Methylation Silencing of the *Apaf-1* Gene in Acute Leukemia

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Abstract

Apaf-1 is important for tumor suppression and drug resistance because it plays a central role in DNA damage–induced apoptosis. Inactivation of the *Apaf-1* gene is implicated in disease progression and chemoresistance of some malignancies. In this study, we attempted to clarify the role of Apaf-1 in leukemogenesis. Apaf-1 mRNA levels were below the detection limit or very low in 5 of 20 human leukemia cell lines (25%) and 5 of 12 primary acute myeloblastic leukemia cells (42%). There were no gross structural abnormalities in the *Apaf-1* gene in these samples. Expression of factors regulating Apaf-1 transcription, such as E2F-1, p53, and Sp-1, did not differ between Apaf-1-positive and Apaf-1-negative cells. Methylation of CpG in the region between +87 and +128 of the *Apaf-1* gene was almost exclusively observed in Apaf-1-defective cell lines. Treatment of these cells with 5-aza-2′-deoxycytidine, a specific inhibitor of DNA methylation, restored the expression of Apaf-1. Furthermore, we showed that the region between +87 and +128 could act as a repressor element by recruiting corepressors such as methylated DNA-binding domain 2 and histone deacetylase 1 upon methylation. Overexpression of Dnmt1, a mammalian maintenance DNA methyltransferase, was associated with *Apaf-1* gene methylation. DNAs from Dnmt1-overexpressing cells were more resistant to digestion with methylation-sensitive enzyme *Hpa*II than those from cells with low Dnmt1 expression, suggesting that Dnmt1 mediates aberrant methylation of multiple genes. In conclusion, methylation silencing is a mechanism of the inactivation of Apaf-1 in acute leukemia, and Dnmt1 overexpression may underlie hypermethylation of the *Apaf-1* gene. (Mol Cancer Res 2005;3(6):325–34)

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Introduction

Apaf-1 is a central component of the intrinsic pathway of apoptosis and is important for cellular responses to DNA damage (1). When DNA is damaged by chemotherapeutic agents, oncogenic stimuli, UV and ionizing radiation, and hypoxia, cytochrome *c* is released from the mitochondria, binds to Apaf-1 in the cytosol, and in association with dATP/ATP, facilitates a conformational change of Apaf-1 to expose its CARD domain (2). Subsequently, Apaf-1 oligomerizes through the exposed CARD domain and catalyzes autoactivation of caspase-9, leading to the serial activation of downstream effector caspases such as caspase-3, caspase-6, and caspase-7, which results in apoptotic cell death (3). Gene-targeting studies revealed that a lack of Apaf-1 greatly affects the execution of the intrinsic pathway-triggered apoptosis (4).

Given the role of Apaf-1 in DNA damage–induced apoptosis, it is reasonable to speculate that a defect in Apaf-1 expression plays a role in oncogenesis and chemoresistance. It has been shown that inactivation of Apaf-1 substantially reduces the number of cells required to form tumors in nude mice transplanted with Myc/Ras-transformed fibroblasts, implying that Apaf-1 actually acts as a tumor suppressor (5). Loss of Apaf-1 expression is observed in approximately half of metastatic melanomas, whereas primary melanoma cases rarely show a decrease in Apaf-1 levels (6). This suggests that Apaf-1 is related to the disease progression and treatment sensitivity of malignant melanoma. Aberrant expression of Apaf-1 with defects in dATP-mediated caspase-3 activation is also detected in male germ cell tumors (7). Recently, Watanabe et al. (8) reported frequent inactivation of Apaf-1 due to loss of heterozygosity in glioblastoma. Furthermore, a frame-shift mutation of the mononucleotide tract in the *Apaf-1* gene is associated with gastrointestinal cancer of the microsatellite mutator phenotype (9). These findings point to the possibility that abnormalities of Apaf-1 are not restricted to melanoma and play a role in the pathogenesis of a wide variety of cancer.

Recently, we have shown that Apaf-1 is a transcriptional target of E2F-1 and mediates E2F-1-induced apoptosis in hematopoietic cells (10). We isolated the promoter region of the *Apaf-1* gene and found an extremely high G/C content with an observed/expected CpG ratio of >0.8, which fulfills the requirement of a CpG island (11). The structural features of the Apaf-1 promoter suggest that methylation of the CpG island serves as the major mechanism of transcriptional regulation of Apaf-1. Indeed, Apaf-1 expression is successfully restored by 5-aza-2′-deoxycytidine, an inhibitor of DNA methylation (12), in Apaf-1-defective melanoma cell lines, implicating aberrant methylation in silencing of the *Apaf-1* gene (6).

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The present study was aimed at clarifying the role of Apaf-1 in leukemogenesis and drug resistance in acute leukemia. We found that Apaf-1 expression was defective in substantial proportions of human leukemia cell lines and primary leukemic cells. We also obtained evidence suggesting that methylation silencing is a mechanism of the inactivation of Apaf-1 and overexpression of Dnmt1 (a mammalian maintenance DNA methyltransferase) underlies hypermethylation of the *Apaf-1* gene.

Results

The Decrease in Apaf-1 *mRNA and Protein Expression in Leukemic Cells*

To investigate the role of Apaf-1 in leukemogenesis, we first screened various human leukemic cell lines for Apaf-1 mRNA expression using Northern blotting. As shown in Fig. 1A, the Apaf-1 transcript was under the detection limit or faintly expressed in 5 of 20 cell lines examined (25%). Apaf-1-defective cell lines include two lines derived from chronic myeloid leukemia in blastic crisis (KU812 and K562) and two lines established from patients with acute lymphoblastic leukemia (ALL)-FAB type L3 (Daudi and Raji). The absence or decreased expression of the Apaf-1 transcript in these cell lines was confirmed by reverse transcription-PCR (RT-PCR) assays (data not shown). We then extended our analysis to determine Apaf-1 expression in primary leukemic cells. Leukemic cells were obtained from 12 patients with acute myeloblastic leukemia (AML; see Table 1 for the characteristics of patients) and subjected to semiquantitative RT-PCR for Apaf-1 mRNA expression. CD34-positive bone marrow mononuclear cells and T lymphocytes from healthy volunteers were simultaneously analyzed as normal controls. Representative results of RT-PCR are shown in Fig. 1B and all the results are summarized in Table 1. Overall, the Apaf-1 transcript was absent in 5 of 12 cases examined (42%), whereas Apaf-1 expression was constantly observed in normal controls. We also

