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Expression of Basic Helix-Loop-Helix Transcription Factors in Explant Hematopoietic Progenitors

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Abstract The basic helix-loop-helix (bHLH) transcription factors form heterodimers and control steps in cellular differentiation. We have studied four bHLH transcription factors, SCL, lyl-1, E12/E47, and Id-1, in individual lineage-defined progenitors and hematopoietic growth factor–dependent cell lines, evaluating mRNA expression and the effects of growth factors and cell cycle phase on this expression. Single lineage-defined progenitors selected from early murine colony starts and grown under permissive conditions were analyzed by RT-PCR. SCL and E12/E47 were expressed in the vast majority of tri-, bi-, and unilineage progenitors of erythroid, macrophage, megakaryocyte, and neutrophil lineages. Expression for E12/E47 was not seen in unilineage megakaryocyte and erythroid or bilineage neutrophil/mast cell progenitors. Lyl-1 showed a more restricted pattern of expression, although expression was seen in some bi- and unilineage progenitors. No expression was detected in erythroid, erythroid-megakaryocyte-macrophage, macrophage-neutrophil, macrophage, or megakaryocytic progenitors. Id-1, an inhibitory bHLH transcription factor, was also widely expressed in all bi- and unilineage progenitors; only the trilineage erythroid-megakaryocyte-macrophage progenitors failed to show expression. Expression of these factors within a progenitor class was generally heterogeneous, with some progenitors showing expression and some not. This was seen even when two sister cells from the same colony start were analyzed. Id-1, but not E12/E47, mRNA was increased in FDC-P1 and MO7E hematopoietic cell lines after exposure to IL-3 or GM-CSF. Id-1, E12, and lyl-1 showed marked variation at different points in cell cycle in isoleucine-synchronized FDC-P1 cells. These results suggest that SCL, lyl-1, E12/E47, and Id-1 are important in hematopoietic progenitor cell regulation, and that their expression in hematopoietic cells varies in response to cytokines and/or during transit through cell cycle.

Key words: basic helix-loop-helix, interleukin-1, interleukin-3, granulocyte-macrophage colony-stimulating factor, progenitor, transcription factor, c-kit ligand

The decision of whether a relatively undifferentiated cell enters cell cycle, continues to proliferate, commits to a specific differentiation pathway, or dies is determined by levels of different transcription factors which, acting largely as homo- or heterodimers, modulate expression of specific gene programs. The transcription factors are characterized by structural homology, common DNA binding sequences, or their ability to heterodimerize with other members of the same group [Williams et al., 1991; Murre et al., 1989a; Beckmann and Kadesch, 1991]. The basic helix-loop-helix (bHLH) transcription factors constitute one of the largest families of transcription factors, including over 30 members [Pabo and Sauer, 1992]. These proteins are characterized by the presence of a basic DNA binding region immediately n-terminal to a helix-loop-helix protein dimerization interface: when two HLH subunits dimerize, the two basic regions are juxtaposed to form a single DNA binding element [Murre et al., 1989b].

The HLH family can be divided into three general classes based on expression patterns and the presence of a second dimerization motif. The f leucine zipper [Murre et al., 1989b] class A

Abbreviations used: bHLH, basic helix-loop-helix; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1, interleukin-1; IL-3, interleukin-3.

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proteins (i.e., E12 and E47) are widely expressed and can readily heterodimerize with class B proteins (i.e., myo D) which are tissue restricted [Murre et al., 1989b]. Class C proteins (i.e., c-myc) can also be called HLH Zip (for helix-loop-helix-zipper) to differentiate them from the simple HLH proteins.

The critical role of bHLH heterodimers in triggering differentiation is perhaps best illustrated by studies on muscle differentiation. Heterodimerization of myo-D and E12/E47 provides a high level of DNA binding with specific activation of genes determining muscle differentiation [Murre et al., 1989b].

The bHLH proteins are expressed in lymphohematopoietic lineages, and three, SCL, lyl-1, and E2A, have been found at characterizing translocations in T or pre-B cell leukemias. E2a was found at the t(1:19) translation in childhood leukemia [Mellentin et al., 1989; Nourse et al., 1990; Kamps et al., 1990], lyl-1 at the t(7:19) translocation in acute T cell lymphoblastic leukemia [Mellentin et al., 1989], and SCL (also known as tal-1 and TCL-5) at breakpoint t(1;14) in patients with T cell ALL [Begley et al., 1989, 1991a; Chen et al., 1990]. E2A is a ubiquitously expressed gene that encodes both transcription factors E12 and E47 by alternative splicing and is expressed in a wide variety of lymphohematopoietic cell lines [Begley et al., 1991b]. SCL is expressed in a more restricted fashion in murine erythroid, megakaryocyte, mast cell, and early myeloid lines [Begley et al., 1991b], and can heterodimerize with the ubiquitously expressed E12 and E47 proteins [Hsu et al., 1991]. Lyl-1 is also expressed in most myeloid, erythroid, and B lymphocyte cell lines [Begley et al., 1991b]. Lyl-1 expressed two alternative size classes of transcripts, the 1.5–1.8 kb size being characteristic of the erythroid lineage while the 2.0–2.3 kb size is seen in the B lineage cells [Begley et al., 1991b].

