mutations result in either loss of one *AML1* allele that leads to decrease in the product, or generation of a mutant AML1 protein that dominantly inhibits the function of the normal AML1 protein. Thus, alterations in the *AML1* gene product are closely associated with human leukemias.

Previously, we cloned the *AML1/Evi-1* fusion gene from a case with blastic crisis of chronic myelogenous leukemia (CML) causing the t(3;21) translocation (Mitani *et al.*, 1994). The *AML1/Evi-1* gene encodes a fusion protein consisting of the N-terminal portion of AML1, including the Runt domain, fused in frame to the complete Evi-1 protein. The *Evi-1* gene is located in human chromosome 3q26, and encodes a transcriptional regulator protein with two zinc finger domains. The Evi-1 protein itself is expressed aberrantly in leukemic cells, and is suggested to have close association with the pathogenesis of myelogenous leukemia (Hirai, 1999; Kurokawa *et al.*, 1998b, 2000). The biological effect of the AML1/Evi-1 chimeric protein in cells has been studied so far. It was shown to exhibit transforming activity on Rat1 fibroblasts (Kurokawa *et al.*, 1995). In hematopoietic cells, it blocks granulocytic differentiation of the 32Dcl3 cell line when stimulated with granulocyte colony-stimulating factor (G-CSF) (Tanaka *et al.*, 1995a). Furthermore, using a retroviral transduction and transplantation approach, the expression of AML1/Evi-1 in bone marrow cells was shown to induce acute myelogenous leukemia in mice (Cuenco *et al.*, 2000). Taken together, AML1/Evi-1 should have a distinct role in leukemogenesis. Several mechanisms by which the expression of AML1/Evi-1 in hematopoietic cells leads to leukemias have been proposed to date. AML1/Evi-1 raises AP-1 activity presumably through elevating the expression of c-*jun* and c-*fos* (Tanaka *et al.*, 1995a). It interacts with Smad3 and interrupts TGFβ-induced signal transduction (Kurokawa *et al.*, 1998a). Moreover, AML1/Evi-1 dominantly inhibits AML1-induced transactivation, which was shown to result from a competitive inhibition. AML1/Evi-1 can interact with PEBP2β subunit more effectively than AML1 does (Tanaka *et al.*, 1998). In addition, the AML1/Evi-1-PEBP2β heterodimer has advantage in interacting with the PEBP2-binding site compared with the AML1-PEBP2β heterodimer (Tanaka *et al.*, 1995a). Recently, we showed that C-terminal binding protein (CtBP) interacts with Evi-1 to repress TGFβ-induced transcription (Izutsu *et al.*, 2001). CtBP was originally identified as a protein which interacts with a C-terminal portion of adenovirus E1A protein (Boyd *et al.*, 1993; Turner and Crossley, 2001). To date, two highly related proteins, termed CtBP1 and CtBP2, have been reported both in mice and humans (Turner and Crossley, 2001). The difference in function between these proteins remains to be elucidated, although their expression pattern in the embryonic and adult tissue has been reported to be slightly different from each other (Katsanis and Fisher, 1998). They have been recognized as corepressor proteins which mediate repression by associating with several transcription factors including basic Krüppel-like factor (BKLF) (Turner and Crossley, 1998), friend of GATA (FOG) (Fox *et al.*, 1999), and T-cell factor (TCF) (Brannon *et al.*, 1999). Although it is not precisely elucidated how CtBP mediates transcriptional repression, it is supposed that histone deacetylase 1 (HDAc1), which was demonstrated to interact with CtBP, may be involved in repression (Sundqvist *et al.*, 1998). Recent studies showed that AML1/ETO(MTG8), a fusion protein which is derived from t(8;21), represses the AML1-driven promoter through the interaction with corepressor proteins: it directly interacts with a mammalian homolog of yeast transcriptional repressor SIN (mSin3) A and nuclear hormone corepressor (NCoR), thereby recruiting HDAc1 (Lutterbach *et al.*, 1998; Wang *et al.*, 1998). Therefore, it is assumed that aberrant recruitment of a corepressor complex to AML1 target genes may have a pivotal role in AML1/ETO(MTG8)-associated leukemogenesis. As for AML1/Evi-1, however, a role for corepressor proteins has not been defined to date. Then, we hypothesized that transcriptional repression by AML1/Evi-1 might be attributed to interaction with a corepressor complex including CtBP.

In this study, we found that AML1/Evi-1 endogenously interacts with CtBP and that the interaction with it may be responsible for transcriptional repression and block in myeloid differentiation by AML1/Evi-1.

Results

AML1/Evi-1 interacts with CtBP

To investigate a potential role of CtBP in AML1/Evi-1-mediated transcriptional repression, we first examined whether AML1/Evi-1 interacts with CtBP. For this purpose, we performed a coprecipitation experiment by overexpressing T7-tagged CtBP1 (T7-CtBP1) and AML1/Evi-1 in COS7 cells. Whole cell lysates were immunoprecipitated with the anti-T7 antibody and the immunoprecipitates were analysed by immunoblotting with the anti-Evi-1 antibody. As shown in Figure 1a, lane 3, AML1/Evi-1 and CtBP were coprecipitated, which indicates the interaction between AML1/Evi-1 and CtBP *in vivo*. We previously determined that Evi-1 interacts with CtBP exclusively through one of the two potential CtBP-binding amino acid sequences, PFDLT and PLDLS (Izutsu *et al.*, 2001). AML1/Evi-1 also retains those two sequences. To determine relative contribution of them to the CtBP binding, we constructed amino acid-substituted mutants for these sequences (Figure 2), and tested their ability to interact with CtBP. In AE(AS/DL) mutant, the amino acid sequence, PFDLT in AML1/Evi-1 (AE) is replaced to PFAST. Likewise, PLDLS is replaced to PLASS in AE(DL/AS), and both of PFDLT and PLDLS are to PFAST and PLASS in AE(AS/AS). AEΔ544-607 is a deletion mutant for AML1/Evi-1 in which the region corresponding to amino acids between 544 and 607 of Evi-1 is deleted (Figure 2).

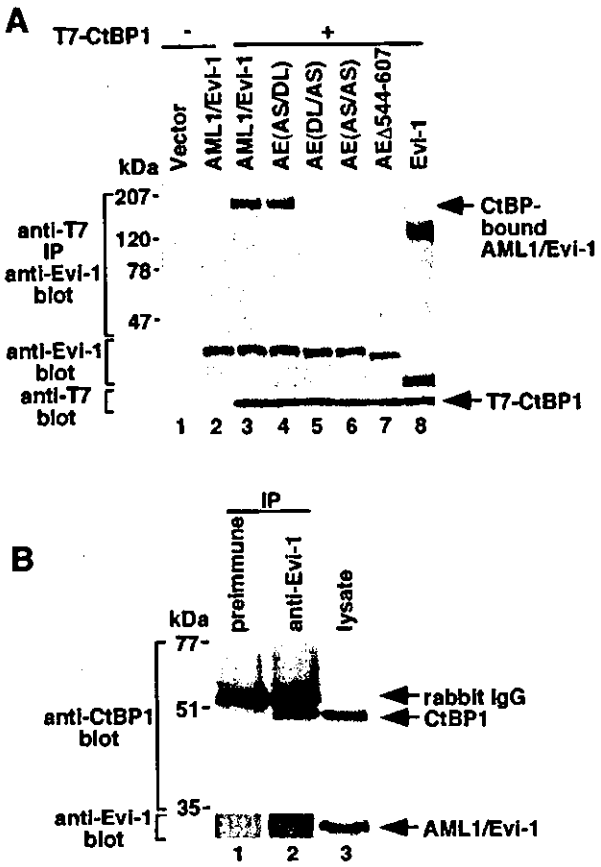

Figure 1 AML1/Evi-1 interacts with CtBP. (a) The pME18S empty vector (lane 1), AML1/Evi-1 (lanes 2 and 3), AE(AS/DL) (lane 4), AE(DL/AS) (lane 5), AE(AS/AS) (lane 6), AEΔ544-607 (lane 7), or Evi-1 (lane 8) in pME18S was transfected into COS7 cells (2×10^6) with the pRc/CMV empty vector (lanes 1 and 2) or T7-CtBP1 in pRc/CMV (lanes 3 to 8). Cells were lysed and subjected to immunoprecipitation (IP) with anti-T7. Immunoprecipitates were subjected to Western blot analysis using anti-Evi-1 (top). Positions of size markers in kilodaltons (kDa) are indicated on the left. Expression of AML1/Evi-1 and T7-CtBP1 was monitored with anti-Evi-1 (middle) and anti-T7 (bottom), respectively. (b) SKH1 cells were lysed and subjected to immunoprecipitation with preimmune serum (lane 1) or anti-Evi-1 (lane 2). Immunoprecipitates (IP) were subjected to Western blot analysis using anti-CtBP1 (top) and anti-Evi-1 (bottom). Expression of endogenous CtBP1 in SKH1 was determined with anti-CtBP1 (lane 3)

As shown in Figure 1a, AE(AS/DL) was coprecipitated with CtBP, whereas AE(DL/AS), AE(AS/AS), or AEΔ544-607 did not. These results indicate that the PLDLS motif is essential for the interaction between AML1/Evi-1 and CtBP, as is also the case with Evi-1.

The association between AML1/Evi-1 and CtBP was also revealed in SKH1 cells derived from megakaryoblastic crisis of chronic myelogenous leukemia, which endogenously overexpress AML1/Evi-1 (Mitani *et al.*, 1994). Total cell lysates from SKH1 cells were subjected to immunoprecipitation experiments using the anti-Evi-1 serum. Figure 1b shows that endogenous CtBP1 is coimmunoprecipitated with endogenous AML1/Evi-1. We tested the involvement of CtBP2 by

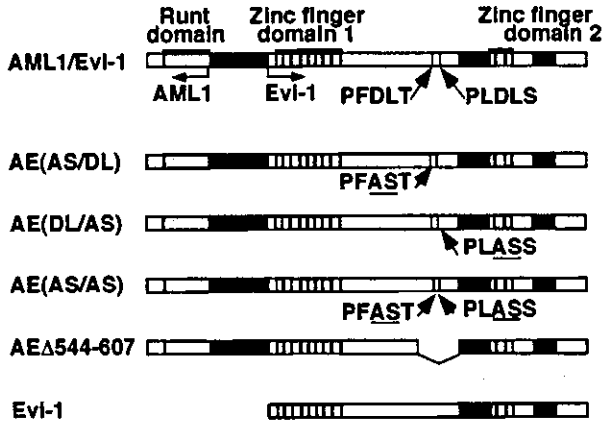

Figure 2 Structures of AML1/Evi-1 and its derivative forms. AML1/Evi-1 and its derivative forms are schematically shown. Amino acid substitutions in AE(AS/DL), AE(DL/AS), and AE(AS/AS) are depicted with underlines. In AEΔ544-607, the region between amino acids 544 and 607 of Evi-1 is deleted

the similar experiment using anti-CtBP2. However, endogenous expression of CtBP2 in SKH1 cells was very faint on the Western blot, and it was barely detected in the immunoprecipitates with anti-Evi-1 (data not shown). Thus, AML1/Evi-1 may form a complex predominantly with CtBP1 at least in SKH1 cells.

