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Expression of AML1-d, a short human AML1 isoform, in embryonic stem cells suppresses *in vivo* tumor growth and differentiation

Revital Ben Aziz-Aloya[1], Ditsa Levanon[1], Heather Karn[1], Debora Kidron[2], Dalia Goldenberg[1], Joseph Lotem[1], Sylvie Polak-Chaklon[3] and Yoram Groner[1,4]

[1] Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot, 76100 Israel
[2] Department of Pathology, Meir Hospital, Kfar-Saba, Israel
[3] Department of Pathology, Tel Hashomer Hospital, Ramat-Gan, Israel
[4] corresponding author: Department of Molecular Genetics. The Weizmann Institute of Science, Rehovot, 76100 Israel. Tel: 972-8-9343972; e-mail: lvgroner@weizmann.weizmann.ac.il

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Abstract

The human AML1 gene encodes a heterodimeric transcription factor which plays an important role in mammalian hematopoiesis. Several alternatively spliced AML1 mRNA species were identified, some of which encode short protein products that lack the transactivation domain. When transfected into cells these short isoforms dominantly suppress transactivation mediated by the full length AML1 protein. However, their biological function remains obscure. To investigate the role of these short species in cell proliferation and differentiation we generated embryonic stem (ES) cells overexpressing one of the short isoforms, AML1-d, as well as cells expressing the full length isoforms AML1-b and AML2. The *in vitro* growth rate and differentiation of the transfected ES cells were unchanged. However, overexpression of AML1-d significantly affected the ES cells' ability to form teratocarcinomas *in vivo* in syngeneic mice, while a similar overexpression of AML1-b and AML2 had no effect on tumor formation. Histological analysis revealed that the AML1-d derived tumors were poorly differentiated and contained numerous apoptotic cells. These data highlight the pleiotropic effects of AML1 gene products and demonstrate for the first time an *in vivo* growth regulation function for the short isoform AML1-d.

Keywords: AML1 short isoform; ES cells induced teratocarcinomas; altered *in vivo* differentiation; enhanced apoptosis

Abbreviations: ES, embryonic stem; CBF, core-binding factor; LIF, leukemia inhibitory factor; TCR, t-cell receptor; MB, methoxybenzamide; EB, embryoid bodies; EC, embryonal carcinoma; TUNEL, terminal deoxynucleotidyltransferase; dUTP, nick end labeling

Introduction

The human chromosome 21 acute myeloid leukemia gene, AML1, is frequently rearranged in the leukemia associated translocations t(8;21), t(3;21) and t(12;21), generating different fused proteins (reviewed in Nucifora and Rowley, 1995). In these translocations, transcription of the fusions is mediated by upstream promoter regions that normally regulate the expression of AML1 (Ghozi *et al*, 1996). AML1 belongs to a gene family of transcription factors sharing high homology with a region of 128 amino acids (a.a.) designated 'runt domain' (RD) present in the *Drosophila* pair-rule gene *runt* (reviewed in Kagoshima *et al*, 1993; Meyers and Hiebert, 1995; Speck and Stacy, 1995; Ito, 1996). In addition to Drosophila *runt*, the family includes the human genes AML1, AML2 and AML3 and their respective mouse homologues (Speck and Stacy, 1995; Ito, 1996). AML proteins bind DNA as heterodimers formed with the β subunit designated core-binding factor β (CBFβ) (Kagoshima *et al*, 1993; Speck and Stacy, 1995). The RD contains regions that are involved in binding to the enhancer DNA sequence PyGPyGGT as well as in the interaction with CBFβ (Kagoshima *et al*, 1993; Meyers and Hiebert, 1995; Speck and Stacy, 1995). The transcriptional activation domain of the AML proteins lies downstream to the RD (Bae *et al*, 1994; Meyers *et al*, 1995a; Speck and Stacy, 1995; Tanaka *et al*, 1995a). The various chimeric AML1 proteins that are formed in t(8;21) and t(3;21) associated leukemias i.e., AML-ETO, AML-EAP, AML-EVI and AML-MDS, lack the transcriptional activation domain, but retain the ability to interact with the core DNA sequence and with CBFβ, and may thereby block the normal function of AML1 (Frank *et al*, 1995; Meyers and Hiebert, 1995; Meyers *et al*, 1995a; Nucifora and Rowley, 1995; 1995a; Ito, 1996; Zent *et al*. 1996).

AML1 mRNA is expressed at high levels in the thymus, fetal liver (day 12), and bone marrow (erythroblasts and/or myeloblasts) (Satake *et al*, 1995; Levanon *et al*, 1996) and in various hematopoietic cell lines (Bae *et al*, 1993; Tanaka *et al*, 1995; Levanon *et al*, 1994,1996). The two promoter regions of AML1 contain several potential binding sites for hematopoietic transcription factors (Ghozi *et al*, 1996). In normal blood cells, five size classes (ranging in size from 2–8 kb) of AML1 mRNAs were observed (Miyoshi *et al*, 1991, 1995; Bae *et al*, 1993; Levanon *et al*, 1994, 1996). Subsequently, several cDNA species corresponding to various AML1 mRNAs were isolated (Miyoshi *et al*, 1991, 1995; Nisson *et al*, 1992; Bae *et al*, 1993, 1994; Nucifora *et al*, 1993b; Levanon *et al*, 1994, 1996; Meyers *et al*, 1995a; Zhang *et al*, 1997). Size differences were mainly due to variations in the 5′ and 3′ untranslated regions. However, additional variability was found in the coding region due to the presence of alternatively spliced stop-codon-containing

