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Cell cycle-dependent initiation and lineage-dependent abrogation of GATA-1 expression in pure differentiating hematopoietic progenitors

(hematopoietic growth factors/erythroid and myeloid development/transcription factors)

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ABSTRACT **The programmed activation/repression of transcription factors in early hematopoietic differentiation has not yet been explored. The DNA-binding protein GATA-1 is required for normal erythroid development and regulates erythroid-expressed genes in maturing erythroblasts. We analyzed GATA-1 expression in early human adult hematopoiesis by using an *in vitro* system in which "pure" early hematopoietic progenitors are induced to gradual and synchronized differentiation selectively along the erythroid or granulocyte–macrophage pathway by differential treatment with hematopoietic growth factors. The GATA-1 gene, though virtually silent in quiescent progenitors, is activated after entrance into the cell cycle upon stimulation with hematopoietic growth factors. Subsequently, increasing expression along the erythroid pathway contrasts with an abrupt downregulation in the granulocyte–macrophage lineage. These results suggest a microenvironment-directed, two-step model for GATA-1 expression in differentiating hematopoietic progenitors that involves (*i*) cycle-dependent initiation and (*ii*) lineage-dependent maintenance or suppression. Hypothetically, on/off switches of lineage-restricted transactivators may underlie the binary fate decisions of hematopoietic progenitors.**

Hematopoiesis is sustained by a pool of stem cells, which self-renew and differentiate into progenitors (1). These progenitors are committed to specific lineages and are functionally defined as colony-forming or burst-forming units (CFU, BFU) of the erythroid series (CFU-E, BFU-E), the granulocyte–monocyte lineage (CFU-GM), and multipotent CFU for the GM, erythroid, and megakaryocytic series (CFU-GEMM) (2). The progenitors in turn differentiate into morphologically recognizable precursors, which mature to terminal forms circulating in peripheral blood.

Proliferation and differentiation of early hematopoietic cells are modulated by specific hematopoietic growth factors (HGFs), termed colony-stimulating factors (CSFs) or interleukins (ILs), which are released by accessory cells (T cells, large granular lymphocytes, monocytes–macrophages, and stromal cells) and exert a multi- or unilineage stimulus (3, 4). Thus, IL-3 (5) and GM-CSF (6) induce differentiation of pluripotent (CFU-GEMM), early erythroid (BFU-E), and GM progenitors (CFU-GM), whereas erythropoietin (Epo) (7), G-CSF (8), and M-CSF (9) specifically trigger differentiation of late erythroid (CFU-E), granulocytic (CFU-G), and monocytic (CFU-M) progenitors, respectively.

Coordinated expression of lineage-specific genes in developing hematopoietic cells is likely to be mediated in part by the programmed activation/suppression and microenvironment-directed expression of tissue- and stage-specific transcription factors. As a transactivator whose presence in mature hematopoietic cells is limited to the erythroid, megakaryocytic, and mast lineages (10, 11), GATA-1 is a prime protein for study in this context. GATA-1, a 50-kDa finger protein, regulates erythroid-expressed genes through core GATA motifs (12) and is required for normal erythroid development as revealed by gene targeting in embryonic stem cells (13). Two mechanisms dependent on GATA-1 favor maturation of erythroid precursors: (*i*) the GATA-1 gene is autoregulated through an upstream GATA element (14); (*ii*) GATA-1 positively regulates the Epo receptor promoter (15) and hence may forestall apoptosis due to Epo starvation (16). While aspects of GATA-1 regulation in maturing erythroid cells are readily reconciled with its proposed role, less is known regarding expression of GATA-1 in early progenitors. The IL-3-dependent multipotential cell lines FDCP1 (17) and 32D (17, 18) have low levels of GATA-1 expression, whereas levels in more mature cell lines [MEL (erythroid), CHRF-288 (megakaryocytic), MC/9 (mast)] are higher (17). However, it is uncertain whether these observations on immortalized cell lines reflect regulation of GATA-1 during normal hematopoiesis.

We analyzed GATA-1 expression during normal hematopoiesis by using an *in vitro* system in which a "pure" population of early progenitors (19) undergoes a gradual, synchronized differentiation selectively along the erythroid or GM pathway in fetal bovine serum (FBS)-free liquid suspension culture.