Interaction with CtBP is required for the repression of AML1-dependent transactivation

We previously showed that AML1/Evi-1 dominantly represses the AML1-induced transactivation by competing with AML1 for binding to PEBP2β subunit and to DNA (Tanaka *et al.*, 1995a). AML1/ETO(MTG8), another dominant negative inhibitor for AML1 (Miyoshi *et al.*, 1993), was shown to recruit a corepressor complex to repress the induction of AML1 target genes (Lutterbach *et al.*, 1998; Wang *et al.*, 1998). However, contribution of corepressor proteins to AML1/Evi-1-mediated repression has not been elucidated. Given that AML1/Evi-1 interacts with CtBP, we made a hypothesis that AML1/Evi-1-mediated repression might depend on the interaction with CtBP. To investigate this, we used the AML1/Evi-1 mutants, which do not interact with CtBP, and tested their ability to repress AML1-induced transactivation. In this study, we used a reporter plasmid that is driven by the M-CSF receptor promoter (pM-CSF-R-luc) (Zhang *et al.*, 1994). The reporter plasmid was transfected into HeLa cells along with the plasmids expressing AML1b, one of the major isoform of AML1 in hematopoietic cells (Miyoshi *et al.*, 1995), and PEBP2β together with those for AML1/Evi-1 or its mutants. Endogenous CtBP1 is expressed in HeLa cells as detected by Western blotting (Figure 3a, lane 1). Coexpression of AML1 and PEBP2β in the cells raised the expression level of the reporter gene about six to eight times as assessed by the luciferase assay. AML1/Evi-1 suppressed the transactivation elicited by AML1

and PEBP2β (Figure 3b), as previously described (Zent *et al*., 1996). As shown in Figure 3c, AE(AS/DL), which interacts with CtBP, showed the equivalent repression activity. However, the repression activity of AE(DL/AS), AE(AS/AS), or AEΔ(544-607), which does not associate with CtBP, was reduced to about 50% or less (Figure 3c). These results suggest that AML1/Evi-1 interacts with endogenous CtBP1 and represses the transcription.

Next, in order to assess the role of CtBP directly, we tested the effect of overexpression of CtBP1 on the repression by AML1/Evi-1. As shown in Figure 3d, overexpression of CtBP1 accentuated the repression by AML1/Evi-1. Overexpression of CtBP1, however, did not affect the repression by AE(DL/AS) that was reduced to about 50% of that by AML1/Evi-1 as mentioned earlier (Figure 3c,d). Taken together, it is indicated that the interaction with CtBP is required for AML1/Evi-1 to fully repress the AML1-induced transactivation.

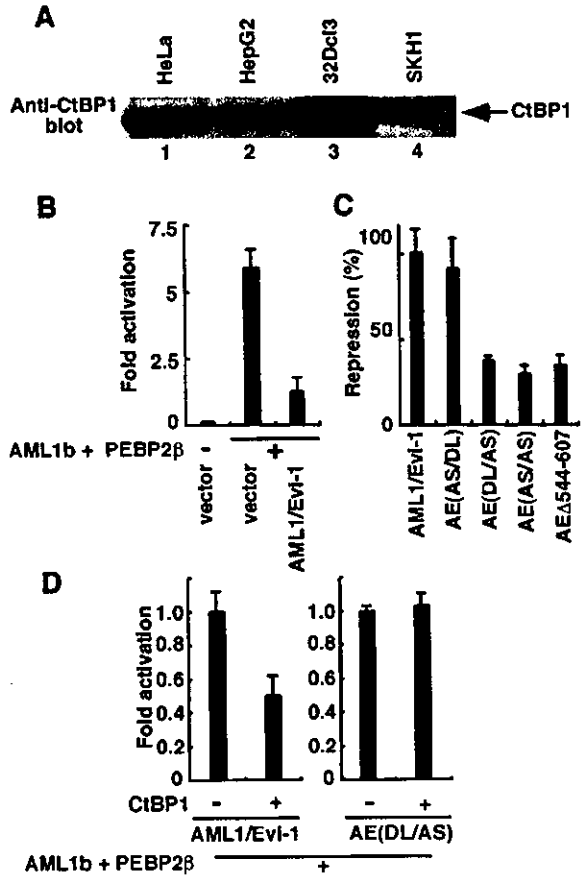

Figure 3 AML1/Evi-1 requires CtBP as a corepressor for dominantly inhibiting AML1-induced transactivation. (**a**) Endogenous expression of CtBP1 in HeLa, HepG2, 32Dcl3, and SKH1 cells were demonstrated by Western blotting using anti-CtBP1. (**b**) HeLa cells were transfected with pM-CSF-R-luc, AML1, and PEBP2β along with AML1/Evi-1 or its derivatives, as indicated. Luciferase activities were measured 30–40 h after transfection, and the values relative to the basal activity of the reporter are presented. (**c**) Similar reporter experiments were performed using AML1/Evi-1 derivatives in place of AML1/Evi-1. Their repression activities relative to that of AML1/Evi-1 are presented in percentages. (**d**) HeLa cells were transfected with the effector plasmids (AML1b, PEBP2β, AML1/Evi-1, AE(DL/AS)) in the presence or absence of CtBP1 along with pM-CSF-R-luc as indicated. The luciferase activities in the presence of CtBP1 relative to those in the absence of CtBP1 are presented. In **b**, **c**, and **d**, the representative data of three independent experiments in duplicate are shown. Values and error bars depict the mean and the standard deviation (s.d.), respectively

A role of HDAc in AML1/Evi-1-mediated repression

Although a full picture of CtBP-mediated transcriptional repression remains elusive, it is supposed that CtBP mediates repression by interacting with HDAc (Sundqvist *et al*., 1998; Turner and Crossley, 2001). Several HDAc proteins have been described in mammalian cells to date. They include class I (HDAc1, HDAc2, HDAc3, and HDAc8), class II (HDAc4, HDAc5, HDAc6, and HDAc7), and several class III HDAc proteins, whose characters are described extensively in the recent review (Khochbin *et al*., 2001). Among them, Sundqvist *et al*. (1998) previously demonstrated that HDAc1 physically interacts with CtBP1 *in vivo* and *in vitro*. We also performed an immunoprecipitation experiment and confirmed the interaction between CtBP1 and HDAc1 *in vivo* (Figure 4a). Next, to investigate a potential role of HDAc in AML1/Evi-1-mediated transcriptional repression, we performed the reporter assay in the presence of histone deacetylase inhibitor, trichostatin A (TSA). For this study, we used another cell line, HepG2 cells, which readily express endogenous CtBP1 (Figure 3a, lane 2),

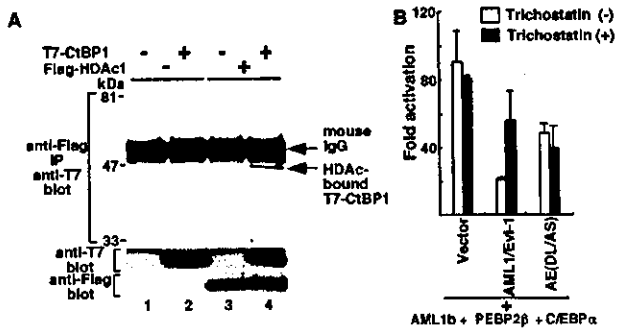

Figure 4 Involvement of the histone deacetylase in the transcriptional repression by AML1/Evi-1. (**a**) The pRc/CMV empty vector (lanes 1 and 3) or T7-CtBP1 in pRc/CMV (lanes 2 and 4) and the pBJ5 empty vector (lanes 1 and 2) or Flag-HDAc1 in pBJ5 (lanes 3 and 4) were cotransfected into COS7 cells (2×10^6). Cells were lysed and subjected to immunoprecipitation (IP) with anti-Flag. The immunoprecipitates were subjected to Western blotting using anti-T7 (top). Positions of size markers in kilodaltons (kDa) are indicated on the left. Expression of T7-CtBP1 and Flag-HDAc1 was monitored with anti-T7 (middle) and anti-Flag (bottom), respectively. (**b**) The pM-CSF-R-luc, AML1b, PEBP2β, and C/EBPα were transfected into HepG2 cells together with AML1/Evi-1 or AE(DL/AS) as indicated. Luciferase activities were measured 30–40 h after transfection following an 8-h treatment with or without 50 ng/ml trichostatin A. Bars represent luciferase activities relative to the basal activity of the reporter. Values and error bars depict the mean and the s.d., respectively

as the viability of HeLa cells was reduced considerably after the treatment with low concentrations of TSA. In HepG2 cells, basal transcription levels of pM-CSF-R-luc were not affected by the treatment with TSA (data not shown). In addition, the transactivation induced by AML1, PEBP2β, and C/EBPα did not change in the presence or absence of TSA (Figure 4b). However, the level of repression, which was observed when we overexpressed AML1/Evi-1 in addition to these transactivators, was considerably alleviated by the treatment with TSA (Figure 4b). In contrast, AE(DL/AS) mutant that does not interact with CtBP was barely affected by TSA (Figure 4b). These findings support a model in which AML1/Evi-1 functions as a repressor in concert with a CtBP-HDAc complex.

