The role of junB as a regulator of erythroid cell survival, proliferation, and differentiation was tested by controlled expression of JunB in erythropoietin (EPO)-dependent erythroleukemia cell line HCD57. JunB induced erythroid differentiation as evidenced by increased expression of the erythroid-specific proteins β-globin, spectrin-α, and TER-119. Expression of JunB for at least 48 h was required for the differentiated phenotype to emerge. Differentiation was accompanied by a slower rate of proliferation and an increase in the expression of the cell cycle inhibitory protein p27. p27 protein expression increased due to reduced turnover without changes in transcription, indicating global changes in cell physiology following JunB induction. JunB expression was also studied in mouse and human primary erythroid cells. JunB expression increased immediately in both primary mouse cells and HCD57 cells treated with EPO and quickly returned to base-line levels, followed by a secondary rise in JunB in primary erythroid cells, but not in HCD57 cells, 36–48 h later. This result suggested that the initial EPO-dependent JunB induction was not sufficient to induce differentiation, but that the late EPO-independent JunB expression in primary erythroid cells was necessary for differentiation. This study suggests that JunB is an important regulator of erythroid differentiation.

Erythropoietin (EPO) is the primary hormone that regulates the production of erythroid cells in mammals. Conflicting theories propose that EPO may be instructive in directing uncommitted cells into erythroid maturation or alternatively that EPO is permissive only in allowing the maturation of cells that spontaneously commit to erythroid development. Whereas it is possible that EPO acts to promote the proliferation, survival, and differentiation of cells committed to erythroid development, some workers have hypothesized that EPO acts primarily as a survival factor for erythroblasts by preventing the cells from undergoing programmed cell death or apoptosis (1). This view is supported by the development of relatively mature erythroid cells at the colony-forming unit-erythroid (cfu-E) stage of differentiation in fetal EPO null or EPO receptor −/− null mice; however, erythroblasts undergo apoptosis in fetal mice with deletion of either EPO or the EPO receptor (2). The results show that EPO is absolutely necessary only during a small window in this process of erythroid maturation: cells at the cfu-E to proerythroblast stage of differentiation. If EPO is absent or suboptimal, these cells undergo apoptosis. If EPO is sufficient, these cells survive to mature to erythroblasts. Erythroblasts will continue to terminally differentiate even if EPO is absent. In murine erythroblasts, this differentiation is accompanied by an increase in the expression of the anti-apoptotic protein Bcl-xL (3). It is not yet clear if intracellular signals from the EPO receptor activated by EPO are limited to promoting survival or whether these EPO-dependent signals are necessary for continued proliferation and later differentiation characterized by induction of genes coding for erythrocyte membrane-specific protein and hemoglobin. Whereas the regulation of the expression of these erythroid-specific proteins by transcription factors such as GATA-1 and erythroid Kruppel-like factor has been described, regulators upstream of these events are not well characterized. A few in vitro EPO-induced erythroid differentiation models exist (4, 5), and erythroleukemic cells respond to chemicals such as Me2SO and hexamethylenebisacetamide by undergoing partial erythroid differentiation (6, 7). Expression of the EPO receptor in Ba/F3 cells results in EPO-dependent transcription of the β-globin gene (8). These observations suggest a possible requirement for EPO-dependent signals that modulate transcription of erythroid genes at some point in erythroid maturation. JunB is a member of the activator protein-1 (AP-1) family of transcription factors that binds to a specific DNA sequence, the 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE), to activate transcription of target genes. The AP-1 family consists of the Fos (c-Fos, Fra-1, Fra-2, and FosB) and Jun (c-Jun, JunB, and JunD) proteins, which are known as "early-response" proteins due to their up-regulation in response to extracellular stimuli affecting cell growth, differentiation, or survival. Inactivation of JunB in mice leads to embryonic lethality between embryonic days 6 and 8 due to vascular defects (9). JunB appears to function as a negative regulator of a number of cell systems, including proliferation in response to negative growth factors (10–13). JunB has also been shown to negatively affect cell cycle progression (14, 15) and cell survival (16). There is also evidence that JunB may play a central role in differentiation and growth arrest during hematopoiesis. In M1 mouse myeloid leukemic cells, induction of differentiation with either chemicals or cytokines is associated with an increase in junB expression (17–19). There is also
Role of JunB in Erythroid Differentiation

some evidence that JunB may play a role in the differentiation of erythroid cells. JunB expression and DNA binding have been detected in Friend murine erythroleukemia-C cells induced to differentiate with Me₂SO or hexamethylenebisacetamide, but not in control cells (6, 7).