exons. This complexity led to production of several isoforms of AML1 proteins that differed at the amino and carboxy termini. Some of them (for example AML1a in Miyoshi *et al* (1995) or AML1-d and AML1-f in Levanon *et al* (1996)) lacked the transactivation domain but retained the RD and hence, the ability to bind DNA and to interact with CBFβ (Bae *et al*, 1994; Meyers *et al*, 1995a; Tanaka *et al*, 1995a; Levanon *et al*, 1996). Significantly, these isoforms are similar to the AML1 segment in the fused protein AML1-ETO of the t(8;21) translocation and were detected in normal blood (Sacchi *et al*, 1994; Levanon *et al*, 1996). Of note, mice heterozygous for a 'knocked in' *AML1-ETO* fusion gene have a severe block in fetal liver hematopoiesis and die in midgestation from hemorrhaging in the central nervous system (Yergeau *et al*, 1997). Although apparently similar to the phenotype of 'knock out' mice lacking AML1 activity (Okuda *et al*, 1996; Wang *et al*, 1996), AML1-ETO mice-derived yolk sac cells could still differentiate *in vitro* into mature macrophages whereas yolk sac cells from AML1$-/-$ mice could not (Yergeau *et al*, 1997). This may suggest that at least *in vitro*, AML1-ETO hematopoietic stem cells retained some hematopoietic capability whereas AML$-/-$ cells have completely lost it. When the short AML1 isoforms were transfected into cells they dominantly suppressed transactivation mediated by the full length proteins (Meyers *et al*, 1995a; Tanaka *et al*, 1995a; Zent *et al*, 1996), and modulated the transfected cells' ability to differentiate (Tanaka *et al*, 1995a; Niitsu *et al*, 1997). Nevertheless, their *in vivo* biological function remained obscure. To investigate the role of these short species in cell proliferation and differentiation we generated ES cells expressing one of the short isoforms, AML1-d, as well as cells expressing the full length AML1-b and AML2. These ES-AML clones were used to investigate the effect of AML1-d on differentiation and tumorigenicity.

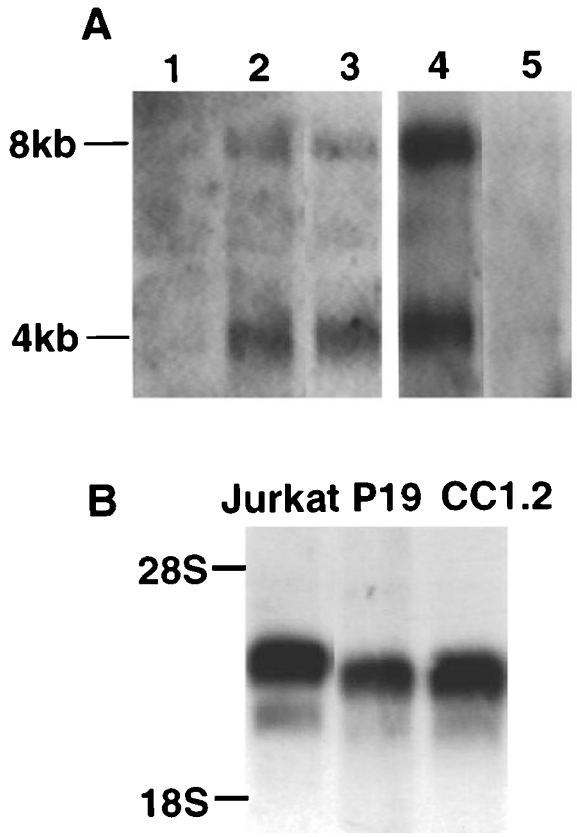

Figure 1 Expression of AML and CBFβ mRNAs in ES cells. (**A**) Expression of *AML* mRNAs in ES during differentiation. Lane 1 ES cells grown in the presence of LIF, Lanes 2 and 3, 3 and 14 days following removal of LIF, respectively. Lane 4, 5 days with RA (10^{-6}M); Lane 5, 8 days with 3-MB (2mM). Lanes 1–3 were probed with mouse *AML1* cDNA and Lanes 4 and 5 were probed with the 3′ coding and UTR regions of AML1. (**B**) Northern blot analysis of *CBFβ* expression. Poly A$^+$RNA, prepared from 150μg of RNA of Jurkat, P19 and ES-CC1.2 cells hybridized with a ^{32}P-labeled *CBFβ* probe. The size difference between mouse and human CBFβ is probably due to species differences

Results

Expression of AML cDNAs in stably transfected CC1.2 ES cells

Expression of *AML* mRNAs in non-transfected undifferentiated ES cells (line CC1.2, Evans and Kaufman, 1981) grown in the presence of leukemia inhibitory factor (LIF), was hardly detected, but became apparent when cells differentiated (Figure 1A). The two more prominent AML1 mRNA species of 8 and 4 kb (Levanon *et al*, 1994) were detected, while the 3 kb mRNA of AML1-d was not seen.

To study the biological effects of the two AML1 isoforms AML1-b and AML1-d and that of AML2, we overexpressed them in CC1.2 ES cells. Clones of ES cells in which expression of these *AML* cDNAs was driven by the PGK promoter were established (Figure 2A). *AML-1b* encodes a protein of 452 a.a. including both the RD and C-terminal transactivation domains, and is, therefore, capable of mediating DNA binding and transactivation. *AML1-d* encodes a shorter isoform of 243 a.a. which binds to the DNA but in contrast to AML1-b, can not transactivate transcription (Bae *et al*, 1994; Frank *et al*, 1995; Meyers *et al*, 1995a; Tanaka *et al*, 1995a). The third vector, encodes the 415 a.a. protein AML2 which contains the RD and transactivation domains (Figure 2A) (Levanon *et al*, 1994).

