Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia

Masakazu Higuchi,¹ Darin O'Brien,¹ Parasakthy Kumaravelu,¹ Noel Lenny,¹ Eng-Juh Yeoh,^{1,2} and James R. Downing^{1,3,4}

¹Department of Pathology ²Department of Hematology and Oncology ³Department of Tumor Cell Biology St. Jude Children's Research Hospital, Memphis, Tennessee 38105 ⁴Correspondence: jim.downing@stjude.org

Summary

The AML1/CBFβ transcription factor complex, a frequent target of chromosomal translocations in leukemia, is essential for the generation of definitive hematopoietic stem cells. Paradoxically, expression of the acute myeloid leukemia-associated AML1-ETO fusion protein in mice results not in leukemia, but in embryonic lethality due to an absence of normal hematopoiesis. To bypass the embryonic lethality, we generated a mouse strain with a conditional *AML1-ETO* knockin allele that contains a *loxP* bracketed transcriptional stop cassette 5' to the *AML1-ETO* fusion site. Activation of this allele in vivo by Cre-mediated recombination resulted in an enhanced replating efficiency of myeloid progenitors, but it did not block their differentiation, nor was it sufficient to induce leukemia. However, induction of cooperating mutations resulted in the development of an acute myeloid disease that mimicked many of the features of human AML1-ETO-expressing leukemia.

Introduction

The core binding factors are a small family of heterodimeric transcription factors that control critical cell fate decisions in a number of different cell lineages (Downing, 1999; Speck et al., 1999; Downing et al., 2000). This family is comprised of one of three different DNA binding α subunits (Runx1–3) with homology to the Drosophila pair rule gene runt, and a single common β subunit (CBFβ). The heterodimeric complex composed of Runx1 (also called AML1, CBFA2, and PEBP2 α B; referred to as AML1 for the remainder of this article) and CBFB is essential for the formation of the entire definitive hematopoietic system (Okuda et al., 1996; Wang et al., 1996a, 1996b; Sasaki et al., 1996). Loss of the genes encoding either subunit results in an embryonic lethal phenotype at the midpoint of embryologic development from a complete absence of definitive hematopoietic stem cells. Thus, AML1/ CBF_B appears to function as a master regulatory switch that establishes a transcriptional cascade that controls critical cell fate decisions necessary for the development of definitive hematopoietic stem cells. This function is mediated through the core enhancer DNA sequence present in the promoters and enhancers of a large number of hematopoietic specific genes. AML1 binds this sequence through its central runt homology domain (RHD), and its DNA binding affinity is increased by heterodimerizing through this domain with CBF β (Wang et al., 1993; Ogawa et al., 1993).

A surprising finding that has emerged from the study of AML1/CBF β has been that the genes encoding this transcription factor complex are some of the most frequent targets of genetic alterations in human acute leukemias (Downing, 1999; Speck et al., 1999; Downing et al., 2000; Friedman, 1999). This complex is altered by chromosomal rearrangements in up to 30% of human acute leukemias, with the AML1 subunit being targeted by the t(8;21), t(3;21), t(16;21), and a variety of other rarer translocations in acute and chronic myeloid leukemias, and by the t(12;21) in pediatric acute lymphoblastic leukemia. Similarly, the CBF β subunit is altered by the inv(16) and t(16;16), found primarily in the acute myeloid leukemia (AML) subtype, M4Eo (Liu et al., 1993).

SIG NIFIC A N C E

One of the more prevalent subtypes of human acute myeloid leukemia is the so-called "core binding factor leukemias," which are characterized by alterations of the genes encoding the AML1/CBF β transcription factor complex. Here we report a murine model of this leukemia subtype. Key to the development of this model was the use of a conditional AML1-ETO knockin allele that mimicked the human t(8;21) translocation. This murine model will serve as a powerful tool to identify the spectrum of mutations that cooperate with AML1-ETO in leukemogenesis, and to assess novel therapies that target either AML1-ETO or components of its downstream signaling pathway. In addition, the experimental strategy employed here should have broad applicability in the generation of other animal models of human malignancies. Through the use of a conditional transcriptional stop cassette, the expression of dominantly acting oncogenes or tumor suppressors can be regulated in both a temporal and lineage-specific manner.



Figure 1. Generation of mice with a conditional AML1-ETO knockin allele

A: Schematic of AML1 cDNA, partial murine AML1 genomic locus, replacement targeting vector, the targeted AML1-ETO-stop knockin allele generated by homologous integration of the vector, and the activated allele with a deleted stop cassette following Cre-mediated recombination. AML1 exons 3 and 4 are indicated by solid boxes. The stop cassette bracketed by loxP recombination sequences, partial human AML1-ETO cDNA, polyadenylation signal (poly A), positive selection neomycin resistance cassette (neo'), negative selection diptheria toxin-A cassette (DT-A), and hybridization probe (5'-probe) are shown. B: Schematic showing the approach used to test for Cre-mediated activation of the AML1-ETO-stop allele. C: Western blot analysis of AML1-ETO. Cells analyzed include fetal liver cells from AML1-ETO heterozygous embryos (lane 1), bone marrow cells from wild-type mice (lane 2), and bone marrow cells from AML1-ETO-stop mice either infected with an empty retrovirus (lane 3) or with the MSCV-Cre-IRES-GFP retrovirus (lane 4).

Each of these chromosomal rearrangements results in the formation of AML1 or CBF β fusion proteins that retain domains necessary for either DNA binding and/or heterodimerization with the wild-type partner protein. Functionally, these fusion proteins appear to act in a dominant negative fashion to inhibit the normal transcriptional activity of AML1/CBF β (Downing, 1999; Speck et al., 1999; Downing et al., 2000). In addition, point mutations within the RHD of *AML1* have been identified as the underlying cause of a familial platelet disorder with a predisposition to AML, and as somatic events in rare cases of de novo AML and myelodysplastic syndrome (Song et al., 1999; Osato et al., 2000; Preudhomme et al., 2000; Imai et al., 2000).

