AML1/RUNX1 Increases During G1 to S Cell Cycle Progression Independent of Cytokine-dependent Phosphorylation and Induces Cyclin D3 Gene Expression

Revised for publication, September 9, 2003, and in revised form, December 31, 2003
Published, JBC Papers in Press, January 27, 2004, DOI 10.1074/jbc.M310023200

Florence Bernardin-Fried‡, Tanawan Kummalue‡, Suzanne Leijen‡, Michael I. Collector‡, Katya Ravid‡, and Alan D. Friedman¶‡

From the §Division of Pediatric Oncology, Johns Hopkins University, Baltimore, Maryland 21231 and the §Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02128

AML1/RUNX1, a member of the core binding factor (CBF) family, stimulates myelopoiesis and lymphopoiesis by activating lineage-specific genes. In addition, AML1 induces S phase entry in 32Dcl3 myeloid or Ba/F3 lymphoid cells via transactivation. We now found that AML1 levels are regulated during the cell cycle. 32Dcl3 and Ba/F3 cell cycle fractions were prepared using elutriation. Western blotting and a gel shift/supershift assay clearly demonstrated that endogenous CBF DNA binding and AML1 levels were increased 2–4-fold in S and G2/M phase cells compared with G0 cells. In addition, G1 arrest induced by mimosine reduced AML1 protein levels. In contrast, AML1 RNA did not vary during cell cycle progression relative to actin RNA. Analysis of exogenous Myc-AML1 or AML1-ER demonstrated a significant reduction in G0 phase cells, whereas levels of exogenous DNA binding domain alone were constant, lending support to the conclusion that regulation of AML1 protein stability contributes to cell cycle variation in endogenous AML1. However, cytokine-dependent AML1 phosphorylation was independent of cell cycle phase, and an AML1 mutant lacking two ERK phosphorylation sites was still cell cycle-regulated. Inhibition of AML1 activity with the CBFβ-SMMHC or AML1-ETO oncoproteins reduced cyclin D3 RNA expression, and AML1 bound and activated the cyclin D3 promoter. Signals stimulating G1 to S cell cycle progression or entry into the cell cycle in immature hematopoietic cells might do so in part by inducing AML1 expression, and mutations altering pathways regulating variation in AML1 stability potentially contribute to leukemic transformation.

Core binding factor (CBF) is a family of transcription factors containing one of three CBFα subunits, RUNX1/AML1, AML2, or AML3, and a CBFβ subunit (1). The CBFα subunits bind a common DNA consensus site via the N-terminal Runt domain, which also mediates heterodimerization with CBFβ (2, 3).

CBFβ does not contact DNA, but increases the DNA affinity of the α-subunits (4, 5). CBF activities are reduced in 30% of acute myeloid leukemia cases, due either to AML1 point mutations, AML1 gene deletion, or, most often, chromosomal abnormalities involving genes encoding AML1 or CBFβ (1).

Mice lacking either AML1 or CBFβ do not develop definitive hematopoiesis, indicating a critical role for these factors in pluripotent hematopoietic stem cells (6–10). During early hematopoiesis AML1 regulates myeloid and lymphoid lineage-specific genes, such as those encoding T-cell receptor δ, myeloperoxidase, and the M-CSF receptor (11–14), and AML1 is down-regulated during terminal neutrophilic differentiation (15).