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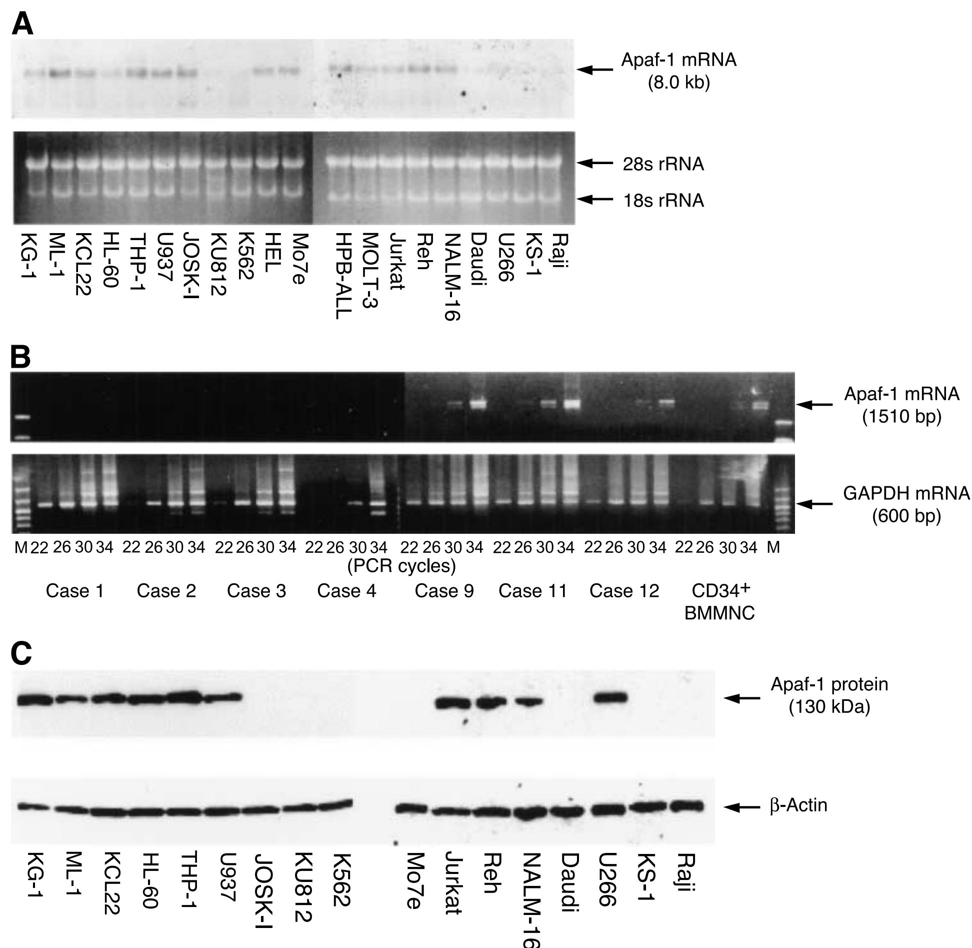

FIGURE 1. Apaf-1 mRNA expression in leukemic cells. **A.** Total cellular RNA was isolated from various leukemia cell lines and subjected to Northern blotting for Apaf-1 mRNA expression. Ethidium bromide–stained 28s and 18s rRNAs are shown as a loading control. **B.** Total cellular RNA was isolated from primary leukemic cells of patients with AML and subjected to a semiquantitative RT-PCR analysis for Apaf-1 mRNA expression as described in Materials and Methods. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was simultaneously amplified as a control. *Bottom*, PCR cycles. We used Bio Marker Low (Bio Ventures, Inc., Murfreesboro, TN) as a molecular size marker (*M*). **C.** Whole cell lysates were prepared from various leukemia cell lines and subjected to immunoblotting for Apaf-1 protein expression. The membrane filters were reprobed with anti-β-actin antibody to verify the equal loading and integrity of samples. Representative data of multiple independent experiments.

examined the expression of Apaf-1 protein in AML cell lines (Fig. 1C). Apaf-1 protein was not detectable in all cases lacking mRNA expression and, additionally, in two cell lines with intact Apaf-1 transcripts (JOSK-I and Mo7e). This suggests that Apaf-1 expression is regulated at both transcriptional and post-transcriptional levels, consistent with a recent report by Fu et al. (13). The sample numbers are too small to analyze the relationship between the expression of Apaf-1 and clinical characteristics of the patients such as FAB subtype, treatment outcome, and chromosomal abnormalities. Therefore, we went on to investigate the mechanisms of the reduced expression of Apaf-1 mRNA in leukemia.

No Structural Abnormalities of the Apaf-1 *Gene in Leukemia*

In a previous study, we reported the isolation and characterization of the promoter region of the *Apaf-1* gene (10). Based on the previous data, we searched for structural abnormalities of the Apaf-1 promoter in leukemic cells. No gross abnormalities were found in the Southern blot analysis using a full-length Apaf-1 cDNA probe in cell lines with defective Apaf-1 expression (Fig. 2A). In addition, deletions and point mutations were not detected in PCR products spanning the region between −1136 and −31 (Fig. 2B). These results suggest that large structural abnormalities such as gene deletions and rearrangements do not underlie the lack of Apaf-1 mRNA expression.

A search for putative binding sites of known transcription factors in the Apaf-1 promoter revealed three E2F consensus sites (−532 to −526, −119 to −113, and −36 to −30), two p53-responsive elements (−765 to −739 and −604 to −572), and several GC boxes (Fig. 3). Given the role of these factors in the regulation of Apaf-1 transcription, we examined the possibility that a lack of E2F-1, p53, and Sp-1 is a cause of the decreased expression of Apaf-1 in leukemic cells. Western blot analysis revealed that E2F-1 and Sp-1 proteins were almost ubiquitously expressed in the leukemic cell lines examined except for the lower levels of Sp-1 in Apaf-1-positive JOSK-I cells (Fig. 4). In contrast, p53 expression was defective in 7 of 20 cell lines, but there was no correlation with the loss of Apaf-1 (Fig. 4). Taken together, the involvement of genetic mechanisms is unlikely in the reduced expression of Apaf-1 in leukemia.