Colonies (30–40) of transfected ES cells were selected in medium containing puromycin and collected 8–10 days after electroporation. Northern blot analysis of 12 randomly selected colonies taken from several electroporation experiments, revealed that more than 50% expressed the vector derived *AML* mRNAs (Figure 2B). Of note, the higher molecular weight RNA bands seen in Figure 2B are probably unprocessed RNA species, since they did not appear in preparations of Poly A$^+$ RNA. Western blot analysis recorded the level of AML proteins in the transfected ES clones (Figure 2C). Protein bands of the expected size were detected with anti-AML1 antibodies only in the transfected cells (ES-AML), i.e., in nuclear extracts of ES-AML1-b (50 kDa, lane 2) and ES-AML1-d clones (27 kDa, lanes 3 and 4). Using immunostaining, the nuclear localization of AML1-b and AML1-d in the transfected ES clones was confirmed (Figure 2D). The much weaker fluorescent

signal seen all over the cell in control ES-puro clones was probably non specific since no AML protein was detected in undifferentiated ES cells.

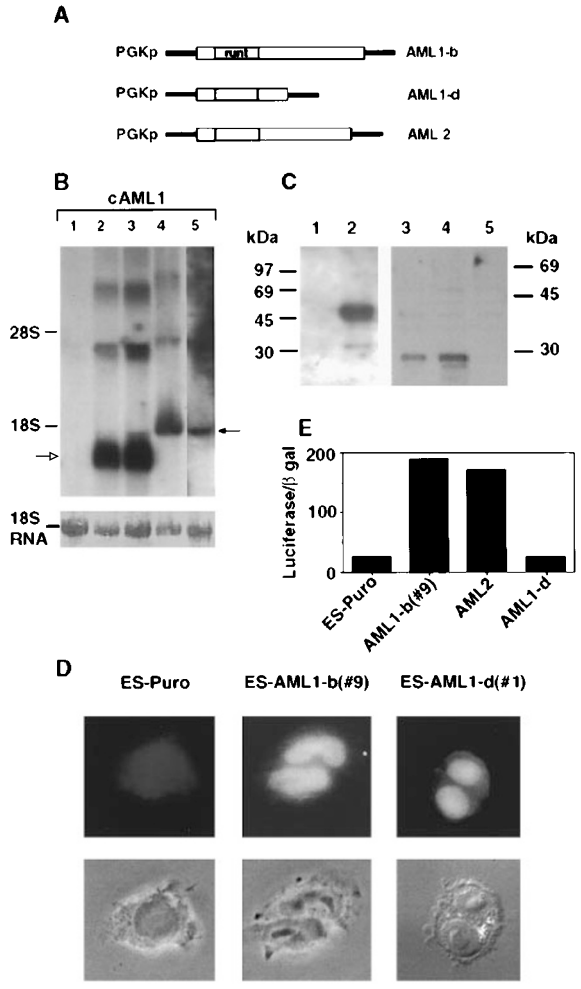

Figure 2 Expression of *AML* mRNAs and proteins in stably transfected ES cells. (**A**) Schematic drawing of the expression vectors harboring *AML* cDNAs. *AML* cDNA expression vectors were constructed by replacing the neo cDNA in a PGK-neo vector (Adra *et al*, 1987) with the *AML1-b*, *AML1-d* or *AML2* coding region (Levanon *et al*, 1996). Expression was driven by the mouse PGK promoter. The PGK poly A signal was added at the 3′ end of the construct. (**B**) Northern blot analysis of stably transfected ES clones. For each lane 20 μg of total RNA were hybridized with *AML1-b* cDNA probe. Electrophoretic migration of 28S and 18S ribosomal RNA was used as an internal size marker. Lane 1 ES-puro, Lane 2 ES-*AML1-d* clone #1; Lane 3 *ES-AML1-d* clone #9; Lane 4 ES-*AML1-b* and lane 5 ES-*AML2*. The open arrow marks *AML1-d* mRNA and the dark arrow *AML1-b* and *AML2* mRNAs. 18 S rRNA is shown as an internal control for loading of RNA per lane. (**C**) Western blot analysis of ES clones. Nuclear extracts (20 μg/lane) of ES-puro (lanes 1 and 5); AML1-b (lane 2), AML1-d clones #9 and #1, (lanes 3 and 4) were analyzed with mouse anti AML1 antibody (lanes 1 and 2) or rabbit anti AML1 antibody (lanes 3, 4 and 5). (**D**) Immunofluorescence labeling of AML1-b and AML1-d proteins in ES cells. Upper panel: Fluorescence image ES-puro, ES-AML1-b and ES-AML1-d cells were treated as described in Materials and Methods. Lower panel: Phase-contrast photomicrographs. (**E**) Transcription activation through the AML1 DNA binding site by ES-AML clones. Values (mean values of two experiments) represent luciferase activities normalized to β-gal activity

Transactivation of TCRβ enhancer-reporter gene by ES-AML clones

Mobility shift assays performed to assess the DNA binding activity of AML proteins in the ES-AML clones have shown that both AML1-b and AML1-d proteins bound to the consensus core DNA sequences (not shown). The β subunit (CBFβ) plays a major role in increasing the DNA binding activity of AML1 proteins (Speck and Stacy, 1995). CBFβ expression in ES cells was analyzed on a Northern blot, high levels of CBFβ mRNA were detected in the ES cells, comparable to the level in mouse teratocarcinoma cell line (P19) and to the human T-cell line Jurkat that were used as reference (Figure 1B). Transient transfection with TCRβ enhancer vector containing luciferase reporter was used to monitor transactivation mediated by ES-AML cells. Expression of AML1-b or AML2 resulted in an 8–9-fold increase in the levels of TCRβ-luciferase expression (Figure 2E). However, there was no effect of AML1-d expression on TCRβ enhancer activity (Figure 2E), as previously reported by others (Bae *et al*, 1994; Meyers *et al*, 1995a; Tanaka, 1995a).

Taken together, the results of nuclear localization, DNA binding and transactivation demonstrate that the ES-AML clones expressed biologically active AML proteins.

