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Dual Functions of the AML1/Evi-1 Chimeric Protein in the Mechanism of Leukemogenesis in t(3;21) Leukemias

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The chromosomal translocation t(3;21)(q26;q22), which is found in blastic crisis in chronic myelogenous leukemias and myelodysplastic syndrome-derived leukemias, produces AML1/Evi-1 chimeric transcription factor and is thought to play important roles in acute leukemic transformation of hemopoietic stem cells. We report here the functional analyses of AML1/Evi-1. It was revealed that AML1/Evi-1 itself does not alter the transactivation level through mouse polyomavirus enhancer-binding protein 2 (PEBP2; PEA2) sites (binding site of AML1) but dominantly suppresses the transactivation by intact AML1, which is assumed to be a stimulator of myeloid cell differentiation. DNA-binding competition is a putative mechanism of such dominant negative effects of AML1/Evi-1 because it binds to PEBP2 sites with higher affinity than AML1 does. Furthermore, AML1/Evi-1 stimulated c-*fos* promoter transactivation and increased AP-1 activity, as Evi-1 (which is not normally expressed in hemopoietic cells) did. Experiments using deletion mutants of AML1/Evi-1 showed that these two functions are mutually independent because the dominant negative effects on intact AML1 and the stimulation of AP-1 activity are dependent on the runt domain (DNA-binding domain of AML1) and the zinc finger domain near the C terminus, respectively. Furthermore, we showed that AML1/Evi-1 blocks granulocytic differentiation, otherwise induced by granulocyte colony-stimulating factor, of 32Dcl3 myeloid cells. It was also suggested that both AML1-derived and Evi-1-derived portions of the fusion protein play crucial roles in this differentiation block. We conclude that the leukemic cell transformation in t(3;21) leukemias is probably caused by these dual functions of AML1/Evi-1 chimeric protein.

Defined karyotypic abnormalities are observed in some types of human leukemias. By these chromosomal abnormalities, various genes encoding transcription factors are rearranged and the resultant fused genes produce chimeric proteins (9, 48). It is well known that the reciprocal translocations t(15;17)(q21;q21), t(8;21)(q22;q22), t(6;9)(p23;q34), and t(1;19)(q23;q13.3) result in formation of chimeric transcription factor proteins PML/retinoic acid receptor α chain (RARα) (12, 26), AML1/MTG8 (ETO) (15, 39, 41), DEK/CAN (68), and E2A/PBX1 (27, 49), respectively. Because these chimeric proteins should play causative roles in leukemogenesis, it is important to investigate both their transcriptional activities and their biological functions.

The t(3;21)(q26;q22) translocation, seen in the blastic crisis phase of chronic myelogenous leukemia and myelodysplastic syndrome-derived leukemia, is thought to cause leukemic cell transformation of hemopoietic stem cells (56, 61). We and others recently reported that t(3;21)(q26;q22) fuses *AML1* and *Evi-1* (ecotropic viral integration site 1) genes and produces AML1/Evi-1 chimeric protein (38, 50) (Fig. 1).

AML1 is a transcription factor which is highly homologous with the *Drosophila* pair-rule gene *runt* product and mouse polyomavirus enhancer-binding protein 2αA (PEBP2αA) and is a human homolog of mouse PEBP2αB or core-binding factor α(CBFα) of the Moloney leukemia virus enhancer (5, 28, 52, 69). From the *AML1* gene, at least three forms of proteins are produced by alternative splicings (40). AML1b has a runt domain, which is responsible for DNA binding to a PEBP2 consensus sequence and heterodimer formation with a human homolog of PEBP2β (CBFβ), and a proline-, serine- and threonine-rich (PST) region, which is a putative transcriptional activation domain (Fig. 1). AML1a has a runt domain but lacks a PST region. Recently we showed that AML1a and AML1b regulate both hemopoietic myeloid cell differentiation and transcriptional activation antagonistically (67). AML1c, which was identified last, differs from AML1b only in the small N-terminal region (40) and probably has the same function as AML1b. We have found that AML1b and AML1c show much higher levels of transcripts than does AML1a in normal bone marrow cells and several hemopoietic cell lines (64). In this report, therefore, we refer to AML1b protein as AML1, if not specified otherwise, and used this form for the functional analyses.

Evi-1, which is not expressed in normal hemopoietic cells (44, 46), is a transcription factor with two separate Cys_2His_2-type zinc finger domains (Fig. 1). *Evi-1* was first identified as a gene existing in a common locus of retroviral integration in myeloid tumors found in AKXD mice (47). In some murine leukemias, inappropriate expression of Evi-1 is caused by retroviral insertions (6, 7). The *Evi-1* gene is transcriptionally activated also in human acute myelogenous leukemias by translocations and inversions involving chromosome 3q26, which is the *Evi-1* gene locus (17, 45, 53). These studies suggest that inappropriate expression of Evi-1 disturbs normal cellular proliferation and differentiation in hematopoiesis, probably resulting in or at least contributing to leukemic transformation of the cells. Recently, we showed that Evi-1 stimulates trans-

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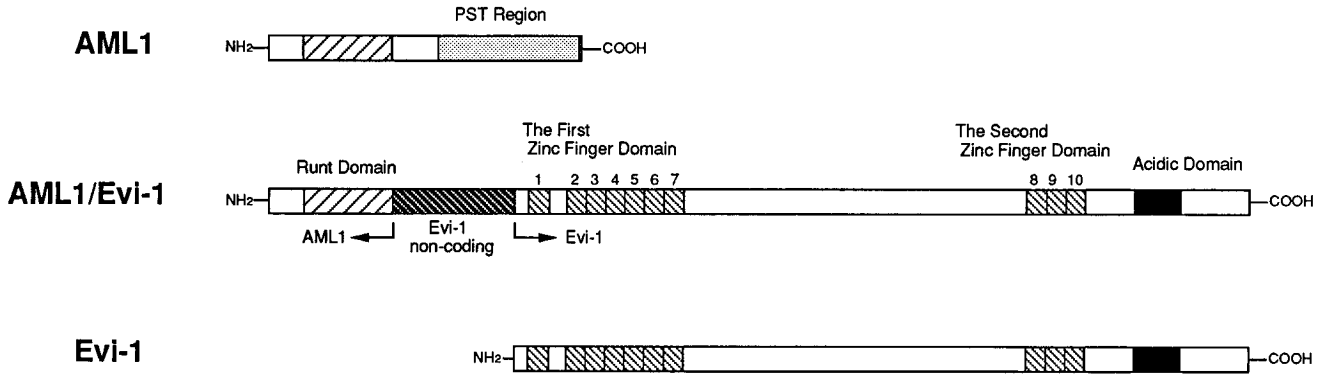

FIG. 1. Structures of AML1, AML1/Evi-1, and Evi-1 proteins. The structures of these proteins are schematically presented (5, 38, 40, 42). The locations of a runt domain, PST region, Evi-1 noncoding region, the first zinc finger domain, the second zinc finger domain, and an acidic (acidic amino acid-rich) domain are shown by boxes.

activation of the c-*fos* promoter, through at least two portions of the promoter, and raises AP-1 activity with dependence on the second zinc finger domain (65).

In AML1/Evi-1 fusion protein, AML1 is disrupted at the end of the runt domain and fused with peptides, which are translated from otherwise *Evi-1* noncoding exons, and the entire Evi-1 protein (38) (Fig. 1). In this study, we investigated the role of AML1/Evi-1 fusion protein as a transcriptional regulator and its biological functions to identify the mechanisms for leukemogenesis in t(3;21) leukemias.

