PU.1 Directly Regulates cdk6 Gene Expression, Linking the Cell Proliferation and Differentiation Programs in Erythroid Cells

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Cell proliferation and differentiation are highly coordinated processes during normal development. Most leukemia cells are blocked from undergoing terminal differentiation and also exhibit uncontrolled proliferation. Dysregulated expression of transcription factor PU.1 is strongly associated with Friend virus-induced erythroleukemia. PU.1 inhibits erythroid differentiation by binding to and inhibiting GATA-1. PU.1 also may be involved in controlling proliferation of erythroid cells. We reported previously that the G1 phase-specific cyclin-dependent kinase 6 (CDK6) also blocks erythroid differentiation. We now report that PU.1 directly stimulates transcription of the cdk6 gene in both normal erythroid progenitors and erythroleukemia cells, as well as in macrophages. We propose that PU.1 coordinates proliferation and differentiation in immature erythroid cells by inhibiting the GATA-1-mediated gene expression program and also by regulating expression of genes that control progression through the G1 phase of the cell cycle, the period during which the decision to differentiate is made.

The production of mature blood cells from multipotent progenitors involves both the acquisition of tissue-specific functions and an increasingly restricted proliferative capacity that normally culminates in cell cycle exit. These two processes are governed by certain master regulatory transcription factors (reviewed in Refs. 1–3). Thus, the gene expression programs initiated by such lineage-determining transcription factors are expected to encompass both tissue-specific genes as well as genes involved in controlling cell proliferation. Whereas much has been learned about the control of tissue-specific gene expression by such transcription factors, their roles in regulating genes that control cell division are less understood.

GATA-1 and PU.1 are two transcription factors that play central roles in the development of several hematopoietic lineages. GATA-1 is a zinc finger DNA binding protein required for the development of erythrocytes and megakaryocytes (reviewed in Refs. 4–6). PU.1, an Ets family transcription factor, is required for the development of myeloid cells and B cells (reviewed in Refs. 7–9). PU.1 and GATA-1 have a particularly close relationship because they direct lineage commitment decisions from shared common multipotential (myeloid) progenitors. Moreover, PU.1 and GATA-1 physically interact and repress each other’s transcriptional activation functions (10–12).

Dysregulation of PU.1 expression is oncogenic in erythroid cells and leads to murine erythroleukemia. Proviral insertions at the PU.1 locus are found in 95% of murine erythroleukemias caused by the spleen focus-forming virus component of Friend leukemia virus (13). The murine erythroleukemia (MEL) cell lines obtained from such tumors are blocked from completing differentiation and have readily detectable levels of PU.1, as well as GATA-1. Certain treatments enable MEL cells to resume terminal erythroid differentiation, whereupon the PU.1 level rapidly declines. However, enforced expression of PU.1 in the cells blocks their reentry into the differentiation program (14).

PU.1 also blocks differentiation of normal erythroid progenitors (11, 15). The block is due, at least in part, to the binding of PU.1 to GATA-1 on DNA, creating a repressive chromatin structure in the vicinity of GATA-1 target genes, thereby inhibiting GATA-1 from promoting its erythroid-specific gene expression program (16, 17). Thus, PU.1 is a potent negative regulator of red blood cell terminal differentiation.

Normally, cell differentiation decisions are coordinated with the cell proliferation program. Both aspects are disrupted in malignant cells. Because PU.1 plays a central role in the development of murine erythroleukemias, we wondered whether, in addition to blocking differentiation, PU.1 might also stimulate proliferation of these cells. Studies of PU.1-deficient mice suggest a positive role for PU.1 in the proliferation of normal erythroid progenitors (18, 19). Interestingly, we reported previously that differentiation of erythroleukemia cells is accompanied by a rapid down-regulation of cyclin-dependent kinase 6 (CDK6), which parallels the decline in PU.1 (20). Moreover, enforced expression of CDK6, like PU.1, blocks MEL cell differentiation. This effect is highly specific, as the closely related CDK4 is not able to block differentiation (20). The unique activity of CDK6 in blocking MEL cell differentiation may not be confined to the erythroid lineage, as there are now two additional reports.