To investigate the mechanistic role of the t(8;21)-encoded AML1-ETO fusion protein in leukemogenesis, we and others used gene targeting to create mice with an *AML1-ETO* knockin allele that mimics the t(8;21) (Yergeau et al., 1997; Okuda et al., 1998). Embryos heterozygous for *AML1-ETO*, like AML1-deficient mice, died around E13.5 from a complete absence of normal fetal liver-derived definitive hematopoiesis. However, in contrast to AML1-deficient embryos, fetal livers from *AML1-ETO*/+ embryos contained either abnormal monocytes (Yergeau et al.,

1997) or dysplastic multilineage hematopoietic progenitors that had an abnormally high self-renewal capacity in vitro (Okuda et al., 1998). Thus, AML1-ETO not only neutralized the normal biologic activity of AML1, but also directly induced aberrant hematopoietic cell proliferation. Importantly, however, AML1-ETOexpressing cells failed to expand within the developing embryos, or to induce leukemia when transplanted into syngeneic or immunocompromised recipients (Yergeau et al., 1997; Okuda et al., 1998). This latter result suggests that AML1-ETO alone is not sufficient to induce leukemia.

The embryonic lethal phenotype that results from the expression of AML1-ETO during murine development precludes the use of this system to explore the mechanism through which AML1-ETO contributes to leukemogenesis. To circumvent this hurdle, we have now generated a murine line with a conditional *AML1-ETO* knockin allele, in which a strong transcriptional stop cassette bracketed by *loxP* sites was placed 5' to the *AML1-ETO* fusion site. While this allele is transcriptionally silent in its intact state, it can be activated following birth through Cre-mediated deletion of the transcriptional stop cassette. Activation results in *AML1-ETO* expression driven off of the endogenous *AML1* regulatory sequences, thus closely mimicking the molecular consequences of the t(8;21) translocation. Using these mice, we now demonstrate that expression of AML1-ETO in adult mice does not block the ability of hematopoietic cells to differentiate, but instead induces an enhanced in vitro replating efficiency of myeloid progenitors. Moreover, AML1-ETO alone was shown to be insufficient to induce leukemia, but following the acquisition of cooperating mutations, efficiently led to the development of an acute myeloid neoplasm that mimicked many of the features of human AML1-ETO-expressing leukemia. These results dramatically demonstrate the value of using a conditional knockin strategy to create a mouse model of human cancer induced by an oncogenic fusion protein.

Results

Generation of a conditional AML1-ETO knockin allele

To circumvent the embryonic lethality that results from expression of AML1-ETO, we generated mice with a conditional AML1-ETO knockin allele. This allele was constructed by fusing human AML1-ETO sequences in frame to murine AML1 exon 4 and inserting a loxP bracketed transcriptional stop cassette (Lakso et al., 1992) 5' to the AML1-ETO fusion in intron 3 (Figure 1A). This cassette results in the efficient termination of transcripts within its sequence, thus preventing the generation of full-length AML1-ETO transcripts. While this allele is transcriptionally silent in the germline, it can be activated following birth through Cre-mediated deletion of the transcriptional stop cassette. Activation results in AML1-ETO expression driven off of the endogenous AML1 regulatory sequences, and thus allows the activated allele to retain the temporal, lineage specific, and quantitative transcriptional control afforded by AML1 transcriptional regulatory sequences. Therefore, this approach should provide a significant advantage over the alternative approach of expressing the AML1-ETO oncoprotein from a transgene containing a heterologous lineage-specific promoter. Targeted ES cells (AML1-ETOstop/+) with normal ploidy were obtained and used to achieve germline transmission of the allele.

In contrast to the embryonic lethal phenotype seen with mice heterozygous for *AML1-ETO*, *AML1-ETO-stop/+* heterozygous mice were viable and were born at a normal Mendelian ratio. Moreover, RT-PCR analysis demonstrated the absence of appropriately spliced *AML1-ETO* transcripts from *AML1* exon 3 through *ETO* sequences, and Western blot analysis failed to detect any AML1-ETO fusion proteins using antibodies specific for the N terminus of AML1 or the C terminus of ETO (data not shown and Figure 1C). Thus, the strategy used effectively blocked expression of the *AML1-ETO* allele during murine development.

To test our ability to activate the *AML1-ETO-stop* allele, we harvested bone marrow (BM) cells from 5-flourouracil treated adult *AML1-ETO-stop/+* mice and infected these cells in vitro with an MSCV-based retrovirus (Persons et al., 1997) that expressed both the Cre recombinase and the green fluorescence protein (GFP) (Figure 1B). Infected hematopoietic cells were flow sorted for GFP positivity and analyzed by Western blot analysis for evidence of expression of AML1-ETO. As expected, infection with the Cre-expressing retrovirus resulted in expression of the appropriately sized AML1-ETO chimeric protein (Figure 1C). Southern blot analysis demonstrated that the floxed stop cassette had been deleted, and sequence analysis of transcripts expressed from this allele demonstrated appropriate splicing be-

tween murine *AML1* exon 3 and the fused murine exon 4-human *AML1-ETO* sequences (data not shown). Taken together, these data suggest that the conditional *AML1-ETO* allele can be efficiently activated through Cre-mediated deletion of the transcriptional stop cassette.

In vivo activation of the conditional *AML1-ETO* allele alters the growth properties of hematopoietic progenitors

To activate the conditional *AML1-ETO* allele in vivo, we crossed the *AML1-ETO-stop/+* mice with a murine line transgenic for a *Cre* recombinase gene driven off of the interferon (IFN)- α/β -inducible *Mx1* promoter (Kuhn et al., 1995). This promoter can be activated in vivo by treatment of mice with either IFN- α/β or the synthetic double-stranded RNA, polyinosinic-polycytidylic acid (pl-pC), which activates the expression of endogenous IFN. Treatment with either agent results in the expression of Cre in cells that contain the receptor for IFN- α/β . Although this results in deletion of the transcriptional stop cassette in a broad range of hematopoietic and nonhematopoietic cells (Kuhn et al., 1995), expression of the activated *AML1-ETO-stop* allele is restricted to cells that normally express *AML1*, thus providing lineage specificity.

As shown in Figure 2, BM cells from pl-pC treated single transgenic *AML1-ETO-stop/+*, and *Mx1-Cre/+* mice, and from untreated double transgenic *AML1-ETO-stop/+ Mx1-Cre^{+/-}* mice showed no significant evidence of deletion of the stop cassette (Figure 2A), and failed to express the AML1-ETO chimeric protein (Figure 2B). By contrast, treatment of *AML1-ETO-stop/+ Mx1-Cre^{+/-}* double transgenic mice with three intraperitoneal injections of pl-pC over a six-day period resulted in the rapid deletion of the transcriptional stop cassette and the efficient expression of AML1-ETO (Figures 2A and 2B).