MATERIALS AND METHODS

Progenitor Purification. Human hematopoietic progenitors were purified from normal human peripheral blood by a four-step procedure (19): step I, a Ficoll cut followed by monocyte removal (plastic adherence and glutamate dimethyl ester treatment); step II, a discontinuous Percoll density gradient to separate the low-density cells; step III, negative selection with immunomagnetic beads and a panel of monoclonal antibodies (mAbs) against T, B, and natural killer lymphocytes and monocytes; step IV, positive selection with immunomagnetic beads and anti-CD34 mAb. The procedure was slightly modified: step I, glutamate dimethyl ester treatment was omitted; step II, the cells were purified on a four-step (rather than three-step) Percoll gradient (3-ml frac-

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Abbreviations: BFU-E, erythroid burst-forming unit(s); CFU, colony-forming unit(s); CSF, colony-stimulating factor; GM, granulocyte–monocyte; Epo, erythropoietin; IL, interleukin; HGF, hematopoietic growth factor; mAb, monoclonal antibody; FBS, fetal bovine serum; RT, reverse transcriptase; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

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tions: 1.052, 1.056, 1.059, and 1.065 g/ml). Cells of density ≤ 1.056 g/ml were recovered and further processed.

Liquid Suspension Culture. Step IV progenitors were grown at 10^4 cells per ml in Iscove's modified Dulbecco's medium (IMDM) without FBS and supplemented (20) with bovine serum albumin (10 mg/ml), human transferrin (1 mg/ml), human low density lipoproteins (40 μg/ml), insulin (10 μg/ml), sodium pyruvate (0.1 mM), L-glutamine (2 mM), rare inorganic elements (ref. 21), ferrous sulfate (40 mM), nucleosides (each at 10 μg/ml), and recombinant human HGFs [for GM differentiation culture, high doses of IL-3 (100 units/ml) and GM-CSF (10 ng/ml); for erythroid differentiation culture, low doses of IL-3 (0.01 unit/ml) and GM-CSF (0.001 ng/ml) and a plateau level of Epo (3 units/ml)]. Cultures were incubated in a fully humidified atmosphere of 5% CO_2/5% O_2/90% N_2 and were periodically counted, harvested, and analyzed for cell cycling, membrane phenotype, and morphology. To analyze the proliferative potential of the progenitors, the cells were periodically plated in FBS-containing methylcellulose cultures, as indicated above (HGFs including saturating doses of IL-3, GM-CSF, and Epo); on day 14 the colonies were scored individually, picked up, washed, resuspended, and counted.

Clonogenic Assay. The assay (19) was performed in FBS-containing methylcellulose culture supplemented with recombinant HGFs [IL-3 (100 units/ml, Genetics Institute, Boston), GM-CSF (10 ng/ml, Genetics Institute), and Epo (3 units/ml, Amgen), as well as c-Kit ligand (10 ng/ml, Immunex), replacing basic fibroblast growth factor].

Cell Surface Markers. Expression of cell surface markers was evaluated by double immunofluorescence using a fluorescein isothiocyanate (FITC)-labeled anti-CD34 mAb (8G12 clone, kindly provided by P. Lansdorp, Terry Fox Laboratories, Vancouver) and various phycoerythrin (PE)-conjugated mAbs, including anti-CD45RO (UCHL-1 clone, Dakopatts, Glostrup, Denmark), anti-HLA-DR (Becton Dickinson), and anti-CD33 (Silenus, Paris). The cells were incubated for 60 min at 4°C in the presence of an appropriate dilution of mAbs, washed three times in cold Hanks' saline solution containing bovine serum albumin (2 mg/ml; fraction V, Sigma), suspended in phosphate-buffered saline with 2.5% (vol/vol) formalin, and analyzed with a FACScan (Becton Dickinson) using the LYSIS program for analysis of double fluorescence.