Our laboratory uses the EPO-dependent murine erythroid cell line HCD57 as a model cell system for the study of erythroid proliferation, survival, and apoptosis. These cells depend on EPO for survival and proliferation, but do not differentiate in the presence of EPO. We have demonstrated that AP-1 DNA-binding activity induced in the growth factor withdrawal state is associated with an increase in junB message, JunB protein, and DNA-binding activity. Stable expression of a dominant-negative AP-1 mutant prevents apoptosis of HCD57 cells in the absence of EPO and prevents the down-regulation of the anti-apoptotic protein Bcl-xL (16). Therefore, in the absence of EPO, junB expression correlates with the induction of apoptosis. To further investigate the role of junB in erythropoiesis, we were interested in the effect JunB expression might have in the presence of EPO. In this study, we show that, in the presence of EPO, overexpression of junB induces differentiation of HCD57 cells. Furthermore, we found that JunB expression is associated with terminal differentiation of both mouse and human primary erythroid progenitors, thereby suggesting an additional role for JunB in the regulation of erythropoiesis.

MATERIALS AND METHODS

Cell Culture and Creation of the HCD57–JunB Cell Line—HCD57(K) cells (described previously (20)) stably expressing the pTET-Off transactivator plasmid were a gift from Dr. Steve Brandt (Vanderbilt University) and were cultured in Iscove’s modified Dulbecco’s medium (Invitrogen) containing 30% fetal calf serum, 50 μg/ml Geneticin (Invitrogen), and 10 μg/ml gentamycin at 37 °C in a 5% CO₂ environment and maintained in 2 units of EPO/ml of medium. To create the tetra-cycline (Tc)-inducible pTRE-JunB plasmid, the human junB gene was excised from the parental pGEM4-JunB cDNA on a SacI/RI restriction fragment. The ends of the fragment were filled in with Klenow DNA polymerase. EcoRI/NorI adaptors (Invitrogen) were ligated onto the fragment, and this fragment was ligated into the unique EcoRI site of the pTRE plasmid. The resulting pTRE-JunB plasmid was cotransfected with the p2.3-K-Hygro retroviral expression vector into the HCD57(K) cells using DIMRIE-C reagent (Invitrogen), and stable clones were selected by limiting dilution in the medium described above supplemented with 400 μg/ml hygromycin and 2 μg/ml tetracycline (to repress junB expression). Hygromycin-resistant cell lines were then cultured for 24 h in the absence of Tc and screened for JunB expression by Northern blot analysis using the junB cDNA as a probe. The clone used in these studies was stably expressing of junB and was designated HCD57-JunB. For each time point, 5 × 10⁶ HCD57-JunB cells were used. For induction of junB expression, the cells were washed three times with serum-free medium and incubated in Iscove’s modified Dulbecco’s medium containing 30% fetal calf serum, 50 μg/ml Geneticin, and 2 units/ml EPO, but in the absence of Tc, for the times indicated in the figure legends. For the electrophoretic mobility shift assay, HCD57-JunB cells were washed to remove Tc as indicated above and then incubated in medium either supplemented with 2 μg/ml Tc (to repress JunB expression) in concentrations of Tc indicated in the figure legends or without Tc to induce JunB expression. For the protein turnover studies, the HCD57-JunB cells were cultured for 48 h in the absence or presence of Tc, followed by treatment with 100 μM cycloheximide for the times indicated. To test the effect of length of JunB expression on differentiation, cells were washed to remove Tc from the medium, and then Tc was re-added to the medium at the times indicated. junB mRNA expression ceased within 4 h of Tc re-addition (data not shown). Nuclear extracts and total cell extracts were prepared as previously described (21), and 20 μg of protein were subjected to Western blot analysis with anti-JunB (N-17), anti-actin (J-19), anti-p16 (M-166), and anti-phospho-p27 (C-20) antibodies, or actin antibodies (Santa Cruz Biotechnology) or mouse monoclonal anti-p27 antibody (Transduction Laboratories). Western-blotted proteins were visualized using enhanced chemiluminescence (Amersham Biosciences, Inc.). The level of p27 protein expression during cycloheximide treatment was quantitated using a scanning densitometer and analyzed using ImageQuant software (Molecular Dynamics, Inc.). For the cell viability studies, cells were washed with medium as indicated above and cultured at 1 × 10⁶ cells/ml in the presence of EPO and in the absence or presence of 2 μg/ml Tc. Cell viability was determined by counting cells in a hemocytometer in the presence of 0.2% trypan blue.

