b* promoter by RUNX1 and PU.1. To determine if CBFβ-SMMHC is capable of suppressing transcription from the *INK4b* promoter directly, a reporter assay was done in U937/MiG and U937/inv16 cells. Initially, a construct was prepared containing the area from −980 to +485 (relative to the translation start) followed by the luciferase gene. This included an upstream CBF-binding site as well as a binding site downstream in the first intron. We found that the amount of luciferase expressed from this construct was extremely low (data not shown). This lack of activity may be due to the artificial character of such construct. The putative RNA expressed from this vector contains the entire exon 1 and part of intron 1 attached to the luciferase gene. Presence of the unspliced intron as well as the p15 translation start could potentially interfere with the proper translation of luciferase. Because of this result, a construct was prepared with an *INK4b* fragment −980 to −29, which included only the upstream putative CBF-binding site (−616; Fig. 4*A*). As shown on Fig. 4*B*, the DNA-binding subunit of CBF, RUNX1, was capable of activating the promoter 1.5- to 3-fold by itself. Other known protein partners of RUNX1, CBFβ, PU.1, and CAAT/enhancer binding protein were evaluated for their cooperativity. Of these

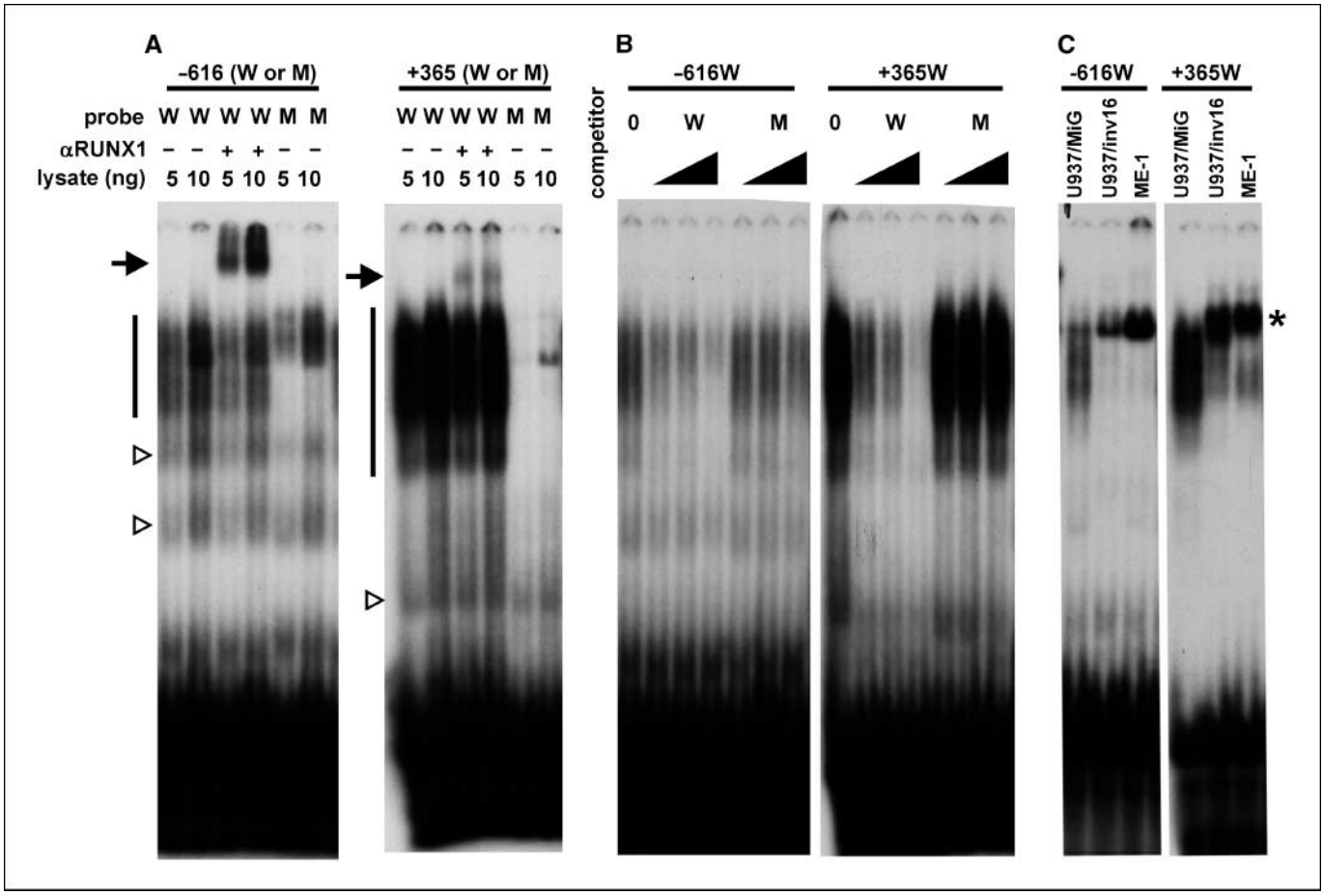

Figure 3. Complexes binding to the *INK4b* promoter *in vitro* contain RUNX1 and are disrupted by the presence of CBFβ-SMMHC. *A,* binding of proteins from U937/MiG cells to the wild-type (*W*) and mutant (*M*) probes corresponding to the CBF-binding sites in *INK4b* locus. Equal amounts of each probe were incubated with two different amounts of lysate from U937/MiG cells at room temperature. *Vertical line,* RUNX1-containing complexes. *Arrowheads,* nonspecific bands. Anti-RUNX1 serum (2 μg) was added to the reaction if indicated. *Arrows,* supershifted bands. *B,* EMSA reaction with indicated wild-type labeled probes and with 5-, 10-, and 50-fold excess of unlabeled wild-type or mutated probes. *C,* EMSA with wild-type probes and equal amounts of lysates from U937/MiG cells and from two CBFβ-SMMHC–overexpressing cell lines, U937/inv16 and ME-1.