Table 1. Characteristics of the Patients in This Study

Case No.	FAB Type	Treatment Courses Required for Achieving CR	Chromosome Abnormalities	Apaf-1 Expression
1	CML-BC*	1	t(9;22)(q34;q11)	−
2	M3	1	t(15;17)(q22;q11)	−
3	M4	1	t(2;17)(q13;q11), +8	−
4	M5b	>3	+8, add(9)(p13), del(11)(q?)	−
5	M3	1	t(15;1 7)(q22;q11)	+
6	M1	2	t(10;11)(p12^14;q21), −16, +mar1	+
7	M4	2	inv(16)(p13;q22)	+
8	M3	1	t(15;1 7)(q22;q11)	+
9	M2	2	−	+
10	M5	1	+8	−
11	M1	1	+mar1	+
12	M4	2	−	+

Abbreviations: CML-BC, chronic myeloid leukemia in blastic crisis; FAB, French-American-British; CR, complete remission.
*Case 1 is chronic myeloid leukemia initially presenting as myeloid blastic crisis.

Methylation Silencing of the Apaf-1 *Gene in Leukemic Cells*

As we previously described (10), the Apaf-1 promoter is extremely GC rich; % (G + C) of the sequence between −776 and +188 is 70% and the ratio of observed/expected CpG is 0.81 (Fig. 3). This indicates that the promoter constitutes a CpG island or is part of a large CpG island. We therefore examined the methylation status of the Apaf-1 promoter by bisulfite sequencing. Briefly, genomic DNA was isolated from nine leukemic cell lines and normal T lymphocytes, treated with sodium bisulfite to convert unmethylated cytosines to uracils, and subjected to direct sequencing after PCR amplification. The quality of bisulfite treatment was verified by methylation-specific PCR for p15/Ink4b and E-cadherin for each sample (data not shown). Using the primer pairs listed in Table 2, we amplified the region between −571 and +184 in four separate segments (−571 to −383, −409 to −70, −96 to +41, and +41 to +184), which contain 10, 44, 12, and 13 CpG sites, respectively. Methylation was not detected in 66 cytosine residues in the regions between −571 and +41 in any cell types tested. In contrast, scattered CpG methylation was observed in the region between +41 and +184 in Apaf-1-negative cell

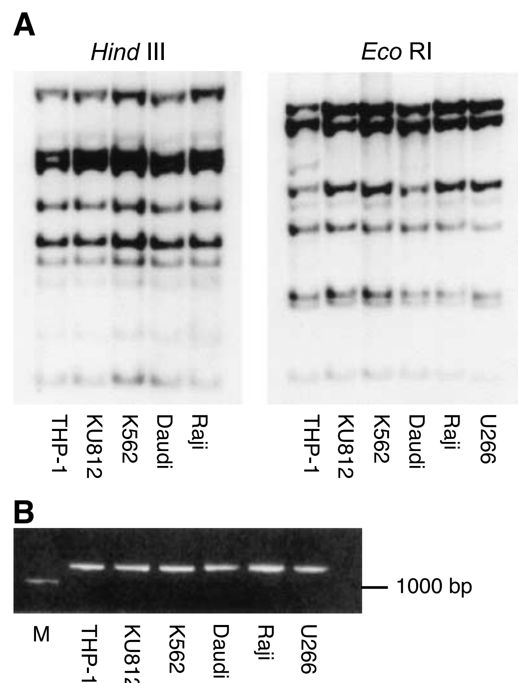

FIGURE 2. Structure of the *Apaf-1* gene in leukemia. **A.** Southern blotting was done with genomic DNA isolated from the indicated cell lines after digestion with *Hind*III and *Eco*RI. Full-length Apaf-1 cDNA was used as a probe. **B.** The Apaf-1 promoter region between −1136 and −31 was amplified by PCR with the same samples used in **A**. The PCR products were purified and subjected to DNA sequencing, which revealed no point mutations or deletions (data not shown). We used Bio Marker Low (Bio Ventures) as a molecular size marker (*M*).

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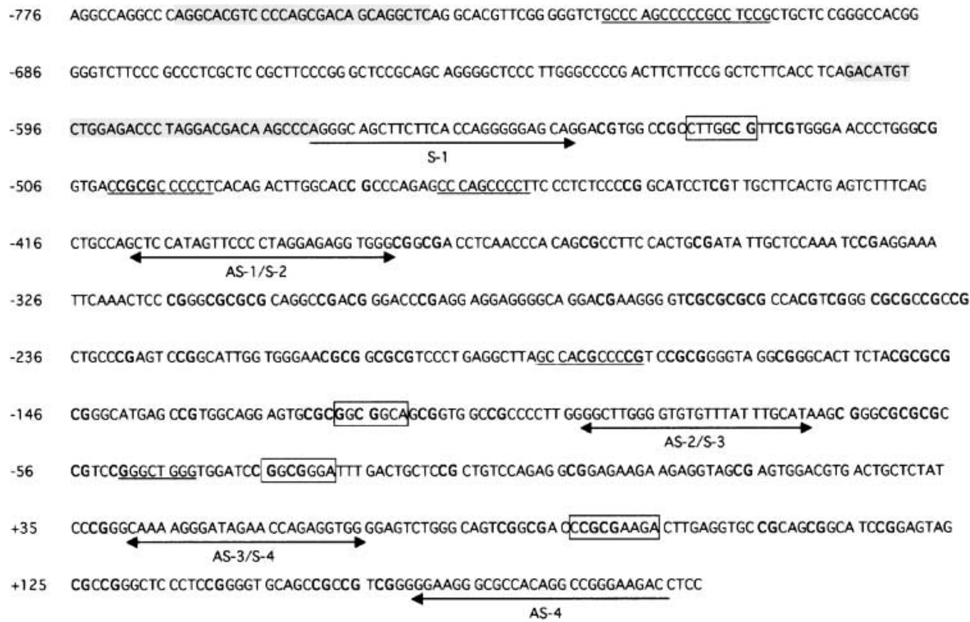

FIGURE 3. Structure of the promoter region of the *Apaf-1* gene. The sequence of the 5′-untranslated region of the *Apaf-1* gene. Position +1 indicates the 5′ end of the full-length cDNA. CpG dinucleotides are in boldface type. Putative binding sites for E2F (*boxed*), p53 (*shaded*), and Sp-1 (*underlined*). Arrows correspond to the sequences used for PCR primers for bisulfite sequencing (see also Table 2). The nucleotide sequence shown here has been deposited in the DDBJ/EMBL/Genbank database under accession no. AB070829.

lines (Raji, K562, Daudi, KU812, and KS-1) but not in Apaf-1 mRNA-expressing cells (T lymphocytes, HL-60, U937, JOSK-I, and Jurkat) except for methylation of cytosine at +128 in T cells from one volunteer (Fig. 5). The cytosine residue at +128 seemed to be a hotspot, because it was methylated in 17 of 27 samples from five AML cell lines lacking Apaf-1 mRNA expression.