MATERIALS AND METHODS

Cell culture. P19 cells were maintained on gelatin-coated dishes in Dulbecco's modified Eagle's medium (high-glucose formulation) supplemented with 10% fetal calf serum (57, 65). NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum. The 32Dcl3 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and 0.25 ng of murine interleukin-3 (IL-3; a generous gift from Kirin Brewery) per ml or cultured in 5 ng of human granulocyte colony-stimulating factor (G-CSF; a generous gift from Kirin Brewery) (instead of IL-3) per ml when indicated.

Plasmid construction. The cDNA of *AML1/Evi-1* was identified and obtained from the SKH-1 cell line (38). The human AML1 (AML1b) cDNA was kindly provided by M. Ohki (National Cancer Research Institute, Tokyo, Japan) (40). The sequence of Evi-1 cDNA, which was obtained from the AML1/Evi-1 fusion cDNA, was confirmed to be essentially identical to the sequence of the cDNA which Morishita et al. (42) obtained (38). The *Eco*RI site was created by site-directed mutagenesis (31) at bp 65, 65, or 33 bp upstream from the AML1/Evi-1, AML1, or Evi-1 translation initiation site, respectively, for subclonings into the expression vectors. These cDNAs were inserted into the *Eco*RI site of pME18S, an SRα promoter-driven expression plasmid (63), in the sense orientation to give pME-AE, pME-AML1, and pME-Evi-1, respectively. Tww-tk-Luc and Tmm-tk-Luc were produced by replacing the *Xho*I-*Hpa*I fragment of Tβ3W4W-tkCAT and Tβ3M4M-tkCAT (kindly provided by Y. Ito, Kyoto University, Kyoto, Japan) (52), respectively, with the *Hin*dIII-*Hpa*I fragment of luciferase cDNA, using a *Hin*dIII linker. Tww-tk-Luc contains the Tβ3-Tβ4 fragment (derived from the T-cell receptor β gene enhancer), which has two PEBP2 (PEA2) sites, inserted immediately upstream of the herpes simplex virus thymidine kinase promoter followed by the firefly luciferase cDNA. In Tmm-tk-Luc, the two nucleotides of each PEBP2 (PEA2) site sequence are changed from the PEBP2 site consensus sequence. The constructions of plasmid (wild TRE)×3-tk-Luc, in which three tandemly repeated 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)-responsive elements (TRE) were inserted immediately upstream of the thymidine kinase promoter followed by the firefly luciferase cDNA, and plasmid (mut. TRE)×3-tk-Luc, in which two nucleotides were replaced in each TRE, were described previously (65). SRα-Luc was constructed by inserting luciferase cDNA downstream of the SRα promoter in an expression plasmid. For construction of AML1/Evi-1 mutants, each new restriction enzyme site, *Apa*I (141) or *Eco*RV (3580) (numbers in parentheses indicate nucleotide numbers from the start site of translation to the cutting site of the enzyme), was created in the AML1/Evi-1 cDNA by site-directed mutagenesis (31). Deletion mutants Δrunt domain and ΔZF (zinc finger)8-10 were constructed by deleting internal fragments from mutagenic *Apa*I (141) to *Apa*I (516) and *Eco*473 (3253) to mutagenic *Eco*RV (3580), respectively. For ΔZF8-10+AD (acidic domain), the *Eco*473 (3253)-*Afl*II (4045) fragment was deleted, filled in with Klenow fragment, and religated. To construct expression plasmids, three mutants generated as described above were inserted into an *Eco*RI site of pME18S. For ΔC-end, the *Asu*II (3600)-*Spe*I fragment of pME-AE was deleted, filled in with Klenow fragment, and religated. The AML1/Evi-1 and ΔZF8-10 cDNAs were inserted also into the *Eco*RI site of pMV7 (8) in the sense orientation to give pMV-AE and pMV-AEΔ, respectively.

Luciferase assay. For the analysis of luciferase activities in P19 cells, reporter and expression plasmids were transfected into the cells by the calcium phosphate precipitation method as described previously (29, 67). In cotransfection with several expression plasmids, equivalent-molar plasmid DNAs were transfected. To keep the transfection efficiency as constant as possible among the samples to be compared, the total amount of DNA in terms of weight was adjusted to be equal by adding plasmid pUC13 DNA. P19 cells were washed twice with phosphate-buffered saline after 10 to 12 h of transfection, cultured for 30 to 36 h and harvested.

NIH 3T3 cells were transfected with expression and reporter plasmids and subjected to the luciferase analyses as previously described (65).

For the luciferase assay, 32Dcl3-derived clones were transfected with reporter plasmids by DEAE-dextran treatment followed by chloroquine exposure (62). The 32Dcl3-derived clones were cultured in IL-3-containing medium 40 to 48 h after transfection and harvested. Transfection of SRα-Luc (3 μg) was also performed for calibrations of cell numbers and other conditions among 32Dcl3-derived clones. Luciferase activities evaluated by transfection of SRα-Luc showed no more than 15% difference among clones, which indicates that similar transfection conditions were maintained among clones, and were used for standardization of measured luciferase activities in each clone.

The luciferase assay system (Promega) and a luminometer (Luminoscan; Labsystems) were used to estimate luciferase activities. In all assays of luciferase activity, a plasmid expressing β-galactosidase (1 μg) was cotransfected as an internal control of transfection efficiency, and the data were normalized to the β-galactosidase activity as described previously (65). All transfection experiments were performed at least twice (similar results were obtained), and representative data are presented.

EMSA. Nuclear extracts were obtained from COS-7 cells (1) which were transfected with pME18S, pME-AE, pME-AML1, or a Δrunt domain construct DNA inserted into pME18S by the DEAE-dextran method (62). Procedures for the electrophoretic mobility gel assay (EMSA) were described previously (59, 67). The M4 probe, which includes a partial A core of the polyomavirus enhancer and a mutated PEBP4 site (the introduced mutation abolishes the binding of PEBP4), was produced by annealing oligonucleotides 5′-GATCTAACTGAC CGCAGCTGTCAGTGCGAG-3′ and 5′-GATCCTCGCACTGACAGCTGCG GTCAGTTA-3′ (21). The M24 probe, in which the sequence of the PEBP2 site in the M4 probe was changed to one different from the PEBP2 consensus sequence, was obtained by annealing oligonucleotides 5′-GATCTAACTCACG GCAGCTGTCAGTGCGAG-3′ and 5′-GATCCTCGCACTGACAGCTGCCG TGAGTTA-3′. For radioisotope labeling, [α-^{32}P]dCTP was incorporated into the M4 probe by incubation with Klenow fragment. The anti-AML1 serum used in EMSA was obtained from a rabbit immunized by *Escherichia coli*-produced maltose-binding protein fused with a partial AML1 protein (corresponding to a cDNA fragment from bp +96, relative to the start site of translation, to the end of translation) as described previously (67). The immunoglobulin G fractions of the serum were obtained with a protein A-Sepharose column (Pharmacia).

Isolation of 32Dcl3 stable transfectants. The 32Dcl3 cells were transfected with pMV7, pMV-AE, or pMV-AEΔ by an electroporation method (55). Following transfection, selection with G418 (800 μg/ml) for 2 weeks and limiting dilution were performed. Surviving clones were screened for expression of AML1/Evi-1 fusion protein or AML1/Evi-1ΔZF8-10 mutant by Western blotting (immunoblotting).