There is another family of proteins which functions to inhibit the activity of other bHLH proteins that are coexpressed in the same cells during the same phase of differentiation; these are termed Id (inhibitor of DNA binding) proteins [Benezra et al., 1990a; Christy et al., 1991; Sun et al., 1991; Deed et al., 1993]. The Id family members appear to inhibit the expression of lineage-specific genes, and alterations in their levels may be an important feature in determining whether a cell commits to differentiate. The Id-1 proteins all contain an HLH protein dimerization domain, but lack a functional DNA binding region [Benezra et al., 1990a; Christy et al., 1991; Sun et al., 1991; Deed et al., 1993]. When Id-1 proteins dimerize with other HLH proteins, they form heterodimers, lacking a complete DNA binding region and therefore incapable of binding effectively to DNA [Benezra et al., 1990a; Christy et al., 1991; Sun et al., 1991]. It would be expected that the HLH family activation proteins such as E12/E47 and myo-D would be rendered functionally inert by heterodimerization with Id proteins when they are coexpressed in the cell, and in fact Id-1 is expressed at high levels in proliferating myoblasts and these levels fall when myoblasts are induced to differentiate by growth factor withdrawal [Benezra et al., 1990a].

We have focused on the lineage expression and modulation of SCL, lyl-1, E12/E47, and Id-1 in both cytokine-dependent hematopoietic cell lines and explant progenitor/stem cells derived from colony starts grown under growth factor-permissive conditions. We have also evaluated the influence of cell cycle on expression of these bHLH mRNAs.

MATERIALS AND METHODS

Probes

Probes specific for the 3′ end of each cDNA were made by reverse transcribing 1 μg of total RNA from mouse tissues or hematopoietic cell cultures, using mixed hexamer primers and AMV reverse transcriptase (Boehringer Mannheim), then priming the polymerase chain reaction with primers for the 3′-most 300 bp of each cDNA, using Vent DNA polymerase (New England Biolabs). The PCR conditions were 95°C for 2′, then 95°C for 1′, 55° for 1′, and 72° for 1′ for 34 cycles. Samples were run on a 4% Nusieve: agarose 3:1 gel (FMC), then the ethidium-stained band (of the appropriate size) was excised from the gel, run into 0.6% low melt agarose (FMC), boiled, labeled by random priming (Boehringer Mannheim kit), and used as a probe.

Gene	Sequence
SCL	ACAGCAACTAGAGTGAGCTG
	AGA GTCACACGCCCGCAGTG
28S	AACGATCAGAGTAGTGGTATTTCACC
Id	ACTGAGGGACCAGATGGACTCCAG
	GCCAGTGATCATTGTAATATACA
Iyl-1	TCAGGACCTCTGGGATCCCAT

Other probes included the E12/E47 probe, which was generously provided by Charles P. Emerson, Jr., and was a 2.5 kb cDNA cloned from a C2C12 mouse myoblast cDNA library which detects both E47 and E12 transcripts; the mouse histone H2a probe, provided by William Marzluff [Harris et al., 1991]; and the lyl-1 mouse genomic DNA probe, provided by Kuo and Cleary [Kuo et al., 1991]. We used the lyl-1 oligonucleotide from the 3′ untranslated region to probe the FDC-P1 isoleucine blot, while the lyl-1 cDNA was used for the other blots. The G3PDH probe was amplified from mouse liver RNA using commercially available primers (Contech). The Id 3′ untranslated RT-PCR product, as described above, was used to probe the hematopoietic precursor polyAPCR blots, whereas the full-length Id cDNA (generously provided by Harold Weintraub) [Benezra et al., 1990b] was used to probe the other blots. An SCL cDNA (courtesy Glenn Begley) was utilized for the FDC-P-1 isoleucine blot, while the RT-PCR'd SCL 3′ untranslated probe was used for the progenitor blots. Probe identity was validated by nucleotide sequencing (E12) and/or hybridization to the expected size RNA species in the appropriate tissue or cell sample. The cDNAs were random prime labeled and used as probes.

Blots

Formaldehyde–1.2% agarose Northern gels were performed by standard methods [Ausubel et al., 1992] using Zetabind (AMF Cuno) as the source of the nylon transfer membrane, and electroblotting as the method of transfer. Membranes were hybridized and washed at 65°C as described [Church and Gilbert, 1984], using 0.1 mg/ml *E. coli* DNA (Sigma) as nonspecific carrier DNA. Blots were exposed to preflashed XAR-5 (Kodak) film with a single Cronex intensifying screen. Signal intensities were quantified on a phosphorimager (Molecular Dynamics) and plotted using Excel software (Microsoft).

Cell Line Culture, Growth Factor Deprivation, and Add-Back

Human megakaryocytic leukemic cells (line MO7E), routinely cultured in the presence of 100 units/ml human GM-CSF (continuous), were deprived of GM-CSF for 18 hr, followed by the addition of 100 units/ml of human GM-CSF, or human IL-3, or no add-back of growth factor (deprived). Six hours later cells were harvested and RNA isolated [Chomcznski and Sacchi, 1987], which was then electrophoresed, blotted, and probed with the indicated cDNA probes. Mouse FDC-P1 cells were cultured similarly (described in more detail below).

^{3}H-Thymidine Incorporation Into DNA of FDC-P1 Cells

At the designated time points FDC-P1 cells were incubated with 5 μCi/ml of ^{3}H-thymidine (ICN Biomedicals, Inc., Costa Mesa, CA) for 60 min. Incorporation of ^{3}H-thymidine into acid-precipitable material was terminated by adding trichloroacetic acid (TCA) to a final concentration of 10%. Cultures were incubated on ice for 15 min and the precipitate formed was recovered by centrifugation at 10,000 rpm for 10 min in a Sorvall RC-5B centrifuge (Dupont Co, Wilmington, DE) using an SM-24 rotor. Precipitate was dissolved in 0.5 ml of 0.4 mol/l NaOH, and reprecipitated by adding TCA to a final concentration of 10% and incubating on ice for 15 min. Each sample was diluted fivefold with ice-cold H_2O and the precipitate was once again recovered by centrifugation. Precipitate was then dissolved in 0.5 ml of 0.4 mol/l NaOH, and 0.1 ml aliquots were counted for radioactivity in 5 ml "Ready Protein" scintillation cocktail (Beckman, Fullerton, CA).