Induction of controlled differentiation programs in the ES-AML clones

When grown under regular conditions, in the presence of LIF, the ES-AML clones maintained the normal undifferentiated morphology of parental ES cells. To determine whether overexpression of AML affected the *in vitro* differentiation program of the ES clones, cultures were exposed to several known differentiation inducing conditions following withdrawal of LIF and monitored by inverted light microscopy (Doetschman *et al*, 1985; Smith, 1991; Keller, 1995). Under these differentiation-promoting conditions, the ES-AML clones formed embryoid bodies (EBs) that contained differentiated cells of the hematopoietic, endothelial, muscle and neuronal lineages. In the presence of retinoic acid (RA), ES-AML cultures consisted predominantly of large fibroblastic cells. Addition of 3-methoxybenzamide (MB) resulted in a uniform monolayer of epithelial cells. Daily microscopic analysis revealed no significant difference in the differentiation pattern between the parental CC1.2 ES, ES-puro cells and the transfected ES-AML clones. *In vitro* hematopoietic differentiation was also studied by growing the clones in methylcellulose for 14 days under hematopoietic differentiation conditions as described (Wulf *et al*, 1993). No significant difference was detected in the number of ES-AML hematopoietic colonies as compared to ES-puro. Overt hemoglobinization manifested by a red color in dark field illumination was seen in all cultures, indicating that the ES clones had undergone hematopoietic differentiation. In addition, expression of several known differentiation markers including c-*kit*, an early hematopoietic progenitor marker; c-*fms*, a macrophage marker and embryonic β *globin* (βH-1-*globin*) an erythroid marker; were detected by RT–PCR with no significant difference between ES-AML, ES-puro and parental CC1.2 colonies (not shown).

Taken together, the results of EB formation, RA and 3-MB induced differentiation and RT–PCR analyses showed no significant differences in the *in vitro* differentiation pattern of the ES-AML and ES-puro clones or CC1.2 cells.

Tumorigenicity and *in vitro* differentiation of ES-AML clones

The ability of AML proteins to affect tumor development *in vivo* was tested by subcutaneous injection of ES-AML and ES-puro clones into syngeneic SV129 mice. Under such conditions wild-type ES cells form teratocarcinomas that contain cells of all three germinal layers (Doetschman *et al*, 1985; Hilberg and Wagner, 1992). Mice injected with control ES-puro clones, as well as with ES-AML clones expressing full length AML1-b and AML2, developed tumors within 14–18 days after injection. In contrast, a dramatic decrease in the number of tumors was observed when two independently derived ES-AML1-d clones, d#9 and d#12 were injected (Figure 3A and B). After 25 days, tumors became evident also in mice injected with the ES-AML1-d clones, but these tumors were much smaller than those obtained with ES-puro or ES-AML1-b clones (Figure 3B). To further substantiate these results, two additional ES-AML1-d clones were tested: AML1-d(#1) and AML1-d(#22), expressing relatively high (#1) and low (#22) levels of AML1-d mRNA. Both showed delayed tumor formation and a smaller size tumors, similar to ES-AML1-d clones d#9 and d#12 (not shown). Using Northern blot analysis we confirmed that the *in vivo* grown tumor cells expressed the corresponding *AML* RNA at a comparable level to that of the injected ES-AML clones. No such mRNAs were detected in the ES-puro derived tumors. The significant decrease in the ability of ES-AML1-d clones to form tumors *in vivo*, led us to examine their *in vitro* growth rate. No significant difference between two ES-AML1-d and ES-puro clones was observed (Figure 4). Therefore, the differences in tumor size *in vivo* were not due to a marked growth deficiency of the ES-AML1-d clones as such. The data suggest that ectopic expression of AML1-d, but not AML1-b or AML2, severely abrogated the ability of the ES cells to form teratocarcinomas in syngeneic mice, implying a role of the short AML1 isoforms, that lack transactivation domain, in cell proliferation *in vivo*.

Histological analysis of several tumors derived from ES-puro, ES-AML1-b, ES-AML2, or AML1-d revealed marked differences in the differentiation pattern between AML1-d tumors and all the others (Figure 5). Tumors derived from ES-puro, ES-AML1-b and AML2 consisted of small nests of undifferentiated embryonal carcinoma (EC) surrounded by differentiated elements of all three germinal layers (Figure 5A and B). The degree of differentiation varied from very immature cells to mature tissue. By histological criteria

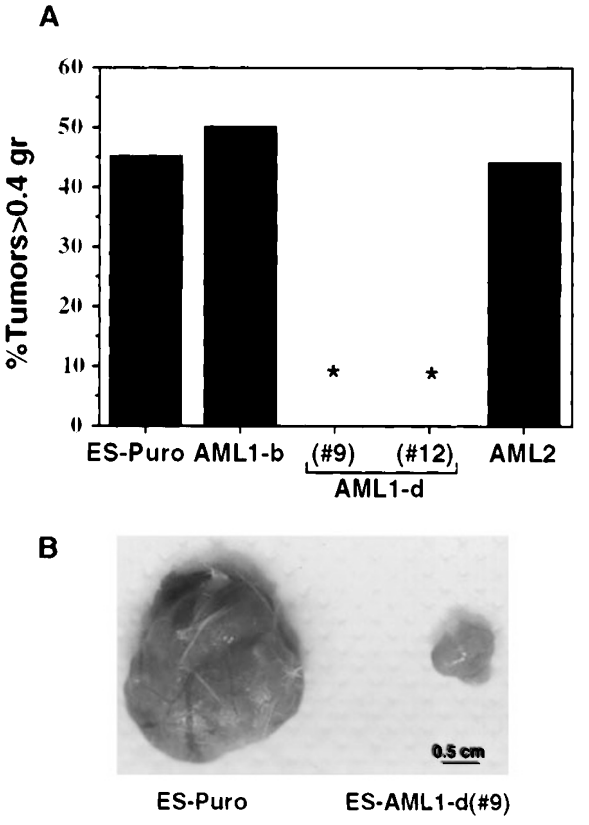

Figure 3 Suppresion of *in vivo* tumorigenicity of ES cells by overexpression of AML1-d. (**A**) Syngeneic SV129 mice (n=8–15) were injected subcutaneously with 2×10^6 ES-AML or ES-puro cells in 0.2 ml PBS. Clearly detectable tumors (i.e. >0.4 gr) were counted 25 days following injection. (*) All tumors of AML1-d clones weighed less than 0.4 gr. The presented data are of a representative experiment out of the three carried out each with a similar number of mice. (**B**) Reduced size of tumors generated by ES-AML1-d compared to ES-puro clones. Mice were sacrificed on day 25 following injection and tumors were removed. Depicted are representative tumors from mice injected with ES-puro and ES-AML1-d clone #9