To assess the in vivo efficiency of Cre-mediated activation of the conditional allele, we measured the level of deletion of the stop cassette by Southern blot analysis in BM cells as a function of time following pI-pC treatment. One week after the last pI-pC injection, 35% of BM cells had undergone deletion (Figure 2C). By two weeks, this percentage increased to greater than 85% and was sustained at this level throughout the life of the animal. Moreover, AML1-ETO expression was easily detected by Western blot analysis and its level of expression directly correlated to the percentage of cells containing a deleted allele (Figure 2B and data not shown). Since Cre expression is maintained for at most 2-3 days following pI-pC injections, the observed increase in AML1-ETO-expressing BM cells over time suggests that these cells may have an in vivo selective growth advantage. The persistent expression of AML1-ETO throughout the life of the mice suggests that Cre-mediated deletion of the stop cassette may be occurring in long-term repopulating hematopoietic progenitors (see below).

Interestingly, following pI-pC injection, 90% of BM and 49% of spleen cells contained an *AML1-ETO-stop* allele with a deleted stop cassette, whereas thymocytes contained only background levels of this activated allele (Figure 2D). Moreover, high levels of *AML1-ETO* mRNA were detected in total BM cells and flow sorted splenic B cells, whereas only trace levels of transcript were detected in isolated T cells, despite the fact that T cells express a high level of wild-type *AML1* (data not shown). Since previous studies have demonstrated that the *Mx1* promoter results in the expression of a functional Cre protein in developing and mature



Figure 2. Cre-mediated recombination leads to efficient in vivo activation of the conditional AML1-ETO knockin allele

A: Southern blot analysis of bone marrow cells from single transgenic AML1-ETO-stop/+, Mx1-Cre^{+/-}, or double transgenic AML1-ETO-stop/+ Mx1-Cre^{+/-} mice either untreated or injected with pl-pC to induce Cre-expression. Deletion of the stop cassette (9.0 kb deleted allele) is only seen in cells from the double transgenic mice following pl-pC injection. B: Western blot analysis of AML1-ETO. C: Southern blot showing a time course in weeks (w) for Cre-mediated deletion following pl-pC injection. The percentage of AML1-ETO-stop (knockin allele) and deleted allele are indicated. D: Southern blot showing the percentage of activated (deleted) allele in different organs from induced and uninduced double transgenic AML1-ETO-stop/+ Mx1-Cre^{+/-} mice.

T cells (Kuhn et al., 1995), these data raise the possibility that expression of AML1-ETO may selectively compromise T cells' survival.

Previous studies have demonstrated that expression of AML-ETO can induce an increase in the self-renewal capacity of hematopoietic progenitors (Okuda et al., 1998). To determine if the level of AML1-ETO expressed from this in vivo activated allele was sufficient to induce this phenotype, BM cells were plated in methylcellulose-containing media in the presence of cytokines, and colonies were counted following seven days of growth. The population of colonies were then pooled, washed, and 1×10^4 cells replated in methylcellulose, and this process was repeated reiteratively every seven days. As shown in Figure 3A, following activation with the *Mx1-Cre* transgene, the AML1-ETO-expressing cells were able to form myeloid colonies long after non-AML1-ETO-expressing cells stopped growing. Importantly, the AML1-ETO-expressing cells were absolutely dependent on the presence of hematopoietic growth factors for both proliferation and survival (data not shown). The majority of the colonies detected in the methylcellulose cultures were granulocyte-macrophage, with only rare erythroid and mixed lineage colonies identified in late passage cultures. In addition, terminally differentiated myeloid cells were present in the cultures, suggesting that AML1-ETO alters the self-renewal capacity of cells without significantly affecting their ability to differentiate.

The identification of mixed lineage colonies in the long-term cultures suggests that Cre-mediated recombination is occurring not only in committed myeloid cells, but also in some immature multi-lineage progenitor. In support of this notion, the expression of the AML1-ETO chimeric transcript was detected by RT-PCR analysis in 25/26 individual multipotential mixed (myeloid, monocytic, erythroid, and megakaryocytic) colonies generated from BM cells obtained from double transgenic mice 4-12 weeks following pl-pC treatment. In addition, AML1-ETO expression was detected in 22/23 multipotential day 12 spleen colony-forming units (CFU-S₁₂) from pI-pC treated double transgenic mice (data not shown). Moreover, when BM cells from pI-pC treated double transgenic mice were transplanted into lethally irradiated recipients, AML1-ETO-expressing cells were detected in both myeloid and B cells of the recipients 6 months following transplantation (data not shown). Importantly, no evidence of leukemia developed in the mice transplanted with these AML1-ETO-expressing BM cells. These data are consistent with Cre-mediated activation of the conditional AML1-ETO allele occurring in multi-lineage hematopoietic progenitors, and possibly long-term repopulating hematopoietic stem cells.

To quantify the effect of AML1-ETO on the replating capacity of hematopoietic progenitors on a clonal basis, individual mixed colonies were harvested from BM cultures derived from wild-type mice (10 colonies), pl-pC treated double transgenic AML1-ETOstop/+ Mx1- $Cre^{+/-}$ mice (10 colonies), and AML1-ETO-expressing fetal liver cultures obtained from mice containing an traditional AML1-ETO knock-in allele (20 colonies, Okuda et al., 1998). As shown in Figure 3B, individual mixed colonies from wild-type cells rapidly lost their in vitro replating capacity, with no colonies observed following the third replating. By contrast, individual AML1-ETO-expressing BM and fetal liver mixed colonies gave rise to long-term colony forming cells at very high efficiency



Figure 3. Expression of AML1-ETO increases the in vitro replating efficiency of adult bone marrow cells

A: BM cells were plated in methylcellulose-containing media in the presence of cytokines and colonies were counted following 1–2 weeks of growth. The total population of colonies was then pooled, washed, and 1×10^4 cells replated in methylcellulose, and this process was repeated reiteratively every seven days. The number of colonies/ 1×10^4 cells as a function of the total days in culture is shown. **B**: Individual mixed colonies), pl-pC treated double transgenic AML1-ETO-stop/+ Mx1-Cre^{+/-} mice (10 colonies), and from AML1-ETO-expressing fetal liver cultures obtained from mice containing an traditional AML1-ETO knockin allele (20 colonies). Colonies obtained following the initial growth of the individual mixed colonies were then serially passed in culture as outlined above. The number of original colonies that were capable of generating new colonies during each in vitro passage is indicated.