Time Course of Expression in Synchronized FDC-P1 Cells, Plus or Minus Growth Factor

FDC-P1 cells [Dean et al., 1987] were cultured in DMEM with 10% fetal calf serum and 25% WEHI-conditioned medium, which supplies the IL-3 the cells require for proliferation. For synchronization, cells were pelleted and resuspended three times and then cultured for 36 hr in DMEM without isoleucine, still containing 10% dialyzed FCS (purchased from Sigma Chemical Co., St. Louis, MO) and 25% dialyzed WEHI-conditioned media (dialyzed against PBS). Following isoleucine deprivation for 36 hr, cells were pelletted twice and resuspended in isoleucine-containing DMEM with 10% fetal calf serum, either plus or minus 25% WEHI-conditioned medium, then cultured until harvest. Control cells were from the same initial flasks of cells, but cultured continuously in isoleucine-containing medium supplemented with FCS and WEHI-conditioned medium, i.e., not synchronized or deprived of growth factor. Cells were harvested at time points by pelleting the cells, washing once in PBS, resuspending in 4 M guanidinum thiocyanate solution, and vortexing to lyse the cells. Lysates were stored at −20°C until further purification of total RNA by the

acid-phenol-guanidium method [Chomcznski and Sacchi, 1987], followed by electrophoresis in formaldehyde–agarose gels, electroblotting to Zetabind, and hybridization to the indicated cDNA probes. Blot signals were quantified on a phosphorimager (Molecular Dynamics).

Hematopoietic Precursor Blots

The polyA PCR for general amplification of polyadenylated cDNA, its application to single hemopoietic progenitor cells, and the preparation and validation of the sample set analyzed in this study are described in detail elsewhere [Brady et al., 1990; Brady and Iscove, 1990]. Briefly, marrow cells from CBA/J mice were cultured in methyl cellulose containing interleukin-1 (IL-1) interleukin-3 (IL-3), c-kit ligand, and erythropoietin. Colony starts containing 4–8 cells were identified at 25–28 hr, and single cells were taken for cDNA amplification. The identity of each sampled cell was determined by analysis of the lineage content of the colonies subsequently obtained from each of its individually cultured sibling cells. For cDNA amplification individual cells were lysed, and reverse transcription was primed with dT. The resulting cDNA strands were tailed with poly (dA) and finally PCR amplified using an oligo (dT)–containing primer. Amplified cDNA samples, 0.5 μg per lane, were electrophoresed in agarose gels, transferred to Hybond N+ nylon membranes (Amersham), and hybridized with the indicated probes. All probes included extreme 3′ sequences as required for cDNA amplified by the polyA PCR technique.

RESULTS

bHLH Transcription Factor Expression in Individual Murine Progenitor/Stem Cells

We evaluated bHLH expression in normal explant murine marrow progenitor/stem cells by probing blots of polyA PCR–amplified cDNAs from single cells harvested from colony starts grown under growth factor–permissive conditions in the presence of IL-3, IL-1, c-kit ligand, and erythropoietin. One or two cells from a colony start were harvested for single cell RT-PCR and the other cells followed for colony phenotype, thus allowing for an assignment of lineage fate to the cells picked for the polyA PCR RNA amplification. The lineage assignments and expression of Id-1, E12/E47, SCL, and lyl-1 mRNA are presented in Figure 1.

SCL is expressed in one or more progenitors for each lineage. While these techniques are not quantitative, the strong expression of SCL in erythroid/megakaryocyte (eryth/meg) (3/6) and erythroid/megakaryocyte/macrophage (eryth/meg/mac) (1/5) is impressive (Fig. 1, Table I). A feature of these results is the relatively large number of negative results in those progenitor classes showing one or more positive cells. In 31 instances there were 20 negatives in the eight lineages which expressed at least one positive. In nine instances two sister cells from the same developing colony were sampled; one from an eryth/meg progenitor, three from macrophage/neutrophil (mac/neut) colony starts, three from macrophage (mac) progenitors, one from a neutrophil (neut) progenitor, and one from an erythroid (eryth) progenitor (Table II). PR 7 and 8 (eryth/meg), PR 13, 14, 17, and 18 (mac/neut), and PR 35 and 36 (neut) were all discordant, with one cell expressing mRNA for SCL and the other not showing expression. These results suggest marked differences in mRNA abundance in progenitors of the same kind under essentially the same culture conditions.

There was expression of E12/E47 in tri-, bi-, and unipotential progenitors with only neutrophil/mast cell (neut/mast) (0/2), eryth (0/1), and megakaryocyte (meg) (0/4) not showing expression. E12/E47 expression also varied within each class of progenitors in which positive expression was seen in at least one instance and showed discordance between sister cells from two of nine colony starts (PR 15 and 16, mac/neut, and PR 35 and 36, neut). PR 15 and 16 were not discordant in the analysis of SCL mRNA.

Id-1 mRNA was expressed in eryth/meg, mac/neut, neut/mast, eryth, mac, neut, and meg lineages, but not in trilineage progenitors (Fig. 1, Table I). Once again there was a wide variation in expression within a specific progenitor group, and discordance was seen in sibling eryth/meg, mac/neut, mac, neut, and eryth progenitors.