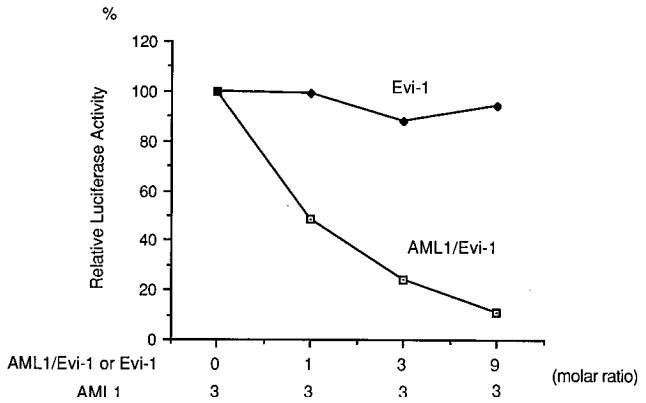

FIG. 2. Dominant negative effects of AML1/Evi-1 fusion protein on transactivation by AML1. P19 cells (3×10^5) were transfected with Tww-tk-Luc (5 μg), pME-AML1 (2.4 μg), and pME-AE in the indicated molar ratios. As a control, pME-Evi-1 was used instead of pME-AE. The vertical axis shows luciferase activities in percentages relative to the activity when only pME-AML1 was transfected with the reporter plasmid.

Western blotting and Northern (RNA) blotting. Anti-Evi-1 serum was obtained and Western blottings were performed as previously described (65). Procedures for Northern blottings were also as described previously (66) except that filters were washed at 55°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

RESULTS

Dominant negative effects of AML1/Evi-1 chimeric protein on transactivation by AML1 through the PEBP2 (PEA2) sites. The *AML1* gene is a human homolog of murine the *PEBP2αB* (*CBFα*) gene (5, 69), whose product was first identified as a transcriptional activator for the PEBP2 (PEA2) sites found in the polyomavirus enhancer (54, 59). Recently we have shown that AML1, as well as murine PEBP2αB, is a transcriptional activator through the PEBP2 (PEA2) site (67). AML1/Evi-1 fusion protein might bind to the PEBP2 (PEA2) site and alter the transactivation level because it has an intact runt domain in its AML1 portion (Fig. 1). Therefore, we investigated the transcriptional activity of AML1/Evi-1 fusion protein through the PEBP2 (PEA2) sites, using P19 murine embryonal carcinoma cell line, which has only very low levels of intrinsic PEBP2 (CBF) activity in its undifferentiated state (4). As controls, AML1 and Evi-1 were also examined for their transactivation abilities. Each expression plasmid was cotransfected with the reporter plasmid Tww-tk-Luc or Tmm-tk-Luc into P19 cells. AML1 showed a 20-fold or more level of transactivation specifically through the PEBP2 site, as we have previously reported (67). However, Evi-1 and AML1/Evi-1 showed no capacity to transcriptionally activate the PEBP2 (PEA2) site (data not shown). These findings are consistent with the fact that AML1/Evi-1 has lost the PST region seen in intact AML1, a putative transcriptional activation domain, by the gene rearrangement (38) (Fig. 1).

Furthermore, we investigated whether AML1/Evi-1 alters the transactivation level raised by AML1. Cotransfection of AML1 and AML1/Evi-1 into P19 cells decreased the transactivation level through the PEBP2 (PEA2) sites compared with the level when only AML1 was expressed (Fig. 2). The increased amount of the transfected AML1/Evi-1 expression plasmid was associated with the decreased transactivation level. When Evi-1 was coexpressed (as a control) with AML1, the transactivation by AML1 was not affected. To rule out the possibility that the cotransfected AML1/Evi-1 expression vector affects the AML1 expression level as a result of a competition for transactivation machineries by the common promoters or unknown mechanisms, we investigated expression levels of AML1 when AML1/Evi-1 was coexpressed. We observed no change of the AML1 expression level in Western blot analysis using an AML1-specific antibody (data not shown). To investigate whether the suppressing activity of AML1/Evi-1 is specific for the transactivation by AML1, we used the GAL4/VP16 transactivation system. GAL4/VP16, a chimeric protein of the GAL4 DNA-binding domain and VP16 transactivation domain, transactivates through a GAL4 site (58). AML1/Evi-1 had no effects on the level of transactivation through the GAL4 sites by GAL4/VP16 fusion protein when it was coexpressed with GAL4/VP16 in P19 cells (data not shown). These findings indicate that AML1/Evi-1 dominantly and specifically suppresses the transactivation by AML1 through the PEBP2 (PEA2) site.

Analyses of binding of AML1/Evi-1 fusion protein to the PEBP2 (PEA2) site. Both AML1 and AML1/Evi-1 possess a runt domain, which is reported to be responsible for binding to the PEBP2 (PEA2) site (35, 52). Recently, we demonstrated that AML1 specifically binds to a PEBP2 (PEA2) site (67). If AML1/Evi-1 also binds to the PEBP2 (PEA2) site, the competition for DNA binding between AML1 and AML1/Evi-1 should be considered a possible mechanism for the suppression of AML1 transactivation by AML1/Evi-1. We used EMSA to investigate whether AML1/Evi-1 binds a PEBP2 (PEA2) site (Fig. 3A). For this assay, a double-stranded oligonucleotide containing a PEBP2 (PEA2) site was used as a probe. When this probe was incubated and electrophoresed with nuclear extract prepared from AML1/Evi-1-expressing COS cells, we observed a distinct band (Fig. 3A, lane 2) which is not seen in the mock lane (lane 1; nuclear extract prepared from empty vector-transfected COS cells). This band contains AML1/Evi-1 protein because the band became faint and showed a slight supershift when an anti-AML1 antibody was added (lane 7). The band provided by AML1/Evi-1 was abolished when an unlabeled probe (containing the same sequence as a labeled probe) was coincubated (lane 3). On the other hand, the intensity of the band was not affected when we used an unlabeled probe in which the PEBP2 (PEA2) site was changed to a sequence different from the PEBP2 (PEA2) site consensus sequence (lane 4). These findings indicate that binding of AML1/Evi-1 to a probe depends on the PEBP2 (PEA2) site.

When we consider the competitive binding of AML1 and AML1/Evi-1 to the PEBP2 (PEA2) site, the DNA-binding affinity of each protein is crucial. DNA-binding affinity was analyzed by adding an unlabeled competitor in various concentrations and evaluating intensities of shifted bands in EMSA (Fig. 3B). This method has been used in some studies (25, 67) and is rationalized by the fact, deduced from the reactant kinetic theory, that intensities of the shifted bands are approximately proportional to the amounts of nonspecific binding [in this case, binding mainly to poly(dI-dC) included in the reaction mixture] of the protein in question in the process of increasing the concentration of the unlabeled competitor. AML1/Evi-1 required a reduced competitor DNA concentration for equivalent declining in binding by AML1, demonstrating that the affinity of AML1/Evi-1 for the PEBP2 (PEA2) site was severalfold higher than that of AML1. These data support the hypothesis that AML1/Evi-1 dominantly suppresses AML1 transactivation by competing with AML1 for binding to the PEBP2 (PEA2) site.

Increased AP-1 activity in the presence of AML1/Evi-1. We have reported that Evi-1 raises the AP-1 activity probably by stimulating transactivation of the c-*fos* gene promoter (65).