In addition, CBF/AML1 regulates the G1 to S cell cycle transition. CBFβ-SMMHC is a CBF oncoprotein capable of sequestering CBFα subunits in multimers, which form via its myosin (SMMHC) domain (16, 17). Expression of CBFβ-SMMHC from the zinc-responsive metallothionein (MT) promoter in the Ba/F3 pro-B lymphoid or 32Dcl3 myeloid cell lines reduces CBF/AML1 DNA binding, leading to the accumulation of hypophosphorylated retinoblastoma (Rb) protein, and slows cell proliferation during G1 (18). N-terminal CBFβ residues required for interaction with CBFα subunits are required for inhibition of proliferation by CBFβ-SMMHC (19). An AML1 DNA binding domain:KRAB transrepression domain fusion protein expressed inducibly in Ba/F3 or 32Dcl3 cells also blocks G1 progression (20). Similarly, AML1-ETO, a CBF oncoprotein, which binds DNA and represses transcription, slows G1 progression in myeloid cell lines, dependent upon its ability to bind DNA (17, 21, 22). Inhibition of proliferation by CBFβ-SMMHC or by KRAB-AML1-ER is overcome by exogenous cdk4, cyclin D2, or c-Myc (20, 23). Also, AML1 accelerates G1 progression when expressed stably in 32Dcl3 cells or when expressed as an AML1-ER fusion protein in Ba/F3 cells (20, 24). When coexpressed with CBFβ-SMMHC, AML1-ER stimulated proliferation more potently, dependent upon integrity of its transactivation domain (25). Finally, genetic changes that accelerate G1, loss of p16INK4a and p19ARF or expression of papillomavirus E7 protein, cooperate with CBF oncoproteins to induce acute leukemia in mice (26, 27).

Based on the conclusion that AML1 stimulates the G1 to S transition, we sought to determine whether AML1 levels are themselves regulated during the cell cycle. 32Dcl3 and Ba/F3 cell cycle fractions were prepared using elutriation, a method that avoids the cytotoxicity of chemical synchronizing agents. Endogenous or exogenous AML1 levels increased during G1 to S cell cycle progression, as did AML1 DNA binding activity. An AML1 variant lacking two ERK phosphorylation sites retained cell cycle variation, and IL-3 induced phosphorylation of endog-
enous AML1 independent of cell cycle phase. In addition, inhibition of AML1 repressed cyclin D3 mRNA expression, and AML1 bound and activated the cyclin D3 gene.

EXPERIMENTAL PROCEDURES

Cell Culture and Generation of Stable Clones—32Dcl3 cells (28) were cultured in Iscove’s modified Dulbecco medium (IMDM) with 10% heat-inactivated fetal bovine serum (HI-FBS), 1 ng/ml murine interleukin-3 (IL-3, Peprotech), Ba/F3 cells (29) were cultured in RPMI 1640 with 10% HI-FBS, and 1 ng/ml IL-3. CRE retroviral packaging cells (30) were cultured in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated calf serum. All cultures contained penicillin-streptomycin. ZipBlue (Invitrogen), or mimosine (Santa Cruz Biotechnology) were added as indicated. 32Dcl3 differentiation was induced by washing twice with phosphate-buffered saline followed by culture in IMDM with 10% HI-FBS, and 20 ng/ml G-CSF (Amgen). 32Dcl3 or Ba/F3 cells were stably transfected by electroporation, and 32Dcl3 cells were transfected using CRE-AML1 (86–217) ER cDNA as described and selected using 1.2 mg/ml G418 (total) or 2 μg/ml puromycin (18, 31). Single cell clones were isolated by limiting dilution. 29T4 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% HI-FBS and were transiently transfected using LipofectAMINE 2000 (Invitrogen), as described (17).

Plasmids—The human AML1 cDNA, the longest splice variant of AML1 (32), was ligated into the polylinker of pmcCB6 (18) to generate pmc-Myc-AML1. pmc-Myc-AML1 was generated by inserting an oligonucleotide encoding Kozak’s rules for translation initiation following a methionine and a Myc tag upstream of the AML1 cDNA. The oligonucleotide sense strand, with a KpnI-compatible 3’-end was 5’-CATCGAGTGGCCGACCACCCCAGTGCG-3’ (AF15788, 405–431), 5’-CTAGACAAAAATTCAGACACCATTTCCTTCCAGGAGTCATCA-3’ (U3844, 1291–1317). Total cellular RNAs were prepared using Trizol reagent (Invitrogen) or RNAeasy (Qiagen) per the manufacturer’s instructions. The RNAs, 10 μg per lane, were subjected to Northern blotting as described (31). Murine cyclin D1, D2, and D3 cDNAs were kindly provided by C. Sherr and the murine AML1 cDNA by Y. Ito (2). Densitometric analysis was carried out with the NIH Image 1.62 program.