Lyl-1 showed much more limited expression. Only three progenitor types, eryth/meg, neut/mast, and neut, were positive. None of the sibling pairs analyzed showed any expression of lyl-1.

Cytokine Modulation of bHLH Transcription Factors

Expression of the helix-loop-helix transcription factor Id-1 is growth factor modulated in myeloid cell lines. Id-1 mRNA levels fall when mouse FDC-P1 factor–dependent myeloid cells are deprived of growth factor (FDC-P1 cells

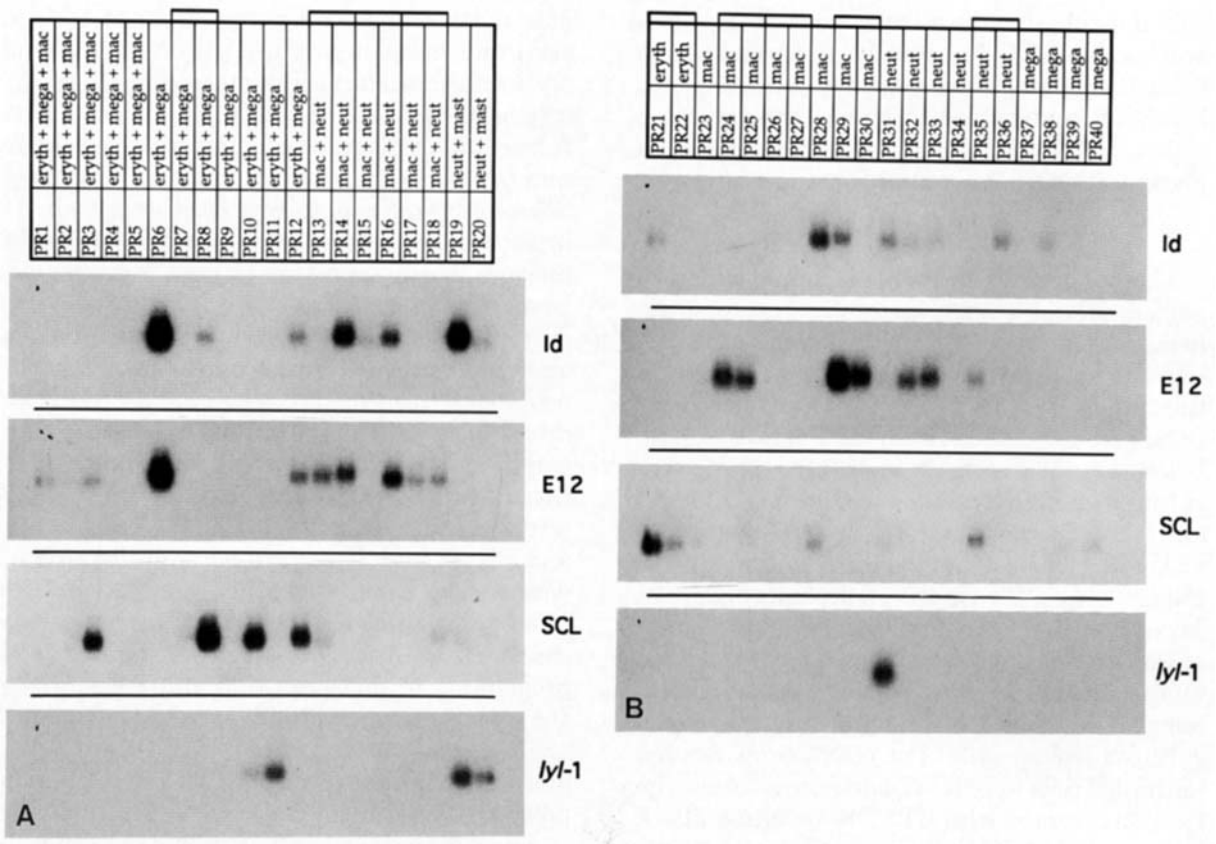

Fig. 1. Southern analysis of expression in individual lineage-defined murine hematopoietic progenitor cells. Southern blots with poly APCR–amplified cDNA from the indicated bone marrow–derived progenitors were hybridized with the designated probes. Abbreviations: eryth, erythroid; meg or mega, megakaryocyte; mac, macrophage; mast, mast cell; neut, neutrophil. **A:** Progenitors 1–20. **B:** Progenitors 21–40. Exposure times were 17 hr Id; 7 hr E12; 7 hr SCL; and 17 hr lyl-1, using XAR-5 film (Kodak).

TABLE I. Heterogeneity of bHLH Expression in Individual Lineage-Determined Hematopoietic Progenitor Cells*

	mRNA expression			
Colony type	SCL	E12/E47	ID	yl-1
Eryth/meg/mac	1/5	2/5	0/5	0/5
Eryth/meg	3/6	2/6	3/6	2/6
Mac/neut	2/3	3/3	2/3	0/3
Neut/mast	1/2	0/2	2/2	2/2
Eryth	1/1	0/1	1/1	0/1
Mac	1/5	2/5	2/5	0/5
Neut	1/5	3/5	4/5	1/5
Meg	1/4	0/4	1/4	0/4

*Scoring number positive over number tested. When sibling pairs from a colony start were both analyzed an n of one was used, and if they were discordant for expression of a bHLH transcription factor this was scored as positive expression.