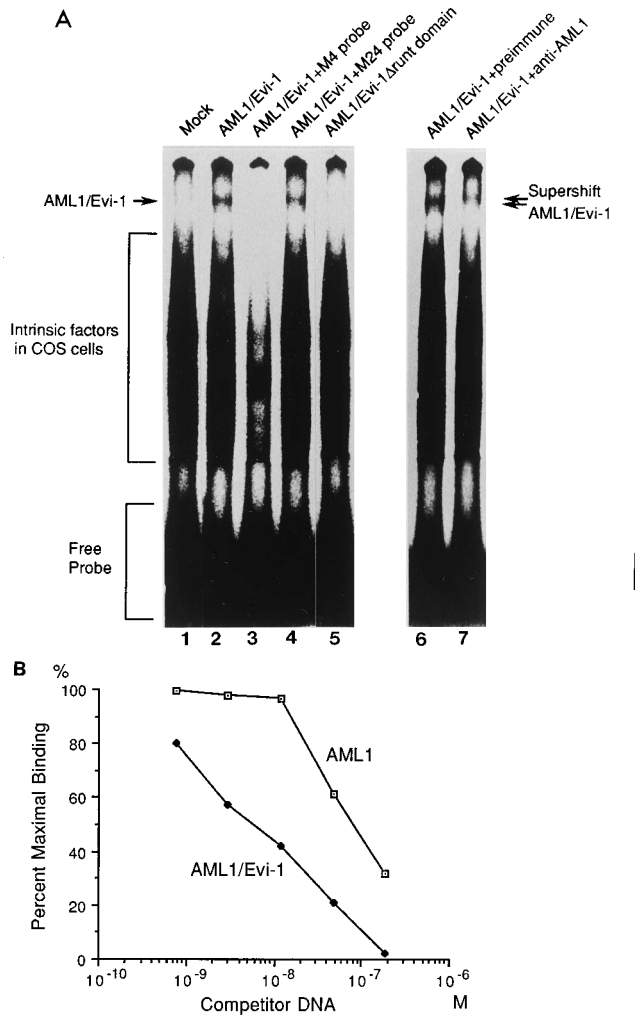

FIG. 3. EMSA. (A) ^{32}P-labeled M4 probe (1 ng) was coincubated with nuclear extracts (containing 10 μg of protein) from COS cells transfected with pME18S (lane 1), pME-AE (lane 2, 3, 4, 6, and 7), or a Δrunt domain construct DNA inserted into pME18S (lane 5). Seventy nanograms of unlabeled M4 (lane 3) or M24 (lane 4) probe was added to the reactions. The immunoglobulin G fraction (containing 6 μg of protein) from preimmune (lane 6) or anti-AML1 (lane 7) serum was also added to the reaction. Arrows indicate the shifted bands including AML1/Evi-1 and its supershift. (B) Analyses of DNA-binding affinity of AML1/Evi-1. The EMSA was performed as for panel A. Competition curves are shown for AML1 and AML1/Evi-1 binding to the PEBP2 site in the presence of increasing concentrations of identical but unlabeled M4 probe. Quantification of bound DNA was evaluated with a computer-controlled imaging system (Fujix BAS 2000). Molar concentrations of competitor DNA are indicated. Data for the AML1 competition curve were shown elsewhere (67) (upper band of AML1b) and are presented here as controls.

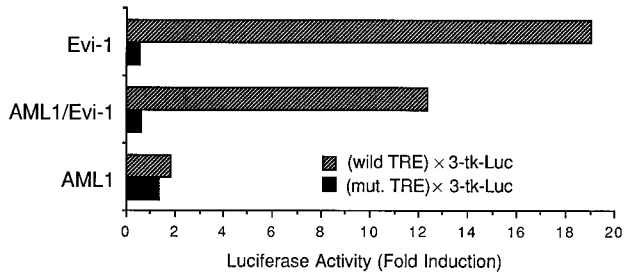

FIG. 4. Evaluation of AP-1 activity in the presence of AML1/Evi-1 chimeric protein. NIH 3T3 cells (3×10^5) were transfected with each expression plasmid, 2.4 μg of pME-AML1 or an equivalent molar pME-AE or pME-Evi-1. The reporter plasmid (5 μg), (wild TRE)×3-tk-Luc or (mut. TRE)×3-tk-Luc, was cotransfected with each expression plasmid. Bars show luciferase activities expressed relative to the levels when control vector pME18S was cotransfected with the corresponding reporter plasmid. Data for Evi-1 were shown elsewhere (65) and are presented here as controls.

Since AML1/Evi-1 fusion protein contains the whole Evi-1 protein amino acid sequence, it is plausible that AML1/Evi-1 could increase the AP-1 activity as does Evi-1. To investigate this possibility, we transfected both the reporter, (wild TRE) ×3-tk-Luc containing three tandemly repeated TREs or (mut. TRE)×3-tk-Luc containing mutated TREs, and Evi-1, AML1/Evi-1, or AML1 an expression plasmid into NIH 3T3 cells, which are frequently used for studies of AP-1 activity (Fig. 4). Expression of AML1/Evi-1, like that of Evi-1, led to marked transactivation specific to TREs, but no significant transactivation was observed when AML1 was expressed. Furthermore, we observed stimulated transactivation of the c-*fos* promoter in the presence of Evi-1 or AML1/Evi-1 but not in the presence of AML1 (data not shown). These findings demonstrate that AML1/Evi-1 expression raises the AP-1 activity as Evi-1 expression does. The AP-1 activation by AML1/Evi-1 should be correlated with cellular transformation, because AML1/Evi-1-expressing fibroblast cells show increased c-*jun* transcripts and anchorage-independent growth in soft agar (32).

Functional analyses of AML1/Evi-1 deletion mutants as transcriptional regulators. So far, we have shown that AML1/Evi-1 fusion protein suppresses transactivation by AML1 and increases AP-1 activity. Are these two functions independent or mutually dependent? To address this question, we constructed AML1/Evi-1 mutants lacking various functional domains as transcription factors (Fig. 5A) and analyzed their functions. When mutant expression plasmids were transfected into NIH 3T3 cells, each mutant protein was sufficiently expressed in the anticipated size (Fig. 5B). First, we investigated whether AML1/Evi-1 deletion mutants have dominant negative effects on transactivation by AML1 (Fig. 5C). Neither ΔZF8-10 nor Δrunt domain showed stimulated transactivation through the PEBP2 (PEA2) site, as was the case with wild-type AML1/Evi-1. When coexpressed with AML1, ΔZF8-10 suppressed the transactivation by AML1 but Δrunt domain did not. ΔC-end and ΔZF8-10+AD showed results similar to those for ΔZF8-10 (data not shown). In EMSA, use of Δrunt domain-containing nuclear extract resulted in disappearance of the shifted band (Fig. 3A, lane 5). These results show that the DNA-binding ability and the dominant negative effect (on transactivation by AML1) are correlated with each other and suggest that the dominant negative effect of AML1/Evi-1 is due to the competition for DNA binding between AML1/Evi-1 and AML1. Second, we evaluated AP-1 activity in the presence of each mutant protein (Fig. 5D). ΔC-end increased the AP-1 activity (we have discussed elsewhere the reason why the acidic domain is unnecessary for increasing AP-1 activity [65]), but ΔZF8-10 and ΔZF8-10+AD did not. These results are the same as those for Evi-1 mutants (65) and consistent with the observation that ΔZF8-10 and ΔZF8-10+AD lose the ability to transform fibroblast cells but ΔC-end can transform them (32). In addition, we found AP-1 activation by Δrunt domain. In short, the dominant negative effect of AML1/Evi-1 (on transactivation by AML1) is dependent on the runt domain but not on the second zinc finger domain, and the AP-1 activation by AML1/Evi-1 is due to the second zinc finger domain but not due to the runt domain. Since these results indicate that AML1/Evi-1 has two independent functions, we can conclude that AML1/Evi-1 is a chimeric transcription factor with dual functions.