(~50%). Moreover, cytokine-dependent myelomonocytic cell lines could be readily established from these cultures (data not shown). Although these cultures were initiated from individual mixed lineage colonies, the majority of colonies in each replated culture were myeloid/monocytic in nature, with only rare erythroid and mixed colonies observed. No change in the type of colonies was observed following the initial replating, and no blast colonies were detected, suggesting that the serial growth was derived from more committed progenitors. Thus, the in vivo activation of the Mx1-Cre transgene results in an increase in the replating capacity of myeloid progenitors such that a high percentage of these cells are able to bypass culture-induced senescence and establish permanent cell lines. Injection of the AML1-ETO BMderived cell lines into SCID or sublethally irradiated syngeneic mice failed to lead to the development of leukemia (data not shown).

Expression of AML1-ETO in vivo induces minimal hematopoietic abnormalities in adult mice

Although AML1-ETO was expressed in a high percentage of hematopoietic cells and altered their in vitro growth characteristics, only minimal alterations were observed in vivo in the hematopoietic systems of these mice. No significant abnormalities were seen in their hematocrits, nucleated blood cell counts, white blood cell or red cell differentiation, or platelet counts (negative data not shown). Moreover, no histological abnormalities were observed in any of the hematopoietic organs. However, when the number of hematopoietic colony forming cells was assessed, AML1-ETO expression appeared to induce a slight increase in the number of granulocyte-monocyte (GM), mixed, and total colonies (Table 1). Similarly, a slight increase was found in the number of CFU-S₁₂ in BM cells from AML1-ETO-expressing mice as compared to uninduced double transgenic *AML1-ETO-stop/+ Mx1-Cre^{+/-}* mice (22.0 ± 3.8 colonies/1 × 10⁵ cells versus 15.6 ± 4.7 colonies/1 × 10⁵ cells, respectively). These data dramatically demonstrate that expression of AML1-ETO fails to significantly impair the differentiation of hematopoietic progenitors.

Importantly, although AML1-ETO was expressed in a high percentage of hematopoietic cells including immature multipotential progenitors, no leukemia or other hematopoietic neoplasms developed during the first 11 months of life in a cohort of 20 AML1-ETO-expressing mice. However, by one year of age, two mice in this group developed hematopoietic neoplasms: one with a T cell lymphoma, and one with an undifferentiated lymphoma (Group 3, Table 2). Each tumor expressed the AML1-ETO protein (data not shown). Taken together, these data suggest that expression of AML1-ETO in adult hematopoietic cells results in a slight increase in the number of multipotential progenitors, but does not significantly alter the in vivo growth of these cells or their ability to terminally differentiate. Moreover, expression of AML1-ETO is insufficient by itself to induce leukemia.

Secondary mutations can cooperate with AML1-ETO to induce myeloid leukemia

The rare occurrence of hematopoietic neoplasms after a long latency in the AML1-ETO-expressing mice suggests that additional genetic event(s) are required to induce the full leukemic transformation of hematopoietic progenitors. To directly test this hypothesis, 5- to 10-week-old AML1-ETO-stop/+ Mx1-Cre^{+/-} mice were treated with pl-pC to induce expression of AML1-ETO, and after two weeks these mice were treated with a single mutagenic dose of the DNA alkylating agent, N-ethyl-N-nitrosourea (ENU, 50 mg/kg). Although this dose of ENU efficiently induces single base mutations (Breuer et al., 1991), only 2 hematopoietic neoplasms, a thymic-derived T cell lymphoblastic lymphoma and a histiocytic lymphoma, developed in the ENU treated control groups that lacked AML1-ETO expression (Table 2). By contrast, 47% (17/36) of the AML1-ETO-expressing mice developed hematopoietic neoplasms 2-10 months after treatment (Table 2). Two distinct types of malignancies were identified: granulocytic sarcoma/acute myeloid leukemia that was highly reminiscent of aspects of the disease seen in patients with t(8;21)-containing AML (11/36, 31%), and thymic derived T cell lymphoblastic lymphomas (6/36, 17%). Of the latter tumors, 4/5 analyzed cases failed to express AML1-ETO by Western blot analysis, suggesting that these are likely to represent ENU-induced thymic lymphomas that may not be a direct result of AML1-ETO expression. The T cell tumors will be discussed below.

The granulocytic sarcomas consisted of solid masses of proliferating myeloblasts arising within the retropertinuem, soft tissue, or bones of the sternum, cranium, or extremities and extending into the adjacent soft tissue (Figures 4A–4H). These tumors were composed of sheets of myeloblasts showing variable expression of myeloperoxidase, admixed with a few maturing myeloid cells. The tumor cells were negative for T cell markers and expressed variable levels of CD34, c-Kit, Sca-1, Mac-1, Gr-1, TER119 and B220 (data not shown). Although distinct tumor masses were evident in mice at presentation, death was rapid and necropsy revealed widespread dissemination of the blasts

Table 1. Bone marrow hematopoietic progenitors										
Genotype			GM	G	м	CFU-E	BFU-E	Meg	Mixed	Total
AML1-ETO-stop	Mx1-Cre	pl-pC								
KI/+	+/-	+	62.7 ± 3.1°	26.3 ± 1.5	3.0 ± 1.0	0.3 ± 0.6	6.0 ± 2.6	1.3 ± 0.6	15.3 ± 2.1	115.0 ± 6.2
KI/+	+/-	-	49.3 ± 0.6	21.3 ± 6.1	4.0 ± 1.7	0.3 ± 0.6	2.7 ± 0.6	0.3 ± 0.6	11.3 ± 1.5	89.3 ± 5.5
KI/+	-/-	+	$38.7~\pm~5.0$	28.0 ± 10.5	4.7 ± 1.5	1.0 ± 0.0	5.0 ± 2.6	0	9.7 ± 1.5	87.0 ± 16.5

 1×10^4 viable cells were plated in triplicate into 35 mm methylcellulose cultures. Numbers represent means plus or minus standard deviations. $^{\circ}P < 0.05$ compared with each of the other groups. GM, granulocyte-macrophage colonies; G, granulocyte colonies; M, macrophage colonies; CFU-E, colony-forming units-erythroid; BFU-E, burst-forming units-erythroid; Meg, megakaryocyte colonies; Mixed, mixed hematopoietic colonies.

with clusters of malignant cells present within the spleen, liver, kidney, and lymph nodes (Figures 4F–4H, and data not shown). In some mice, an increase in myeloblasts was also noted within the BM (Figure 4E). Although the peripheral blood counts of myeloid tumor-bearing mice showed marked variability, four mice had anemia coupled with markedly increased WBC counts (between 15,000–50,000 cells/ μ I), with easily identified circulating leukemic blasts.

