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The chimeric genes *AML1/MDS1* and *AML1/EAP* inhibit AML1B activation at the *CSF1R* promoter, but only *AML1/MDS1* has tumor-promoter properties

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ABSTRACT **The (3;21)(q26;q22) translocation associated with treatment-related myelodysplastic syndrome, treatment-related acute myeloid leukemia, and blast crisis of chronic myeloid leukemia results in the expression of the chimeric genes *AML1/EAP*, *AML1/MDS1*, and *AML1/EVI1*. *AML1* (*CBFA2*), which codes for the α subunit of the heterodimeric transcription factor CBF, is also involved in the t(8;21), and the gene coding for the β subunit (*CBFB*) is involved in the inv(16). These are two of the most common recurring chromosomal rearrangements in acute myeloid leukemia. CBF corresponds to the murine Pebp2 factor, and CBF binding sites are found in a number of eukaryotic and viral enhancers and promoters. We studied the effects of AML1/EAP and AML1/MDS1 at the AML1 binding site of the *CSF1R* (macrophage-colony-stimulating factor receptor gene) promoter by using reporter gene assays, and we analyzed the consequences of the expression of both chimeric proteins in an embryonic rat fibroblast cell line (Rat1A) in culture and after injection into athymic nude mice. Unlike AML1, which is an activator of the *CSF1R* promoter, the chimeric proteins did not transactivate the *CSF1R* promoter site but acted as inhibitors of AML1 (CBFA2). *AML1/EAP* and *AML1/MDS1* expressed in adherent Rat1A cells decreased contact inhibition of growth, and expression of *AML1/MDS1* was associated with acquisition of the ability to grow in suspension culture. Expression of *AML1/MDS1* increased the tumorigenicity of Rat1A cells injected into athymic nude mice, whereas *AML1/EAP* expression prevented tumor growth. These results suggest that expression of *AML1/EAP* and *AML1/MDS1* can interfere with normal AML1 function, and that AML1/MDS1 has tumor-promoting properties in an embryonic rat fibroblast cell line.**

The (3;21)(q26;q22) translocation is a recurring chromosomal abnormality associated with treatment-related acute myeloid leukemia (t-AML), treatment-related myelodysplastic syndrome (t-MDS), and blast crisis of chronic myeloid leukemia (1). The gene on chromosome 21 located at the translocation breakpoint is *AML1* (2), which codes for the CBFA2 subunit of the transcription factor CBF. AML1 belongs to a family of CBFA subunits characterized by a 5′ region that encodes a domain with both DNA-binding and protein dimerization properties which is homologous to the DNA-binding domain of the *Drosophila melanogaster* protein encoded by the segmentation gene runt (3, 4). The DNA-binding site of AML1 targets the sequence TGYGGTY (5), which has been identified in regulatory regions of several essential hematopoietic genes (6–8). The runt homology region of AML1 allows binding to CBFB to form a heterodimer that has increased DNA binding affinity (4). The 3′ sequence of *AML1* codes for a transactivation domain (9). AML1 is the human homolog [99% identity of the deduced amino acid (aa) sequence for the first 241 aa residues] of the α subunit of murine Cbf (10). Murine Cbf2 (Pebp2) has been shown to bind and to transactivate the murine myeloperoxidase gene enhancer (6); it is expressed in some T-cell subpopulations in which it may have a role in the regulation of T-cell-specific gene expression (11). AML1 has several isoforms, four of which have been cloned. Two of the isoforms are smaller proteins of 250 aa (2) and 258 aa (1) that consist mainly of the runt homology domain. One of these smaller proteins, AML1A, has been shown to bind the *TCRB* enhancer without causing transactivation (9). The other two cDNAs encode larger proteins of 472 aa (12) and 479 aa that include a transactivation domain (9). One of them, *AML1B*, coding for the 479-aa protein induces modest transactivation of the human *TCRB* (9) and *CSF2* (granulocyte/macrophage-colony-stimulating factor) (7) regulatory regions. Higher levels of transactivation by AML1B may require the synergistic action of other proteins at the enhancer/promoter site, as demonstrated by the increase in Cbfa2 transactivation of the *TCRA* enhancer by Ets-1 (13).