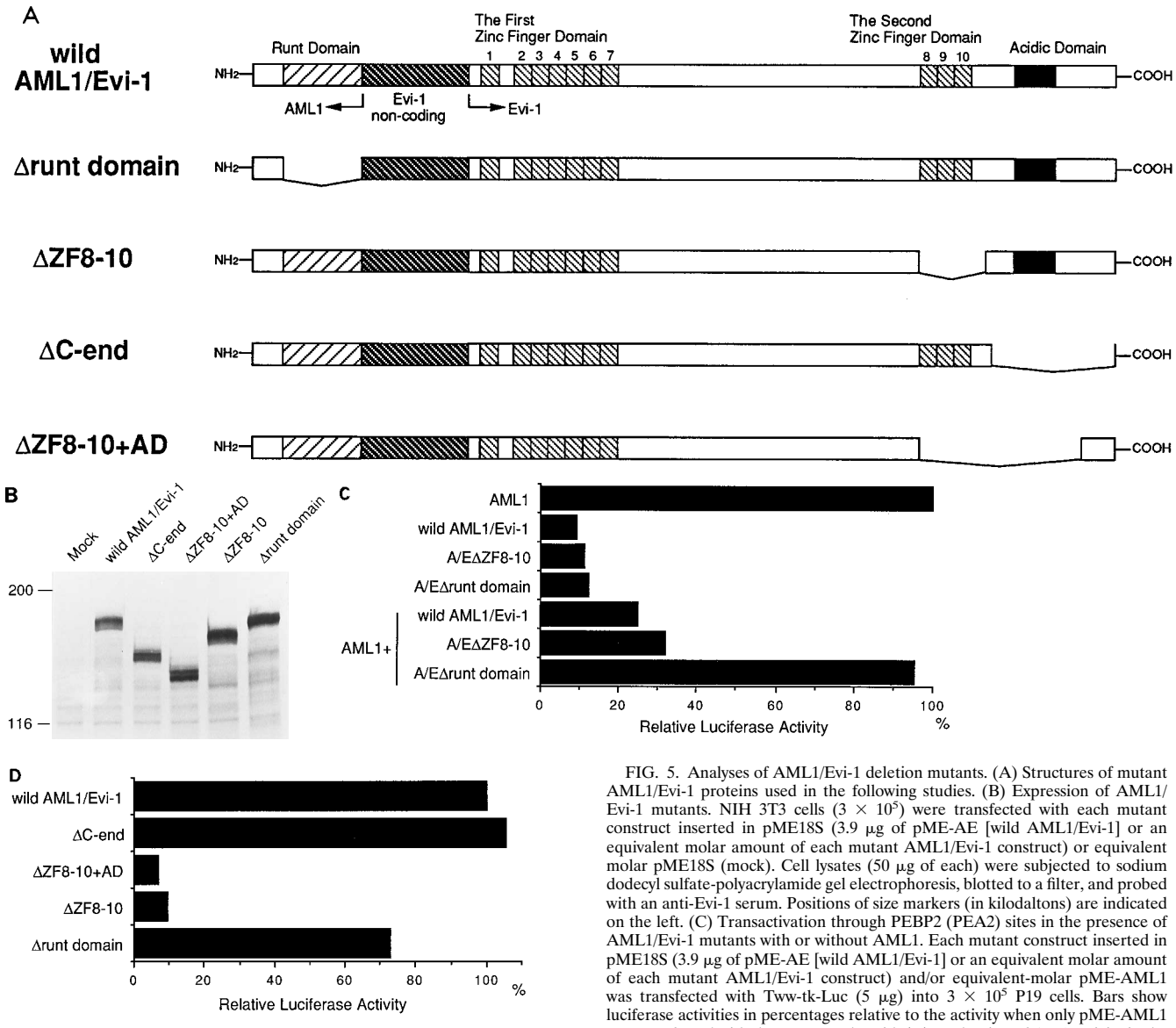

FIG. 5. Analyses of AML1/Evi-1 deletion mutants. (A) Structures of mutant AML1/Evi-1 proteins used in the following studies. (B) Expression of AML1/Evi-1 mutants. NIH 3T3 cells (3×10^5) were transfected with each mutant construct inserted in pME18S (3.9 μg of pME-AE [wild AML1/Evi-1] or an equivalent molar amount of each mutant AML1/Evi-1 construct) or equivalent molar pME18S (mock). Cell lysates (50 μg of each) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted to a filter, and probed with an anti-Evi-1 serum. Positions of size markers (in kilodaltons) are indicated on the left. (C) Transactivation through PEBP2 (PEA2) sites in the presence of AML1/Evi-1 mutants with or without AML1. Each mutant construct inserted in pME18S (3.9 μg of pME-AE [wild AML1/Evi-1] or an equivalent molar amount of each mutant AML1/Evi-1 construct) and/or equivalent-molar pME-AML1 was transfected with Tww-tk-Luc (5 μg) into 3×10^5 P19 cells. Bars show luciferase activities in percentages relative to the activity when only pME-AML1 was transfected with the reporter plasmid. (D) Evaluation of AP-1 activity in the presence of AML1/Evi-1 mutants. Each mutant construct inserted in pME18S (3.9 μg of pME-AE [wild AML1/Evi-1] or an equivalent molar amount of each mutant AML1/Evi-1 construct) was transfected with (wild TRE)×3-tk-Luc (5 μg) into 3×10^5 NIH 3T3 cells as previously described (65). Bars show luciferase activities in percentages relative to the activity when wild-type AML1/Evi-1 was transfected with the reporter plasmid.

Biological effects of AML1/Evi-1 fusion protein in a 32Dcl3 myeloid cell line. Does AML1/Evi-1 chimeric protein induce any biological effects which are correlated with dual functions as a transcriptional regulator? To address this question, we used the 32Dcl3 murine IL-3-dependent myeloid cell line, which clearly differentiates to mature granulocytes when treated with G-CSF (36). Recently it was demonstrated that overexpessed AML1a (a short form of AML1), which dominantly suppresses AML1 (AML1b)-induced transactivation as well as AML1/Evi-1, blocks granulocytic differentiation of 32Dcl3 cells treated with G-CSF and induces G-CSF-dependent proliferation (67). We experimentally confirmed that Evi-1-overexpressing 32Dcl3 cells also block granulocytic differentiation as do cells overexpressing AML1a but have an increased loss of cellular viability, in contrast to AML1a-expressing cells, when cultured in G-CSF, as previously reported (43) (data not shown). We expected that similar biological effects may be induced by AML1/Evi-1 as well. Therefore, we established several 32Dcl3 stable cell clones expressing AML1/Evi-1 fusion protein by transfecting the AML1/Evi-1 expression plasmid (pMV-AE) carrying the Neo^r gene and selecting the transfected cells for G418 resistance. AE-51 and AE-53 are representative clones showing stable expression of AML1/Evi-1 (Fig. 6). Two control 32Dcl3 clones, which were transfected with the empty vector, were designated O-11 and O-22. First, we investigated functions of AML1/Evi-1 as a transcriptional regulator in 32Dcl3 cells. As shown in Fig. 7A, significant transactivation through PEBP2 (PEA2) sites was observed in O-11 and O-22, but such transactivation was reduced in AE-51 and AE-53 cells. These effects are probably explained by the dominant negative effects of AML1/Evi-1 protein on the intrinsic PEBP2 activity in 32Dcl3 cells. The increased AP-1 activity (two- to threefold) was also detected in AE-51 and AE-53, although it was not seen in control clones (Fig. 7B). In

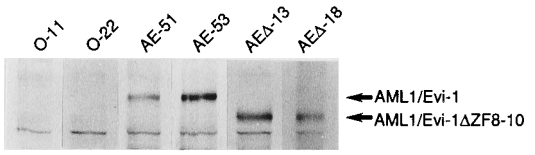

FIG. 6. Expression of the AML1/Evi-1 fusion protein or AML1/Evi-1ΔZF8-10 mutant protein in 32Dcl3 cell-derived clones. All clones were established from 32Dcl3 cells which were transfected with the expression vectors and survived G418 selection. To obtain O-11/O-22, AE-51/AE-53, and AEΔ-13/AEΔ-18 clones, cells were transfected with pMV-7 empty vector, pMV-AE, and pMV-AEΔ, respectively. Lysates prepared from these clones were subjected to Western blot analysis using the anti-Evi-1 serum (65). Arrows indicate the AML1/Evi-1 fusion protein or AML1/Evi-1ΔZF8-10 mutant protein.