TABLE II. Fraction of Sibling Pairs Showing Discordant Expression of Each bHLH Transcription Factor

Sibling pair colony type	Id1	E12	SCL	lyl-1
Eryth/meg	1/1[a]	0/1	1/1	0/1
Mac/neut	1/3	1/3	2/3	0/3
Mac	1/3	0/3	0/3	0/3
Neut	1/1	1/1	1/1	0/1
Eryth	1/1	0/1	0/1	0/1
Overall % discordance	56	22	44	0

[a]Scoring the number of pairs discordant over the total number of sibling pairs.

require either IL-3 or GM-CSF to grow; WEHI-cm provides mostly IL-3) and then return when the growth factor is added back (Fig. 2). In this experiment cells were harvested and RNA isolated 6 hr after the add-back time. In contrast, E12/E47 mRNAs are only slightly modulated when deprived and refed with growth factor (Fig. 2).

Id-1 mRNA levels are also growth factor–modulated in the human MO7E megakaryocytic leukemic cell line (Fig. 3). This experiment shows a time course, harvesting the cells for RNA isolation at the indicated times after growth factor add-back. Expression of Id-1 falls off significantly in cells deprived of growth factor, then

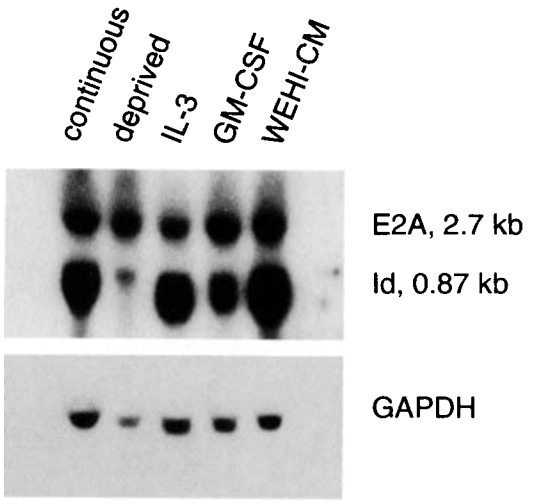

Fig. 2. Northern analysis of Id-1 and E12/E47 expression in the FDC-P1 murine hematopoietic factor–dependent cell line following growth factor deprivation and add-back of growth factor. Murine FDC-P1 cells routinely grown in the presence of 25% WEHI-conditioned medium (WEHI-CM) were deprived of WEHI-CM for 18 hr, followed by the addition of 100 units/ml GM-CSF, 8 units/ml IL-3, or 25% WEHI-CM, or no add-back of growth factor (deprived). Six hours later cells were harvested and RNA isolated. Ten micrograms per lane of RNA was electrophoresed on a formaldehyde–agarose gel, electroblotted to Zetabind, probed with the indicated probes, and autoradiographed.

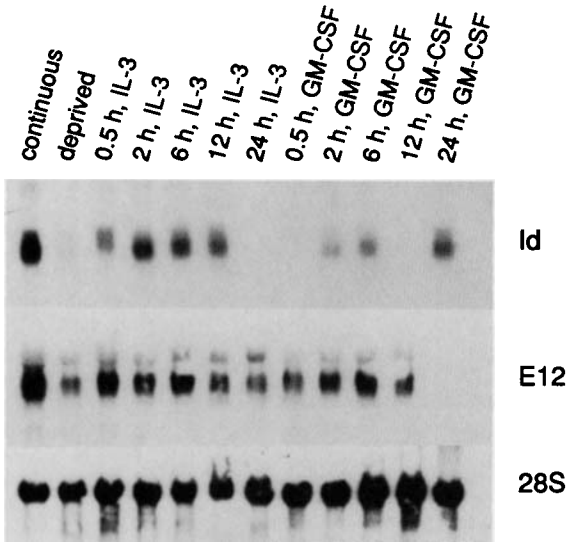

Fig. 3. MO7E human leukemic cell line, growth factor deprivation, and add-back. Human megakaryocytic leukemic cells (line MO7E), routinely cultured in the presence of 100 units per ml of human GM-CSF, were deprived of GM-CSF for 18 hr, followed by the addition of 100 units/ml human GM-CSF or 8 units/ml human IL-3, or no add-back of growth factor (deprived). At the indicated time points, cells were harvested and RNA isolated. Ten micrograms per lane was electrophoresed and gels were electroblotted to Zetabind (AMF Cuno), probed with the indicated cDNA probes, and autoradiographed.

begins to return rapidly after growth factor readdition, with a significant increase evident at 0.5 hr. These data show that the return of Id-1 mRNA expression is more rapid with IL-3 restimulation than with GM-CSF, since there is more signal at 0.5 hr with IL-3 add-back than with GM-CSF. Twenty-four hours after add-back Id-1 levels are again low in the IL-3 add-back group, but are actually enhanced in the GM-CSF group, suggesting a differential regulation of Id by different cytokines, which could, in part, be related to the cell cycle progression induced by each factor. The Id-1 blot was reprobed with a 28S rRNA oligonucleotide probe, confirming that each lane of total RNA had been approximately equally loaded.

E12/E47 mRNA levels, unlike Id-1, are only modestly growth factor–modulated (Fig. 3). There is a decrease in E12/E47 mRNA when the cells are deprived of growth factor. E12/E47 mRNA levels return rapidly after growth factor add-back. These data indicate that the return of E12/E47 mRNA expression is also more rapid with IL-3 restimulation than with GM-CSF, since there is more signal at 0.5 hr with IL-3 than GM-CSF. The 24 hr GM-CSF lane contained no RNA on the E12-probed blot.