Cells isolated from the granulocytic sarcomas expressed AML1-ETO (8/8 tumors analyzed, representative results shown in Figure 5C) grew efficiently in vitro in liquid cultures and formed colonies in methylcellulose-containing media that were composed of blasts admixed with rare maturing myeloid and erythroid cells. These cells readily established immortalized AML1-ETOexpressing immature myeloid cell lines that showed variable expression of CD34, c-Kit, Sca-1, TER119, Mac-1, and Gr-1, and continued to give rise to rare mature myeloid and erythroid cells in culture (Figures 5A and 5B). These AML1-ETO-expressing lines contained a diploid content of DNA when analyzed by flow cytometry after propidium iodide staining and lacked evidence of recurrent chromosomal rearrangements as assessed by routine cytogenetic analysis and spectral karyotyping (data not shown and Figure 5D). Importantly, one tumor was found to have a trisomy of chromosome 17 in all analyzed metaphases, suggesting a clonal neoplasm.

The granulocytic sarcomas were readily transplantable into recipient mice. Cells from tumors were injected subcutaneously into SCID mice, and granulocytic sarcomas developed at the site of injection in each recipient. Similarly, cells were injected intravenously into syngeneic recipients and AML1-ETO-expressing myeloblastic proliferations were detected within 3–14 weeks. Interestingly, in some of the mice injected intravenously, solid masses of proliferating myeloblasts developed near the site of injection. For example, injection into the retro-orbital sinus resulted in large orbital tumors, whereas injection into the tail vein typically resulted in tumor masses at the base of the tail. At necropsy, however, recipient mice had widespread dissemination of the leukemic myeloblasts with involvement of BM, spleen, lymph node, and liver. The granulocytic sarcomas in the recipient mice were histologically identical to those seen in the primary animals, with the majority of cells consisting of myeloblasts admixed with rare maturing granulocytes. Cells isolated from the tumors of transplanted mice readily grew as immature myeloid cell lines in liquid culture.

Altered growth factor signaling pathways cooperate with AML1-ETO to induce leukemia

Pinpointing the critical ENU-induced mutation(s) that cooperate with AML1-ETO to induce leukemia will be difficult since multiple mutations are likely to have resulted from the dose of ENU used. Nevertheless, since the transformation of murine cells frequently involves alterations of the p19^{Art}/Mdm2/p53 or p16^{INK4A}/pRb cell cycle checkpoint responses, we examined several components of these pathways to see if they had been altered either directly by AML1-ETO or by ENU mutagenesis. No mutations were detected in the DNA binding domain of p53 by direct sequence analysis in 8/8 AML1-ETO-expressing myeloid tumors. Moreover, by Western blot analysis, the p53 transcription factor was rapidly induced following X-ray irradiation, and resulted in the induction of the p21 cyclin-dependent kinase inhibitor and apoptosis (Figure 6A and data not shown). These results suggest that the AML1-ETO-expressing cells maintain an intact p53-dependent checkpoint. Expression of both p19^{Arf} and p16^{INK4A} is known to be progressively induced following serial passage of wild-type BM cells in culture and is frequently lost in immortal BM-derived cell lines (Sherr, 2001; Randle et al., 2001). Surprisingly, p19Arf and p16^{INK4A} expression was maintained in 8/8 AML1-ETO-expressing myeloid tumors (Figure 6B), suggesting that the expression of these cell cycle regulators is unlikely to be significantly effected by AML1-ETO expressed from the conditional allele. Despite the persistent expression of these cell cycle inhibitors, the tumor cells continue to proliferate both in vitro and in vivo.

Group	Genotype			No. of evaluable	Granulocytic	Tcell		
	AML1-ETO-stop	Mx1-Cre	pl-pC	ENU	mice	sarcoma	lymphoma	other tumor
1	KI/+	+/-	+	+	36	11	6	0
2	KI/+	+/-	_	+	13	0	0	0
3	KI/+	+/-	+	_	15	0	1	10
4	KI/+	+/-	_	_	13	0	0	0
5	KI/+	-/-	+	+	15	0	1	1 ^b



An important observation that might provide insight into the nature of the cooperating mutations was the marked difference in growth factor requirements of the AML1-ETO-expressing nontumorigenic cell lines versus those derived from the AML1-ETOexpressing myeloid tumors. Specifically, all of the immortal myeloid cell lines generated from AML1-ETO expressing BM were absolutely dependent on cytokines for both their growth and survival, whereas the cell lines derived from the AML1-ETO-expressing myeloid neoplasms grew efficiently in media that contained fetal bovine serum and insulin, but lacked other exogenous cytokines (data not shown). This cytokine independent growth of the tumor-derived lines suggest that the mutations that cooperate with AML1-ETO to induce leukemia might involve critical alterations in growth factor signaling pathways that allow cells to proliferate in the face of persistent p19^{Arf} and p16^{INK4A} expression. Consistent with this hypothesis, the AML1-ETO-expressing myeloid tumors showed an increase in the level of expression of both CDK4 and cyclin D2 as compared to the levels observed in the AML1-ETO nontumor cell lines (Figure 6C). Moreover, the tumors

show a marked increase in the phosphorylated form of the retinoblastoma protein (Rb), consistent with its functional inactivation (Figure 6C). No evidence of *CDK4* gene amplification was seen, suggesting that its increased expression is transcriptional or posttranscriptional in nature (data not shown).

The cytokine-independent growth of the tumors does not appear to be the result of an autocrine production of growth factors, since conditioned media from cultures of tumor cells failed to support the growth of AML1-ETO immortal nontumor cell lines (data not shown). In addition, no mutations were detected in *N-ras*, *K-ras*, or *H-ras* by direct sequence analysis in 8/8 tumors. Thus, although cytokine signaling pathways are clearly altered in the AML1-ETO tumors, the nature of the specific mutations remains to be defined.