The abbreviations used are: MEL, murine erythroleukemia; ES-EP, embryonic stem cell-derived erythroid progenitor; FL-EP, fetal liver-derived erythroid progenitor; HA, hemagglutinin; ER, estrogen receptor; GST, glutathione S-transferase; CHIP, chromatin immunoprecipitation; siRNA, short interfering RNA; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter.
showing that CDK6 can block differentiation of an osteoblast cell line (21) and a myeloid cell line (22).

After finding that PU.1, a transcription factor, and CDK6, a G1 phase-specific cell cycle kinase, both can block erythroid differentiation, we were prompted to ask whether PU.1 controls cdk6 gene expression. We report here that PU.1 directly regulates transcription of the cdk6 gene in both normal erythroid progenitors and MEL cells, as well as in macrophage cell lines. Thus, PU.1 has a dual action in erythroid cells; (i) it binds to and inhibits GATA-1 from promoting erythroid differentiation; and (ii) it directly stimulates transcription of cdk6, an important component of the cell cycle machinery that promotes passage through the G1 phase of the cell cycle, the period when commitment to terminal differentiation occurs. These results illustrate how a master transcriptional regulator coordinates proliferation and differentiation in immature hematopoietic cells by regulating both tissue-specific and cell proliferation gene expression programs in these cells. The results also provide further insight into the central role of PU.1 in murine erythroleukemia, and they help to explain the proposed role for PU.1 in regulating proliferation of erythroid progenitors during stress erythropoiesis (18).

EXPERIMENTAL PROCEDURES

Cell Culture, Lentivirus Infection, and Differentiation—MEL cells (clone DS19), MEL cell transfectants PU.1-HA (clone 51) (11) and PU.1-ER (clone 9) (20), BAC-1.2F5 cells, and RAW 264.7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. 3,000 units/ml of 2u/ml epogen (Amgen), and 0.1% 26 M hexamethylene bisacetamide and treatment with 17 estradiol were as described previously (11, 20). Fetal liver erythroid progenitors (FL-EPs) were isolated, and FL-EP and ES-EP were prepared as detailed in supplemental “Experimental Procedures”. Reporter assays were performed by transfection of DNA constructs into HeLa cells essentially as described previously (25), with further details described in supplemental “Experimental Procedures”.

Electromobility Shift Assays—cdk6 promoter DNA fragments used for electromobility shift assays were generated by annealing complementary oligonucleotides containing either a wild-type cdk6 promoter sequence or ones in which the putative PU.1 binding site (in boldface type) was mutated (K6 wild-type: 5’-GTT-GCC-GCT-GCA-GAA-GCT-GGA-TGG-AG; K6 mutant: 5’-GTT-GCC-GCT-GCA-TTC-GCT-GGA-TGG-AG) and end-labeling with γ-32PATP (PerkinElmer Life Sciences) using polynucleotide kinase. 20 μl reaction mixtures contained 4 μl of 5× binding buffer (60% glycerol, 60 mM HEPES pH 7.5, 20 mM Tris, pH 8.0, 250 mM KCl, 5 mM EDTA, and 5 mM DTT), 2.5 μg of poly(dI-dC) (Amersham Biosciences), and 50 ng of purified glutathione S-transferase (GST) proteins (11). The reaction mixtures were incubated at 4 °C for 20 min; 100,000 cpm of labeled probe was added, and the incubation was continued at 4 °C for 30 min. 5 μl of 60% glycerol were added, and the mixtures were electrophoresed in 5% acrylamide gels in 10 mM Tris, pH 8.0, 2.5 mM EDTA for 4 h at 200 V at 4 °C, and radioautography was performed.