The *CSF1R* promoter has binding sites for CBF and PU.1 (a member of the Ets family) (8, 14), and *CSF1R* mutations resulting in ligand-independent upregulated kinase activity are associated with cellular transformation (15, 16) and with human myeloid leukemia and MDS (17). Abnormalities in CSF1R structure and function may thus have an important role in leukemogenesis.

The (3;21) translocation gives rise to the chimeric fusion genes *AML1/MDS1* and *AML1/EAP* (18–20). In addition, a third fusion gene, *AML1/EVI1*, has been detected in some cases of t(3;21) (18, 21). *EAP* codes for the ribosomal protein L22. However, the *EAP* reading frame is not maintained in the fusion with *AML1* and translation of *AML1/EAP* stops after the addition of 17 non-EAP-related amino acid residues to the runt domain of AML1 (18, 20).

MDS1 is a small gene that is centromeric to EAP and encodes a protein of 170 aa (18). *AML1/MDS1* contains the same 5′ *AML1* region as that found in the *AML1/EAP*, fused in frame to *MDS1*. The normal function of MDS1 is unknown.

Fig. 1 provides a diagrammatic summary of the structure of the isoforms of AML1 and the chimeric fusion proteins produced as a result of the (3;21)(q26;q22) and (8;21)(q22;q22) translocations.

We report results showing that AML1/EAP and AML1/MDS1 inhibit AML1B transactivation of the *CSFR1* promoter. This inhibition is not reversed by expression of increased amounts of CBFB. Expression of AML1/EAP and AML1/MDS1 caused changes characteristic of transformation in

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Abbreviations: AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; t-, treatment-related; FBS, fetal bovine serum.

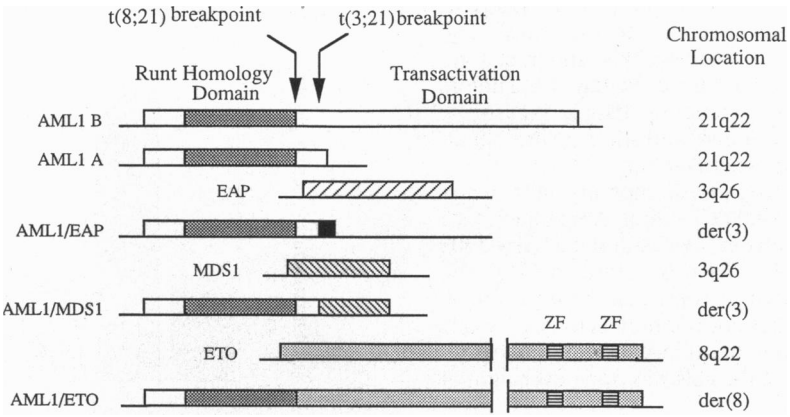

FIG. 1. Comparison of AML1A and AML1B and the chimeric AML1/MDS1, AML1/EAP, and AML1/ETO. *AML1B* encodes a DNA-binding and dimerization (runt homology) domain (cross-hatched box) at the 5′ end and a transactivation domain (open box) at the 3′ end. In the chimeric *AML1/MDS1*, the 5′ part of *AML1*, including the intact runt domain, is fused to the 3′-terminal codons of *MDS1*. *AML1/EAP* contains the 5′ part of *AML1* fused to 17 codons from the out-of-frame fusion with *EAP*. *AML1/ETO* contains the 5′ part of *AML1* fused to almost the entire *ETO*, which encodes two zinc finger (ZF) motifs. Arrows indicate the breakpoints on chromosome 21 in the t(3;21) and t(8;21). Single lines indicate 5′ and 3′ untranslated sequence. Open reading frames are indicated by boxes.

cultured fibroblasts, and injection of transfected cells into athymic nude mice demonstrated significant tumor growth enhancement by AML1/MDS1.