Lastly, since biallelic inactivating mutations of *AML1* have been identified in rare sporadic cases of human acute myeloid leukemia with minimal differentiation (Song et al., 1999; Osato et al., 2000; Preudhomme et al., 2000; Imai et al., 2000), we also sequenced the wild-type *AML1* allele in the AML1-ETO-express-



Figure 5. AML1-ETO-expressing tumor derived cell lines

A and B: Cells from granulocytic sarcomas were cultured in the absence of cytokines for 4 weeks prior to morphologic analysis. Cytocentrifuge preparations were stained with Wright-Giemsa. Arrows indicate mature myeloid and erythroid cells. C: Western blot analysis for AML1-ETO. Samplesinclude fetalliver cells from AML1-ETO heterozygous embryos (AML1-ETO-KI), BM cell from a wild-type mouse (wild-type), primary tumor tissue from a granulocytic sarcoma (GS-1), and cultured cells from a granulocytic sarcoma (GS-2). D: Spectral karyotype of cells from granulocytic sarcomas.

ing tumor lines to determine if ENU mutatgenesis might have targeted this allele. No mutations were detected in the DNA binding runt homology domain of *AML1* in 8/8 tumors. The complete absence of mutation in five different genes in each of the analyzed tumors suggests that the expression of AML1-ETO is unlikely to be inducing global genetic instability. Further supporting this interpretation is the fact that both the AML1-ETO leukemias and nontumor cell lines are diploid and lack structural chromosomal abnormalities.

Discussion

By using a knockin strategy that couples a lox-P bracketed transcriptional stop cassette with an inducible Cre transgene, we generated a mouse in which lineage specific expression of AML1-ETO can be temporally controlled. This strategy allows us to bypass the embryonic lethal phenotype that results from the expression of AML1-ETO and to directly define the contribution of this fusion protein to the development of leukemia in the adult mouse. Following AML1-ETO expression, a slight increase was noted in the number of multipotential progenitors; however, the in vivo growth of these cells appeared normal, with no significant alterations in the numbers of hematopoietic cells or their extent of differentiation. Despite showing minimal in vivo growth abnormalities, these AML1-ETO-expressing BM cells showed a markedly enhanced in vitro replating efficiency, and readily formed immortal cytokine-dependent myeloid cell lines. Thus, although expression of AML1-ETO directly alters the growth characteristics of hematopoietic progenitors, the resultant cells remain responsive to normal in vivo homeostatic controls. Importantly, only a rare AML1-ETO-expressing mouse spontaneously developed a hematopoietic neoplasm after a long latency (12 months), demonstrating that expression of AML1-ETO alone is not sufficient to induce the transformation of hematopoietic cells. By contrast, treatment of the AML1-ETO-expressing mice with the

chemical mutagen ENU induced a malignant proliferation of myeloblasts in 31% of animals. Although these neoplasms presented initially as granulocytic sarcomas similar to those seen in some human patients with t(8;21)-containing AML, the disease rapidly disseminated throughout the animals. In addition, the tumors were transplantable into primary recipients and formed immortalized factor-independent cell lines confirming their malignant nature. Thus, this murine model provides direct experimental evidence that contrary to current models, expression of AML1-ETO in adult myeloid progenitors does not block differentiation, but instead contributes to their immortalization. Moreover, AML-ETO-induced leukemia is a multistep process, in which secondary genetic alterations cooperate with AML1-ETO to alter differentiation and induce full transformation.

The mechanism through which AML1-ETO increases the self-renewal capacity of hematopoietic progenitors remains to be defined. Although an increase in replating efficiency was observed, only half of the individual multi-lineage progenitors were able to give rise to long-term colony growth and immortal cyto-kine-dependent cell lines. These data suggest that AML1-ETO may not directly induce hematopoietic cells to bypass culture-induced replicative senescence, but instead may result in enhanced or prolonged growth of the cells in vitro, from which subclones can emerge that have escaped senescence.

The inability of AML1-ETO expressed from the endogenous *AML1* transcriptional regulatory sequences to significantly alter differentiation of hematopoietic progenitors is in stark contrast to results obtained using a variety of cell lines (Kohzaki et al., 1999; Burel et al., 2001; Sykes and Kamps, 2001). In most of the latter experiments, *AML1-ETO* was constitutively expressed from heterologous promoters, including retroviral LTRs. These situations fail to mimic the normal marked downregulation of AML1 expression, and presumably AML1-ETO expression, that occurs during normal myeloid differentiation. Moreover, the level of AML1-ETO expressed from our conditional allele was 5- to 10-fold less than



Figure 6. Intact p53 checkpoint, persistent p19^{Arf} and p16^{INK4a} expression, and functional pRb inactivation in AML1-ETO-expressing tumors

A: AML1-ETO-expressing tumor cells were grow as growth factor independent cell lines and irradiated at dose of 5.6 G at 760 rads/min. Following irradiation, the cells were then grown for the indicated times, lysed, and analyzed by Western blot for expression of p53 and p21. Results from two representative cell lines are shown. **B:** AML1-ETO-expressing tumors were analyzed by Western blot for expression of p19^{Art} and p16^{INK4a} **C:** AML1-ETO-expressing immortal bone marrow cells (A-E Im. BM) and tumors were analyzed by Western blot for expression of CDK4, Cyclin D2, and phosphorylated pRb.

the levels achieved using a retrovirus (J.D. and N.L., unpublished data). These differences are likely to have profound biologic consequences and highlight the need to closely mimic the situation seen in the human disease so that correct biologic insights can be made.

A clear result that emerges from our data is that the expression of AML1-ETO by itself is not sufficient to induce leukemia. However, by inducing cooperating mutations with ENU, we were able to efficiently develop acute myeloid leukemia/granulocytic sarcoma. The inability of AML1-ETO to induce leukemia by itself is consistent with recent clinical data from patients with t(8;21)containing AML (Nucifora et al., 1993; Miyamoto et al., 1996; Saunders et al., 1997; Miyamoto et al., 2000). In these patients, it is common to find persistent low numbers of *AML1-ETO*expressing cells in the BM during clinical remission. These *AML1-ETO*-expressing cells retain their capacity to differentiate to mature cells of the myeloid, erythroid and B cell lineages, and can persist within the patient for greater than 10 years. Thus, these cells are not leukemic, but rather appear to represent a preleukemic population that must acquire additional mutations in order to transform to overt leukemia. Similar observations have recently been made in other models of core binding factor leukemias. In experiments using chimeric mice containing a knockin allele that mimics the inv(16)-encoded $CBF\beta$ –MYH11, acute myelomonocytic leukemias develop after ENU-induced mutagenesis (Castilla et al., 1999). Similarly, in mice containing an AML1-ETO transgene driven off of the myeloid specific MRP8 promoter, a myeloid disease developed following ENU mutagenesis; however, the latter cells were not shown to be transplantable, nor were their in vitro growth potential assessed (Yuan et al., 2001). Lastly, in a recent mouse model that induced Cre/LoxP mediated interchromosomal recombination between AML1 and ETO, the efficiency of the recombination was too low to allow a direct assessment of the leukemic potential of AML1-ETO (Buchholz et al., 2000).