Isolation and Culture of Primary Erythroid Cells—Primary murine erythroid progenitors infected with the anemia-inducing strain of the Friend virus (FVA cells) were isolated as previously described (3), and 10⁶ FVA cells were cultured in EPO for 0, 2, 4, 8, 24, 36, and 48 h. Primary human erythroid progenitors were derived by in vitro culture of CD34° cells isolated from peripheral blood. Growth factor-mobilized peripheral blood collected from normal donors was purchased from the California Research Institute (Berkeley, CA). CD34° cells were isolated from growth factor-mobilized peripheral blood cells using the antibody-coated paramagnetic microbeads in the CliniMACSTM cell isolation device (Miltenyi Biotec, Inc., Auburn, CA). The isolated cells were >95% positive for CD34° as determined by flow cytometry and were cultured for 7–8 days to obtain highly purified erythroid progenitors that were at the cfu-E stage of differentiation. The cell culture medium contained 15% fetal calf serum, 15% human AB serum, Iscove’s modified Dulbecco’s medium, 100 units/ml penicillin, 10 ng/ml streptomycin, 10 ng/ml interleukin-3, 2 units/ml EPO, 50 ng/ml stem cell factor, and 50 ng/ml insulin-like growth factor-1. To facilitate terminal differentiation, stem cell factor was omitted from the culture medium beyond day 5 of culture. >90% of these cells were positive for CD71 and glycosphingolipids of type A produced by flow cytometry and were then isolated in methylcellulose. Day 7, 9, and 14 cfu-E cells are at the basophilic, polychromatophilic, and orthochromatoc stages of differentiation, respectively.

Northern Blot Analysis—Following treatment of the cells, total RNA was isolated using the RNeasy miniprep kit (QiAGEN Inc.), and 10 μg of total RNA were subjected to Northern blot analysis as previously described (16). cDNA probes to junB or β-globin (kindly provided by Dr. Joyce Lloyd, Virginia Commonwealth University) were labeled with α³²PdCTP using the random priming method (Strategene). Filters were hybridized to the probes as previously described (16). The blots were washed under high stringency conditions and visualized with autoradiography for 3 days at −80 °C. Changes in mRNA expression were quantified using the Cyclone phosphomager and OptiQUEST Image analysis software (Becton Dickinson).

RbNase Protection Analysis—For RbNase protection of AP-1 and bcl family members, the murine AP-1 and APO-2 RbNase protection templates, respectively (both from BD Pharmingen), were transcribed in vitro using the Riboquant in vitro transcription kit (BD Pharmingen). 5 μg of total RNA isolated from HCD57-JunB cells were subjected to RbNase protection kit (BioTech, Inc.) and resolved on a 5% polyacrylamide gel containing 7 M urea and 0.5% Triton X-100.