METHODS

Plasmid Construction. The plasmid pCMV5-AML-1B, expressing AML1B (gift of Scott Hiebert) has been described (9). *AML1/EAP* and *AML1/MDS1* cDNAs (18) were cloned in the *Not* I–*Xho* I sites of the vector pREP4 (expressing hygromycin resistance) and pREP9 (expressing neomycin resistance, *AML1/MDS1* only) (Invitrogen) for expression in eukaryotic cells. The plasmid pCC2, expressing normal human *CBFB* cDNA, has been described (22).

Cell Lines, Cell Transfection, and Reporter Gene Assays. The embryonic rat fibroblast cell line Rat1A was maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO/BRL) with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO/BRL). The embryonic murine teratocarcinoma cell line P19 was maintained in minimal essential medium alpha (MEMα) (GIBCO/BRL) with 10% FBS. Cell lines were transfected with a calcium phosphate precipitation kit (Invitrogen) according to the manufacturer's instructions and were selected by addition of hygromycin B (200 μg/ml; Boehringer Mannheim) or both hygromycin and Geneticin (500 μg/ml; Sigma) to the culture medium as needed.

To avoid gene expression effects due to integration sites, we combined stably transfected single clones of similar morphology and growth characteristics to provide a mixed cell population expressing either the vector (vector cells), or *AML1/EAP* (AML1/EAP cells), or *AML1/MDS1* (AML1/MDS1 cells), or both *AML1/EAP* and *AML1/MDS1* (AML1/EAP+AML1/MDS1 cells).

For luciferase assays, P19 cells (2.5×10^5) were grown overnight to about 20% confluence in 100-mm-diameter tissue culture plates. P19 cells were used for the assay because they have been reported to have a low level of endogenous expression of AML1 (3). The reporter gene construct CSF1R-luc or the mutated negative control pM-CSF-R(MB)-luc (binding sequence mutated to TCTAAGG) was used at 10 μg of plasmid per 100-mm-diameter plate. These plasmids have been described (8). pCMV-AML1B was used at 10 μg of plasmid per plate, and the *AML1/MDS1* and *AML1/EAP* plasmids were used at amounts from 1.25 to 20 μg per plate. pCC2 was used at between 1.25 and 20 μg per plate. All cells were also transfected with plasmid pCH110 (7 μg per plate; Pharmacia), coding for β-galactosidase to allow correction for efficiency of transfection. For the transient transfection assays, we harvested cells 48 hr after the addition of DNA. The luciferase assay system (Promega) was used according to the manufacturer's instructions.

Growth Assays. Growth rate assays using direct cell counts, as well as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (23), have been described. Cells detaching from the adherent monolayer were cultured in suspension in RPMI 1640 (GIBCO/BRL) with 10% FBS and hygromycin B (200 μg/ml).

Athymic Nude Mouse Studies. Six-week-old female athymic nude mice (Charles River Breeding Laboratories) were injected subcutaneously in each flank with 4×10^5 (left side) and 8×10^5 (right side) of Rat1A cells or with stably trans-