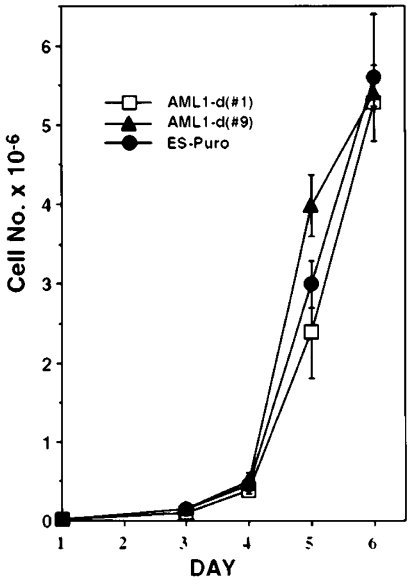

Figure 4 Similar growth rates of ES-puro and ES-AML1-d clones *in vitro*. Cells (1×10^5) were plated onto tissue culture plates (6 cm) and counted in hemocytometer daily for 5 days. Each point represents the mean (±S.D.) of duplicated culture plates

(Talerman, 1994), such tumors are classified as immature teratomas grade III. In contrast, tumors derived from ES-AML1-d clones showed wide areas (approaching 95% of the section) of homogeneously appearing undifferentiated tumor, occasionally interrupted by small foci of differentiated elements (Figure 5C and D). Tumor cells showed vesicular nuclei, with prominent eosinophilic nucleoli which were centrally located. Also noted were numerous mitotic figures, as well as pyknotic and fragmented nuclei and nuclear debris, suggestive of apoptosis (Figure 5D insert). All in all, the ES-AML1-d tumors can be classified as mixed germ cell tumor with EC and foci of immature teratoma.

The degree of apoptosis in the tumors, was evaluated by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay (Figure 6A). Sections of ES-AML1-d tumors contained almost 10 times more TUNEL positive cells than the ES-puro sections (Figure 6B). Of note, TUNEL staining in ES-AML1-d tumors was detected throughout the tumor area, whereas in the ES-puro sections apoptotic cells were closely confined to the undifferentiated regions intermixed within the differentiated area (Figure 6A). The data show that cells within the ES-AML1-d tumors undergo accelerated apoptosis, compared to cells in ES-AML1-b or ES-puro tumors. Taken together, the results of reduced tumor size, poorly differentiated tumors and enhanced apoptosis demonstrate that under *in vivo* conditions overexpression of AML1-d in ES cells caused enhancement of apoptosis which abrogated the normal growth and differentiation program of the ES cells *in vivo*.

Discussion

In the present study we found that stable overexpression of AML1-d in ES cells significantly affected their ability to form teratocarcinomas in syngeneic mice, while similar overexpression of AML1-b and AML2 had no effect on tumor formation. As noted before, the AML1-d isoform belongs to the group of AML1 proteins missing most of the C-terminal amino acids required for transactivation. Several variants of this group were identified which differ from each other in their N- or C-terminal amino acids (Nucifora *et al*, 1993b; Miyoshi *et al*, 1991; Levanon *et al*, 1996). In contrast to the effect of AML1-d on *in vivo* tumorigenicity, neither AML1-d nor the full length AML1-b or AML2 affected the *in vitro* growth rate and differentiation potential of the transfected ES cells. These data highlight the pleiotropic effects of AML1 gene products and demonstrate for the first time an *in vivo* growth regulation function for the short isoform, AML1-d. It also shows that *in vivo* the ES cells were exposed to surrounding conditions that

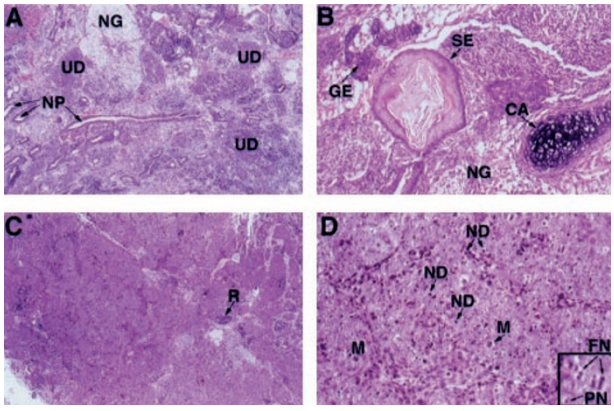

Figure 5 Histological sections of ES-AML1-d and ES-puro teratocarcinomas. ES cells (2×10^6) were injected into SV129 mice and tumors were explanted and processed as described in Materials and Methods. (**A** and **B**) ES-puro tumor: (**A**) Low magnification ($\times 40$): Tumor nodule with complex morphologic elements, predominantly showing neuro-epithelial elements (NP) and neuroglial elements (NG). Sporadic small nests of undifferentiated cells (UD) are distributed throughout the tumor. (**B**) High magnification ($\times 250$): Differentiating and differentiated tissues; squamous epithelium (SE), cartilage (CA), glandular elements (GE) and neuroglial elements (NG). (**C** and **D**) ES-AML1-d(#9) tumor: (**C**) Low magnification ($\times 40$): Confluent areas of homogeneously appearing undifferentiated tumor cells, focally interrupted by sparse neuroepithelial elements with rosette (R) formation. (**D**) High magnification ($\times 400$): undifferentiated cells with large vesicular nucleus, prominent nucleolus, indistinct cytoplasmic borders, numerous mitotic figures (M) and abundant nuclear debris (ND). insert-($\times 1000$) several pyknotic nuclei (PN) and fragmented nuclei (FN) are shown