Several features of the acute myeloid disease/leukemia that develop in these mice accurately mimic those seen in human t(8;21)-containing leukemia. One of the perplexing findings in the human disease has been the tendency for the leukemic cells to frequently grow as solid tumors (Abe et al., 1986; Hagihara et al., 1991; Tallman et al., 1993). Although granulocytic sarcomas are seen in other types of leukemia, in acute leukemias they are most prevalent in the t(8;21) AMLs, being observed either at presentation or during the course of the disease in up to 22% of the patients (Abe et al., 1986; Hagihara et al., 1991; Tallman et al., 1993; Neiman et al., 1981; van Veen et al., 1991; Bitter et al., 1987). The predominant formation of granulocytic sarcomas in the murine model suggests that expression of AML1-ETO must directly result in the development of leukemic cells that have cell surface features that foster growth as adhesive masses. Although the reason for this type of growth remains unknown, genome-based gene expression analysis of the neoplastic cells should provide critical insights into the expression of adhesion or homing molecules that contribute to this pattern of growth. A second feature of the murine disease that resembles that seen in humans is the persistent ability of the leukemic blasts to undergo some terminal differentiation. Patients with t(8;21) leukemias frequently have mature myeloid cells including neutrophils that are part of the leukemic clone (Berger et al., 1982; Swirsky et al., 1984).

An important observation from our studies is that the nonleukemic AML1-ETO-expressing cell lines were cytokine-dependent, whereas cell lines derived from AML1-ETO-expressing leukemias were factor-independent. These data strongly suggest that one signaling pathway that may collaborate with AML1-ETO is cytokine-mediated proliferation or survival. Support for this hypothesis comes from the recent identification of a case of TEL-PDGF_BR-expressing chronic myelomonocytic leukemia (CMML) that underwent blastic transformation to AML following acquisition of the t(8;21), and a second case of a patient with BCR-ABLexpressing CML, who during accelerated phase was found to have acquired a t(8;21) (Kojima et al., 2000). In both examples, coexpression of AML1-ETO with a constitutively active tyrosine kinase correlated with the development of myeloblastic disease. Similarly, recent studies from our own laboratory, as well as others, have identified a high frequency of N-RAS and C-KIT mutations in core bind factor leukemias (Beghini et al., 2000). Coupled with the data from our murine model, these studies suggest that altered growth factor signaling pathways are likely to be a frequent collaborating event in AML1-ETO mediated leukemogenesis.

The occurrence of several T cell lymphoblastic lymphomas in the AML1-ETO-expressing mice was an unexpected finding, considering the near complete absence of the activated allele in normal T cells. Moreover, expression of the AML1-ETO fusion protein was not detected in the majority of the T cell tumors analyzed (4/5). Although we cannot rule out the possibility that AML1-ETO expression was extinguished in these tumors, its absence suggests that the formation of these T cell tumors may be an indirect consequence of AML1-ETO induced T cell toxicity. It is important to note that the predominant tumor type induced following ENU treatment of normal mice is T cell lymphoma (Breuer et al., 1991). In addition, it has been established that pI-pC treatment induces a rapid decrease in the number of thymoctyes by over 50% (Kuhn et al., 1995). The T cells that remain following plpC treatment and activation of the AML1-ETO-stop allele may have an increased susceptibility to transformation as a consequence of compensatory proliferation. Experiments to achieve lineage specific activation of the AML1-ETO-stop allele in T cells through the use of the Ick-Cre transgene should help to further define the role of this fusion protein in the development of these T cell tumors.

In summary, this murine model of t(8;21)-containing AML should serve as a powerful tool to aid in the determination of the spectrum of mutations that cooperate with AML1-ETO to induce leukemia. Moreover, this model should prove valuable in the assessment of novel therapies targeted toward either the AML1-ETO chimeric oncoprotein, interactive partners proteins such as the nuclear corepressors complex, or integral components of the downstream signaling pathway altered by expression of AML1-ETO. Lastly, the experimental strategy employed to make the AML1-ETO conditional allele should have broad applicability in the generation of other models of human malignancies. Through the use of a conditional transcriptional stop cassette, the expression of dominantly acting oncogenes or tumor suppressors can be regulated in both a temporal and lineage-specific manner. This level of control provides the ability to accurately direct expression of these genes to the appropriate cellular targets needed to model the desired human neoplasm.

Experimental procedures

Generation of mice with a conditional AML1-ETO allele

To generate a conditional AML1-ETO allele, a targeting vector was constructed in which a fragment of the human AML1-ETO cDNA, which encodes 62 bp of AML1 exon 4, all of exon 5, and the entire fused portion of ETO, was inserted in frame into the murine AML1 exon 4 as previously described (Okuda et al., 1998). To efficiently inhibit expression of the fused AML1-ETO sequences, we inserted a 1.5 kb DNA fragment containing a 1.3 kb transcription termination stop cassette flanked by loxP sites (Lakso et al., 1992) (Life Technologies Inc, Gaithersburg, MD) into an Sfil site into AML1 intron 3. This cassette contains a strong SV40 polyadenylation sequence, and thus prevents the generation of full-length transcripts. Although the use of this stop cassette resulted in a complete abrogation of the production of AML1-ETO protein, trace levels of an alternatively processed AML1-ETO transcript were detected by RT-PCR analysis. This trace transcript resulted from low levels of splicing of AML1 exon 3 into the stop cassette and then to the fused AML1 exon 4. Importantly, however, this chimeric transcript did not result in the production of detectable protein. AML1-ETO-stop/+ ES cell clones with undifferentiated morphology and normal karyotypes were injected into C57BL/6 blastocysts and male chimeras were bred with C57BL/6 females to achieve germline transmission

Double transgenic *AML1-ETO-stop/+ Mx1-Cre*^{+/-} mice were generated by crossing these mice with a line transgenic for an *Mx1-Cre* gene (kindly provided by Dr. Werner Müller, University of Cologne, Germany) (Kuhn et al., 1995). Expression of Cre was induced by injecting mice intraperitoneally with 250 μ g of polyinocinic-polycytidylic acid (pI-pC, Sigma, St. Louis, MO) three times at 2-day intervals as previously described (Kuhn et al., 1995). Mice were monitored daily for evidence of disease and peripheral blood cell counts with a differential were performed every three weeks.