AML1/Evi-1 inhibits granulocytic differentiation in a CtBP-dependent manner

We previously reported the effect of the AML1/Evi-1 chimeric protein when expressed in 32Dcl3 cells, a murine IL-3-dependent myeloid cell line (Tanaka *et al*., 1995a). The 32Dcl3 cells differentiate into mature granulocytes when treated with G-CSF. The 32Dcl3 cells stably expressing AML1/Evi-1 show block in differentiation into mature granulocytes in the presence of G-CSF. Given that CtBP is readily detected in 32Dcl3 cells (Figure 3a, lane 3), CtBP would be potentially implicated in AML1/Evi-1-induced effect on 32Dcl3 cells. For the purpose of investigating the effect of CtBP on AML1/Evi-1-mediated differentiation block, 32Dcl3 cells were transfected with pCXN2-AML1/Evi-1 or pCXN2-AE(DL/AS), in which the expression of AML1/Evi-1 or AE(DL/AS) is driven by the β-actin promoter (Niwa *et al*., 1991). Subsequently, the cells were selected for G418 resistance and cloned with limiting dilution. A19 and A23 are representative clones which express AML1/Evi-1, whereas B15 and B56 are clones expressing AE(DL/AS) (Figure 5a). M1 and M3 are control clones that were transfected with the empty vector. Again, expression of CtBP1 was determined with anti-CtBP1 in each stable clone, and comparable levels of expression were observed (data not shown). When M1, M3, and parental 32Dcl3 cells were cultured with G-CSF instead of IL-3 for several days, the cells differentiated to mature granulocytes that are characterized by cytoplasmic granules and a segmented or circular nucleus (Figure 5b,c). In these G-CSF-treated cells, robust induction of mRNA for MPO was observed by Northern blot analyses (Figure 6). In A19 and A23, which overexpress AML1/Evi-1, immature morphological features characterized by a large un-segmented nucleus were maintained in the presence of G-CSF (Figure 5b,c). Consistently, mRNA for MPO was induced poorly, if any, in A19 and A23 (Figure 6). Thus, overexpression of AML1/Evi-1 blocks G-CSF-induced differentiation to mature granulocytes of 32Dcl3 cells. On the contrary, in B15 and B56 which carry AE(DL/AS), a mutant form of AML1/Evi-1 defective in interaction with CtBP, morphological features and induction of MPO mRNA

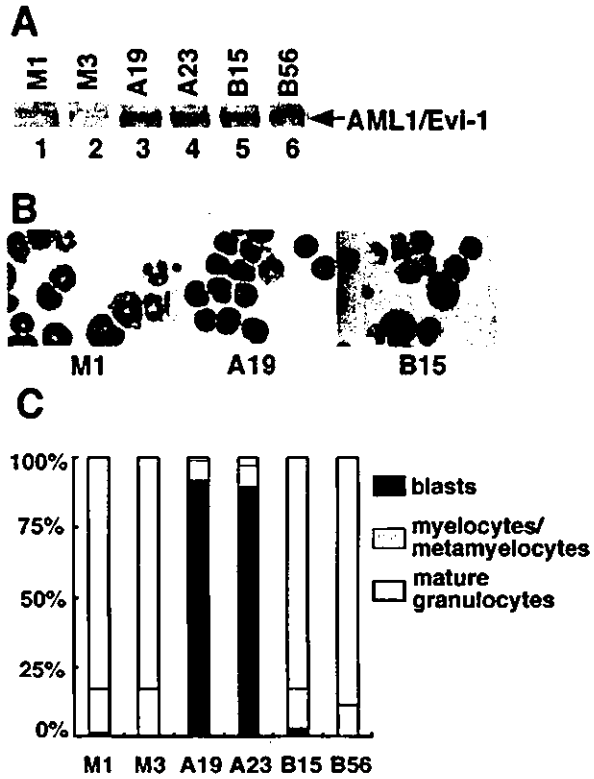

Figure 5 AML1/Evi-1 blocks G-CSF-induced granulocytic differentiation of 32Dcl3 clones in a CtBP-dependent manner. (a) Expression of the AML1/Evi-1 or AE(DL/AS) in 32Dcl3 clones. Clones A19 (lane 3) and A23 (lane 4) were established from cells transfected with AML1/Evi-1, while B15 (lane 5) and B56 (lane 6) were from cells transfected with AE(DL/AS). M1 (lane 1) and M3 (lane 2) are mock clones transfected with the pCXN2 empty vector. Lysates prepared from these clones were subjected to Western blot analysis using anti-Evi-1. The arrow indicates the migration of the AML1/Evi-1 or AE(DL/AS) protein. (b) Cell morphology of the 32Dcl3 clones when cultured in medium containing G-CSF for 7 days. Cytospin preparations were made and subjected to Wright-Giemsa staining. (c) Hemocytogram of 32Dcl3 clones after exposure to G-CSF for 7 days. Values in percentage were obtained by counting 300 cells in each clone. Morphological features for granulocytic differentiation were classified as blasts, myelocytes/metamyelocytes, and mature granulocytes which are demonstrated by closed, semi-closed, and open boxes, respectively

were almost similar to those of mock clones in the presence of G-CSF. These results suggest that AML1/Evi-1 blocks G-CSF-induced granulocytic maturation with dependence on the interaction with CtBP. Taken together, CtBP-dependent repression of AML1 target gene transcription should be one of the mechanisms for AML1/Evi-1-mediated block in granulocytic differentiation.

Discussion

The transcription factor AML1 plays an essential role in regulating growth and differentiation of hematopoietic cells. AML1 has been shown to induce the expression of genes which are essential for development

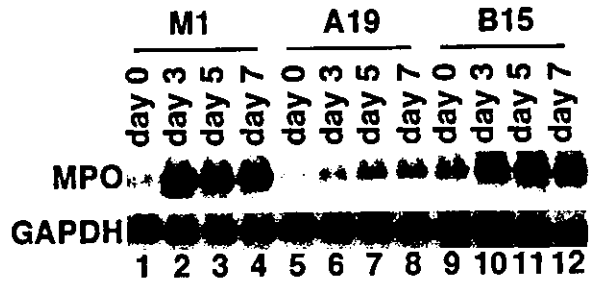

Figure 6 Northern analysis showing expression of the MPO transcript in 32Dcl3 clones. The 32Dcl3 clones were exposed to G-CSF for the indicated periods. Subsequently, total RNAs were prepared from these cells. Aliquots of total RNAs (15 μg per lane) were electrophoresed, transferred to a nylon membrane, and hybridized with a murine myeloperoxidase (MPO) cDNA (top). As a control for RNA loading, the membrane was also hybridized with a mouse glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA (bottom)

of hematopoietic system. Alterations in the *AML1* gene are closely associated with human hematological disorders, including AML, MDS, blastic phase of CML, and acute lymphoblastic leukemia. Chromosomal translocations involving the *AML1* gene have been repeatedly found in cases with leukemia (Look, 1997). AML1/ETO(MTG8), TEL/AML1, and AML1/Evi-1 chimeric proteins are derived from these translocations. These proteins have been shown to repress the AML1-induced transactivation in a dominant negative fashion. The gene encoding M-CSF receptor is accepted as one of the AML1 target genes, and repression of this gene might be involved in the pathogenesis of AML1/Evi-1-induced leukemia (Zhang *et al.*, 1996). M-CSF induces myelocytic or monocytic differentiation of hematopoietic cells, so that decreased expression of its receptor would confer refractoriness to differentiation stimuli in the leukemic cells (Fixe and Praloran, 1998). Besides M-CSF receptor, it is reasonable to assume that the AML1/Evi-1 represses expression of various AML1 target genes, which are critical for differentiation of the hematopoietic system, but have not been identified yet.

The mechanism of transcriptional regulation by AML1 has been rigorously studied so far. It was shown that AML1 associates with a coactivator complex including p300/CBP, and this association is required for the AML1-induced transactivation (Kitabayashi *et al.*, 1998). AML1/ETO(MTG8) not only loses p300/CBP-interacting portion of AML1, but also associates with a corepressor complex which contains N-CoR, mSin3A, and HDAc1 via the ETO(MTG8) portion (Lutterbach *et al.*, 1998; Wang *et al.*, 1998). TEL/AML1 was also shown to interact with a corepressor complex through the TEL portion (Fenrick *et al.*, 1999; Guidez *et al.*, 2000). These studies indicate that AML1/ETO(MTG8) and TEL/AML1 are not only a competitor for AML1, but also behave as an active repressor for transcription.

In this study, we demonstrate that AML1/Evi-1 physically interacts with CtBP and its association is required for AML1/Evi-1 to fully repress AML1-induced transactivation. CtBP is a distinctive type of corepressor protein, whose mechanisms for repression remain to be fully elucidated. Several transcriptional repressor proteins including FOG (Fox *et al.*, 1999), TCF (Brannon *et al.*, 1999), and Net (Criqui-Filipe *et al.*, 1999) have been reported to require CtBP as a corepressor. CtBP was shown to interact with HDAc1 *in vivo* (Sundqvist *et al.*, 1998), and some of these transcription factors are susceptible to TSA (Criqui-Filipe *et al.*,' 1999). Recently, CtBP was also reported to interact with class II HDAc proteins (Dressel *et al.*, 2001; Zhang *et al.*, 2001). It is assumed that class I and class II HDAc form a complex to repress transcription whose constituents differ according to genes to be regulated (Khochbin *et al.*, 2001). Taken together, the CtBP-HDAc complex might be responsible for the transcriptional repression by these transcription factors. The repression by AML1/Evi-1, as we demonstrate here, is also sensitive to TSA, suggesting the involvement of the CtBP-HDAc complex.

An overwhelming majority of the studies on transcriptional regulation has been carried out with experiments using transiently transfected reporters. They have led to the current concept whereby histone acetyl transferases (HAT) and HDAc may play important roles in transcriptional regulation (Hassig *et al.*, 1997; Zhang *et al.*, 1997). Derepression by HDAc inhibitors has also been demonstrated and tested using these experiments (Hassig *et al.*, 1997). However, there seems to be concerns about authenticity of these experiments because these reporter constructs may lack nucleosome structures. Some studies including that by Jin and Scotto, (1998) addressed these concerns. They demonstrated that the HDAc inhibitor led to derepression of both the transiently and the stably transfected *MDR1* promoter assessed by reporter assays, and that these results corresponded well with those from the Northern blot analyses which showed the induction of the intrinsic *MDR1* gene expression by the HDAc inhibitor. Another report also supports these observations (Nagy *et al.*, 1997). Although the underlying mechanisms remain to be fully elucidated, acetylation and deacetylation of non-histone proteins including basal transcription factors, which have been recently reported, may contribute to these results (Burke and Baniahmad, 2000; Imhof *et al.*, 1997). The acetylation status of these proteins, which is potentially regulated by HAT and HDAc mutually, may affect transcriptional initiation from transfected reporter constructs as well as from intrinsic promoters. Thus, we believe that experiments using transiently transfected reporters, as in this study, provide a good model for assessing transcriptional regulation by HAT and HDAc and for assessing the effect of HDAc inhibitors.

Provided that the complex consisted from AML1/Evi-1, CtBP, and HDAc blocks granulocytic differentiation in 32Dcl3 cells, it is tempting to speculate that HDAc inhibitors would alleviate the block in differentiation. However, the 32Dcl3 cells cultured in

the medium containing G-CSF, irrespective of parental cells, mock clones, or AML1/Evi-1-transfected clones, lost viability and died completely within 72 h showing morphological evidence of apoptosis in the presence of 5 ng/ml or above TSA (data not shown). Previous studies show that TSA ranging from 5 to 50 ng/ml or above is required for assessing its *in vivo* effect in hematopoietic cells (Ferrara *et al.*, 2001; Kosugi *et al.*, 1999). At lower concentrations, TSA did not affect the G-CSF-induced differentiation of either 32Dcl3 clone (data not shown). Generally, the HDAc inhibitors including TSA are known to induce growth arrest and apoptosis of cells (Marks *et al.*, 2000). Reportedly, one of the mechanisms is inducing the expression of $p21^{WAF1/Cip1}$ through the HDAc inhibitor activity (Sowa *et al.*, 1999). The 32Dcl3 cells may be vulnerable to these cytotoxic activities of TSA, so that we could not evaluate its effect on AML1/Evi-1-dependent differentiation block in this system. We suppose that the threshold of TSA to release the transcriptional repression mediated through AML1/Evi-1 might be higher than that to induce apoptotic cellular response, at least in 32Dcl3 cells. Nevertheless, it is tempting to assess a potential value of HDAc inhibitors in the therapy for AML1-associated leukemias, including AML1/Evi-1-induced leukemia (Minucci *et al.*, 2001; Wang *et al.*, 1999).