the presence of IL-3, O-11, O-22, AE-51, AE-53, and parental 32Dcl3 cells showed comparable viabilities and proliferative abilities (data not shown). In the absence of IL-3, these four clones and 32Dcl3 cells lost viability and died completely within 3 days (data not shown). When O-11, O-22, and parental 32Dcl3 cells were cultured in G-CSF instead of IL-3, they underwent terminal differentiation to morphologically mature granulocytes and showed a gradual decline in viable cell number (Fig. 8A and B). In contrast, AE-51 and AE-53 rapidly lost viability and mostly died within a week without obvious differentiation to morphologically mature granulocytes (Fig. 8A and B). Myeloperoxidase (MPO) is an enzyme which is expressed specifically during terminal differentiation into granulocytes. The commitment of 32Dcl3 cells to granulocytic differentiation is associated with increased transcripts for MPO (3). We investigated MPO transcripts to confirm whether the terminal differentiation is induced upon G-CSF treatment of established 32Dcl3 clones (Fig. 8C). In parental 32Dcl3 cells and control O-11 and O-12 clones, MPO transcripts were detected within 2 days following transfer to G-CSF and increased afterwards. In contrast, AE-51 and AE-53 showed no detectable levels of MPO transcripts within 4 days. (At day 6 or later, we could not obtain RNA from the AE-51 or AE-53 clone for analyses because of viability loss.) Figure 8C shows representative data of these findings. These results demonstrate that the AML1/Evi-1 fusion protein blocks terminal differentiation to mature granulocytes and stimulates the loss of cellular viability in 32Dcl3 cells treated with G-CSF. These biological effects are similar to those seen in Evi-1-overexpressing 32Dcl3 cells (43).

Biological effects of AML1/Evi-1ΔZF8-10 mutant protein in a 32Dcl3 myeloid cell line. The biological effects induced by the fusion protein in 32Dcl3 cells appeared to be explained by the function of Evi-1. However, it was possible that the dominant negative effects of AML1/Evi-1 on transactivation by AML1 also played a role in the block of granulocytic differentiation, because dominant negative effects (on AML1 transactivation) accompany the differentiation block in 32Dcl3 cells, at least in AML1a (67). We hypothesized that the differentiation block is induced independently by dual functions of AML1/Evi-1 and that the loss of cellular viability is induced because Evi-1's functions are dominant, as for cellular viability, over the effects of the AML1 portion, which could probably induce G-CSF-dependent cell growth as seen for AML1a. Such hypotheses prompted us to investigate the effect on 32Dcl3 cells of an AML1/Evi-1 mutant protein which lacks Evi-1's function. The second zinc finger domain of Evi-1 is essential for the activation of AP-1 (65) and highly conserved in evolution compared with other portions in Evi-1. For instance, it is well conserved in *Caenorhabditis elegans* and *Drosophila melanogaster* (20, 22). In addition, ΔZF8-10 is a minimum alteration for lacking Evi-1

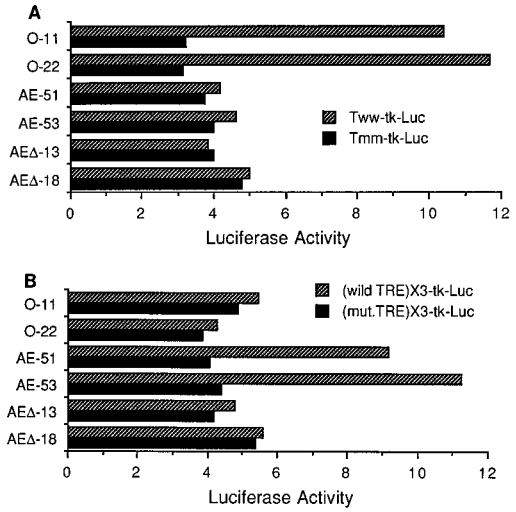

FIG. 7. Luciferase assays using 32Dcl3-derived clones. (A) Evaluation of transactivation through the PEBP2 (PEA2) sites. O-11, O-22, AE-51, AE-53, AEΔ-13, and AEΔ-18 clone cells (1.0×10^6 of each) were transfected with the reporter plasmid (7 μg), Tww-tk-Luc or Tmm-tk-Luc, and subjected to luciferase assays. Bars show luciferase activities expressed in arbitrary units. (B) Evaluation of AP-1 activity. Each clone was transfected with the reporter plasmid (5 μg), (wild TRE)×3-tk-Luc or (mut. TRE)×3-tk-Luc, and subjected to luciferase assays. Bars show luciferase activities expressed in arbitrary units.

function and probably minimizes conformational changes of the whole chimeric protein. Therefore, we transfected plasmid pMV-AEΔ into 32Dcl3 cells, screened for the expression of AML1/Evi-1ΔZF8-10 mutant protein, and obtained several clones expressing it. AEΔ-13 and AEΔ-18 are representative stable clones that we have obtained (Fig. 6). In AEΔ-13 and AEΔ-18, the PEBP2 (PEA2) site-dependent transactivation was suppressed as seen in AE-51 and AE-53 (Fig. 7A). Increased AP-1 activity was not observed in AEΔ-13 and AEΔ-18, in contrast to AE-51 and AE-53 (Fig. 7B). These observations are compatible with those when ΔZF8-10 is transiently expressed in P19 or NIH 3T3 cells. In the presence of IL-3, AEΔ-13 and AEΔ-18 clones showed proliferative abilities comparable to those of AE-51, AE-53, and control clones (O-11 and O-22) (data not shown). AEΔ-13, AEΔ-18, O-11, and O-22 clones similarly lost viability when deprived of IL-3 (data not shown). When cultured in G-CSF, AEΔ-13 and AEΔ-18 clones exponentially proliferated for at least 1 month without losing viability, in contrast to AE-51 and AE-53 (Fig. 8A). AEΔ-13 and AEΔ-18 showed no morphological differentiation to granulocytes and almost no detectable levels of MPO transcripts in the presence of G-CSF (Fig. 8B and C). These observations support our hypotheses and strongly suggest that both AML1-derived and Evi-1-derived portions of the fusion protein play crucial roles for the block of granulocytic differentiation in 32Dcl3 cells.

DISCUSSION

Our studies revealed that AML1/Evi-1 fusion protein generated by t(3;21)(q26;q22) has dual functions, dominant suppression of transactivation by AML1 and activation of AP-1, as a chimeric transcription factor. These effects are dependent on the runt domain and the second zinc finger domain of Evi-1, respectively.

AML1 should have important roles in hemopoietic differentiation because AML1 is transcriptionally active in hemo-