Transcription Factor mRNA Levels in Synchronized Cells

Having found that expression of certain transcription factors is growth factor–modulated, we measured whether expression varied during the cell cycle (Figs. 4, 5). We evaluated transcription factor mRNA expression levels during the cell cycle in the presence of growth factor, and as the cells withdraw from cycle and undergo apoptosis in the absence of growth factor (Kittler et al., unpublished observations). FDC-P1 cells were synchronized in early G1 using isoleucine deprivation [Reddy et al., 1992], the cells then refed with isoleucine-replete medium either with or without growth factor, and RNA isolated at various times at and after refeeding. Northern blots were probed with various transcription factor cDNA probes (Fig. 4). Both synchronized and unsynchronized control FDC-P1 cells expressed Id-1, E12, SCL, lyl-1, and H2a at the zero time point of the experiment. We noted that Id-1 mRNA showed increased expression between the start of the manipulations and the zero time point, which is the time the cells are returned to the incubator (or harvested for the zero time point), about 30–40 min after begin-

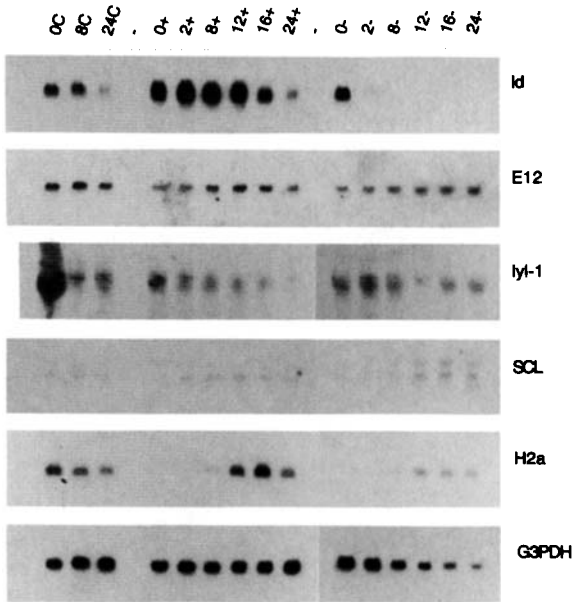

Fig. 4. Synchronization with isoleucine deprivation of FDC-P1 cells. FDC-P1 cells were switched from normal growth medium plus 25% WEHI-CM to growth medium with 25% WEHI-CM, but without isoleucine (dialyzed fetal calf sera), maintained under these conditions for 36 hr, then refed with isoleucine-replete medium and WEHI-CM, and evaluated for cell cycle status at time of isoleucine add-back and at various times after add-back as indicated. Cell cycle status was determined by uptake of tritiated thymidine (3Htdr) or expression of H2a as described in Materials and Methods.

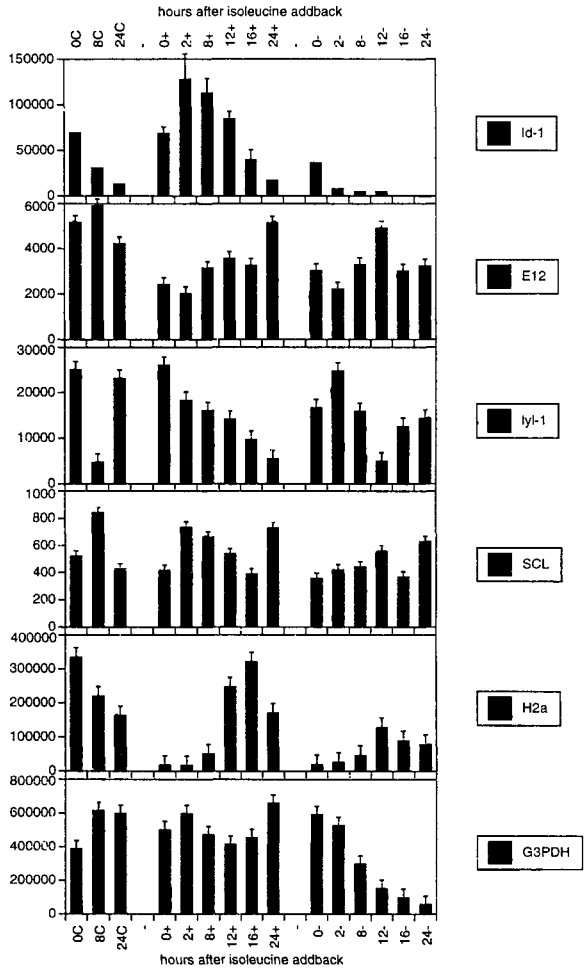

Fig. 5. Northern analysis of expression in synchronized FDC-P1 cells plus or minus growth factor. FDC-P1 cells were synchronized as described, cells harvested, and RNA isolated at the indicated times after isoleucine add-back with (+) or without (−) WEHI-CM (growth factor). C, cells maintained in normal growth media with WEHI-CM throughout the experiment; +, cells which were isoleucine deprived and then repleted in the presence of WEHI-CM; −, cells which were isoleucine deprived and then repleted, but without WEHI-CM in the growth medium. Northern blots containing 10 μg of total RNA per lane were hybridized to the indicated cDNA probes and autoradiographed. Exposure times were 12.5 hr Id; 48 hr E12; and 48 hr lyl-1, to Kodak XAR-5 film with one Cronex intensifying screen. Blot signals were quantified on the phosphoimager (Molecular Dynamics) and plotted.

ning to wash cells back into isoleucine-containing media (data not shown). Cells released from the isoleucine block in the presence of growth factor showed significant increases in the levels of Id-1 as the cells progressed through cell cycle, peaking in mid-G1 (Figs. 4, 5). Id-1 levels increased to a maximum 2 hr after release, then decreased as the cells continued through S at 12–16 hr [Reddy et al., 1992].