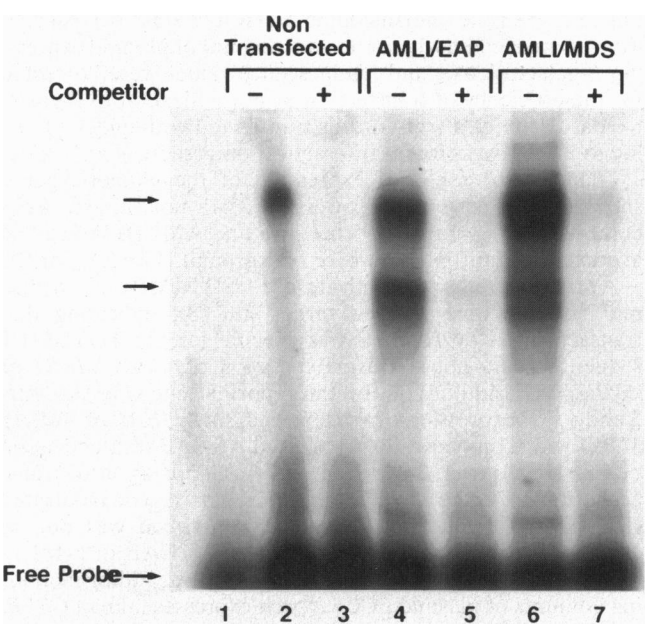

FIG. 2. AML1/EAP and AML1/MDS1 bind specifically to a CBF site. Two microliters of whole cell extract from parental Rat1A cells or Rat1A cells transfected with *AML1/EAP* or with *AML1/MDS1* was incubated with [α-^{32}P]dCTP end-labeled ΔF9-5000 probe (24) without (lanes 2, 4, and 6) or with (lanes 3, 5, and 7) 100× excess unlabeled ΔF9-5000. After separation of the protein–DNA complexes in a 9% polyacrylamide gel, a band (upper arrow) in all cells and an additional band (lower arrow) unique to the transfected cells were detected by autoradiography. Both bands were eliminated by unlabeled competitor.

fected cells suspended in serum and antibiotic-free DMEM. Cells of each population were injected into four mice. The tumor diameter was measured twice weekly under general anesthesia. The animals were sacrificed on day 40. Tumors were dissected from the subcutaneous tissue, measured, weighed, fixed in formalin, sectioned, and stained with hematoxylin and eosin for histologic examination.

DNA Binding Assay. Electrophoretic mobility-shift assays (EMSAs) used [α-^{32}P]dCTP (6000 Ci/mmol, Amersham; 1 Ci = 37 GBq) end-labeled polyoma virus-derived ΔF9-5000 probe and 2 μl of whole cell extract as described (24). The reaction mixture was incubated at room temperature for 30 min with or without 100× excess of nonradioactive ΔF9-5000 and electrophoresed for 90 min at 40 mA in a 9% polyacrylamide gel. Autoradiography of the gels was done overnight at −70°C.

RESULTS

AML1/EAP and AML1/MDS1 Bind Specifically to the CBF Site and Inhibit AML1B Transactivation of the *CSFR1* Promoter. *EMSAs.* Fig. 2 shows the results of the EMSAs with extracts of Rat1A cells or AML1/EAP or AML1/MDS1 cells and the ΔF9-5000 probe. Rat1A extracts contained a protein or proteins that bound specifically to the probe (lanes 2, 4, and 6, upper band). This protein–probe complex was disrupted by addition of 100× excess unlabeled ΔF9-5000. The cell extracts obtained from cells expressing the chimeric genes formed an additional faster-moving complex that could also be disrupted by an excess of unlabeled ΔF9-5000 (lanes 5 and 7).

Reporter gene assay. AML1B binds to the promoter or enhancer regions of several genes that regulate myeloid cell growth and differentiation. To determine whether AML1/MDS1 and AML1/EAP had transactivation properties or could affect AML1B-dependent transactivation, we performed assays using the reporter gene construct CSF1R-luc, in which the *CSF1R* promoter containing the CBF consensus binding site TGTGGTT as well as binding sites for PU.1 is linked to the gene (*luc*) encoding firefly luciferase (8). All cells were cotransfected with a constant amount of plasmid expressing β-galactosidase, and luminescence values were corrected for the efficiency of transfection by normalization to β-galactosidase activity. We used cells transfected with only CSF1R-luc to assess background transactivation activity (Fig. 3*A*, bar 1). The vector used for expression of the chimeric genes showed no effect on *luc* expression (data not shown). Cells cotransfected with the reporter gene and AML1B showed an average 1.75-unit increase over background (Fig. 3*A*, bar 2).