To corroborate the involvement of methylation in the suppression of Apaf-1, we cultured Apaf-1-defective cells with 5-aza-2′-deoxycytidine, a specific inhibitor of DNA methylation (12), and examined Apaf-1 mRNA expression and promoter methylation using Northern blotting and bisulfite sequencing, respectively. As anticipated, treatment with 5-aza-2′-deoxycytidine restored Apaf-1 transcription in Daudi (Fig. 6A), KU812, K562, KS-1 (Fig. 6B), and Raji (data not shown), whereas no effect was observed in Apaf-1-expressing cell lines except for a modest increase in Jurkat. Demethylation of cytosine residues in the region between +87 and +128 was concomitantly induced by 5-aza-2′-deoxycytidine in all cell lines in which Apaf-1 expression was restored (Fig. 6C).

To further confirm the causative role of methylation of this region in silencing of the *Apaf-1* gene, we carried out two

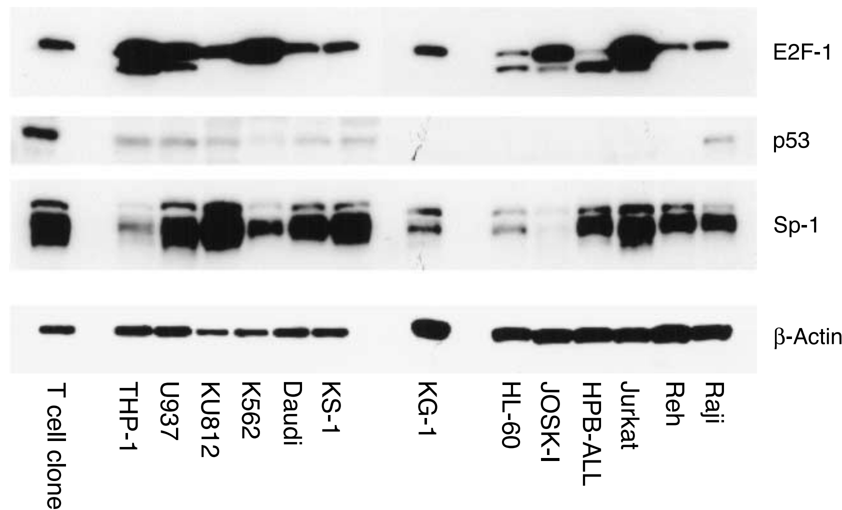

FIGURE 4. Expression of transcription factors regulating Apaf-1 transcription in leukemic cell lines. Whole cell lysates were isolated from various leukemia cell lines and subjected to immunoblot analysis for E2F-1, p53, and Sp-1. We used a human T-cell clone as a positive control. The same samples were used for β-actin immunoblotting to verify the equal loading and integrity of samples. Representative data of multiple independent experiments.

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Table 2. Primers Used for Analyzing the Methylation Status of the Apaf-1 Promoter

Primers	Positions	Sequences*
S-1	−571/−545	AGG**G**TA**G**TT**T**T**T**T**AT**TAGGGGGA**G**TA
AS-1	−383/−409	CCCACCTCTCCT**AAAA**AAACTA**TAAAA**
S-2	−409/−383	T**TT**TATAGT**TTTTT**TAGGAGAGGTGGG
AS-2	−70/−96	A**T**ACAAATAAACACACCCCA**A**ACCCC
S-3	−96/−70	GGG**G**TTTGGGGTGTGTTTATTT**G**TAT
AS-3	+64/+41	CCACCTC**TA**ATTCTATCCCTTT**TA**
S-4	+41/+64	TAAAAGGGATAGA**AT**TAGAGGTGG
AS-4	+184/+159	ATCTTCC**CA**ACC**T**A**TA**A**C**ACCCTTCCC

Abbreviations: S, sense; AS, antisense.
*Cytidine residues modified by bisulfite treatment are shown in boldface type.

additional experiments. First, we investigated whether the region between +41 and +184 could act as a repressor element upon methylation in a reporter assay. To this end, we subcloned the bisulfite-treated fragments of the region between +41 and +184 from KU812 and U937 cells into a chloramphenicol acetyltransferase (CAT) reporter plasmid, transfected them into K562 cells, and measured CAT activity after 14 days. As shown in Fig. 7A, CAT activity was significantly decreased by the insertion of the KU812-derived fragment, which retained CpG sequences, whereas the U937-derived fragment, in which C was replaced by T after bisulfite treatment, did not affect the reporter activity of pCAT-enhancer vector. Second, we did chromatin immunoprecipitation assays and showed the binding of transcriptional corepressors, methylated DNA-binding domain 2 and histone deacetylase 1, to the region between +41 and +184 in three cell lines with defective Apaf-1 expression but not in three cell lines with intact Apaf-1 expression (Fig. 7B). Taken together, these data support the notion that DNA methylation–dependent formation of the repressor complex in this region contributes to inactivation of the *Apaf-1* gene.

Dnmt1 Overexpression in Leukemic Cells

Finally, we tried to elucidate the mechanisms behind the altered methylation of the *Apaf-1* gene in leukemia. In mammals, methylation of CpG dinucleotides is mediated by three DNA methyltransferases: Dnmt1, Dnmt3a, and Dnmt3b (14). Dnmt1 has a preference for hemimethylated DNA as a substrate and is considered a maintenance methyltransferase that restores methylation patterns after DNA replication (15). In contrast, Dnmt3a and 3b are responsible for *de novo* methylation, which occurs during early development (16). We carried out Western blot analysis for the expression of DNA methyltransferases in leukemic cell lines. As shown in Fig. 8, Dnmt1 protein was apparently more abundant in Apaf-1-defective cell lines than in Apaf-1-expressing cells, whereas Dnmt3a and 3b were not detectable or very weakly expressed in all cell lines examined. χ^2 test revealed that the inverse correlation between the expression of Dnmt1 and Apaf-1 was statistically significant (χ^2 = 11.15, P < 0.01).

To investigate whether Dnmt1 overexpression is implicated in the overall level of DNA methylation, we compared the susceptibility of genomic DNA from Dnmt1-overexpressing cells to the methylation-sensitive restriction endonuclease *Hpa*II with that of DNA from cell lines with low Dnmt1 expression, as reported by Bourc'his et al. (17). We used the methylation-insensitive isoschizomer *Msp*I as a control. As shown in Fig. 9, DNAs from Dnmt1-overexpressing cells were more resistant to digestion with *Hpa*II than those from cells with low Dnmt1 expression, suggesting that Dnmt1 mediates aberrant methylation of multiple genes. This notion was supported by the finding that Dnmt1-overexpressing cells possessed altered methylation of multiple genes, including *E-cadherin* and *p15/Ink4b*, in addition to *Apaf-1* (data not shown). Although these results imply that overexpression of Dnmt1 is related to methylation silencing of the *Apaf-1* gene and possibly several other tumor suppressor genes in leukemia, further investigation is required to confirm the implication of causality.