RESULTS

Endogenous CBF DNA Binding and AML1 Increase During G1 to S Progression—We employed counterflow elutriation to fractionate 32Dcl3 and Ba/F3 cells into cell cycle fractions based on density. In this procedure, the flow of the elutriation media opposes the centrifugal force on the cells, establishing an equilibrium. As the flow rate is increased, denser cells come into position to exit the elutriation chamber. Use of elutriation allowed us to study cells in active, physiologic cell cycle, which did not prove possible by synchronizing cells followed by release of the chemical block as 32Dcl3 cells treated in this manner underwent apoptosis rather than continued cell cycle progression (not shown). A typical cell cycle fraction profile and the proportion of cells in each fraction are shown (Fig. 1). Fr 18–20 contains G1 phase cells, Fr 21–24 contains late G2/early S cells, Fr 25–30 contains mainly S phase cells, and Fr 31–34 mainly G0/M phase cells. Cell cycle fractionation was monitored in each experiment. In some experiments the cells began to elute at 20 ml/min rather than 18 ml/min, as indicated in each figure.

AML1 is expressed entirely in the cell nucleus (34). We first employed a gel shift assay to assess CBF DNA binding activity during the cell cycle, utilizing an equivalent amount of nuclear protein per sample (Fig. 2, A and B, left panels). Based on densitometric analysis, CBF DNA binding activity increased almost 4-fold as 32Dcl3 or Ba/F3 cells progressed from G1 to S. Of note, micrograms of nuclear protein per cell were not significantly different between the G1 and S phase samples. This pattern of CBF gel shift activity was reproduced in four separate experiments. To confirm that the indicated bands contain members of the CBF family, the peak fractions for each cell line were subjected to competition with increasing amounts of unlabelled wild-type CBF oligonucleotide or with an oligonucleotide carrying clustered point mutations in the CBF binding site (Fig. 2C). Specific competition was observed with the CBF complexes. To resolve AML1-specific DNA from among the CBF binding activity, equal amounts of the 32Dcl3 nuclear extracts were subjected to a supershift assay with normal rabbit serum, AML1 antisera, or AML1 antisera in the presence of its specific peptide (Fig. 2A, right panel). A specific supershift band was obtained from each fraction (arrow) which increased 2.3-fold as cells progressed from G1 to S. Gel shift assay indicated that Ba/F3 cells have substantially less overall CBF DNA binding activity than 32Dcl3 cells (Fig. 2B), perhaps accounting for our inability to detect a supershifted AML1 species with the Ba/F3 extracts, although the antibody did reduce the intensity of the CBF bands (Fig. 2B, right panel).

We next prepared total cellular proteins from 32Dcl3 and Ba/F3 cell cycle fractions and analyzed these for AML1 expression by Western blotting (Fig. 3, A and B). A slowly migrating nonspecific band was evident in the 32Dcl3 samples, and Coo- massie Blue dye-stained protein fractions serve as a further control for protein loading. Densitometric analysis indicated that total cellular proteins increased 2-fold during the cell cycle, consistent with doubling of cell size. AML1 levels increased 2.7- or 2.6-fold from G1 (Fr 18–19) to S (Fr 28–29) or G0/M (Fr 32–33) in the 32Dcl3 fractions, relative to total cellular proteins, and increased ~1.8- or 1.9-fold in the same Ba/F3
fractions. Additional repetitions of this analysis with 32Dcl3 cells demonstrated a 3.0- or 3.5-fold increase (for an average of 3.0-fold) in AML1 expression during the cell cycle (see Fig. 8B).

This degree of cell cycle variation is consistent with the EMSA studies for 32Dcl3 cells, but the increase in CBF DNA binding was greater than that for AML1 in Ba/F3 cells, perhaps reflecting a greater contribution of AML2 and AML3 to CBF cell cycle variation in these cells.