AP-1 DNA Binding Studies and Supershift Assay—Electrophoretic mobility shift assays and supershift assays were conducted as previously described (16) using an electrophoretic mobility shift assay (Stratagene). 10 μg of nuclear extract were incubated with an [γ-³²P]ATP-labeled DNA fragment corresponding to the TRE (sense strand, 5′-CTAGTGATGACTGACGCGACGTAC-3′) at 4 °C in the absence of presence of 10× unlabeled TRE and subjected to electrophoresis at 25 mA for 2.5 h at 4 °C on 7% polyacrylamide gels. The gels were dried in vacuo for 1.5 h and exposed to x-ray film overnight at room temperature. For the supershift assay, 5 μg of nuclear extract were preincubated with 4 μg of anti-JunB antibody (Genexa Biotechnologies, Inc.) for 1 h at 4 °C prior to incubation with the [γ-³²P]ATP-labeled TRE for 15 min at 4 °C and electrophoresis at 4 °C as described above. Dried gels were exposed to x-ray film for 2–3 days at −80 °C.

Flow Cytometry Analysis—Differentiation of HCD57-JunB cells was measured by detection of the mature erythro-specific marker TER-119 using flow cytometry. 2 × 10⁶ HCD57-JunB cells cultured with junB induced or uninduced for 96 h were washed once with fluorescence-activated cell sorter buffer (1× phosphate-buffered saline, 2% fetal calf serum, and 0.1% sodium azide) and preincubulated with 20 μg/ml anti-Fcγ receptor II blocking antibody 2.4G2 (BD Pharmingen) for 15 min at 4 °C. The cells were then incubated with either phycoerythrin-labeled rat IgG (BD Pharmingen) or phycoerythrin-labeled rat anti-TER-119 antibody (BD Pharmingen) at a final concentration of 2 μg/ml for 30 min at 4 °C. The cells were washed twice with fluorescence-activated cell sorter buffer and then subjected to flow cytometry using the FACSscan flow cytometer.

DNA Fragmentation Studies—For DNA fragmentation studies, cells were cultured at 1 × 10⁶ cells/ml in the absence or presence of 1 unit/ml EPO or in the presence of EPO and in the absence or presence of Tc. 2 × 10⁶ cells were harvested at 24-h intervals, and genomic DNA was...
isolated from the cells using the Omniprep genomic DNA isolation kit (Genotech, Inc). 10 μg of genomic DNA were resolved on a 2.25% agarose gel containing 1× Tris acetate/EDTA and 300 ng/ml ethidium bromide. DNA ladder ing indicative of apoptosis was visualized using ultraviolet light.

In Vitro Kinase Assay—HCD57-JunB cells were cultured in the presence of 2 units/ml EPO and in the absence (junB expression-induced) or presence (junB expression-suppressed) of Tc for 48 h. 500 μg of cell extracts isolated from 5 × 10⁶ cells were subjected to immunoprecipitation as previously described (22) with anti-Cdk2 (M-2) or anti-cyclin E (M-20) antibody (Santa Cruz Biotechnology) and protein A-agarose (Transduction Laboratories). Anti-Cdk2 and anti-cyclin E antibody-immunoprecipitated proteins were concentrated in 25 μl of lysis buffer and subjected to an in vitro kinase assay as previously described (22) using 5 μg of histone H1 (Sigma) as a substrate. Following electrophoresis of the samples on a 12% SDS-polyacrylamide gel, the proteins were transferred to nitrocellulose and exposed to autoradiography for 18 h at −80 °C with an intensifying screen to visualize phosphorylated histone H1. The blots were then probed with monoclonal anti-p27 antibody and finally with anti-Cdk2 or anti-cyclin E antibody to ensure equal loading of proteins.