Quantitative Chromatin Immunoprecipitation (ChIP)—ChIP was carried out essentially as described previously (17) with the following modifications. Sonicated chromatin from 2.5 × 10⁶ cells was precultured for 2 h at 4 °C with Protein Aagarose beads (Roche). The mixture was centrifuged in a tabletop centrifuge at max speed at 4 °C for 5 min and incubated overnight at 4 °C with 2 μg of antiserum against PU.1 (Santa Cruz Biotechnology T-21) or HA (Santa Cruz Biotechnology Y11). The mixture was then centrifuged as in the preceding step and chromatin-antibody complexes were collected by incubation with Protein A-agarose beads for 90 min at 4 °C. Washes and isolation of DNA were performed as described previously (17). Quantitative PCR conditions and primers are described in supplemental “Experimental Procedures”.

siRNA Treatment—A 21-bp double stranded RNA oligonucleotide targeting PU.1 (5’-AAG-GAG-GUG-UCU-GAU-GGA-GAA-3’) and a control siRNA (5’-AAG-AGG-UAG-GGG-AAG-AGC-UAU-3’) were obtained from Qiagen. Cells were plated in 6-well plates at the concentration of 1 × 10⁵ cells/well. Transfection was carried out with 4 μl of oligofectamine (Invitrogen) and siRNA at 200 nm as described previously (26). RNA and protein extracts were prepared 24 h after transfection.

RESULTS

CDK6 Is the Dominant D-Type CDK in Proliferating Erythroid Cells—In previous work, we showed that CDK6 is the active D-type cyclin-dependent kinase in proliferating MEL blasts (20, 27). In contrast, CDK4 drives the final terminal cell divisions once the MEL cells have committed to reenter their differentiation program (28). To determine whether the same relationship between CDK6 and CDK4 exists in normal erythroid cells, we studied their properties in erythroid progenitors derived from murine embryonic stem cells (ES-EP) (23) and in
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**PU.1 Controls cdk6 Expression in Erythroid Cells**—During generation of erythroleukemia cells, expression of PU.1 is dysregulated by provirus insertion, leading to malignant transformation of erythroblasts. Surprisingly, proliferating ES-EP and FL-EP contain levels of PU.1 comparable to those in MEL cells (Fig. 1A and supplemental Fig. 1A). Transfer of FL-EP, ES-EP, or MEL cells to differentiation conditions leads to a rapid decline in the levels of PU.1 (Figs. 1A and 2A and supplemental Fig. 1A) (14). The level of CDK6 also declines during this period (Figs. 1A and 2A and supplemental Fig. 1A) (20, 28). To determine whether the decline of CDK6 is linked to the decline of PU.1, we examined cdk6 expression in MEL cell transfectants (MEL-PU.1-HA) that stably express exogenous HA-tagged PU.1. These transfectants express approximately equal amounts of exogenous PU.1-HA and endogenous PU.1, but because a constitutively active promoter was used for expression of exogenous PU.1-HA, the level of total PU.1 in these cells is maintained at a relatively high level in differentiation conditions (medium containing hexamethylene bisacetamide), and hence, the transfectants are blocked from differentiating (11). As shown in Fig. 2, A and B, in contrast to the response in MEL cells, the levels of CDK6 protein and mRNA do not decline in differentiation conditions in MEL-PU.1-HA transfectants, indicating that in MEL cells, cdk6 gene expression is dependent on the level of PU.1. Moreover, in these MEL-PU.1-HA transfectants even in proliferation conditions (t = 0), the CDK6 protein level is significantly higher, and the CDK6 mRNA level is markedly higher than in untransfected MEL cells. The observed effects of exogenous PU.1 on CDK6 mRNA and protein are specific, as the described effects were not observed for the mRNA of the highly related cyclin D-dependent kinase, CDK4 (Fig. 2, B and C). We also found that the CDK6 mRNA level in MEL cells declines when PU.1 synthesis is inhibited by RNA interference (see below).

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PU.1-activated transcription. Once again, the effect of PU.1-ER on the CDK6 mRNA level is specific, because treatment of untransfected MEL cells with estrogen did not lead to a change in the level of CDK6 mRNA, and treatment of PU.1-ER MEL cell transfectants with estrogen did not cause a change in the level of CDK4 mRNA (Fig. 2 C).