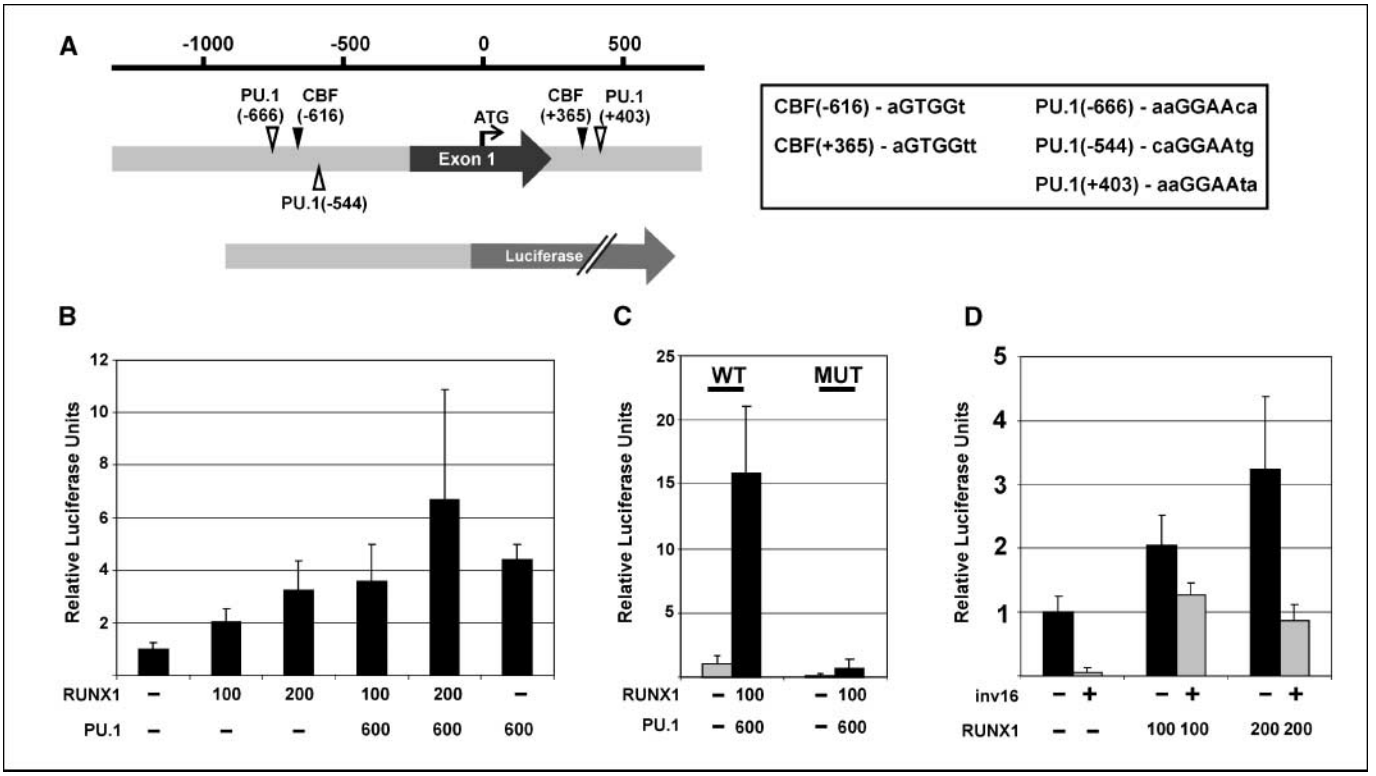

Figure 4. Luciferase reporter assays showing regulation through the CBF-binding site in the *INK4b* promoter and inhibition by CBFβ-SMMHC. *A,* map showing putative CBF- and PU.1-binding sites in the human *INK4b* promoter. *Number in parentheses,* position of the binding site relative to the start codon. *Arrowheads,* strand on which the binding site is located. *Right,* sequences of particular binding sites with capitalized bases representing core sequence. *Bottom right,* structure of the reporter construct used in the luciferase assay. *B,* transcriptional activity of the *INK4b* promoter in U937/MiG cells in the presence of various amounts of expression vectors encoding RUNX1 and PU.1. *C,* activity of wild-type (*WT*) and mutant (*MUT*) *INK4b* promoters in the presence and absence of RUNX1 and PU.1. *D,* activity of *INK4b* promoter in the U937/MiG and U937/inv16 cells. *Bars*, SD.

transcription factors, PU.1 resulted in the greatest increase in combination with RUNX1 (Fig. 4*B*; data not shown). This finding is consistent with the presence of two putative PU.1-binding sites in close proximity of the CBF site and with our previous observation that PU.1 is an *INK4b* activator in myeloid cells (12). Figure 4*C* shows that a mutation in the CBF-binding site resulted in decreased basal activity and a failure of the promoter fragment to be activated by RUNX1 and PU.1. Finally, an experiment comparing the activity of the *INK4b* promoter construct in U937/MiG and U937/inv16 cells showed a negative effect of CBFβ-SMMHC on the *INK4b* promoter and on its ability to be activated by RUNX1 (Fig. 4*D*).