Cell Cycle Analysis. The *in vitro* percent killing of progenitors after incubation with [^{3}H]thymidine was evaluated as described (22, 23). In brief, 10^3 cells from each sample were divided into four tubes containing IMDM. Tubes II and III contained 20 and 100 μCi, respectively, of [*methyl*-^{3}H]thy-

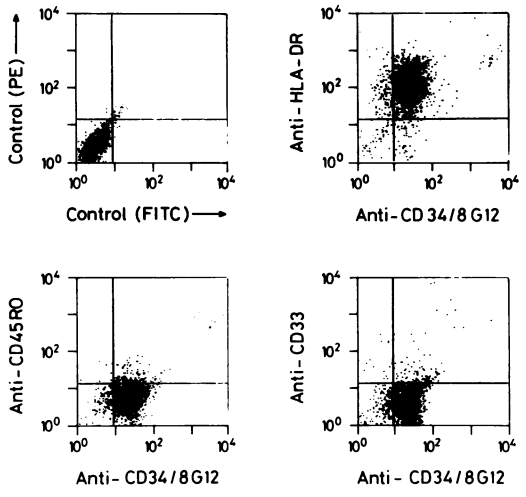

FIG. 1. Membrane phenotype of pure (step IV) hematopoietic progenitors from human adult peripheral blood (19) as evaluated by double immunofluorescence. The purification index of the progenitors (CFU-GEMM, BFU-E, CFU-GM), as evaluated in FBS-containing methylcellulose culture (100 cells per dish in the presence of c-Kit ligand, IL-3, GM-CSF, and Epo) was 88% (cf. refs. 19 and 25). The progenitors are essentially quiescent ([^{3}H]thymidine suicide index, <5%; see Fig. 2) and give rise to large colonies in clonogenetic culture even without addition of c-Kit ligand (see Fig. 2). The progenitors were then cultured in liquid phase for selective erythroid or GM differentiation (Figs. 2–4) and finally analyzed by RT-PCR for GATA-1 mRNA expression (Fig. 5). The results were consistently confirmed in several separate experiments.