To determine whether the variation in AML1 protein levels during the cell cycle is paralleled by variation in AML1 RNA levels, total cellular RNAs were prepared from 32Dcl3 and Ba/F3 cell cycle fractions and 10 H9262 g of each sample was subjected to Northern blotting (Fig. 4). AML1 mRNA increased only 1.3-fold in both Ba/F3 cells and 32Dcl3 cells from early G1 to late S phase, relative to β-actin mRNA. Of note, RNA per cell increased 2-fold, on average, during the cell cycle. Thus, relative to total RNA, AML1 RNA increased 2.6-fold in each line. Comparison to β-actin may be more relevant, as total RNA largely represents ribosomal RNA.

AML1 Protein Levels Are Reduced in Cells Arrested in G1—Decreased expression of AML1 in early G1 suggests that cells arrested in G1 would have reduced levels of AML1 compared with proliferating cells. 32Dcl3 or Ba/F3 cells were exposed to mimosine, an agent, which induces the p27 cyclin-dependent kinase in hematopoietic cells (35). Mimosine was employed at 0.2 or 0.4 mM for 6 h, as exposure for longer times led to substantial apoptosis. At 0.4 mM, the G1/S ratio increased from 1.4 to 2.2 in Ba/F3 cells and from 1.1 to 1.6 in 32Dcl3 cells, with minimal apoptosis (Fig. 5, A and B). Total cellular proteins from these cultures were analyzed for endogenous AML1 expression by Western blotting (Fig. 5B). Exposure to 0.4 mM mimosine for 6 h reduced endogenous AML1 levels 1.7-fold in Ba/F3 cells and 2.5-fold in 32Dcl3 cells, relative to total protein content.

**Fig. 1.** Cell cycle fractionation of 32Dcl3 cells. A, 2 x 10^6 32Dcl3 cells were subjected to counterflow elutriation. The cell cycle profile of the total cell population and of isolated fractions are shown. Approximately 80% of the input cells were recovered. B, proportion of the isolated cells in each fraction and their cell cycle phases are shown.
For comparison, 32Dcl3 cells were transferred from IL-3 to G-CSF, which induces differentiation concomitant with a potent G1 arrest (31). The G1/S ratio increased from 1.1 to 5.0 after 1 day (D1) and to 8.9 after 2 days (D2), without evident apoptosis (Fig. 5, A and B). AML1 levels were reduced 3-fold after 1 day and more than 10-fold after 2 days in G-CSF.

To determine whether reduced AML1 levels associated with G1 arrest are due to reduced AML1 RNA levels, total cellular RNAs were prepared from 32Dcl3 cells cultured in IL-3 or in G-CSF for 1 or 2 days and from 32Dcl3 or Ba/F3 cells cultured in 0.4 mM mimosine for 6 h. These RNAs were subjected to Northern blotting for AML1 and β/γ2-actin (Fig. 5C). Relative to β-actin, AML1 RNA increased in 32Dcl3 cells in response to exposure to mimosine for 6 h or to G-CSF for 2 days and only decreased 10% in Ba/F3 cells in response to mimosine.

Exogenous AML1 Levels Also Increase During G1 to S Progression—Based on our finding that endogenous AML1 levels are regulated during the cell cycle, we predicted that exogenous AML1 protein levels might be controlled similarly. To clearly distinguish exogenous AML1, we positioned a 17-residue tag, MEQKLISEEDLASTEF (the Myc epitope is underlined), at

FIG. 2. CBF and AML1 DNA binding activity increases during G1 to S progression. A, nuclear extracts, 12 μg of total protein per lane, from the indicated 32Dcl3 cell cycle fractions were subjected to gel shift assay with a radiolabeled AML1 binding site from the myeloperoxidase gene, WTMPO, either alone (left panel) or in the presence of 1 μl of normal rabbit serum (R), AML1 antiserum (Ab), or AML1 antiserum, and 4 μg of its specific peptide (P) (right panel). The relative intensity of the CBF shift or the AML1 supershift bands between fractions is shown. B, nuclear extracts from the indicated Ba/F3 fractions were subjected to gel shift assay similarly. The right panel was exposed for a shorter time. C, 32Dcl3 fraction 25–28 and Ba/F3 fraction 24–25 were subjected to gel shift assay in the absence of competitor (−) or in the presence of 10-, 50-, and 200-fold excess of unlabelled wild-type (WT) or mutant (Mut) competitor. The two panels were exposed for similar times.