Our study, which suggests an important role of CtBP in AML1/Evi-1-mediated transcriptional repression and inhibition of granulocytic differentiation, strengthen the recent finding that aberrant recruitment of a corepressor complex to AML1 target genes might play a central role in leukemogenesis (Lutterbach *et al.*, 1998; Wang *et al.*, 1998).

Materials and methods

Cell culture and establishment of stable clones

COS7, HeLa, and HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin, and 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 incubator. SKH1 cells were maintained in RPMI 1640 with 10% FCS. 32Dcl3 cells were maintained in RPMI 1640 medium supplemented with 0.25 ng of murine interleukin-3 (IL-3) per ml and 10% FCS.

Plasmids

The pME18S-AML1/Evi-1, in which the AML1/Evi-1 complementary DNA (cDNA) was inserted into the *Eco*RI site of the pME18S vector, was used for transient transfection (Takebe *et al.*, 1988; Tanaka *et al.*, 1995a). The deletion mutant, pME18S-AEΔ544-607 was constructed substituting the 2.3 kb *ApaI-PmaCI* fragment of pME18S-AML1/Evi-1 with that of pME18S-Evi-1Δ544-607 (Izutsu *et al.*, 2001). Other mutant forms of AML1/Evi-1, AE(DL/AS), AE(AS/DL), and AE(AS/AS), were generated in the same manner using the corresponding mutants of Evi-1 (Izutsu *et al.*, 2001). The AML1 and PEBP2β cDNAs were inserted into the *Eco*RI site of pME18S (Tanaka *et al.*, 1995a,b).

Transcriptional response assay

Transcriptional response assays were performed with HeLa cells as described previously with minor modifications (Imai *et al.*, 2000). Briefly, the cells were seeded in 12-well plate at 4×10^4 cells per well. For each well, 1 μg of the reporter and typically 500 ng of the effector plasmid were transfected. As a control of transfection efficiency, a plasmid expressing β-galactosidase was cotransfected, and the data were normalized to the β-galactosidase activity. For the experiment using the HDAc inhibitor, HepG2 cells were seeded in 12-well plate at 4×10^4 cells per well, and were transfected using SuperFect (Qiagen). The cells were incubated for 30 to 35 h after the transfection and were treated with 50 ng/ml trichostatin A (Waco) for 8 h before harvesting.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed as described previously (Izutsu *et al.*, 2001). For the immunoprecipitation analysis using SKH1 cells, they were lysed in the TNE buffer (Kurokawa *et al.*, 1998b). Lysates were incubated with anti-Evi-1 (Tanaka *et al.*, 1994) or with preimmune rabbit serum for 6 h at 4°C. Then the samples were incubated with protein-A-Sepharose (Sigma) for 6 h at 4°C. The precipitates were washed five times with the TNE buffer, and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and analysis by Western blotting. Immunoblotting was performed with anti-CtBP1 or anti-CtBP2 (Santa Cruz Biotechnology) using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). The anti-Flag M2 antibody (Sigma) was used for immunoprecipitation of Flag-tagged HDAc1.

Establishment of 32Dcl3 stable transfectants and granulocytic differentiation assay

To generate stable clones of 32Dcl3 overexpressing AML1/Evi-1 and its mutant, the constructs subcloned into pCXN2 vector (Niwa *et al.*, 1991), which has neomycin resistance gene, were transfected by the electroporation method as described previously (Tanaka *et al.*, 1995b). These cells were selected in medium containing G418 (800 μg/ml). G418-resistant clones were screened for expression of AML1/Evi-1 by Western blotting. For each construct, two independent clones with comparable expression were used in further studies. For the induction of granulocytic differentiation, 32Dcl3 cells were washed twice with phosphate-buffered saline and placed in RPMI 1640 medium supplemented with 10% FCS and 5 ng of recombinant human granulocyte colony stimulating factor (G-CSF) (Kirin Brewery) per ml, instead of murine IL-3. After 7 days, morphological studies were performed on cytospin preparations with Wright-Giemsa staining.

RNA isolation and Northern analysis

Total cellular RNA was prepared according to the acid guanidium thiocyanate-phenol-chloroform method. Aliquots of 15 μg of the total RNA per lane were electrophoresed in 1.0% agarose gels and were transferred to nylon membranes (Hybond-N, Amersham Pharmacia Biotech). Membranes were prehybridized for 4 h at 42°C. The probes for murine myeloperoxidase (MPO) or murine glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were amplified using reverse transcriptase-polymerase chain reaction (RT–PCR) (Tanaka *et al.*, 1995b), and were labeled by a random primer method

using the Megaprime DNA labeling system (Amersham Pharmacia Biotech) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech). The membranes were subjected to hybridization with the labeled probes at 42°C overnight. Washed membranes were subjected to detection with autoradiography.

Acknowledgments

We thank M Ohki (National Cancer Center Research Institute, Tokyo, Japan) for providing AML1, Y Ito (Institute for Virus Research, Kyoto University, Japan) for PEBP2β, DE Zhang (The Scripps Research Institute, La Jolla, CA, USA) for pM-CSF-R-luc, SL McKnight (Johns Hopkins University, Baltimore, MD, USA) for C/EBPα, SL Schreiber (Harvard University, Cambridge, MA, USA) for Flag-HDAc1, G Chinnadurai (St. Louis University, MO, USA) for T7-hCtBP1, K Arai (The Institute of Medical Science, University of Tokyo, Japan) for pME18S, and J Miyazaki (Osaka University, Japan) for pCXN2. The murine IL-3 is a generous gift from Kirin Brewery (Tokyo, Japan). This study is supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare and from the ministry of Education, Culture, Sports, Science and Technology of Japan.

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Leukemia & Lymphoma, 2002 VOL. 43 (3), pp. 617–621

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Mutational Analyses of the AML1 Gene in Patients with Myelodysplastic Syndrome

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(Received 10 August 2001)

The *AML1* gene is the most frequent target of translocations associated with human leukemias. We recently found somatic point mutations of the *AML1* gene, V105ter and R139G, in two cases of myelodysplastic syndrome (MDS). Both mutations are present in the region encoding the Runt domain of AML1, and cause loss of the DNA-binding ability of the resultant products. Of these mutants, V105ter has also lost the ability to heterodimerize with PEBP2β/CBFβ. On the other hand, the R139G mutant acts as a dominant negative inhibitor through competing with wild-type AML1 for interaction with PEBP2β/CBFβ. In this review, we summarize mutational changes of the *AML1* gene in hematological malignancies, especially in MDS and discuss the mechanism whereby the mutant acts as a dominant negative inhibitor of wild-type AML1.

Keywords: AML1; MDS; Mutation; Leukemia

INTRODUCTION

The human *AML1* gene encodes DNA-binding protein that contains the Runt domain and is the major subunit, α, of heterodimeric transcription factor PEBP2/CBF [1]. *AML1* is found at the breakpoints of some translocations associated with leukemias. Furthermore, some studies indicate that the structural alterations of AML1 caused by non-translocation-generated mutations may also play a role in leukemogenesis. However, no mutations have been described in sporadic cases of preleukemic diseases. Myelodysplastic syndrome (MDS) is a preleukemic state in which multistep progression to acute myelogeneous leukemia (AML) is documented by serial acquisition of genetic abnormalities associated with progression of disease [2,3]. In this review, we discuss the mutations of AML1 found in patients with MDS and their functional implications in pathogenesis.

The AML1 Transcription Factor is Essential for Definitive Hematopoiesis

We have shown that AML1 regulates myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms, AML1a and AML1b, suggesting that the transcriptional property of AML1 is necessary for myeloid cell differentiation [4]. It has also been reported that AML1 regulates the transcription of various genes which are important in hematopoiesis, such as those for myeloperoxidase, neutrophil elastase, the receptor for macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor, and T cell receptors (TCRs) [5–11]. Furthermore, we have revealed that AML1 can cause neoplastic transformation when overexpressed in fibroblast, suggesting a potential role for AML1 in promoting cellular proliferation [12]. The Runt domain is an evolutionally conserved protein motif that is responsible for both DNA binding and heterodimerization with a non-DNA-binding regulatory subunit, β [13]. AML1 includes three alternative splicing forms: AML1a, AML1b, and AML1c [14]. AML1b is known to be a transcriptionally active form, which we refer to as AML1 in this manuscript. It was shown that mice lacking *AML1* die during mid-embryonic development, secondary to the complete absence of definitive hematopoiesis [15,16].

Translocation- and Non-translocation-generated Mutations of the AML1 Gene

The human *AML1* gene was first identified on chromosome 21 as the gene that is disrupted in the

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ISSN 1042-8194 print/ISSN 1029-2403 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10428190290012155

(8;21)(q22;q22) translocation, which is one of the most frequent chromosome abnormalities associated with human acute myelogeneous leukemia [17,18]. In this translocation, the rearrangement results in the production of the AML1/MTG8 (ETO) fusion protein [19–21]. The *AML1* gene is also disrupted in t(3;21)(q26;q22), which is found in the blastic crisis phase of chronic myelogeneous leukemia and therapy-related acute myelogeneous leukemia [22–26]. PEBP2β/CBFβ, a heterodimeric partner of AML1, is known to be disrupted in the inv(16)(p13;q22) chromosome abnormality associated with AML [27]. These findings suggest that the structural alteration of AML1 caused by translocation-generated mutations triggers leukemic transformation and that intact AML1 may play important roles in hematopoietic cell differentiation and proliferation.

Furthermore, biallelic and heterozygous point mutations in the Runt domain of the *AML1* gene were found in 8 of 160 (5%) leukemia patients: two silent mutations, four heterozygous missense mutations, and two biallelic nonsense or frameshift mutations [28]. The other group reported that a point mutation of the *AML1* gene was found in 14 cases, including nine patients with AML of the M0 type and five myeloid malignancies with acquired trisomy 21: one M1AML, two M2AML, one essential thrombocythemia (ET) and one chronic myelogeneous leukemia (CML) [29]. These results indicate that the structural alterations of AML1 caused by non-translocation-generated mutations may also play a role in leukemogenesis.