Discussion

Given the central role of Apaf-1 in DNA damage–induced apoptosis, it is reasonable to speculate that a defective Apaf-1 expression underlies carcinogenesis and drug resistance. Indeed, inactivation of Apaf-1 and its relationship to tumor development and chemoresistance have been reported in malignant melanoma (6, 18). In this study, we examined the expression of Apaf-1 in acute leukemia and found that the Apaf-1 mRNA level was under the detection limit or very low in 25% and 42% of leukemic cell lines and primary AML cells, respectively. There were no gross structural abnormalities in the *Apaf-1* gene and its promoter regions in our analysis. Expression of factors regulating Apaf-1 transcription, such as E2F-1, p53, and Sp-1, did not differ between Apaf-1-positive and Apaf-1-negative leukemic cells. Methylation of cytosine residues in the region between +87 and +128 of the *Apaf-1* gene was almost exclusively observed in Apaf-1-defective cell lines. In addition, treatment of these cells with 5-aza-2′-deoxycytidine, a specific inhibitor of DNA methylation (12), restored the expression of Apaf-1. Furthermore, we showed that this region could act as a repressor element by recruiting corepressors such as methylated DNA-binding domain 2 and histone deacetylase 1 upon methylation. These results suggest that methylation silencing contributes at least in part to the in activation of Apaf-1 in acute leukemia. Finally, we investigated the mechanisms of hypermethylation and found that overexpression of Dnmt1, a mammalian maintenance DNA methyltransferase (19), was associated with the methylation of the *Apaf-1* gene and possibly other tumor suppressor genes, including *E-cadherin* and *p15/Ink4b*.

Abnormal methylation events, including hypermethylation of CpG islands, have been observed in several human tumors (20). DNA methylation can confer a selective growth advantage to cells when it occurs in the promoter regions of genes involved in growth regulation and DNA damage responses, which results in the development of cancer (21). Similarly, DNA methylation may affect treatment outcome by modulating the expression of genes affecting drug responses (22). This is the case with hematologic malignancies. For

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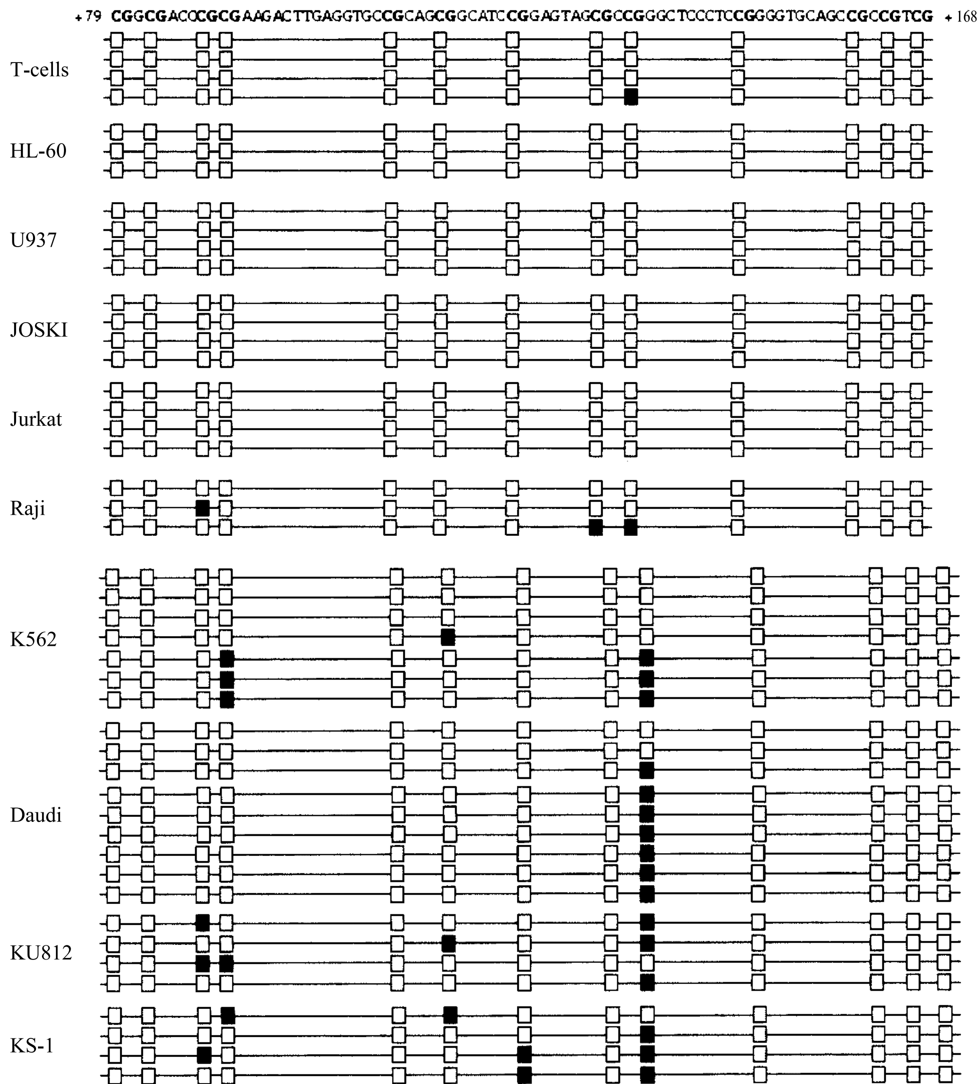

FIGURE 5. Methylation status of the *Apaf-1* gene in leukemia. Methylation status of the *Apaf-1* gene between nucleotides +79 and +168 was analyzed by bisulfite sequencing using DNA from Apaf-1-positive (normal T lymphocytes, HL-60, U937, JOSK-I, and Jurkat) and Apaf-1-negative (Raji, K562, Daudi, KU812, and KS-1) cells. The nucleotide sequence in this region (*top*) and CpG dinucleotides (*boldface type*). Methylated (■) and unmethylated (□) cytosines. Each line shows the results from different individuals in the case of T lymphocytes and independent analyses of cell lines.