RUNX1 binds to the endogenous *INK4b* promoter in the absence, but not presence, of CBFβ-SMMHC. In support of the hypothesis that the RUNX1 is involved in regulating expression of the endogenous *INK4b* gene, a ChIP assay showed that RUNX1 is bound to the *INK4b* promoter in U937/MiG cells (Fig. 5*A*). Binding was specifically detected using primers located between the two CBF recognition sequences but not with primers designed upstream of both sites (Fig. 5*A*). Interestingly, the RUNX1 binding was not detected in cells expressing CBFβ-SMMHC (U937/inv16; Fig. 5*B*). The possibility that there was a difference in the RUNX1 protein level in U937/MiG and U937/inv16 cells to explain this result was ruled out because there was an equal amount of protein detected in a Western blot (Fig. 5*C*). This suggests that the chimeric protein either prevented RUNX1 from binding to this region or its presence resulted in inaccessibility of the RUNX1 protein to antibodies. This finding is consistent with our results from EMSA, where presence of CBFβ-SMMHC caused major change in the composition and migration of transcription factor complexes bound to the *INK4b* promoter (Fig. 3*C*).

Discussion

Our study shows that AML subtypes, based on their cytogenetic abnormalities, can vary dramatically in their frequency of methylation at the *INK4b* locus. Most notable is the low level of methylation in leukemias with inv(16), in contrast to those with t(15;17) and t(8;21). This observation led to an investigation of a mechanism that could explain low *INK4b* expression in the absence of hypermethylation in inv(16) AML. In our experiments, we discovered a new form of regulation of the *INK4b* promoter involving CBF-binding sites. Through at least one of these sites, RUNX1 exerts transcriptional activation, which can be reversed by the chimeric protein CBFβ-SMMHC encoded by inv(16). We propose that repression caused by the aberrant transcription factor may be sufficient to maintain very low levels of expression of *INK4b* in leukemic cells. This would therefore preclude the necessity for silencing through CpG hypermethylation frequently found in AML with other cytogenetic abnormalities.