AML1B did not transactivate pM-CSF-R(MB)-luc, a plasmid containing a mutated target site (8), indicating that transactivation by AML1B was specific for the TGTGGTT sequence (data not shown). Expression of *AML1/MDS1* or *AML1/EAP* did not activate the reporter gene (Fig. 3*A*, bars 3 and 4). Coexpression of *AML1B* and *AML1/EAP* or *AML1/MDS1* in a 1:1 molar ratio suppressed AML1B transactivation of CSF1R-luc (Fig. 3*A*, bars 5 and 6). Suppression of transactivation by AML1B with the chimeric genes could be titrated (Fig. 3*B*). To determine whether suppression was due to competition between AML1B and AML1/EAP or AML1/MDS1 for CBFB, we repeated the experiment, adding increasing amounts of plasmid pCC2, which expresses human *CBFB*. Addition of CBFB not only failed to restore activation of *luc* repressed by each of the chimeric genes but also suppressed *luc* transactivation by AML1B (Fig. 3*C*).

Effect of AML1/EAP and AML1/MDS1 on Cell Growth. *Growth assays.* Stably transfected vector, AML1/EAP, AML1/MDS1, and AML1/EAP+AML1/MDS1 cells maintained in subconfluent culture had a microscopic morphology indistinguishable from that of non-transfected Rat1A cells. The growth rate of the Rat1A cell line and that of stably transfected cell populations did not differ significantly. To determine contact inhibition of growth, we transfected Rat1A