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example, hypermethylation is frequently observed in CpG islands of various cancer-related genes, such as *p16/Ink4a, p15/ink4b, Rb, E-cadherin, calcitonin*, *glutathione S-transferase-π* (*GST-π*), *estrogen receptor*, and *HIC-1* (hypermethylated in cancer-1), in primary AML cells (23). According to Chim et al. (24), aberrant methylation of *p15* is most frequently observed in acute promyelocytic leukemia, and the genes encoding estrogen receptor, retinoic acid receptor-β (RAR-β), p16, and E-cadherin are also methylated in substantial numbers of cases, whereas there is no methylation in *p73*, *VHL (von Hippel-Lindau), caspase-8,* and O^6-methylguanine-DNA methyltransferase. In addition, *p15* methylation was inversely correlated with the disease-free survival of the patients and remained the sole prognostic factor in a multivariate analysis (24). Similarly, prognostic effect of methylation of the *p21/Waf1* and *p57/Kip2* genes has been shown in acute lymphoblastic leukemia (25, 26). Our present finding, along with a recent report by Fu (13), defines Apaf-1 as another target of methylation silencing in acute leukemia, although its clinical significance is to be determined (27).

Increased Dnmt1 expression has been reported in a number of human cancers, including hepatocellular carcinoma and pancreas and colon cancer (28, 29). In these tumors, the increase in Dnmt1 activity is believed to be responsible for the repression of tumor suppressor genes such as *p16, p15, E-cadherin*, and *HIC-1*, indicating a role for aberrant Dnmt1 expression in carcinogenesis. Furthermore, one study clearly showed a relationship between Dnmt1 expression and prognosis in hepatocellular carcinoma; disease-free and overall survival was significantly lower in patients with

Dnmt1-expressing hepatocellular carcinoma than those with Dnmt1-negative hepatocellular carcinoma (30). In hematologic malignancies, Di Croce et al. (31) reported that PML/RAR-α leukemic fusion protein causes hypermethylation and silencing of the tumor suppressor gene *RAR-β2* by recruiting Dnmt1 to its promoter region in acute promyelocytic leukemia. Although the tethering of Dnmt1 by leukemic fusion proteins is a mechanism of aberrant DNA methylation in leukemias with chromosome translocation, it is likely that distinct mechanisms are involved in the methylation events of other leukemias. In our analysis, Dnmt1 overexpression was suggested to be a cause of the altered methylation of Apaf-1 and possibly other tumor suppressor genes, although further investigation is required to confirm the implication of causality. Taking in to account the methylation of multiple genes in same cases (23), however, Dnmt1 overexpression may be a common mechanism of hypermethylation in leukemias and thus play an important role in leukemogenesis. We are currently investigating the underlying mechanisms of Dnmt1 overexpression in acute leukemia and trying to develop a therapeutic strategy with Dnmt1 as a molecular target.