FIG. 3. Endogenous AML1 protein levels increase during the cell cycle. A, cellular proteins isolated from unfractionated cells (T) or from the indicated cell cycle fractions were subjected to Western blotting for AML1. The positions of molecular weight markers are shown (top panel). Equal volumes of each fraction were electrophoresed on a second gel and stained with Coomassie Blue dye (bottom panel). B, cell cycle fractions from Ba/F3 cells were analyzed similarly. Relative expression of AML1 in each sample based on densitometry, normalized for loading, is shown for both blots.

FIG. 4. Endogenous AML1 RNA levels do not vary during the cell cycle. Total cellular RNA prepared from the indicated Ba/F3 and 32Dcl3 cell cycle fractions, 10 μg per sample, were subjected to Northern blotting for murine AML1, cyclin D3, and β-actin. Actin is 1892 and D3 is 1905 bp. Expression of AML1 RNA relative to β-actin in each sample is shown.
FIG. 5. Endogenous AML1 levels are reduced by G1 cell cycle arrest. A, cell cycle profiles of 32Dcl3 or Ba/F3 cells exposed to 0.4 mM mimosine (mim) for 6 h or of 32Dcl3 cells transferred from IL-3 to G-CSF for 1 or 2 days (D1, D2). B, total cellular proteins prepared from Ba/F3 or 32Dcl3 cells exposed to the indicated doses of mimosine for the indicated times were subjected to Western blotting for AML1 and to Coomassie Blue dye staining (left panels). Similar analysis was carried out on 32Dcl3 cells cultured in IL-3 or after transfer to G-CSF for 1 or 2 days (right panels). The G1/S ratio and the relative expression of AML1, corrected for loading, is shown for each sample. C, total cellular RNAs prepared from 32Dcl3 cells cultured in IL-3 or in G-CSF for 1 or 2 days and from 32Dcl3 and Ba/F3 cells cultured in 0.4 mM mimosine for 6 h were subjected to Northern blotting for murine AML1 and β-actin. Expression of AML1 RNA relative to β-actin in each sample is shown.
AML1 Varies during the Cell Cycle

Fig. 6. Exogenous AML1 protein levels increase during the cell cycle. A, total cellular proteins prepared from 1 × 10⁶ 32Dcl3 (32D) cells or from 32D-Myc-AML1 cells exposed to 100 μM zinc chloride for 16 h were subjected to Western blotting using an AML1 antisera (left panel). Extracts from 32D-Myc-AML1 cells exposed to the indicated concentrations of zinc chloride (μM) for 16 h were subjected to Western blotting using a Myc tag antibody. B, total cellular proteins prepared from the indicated 32D-Myc-AML1 cell cycle fractions, obtained after culture in 75 μM zinc chloride for 16 h, were subjected to Western blotting for myc-AML1 (using the Myc tag antibody), cyclin D3, or cyclin E. Coomassie Blue dye staining of these fractions is also shown (bottom panel). C, total cellular RNAs prepared from the indicated 32D-Myc-AML1 cell cycle fractions were subjected to Northern blotting for Myc-AML1 (using a human AML1 probe) and β-actin. D, total cellular proteins prepared from the indicated 32D-AML1ER cell cycle fractions, obtained after culture for 16 h in 200 nM 4HT, were subjected to Western blotting for AML1-ER (using ER antisera), CBFβ, and cyclin D3. Myc-AML1 or AML1-ER expression in each sample, corrected for loading, is shown in parts B, C, and D.