In vitro activation of the conditional AML1-ETO allele

BM cell from 5-fluorouracil treated *AML1-ETO-stop/+* mice were infected with a MSCV retrovirus (Persons et al., 1997) engineered to express *Cre* from the LTR followed by the encephalomyocarditis virus internal ribosome entry site and a cDNA encoding the enhanced green fluorescent protein (MSCV-Cre-IRES-GFP). Infection of BM cells was carried out by coculturing with retroviral producer cell lines (Persons et al., 1997), and after 10 days in culture, GFPexpressing cells were sterilely sorted and subsequently expanded.

Hematopoietic progenitor assays

Hematopoietic cells were cultured in methylcellulose-containing media as previously described (Okuda et al., 1996) using MethoCult[™] GF M3434 media (StemCell Technologies, Vancouver, British Columbia, Canada), which contains 50 ng/ml murine stem cell factor, 10 ng/ml murine interleukin (IL)-3, 10 ng/ml human IL-6, and 3 units/ml human erythropoietin. CFU-S₁₂ were assessed by injecting intravenously 2–5 × 10⁴ BM cells from untreated or pl-pC treated *AML1-ETO-stop/+ Mx1-Cre^{+/-}* mice into recipient mice that had been previously exposed to a split dose of 950 cGy. Transplanted mice were sacrificed 12 days postinjection and spleen colonies were counted, and then excised and analyzed for expression of *AML1-ETO*.

ENU mutagenesis

5- to 10-week-old *AML1-ETO-stop/+ Mx1-Cre^{+/-}* mice were treated with plpC as described above, and then 6 days after the last dose were injected with 1 µg of human G-CSF (NEUPOGEN; Amgen, Thousand Oaks, CA) subcutaneously for 4 consecutive days. On the fifth day, mice were injected intraperitoneally with a single sublethal dose of 50 mg/kg N-ethyl-N-nitrosourea (ENU; Sigma, St. Louis, MO).

Western blot and immunophenotypic analysis

Western blot was performed as previously described (Okuda et al., 1998) using affinity purified AML-1 N-terminal peptide antiserum, affinity purified ETO N-terminal peptide antiserum, or commercial antibodies against p53 (Ab-7, Oncogene Research Products, Boston, MA), p21 (c-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA), p16^{NK4A} (M-156, Santa Cruz Biotechnology Inc.), p19^{Ar} (Abcam Limited, Cambridge, UK), CDK4 (H-22, Santa Cruz Biotechnology Inc.), Cyclin D2 (M-20, Santa Cruz Biotechnology Inc.), Actin (c-11, Santa Cruz Biotechnology Inc.), and phosporylated pRb (Cell Signaling Technology, Beverly, MA). Cell surface antigens expression was detected using phycoerytrhrin-conjugated monoclonal antibodies (MoAb) from PharMigen (San Diego, CA) to CD34, c-Kit, Sca-1, Gr-1, (Mac-1), B220, CD4, CD8, and TER119.

Histology and spectral karyotyping

Tissues were fixed in 10% buffered formalin, embedded in paraffin, and sections stained with hematoxylin and eosin or by the avidin-biotin-peroxidase method using anti-Myeloperoxidase, CD3, lysozyme (DAKO, Carpinteria, CA), and B220/CD45R (PharMingen, San Diego, CA) antibodies. Peripheral blood was collected in 10 mmol/L EDTA and smears stained with Wright-Giemsa. Multicolor spectral karyotyping was performed using standard methodologies.

Transplantation analysis

Tumor cells from *AML1-ETO-stop/+ Mx1-Cre^{+/-}* mice ($2.5-6 \times 10^6$) were expanded in short term cultures in the presence of cytokines (1 week) and then transplanted into either syngeneic or C.B-17 SCID mice. For transplants into syngeneic mice, cells were injected intravenously into both sublethally irradiated (450 cGy in a split dose) or nonirradiated recipients. Injections into SCID mice were similarly performed into irradiated (350 cGy) or nonirradiated mice. However, to expand our analysis, nonirradiated SCID mice were injected both intravenously and subcutaneously. Recipient mice were 5 to 28 weeks of age at the time of transplantation. Mice were monitored every day for sickness and sacrificed when moribund.

RT-PCR and sequence analysis

For RT-PCR analysis, RNA was extracted and amplified as previously described (Okuda et al., 1998). Detection of *AML1-ETO* mRNA was performed using primers to murine *AML1* exon 3 (5'-ACTTCCTCTGCTCCGTGCTA-3') and human *ETO* (5'-TTGAGTAGTTGGGGGAGGTG-3'). Amplification was carried out with parameters of 2 min at 94°C for denaturation followed by 30 cycles of 1 min at 94°C, 1 min at 62°C, 40 s at 72°C, ending with 6 min at 72°C. For mutation screening of *N-ras*, *H-ras*, *K-ras*, and *AML1*, individual exons were amplified and directly sequenced using standard methodologies. For p53, sequence analysis was performed on amplified mRNA. The sequence of the primers used for amplification and sequencing are included in the Supplemental Data at http://www.cancercell.org/cgi/content/full/1/1/63/DC1.

Acknowledgments

The authors would like to thank Drs. Susan Mathew for spectral karyotype analysis, P. Liu for advice on ENU mutagenesis, S. Hiebert for antibodies to AML1 and ETO, and Shouli Yang, Kent Williams, and Dorothy Bush for technical support. This work was in part supported by National Institutes of Health (NIH) grants P01 CA71907-06, U01 CA84221-03, and the Cancer Center CORE Grant CA-21765, and by the American Lebanese and Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital.

Received: October 29, 2001 Accepted: December 5, 2001

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