Furthermore, it was reported that haploinsufficiency of AML1 caused by the mutations of the *AML1* gene in one allele results in familial platelet disorder with predisposition to acute myelogeneous leukemia (FPD/AML) [30]. FPD is a hereditary disease characterized by qualitative and quantitative platelet defects with propensity to develop AML. These results suggest that altered transcriptional regulation of AML1 caused by non-translocation-generated mutations may cause a preleukemic disease.

Mutations of the AML1 Gene in Myelodysplastic Syndrome

In these lines, we investigated mutational changes of the *AML1* gene in the patients with MDS, a sporadic preleukemic disease [2,3]. We screened 37 MDS patients for mutations in four exons (exons 3, 4, 5, and 6) of the *AML1* gene, which include the Runt domain, using the reverse transcriptase-polymerase chain reaction-single-strand conformation polymorphism (RT-PCR-SSCP) and sequencing analyses [31]. The 37 patients are composed of 18 refractory anemia (RA), two refractory anemia with excess of blasts (RAEB), five chronic myelomonocytic leukemia (CMMoL), two refractory anemia with excess of blasts in transformation (RAEB in T), and 10 leukemia secondary to MDS. Abnormally migrating bands were detected on the RT-PCR-SSCP analyses in two patients with MDS; one was a patient with CMMoL and the other was a patient with AML secondary to RA. The sequencing analyses showed nucleotide alterations of the *AML1* gene in exon 4 in both patients. The mutation found in the patient with CMMoL was a GT insertion at codon 105 resulting in V105 termination (V105ter) (single-letter amino acid code). The other patient had a missense mutation at codon 139 (CGA to GGA), which lead to a change of amino acid, R139G. From the sample of the patient with CMMoL, the normal and the mutated sequences were obtained. On the other hand, only the abnormal sequence was obtained from sequencing of the PCR product of the other MDS patient. These results suggest that an allelic loss of the Runt-domain-encoding region also exists in this patient. To determine whether the *AML1* gene is mutated at the germ line or somatic level, we examined the genomic DNA sequences of the formalin-fixed and paraffin-embedded specimen of the rectum from the patient with CMMoL and the lung and liver from the patient with AML secondary to RA. Both of the genomic DNA sequences of the corresponding regions of *AML1* in these patients were normal; this reveals that the AML1 mutations are somatic events.

These findings suggest that altered regulation by AML1 caused by mutational changes of the *AML1* gene may cause a predisposition for acquisition of additional mutations leading to leukemia.

The Functional Analyses of the AML1 Mutants Found in MDS

AML1 has been shown to regulate expression of several hematopoietic lineage-specific genes by affecting transcription from the cognate promoters or enhancers [5,32–35]. Among them is that of the receptor for macrophage colony-stimulating factor (M-CSF). To elucidate functional alterations of AML1 in preleukemic states, we investigated transcriptional activities of the AML1 mutants found in MDS [31]. In the transcriptional response assays with a reporter plasmid containing an M-CSF receptor promoter, those two mutants of AML1 found in MDS lacked transcriptional activities. Concomitant expression of the V105ter mutant with wild-type AML1 did not affect transcriptional activation induced by wild-type AML1. In contrast, the R139G mutant repressed the transcriptional activity of wild-type AML1 in a dose-dependent manner. These results suggest that R139G could act as a dominant negative inhibitor for wild-type AML1.

The Runt domain of AML1 is reported to be responsible for binding to the specific sequence TGT/cGGT, which is a consensus DNA sequence for AML1 binding, called the PEBP2 site [13,33]. We previously demonstrated that AML1 specifically binds to the PEBP2 site by means of electrophoretic mobility shift assay (EMSA) and that the DNA-binding is required for AML1-induced transactivation [36]. However, both of the mutants, V105ter and R139G, failed to bind to the PEBP2 site when assessed by

EMSA. These findings account for loss of the transcriptional activity of these two mutants in the transcriptional response assays. The DNA-binding ability of wild-type AML1 was not affected when the V105ter mutant was coexpressed. However, when the R139G mutant was coexpressed with wild-type AML1, there was a marked reduction of the DNA-binding ability of wild-type AML1. These results suggest that the R139G mutant blocks binding of wild-type AML1 to the PEBP2 site. Because AML1-induced transcription is dependent on binding to the PEBP2 site, these findings are compatible with the results that the R139G mutant acts as a dominant negative inhibitor of wild-type AML1 in the transcriptional response assays.

AML1 is known to heterodimerize with PEBP2β/CBFβ, and heterodimerization with PEBP2β/CBFβ enhances the DNA-binding ability of AML1, resulting in the enhanced transcriptional potency of the AML1–PEBP2β/CBFβ complex [37]. The recent study showed that dimerization with PEBP2β/CBFβ protects AML1 from ubiquitin-proteasome-mediated degradation [38]. Thus, association with PEBP2β/CBFβ is one of the key determinants for AML1 functions. In these lines, we previously demonstrated that the chimeric products of AML1 in t(8;21) and t(3;21) leukemias inhibit the transcriptional activity of AML1 by sequestering PEBP2β/CBFβ from AML1 [39]. As for the current two mutants, we found that V105ter has lost the ability to heterodimerize with PEBP2β/CBFβ while R139G can associate with PEBP2β/CBFβ. Remarkably, the R139G mutant exhibits an enhanced binding affinity with PEBP2β/CBFβ and competes with wild-type AML1 for heterodimerization with PEBP2β/CBFβ, resulting in a reduced DNA-binding and a transactivational ability of wild-type AML1. These results provide a potential mechanism by which the R139G mutant acts as a dominant negative inhibitor of wild-type AML1 (Fig. 1).

A recent study of the crystal structure of AML1 by Tahirov *et al.* revealed the distinct regions involved in DNA binding or heterodimerization with PEBP2β/CBFβ [40]. They describe that Arg139 is located in L9 and the interaction between L9 and minor groove of DNA seems to be critical for DNA binding. On the other hand, L9 is shown not to be involved in heterodimerization with PEBP2β/CBFβ. These results are consistent with our findings about R139G. Functional analyses of the mutations found in hematological malignancies by us and others are summarized in Table I [28–31,41].

Mutational Changes of the AML1 Gene and Predisposition to Leukemia

Our findings about the mutants found in cases with MDS suggest that altered transcriptional regulation by mutated AML1 may cause a predisposition for acquisition of additional mutations leading to leukemias. Some investigations about the mice harboring an abnormal fusion protein containing a part of AML1 support this hypothesis.

Expression of the AML1/MTG8 (ETO) fusion protein using a "knock-in" strategy in mice leads to generation of dysplastic hematopoietic progenitors in the fetal liver [42]. However, the transgenic mice in which the expression of AML1/MTG8 (ETO) is strongly induced in the bone marrow under the control of a tetracycline-inducible system have not developed leukemia [43]. In spite of this, abnormal maturation and proliferation of progenitor cells have been observed from these animals. These results suggest that, though dysfunction of AML1 *per se* may cause dysplasia of hematopoietic cells, additional mutation or abnormal expression of other genes may be necessary to promote leukemogenesis. In fact, two of the three cases of AML having mutations in the Runt domain also harbored translocation-generated mutations [28].

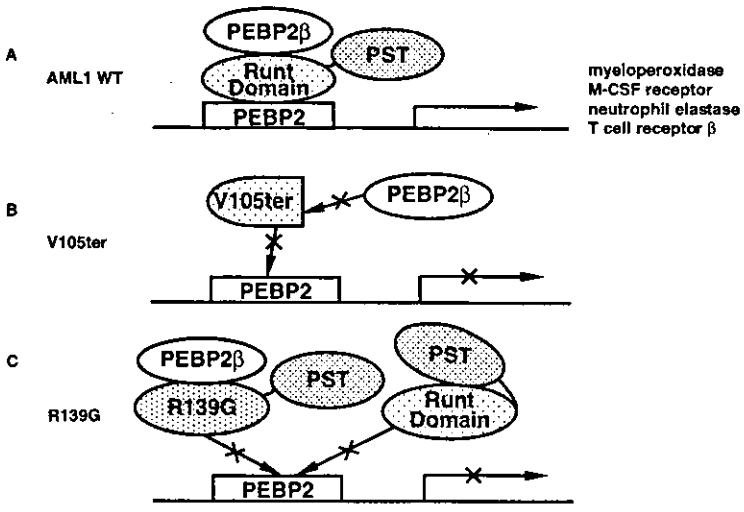

FIGURE 1 Models for functions of the AML1 mutants found in MDS. (A) A model for transactivation induced by wild-type AML1. (B) and (C) Potential mechanisms of dysfunction of V105ter (B) and R139G (C). PST: the PST region of AML1.

TABLE I Functional analyses of the mutant AML1 proteins found in patients with hematological malignancies (CML BC, blastic crisis phase of chronic myelogeneous leukemia; ND, not determined)

Mutation	Diagnosis	DNA binding	Heterodimerization	Nuclear accumulation	Transcriptional activity
His58Asn	AML M0	+	ND	+	+
Arg80Cys	CML BC	−	+	+	−
Lys83Asn	AML M3 relapse	−	+	+	−
Val105ter	MDS	−	−	−	−
Ser114ter	AML M0	−	−	−	−
Arg135Gly	AML M0	−	+	ND	ND
Gly138Asp	AML M0	−	+	ND	ND
Arg139Gln	FPD	−	+	ND	ND
Arg139Gly	MDS	−	+	+	−
Asp171Gly	AML M0	−	+	ND	ND
Arg174Gln	FPD, ET	−	+	ND	ND
Arg177ter	AML M0	+	ND	−	−
Arg177Gln	AML M5a, AML M2	+	ND	−	−

Detailed mechanisms whereby the second mutations are promoted to occur through dysfunction of AML1 are to be elucidated.

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Dev Genes Evol (2002) 212:293–297
DOI 10.1007/s00427-002-0244-1

SHORT COMMUNICATION

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An orphan PRD class homeobox gene expressed in mouse brain and limb development

Received: 18 December 2001 / Accepted: 21 March 2002 / Published online: 24 May 2002

Abstract We report the cDNA sequence and expression of a mouse homeobox gene, *Dmbx1*, from the PRD class and comparison to its human orthologue. The gene defines a new homeobox gene family, Dmbx, phylogenetically distinct from the Ptx, Alx, Prx Otx, Gsc, Otp and Pax gene families. The *Dmbx1* gene is expressed in the developing mouse diencephalon, midbrain and hindbrain, and has dynamic expression during forelimb and hindlimb development. Unusually for homeobox genes, there is no orthologue in the *Drosophila* or *Caenorhabditis* genomes; we argue this reflects secondary loss.