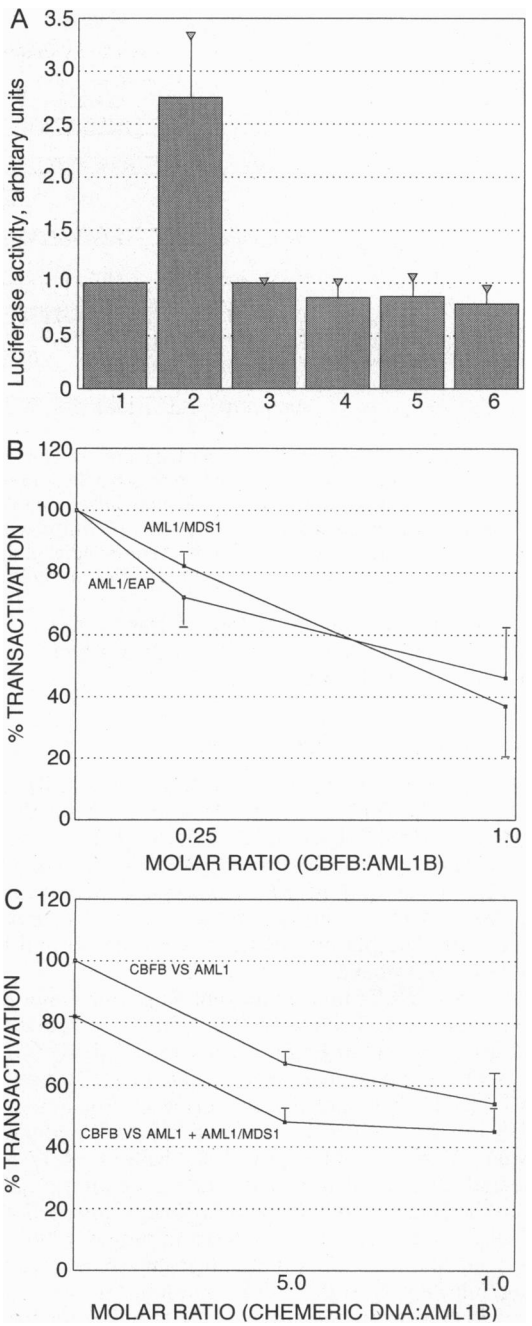

FIG. 3. AML1/EAP and AML1/MDS1 repress the AML1B-dependent activation of CSF1R-luc. Overexpression of *CBFB* increases repression. We transfected P19 cells with the reporter gene construct CSF1R-luc to determine the effect of AML1B and chimeric proteins at the *CSF1R* promoter site. The mean and standard error of three or more experiments are shown. (*A*) Background activity of control cells, transfected with only the CSF1R-luc construct, was arbitrarily assigned the value of 1 (bar 1). Cotransfection with *AML1B* increased transactivation by 1.75 units above background levels (bar 2). Expression of *AML1/MDS1* (bar 3) or *AML1/EAP* (bar 4) did not increase the transactivation of *luc*. Coexpression of *AML1B* and equimolar amounts of *AML1/MDS1* (bar 5) and *AML1/EAP* (bar 6) inhibited transactivation by AML1B to background levels. (*B*) Transactivation by AML1B can be titrated by AML1/EAP and AML1/MDS1. The transactivation by AML1B was arbitrarily assigned the value of 100%. An increase in the molar ratio of chimera to AML1B resulted in a progressive decrease in transactivation. (*C*) *CBFB* expression inhibits AML1B transactivation. Inhibition of transactivation by *AML1/MDS1* (fixed dose of 5 μg per plate) can be titrated by overexpression of *CBFB*.

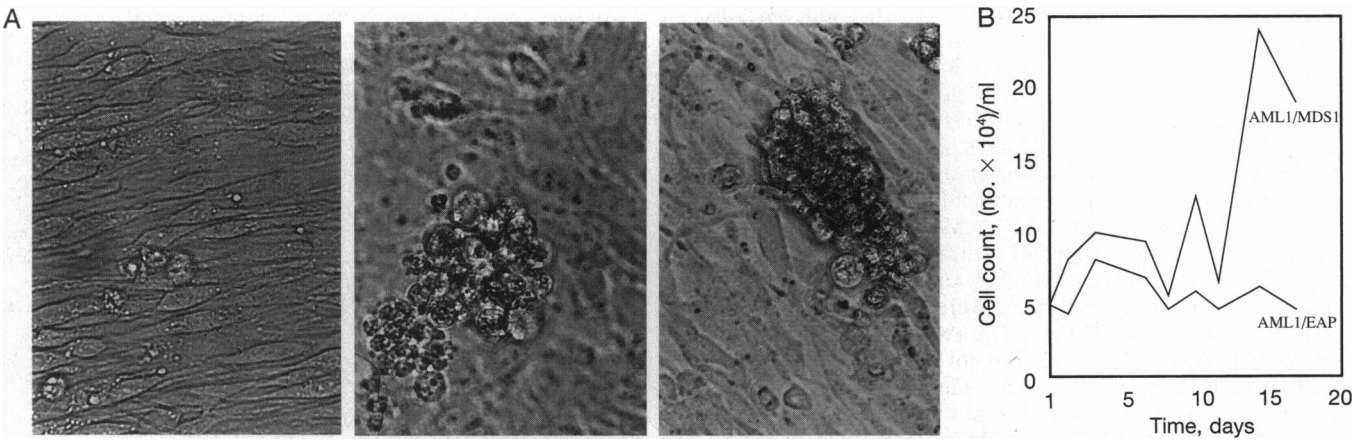

FIG. 4. Loss of contact inhibition of growth in Rat1A cells transfected with *AML1/EAP* and *AML1/MDS1*. (*A*) Cells were transfected by the calcium phosphate precipitate method and grown to confluence without antibiotic selection. Photomicrographs of cultures (×400) were taken at 21 days. Rat1A cells transfected with vector plasmid (*Left*) grew as an adherent monolayer and were indistinguishable from Rat1A cells. Cells transfected with *AML1/EAP* (*Center*) and *AML1/MDS1* (*Right*) plasmids produced multiple foci, above the confluent monolayer. (*B*) Cells were harvested from the supernatant of AML1/MDS1 or AML1/EAP cell cultures prior to confluence of the adherent monolayer and resuspended at 5×10^4 cells per ml in RPMI 1640 medium with 10% FBS. Cell growth was followed by counting viable cells (by trypan-blue exclusion) with a hemocytometer. Rat1A and vector cells were not viable (data not shown), and AML1/EAP cells remained viable, but did not grow. In contrast, AML1/MDS1 cells showed sustained growth in culture, and logarithmic-phase growth could be induced by resuspension in fresh medium (days 8 and 11).

cells with the various plasmids and cultured them for 21 days without antibiotic selection. After reaching confluence, cells were examined microscopically for the development of abnormal cell foci. Only the cells transfected with each chimeric gene (Fig. 4*A Center* and *Right*) or with both chimeric genes (data not shown) grew over the monolayer, forming distinct foci.