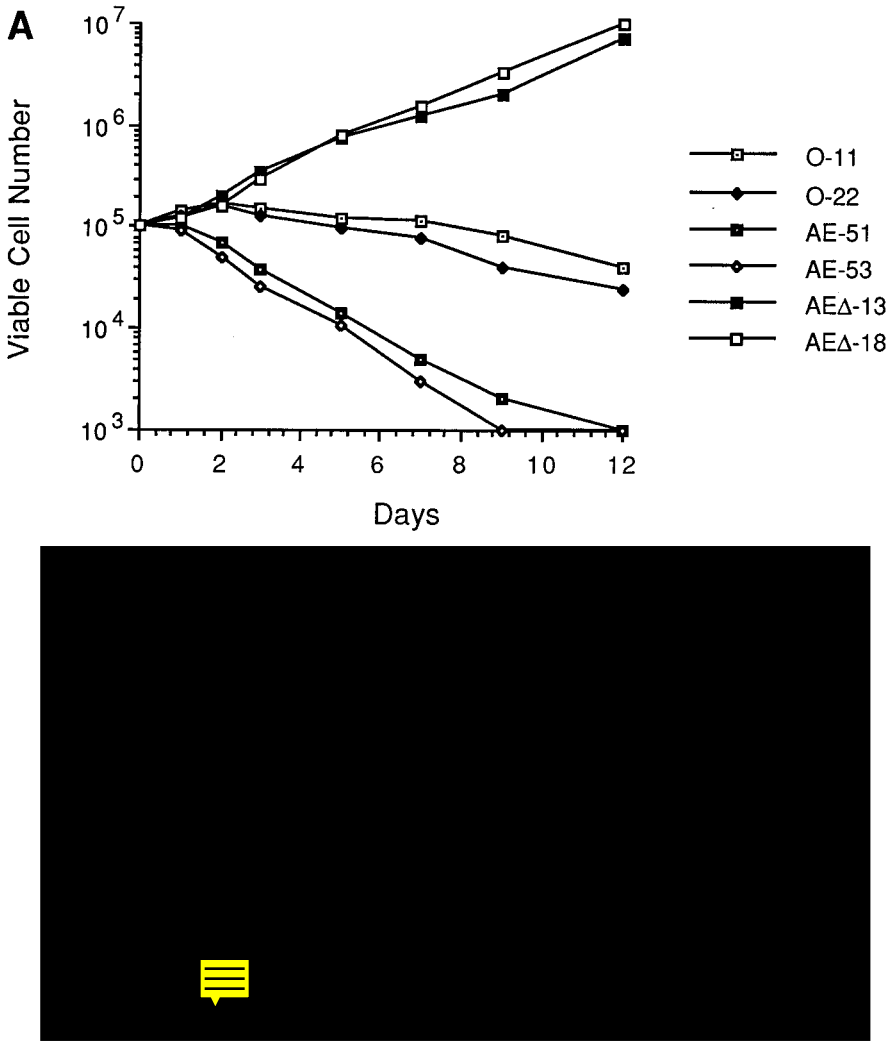

FIG. 8. Analyses of G-CSF-induced differentiation using 32Dcl3-derived clones. (A) Time course of viable cell numbers in the presence of G-CSF. O-11, O-22, AE-51, AE-53, AEΔ-13, and AEΔ-18 clones were washed free of IL-3 and subsequently cultured in medium containing 5 ng of human G-CSF per ml. Cultures were diluted when the cell number reached 10^6/ml. At several time points, the viable cell numbers were counted. Cell viability was assessed by trypan blue exclusion. Three trials gave similar results; representative data are shown. (B) Cell morphology of 32Dcl3 clones when cultured in medium containing IL-3 or G-CSF. Cytospin preparations were made and stained with May-Grunwald-Giemsa solution. (C) Northern blot analysis showing expression of MPO transcripts in 32Dcl3 clones. Total RNAs (15 μg) were prepared from cells treated with G-CSF for the indicated number of days, blotted, and hybridized with mouse MPO cDNA. As a control for RNA loading, the blots were also hybridized with a rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probe (18).

poietic organs (39) and because PEBP2s (CBFs) (including a mouse homolog of AML1) show a variety of patterns of gel shift bands among mouse hemopoietic lineages (60). In fact, we have indicated that AML1a and AML1b show antagonistic actions on granulocytic differentiation in 32Dcl3 cells and that AML1b can stimulate myeloid differentiation (67). The alteration of AML1 functions is known to induce leukemic cell transformation in some myelogenous leukemias with karyotypes such as t(8;21)(q22;q22), t(3;21)(q26;q22), and inv (16)(p13q22). In inv(16)(p13q22), CBFB, which is a human homolog of PEBP2β (CBFβ) (51) and heterodimerizes with AML1, is disrupted and forms a chimeric protein with smooth muscle myosin heavy chain (SMMHC) (33). These facts also suggest that AML1 has essential roles in regulation of myeloid cell differentiation and proliferation. Our data indicate that AML1/Evi-1 fusion protein has dominant negative effects on transactivation by AML1. The runt homology domain of AML1 is intact in AML1/Evi-1 fusion protein. This domain is shown to function as a DNA-binding domain and to be also responsible, at least in PEBP2αA, for dimerization with PEBP2β (CBFβ) (35, 52). PEBP2β (CBFβ) shows ubiquitous expression in various mammalian cell lines, and heterodimerization with PEBP2α (CBFα) causes a marked increase in the

intrinsic DNA-binding affinity of the α subunit (51, 69). We have not clarified completely the mechanism by which AML1/Evi-1 dominantly suppresses transcriptional activation through the PEBP2 (PEA2) site by AML1. However, the most probable mechanism is a competition for DNA binding between AML1 and AML1/Evi-1. This mechanism is strongly supported by our observations that AML1 and AML1/Evi-1 bind the same DNA sequence and AML1/Evi-1 shows higher affinity than AML1. Another possible mechanism is a competition between AML1 and AML1/Evi-1 for heterodimer formation with PEBP2β (CBFβ).

On the other hand, the transcription factor AP-1 represents a prototype of regulatory protein that converts extracellular signals into changes in the gene expression program (2). AP-1 is activated by growth stimuli, including growth factors, phorbol esters such as 12-*O*-tetradecanoylphorbol 13-acetate, and various transforming oncogene products. There have been many observations that the AP-1 activity is important for cellular differentiation and proliferation. Overexpressed c-Fos and c-Jun, component proteins of the AP-1 complex, have transforming effects on a variety of cells (24, 37). Increased c-*fos* and c-*jun* expression is also accompanied by the apoptotic cell death and suggested to induce apoptosis in conditions in which cells cannot proliferate any more (10, 23, 34). Recently we have revealed that Evi-1 raises AP-1 activity with dependence on the second zinc finger domain (65). Stimulated transactivation of the c-*fos* promoter by Evi-1 is probably the mechanism for the increase in AP-1 activity. It was also indicated that AML1/Evi-1 increases AP-1 activity and elevates the transactivation level of the c-*fos* promoter. Intact Evi-1 protein in the AML1/Evi-1 chimera is responsible for these effects, because they are not observed without the second zinc finger domain of AML1/Evi-1, as is the case of Evi-1. We have also shown that AML1/Evi-1-expressing fibroblast cells show increased c-*jun* transcripts with dependence on the second zinc finger domain of Evi-1 (32). Expression of AML1/Evi-1 fusion protein should be regulated by the AML1 promoter in t(3;21)(q26;q22) leukemia cells. In contrast, normal hemopoietic cells express neither AML1/Evi-1 nor Evi-1 (44). Therefore, AML1/Evi-1 fusion protein may increase the AP-1 activity in t(3;21)(q26;q22) leukemic cells.

Our studies have shown that the AML1/Evi-1 fusion protein blocks terminal differentiation to mature granulocytes in 32Dcl3 cells treated with G-CSF. These biological effects are similar to those seen in Evi-1-overexpressing 32Dcl3 cells. It was also indicated that AML1/Evi-1ΔZF8-10 mutant protein blocks granulocytic differentiation and, in contrast to wild-type AML1/Evi-1, induces G-CSF-dependent exponential cell proliferation. It is feasible to assume that both AML-1-derived and Evi-1-derived portions independently play crucial roles in the block of granulocytic differentiation in 32Dcl3 cells, because the differentiation block is induced both by Evi-1 and by AML1a, which contains the almost same portion of AML1 as AML1/Evi-1 chimeric protein and dominantly suppresses the transactivation by AML1 as the chimeric protein does (43, 67). As for maintenance of cellular viability when 32Dcl3 cells are cultured in G-CSF, it appears that Evi-1's function (which decreases cell viability) is dominant over the suppressive effect (which induces G-CSF-dependent cell proliferation) on AML1. Both the activation of AP-1 and the increased loss of cellular viability in the presence of G-CSF are dependent on the second zinc finger domain of Evi-1. It is possible, although not definite, that the former induces the latter because the increased expression of c-*fos* and c-*jun* stimulates apoptosis in some conditions, as described above. In fact, the decreased cell viability was also observed when AE-51 and AE-53 were cultured in a lower concentration (0.25 pg/ml) of IL-3 (64), although complete IL-3 deprivation showed no significant difference from controls (possibly because of full stimulation of apoptosis even in controls). The 32Dcl3 cells appear to show exponential proliferation when only the differentiation signal through the G-CSF receptor is blocked, since G-CSF-dependent exponential cell proliferation is induced in 32Dcl3 cells which express the mutant G-CSF receptor lacking the C terminus, responsible for the G-CSF-induced differentiation, of the cytoplasmic domain (13, 19). Therefore it is suggested that Evi-1 and AML1/Evi-1 can not only block differentiation signal but also decrease the viability of 32Dcl3 cells when the cells are treated with G-CSF. Thus, probably both AML1-derived and Evi-1-derived portions are independently effective for the differentiation block in 32Dcl3 cells. It is possible that these two portions cooperate to establish the complete block of granulocytic differentiation, since our preliminary results suggest that AML1/Evi-1 completely blocks the MPO induction by G-CSF but AML1/Evi-1ΔZF8-10 allows a low level of MPO induction in 32Dcl3 cells (64).