Synchronized cells deprived of growth factor evidenced suppression of Id-1 expression as the cells were released from the isoleucine block and presumably underwent apoptosis in the absence of growth factor; Id-1 levels dropped within the first 2 hr. The timing of cell cycle was confirmed by separate determination of the rate of ^{3}H-thymidine incorporation into cells (data not shown) and by reprobing the blots with the mouse histone H2a probe, known to be upregulated as cells move from G1 into S phase [Harris et al., 1991], which showed significant hybridization 12–24 hr after isoleucine block release (Figs. 4, 5).

SCL expression remained roughly constant, with relatively low levels of expression throughout the time course in the presence or absence of growth factor. The pattern of expression of lyl-1 mRNA in the presence or absence of growth factors was distinct from the patterns seen with either Id-1 or SCL. In the presence of cytokines, lyl-1 showed a steady decrease in expression from time zero and into S phase; in the absence of cytokines lyl-1 expression fluctuated, with a decrease at 12 hr postrefeed. E12/E47 expres-

sion was relatively stable in the presence of growth factor, with an increase seen at 24 hr, and was also stable without growth factor, with an increase observed at 12 hr. Multiple blots from additional repetitions of the cell synchrony experiments were probed in this series to confirm the results. The phosphorimager profiles shown in Figure 5 represent the average of 2–3 Northern blot experiments from a single cell synchrony series, with error bars showing the standard deviations calculated for the averages of Id-1 and E12/E47 and standard error bars for lyl-1, SCL, H2a, and G3PH.

DISCUSSION

The study of events at early lymphohematopoietic progenitor/stem cell levels has been limited by the rarity of the cells and the difficulty in obtaining significant numbers of them for study. The culture of hematopoietic stem cells, in part, represents a functional purification, and the evaluation of single cells in colony starts a unique opportunity to assess events at early progenitor/stem cell levels. The nature of these cells and their lineage fate is, of necessity, assessed by "the company they keep," but the evaluation of the differentiation fate of the nonselected colony cells allows for a reliable assignment of lineage phenotype for those cells subjected to the single-cell RT-PCR analysis.

These data represent the first demonstration of expression of SCL, Id-1, lyl-1, and E12/E47 in normal individual explant progenitor cells, and suggest that they may play a regulatory role in hematopoietic proliferation and differentiation. Each bHLH transcription factor evidenced different patterns of expression in progenitor/stem cells, with expression within a lineage, such as erythroid-megakaryocyte progenitors, differing between individual progenitors. In the latter case, isolated expression of lyl-1, SCL, and E12 was seen in different individual erythroid-megakaryocyte progenitors.

SCL in Hematopoietic Progenitors

Our results showing SCL expression in individual progenitor cells from each of the lineage classes is consistent with previous reports of expression of SCL in erythroid, mast cell, megakaryocyte, and some "early myeloid" cell lines, and in normal mast cells [Begley et al., 1991b; Green et al., 1991a, 1992; Elwood et al., 1994]. Coordinate expression of SCL mRNA with GATA-1 mRNA was seen in multiple hemopoietic cell lineages. In nonhemopoietic tissue SCL was detected in adult and developing brain [Green et al., 1992]. A nonrandom site-specific SCL rearrangement or deletion occurs in several T cell acute lymphoblastic leukemia cell lines and in de novo T cell acute lymphoblastic leukemias [Aplan et al., 1992; Macintyre et al., 1992], although previous studies have indicated that expression of SCL is low or undetectable in normal T cell populations [Green and Begley, 1992]. Studies on hematopoietic cells from normal human marrow and peripheral blood, employing both in situ hybridization and RT-PCR techniques, have shown expression of SCL in erythroblasts (derived from BFU-E), megakaryocytes (liquid culture), and separated CD34+/CD38+ cells [Mouthon et al., 1993]. Basophils from the peripheral blood of a patient with chronic myelogenous leukemia were also positive by in situ hybridization. Neutrophils, eosinophils, T and B cells, monocytes, and CD34+/CD38− cells were negative by in situ hybridization. Studies with RT-PCR also showed expression of SCL in 12 and 22 week fetal liver, platelets, and peripheral blood granulocytes. This latter finding was interpreted as being due to the basophil component of the granulocytes [Mouthon et al., 1993]. This study also indicated that SCL mRNA expression decreased during both erythroid and megakaryocyte differentiation. Studies on human and murine leukemia cell lines have given divergent results with regard to changes in SCL mRNA expression with differentiation. Levels of SCL mRNA increase during DMSO-induced differentiation of murine erythroleukemia cell lines [Begley et al., 1991b], while in the human erythroleukemia cell line, K562, SCL appears to play a role in proliferation and its inhibition by antisense was associated with differentiation [Green et al., 1991b]. Studies on the murine M1 leukemia cell line have indicated that cytokine-induced differentiation was associated with decreased SCL protein and mRNA [Tanigawa et al., 1993].

Our present results show expression of SCL mRNA in varying percentages of all tri-, bi-, and unilineage progenitors assessed, and are most consistent with a role of SCL in progenitor cell proliferation and differentiation prior to the terminal differentiation events into erythroid, megakaryocyte, mast cell, neutrophil, and macrophage lineages. This indicates a less restricted expression of SCL than was previously reported. This is not in conflict with previous data since

this is the first assessment of individual normal progenitors with defined lineage potentials.