There was a marked increase in the number of cells detaching from the adherent culture both before and after reaching confluence in AML1/EAP, AML1/MDS1, or AML1/EAP+AML1/MDS1 cells (but not vector-transfected cells) grown to confluence in medium with hygromycin (and Geneticin as required). These cells were viable when tested for trypan-blue exclusion. They were collected from the supernatant and were resuspended in RPMI 1640 with 10% FBS at a concentration of 5×10^4 cells per ml. Detached cells collected from Rat1A and vector cell cultures were not viable in suspension culture and died within 72 hr. AML1/EAP cells as well as AML1/EAP+AML1/MDS1 cells remained viable, but did not grow in suspension culture. AML1/MDS1 cells were capable of sustained growth in suspension culture (Fig. 4*B*).

Athymic nude mice. Rat1A cells, as well as the transfected AML1/EAP, AML1/MDS1, or AML1/EAP+AML1/MDS1

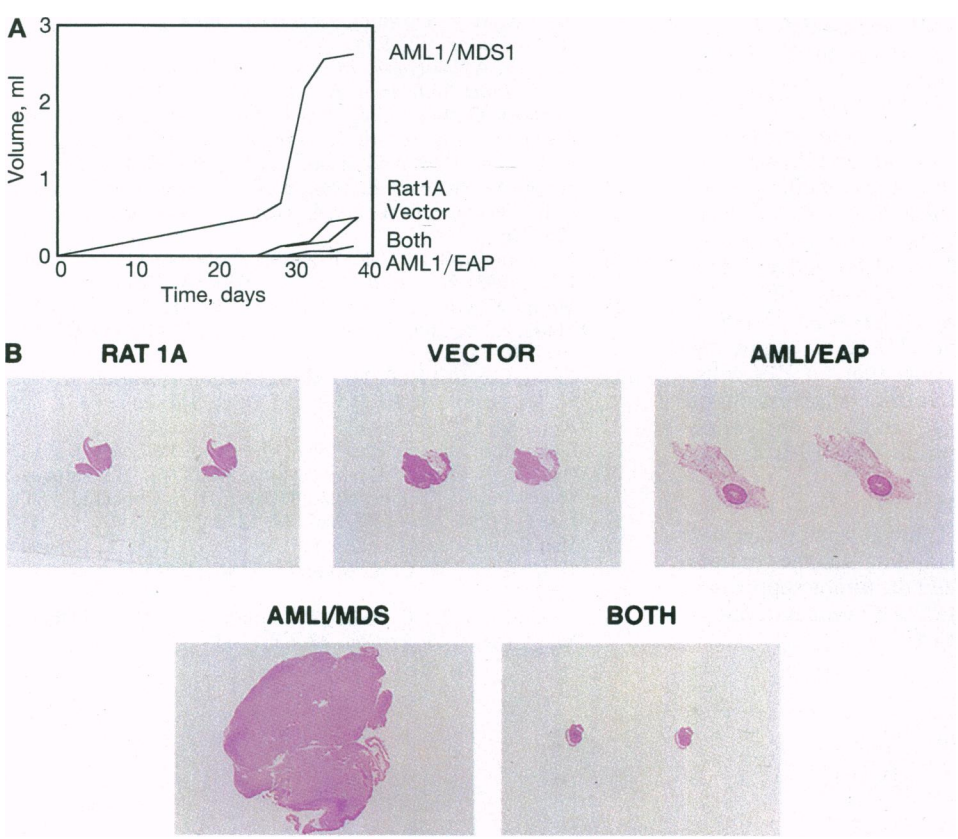

FIG. 5. Tumor size. Athymic nude mice were injected subcutaneously with 8×10^5 Rat1A cells, vector cells, AML1/EAP cells, AML1/MDS1 cells, or AML1/EAP+AML1/MDS1 cells (both) and tumor growth was measured for 40 days. *A* represents tumor growth with time and *B* compares sections of fixed tumor tissue. The vector-transfected cells produced tumors of the same size as Rat1A-injected animals (*B Upper Left* and *Center*). Cells transfected with *AML1/EAP* developed masses which contained only reactive lymphoid tissue. No malignant cells were seen on microscopic examination (*B Upper Right*). Cells transfected with *AML1/MDS1* produced tumors significantly larger than those produced by vector-transfected cells (*B Lower Left*). AML1/EAP+AML1/MDS1 cells had tumors significantly smaller than those produced by vector-transfected cells (*B Lower Right*). (×2.)

cells were injected subcutaneously into athymic nude mice to assess tumorigenicity. An initial study demonstrated that Rat1A and vector cells injected at a dose of 1×10^6 cells per animal produced small and nondisabling tumors at 6 weeks. The tumorigenicity of Rat1A cells has been described previously (25, 26). Four and 8×10^5 cells per animal were used in this study. Control mice were injected with either Rat1A or vector cells, and the experimental mice with AML1/EAP, AML1/MDS1, or AML1/EAP+AML1/MDS1 cells. One mouse injected with AML1/MDS1+AML1/EAP cells died unexpectedly and without a known cause within a week of injection. The remaining 19 mice were monitored and were sacrificed electively at 40 days. The swellings in the animals injected with AML1/EAP cells did not grow, whereas those in all other animals enlarged