Interestingly, although CBFβ-SMMHC was discovered some time ago, there are not many known transcriptional targets for

the protein. The fact that CBFβ-SMMHC can repress the promoter of the CDKI gene *INK4b* is an important finding. It is becoming clear that, for neoplastic transformation to occur, the activity of several cell cycle inhibitors, including those in the INK4 and CIP/KIP families of proteins, needs to be inactivated. It is only in rare instances that they are bypassed by transcriptionally deregulated positive regulators, such as *c-myc*, as shown in cultured cells and observed in leukemia (16, 30). Until recently, the chimeric protein CBFβ-SMMHC had been known primarily for its ability to block differentiation presumably by inhibiting transcription of myeloid-specific genes through dominant repression of CBF transcription factors (31, 32). Very recently, it was shown that CBFβ-SMMHC can suppress expression of *ARF* by impairing RUNX1-mediated activation of the promoter in HeLa cells (33). These data along with ours suggest that CBFβ-SMMHC induces leukemia in part by affecting the cell cycle and apoptosis through inhibition of the p53 and the Rb pathways.

Several studies have indicated that CBFβ-SMMHC slows proliferation when ectopically expressed in myeloid cells and primary human and mouse progenitor cells in culture (34–36). In addition, a conditional knockin of *CBFB-MYH11* in the mouse resulted in appearance of abnormal myeloid progenitors deficient in proliferation capacity (37). In regard to this slowing of proliferation, it was reported that loss of *INK4b* in bone marrow mononuclear cells did not prevent slowing of proliferation by CBFβ-SMMHC (34). Our results provide an explanation for the latter observation because we have shown that expression of CBFβ-SMMHC itself blocks expression of the tumor suppressor,

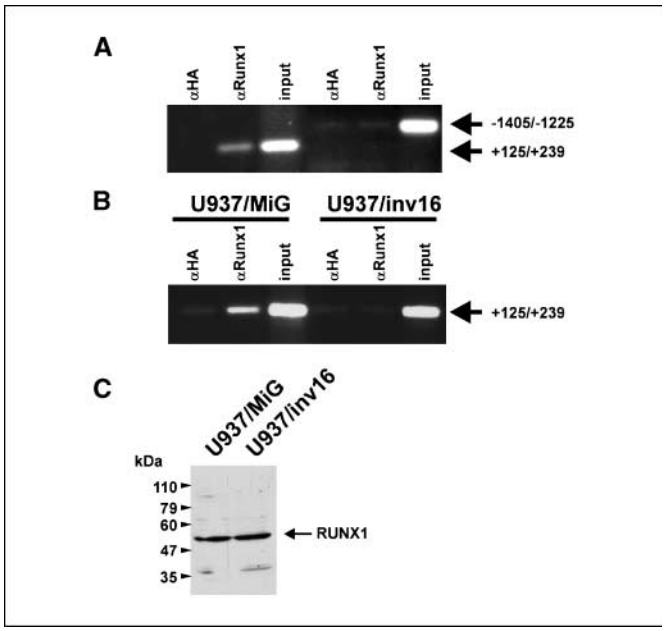

Figure 5. CBFβ-SMMHC disrupts CBF-containing complexes that bind to the *INK4b* promoter. *A,* ChIP assay showing the binding of RUNX1 to the endogenous promoter in U937/MiG cells. Cross-linked protein-DNA complexes were precipitated by addition of antibodies against RUNX1 to the cell lysate and analyzed by PCR using the primers located between the putative CBF-binding sites (*+125/239*). Primers located upstream of both sites (*−1,405/−1,225*) were used as a negative control. Isotypic anti-HA antibody was used as an additional negative control. *B,* ChIP assay comparing the binding of RUNX1 to the *INK4b* promoter in U937/MiG and U937/inv16 cells. *C,* Western blot confirming equal expression of RUNX1 in U937/MiG and U937/inv16 cells.

and therefore, knocking it out should have no additional effect. The observed slowing of proliferation by the aberrant protein must be due to another cell cycle inhibitor pathway(s), which needs to be overcome for full-blown leukemia. Indeed, cooperating events have been observed in animal models using ethylnitrosourea and retroviral insertional mutagenesis, and such events seem to overcome the negative effect of CBFβ-SMMHC on proliferation (37–39).