To determine whether PU.1 also regulates cdk6 transcription in normal erythroid cells, we studied cdk6 gene expression in ES-EP exposed to differentiation medium after they were infected with a lentivirus encoding PU.1 (and green fluorescent protein (GFP)) and in control cells infected with a lentivirus encoding only GFP. Expression of additional, exogenous PU.1 in ES-EP blocks the cells from differentiating, just as it does in MEL cells (supplemental Fig. 2). We used quantitative reverse transcription-PCR assays to quantitate the levels of CDK6 and CDK4 mRNAs in FACS-sorted GFP-positive cells (Fig. 3). In

cyte-macrophage colony stimulating factor receptor gene promoter, that was shown previously to be regulated by PU.1 (30), was used as a control. It was stimulated ~3-fold by PU.1, consistent with published data. A second control reporter plasmid consisted of a DNA fragment containing five copies of a PU.1 binding sequence (PU5) from the SV40 enhancer (31). Luciferase production from this reporter was stimulated by PU.1 ~20 fold (Fig. 4A). Among the cdk6 promoter-reporter constructs, the largest fold increase in PU.1-stimulated luciferase production was obtained with the 0.5-kb cdk6 reporter, which was stimulated up to 8.7 fold by PU.1, producing levels of luciferase considerably higher than that produced from the two control PU.1 reporter constructs. We also observed that the basal activity of the 4.8-kb cdk6 reporter is significantly lower than that of 1.5-kb cdk6 reporter, suggesting that there may be a repressive element located between −1.5 and −4.8 kb of the

FIGURE 2. The levels of CDK6 mRNA and protein in MEL cells are dependent on PU.1 expression. MEL cells and transfected MEL cells stably expressing exogenous PU.1-HA (clone 51) were cultured in media containing 5 mM hexamethylene bisacetamide. At the indicated times, total RNA and protein extracts were prepared and analyzed for the levels of the indicated proteins by immunoblotting (A) and the indicated RNA transcripts by RNA blot hybridization (B) as described under “Experimental Procedures.” MEL cells and transfected MEL cell stably expressing an exogenous PU.1-ER fusion protein (clone 9) were cultured in medium containing 17β-estradiol (C). At the indicated times, total RNA was prepared and analyzed for the indicated transcripts as in B. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PU.1 Regulates cdk6 – The preceding results suggest that transcription of the cdk6 gene is regulated by PU.1. To determine whether PU.1 directly activates the cdk6 promoter, we constructed a luciferase reporter plasmid containing the cdk6 gene promoter and then studied the effect of PU.1 on reporter gene activity in HeLa cells. A 4.8-kb DNA fragment containing the region upstream of the cdk6 gene and 50 bp of the 5’ untranslated region of the CDK6 mRNA was isolated from a BAC clone and cloned into the pGL3-basic luciferase reporter plasmid that lacks a transcriptional promoter. Two smaller fragments of the cdk6 5’-upstream region, 1.5 and 0.5 kb, were also subcloned into the pGL3-basic vector.

Cotransfection of any of the three reporter plasmids with various amounts of a plasmid encoding PU.1 led to dose-dependent stimulation of luciferase production (Fig. 4A). A luciferase reporter plasmid containing 400 bp of the granulocyte-macrophage colony stimulating factor receptor gene promoter, that was shown previously to be regulated by PU.1 (30), was used as a control. It was stimulated ~3-fold by PU.1, consistent with published data. A second control reporter plasmid consisted of a DNA fragment containing five copies of a PU.1 binding sequence (PU5) from the SV40 enhancer (31). Luciferase production from this reporter was stimulated by PU.1 ~20 fold (Fig. 4A). Among the cdk6 promoter-reporter constructs, the largest fold increase in PU.1-stimulated luciferase production was obtained with the 0.5-kb cdk6 reporter, which was stimulated up to 8.7 fold by PU.1, producing levels of luciferase considerably higher than that produced from the two control PU.1 reporter constructs. We also observed that the basal activity of the 4.8-kb cdk6 reporter is significantly lower than that of 1.5-kb cdk6 reporter, suggesting that there may be a repressive element located between −1.5 and −4.8 kb of the
**PU.1 Regulates cdk6**

**A.**

|       | CDK6                      |
|-------|---------------------------|
| GFP   |                           |
| PU.1(GFP) | 3 *****                   |
| Time in Differentiation Medium (Hours) | 0 24 36 |

**B.**

|       | CDK4                      |
|-------|---------------------------|
| GFP   |                           |
| PU.1(GFP) | 3 *****                   |
| Time in Differentiation Medium (Hours) | 0 24 36 |

**FIGURE 3. PU.1 stimulates cdk6 gene expression in normal erythroid cells.** ES-EP were cultured in erythroid expansion medium and infected with lentiviruses encoding PU.1 and GFP (PU.1(GFP)) or only GFP as described under “Experimental Procedures.” Three days after infection, cells were transferred to differentiation medium and, at the indicated times, GFP-positive cells were isolated by FACS, and total RNA was prepared. mRNA levels were analyzed by quantitative reverse-transcription-PCR as described under “Experimental Procedures.” Error bars indicate the S.D. of duplicate experiments. Single and triple asterisks indicate p values of <0.05 and <0.0005, respectively, using Student’s t test.