rapidly (Fig. 5*A*). On day 40, the tumors were carefully dissected from the skin and subcutaneous tissue and weighed. The weight of tumors produced by vector-transfected cells was similar to that of tumors produced by Rat1A cells. AML1/MDS1 cells produced tumors that weighed significantly more ($P = 0.043$; data not shown), whereas the tumors produced by AML1/MDS1+AML1/EAP cells weighed significantly less ($P = 0.033$; data not shown) than those produced by vector cells. Representative sections of each tumor type are shown in Fig. 5*B*.

Tissue from all tumors derived from cells initially selected in hygromycin (and Geneticin) were grown *in vitro* in medium containing the appropriate antibiotic, confirming that the tumors arose from the antibiotic-resistant injected cells.

Light microscopic examination of fixed tumor sections showed morphologically indistinguishable undifferentiated fibrosarcomas in all resected tissues except that derived from AML1/EAP cells, which consisted of enlarged subcutaneous lymph nodes with features of acute and chronic inflammatory changes, suggesting a sustained immune response (Fig. 5*B*).

DISCUSSION

Recurring nonrandom chromosomal rearrangements involving the genes coding for CBF are found in AML, blast phase of chronic myeloid leukemia, and t-AML and t-MDS. Two of the most common rearrangements in AML are t(8;21), giving rise to an *AML1/ETO* fusion gene, and inv(16), fusing *CBFB* to one of the myosin heavy-chain genes (*MYH11*). The presence of both *AML1/MDS1* and *AML1/EAP* transcripts in all five patients with a t(3;21) studied by our group (18) suggests that MDS may develop only as a result of the combined effect of both genes. We have now demonstrated that *AML1/EAP* and *AML1/MDS1* derived from one of these five patients may have the potential to interfere with AML1B activity. The mechanism by which chimeric AML1 proteins inhibit the normal function of AML1 is unknown. It has been proposed that the chimeric proteins may inhibit AML1B by competing for CBFB binding (10). Our data show that, in P19 cells, overexpression of CBFB results in further suppression of AML1B transactivation of the *CSF1R* promoter, suggesting that mechanisms other than competition for CBFB are involved.

Interference with normal AML1B function may have an important role in the pathogenesis of the associated myeloid malignancies, and a better understanding of the tumor-promoter function of AML1/MDS1 and the tumor-suppressor function of AML1/EAP could facilitate the rational development of improved treatment modalities.

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