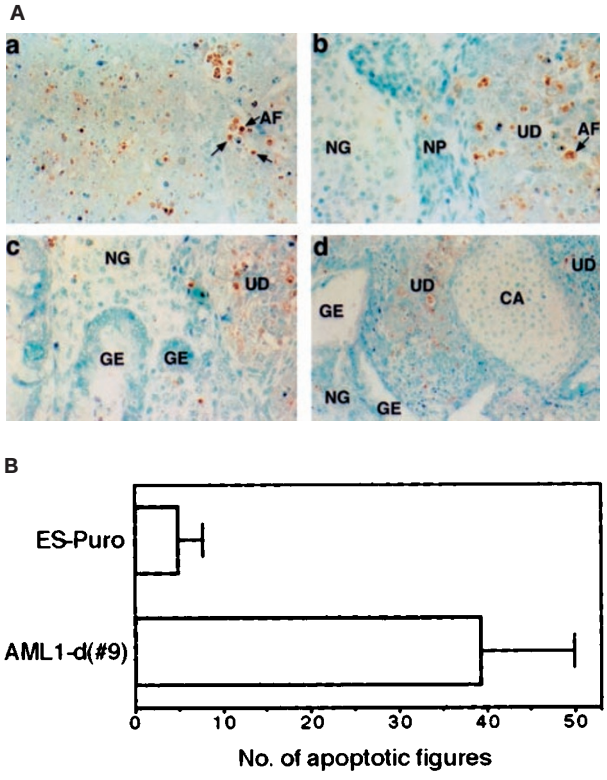

Figure 6 Enhanced apoptosis in ES-AML1-d tumors. (**A**) Histologic sections, *in situ* TUNEL method. Apoptotic cells are identified by an intense brown/red staining. (a) ES-AML-1d(#9) tumor $\times 400$, (b–d) ES-puro tumors, (b $\times 400$; c and d $\times 250$). AF, apoptotic figures; NG, neuroglial elements; NP, neuroepithelial elements; UD, undifferentiated cells; GE, glandular elements; CA, cartilage. (**B**) Quantitative analysis of apoptotic cells. Histogram of mean and standard deviation of number of TUNEL positively stained apoptotic figures per microscopic high power field (n=10) in ES-puro and AML1-d (#9) tumors

markedly differed from those present *in vitro* in cell culture. A somewhat analogous observation was previously made in ES cells lacking activity of c-*jun*. The disruption of both copies of c-*jun* had no effect on ES cell viability, growth rate or differentiation *in vitro* but injection into syngeneic mice showed a drastic reduction in tumorigenicity of the c-*jun*−/− ES cells (Hilberg and Wagner, 1992).

Several lines of evidence suggest that the human *AML1* gene plays crucial roles in hematopoiesis. *AML1* knock out homozygous mice suffer from a block in development of all definitive hematopoietic lineages leading to fetal death by E12.5 (Okuda *et al*, 1996; Wang *et al*, 1996). Consistent with this, the list of presently identified AML1 target genes includes a variety of proteins expressed in hematopoietic cells, such as cytokines, cell surface differentiation markers and myeloid specific genes (Reviewed in: Speck and Stacy, 1995). The *AML1* gene is regulated by two promoters (Ghozi *et al*, 1996) and encodes several protein isoforms that differ at the N- and C-termini (Miyoshi *et al*, 1991, 1995; Bae *et al*, 1993, 1994; Nucifora *et al*, 1993b; Sacchi *et al*, 1994; Meyers *et al*, 1995a; Levanon *et al*, 1996; Zhang *et al*, 1997). The various AML1 isoforms may regulate an array of biological events. Indeed, AML1 is involved in control of cell growth and differentiation of both hematopoietic and nonhematopoietic cells (Tanaka *et al*, 1995a; Kurokawa *et al*, 1996; Levanon *et al*, 1996; Niitsu *et al*, 1997). Reported observations indicated that overexpression of the full length AML1 protein, that contains the C-terminal transactivation domain, causes neoplastic cell transformation in NIH3T3 cells, presumably by enhancing transcription of AML1 target genes (Kurokawa *et al*, 1996). It is assumed that in these cases AML1 acts as a positive transcription factor, that binds to the consensus core DNA element PyGPyGGT and activates transcription of genes residing in the vicinity of this element. The short isoform AML1a, that did not contain the transactivation domain, did not cause transformation of NIH3T3 cells (Kurokawa *et al*, 1996). However, other studies indicated that the short isoforms can have biological consequences when overexpressed in cells. For example, the fused protein product AML1-ETO and the short AML1 isoforms can alter AML1-dependent transactivation (Reviewed in: Meyers and Hiebert, 1995; Nucifora and Rowley, 1995; Speck and Stacy, 1995; Ito, 1996) and affect differentiation of leukemia cells (Tanaka *et al*, 1995a; Niitsu *et al*, 1997), presumably by blocking the normal AML1-dependent transcription activity. Other studies showed that overexpression in Rat1A cells of chimeric AML1/MDS1 protein, that lacks transactivation domain, increased their tumorigenicity in nude mice, whereas expression of AML1/EAP prevented tumor growth (Zent *et al*, 1996). Of note, the AML1-d isoform described here terminates at a position similar to where the t(3;21) translocation disrupts the AML1 coding region to form the AML1/MDS1 and AML1/EAP fusions (Nucifora *et al*, 1993a, 1994; Sacchi *et al*, 1994). Moreover, in the AML1/EAP fusion, EAP adds merely 17 amino acids to the AML1 coding region (Nucifora *et al*, 1993a; Sacchi *et al*, 1994) thus generating an AML1-d like protein.