The N terminus of the 480-residue AML1 cDNA just downstream of a segment containing Kozak’s rules for optimum translational initiation. The Myc-AML1 cDNA, lacking endogenous AML1 5′- or 3′-untranslated regions (UTRs), was then positioned downstream of the zinc-responsive metallothionein promoter in pMTCB6 to generate pMT-myc-AML1. This plasmid also contains the G418-resistance gene linked to the SV40 promoter. pMT-myc-AML1 was linearized and electroporated into 32Dcl3 cells. Two G418-resistant subclones expressing myc-AML1 in the presence of zinc chloride were identified by Western blotting. When a total cellular extract from zinc-treated 32D-Myc-AML1 cells was analyzed by Western blotting using an AML1 antibody, two doublets were detected, with the lower doublet co-migrating with AML1 detected from parental 32Dcl3 cells (Fig. 6A, left panel). Presence of a doublet may reflect AML1 phosphorylation (36). The increased size of exogenous myc-AML1 is due to the 17-residue Myc tag and to the fact that exogenous human AML1 contains 27 residues at it N terminus lacking in the endogenous murine protein. Of note, the remainder of human and murine AML1 are 97% identical (32). We observed that myc-AML1 was expressed at ~3-fold higher level than endogenous AML1. Use of the zinc-responsive MT promoter not only avoids generation of cell lines perturbed during isolation by high basal activity of AML1 but also allows control of myc-AML1 expression via use of suboptimal zinc concentrations (Fig. 6A, right panel). Even in the absence of zinc, basal expression of myc-AML1, especially the lower band of the doublet, is evident. 75 μM zinc chloride led to expression 2–3-fold below that detected in cells exposed to 100 μM zinc, presumably similar to endogenous AML1 levels. 32D-Myc-AML1 cells were therefore exposed to 75 μM zinc and subjected to elutriation. Cell cycle fractions were analyzed by Western blotting for myc-AML1, cyclin D3, and cyclin E (Fig. 6B). Compared with early G1 cells (Fr 18–20), Myc-AML1 levels increased 2.3-fold by mid-S phase (Fr 27–28) and 3.0-fold by late-S/G2/M phase (Fr 29–30). Cyclin D3 and E levels increased similarly. Analysis of a second 32Dcl3 subclone demonstrated a 3.4-fold increase in exogenous Myc-AML1 during cell cycle progression (not shown). Northern blot analysis indicated that AML1 RNA increased 1.8-fold relative to β-actin, consistent with both transcriptional and post-transcriptional regulation of AML1 during G1 to S progression (Fig. 6C).

To evaluate the effect of cell cycle phase on the expression of exogenous AML1 by an independent strategy we utilized 32Dcl3 cells expressing AML1-ER from the MMLV retroviral LTR in pBabePuro (20). Western blot analysis of cell cycle fractions demonstrated that AML1-ER increased 1.9–2.3-fold by S phase (Fr 25–28 and Fr 29–31) and 4.5-fold by G2/M phase (Fr 32–34) relative to CBFβ levels, which remained constant throughout the cell cycle (Fig. 6D). Cyclin D3 levels again increased during G1 to S progression. Thus, exogenous AML1 levels varied similar to endogenous AML1 during the cell cycle.
**AML1 Varies during the Cell Cycle**

Despite lack of the AML1 promoter, expected to control transcription, or the AML1 5′- and 3′-untranslated RNA segments, expected to control translation efficiency and RNA stability.

Exogenous AML1 DNA Binding Domain Does Not Vary during the Cell Cycle—To begin to map the domain of AML1 responsible for its cell cycle variation, 32Dcl3 lines stably expressing either Myc-AML1(1–217) from the MT promoter, retaining only the DNA binding domain and 87 N-terminal residues, or AML1-(86–217)-ER from the pBabePuro retroviral vector, retaining only the DNA binding domain, were subjected to elutriation and Western blot analysis (Fig. 7, A and B). In contrast to Myc-AML1 or AML1-ER, which increased 3.0- or 4.5-fold, Myc-AML1-(1–217) increased 1.5-fold and AML1-(86–217)-ER increased 1.6-fold during the cell cycle. Analysis of a second subclone expressing AML1-(86–217)-ER gave similar results (not shown).