*cdk6* promoter. Because PU.1-stimulated luciferase production was highest with the 0.5-kb *cdk6* reporter, we reasoned that this region must contain one or more sites that can bind PU.1 and allow it to stimulate transcription.

To identify the PU.1-responsive region within the 0.5-kb *cdk6* promoter fragment, progressively smaller subfragments of the region were cloned into the reporter plasmid using a PCR-based strategy. We found that a reporter plasmid containing as little as 150 bp of the *cdk6* upstream region is stimulated by PU.1 to nearly the same extent as the 500-bp reporter plasmid (Fig. 4B). The basal activity of this construct is somewhat lower than the 500-bp reporter construct, but the fold activation by PU.1 is somewhat higher. Analysis of the DNA levels in the 150-bp region identified one potential PU.1 binding site (5’-AGAA-3’) 40 bp upstream of the transcription start site. PU.1 was first noted to bind to DNA sequences that include a purine-rich core (GGAA) (31). However, even the core sequence is not invariant, since it has been reported that several promoters, including the immunoglobulin J-chain gene (32), are regulated by PU.1 via a core sequence consisting of 5’-AGAA-3’. We changed the core sequence of the putative PU.1 binding site at −40 bp from AGAA to ATTC in the reporter construct containing the −150-bp fragment. This change caused a marked reduction in the PU.1-mediated stimulation of the promoter, from 11- to 2-fold (Fig. 4B). The residual 2-fold stimulation of the mutated reporter by PU.1 is likely a nonspecific effect as a control reporter plasmid, pGL3-SV40, containing the minimal SV40 early promoter without its enhancer, was stimulated to a similar extent.

To determine whether the sequence at −40 bp in the *cdk6* promoter actually binds PU.1, electrophoretic mobility shift assays were performed with a double stranded oligonucleotide probe corresponding to the sequence in this region. GST-tagged PU.1 (GST-PU.1), purified from bacteria, readily bound the probe, whereas it did not bind a mutated probe in which the AGAA core sequence was changed to ATTC (Fig. 4C). Control experiments showed that the binding was specific, as GST itself did not form a complex with the probe, and the complex with GST–PU.1 could be competed by addition of excess unlabeled probe.

**PU.1 Occupies the cdk6 Promoter in Normal Erythroid Progenitors and Erythroleukemia Cells**—The reporter assays presented in the previous section strongly indicate that PU.1 can directly regulate transcription of the *cdk6* gene. To prove that PU.1 actually occupies the *cdk6* promoter in erythroid cells, we carried out quantitative ChIP experiments with a PU.1 antisem. Quantitative ChIP was performed with chromatin from both ES-EP and MEL cells. In both types of cells, we observed occupancy of PU.1 in the region of the *cdk6* promoter just upstream of the transcription start site (Fig. 5). PU.1 was not found in regions 4 kb on either side of the *cdk6* promoter. It was also not found in the region of the myogenin gene promoter. We conclude that PU.1 occupies the *cdk6* promoter region in both normal and malignant erythroblasts.