The mechanism by which AML1-d inhibits ES tumorigenicity *in vivo* remains unknown. Nevertheless, its ability to inhibit tumorigenicity, abrogate differentiation and increase apoptosis in the ES derived tumors, raise the possibility that the AML proteins regulate growth, differentiation and viability functions in the ES derived tumors. The increased apoptosis in the ES-AML1-d derived tumors may be related to the block in fetal hematopoietic development seen in mice heterozygous for knocked in AML1-ETO fusion (Yergeau *et al*, 1997). Of note, these mice exhibited increased cell death in the vicinity of the blood vessels in hemorrhaging areas (Yergeau *et al*, 1997). It will be interesting to determine whether overexpression of AML-ETO in ES cells results in a similar reduced tumorigenicity phenotype. The *in vivo* effects of AML1-d overexpression may be due to competition with the full length isoforms for the DNA-binding site of target genes. In fact, AML1-d can interfere with the activity of all three mammalian AML gene products due to its high affinity for the consensus DNA binding site (Meyers *et al*, 1995b). Our analysis revealed that expression of AML mRNAs is turned on during *in vitro* differentiation of ES cells (Figure 1). It is therefore conceivable that the first stages of tumor formation involve expression of the AML genes.

Alternatively, AML1-d may abrogate the *in vivo* differentiation program, promote apoptosis and inhibit tumorigenicity of ES cells by interacting with other transcription factors that participate in higher order protein-DNA complexes on AML target genes. AML proteins are known to cooperatively interact with Ets, Myb and C/EBP, through adjacent binding sites, to stimulate activity of reporter genes (Reviewed in: Speck and Stacy, 1995; Ito, 1996; Zhang *et al*, 1996). In addition, AML proteins cooperatively interact with the non DNA binding proteins CBFβ and ALY (Reviewed in: Speck and Stacy, 1995; Ito, 1996; Bruhn *et al*, 1997). Of note, the interactions with Ets, C/EBP and the CBFβ are mediated through the runt domain present in the short isoforms (Giese *et al*, 1995; Speck and Stacy, 1995; Ito, 1996; Zhang *et al*, 1996). Interestingly, a mutant of the hematopoietic transcription factor PU.1 that lacks transactivation domain, has been shown to efficiently stimulate enhancer activity in the presence of other enhancer binding proteins (Pongubala and Atchison, 1997). It is tempting to speculate that AML1-d, too, may play an architectural role in the interaction with other transcriptional factors, a process for which the transactivation domain is not needed, and thereby activate genes that block differentiation and promote apoptosis. Interestingly, overexpression of PU.1 in erythroleukemia cells inhibits growth and differentiation and induces apoptosis (Yamada *et al*, 1997). Future work on the activity of the short AML1 isoforms will hopefully allow better elucidation of their biological significance.

Materials and Methods

Maintenance and differentiation of ES cells in culture

ES cells, line CC1.2 (Evans and Kaufman, 1981), were cultured at 37°C/5% CO_2 in an enriched DMEM (DMEM-ES), supplemented with L-Alanine (8.9 mg/ml), L-Asparagine (13.2 mg/ml), L-Aspartic acid

(13.3 mg/ml), L-Proline (23 mg/ml), sodium pyruvate (110 mg/ml), 15% Fetal Calf Serum (FCS), and 0.1 mM β-mercaptoethanol. Cells were adapted to grow on gelatinized plates, in the presence of 10^3 u/ml leukemia-inhibitory factor (LIF, Gibco BRL) without feeder cells and were examined daily and passaged by trypsinization, every 2–3 days. To determine the *in vitro* growth rate, cells (5×10^4) were plated onto 6 cm tissue culture plates and were trypsinized and counted daily in a hemocytometer. For differentiation in liquid cultures, cells were generally grown in DMEM-ES medium (10% FCS) in the absence of LIF. 2×10^6 cells were grown in bacteriological plates (for suspension culture) or in gelatin-coated tissue culture plates (for adherent culture) up to 14 days with daily changes of medium. For differentiation in the presence of retinoic acid (RA, 10^{-6} M) or 3-methoxybenzamide (3-MB, 2 mM), 10^4 cells/cm^2 were seeded onto gelatin-coated tissue culture plates in DMEM-ES medium (10% FCS) supplemented with RA and 10 U/ml LIF, or with 3-MB and 100 U/ml LIF as detailed in Smith (1991), and therafter examined daily under the microscope. For differentiation in methyl cellulose we modified the method described in Wulf *et al*. (1993). Exponentially growing ES cells were adapted to grow in IMDM (Gibco BRL+15% FCS). Cells (1.5×10^3) were cultured in 35 mm bacteriological plates (Falcon) in a final volume of 1.5 ml of 0.8% methyl cellulose (Fluka) in IMDM, supplemented with 10 mg/ml Insulin (Sigma), freshly prepared 4.5×10^{-4} M monothioglycerol (Sigma), 0.45 mg/ml iron saturated transferrin (Boehringer Mannheim), 2 U/ml erythropoietin (EPO) and 50 mg/ml ascorbic acid.

Transfection of ES cells, Northern blotting and RT–PCR

PGK-AML expression vectors were constructed by replacing the *neo* cDNA cassette in the PGK-neo vector (following digestion by *Pst*I) (Adra *et al*, 1987) with *AML* cDNAs. To generate PGK-AML1-d, the 1.2 kb, *Bsa*I-*Pst*I fragment containing the *AML1-d* coding sequence, was used (Levanon *et al*, 1996, Figure 1). PGK-AML1-b was constructed using the 1.7 kb *Bsa*I-*Xho*I fragment of *AML-1b* cDNA (Levanson *et al*, 1996, Figure 1). For construction of PGK-AML2, a 1.8 kb *Eco*RI fragment was isolated from the *AML2* cDNA (Levanon *et al*, 1994, Figure 1). The PGK-Puro plasmid encoding the mouse puromycin resistance gene under the regulation of PGK promoter was a gift from Alan Bradley (University of Cambridge, Cambridge, UK).