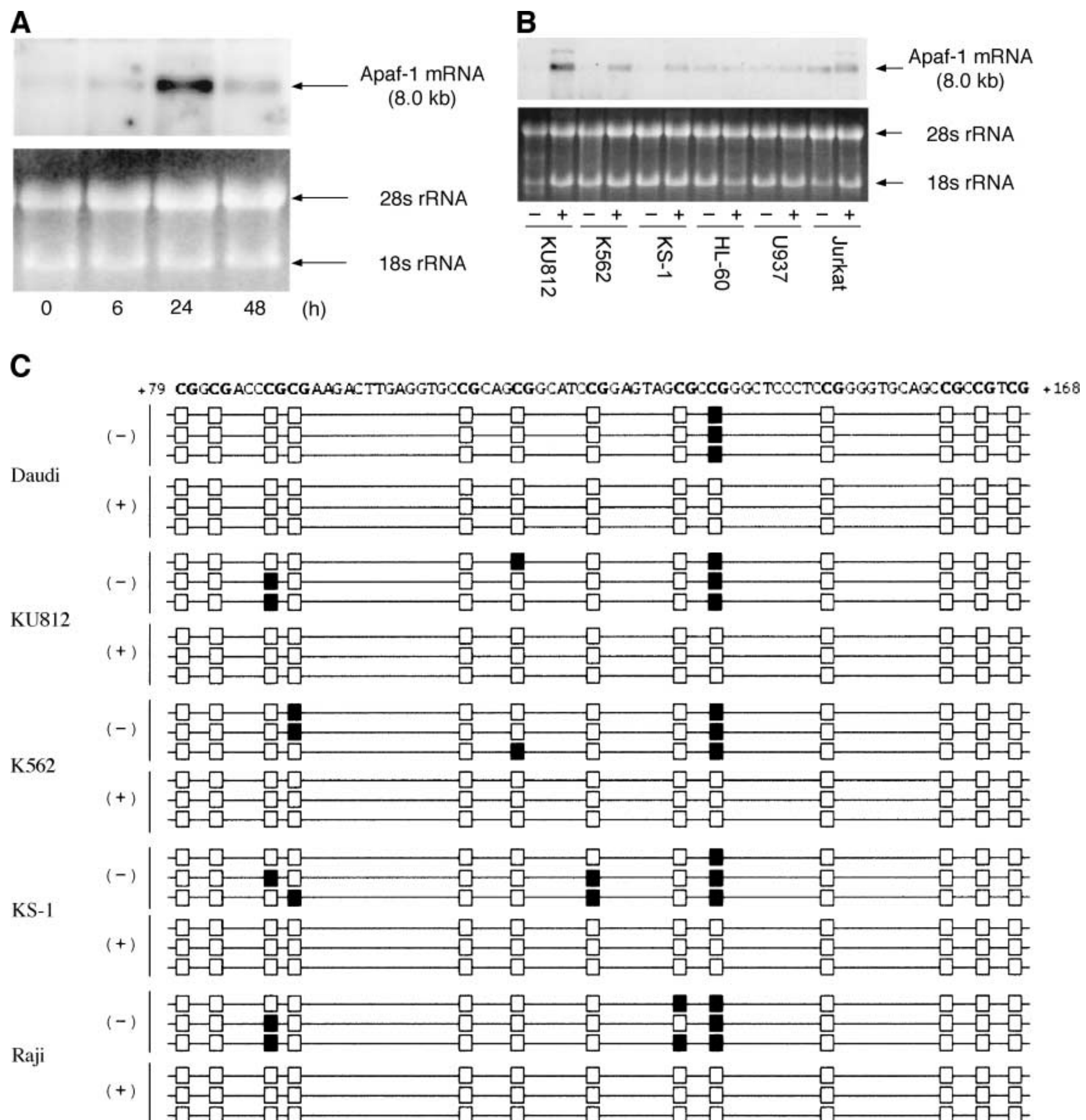

FIGURE 6. Effects of a specific inhibitor of DNA methylation on Apaf-1 mRNA expression in leukemic cells. **A.** Total cellular RNA was isolated from Daudi cells cultured in the presence of 1 μmol/L 5-aza-2′-deoxycytidine (Sigma) at the indicated time points. Northern blotting was carried out as described in the Fig. 1 legend. **B.** The same experiments were done using Apaf-1-negative (KU812, K562, and KS-1) and Apaf-1-positive (HL-60, U937, and Jurkat) cell lines cultured in the absence (−) or presence (+) of 1 μmol/L 5-aza-2′-deoxycytidine for 24 hours. **C.** Methylation status of the *Apaf-1* gene between nucleotides +79 and +168 was analyzed by bisulfite sequencing in Apaf-1-negative cell lines cultured in the absence (−) or presence (+) of 1 μmol/L 5-aza-2′-deoxycytidine for 24 hours. Representative data of multiple independent experiments.

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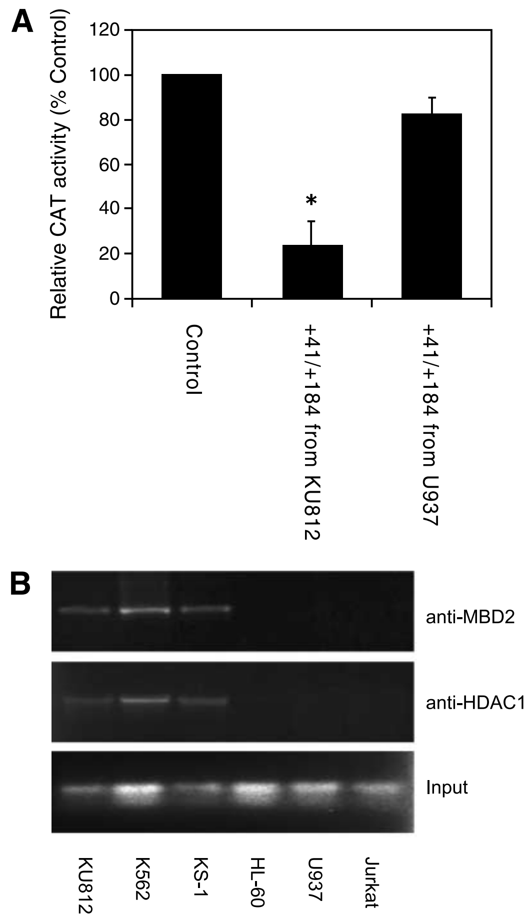

FIGURE 7. Role of DNA methylation in the region between +41 and +184 for Apaf-1 silencing. **A.** K562 cells were transfected with test plasmids (pCAT-enhancer vector [*Control*], pCAT-enhancer vector containing the bisulfite-treated fragment of the region between +41 and +184 of the *Apaf-1* gene from KU812 cells [+41/+184 from KU812], and pCAT-enhancer vector containing the same fragment from U937 cells [+41/+184 from U937]) and pSV2-neo vector and cultured in the presence of 1,000 μg/mL G418. After 14 days, cells were harvested for CAT assays. Relative CAT activity was expressed as %control with the activity of pCAT-enhancer vector (*Control*) set at 100%. Columns, means of three independent experiments; bars, ±SD. *, $P < 0.01$ (paired Student's t test). **B.** After cross-linking with formaldehyde, chromatin suspensions were prepared from the indicated cell lines and subjected to immunoprecipitation with antibodies against methylated DNA-binding domain 2 (*MBD2*) and histone deacetylase 1 (*HDAC1*). The resulting precipitants were subjected to PCR using a specific primer pair corresponding to nucleotide positions +41 to +184 of the *Apaf-1* gene. PCR was carried out for 30 cycles, and the amplified products were visualized by ethidium bromide staining after 2% agarose gel electrophoresis. Input, before the immunoprecipitation, 1 of 40 of the sonicated cell suspension was saved and used for PCR after reversal of the cross-linking.

Materials and Methods

Patients

Mononuclear cells were isolated from the peripheral blood or bone marrow aspirates of 11 patients with AML and one case of chronic myeloid leukemia in blastic crisis at diagnosis by sedimentation on Ficoll-Hypaque density gradients. Clinical characteristics of the patients are listed in Table 1. Informed consent was obtained from all subjects in accord with requirements of the institutional review board. Samples were selected for the study only when they contained >80% leukemic cells. As controls, we used CD34-positive normal bone marrow mononuclear cells purchased from Bio Whittaker, Inc. (Walkersville, MD) and T lymphocytes isolated from the peripheral blood of healthy volunteers.

Cell Lines

The following human leukemia-lymphoma cell lines were used in this study (their origins are shown in parentheses): KG-1 (AML-M1), ML-1 (AML), KCL22 (chronic myeloid leukemia in blastic crisis), HL-60 (AML-M2), THP-1 (AML-M5), U937 (histiocytic lymphoma), JOSK-I (AML-M4), KU812 (chronic myeloid leukemia in blastic crisis), K562(chronic myeloid leukemia in erythroid crisis), HEL (AML-M6), Mo7e (AML-M7), HPB-ALL (T-ALL), MOLT- 3 (T-ALL), Jurkat (T-ALL), Reh (B-ALL), NALM-16 (B-ALL), Daudi (Burkitt's lymphoma/ALL-L3), U266 (multiple myeloma), KS-1 (multiple myeloma), and Raji (Burkitt's lymphoma/ALL-L3).

Northern Blotting

We isolated total cellular RNA using cesium chloride ultracentrifugation and did RNA blotting according to the standard protocol.

RT-PCR Assay

Because of the limited amounts of RNA available from primary cells, we used semiquantitative RT-PCR to determine the expression of the Apaf-1 transcript instead of Northern blotting (32). The following primers were used: sense, 5′-GTCATCATCTTCTCTTAGCC-3′ and antisense, 5′-GTAGAAGGTCTGTGAGGATTCC-3′, which amplify the 1,511-bp fragment corresponding to nucleotides 2099 to 3609. Glyceraldehyde-3-phosphate dehydrogenase mRNA was simultaneously amplified using the following primers and served as a control: sense, 5′-CCACCCATGGCAAATTC-CATGGCA-3′ and antisense, 5′-TCTAGACGGCAGGT-CAGGTCCACC-3′, which generate the 600-bp fragment corresponding to nucleotides 212 to 811.