RESULTS

To investigate the role of JunB in erythropoiesis, the junB gene was cloned downstream of a Tc-inducible promoter and stably transfected into HCD57(K) cells, which undergo massive apoptosis within 24 h of EPO withdrawal (20). We have designated the cell line used in this study HCD57-JunB. Removal of Tc from the cells resulted in rapid up-regulation of the junB message. junB expression was detected within 3 h of Tc removal and reached its maximum 12 h after removal of Tc (Fig. 1A). Overexpression of this Northern blot detected low endogenous junB expression in this cell line (data not shown) comparable to that seen in our previous studies (16). Western blot analysis revealed an increase in JunB protein that correlated with the increase in junB mRNA expression; we consistently observed an ~10-fold increase in JunB protein 24 h after Tc withdrawal (Fig. 1B, lane E). An electrophoretic mobility shift assay carried out with HCD57-JunB cells washed and cultured in fresh medium containing EPO in the absence (lanes 1–6) or presence (lanes 7–12) of JunB induction (JunB (ind.) by removal of Tc. The solid arrow indicates the serum-stimulated AP-1 shift. Brackets indicates shifts produced by induction of JunB expression. cc indicates the addition of cold (unlabeled) competitor to the reaction (lanes 6 and 12). Supershift analysis was carried out with nuclear extracts from HCD57-JunB cells cultured in the presence (lanes 13 and 14) or presence (lanes 15 and 16) of JunB induction for 24 h and incubated with normal rabbit serum (lanes 13 and 15) or a junB-specific antibody (ab; lanes 14 and 16). Dashed arrow indicates the supershift of the lower two bands of the AP-1 complex. The asterisk indicates loss of the uppermost shifted band in the presence of antibody.
that associates with glycophorin A (23), was investigated. Flow cytometry revealed that the entire population of HCD57 cells expressing junB exhibited an increase in the surface expression of TER-119 (Fig. 2A, arrow).

The differentiated phenotype of HCD57-JunB cells was further explored by assessing the expression of three markers of erythroid differentiation: /H9252-globin, a key component of the hemoglobin molecule, and spectrin-α and spectrin-β, two proteins necessary for the assembly of the erythrocyte membrane. Northern blot analysis revealed that /H9252-globin expression was strongly induced by 48 h following JunB induction, with expression still increasing at 96 h when the experiment was stopped (Fig. 2B). Varying the expression of JunB by varying the concentration of Tc in the culture medium revealed that the increased expression of /H9252-globin correlated with the level of JunB expression (Fig. 2C). Western blot analysis revealed that spectrin-β expression was high in the absence and unaltered by the induction of junB expression (Fig. 2D, lower panel); by contrast, the expression of spectrin-α was induced by junB expression with the same kinetics as the emergence of /H9252-globin expression (upper panel). Therefore, HCD57 leukemic cells express some erythroid-specific markers (spectrin-β, /H9252-globin, and TER-119).

junB, like all AP-1 genes, is thought of as an “immediate-early” gene, having its effect soon after its activation by growth factors or stress. We were therefore interested in whether the induction of differentiation was caused by immediate-early induction of junB expression or whether a longer period of junB expression was necessary for the differentiated phenotype. When junB expression was induced for 7 h and then suppressed by re-addition of Tc to the medium, the HCD57-JunB cells did not differentiate, as shown by a lack of increase in /H9252-globin expression (Fig. 2E). junB had to be expressed for at
least 48 h for maximum β-globin expression to occur (Fig. 2D, lane E). Therefore, it appears that long-term junB expression is necessary for differentiation of HCD57 cells.

The effect of long-term expression of junB on the erythroleukemic cells led us to investigate whether similar expression patterns might be seen in differentiating primary erythroid cells. junB expression was assessed in primary murine erythroid progenitors infected with the anemia-inducing strain of the Friend spleen focus-forming virus (FVA cells), which begin terminal differentiation within 48 h after treatment with EPO. Northern blot analysis showed that FVA cells induced to differentiate in the presence of EPO exhibited both an initial increase in mRNA expression of junB and a later increase 36–48 h after addition of EPO (Fig. 3A). By contrast, HCD57 cells deprived of EPO and then stimulated with EPO showed a similar initial increase in junB expression 1 h after EPO induction (Fig. 3A, lane I); this increased junB expression rapidly decreased and did not increase again. This experiment was repeated using RNase protection analysis, and the expression of junB and the housekeeping gene gapdh was quantified. Immediately following treatment with EPO, junB expression increased ~5-fold in both FVA and HCD57 cells. During terminal differentiation of FVA cells (24–48 h after EPO addition), junB expression increased ~5-fold relative to total RNA, whereas gapdh expression decreased to 30% of the starting levels. HCD57 erythroleukemic cells showed no such increase in junB expression during this time period. We then investigated junB expression in normal human colony-forming cells cultured in EPO. For this experiment, CD34+ early hematopoietic cells were cultured under conditions that promote the development of cfu-E cells. The cfu-E (day 7) cells were then further cultured to allow terminal differentiation to occur. junB expression was low during the early cfu-E stage of differentiation (the proerythroblast stage), but increased by day 9 (the polychromatophilic stage) and was still elevated in day 14 terminally differentiated cells (the orthochromatase stage) (Fig. 3B). Therefore, in both murine and human primary erythroid progenitors, elevated junB expression is observed during differentiation of the cells.