**DISCUSSION**

PU.1 is a DNA binding transcription factor that is required for the development of myeloid cells and B cells. It has many well established gene targets that it regulates in these cells (reviewed in Ref. 7–9). Many of these PU.1-responsive genes are lineage-specific, in that they are ordinarily expressed primarily or exclusively in myeloid and/or B cells. On the other hand, we know relatively little about the role of PU.1 in control-
FIGURE 4. Identification of a PU.1-responsive binding sequence at −40 bp in the cdk6 gene promoter. A and B, a series of luciferase reporter plasmids containing various lengths of the cdk6 promoter region from −4.8 kb to −150 bp were constructed in pGL3-basic luciferase as described under “Experimental Procedures.” Luciferase reporter assays were carried out in the HeLa cells transfected with the indicated cdk6 promoter-reporter construct and the indicated amounts of DNA of pXM-PU.1 expressing PU.1 or the control empty vector (pXM). In B, 150 bp PU.1 mut indicates use of the luciferase reporter plasmid containing 150 bp of the cdk6 promoter in which a putative PU.1 response element (5′-AGAA-3′) was mutated to (5′-ATTC-3′) (mut). GM-CSFR, PU.1 × 5, and SV40 indicate use of control luciferase reporters as described under “Experimental Procedures.” Numbers above the filled bars indicate the fold stimulation by PU.1 over values without PU.1. C, electrophoretic mobility shift assays were performed as described under “Experimental Procedures” with the indicated GST proteins and 25-bp 32P-end labeled DNA probes. The probes corresponded to the sequence around −40 bp of the cdk6 promoter (wild-type) or that sequence in which a putative PU.1 response element (5′-AGAA-3′) was mutated to (5′-ATTC-3′) (mut). Where indicated, a 25-fold excess of unlabeled probe with the wild-type (WT) sequence was included in the reaction mixtures.
ling genes that are widely expressed, including genes involved in cell cycle regulation.

Several lines of evidence indicate that PU.1 plays an important role in regulating the proliferation versus differentiation decision in erythroid cells. Proviral insertions and dysregulation of PU.1 expression are found in 95% of murine erythroleukemias caused by the spleen-focus-forming virus component of Friend leukemia virus (13). These tumor cells are blocked from differentiating and exhibit uncontrolled proliferation. Reducing PU.1 levels in MEL cells by RNA interference is sufficient to cause the cells to resume differentiation and undergo terminal arrest (26, 34). Data reported here (Fig. 1 and supplemental Fig. 1) show that PU.1 levels in normal, proliferating erythroid progenitors are comparable to those in erythroleukemia cell lines, suggesting a normal role for PU.1 in immature erythroid cells. Consistent with this suggestion, adult mice having only a single functional allele of PU.1 are defective in stress erythropoiesis and PU.1-null fetal erythroid progenitors lose self-renewal capacity and undergo proliferation arrest, premature differentiation, and apoptosis (18). Other studies support a role for PU.1 in proliferation of erythroid progenitors in adult bone marrow (19). However, all of these effects of PU.1 in immature normal or malignant erythroid cells could be explained by its well established ability to bind to and inhibit the transcriptional activity of GATA-1. Moreover, GATA-1 has been shown to promote not only erythroid differentiation but also cell cycle arrest, including down-regulation of cdk6 (35). Thus, PU.1 could negatively regulate both erythroid differentiation and terminal cell division simply by inhibiting GATA-1. On the contrary, our results demonstrate that PU.1 directly activates the cdk6 gene, encoding the dominant, G1 phase D-type CDK in proliferating erythroid cells. Thus, we conclude that PU.1 has a dual action in proliferating erythroid cells and erythroleukemia cells (Fig. 7); (1) it associates with GATA-1 and inhibits GATA-1-mediated transcription; and (2) it binds to PU.1 binding sites.
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A. Proliferation
   - Erythroid Progenitors
   - Differentiating Erythroid Cells

B. Proliferation
   - Erythroid Progenitors
   - Differentiating Erythroid Cells

FIGURE 7. PU.1 has a dual action in proliferating erythroid cells and erythroleukemia cells. A, in proliferating erythroid cells and erythroleukemia cells, (i) PU.1 binds to GATA-1 on GATA-1 gene targets (e.g. globin genes) and inhibits GATA-1-mediated transcription, and (ii) PU.1 binds to and regulates direct PU.1 gene targets (e.g. cdk6). The dual action of PU.1 causes inhibition of differentiation and stimulation of proliferation. B, in differentiating erythroid cells, PU.1 levels decline leading to relief of PU.1-mediated inhibition of GATA-1 and loss of regulation of direct PU.1 gene targets. The absence of PU.1 results in terminal differentiation and loss of proliferation. See text for details.

in the promoters of certain genes, directly affecting transcription of these genes. A corollary of this hypothesis is that some of these genes encode molecules that promote cell proliferation, and some may also have the capacity to block differentiation. Interestingly, the cdk6 gene satisfies both criteria in erythroid cells (20).