Our ability to show that CBFβ-SMMHC represses the *INK4b* promoter was dependent on our first showing that the promoter could be activated by RUNX1 and PU.1. This cooperation was not surprising because we had previously shown that PU.1 activates the murine *INK4b* promoter, and it was also shown by others that AML1(RUNX1) can synergize with PU.1 to activate the myeloid-specific gene *c-fms* (40). As shown here, the two proteins can together activate *INK4b* transcription by ~6-fold. This activity, however, was completely dependent on the presence of an intact CBF-binding site, indicating that PU.1 by itself was not capable of activating the −980/−29 region of the human promoter. The ability of PU.1 to activate wild-type promoter by itself (Fig. 4*B*) could be explained by the presence of endogenous RUNX1 in the U937 cells (Fig. 5*C*).

Although a mechanism to explain transcriptional suppression by the CBFβ-SMMHC fusion protein has been extensively investigated recently, it is still not completely clear how the repression of CBF target genes works. One of the proposed mechanisms is based on the ability of SMMHC protein to form filamentous multimers. It was suggested that such structures could sequester RUNX1, making it unavailable to its target genes (28, 41–43). A second model suggests that the RUNX1/CBFβ-SMMHC complex binds to the promoter, but its presence would cause steric hindrance altering the assembly of transcription factors (44). More recently, it was shown that the SMMHC contains a cryptic repression domain, capable of recruiting transcriptional corepressors, which would strengthen the negative effect of the chimeric protein (45). The data from our ChIP experiments provide the support for the first hypothesis since in the U937/inv16 cells, the RUNX1 seems to be sequestered from the *INK4b* promoter. However, we cannot completely exclude the possibility that the interaction of RUNX1 with CBFβ-SMMHC caused formation of a complex that made RUNX1 inaccessible to antibody. Regardless, our *in vitro* and *in vivo* experiments revealed that the presence of CBFβ-SMMHC causes a major change in the RUNX1-containing transcription factor complexes resulting in transcriptional suppression.

Our study, which determined a subset of AML that does not undergo methylation at the *INK4b* locus, is a step in the process of developing predictions as to which AML patients will or will not respond to inhibitors of methylation. Such inhibitors have been shown to reactivate *INK4b* with CpG island hypermethylation, suggesting that it may be an important target for reactivation in cases where inhibitors have been effective in clinical trials for AML (18, 46, 47). Previously, it was thought that *INK4b* mRNA expression correlates with methylation density, and certainly, this seems to be true for leukemia with AML1-ETO (48). Our data suggest that this is not always the case and the subgroup of AML with inv(16) is an example. Leukemias belonging to this group show a low overall level of methylation at the *INK4b* locus accompanied by a low level of mRNA. Based on this, we wondered if methylation inhibitors would be unable to affect *INK4b* expression directly. Recently, it was shown that

methylation inhibitors can sometimes induce tumor suppressors by methylation-independent mechanisms (49). However, treatment of ME-1 cells [cell line derived from an AML with inv(16)] with various amounts of 5-aza-2′-deoxycytidine did not significantly increase expression of *INK4b* (data not shown). If *INK4b* is substantiated to be a critical target, it will be imperative that clinicians know which subtypes of AML are most likely to respond. Therapies using demethylation drugs, such as 5-aza-2′-deoxycytidine (decitabine), have been less successful in the treatment of AML than in the treatment of MDS, and one of the reasons for this may be a lack of knowledge about which patients will be most likely to respond to treatment. More information is required to determine subtypes in which *INK4b* is hypermethylated as well as to determine how demethylation of *INK4b* and other genes will affect the therapeutic outcome in specific subtypes.

In conclusion, the findings described herein raise the question of whether there are other interactions between aberrant transcription factors and cell cycle regulatory genes in leukemia. In this regard, it was also shown that AML-ETO can suppress transcription of the p53 pathway gene encoding $p14^{ARF}$ (50). This feature of chimeric transcription factors in leukemia may be more common than previously thought.

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