Other hallmarks of differentiation include suppression of proliferation and potential changes in cell cycle regulatory proteins. We therefore investigated the expression of c-jun in the HCD57-JunB cells because we have previously shown a role for c-jun in the proliferation of HCD57 cells (16). RNase protection analysis of the expression of all jun and fos family members during the induction of junB revealed a transient increase in the expression of c-jun, junD, and junB 1 h after fresh medium was added, with no significant changes in expression thereafter and an increase in c-fos expression during the first 48 h after removal of Tc (Fig. 4). The stably transfected junB gene is the human junB gene, so only the native murine junB expression induced was observed in this experiment. No changes in other AP-1 family members (fra-1, fra-2, and fosB) were detected (data not shown). An examination of the growth properties of HCD57-JunB cells cultured in the absence and presence of the induction of junB expression revealed that these cells proliferated more slowly in response to EPO when junB was expressed (Fig. 5A). 48 h after junB expression was induced, proliferation decreased ~50% compared with cells cultured in the absence of JunB. This 50% decrease was maintained throughout the rest of the time course. Therefore, JunB expression does not simply result in the expression of markers of mature erythroid cells, but reflects other aspects of differentiation, including inhibition of proliferation of HCD57 cells.

When we examined the cell cycle state of junB-expressing HCD57 cells using flow cytometry analysis of propidium iodide-stained cells, no cell cycle arrest was detected; however, a slight increase in the percentage of cells in G2/M led us to investigate potential changes in the expression of cell cycle regulators. Proteins were screened for changes in expression during the induction of JunB expression by Western blot analysis. No changes in expression were detected in the p16 protein (Fig. 5B) or in the p21, p57, and p36 proteins (data not shown). By contrast, an increase in p27 protein expression was detected 24 h after JunB expression was induced (Fig. 5B, upper panel). Therefore, one of the ways that junB may slow cell cycle progression is to increase the expression of p27, which is known to
inhibit the activity of cyclin E and its associated cyclin-dependent kinase Cdk2 and Cdk4. An in vitro kinase assay using anti-Cdk2 and anti-cyclin E immunoprecipitates and histone H1 as a substrate revealed that JunB expression resulted in a decrease in Cdk2- and cyclin E-associated kinase activity (Fig. 5C, lanes B and H) and an increase in the association of p27 with these proteins (lanes D and J). These results suggest that one mechanism for the retardation of proliferation seen in junB-expressing cells is inhibition of Cdk2 kinase activity.

p27 expression is regulated at both the transcriptional and post-transcriptional levels (24–27). The mechanism by which junB regulates p27 expression in HCD57-JunB cells was therefore explored. No changes in the mRNA expression of p27 upon junB induction were detected (data not shown). Potential post-transcriptional changes in p27 expression were then investigated by treatment of junB- and non-junB-expressing HCD57 cells with cycloheximide and measuring the rate of protein turnover. HCD57 cells expressing JUNB for 48 h showed a much slower rate of p27 turnover compared with cells in which JUNB was not expressed (Fig. 5D). The half-life of p27 in cells cultured without JUNB expression was ~20 min, whereas the half-life of p27 cultured in the presence of JUNB expression was ~120 min. Therefore, it appears that one mechanism by which junB increases p27 protein levels is by protein stabilization.