One of the striking features of these results is the heterogeneity of expression of SCL in progenitors of the same defined lineage. The number of positive cells in the tri- and bilineage progenitors ranged from 20 to 67%, indicating that in most progenitor phenotypes cells were found which expressed SCL, and that there must be marked differences in mRNA abundance in progenitors of the same kind. The macrophage and neutrophil single-lineage progenitors gave similar results, with 20% of the cells showing expression of SCL mRNA. Only the single-lineage erythroid cells showed a 100% expression and this was based on only one colony. The single-lineage megakaryocyte progenitors, surprisingly, showed no expression of SCL mRNA.

The above noted heterogeneity of expression extended to the sister cells picked from the same colony. Nine sister pairs were analyzed and in four instances there was total discordance of expression of SCL mRNA; this occurred in an erythroid/megakaryocyte colony start, two of three macrophage/neutrophil colony starts, and one of three macrophage colony starts. Thus progenitors from the same colony start could show strong or no expression of this particular bHLH transcription factor.

This heterogeneity of expression could relate to rapid action of these transcription factors at very specific points in the life cycle of a progenitor either related to stage in cell cycle or level of differentiation. Details of cytokine exposure such as the relative concentration of cytokines (in serum or added to culture) or the sequence of cytokine receptor interactions could also influence the expression of SCL at different points in time. Fluctuation of SCL mRNA expression, albeit at relatively low levels, was seen in synchronized FDC-P1 cells, although growth factor modulation under the conditions of these experiments was not apparent.

E12/E47 in Hematopoietic Progenitors

The E2A gene encodes the immunoglobulin enhancer binding proteins E12 and E47. As noted above, E2A is rearranged in t(1:19) Pre-B cell leukemias and is expressed in a wide variety of cell lines, including those characterized as (or derived) from B cell lymphoma, T cells, neuroblastoma, long-term murine hematopoietic-cultured cells, macrophage, erythroid, plasmacytoma, fibroblast, stroma, and osteoblast [Begley et al., 1991b; Murre et al., 1991; Murray et al., 1992]. E12/E47 proteins also seem to play a role in myogenesis and pancreatic B cell differentiation [Murre et al., 1989b, 1991].

E12 also showed widespread expression in the different hematopoietic progenitor classes: only unilineage megakaryocyte/erythroid and bilineage neut/mast progenitors failed to show expression. Heterogeneity within progenitor cell classes and between sister cells of an individual colony start were again seen. As with SCL, E12 showed cell cycle fluctuation in mRNA expression but only minimal cytokine effect.

Lyl-1 in Hematopoietic Progenitors

The Lyl-1 gene was detected at a chromosome translocation breakpoint, t(7;19), in a T-cell leukemia cell line [Mellentin et al., 1989]. It has been found to be expressed in most myeloid, erythroid, and B lymphocyte cell lines, displaying two different sizes of transcripts, the smaller being typical of erythroid lines and the larger typical of the B cell lineage [Begley et al., 1991b]. Our results with lyl-1 expression were in contrast to that seen with SCL and E12/E47. A very restricted expression was seen, with one neutrophil progenitor and several neut/mast and eryth/meg progenitors showing expression. There was no expression in the trilineage progenitors. These data suggest that lyl-1 expression may play more of a role in lymphoid differentiation and more mature hematopoietic cells. Expression of lyl-1 mRNA also showed cell cycle–related fluctuation with little evidence of cytokine influence.

Id-1 in Hematopoietic Progenitors

Id-1 mRNA is expressed in a wide variety of cell lines including murine erythroleukemia and nondifferentiating variants, mouse embryo fibroblasts (C3H10T1/2), NIH3T3 cells, F3 (a myoblast cell line derived from 10T1/2), Abelson MuLV-transformed pre-B-cell line 18-8, melanoma cell line B16, a rat hepatoma cell line, FTO2b, and embryonal carcinoma cells [Benezra et al., 1990a]. Id-1 was also found to be induced shortly after serum induction in growth-arrested NIH 3T3 cells. Furthermore, decreasing the percent serum in the growth media for these cells from 10 to 0.5% resulted in a 50% reduction of Id-1 mRNA within 1 hr. Platelet-derived growth factor or fetal calf serum stimulated growth of the NIH3T3 cells, and this stimulation was blocked by antisense to Id-1 [Barone

et al., 1994]. These results suggested that Id-1 was an early response gene and that it might function in this setting to bind and inhibit a growth-inhibition bHLH transcription factor.

Our results indicate no expression of Id-1 in trilineage progenitors, but expression in all other lineage phenotypes. There were varying levels of expression in a varying percentage of all the bi- or unilineage progenitors analyzed. Id-1 showed relatively dramatic cell cycle fluctuation and cytokine dependence. Heterogeneity of expression within a progenitor class, perhaps in part related to these cell cycle fluctuations or to cytokine effects, was also seen.

These data are consistent with Id-1 playing a role in the proliferative regulation of bi- and unilineage progenitors. One might speculate that decreases in Id-1 as the progenitor progresses through cell cycle, as was seen with the FDC-P1 cells, may provide the appropriate condition for the progenitor to commit to differentiation in the presence of the requisite inductive signals.

Altogether these data indicate a complex transcriptional regulation of hematopoietic progenitor cells by different members of the bHLH transcription factor family, presumably acting differentially at different points in cell cycle and in response to different cytokine signaling events. Identification of specific hematopoietic dimer partners will be a crucial next step in our understanding of the transcriptional control of hematopoietic progenitors.

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