The 32Dcl3 clones, which overexpress AML1/Evi-1 chimeric protein, showed neither abrogation of the IL-3 requirement for growth nor an increased growth rate in the presence of IL-3. Thus, it does not appear that the increased AP-1 activity is correlated with growth promotion in these cells. However, the AP-1 activity stimulated by AML1/Evi-1 is accompanied by increased cell proliferation in fibroblast cells, since AML1/Evi-1 increased c-*jun* transcripts and induced cell proliferation in soft agar with dependence on the second zinc finger domain (32). Whether the differentiation block or stimulated proliferation is induced by the chimeric protein may depend on the source of cells used in the experimental system.

It is intriguing to hypothesize that AML1/MTG8 (ETO) and PEBP2β (CBFB)/SMMHC also exert dominant negative effects on AML1. AML1/MTG8 (ETO) fusion protein, generated by t(8;21)(q22;q22), is similar in structure to AML1/Evi-1; AML1 protein is disrupted at the end of the runt domain and fused with almost intact MTG8 (ETO) (39). AML1/MTG8 (ETO) possibly suppresses AML1 transactivation by the competition for DNA binding or heterodimer formation with PEBP2β (CBFB). It is also possible that PEBP2β (CBFB)/SMMHC forms a heterodimer with AML1 and the resulting heterodimer suppresses its function by mechanisms such as inhibition of DNA binding or transactivation. We may safely propose that suppression of AML1's functions is probably essential for leukemogenesis in such cases. PML/RARα protein, produced by t(15;17)(q21;q21), is also a transcription factor with dominant negative effects on PML and RARα; it suppresses PML-induced macromolecular organization in the nucleus and transactivation by RARα under some conditions (11, 12, 14, 26, 30, 70). It may be a consistent mechanism for leukemogenesis that a chimeric protein generated by a chromosomal abnormality exerts a dominant negative effect on the original protein, since the proteins encoded on both alleles are eventually inactivated by the structural alteration and the functional repression.

AML1/Evi-1 is a novel chimeric transcription factor with two independent functions; it dominantly suppresses the function of the original protein (AML1), which should be expressed from another allele, and it plays an equivalent role with respect to the other (Evi-1), which otherwise shows no detectable expression in hemopoietic cells. Our experimental results indicate that these dual functions should contribute to induce leukemic cell transformations. Oncoproteins with dual functions are possibly advantageous to oncogenesis because multistep functional abnormalities of oncogenes are accumulated

and finally result in the oncogenic transformation (16). Thus, AML1/Evi-1 should trigger at least two steps of functional abnomalities required for leukemogenesis.

Functional analyses of AML1/Evi-1 fusion protein have provided new insights into mechanisms for leukemogenesis mediated by structural and therefore functional alterations of transcription factors. Further investigations are required for a comprehensive understanding of leukemogenesis from aspects of abnormal transcriptional controls.

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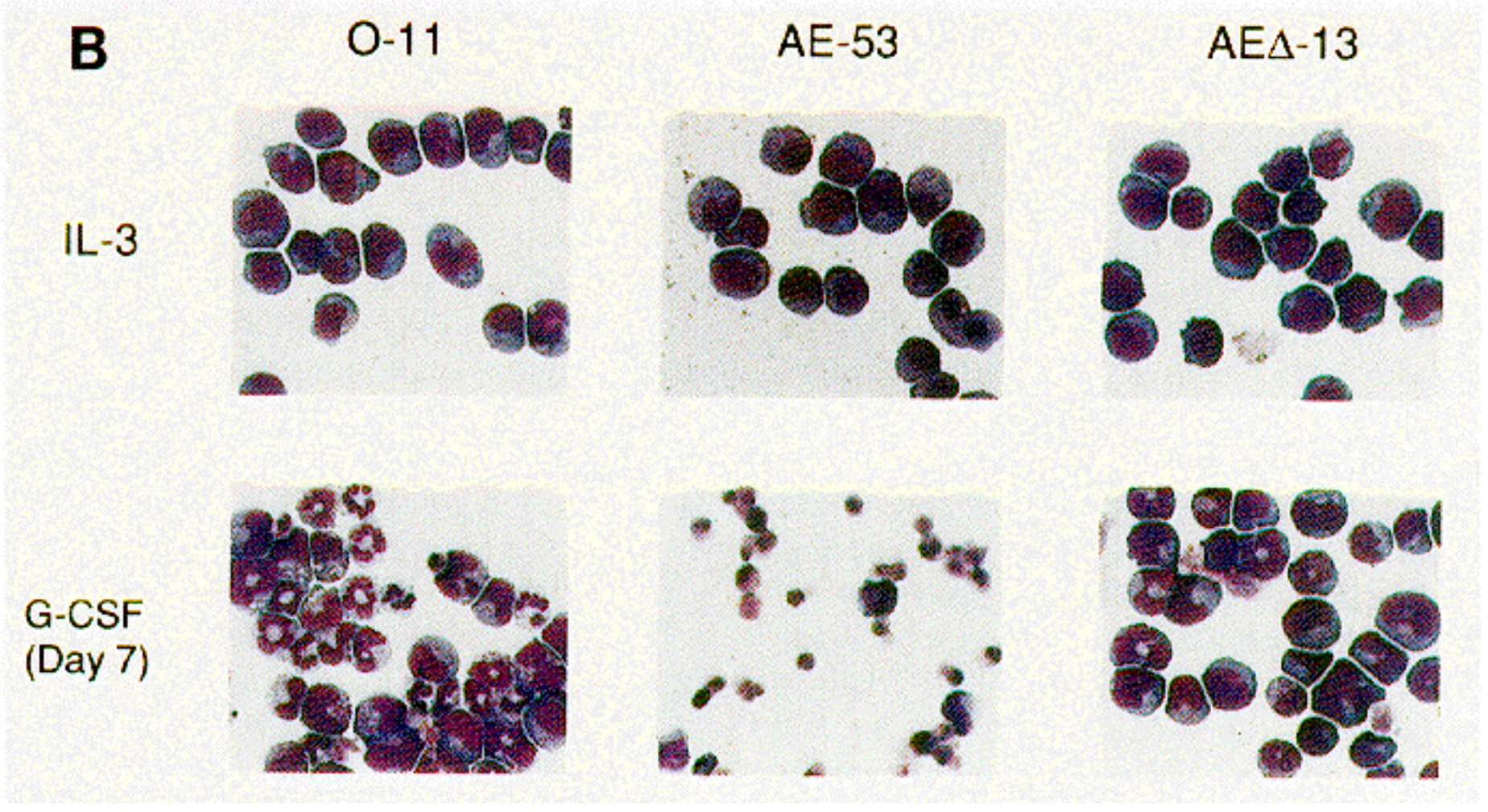
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