In the absence of EPO, the induction of apoptosis of erythroid cell expression is associated with an increase in junB expression and a decrease in the expression of the anti-apoptotic protein Bcl-XL (16). To investigate the apoptotic state of the junB-expressing HCD57 cells cultured in EPO, genomic DNA was isolated from cells cultured in the presence of EPO with JunB expression over a 96-h time period and examined by agarose gel electrophoresis. HCD57-JunB cells deprived of EPO for 24 h exhibited the characteristic DNA laddering, indicating that the cells underwent apoptosis rapidly in the absence of EPO (Fig. 6A, lane B). junB expression for 96 h induced only a small amount of DNA laddering (Fig. 6A, lane G). Additional flow cytometry experiments quantified the increase as ~10% of the total DNA with sub-G0/G1 DNA (data not shown). Expression of the anti-apoptotic gene bcl-XL increased ~8-fold as junB was expressed (Fig. 6B, lane J, upper arrow); no changes in mRNA expression were observed in other bcl family members, including the pro-apoptotic gene bad (Fig. 6B, lower arrow). Taken together, these results indicate that, in the presence of EPO, JunB does not appear to directly induce apoptosis.

**DISCUSSION**

We have created a system in which junB expression can be tightly regulated in an erythroid cell line, allowing us to study the role of JunB in erythropoiesis. Culture of HCD57-JunB cells in the absence of Tc led to a rapid and sustained increase in AP-1 DNA-binding activity due to the presence of JunB in the AP-1 complex. The weak AP-1 DNA binding observed 3 h after medium with Tc was added to the HCD57-JunB cells (Fig. 1C, lane 3) may have been due to transient increases in c-jun, junD, and junB expression induced by the addition of fresh serum (Fig. 4).

Expression of JunB induced differentiation of HCD57 cells as evidenced by increases in the expression of known important markers of erythroid differentiation, including β-globin and spectrin-α. Decreasing the expression of JunB resulted in a corresponding decrease in β-globin expression (Fig. 2C), thus correlating the expression of JunB with the degree of differentiation. Spectrin-β, another erythroid protein, was already present in the HCD57-JunB cells and did not increase upon JunB expression. Therefore, this erythroleukemic cell line has retained some ability to differentiate, but cannot synthesize hemoglobin or express spectrin-α, functions that are restored by the reintroduction of JunB expression. The HCD57-JunB cells did not enucleate (data not shown); therefore, JunB expression did not cause terminal differentiation of HCD57 cells, but restored part of the differentiation program to the erythroleukemic cells.

A number of results imply a correlation between long-term junB expression and erythroid differentiation. First, both human and murine primary erythroid progenitors differentiating in the presence of EPO exhibited long-term junB expression during terminal differentiation. Second, the early, EPO-dependent junB expression detected in HCD57(K) erythroleukemic cells was not sufficient to induce differentiation. Third, expression of junB was required for at least 48 h for maximum β-globin expression to occur in the HCD57-JunB cells (Fig. 2E). Therefore, it was the reintroduction of prolonged JunB expression that partially restored the differentiation program in the HCD57 erythroleukemic cells. The biphasic nature of junB expression in the FVA cells suggests that there may be two modes of junB induction: an early, EPO-dependent induction and a later induction. This later induction may not be EPO-dependent because FVA cells no longer respond to EPO during the terminal stages of differentiation. Therefore, the long-term junB expression may be part of the differentiation program and not induced directly by EPO. It cannot be ruled out that JunB expression increased as a result of differentiation in FVA and cfu-E cells and did not cause differentiation in these cells. However, induction of differentiation by human junB did not cause an increase in endogenous murine junB expression in HCD57 cells (Fig. 4, lanes G and H), suggesting that an increase in junB expression is not a by-product of differentiation. Because the differentiation is incomplete, however, we cannot discount the possibility that junB expression may increase as a result of an additional differentiation mechanism.