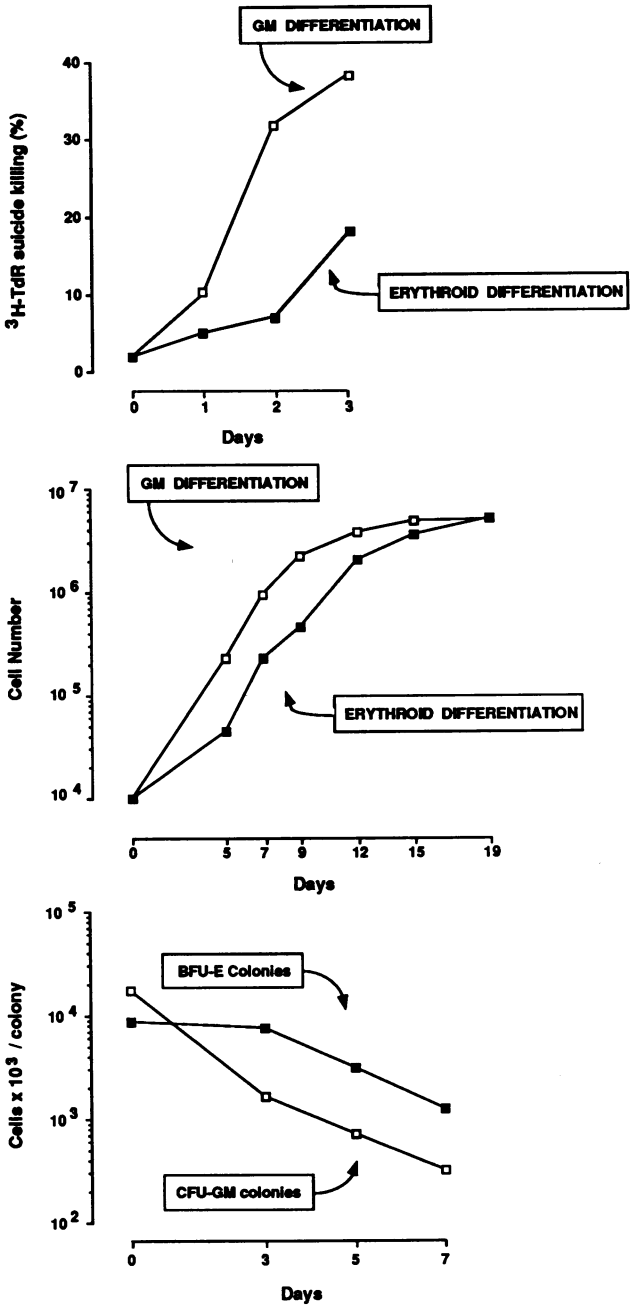

FIG. 2. Pure (step IV) progenitors undergo a wave of gradual/synchronous differentiation selectively along the erythroid or GM pathway in FBS-free liquid suspension culture supplemented with appropriate HGFs at day 0 (see below). (*Top*) [^{3}H]Thymidine (^{3}H-TdR) suicide index (%) of progenitors in the erythroid (■) and GM (□) differentiation cultures at days 1, 2, and 3 after HGF stimulus. (*Middle*) Growth curve in erythroid (■) and GM (□) differentiation cultures. (*Bottom*) Size of colonies (cells per colony) generated by erythroid (BFU-E, ■) and GM (CFU-GM, □) progenitors obtained at sequential days from the erythroid and GM differentiation cultures.