AML vectors were cotransfected with PGK-*Puro* (at a 10 : 1 molar ratio, total of 25 μg DNA) into 1×10^7 ES cells by electroporation (Bio-Rad gene pulser, 250 V, 500 mF, 10 ms time constant). Two days following transfection, growth medium was replaced with fresh medium containing puromycin (3 μg/ml, Sigma). After 7–10 days, single puromycin-resistant colonies were picked, expanded and analyzed for *AML* RNA expression by Northern blotting using 20 μg total RNA as described (Levanon *et al*, 1996). For RT–PCR analysis, total RNA was isolated from developing EBs using the RNAzol method (Cinna/Biotec Laboratories, Inc.). Random primed cDNA was prepared from 1–2 μg of total RNA, using MMLV reverse transcriptase (Gibco BRL) in a 20 μl volume. 2–10 μl cDNA was used for PCR amplification. PCR conditions for *HPRT*, *vav*, *bH1* and KL were as described by Keller *et al* (1993) for c-*kit* (5′: 1750–1774, and 3′: 2018–2042), (Qiu *et al*, 1988) and c-*fms* (5′: 1441–1465 and 3′: 1865–1889) (Rothwell and Rohrschneider, 1987), all used at an anealing temperature of 50°C.

Protein Western blotting and DNA binding analysis

For whole cell protein extracts, cells were solubilized in RIPA lysis buffer (10 mM Tris HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate (DOC) and 5 mM EDTA), supplemented with protease inhibitors (0.1 mg/ml leupeptin), 1.5 mg/ml pepstatin, 4 mg/ml aprotinin, 2 mg/ml chymostatin, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate).

For nuclear extracts, confluent cultures were grown for 6 h in the presence of 50 μg/ml of calpain inhibitor I (Boehringer Mannheim). Cell pellets were washed and resuspended in hypotonic buffer (25 mM Tris pH 7.4, 2 mM $MgCl_2$, 0.3% Triton X-100), kept for 5 min on ice, and spun down (400 *g*, 8 min, at 4°C). The pelleted nuclei were resuspended in ice cold lysis buffer (50 mM Tris pH 8, 120 mM NaCl, 0.5% NP-40, 0.05% SDS) with the above mentioned protease inhibitors, and including 5 μg/ml of calpain inhibitor I. Following 20 min incubation on ice, tubes were centrifuged for 10 min and the supernatant was used for Western blot analysis. Whole cell or nuclear extract proteins (20 μg) were subjected to SDS-gel electrophoresis, blotted onto nitrocellulose membranes and incubated with anti-AML1 antibody. The antibody complex was visualized by incubation with ^{125}I-labeled protein A and exposure to an X-ray film. For electrophoretic mobility shift assay (EMSA), nuclear extracts from the indicated stably transfected ES clones were prepared as described (Haviv *et al*, 1995). A double stranded oligonucleotide probe, containing a high-affinity AML binding site, was prepared according to Furukawa *et al* (1990). Binding assays were performed on ice for 30 min under the conditions described in Bae *et al* (1994).

For assessment of transactivation by AML proteins in stably transfected ES-AML clones, a reporter construct was used that contained a 550 bp fragment of the mouse TCRβ enhancer, encompassing three AML binding sites (Krimpenfort *et al*, 1988) linked to SV40 promoter-luciferase coding region. The reporter gene (2 μg) was cotransfected with β-gal plasmid (0.2 μg) (Eustice *et al*, 1991) into 3×10^5 ES cells (in 35 mm tissue culture plates, 60–80% confluent), using lipofectamine (Gibco-BRL). The ratio between luciferase activity in the ES-AML clone and in the ES-puro control clone was used as an indicator of AML-specific transactivation.

AML antibodies and immuno fluorescence staining

For preparation of anti AML1 antibody, the C-terminal coding region of *AML1-b*, downstream from the RD, (885 bp *Sma*I fragment encoding a.a. 190-452), was cloned into a pRSET-C vector (*in vitro* gene, Netherlands). Recombinant protein was produced and utilized for production of polyclonal antibodies in rabbits and mice. For immuno staining, ES cells were seeded onto tissue culture plates containing gelatinized cover slips. One day later, cells were rinsed in PBS, fixed in 3% (v/v) N. Paraformaldehyde in PBS for 20 min at 37°C, and permeabilized with 0.25% Triton X-100, for 5 min, at room temperature (RT). Blocking of non specific binding was conducted with 5% (v/v) normal goat serum (Gibco BRL) and 1 mg/ml of bovine serum albumin (Sigma, USA) for 1 h at RT. Cells were then incubated with the primary antisera (1 : 100 in a blocking solution) for 1 h at RT, rinsed three times in PBS and incubated with Lissamine Rhodamine (LRSC)-conjugated goat anti mouse IgG, or goat anti rabbit IgG (Jackson Laboratories) for 1 h at RT. Following washing in PBS, coverslips were mounted on microscope slides with 50% glycerol in PBS (w/w), and cells were visualized and photographed with a fluorescence microscope.

Tumorigenicity assay and *in situ* detection of apoptosis

SV129 mice (Jackson Laboratories) (5–6 weeks old males) were injected subcutaneously with 2×10^6 cells in 0.2 ml PBS. Mice were examined daily for tumor formation. Tumors were excised at different

times after cell inoculation, fixed in 4% buffered formalin for 24 h and entirely submitted for histological processing. Four μm thick sections were prepared and stained with Hematoxylin and Eosin (H&E). The degree of apoptosis was determined by the TUNEL (*T*erminal deoxynucleotidyltransferase-mediated d*U*TP *N*ick *E*nd *L*abeling) procedure (Gavrielli *et al*, 1992). The ApoTag *in situ* apoptosis detection kit (Oncor) was used according to manufacturer's instructions. The degree of apoptosis was quantified by counting the number of positively stained cells per high power field (HPF) in 10 HPFs.

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