The fact that junB expression affects not only the expression of markers of erythroid differentiation, but also the proliferative state of the cells, is further evidence that JunB promotes differentiation on a global scale. The accumulation of p27 protein during erythroid cell differentiation is consistent with previous reports on the differentiation of primary FVA cells (28) and on an EPO-dependent cell line (4). The results presented here imply an increase in JunB expression upstream of the p27 protein accumulation due to a decreased rate of protein turnover. It is interesting that we also observed no increase in p16 expression given the recent observations that JunB can

![Fig. 4. Effect of junB on the expression of other AP-1 family members.](Image 92x613 to 254x729)
inhibit proliferation by increasing p16 mRNA expression in fibroblasts (14). This discrepancy may be explained by differences in the expression of junB binding partners between the cell lines or by cell type-specific regulation of p16 expression. An alternative explanation could be that, in erythroid cells, JunB appears to be part of a broader differentiation program and therefore may not directly affect the expression of cell cycle-related genes. A recent report showed that reintroduction of junB into junB−/− mice rescues the embryonic lethality phenotype of the knockout mice, but the adult mice develop a hyperproliferative disease due to loss of the junB transgene in myeloid cells (29). JunB was not lost in erythroid cells, so it is difficult to say what the effect loss of junB might have on adult erythropoiesis in these transgenic mice.

JunB clearly has different effects on erythroid cells in the presence of EPO compared with its absence. HCD57(K) cells underwent apoptosis rapidly in response to EPO withdrawal (Fig. 6A). Overexpression of junB for 48 h prior to EPO withdrawal had a minimal effect on the number of apoptotic cells 6–12 h after EPO was withdrawn compared with cells that did not express junB (data not shown). Because junB expression is induced when EPO is withdrawn from these cells (16), it is probable that this normal amount of junB is sufficient to induce apoptosis and that additional induction of junB does not further contribute to cell death. By contrast, a low level of significant and reproducible apoptosis (≤10%) was observed when JunB was induced in the presence of EPO (Fig. 5A); this low level of apoptosis may occur as a result of cells failing to complete differentiation. Furthermore, bcl-xL expression decreased during the induction of apoptosis upon EPO withdrawal, yet bcl-xL mRNA levels increased when JunB was induced in the presence of EPO. One explanation for these differences could be the availability of binding partners for JunB in the absence and presence of EPO: in the absence of
EPO, only FosB and JunD were available (Fig. 2B) (16), whereas c-Fos, c-Jun, and activating transcription factor-1 and -2 were also available for association with JunB in the presence of EPO. These observations raise the interesting possibility that junB may have a dual role in the regulation of erythroid cell maturation and survival. In the presence of EPO, JunB may be an inducer of apoptosis by inhibiting the expression of survival proteins such as Bcl-xL. In the presence of EPO, JunB may function as part of the differentiation program. Overexpression of junB may restore a loss of prolonged JunB expression necessary for the differentiation of HCD57 cells. Alternatively, prolonged junB expression may replace the loss of another transcription factor that complexes with AP-1 family members and is important in erythroid differentiation. Given that JunB may act as a potent inhibitor of c-Jun transactivation and transformation (30), JunB may promote differentiation by inhibiting the activity of c-Jun, which we have shown to be important in EPO-induced proliferation (16), or by antagonizing the activity of c-Jun at the promoter of cell cycle regulatory genes that have been shown to be activated by c-Jun (31–33). Conversely, JunB may cooperate with c-Jun and other AP-1 family members to activate the transcription of genes necessary for differentiation.

The question remains whether EPO has a directive or permissive role in erythropoiesis. The facts that 1) HCD57 cells do not undergo apoptosis due to JunB expression but are protected from apoptosis by EPO and 2) the long-term increase in junB expression during terminal differentiation may be EPO-independent suggest that, rather than directing JunB to promote differentiation, EPO permits the cells to survive, thus allowing the differentiation program (including JunB expression) to promote differentiation.

In summary, our data support a growing amount of evidence that junB is an important regulator of differentiation of hematopoietic cells. HCD57 cells are derived from a leukemic mouse; therefore, by their very nature, these cells do not differentiate. We have been able to partially differentiate these cells. This is a significant accomplishment and may give clues as to how these cells became leukemic and how the leukemic phenotype might be reversed.

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