**Phosphorylation of AML1 by ERK Does Not Mediate Its Cell Cycle Variation**—Serines 276 and 293 are targets of cytokine-dependent phosphorylation occurs during each cell cycle stage (Fig. 8). This AML1 mutant increased more than 3-fold during cell cycle progression (Fig. 8B), similar data were obtained when this experiment was repeated. Thus, cytokine-mediated phosphorylation of AML1 does not account for the increase in endogenous AML1 observed during G1 to S phase.

The Cyclin D3 Promoter Is Activated by AML1—Cyclin D3 mRNA levels did not vary, relative to β-actin, in 32Dcl3 or Ba/F3 cell cycle fractions (Fig. 4). However, E2F1:DP-1, a heterodimeric factor with peak activity in late G1/early S phase, potently activated the murine cyclin D3 promoter, as predicted from the presence of an E2F consensus site (33). Therefore, we considered the possibility that the peak in CBF DNA binding activity in late G1/early S might be designed similarly to enable maximal cyclin D3 expression when needed in hematopoietic cells. We first examined Ba/F3 cells expressing dominant inhibitory AML1 oncoproteins (17, 18), CBFβ-SMMHC (INV) or AML1-ETO (A-E), from the MT promoter to assess the consequences of CBF inhibition on cyclin D1, D2, and D3 mRNA expression (Fig. 9A). Ba/F3 lines were employed rather than 32Dcl3 lines for this purpose as the MT promoter is leakier in 32Dcl3 cells, making it difficult to maintain high-level expression of these cell cycle-inhibitory oncoproteins. Cyclin D1 expression was not detected, as has been described previously for hematopoietic cell lines (37). Zinc did not affect cyclin D2 or D3 mRNA expression in the Ba/F3 cell line; their expression was inhibited by both the INV or A-E proteins by 7 h, with D3 expression being affected the greatest. We selected candidate AML1 binding sites from the murine D1, D2, and D3 promoters based on the AML1 consensus, 5′-PuACCPuCA-3′, and assessed their affinity for AML1 (Fig. 9B). In this assay, a strong AML1 site from the MPO promoter, WTMO, was radiolabeled and competed with 5-, 10-, and 25-fold excess of unlabelled WTMO, a version of this oligonucleotide mutant in the AML1 site, MutMO, and each of the oligonucleotides derived from the cyclin D promoter regions. The best available site from the D1 promoter region, 5′-AACACCC-3′, did not compete, from the D2 promoter region, 5′-AACCACA-3′, competed modestly, and from the D3 promoter, 5′-GACCA-3′, competed to an even greater extent, almost as well as the WTMO oligonucleotide. Differences in the affinities of the D2–541 and D3–383 sites may reflect affects of flanking sequences. To determine whether AML1 can transactivate the murine cyclin D3 promoter, a 447 bp promoter fragment, including the transcription start site was linked to luciferase and co-transfected with pCMV-AML1 and pCMV-CBFβ in 293T cells (Fig. 9C). AML1 activated the cyclin D3 promoter 2.3-fold in this context in multiple repetitions, and mutation of the AML1 binding site at −383 reduced this activation to 2.3-fold.
1.6-fold, suggesting a role for this binding site, although the differences in activity did not reach statistical significance.

**DISCUSSION**

A key finding of this study is that endogenous CBF DNA binding and AML1 protein levels reproducibly increase 3-fold, when normalized to total protein content, during the G1 to S cell cycle transition. In addition physiologic levels of exogenous AML1, expressed inducibly to avoid biasing cell cycle distribution, increased similarly, supporting the additional conclusion that AML1 protein stability is regulated during the cell cycle. Reduced expression of AML1 in G1-arrested cells is consistent with this conclusion. Regulation of AML1 transcription may also occur during cell cycle progression. These findings were confirmed in two growth factor-dependent hematopoietic cell lines. We have focused our studies on hematopoietic cells as these are most physiologically relevant in view of the finding that AML1-null mice specifically lack hematopoiesis (6, 7). To put the degree of AML1 variation in perspective, mdm2 levels increase 4-fold and cyclin B1 varies 10-fold during cell cycle progression in HeLa cells (38). AML1 levels remained high in G2/M phase compared with S phase cells, whereas CBF and AML1 DNA binding was reduced, suggesting that AML1 DNA binding activity is itself regulated during S to G2/M progression.