Western Blotting

Immunoblotting was carried out according to the standard method using the following antibodies: anti-Apaf-1 (NT; Millennium Biotechnology, Ramona, CA), anti-E2F-1 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53 (clone 80; BD Transduction Laboratories, San Jose, CA), anti-Sp-1 (PEP2; Santa Cruz Biotechnology), anti-Dnmt1 (60B1220; Alexis Biochemicals, Carlsbad, CA), anti-Dnmt3a (Calbiochem, San Diego, CA), anti-Dnmt3b (H-230; Santa Cruz Biotechnology), and anti-β-actin (C4; ICN Biomedicals, Aurora, OH).

Southern Blotting

Southern blotting was done according to the standard procedure using full-length Apaf-1 cDNA as a probe (33).

Methylation Status of Apaf-1 Promoter

We used direct PCR sequencing of sodium bisulfite–treated DNA to analyze the methylation status of the Apaf-1 promoter. Briefly, DNA was isolated from cells with phenol/

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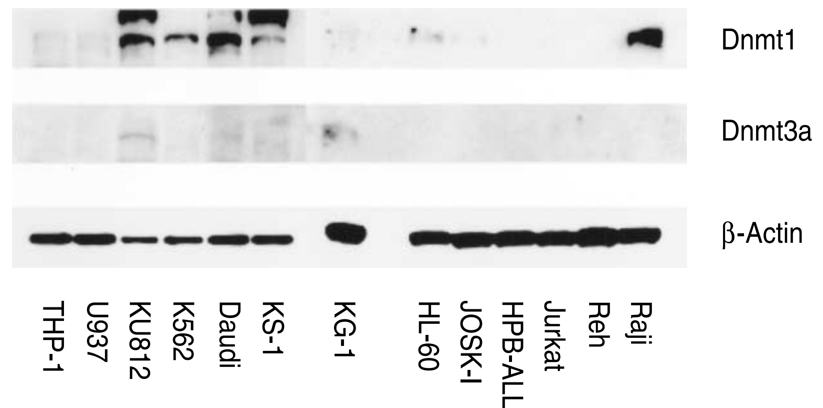

FIGURE 8. Expression of DNA methyltransferases in leukemia cell lines. Whole cell lysates were isolated from various leukemia cell lines and subjected to immunoblot analysis for Dnmt1 and Dnmt3a. The same samples were used for β-actin immunoblotting to verify the equal loading and integrity of samples. Representative data of multiple independent experiments.

chloroform extraction, denatured with NaOH, and incubated with 3.1 mol/L sodium bisulfite/0.5 mmol/L hydroquinone at 50°C for 16 hours (34). After being purified with DNA purification resin, DNA samples were subjected to PCR amplification for given Apaf-1 promoter regions (see Table 2 for primer sequences). The PCR products were subcloned into the pCRII vector (Invitrogen, Carlsbad, CA) for sequencing. To verify the quality of the bisulfite treatment, methylation status of p15/Ink4b and E-cadherin promoters was examined in each DNA sample by methylation-specific PCR using CpG WIZ amplification kits (Intergen, Purchase, NY).

Reporter Assay

The bisulfite-treated fragments of the region between +41 and +184 from KU812 and U937 cells were subcloned into the multicloning site, which is located upstream of SV40 promoter, of pCAT-enhancer vector (Promega, Madison, WI). The test plasmids were transfected into K562 cells along with pSV2-neo vector using electroporation as previously described (35). Cells were cultured in the presence of 1,000 μg/mL G418 and harvested for the measurement of CAT activity after 14 days.

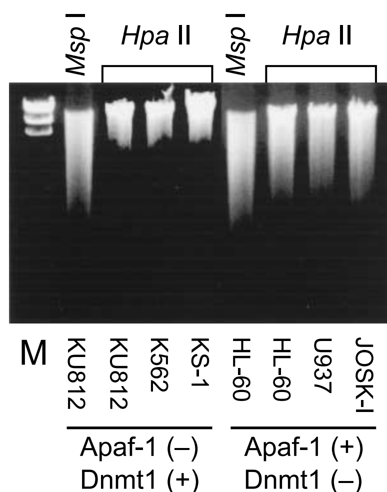

FIGURE 9. Sensitivity of genomic DNA from leukemia cell lines to a methylation-sensitive restriction endonuclease. Genomic DNA was isolated from cell lines with high Dnmt1 expression (*KU812*, *K562*, and *KS-1*) and low Dnmt expression (*HL-60*, *U937*, and *JOSK-I*), and digested with the methylation-sensitive restriction endonuclease *Hpa*II and its methylation-insensitive isoschizomer *Msp*I. The samples were visualized by ethidium bromide staining after 0.8% gel electrophoresis. We used *Hin*dIII-digested λ DNA as a molecular size marker (*M*).

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation assay was done as reported (36). Approximately 1×10^6 cells were resuspended in PBS, fixed with 1% formaldehyde at 37°C for 10 minutes, resuspended in 200 μL of SDS-lysis buffer [50 mmol/L Tris-HCl (pH 8), 10 mmol/L EDTA, and 1% SDS], and sonicated on ice with 10-second pulses four times to disrupt chromatin at an average length of 500 to 1,000 bp. Sonicated cell suspensions were centrifuged at 13,000 rpm for 10 minutes, and 20 μL of each supernatant were heated at 65°C for 4 hours after the addition of 0.8 μL of 5 mol/L NaCl, which was used as an input. The rest of the supernatant was added to 1.8 mL of chromatin immunoprecipitation dilution buffer [167 mmol/L NaCl, 16.7 mmol/L Tris-HCl (pH 8), 1.2 mmol/L EDTA, 0.01% SDS, 1.1% Triton X- 100, 20 μg/mL salmon sperm DNA, and 50 μg/mL yeast tRNA] containing 10 μg of either anti–methylated DNA-binding domain 2 antibody (Upstate Biotechnology, Lake Placid, NY) or anti–histone deacetylase 1 antibody (Sigma, St. Louis, MO). After incubation at 4°C for 16 hours, the mixtures were further rocked with 60 μL of protein A agarose beads in the presence of bovine serum albumin at 15 μg/mL and salmon sperm DNA at 12 μg/mL for 1 hour. The immunoprecipitates were washed thrice each with four different buffers and eluted with 0.1 mol/L $NaHCO_3$ and 1% SDS. The eluents were heat treated, digested with proteinase K, extracted with phenol/chloroform, ethanol precipitated, and finally resuspended in 20 μL of TE (pH 8). We used 5 μL of the final suspension for PCR amplification of the region between +41 and +184 of the *Apaf-1* gene.

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