Keywords Homeobox · Brain development · Limb development · K50 homeodomain · Paired

Introduction

Homeobox genes comprise a diverse and ancient gene superfamily. Molecular phylogenetic analyses of the homeodomain have identified two main "classes" (also called superclasses) of animal homeobox genes – ANTP and PRD – and several more divergent lineages (e.g. TALE, LIM, POU; Galliot et al. 1999). The ANTP and PRD classes can be subdivided, in turn, into numerous distinct gene families, again using homeodomain sequence comparisons. Examples include the Emx, En, Evx, Mnx or Msx families (in the ANTP class) and the Otx, Otp, Ptx, Pax3/7, Pax 1/9, Pax 2/5/8 or Prx families (in the PRD class). Each family includes one or more vertebrate genes and one or more genes from *Drosophila* and/or *Caenorhabditis elegans*. In practice, a gene family usually encompasses all the genes descendent from a single precursor gene in the most recent common ancestor of the Bilateria. The principal exception is the Hox genes, which are often considered a single family, even though the bilaterian ancestors certainly possessed multiple Hox genes (albeit in one cluster). Secondary loss of homeobox gene families has been noticed; for example, loss of Gsx genes in *C. elegans* or loss of Xlox in *Drosophila* and *C. elegans* (Ferrier and Holland 2001). Here we describe cloning and expression of a new member of the PRD class from mouse; this gene is not a member of any previously recognised homeobox gene family. The gene has a clear orthologue in the draft human genome sequence, but no orthologue in *Drosophila* or *C. elegans*.

Edited by B. Herrmann

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Materials and methods

Degenerate primers were designed to two motifs conserved in a subset of PRD-like homeodomains (RRSRTTF and QVWF(K/S)NRR). These were used in RT-PCR reactions using a cDNA template from c-kit+ cells immunopurifed from the aorta-gonad-mesonephros region of 11.5 days post coitum (E11.5) C57B/6 mouse embryos. The expected 170-bp amplified product was cloned and nine recombinants sequenced. Eight derived from known genes (*Phox1, Pax3, S8*); one novel clone was used to screen an unamplified E9.5 mouse embryo cDNA library that we constructed in lambda ZAP II (Stratagene). Two 6-kb and four 4-kb independent cDNA clones were obtained; these all derived from the same gene. BLAST searches showed this to be a novel homeobox gene. The longer sequence is reported here. During preparation of this manuscript, another laboratory deposited a 1-kb sequence onto GenBank that is internal to our cDNAs, and named this *Dmbx1* (accession AF421858) (Ohtoshi et al. 2002, Miyamoto et al. 2002). To avoid confusion in the literature,

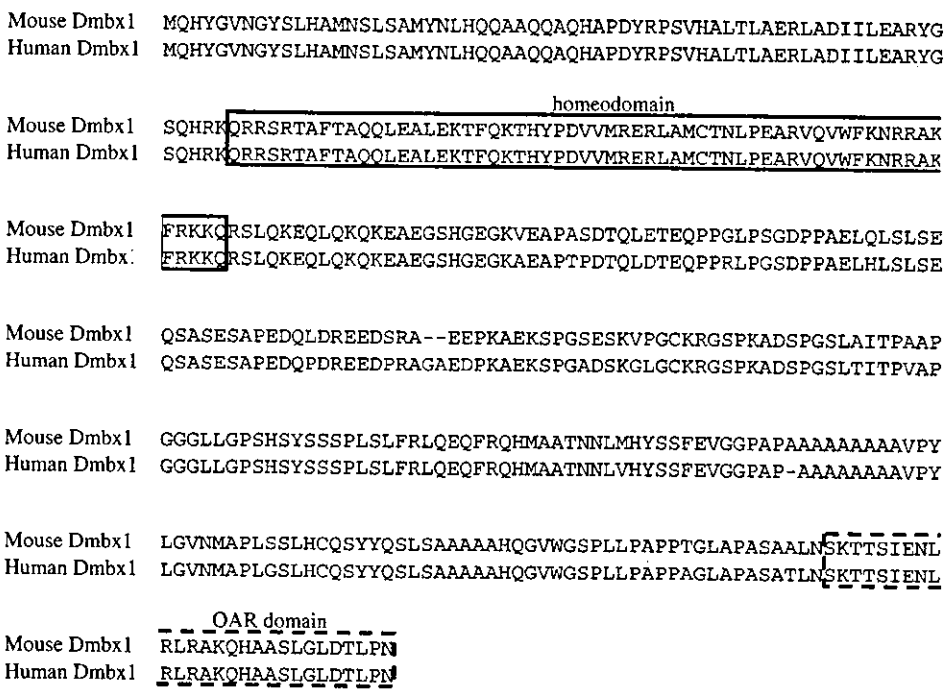

Fig. 1 Deduced protein sequence of mouse Dmbx1 aligned with its human orthologue predicted from genome sequence. *Dashes* indicate gaps inserted to maximise alignment. The *box* marks the homeodomain; the *dashed box* indicates the OAR domain

we follow this nomenclature in the work presented here. Our sequence is deposited with GenBank and given accession number AF499446. Phylogenetic analysis of amino acid sequence was performed using the Neighbour-Joining method implemented in ClustalX (Thompson et al. 1997), with outputs displayed using TreeView (Page 1996). We restricted analysis to the homeodomain to enable maximal representation of PRD class genes; analysis of a longer alignment including the homeodomain plus OAR domain gave similar results. The alignment and list of sequences used is available at http://www.rubic.rdg.ac.uk/amphioxus. Whole-mount in situ hybridisation to mouse embryos (strain CD-1) was performed as described by Nieto et al. (1996) with slight modifications using digoxygenin-labelled riboprobes from the complete 4-kb cDNA or a 990-bp subclone covering most of the open reading frame. The two probes gave identical results. After staining, hindbrains were dissected and flat-mounted under coverslips for photography.

Results and discussion

Using RT-PCR followed by cDNA library screening, we cloned a novel homeobox gene from c-kit+ cells isolated from the developing aorta-gonad-mesonephros (AGM) region of mouse embryos. These cells were chosen because we aimed to isolate genes for transcription factors involved in controlling haematopoiesis; the AGM region gives rise to definitive haematopoietic stem cells (Medvinsk and Dzierzak 1996). As described below, the gene we isolated is likely to have broader developmental regulatory roles. The longest cDNA has the potential to code for a 40.6-kDa protein of 376 amino acids, including a Paired-like homeodomain followed by an OAR domain. The gene, designated *Dmbx1*, has a clear human homologue in the draft human genome sequence, located on chromosome 1 (GenBank accession AL137797). Alignment of the mouse and human deduced proteins reveals 100% identity over the homeodomain and OAR domain, and 94% identity across the entire protein (Fig. 1). This is far higher than expected for paralogues within a gene family, and suggests these genes are direct orthologues. Using genome sequences accessible through FlyBase and WormBase, we did not detect an orthologue in either the *D. melanogaster* or the *C. elegans* genome.

The Dmbx1 homeodomain is equidistant from a range of genes in the PRD class, notably members of the Otx, Prx, Ptx and aristaless families. The presence of diagnostic P26, D27, E32, R44, O46 and A54 residues in the Dmbx1 homeodomain is diagnostic for the PRD class. The residue at homeodomain position 50 is important in determining DNA-binding specificity; some authors use its identity to subdivide the PRD class (Treisman et al. 1989), although these subdivisions are not monophyletic (Galliot et al. 1999). Dmbx1 has a lysine at this position, assigning it to the K50 Paired-like genes.

To investigate the evolutionary origin and relationships of *Dmbx1*, we conducted molecular phylogenetic analyses (Fig. 2). These confirmed that *Dmbx1* is not a member of any known homeobox gene family; indeed the gene is equidistant from several gene families. Considering the absence of a closely related gene in *Drosophila* or *C. elegans*, this could be interpreted as the gene having emerged during deuterostome, chordate, or vertebrate evolution. We suggest this is unlikely, because the phylogenetic node separating the *Dmbx1* lineage from other genes is deeper than the nodes separating *Drosophila*, nematode and vertebrate homologues *within* each known gene family. This implies that *Dmbx1* defines a new gene family within the PRD class, designated Dmbx, and that this gene family is as ancient as the Otx, Prx, Ptx, Gsc, al, Otp and Pax3/7 gene families, each of which have vertebrate and invertebrate members. We conclude that the origin of the Dmbx gene family

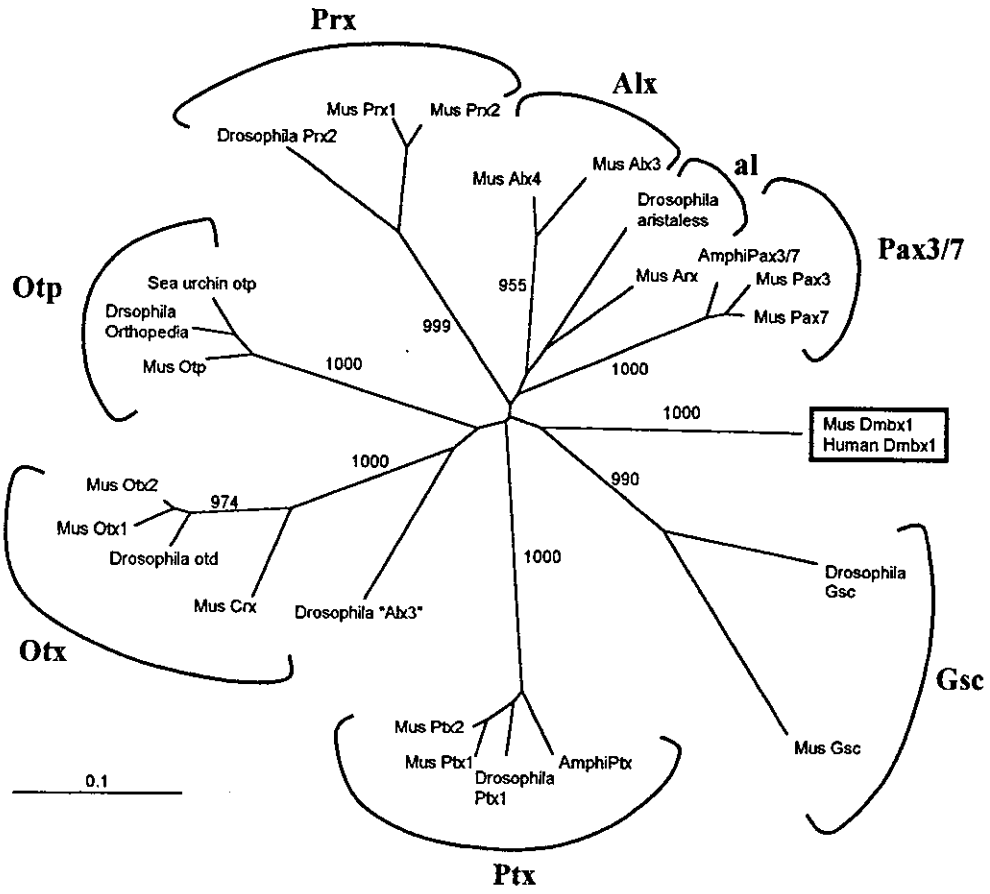

Fig. 2 Unrooted phylogenetic tree constructed from Paired class homeodomains. *Figures on nodes* indicate support values from 1,000 bootstrap resamplings of the data

pre-dates the divergence of arthropods, nematodes and vertebrates, and that Dmbx genes have been secondarily lost on the evolutionary lineages leading to *D. melanogaster* and *C. elegans*. We also note that the Dmbx gene family is (thus far) represented by only a single gene in the human and mouse genomes; most other homeobox families have two to four members (there are a few singletons, such as Xlox). If we accept the emerging view that the early vertebrate genome expanded by two rounds of whole genome duplication (Furlong and Holland 2002), then gene loss must also have occurred in the vertebrate Dmbx gene family.