Several lines of experimentation using the 32Dcl3 and Ba/F3 cell lines had demonstrated that gene activation by AML1 stimulates their G1 to S progression. In particular, transrepression of AML1 target genes slowed G1 progression, an effect overcome by co-expression of regulators known to stimulate S phase entry, and exogenous AML1 accelerated the G1 to S transition, dependent upon the AML1 transactivation domain (18–20, 23, 25). It is therefore not surprising that hematopoietic cells require increased AML1 during late G1 and have developed mechanisms to reduce AML1 during the G1 arrest associated with differentiation. As differentiation is a multistep process, the reduced AML1 protein levels evident after G-CSF treatment may reflect mechanisms other than those utilized to regulate AML1 during the cell cycle.

It is intriguing that hematopoietic cells predominantly express cyclin D2 and D3, rather than D1, and that we have identified cis elements within the D2 and D3 promoters which bind AML1. On its own, AML1 only activated the D3 promoter 2.3-fold, perhaps reflecting the need to cooperate with other proteins to contribute most effectively. And mutation of the AML1 binding site at $$11002^383$$ only partially abrogated this activation, perhaps indicating contribution from additional AML1 binding sites. AML2 or AML3 may also contribute to regulation of the cyclin D3 promoter, as supershifting with AML1 antibody did not eliminate cell cycle variation of residual CBF DNA binding activity (Fig. 2A), perhaps accounting in part for our inability thus far to detect interaction of AML1 with the cyclin D3 promoter by chromosomal immunoprecipitation. In-
Interestingly, the C-terminal regions of AML2 or AML3 when knocked into the AML1 locus allow nearly normal in vivo hematopoietic (39). Future experiments will further characterize regulation of the cyclin D3 gene by CBF family members.

As AML1 is expressed in pluripotent and lineage-restricted hematopoietic stem cells (40, 41), we speculate that stabilization of AML1 protein stability or RNA expression also plays a role in the entry of these cells into cell cycle from G0, as required to maintain hematopoietic homeostasis or during cytokine-mediated stress responses. In this regard, both c-Myc and B-Myb RNA levels increase when cells transition from G0 to G1, and correlated with stress responses. In this regard, both c-Myc and B-Myb maintain hematopoietic homeostasis or during cytokine-mediated stress responses. In this regard, they are likely insufficient to characterize the ubiquitination or phosphorylation of a particular AML1 residue during the cell cycle as the unstable modified or unmodified form may be rapidly degraded. Rather, AML1 mutants resistant to modification might be identified which remain at high, constant levels throughout the cell cycle.

In a subset of acute myeloid leukemias, AML1 activities are reduced due to mutation or expression of dominant oncoproteins, potentially favoring transformation due to inhibition of differentiation and/or reduced apoptosis (13, 54, 55). Perhaps additional leukemias harbor mutations, which reduce AML1 level via protein modification, using the same biochemical pathway employed to reduce AML1 expression in G1 phase cells. In these leukemias, additional mutations stimulating G1 progression may be required (26, 27). Other acute leukemias harbor increased numbers of AML1 genes due to gene amplification or to trisomy 21 (56-59). In these leukemias, AML1 may facilitate transformation by stimulating cell cycle progression. Perhaps additional leukemias harbor alterations, which increase AML1 levels indirectly. Investigating the mechanisms underlying AML1 cell cycle variation might therefore uncover novel pathways contributing to leukemogenesis.

Acknowledgments—We thank Saul Sharkis for helpful discussion, H. Hira for the AML1(R517K/S519A) cDNA, C. Sherr for the cyclin D cDNAs, and H. Drabkin, N. Speck, and S. Hiebert for antisera.

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*J. Biol. Chem.* 2004, 279:15678-15687.
doi: 10.1074/jbc.M310023200 originally published online January 27, 2004

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