The evidence presented here indicates that PU.1 directly regulates cdk6 gene expression in erythroid cells. Furthermore, we present data that PU.1 also controls cdk6 expression in myeloid cells. The evidence includes: (1) finding that CDK6 mRNA and protein levels are dependent on PU.1; (2) reporter assays showing that the cdk6 promoter is highly stimulated by expression of PU.1 in heterologous cells; (3) electromobility shift assays showing that PU.1 can bind to a purine-rich sequence (5’-AGAA-3’) 40-bp upstream of the cdk6 transcription start site and reporter assays showing that PU.1-mediated stimulation of transcription is strongly dependent on this sequence; (4) ChIP assays showing that PU.1 occupies this region in normal, proliferating erythroid progenitors and MEL cells. Although the PU.1-responsive sequence (5’-AGAA-3’) in the cdk6 promoter differs from the purine-rich core sequence (5’-GGAA-3’) found in many lineage-specific PU.1-responsive promoters, there are several other promoters that have been shown to be regulated by PU.1 via a (5’-AGAA-3’) sequence, including the lineage-specific promoters in the immunoglobulin J chain gene (32), the CD11b receptor gene (36), the macrophage scavenger receptor gene (37), the monocyte/neutrophil elastase inhibitor (38), the lge gene (39), and CD20 (40).

We think it is very likely that, besides cdk6, PU.1 also regulates many other widely expressed genes in erythroid cells, including genes that promote passage through the G1 phase of the cell cycle. Other important candidates for PU.1-mediated regulation are the c-myc and cyclin D2 genes, both of which were found to be down-regulated during GATA-1 stimulated red blood cell differentiation (25, 35). It is noteworthy that c-Myc, like CDK6, can block MEL cell differentiation (41–44). It is also interesting that CDK6 is primarily associated with cyclin D2 in proliferating erythroid progenitors and that it is down regulated along with CDK6 as the cells differentiate (Fig. 1 and supplemental Fig. 1). We reported previously that c-myc expression is dependent on PU.1 levels in MEL cells (14), and preliminary ChIP experiments indicate that PU.1 occupies the c-Myc and cyclin D2 promoters in proliferating erythroid progenitors and MEL cells.5

The ability of PU.1 to regulate the synthesis of key G1 phase cell cycle regulators, like CDK6, is consistent with the role of PU.1 in inhibiting erythroid differentiation. Numerous studies have suggested that the decision to differentiate is often made in the G1 phase (45–47) (47), and erythropoietin-induced differentiation has been reported to require prolongation of the G1 phase (48). Moreover, we reported previously that p21 can trigger MEL cell differentiation, which involves inhibition of CDK6 activity, but only when the cells are in the G1 phase of the cell cycle (27). The results reported here deepen our understanding and appreciation for the normal roles of PU.1 in erythroid cells. They indicate that PU.1 does not simply antagonize the actions of GATA-1 in promoting erythroid differentiation. Rather, we believe that PU.1 plays an active role in erythroid cells by stimulating the synthesis of factors that promote passage through the G1 phase, when the proliferation versus differentiation decision is made. Our results provide a molecular basis for understanding how PU.1 contributes to self-renewal of erythroid progenitors and their proliferation in response to specific cytokine signaling and erythropoietic stress (18, 19).

Although high levels of PU.1 promote macrophage differentiation, our finding that PU.1 also controls cdk6 gene expression in two macrophage cell lines suggests that PU.1 may stimulate proliferation at specific stages of myeloid development. Indeed, PU.1 has been reported to regulate cytokine-dependent proliferation of myeloid progenitors and macrophage through effects on the expression of cytokine receptors (33, 49). Further studies of PU.1-directed gene expression in both erythroid and myeloid cells should reveal additional ways in which PU.1 regulates the proliferation program, and possibly other general cellular programs, in both hematopoietic lineages.

5 S. N. Wontakal and A. I. Skoultchi, unpublished observations.
PU.1 Regulates cdk6

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