Using whole-mount in situ hybridisation, we examined the spatiotemporal pattern of *Dmbx1* expression during mouse development. In mouse embryos at embryonic day (E) 7.5–8, expression of *Dmbx1* is detected around the prospective midbrain region (Fig. 3A). By E8–8.5, the expression becomes more definite and limited to the prospective midbrain region exclusively (Fig. 3B). In mouse embryos at E9.5, the domain of midbrain expression has expanded, extending partly into the optic eminence (Fig. 3C). In mouse embryos at E10, the clearest site of *Dmbx1* expression is still the midbrain, where mRNA is detected across all of the developing structure, and rostrally into the diencephalon (Fig. 3D). Clear expression also appears in the medial and lateral nasal pits, the dorsal half of the optic cup, and parts of the hindbrain. By E11, the hindbrain expression can be resolved into two anteroposteriorly oriented stripes along the lateral edges of rhombomeres, on each side of the midline (Fig. 3E). A small but distinct region of expression is also detected in the posterior, distal region of the forelimb buds (Fig. 3E, G). This expression is located near the distal edge of the *Sonic hedgehog*-expressing domain, but subjacent to the *Fgf4*-expressing domain in the posterior region of the apical ectodermal ridge. No expression is observed in hindlimb buds at this stage (Fig. 3H). Expression in the midbrain, diencephalon and hindbrain persists at E11.5, and the nasal expression in now detected on either side of the naso-lacrimal groove (Fig. 3F). Close examination reveals that the midbrain and hindbrain expression is not contiguous; *Dmbx1* transcripts are absent directly at the site of the midbrain-hindbrain boundary or MHB. At this stage, the expression in the optic cup has weakened. The expression in limbs also displays a dynamic pattern. Expression in forelimb buds observed half a day earlier has now disappeared (Fig. 3I); instead, *Dmbx1* transcripts are now detected along the distal edge of the hindlimb buds, subjacent to the apical ectodermal ridge (Fig. 3J). RT-PCR analysis detects *Dmbx1* expression in c-kit+ cells of the AGM region at this stage (data not shown). By flat-mounting the stained hindbrain at E11, the hindbrain expression can be seen to be in two parallel, bilateral stripes of cells running longitudinally (Fig. 3K, L). The more medial stripe runs from rhombomere 2 (r2) to the

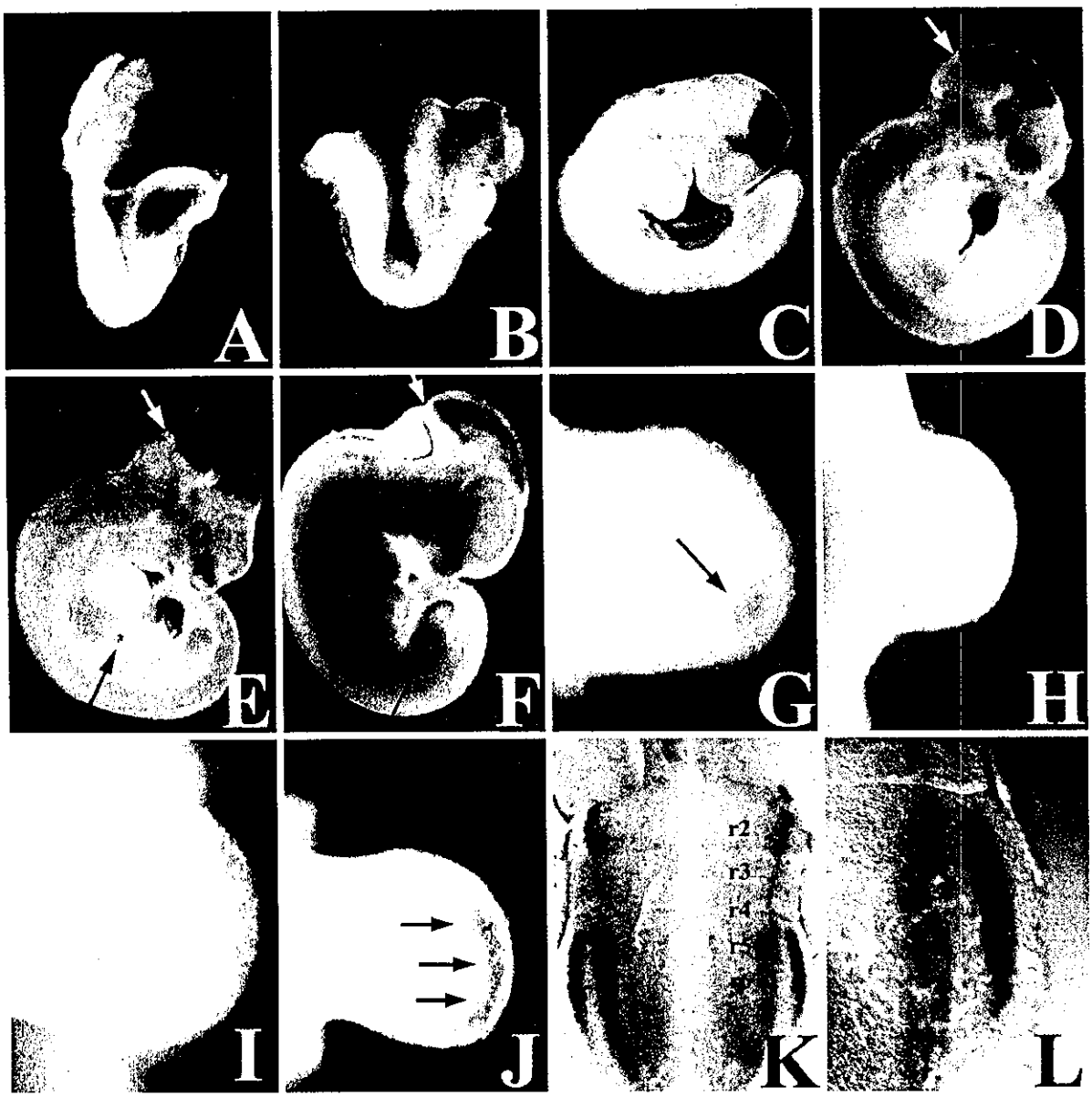

Fig. 3A–L Expression of *Dmbx1* in developing mouse embryos. **A–F** Whole-mouse in situ hybridisation showing *Dmbx1* expression at E7.5–8 (**A**), E8–8.5 (**B**), E9.5 (**C**), E10 (**D**), E11 (**E**) and E11.5 (**F**). **G**, **H** *Dmbx1* expression in limb buds at E11. **G** Forelimb and **H** hindlimb. **I**, **J** *Dmbx1* expression in limb buds at E11.5. **I** Forelimb and **J** hindlimb. **K**, **L** Dissected and flat-mounted hindbrain at E11 viewed from the dorsal surface, showing rhombomere identities. *White arrows* indicate the midbrain-hindbrain boundary and *black arrows* indicate limb bud expression (*e* eye, *np* nasal process)

spinal cord, changing its dorsoventral level and width. The more lateral stripe is more caudal, limited to r5 and r6. In adult mice, northern blot analysis detects a predominant 4.7-kb transcript in brain and stomach (data not shown).

In summary, we report the *Dmbx1* mouse homeobox gene and propose it as the founding member of the Dmbx family of PRD class genes. We infer that the gene family has an ancient origin in animal genomes, but has been secondarily lost from the *Drosophila* and *C. elegans* genomes. Paralogues of *Dmbx1* have also been lost from mammals. The broad expression in midbrain and diencephalon is compatible with a role in specification of regional territories. In contrast, the localised expression in hindbrain longitudinal stripes with distinct anterior and/or posterior boundaries suggests a more restricted developmental role in this structure, possibly in relation to specific neuronal populations as described for some other homeobox genes (Logan et al. 1998; Pattyn et al. 1997). The gene shows an intriguing temporal pattern of expression in limb development, being first restricted to forelimbs and subsequently to hindlimbs. The initial cloning of this gene from rare c-kit+ cells, and subsequent RT-PCR confirmation, is also consistent with a role in haematopoiesis.

Acknowledgements We thank C. Boorman and R. Furlong for help with phylogenetic analysis, C. Perriton and R. Freitas for assistance with in situ hybridisation, A. Miyajima for technical support on AGM excision and A. Graham for useful advice on hindbrain anatomy. This research was funded by the Japan Society for the Promotion of Science and the BBSRC.

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HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo

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Mouse long-term hematopoietic reconstituting cells exist in the c-Kit$^+$Sca-1$^+$Lin$^-$ (KSL) cell population; among them, CD34$^{low/-}$ cells represent the most highly purified population of hematopoietic stem cells in the adult bone marrow. Here, we demonstrate that retrovirus-mediated transduction of CD34$^{low/-}$c-Kit$^+$Sca-1$^+$Lin$^-$ (34$^-$KSL) cells with the *HES-1* gene, which encodes a basic helix-loop-helix transcription factor functioning downstream of the Notch receptor, and is a key molecule for the growth phase of neural stem cells in the embryo, preserves the long-term reconstituting activity of these cells in vitro. We also show that cells derived from the *HES-1*–transduced 34$^-$KSL population produce progenies characterized by negative Hoechst dye staining, which defines the side population, and by CD34$^{low/-}$ profile in the bone marrow KSL population in each recipient mouse at ratios 3.5- and 7.8-fold those produced by nontransduced 34$^-$KSL-derived competitor cells. We conclude that *HES-1* preserves the long-term reconstituting hematopoietic activity of 34$^-$KSL stem cells ex vivo. Up-regulation of HES-1 protein in the 34$^-$KSL population before unnecessary cell division, that is, without retrovirus transduction, may represent a potent approach to absolute expansion of hematopoietic stem cells. (Blood. 2003;101:1777-1783)

Introduction

Hematopoietic stem cells (HSCs) are generated during ontogeny and supply all mature hematopoietic lineages throughout life with their self-renewal and multilineage differentiation capacity.[1] Efforts have been made to expand HSCs ex vivo without loss of their original potency. Long-term reconstitution capacity of mouse and human HSCs is maintained for up to 2 to 3 weeks by coculture with certain stromal cells.[2-4] For expansion of HSCs without stromal cells, various combinations of cytokines that are active for immature hematopoietic progenitors have been surveyed.[5-9] Of interest are approaches using Notch signaling, since it has been shown to inhibit differentiation of diverse types of cells in vertebrates.[10-14] Notch signals are mediated by interactions between Notch receptors and their membrane-anchored ligands expressed in adjacent cells.[15] In the hematopoietic compartment, Notch receptors and ligands are expressed in hematopoietic progenitors and certain stromal cells, respectively.[16-19] It was recently reported that the Notch ligand Jagged-1 maintained the severe combined immunodeficiency (scid)–repopulating activity of human cord blood–derived CD34$^+$CD38$^-$ cells in vitro significantly longer than the control.[20] Further evidence implying the potential usefulness of Notch signaling in HSC expansion comes from the establishment of a line of cytokine-dependent cells which differentiate into myeloid and lymphoid lineages in vivo when transplanted into syngeneic mice, by retroviral transduction of stem cell–enriched bone marrow cells with an activated form of Notch-1.[21]

In these previous investigations, however, it was not certain whether HSC expansion was achieved without loss of the original biologic phenotype, partly because unpurified cell populations were used as the starting materials. Mouse HSCs are enriched in the c-Kit$^+$Sca-1$^+$Lin$^-$ cell population (KSL). Further enrichment, in steady-state mouse bone marrow, showed that the highest purification was obtained with the CD34$^{low/-}$ population (34$^-$KSL). In fact, a single 34$^-$KSL cell was able to repopulate all hematopoietic lineages.[22] Tracking of 34$^-$KSL, therefore, after culturing in vitro and growing in recipient mice, may provide a better answer to the issue of HSC expansion.

Here, we used retrovirus-mediated transduction of 34$^-$KSL with the *HES-1* (hairy enhancer of split-1) gene.[23] *HES-1* is known to code for a basic helix-loop-helix transcription factor functioning downstream of the Notch receptor,[24-27] and together with HES-5, is a key molecule for the growth phase of neural stem cells in the developing mouse.[28] Although it has also been suggested that HES-1 plays an important role in the development of perinatal T cells[19] and myocytes,[29] virtually no information is available about whether HES-1 plays a significant role in hematopoietic stem cell expansion. We demonstrate here that the introduction of *HES-1* into 34$^-$KSL significantly preserves the long-term reconstituting activity of these cells during culture. Moreover, the ratios of the Hoechst dye-staining–defined side population (SP)[30-32] and CD34$^{low/-}$ cells in HES-1$^+$ KSL are significantly higher than those in competitor-derived HES-1$^-$KSL in the bone marrow of each recipient mouse. Given that retroviral transduction in vitro inevitably requires cell division, which typically reduces long-term reconstituting potency,

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Submitted July 10, 2002; accepted October 4, 2002. Prepublished online as *Blood* First Edition Paper, October 24, 2002; DOI 10.1182/blood-2002-07-2051.

Supported in part by grants-in-aid from the Ministries of Education, Culture, Sports and Technology, and Health, Labour and Welfare of Japan.

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these observations indicate that HES-1 is a highly potent molecule for the ex vivo expansion of the most primitive hematopoietic stem cells.

Materials and methods

Mice

C57BL/6 (B6-Ly5.2) mice were purchased from SLC (Tokyo, Japan). Mice congenic for Ly5 locus (B6-Ly5.1) were bred and maintained at the University of Tsukuba Animal Research Center (Tsukuba, Japan).

Antibodies and cytokines

The following materials were purchased from PharMingen (San Diego, CA): both biotinylated and unmodified sets of rat IgG2b anti-lineage markers Gr-1 (RB6-8C5), B220 (RA3-6B2), CD4 (GK1.5), CD8 (53-6.7), Mac1 (M1/70), and Ter119 (TER119); fluorescence-labeled antibodies phycoerythrin (PE)–Gr-1, PE-Mac1, allophycocyanin (APC)–B220, APC-Thy1.2, PE-Ly5.1 (A20), fluorescein isothiocyanate (FITC)-Ly5.1, FITC-Ly5.2 (104), PE-Sca-1 (D7), APC-c-Kit (2B8), and FITC–antimurine CD34 (RAM34); biotinylated antimurine CD34; and peridinin chlorophyll protein (PerCP)–Cy5.5-streptavidin. Energy-coupled dye (ECD)–streptavidin was from Beckman Coulter (Fullerton, CA). All cytokines were formulated at Kirin Brewery Research Laboratory (Takasaki, Japan), except Flt3 ligand (FL), which was purchased from Genzyme (Boston, MA).

Stem cell purification

Bone marrow cells were obtained from 8- to 12-week-old mice and fetal liver cells from E14 embryo (B6-Ly5.1). Adult bone marrow– and fetal liver–derived KSL (B-KSL and L-KSL, respectively) and 34^-KSL were sorted in accordance with a previously described protocol.[22] Briefly, lineage depletion from low-density cells isolated on Histpaque (Sigma, St Louis, MO) was performed with biotinylated rat IgG2b anti–lineage markers Gr-1, B220, CD4, CD8, Mac1, and Ter119, and streptavidin-conjugated magnetic beads (Biomag Binding Streptavidin; Polysciences, Warrington, PA). These cells were stained with ECD-streptavidin, PE-Sca-1, APC-c-Kit, and FITC–antimurine CD34, and analyzed and sorted with a FACS Vantage (Becton Dickinson, Franklin Lakes, NJ). The sorted cells were used for virus infection and cultivation as described below.

Retrovirus production and infection

A cDNA fragment for mouse *HES-1*[33] was subcloned into a retrovirus vector, pMY/IRES-EGFP (a gift from T. Kitamura, IMSUT, Tokyo, Japan). The resulting pMY/HES-1-IRES-EGFP and pMY/IRES-EGFP were transfected into ψMP34 cells[34] (a gift from Wakunaga Pharmaceuticals, Hiroshima, Japan; the resulting viruses were defined as HES-1Gv and GFPv, respectively), which were single cell–sorted for enhanced green fluorescence protein (GFP) with the FACS Vantage. Clones giving the highest infection efficiency, namely 4.5×10^8/mL for NIH/3T3 in both HES-1Gv and GFPv, were used for the rest of the experiments.

Either of the above-sorted KSL or 34^-KSL was deposited into a single well of a 24-well dish coated with a fragment of RetroNectin (Takara, Shiga, Japan), at 1×10^4 to 5×10^4 per well, and cultured in a 1:2 mixture of the supernatant of the virus-producing ψMP34 clones and serum-free StemPro34 medium (Invitrogen, San Diego, CA; final serum concentration, 3.3%) containing 50 ng/mL mouse stem cell factor (SCF), 20 ng/mL mouse thrombopoietin (TPO), and 20 ng/mL human FL (for KSL) or 100 ng/mL SCF and 30 ng/mL TPO (for 34^-KSL). After 24 hours, the culture medium was removed and the same medium containing freshly prepared supernatant of ψMP34 was furnished for an additional 24 hours.

RT-PCR analysis

Total RNA was isolated using RNeasy (QIAGEN, Hilden, Germany) from 1.5×10^4 to 2.5×10^4 of GFP^+-sorted cells after culture, and used for semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR). Primer pairs were as follows: glyceraldehyde phosphate dehydrogenase (GAPDH), 5′-GCATTGTGGAAGGGCTCATG-3′ and 5′-TTGCTGTTGAAGTCGCAGGAG-3′; HES-1, 5′-CGGCATTC-CAAGCTAGAGAAGG-3′ and 5′-GGTAGGTCATGGCGTTGATCTG-3′.

Colony assay

GFP^+ KSL-derived cells were sorted at the end of the 48-hour infection period. Soon after this and after a further 3 days of culture in the presence of SCF, TPO, and FL, the cells were subjected to a colony assay using methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada).

Noncompetitive and competitive long-term reconstitution assay

For long-term reconstitution assay (LTRA) using the KSL-derived cells, Ly5.2 mice were exposed to 7.5 Gy (defined as "sublethal dose") irradiation before injection of 1000 KSL-derived GFP^+-sorted cells (Ly5.1) into the tail vein. At each time point, chimerism of GFP^+ (Ly5.1) and GFP^- (Ly5.2) cells in the blood of recipients was analyzed. For competitive LTRA using the 34^-KSL-derived cells, Ly5.2 mice were exposed to 9.5 Gy ("lethal dose") irradiation and injected with 1000 pMY/HES-1-IRES-EGFP– or pMY/IRES-EGFP–transduced 34^-KSL-derived GFP^+-sorted cells (Ly5.1) together with the same number of nontransduced 34^-KSL-derived cells (Ly5.1) that were cultured for 2 days in the same manner except for the absence of the virus. At each time point, chimerism of $GFP^+Ly5.1^+$ and GFP^- $Ly5.1^+$ cells in the blood of recipients was analyzed. Decrease of $Ly5.2^+$ (GFP^-) cells was simultaneously confirmed.

Identification of SP and $CD34^{low/-}$ cells in the recipient bone marrow KSL cells

Analysis of SP in the competitive LTRA recipient's bone marrow KSL population was performed as previously described[30,31] with Hoechst 33 342 (Sigma).

For analysis of $CD34^{low/-}$ cells in the competitive LTRA recipient's bone marrow KSL population, a staining strategy different from the usual strategy that is described above was used because GFP occupied the FITC wave length. Briefly, lineage depletion was executed by the same set of, but unmodified series of, lineage marker antibodies used for the initial cell sorting, and anti–rat IgG beads (Dynabeads M-450; Dynal, Oslo, Norway). The lineage-depleted cells were stained with PE-Sca-1 (D7), APC-c-Kit (2B8), and biotinylated antimurine CD34 plus PerCP-Cy5.5-streptavidine, after confirmation of lineage depletion with a portion of the cells.

Results

Retrovirus containing HES-1 preserves immature progenitors in bone marrow– and fetal liver–derived KSL

We placed *HES-1* cDNA in the retroviral vector pMY/IRES-EGFP, which drives expression of a cDNA of interest and of GFP as a marker from a single bicistronic mRNA (Figure 1A).[35,36] The infection efficiencies of the resulting HES-1Gv and GFPv in B-KSL and L-KSL (Figure 1B) after 48-hour culture were 20% to 75% and 40% to 90%, respectively, in the presence of SCF, TPO, and FL (Figure 1C).

Next, the sorted GFP^+ cells were subjected to the colony-forming assay before and after an additional 3-day culture in the presence of SCF, TPO, and FL. Results showed that the numbers of mature progenitor-derived colonies such as granulocyte colonies and erythroid colony-forming unit–derived colonies were similar between the HES-1gv–transduced and GFPv-transduced B-KSL–derived cells. However, the number of high-proliferative-potential-mix (HPP-mix)–derived colonies was greater in the HES-1gv–transduced than in the GFPv-transduced B-KSL–derived cells,