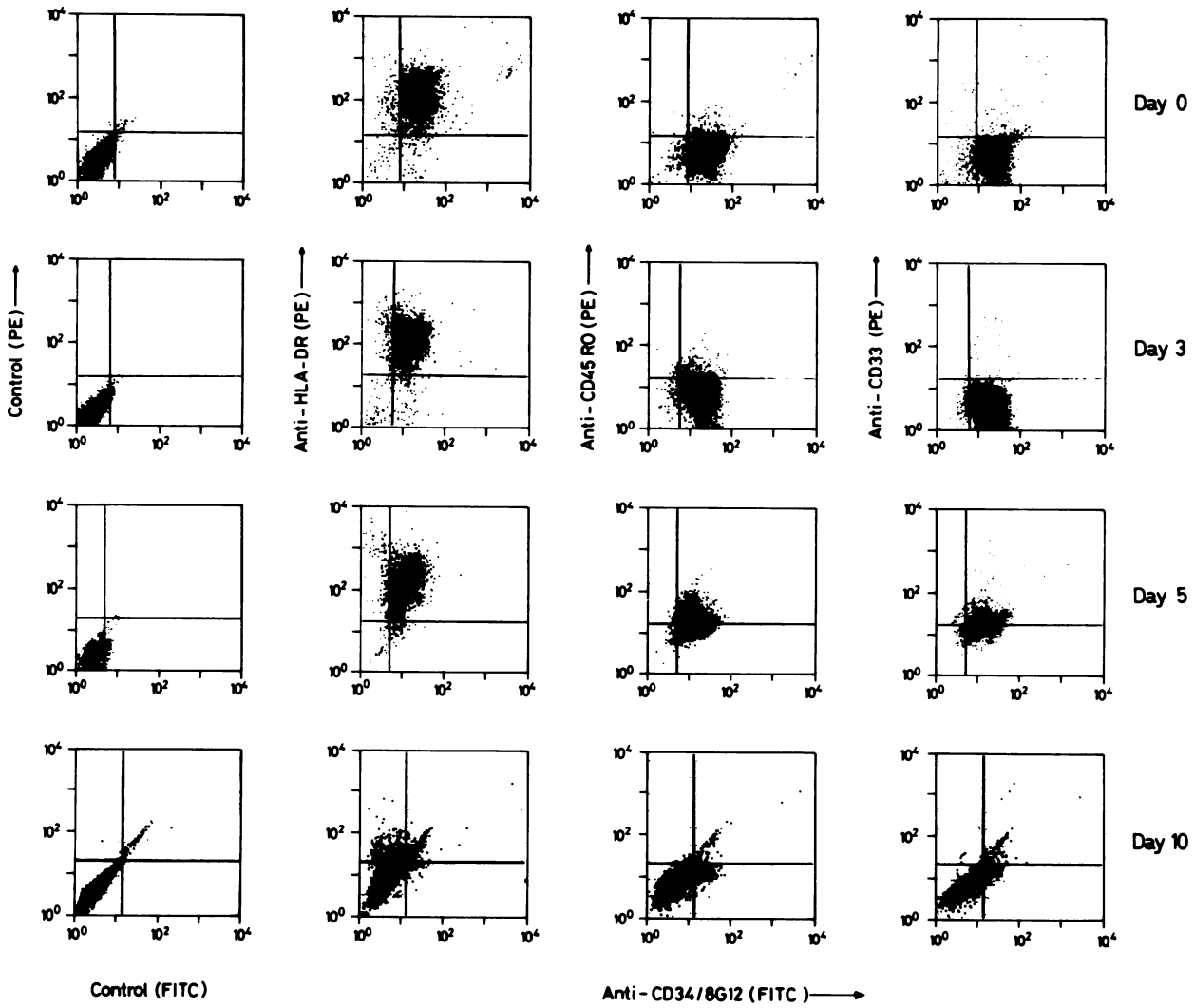

FIG. 3. Membrane phenotype of progenitors (Fig. 1) grown in the erythroid liquid culture (Fig. 2 legend), harvested at sequential days, and analyzed by double immunofluorescence (Fig. 1 legend). Similar results are observed in the liquid GM culture system (data not shown).

midine (20 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq), and tube IV contained 200 μg of nonradioactive thymidine (Serva) and 20 μCi of [^{3}H]thymidine. After 20 min of incubation in a water bath, cells were washed twice in the presence of thymidine (200 and 40 μg/ml) with IMDM containing 2% FBS. There was no significant difference in cell death between tubes II and III or tubes I and IV. The cells from each tube were plated (100 cells per dish) for assay of progenitors in FBS-containing methylcellulose cultures as indicated above. The fraction of precursors killed by [^{3}H]thymidine was calculated as $1 - T/C$ (T, total colonies from dishes II and III; C, total colonies from dishes I and IV).

Reverse Transcriptase–Polymerase Chain Reaction (RT–PCR) Analysis. Total RNA extracted by the guanidinium isothiocyanate/CsCl method (24) from the same number of cells in the presence of 12 μg of *Escherichia coli* rRNA was quantitated by dot hybridization with a human rRNA probe. After densitometric analysis the normalized amount of RNA was reverse-transcribed (Moloney murine leukemia virus RT, BRL) with oligo(dT) as primer. An aliquot of each RT reaction mixture was incubated in the presence of [α-^{32}P]dCTP tracer and the reverse-transcribed RNA·DNA was normalized accordingly. Amplification within the linear range was achieved by 20 cycles of PCR consisting of denaturation at 95°C for 1.5 min, annealing at 52°C for 1.5 min, and extension at 72°C for 1.5 min. Primers for GATA-1 (25) were 5′-TTAGCCACCTCATGCCTT-3′ and 5′-ACATCGGTCTTAAGACCT-3′ and generated a PCR product of 194 base pairs. PCR was performed in a total volume of 100 μl, and 10 μl of each sample was electrophoresed in a 2% agarose gel and transferred to a nylon filter for hybridization with an internal 40-mer GATA-1 probe (5′-TACTGTGGTGGCTCCGCTCAGCTCATGAGGCACAGAGCA-3′). In control experiments, serial dilutions of each sample were amplified; the dose–response curves showed linearity for all points. Relative intensities of bands were quantified by scanning with a laser densitometer (LKB).

GATA-1 Gel Shift Assay. Since this analysis requires a large number of cells, step III progenitors were utilized (see refs. 19 and 25). Nuclear extract preparation (26) and gel shift analysis (15) were performed with 0.27–1 × 10^6 cells per time point. Incubation mixtures (20 μl) contained 2 μg of poly(dI-dC), Epo receptor promoter probe (5–10 × 10^3 dpm/ng) (15), and 2–6 μg of nuclear extract. DNA–protein complexes were resolved in 5% polyacrylamide gels (15). Bands were quantitated with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

Phenotype of Purified Progenitors. Hematopoietic progenitors were purified from normal adult peripheral blood by a four-step procedure (19): the final index of purification is ≈90% and the yield is ≈20% (ref. 19 and legend to Fig. 1).

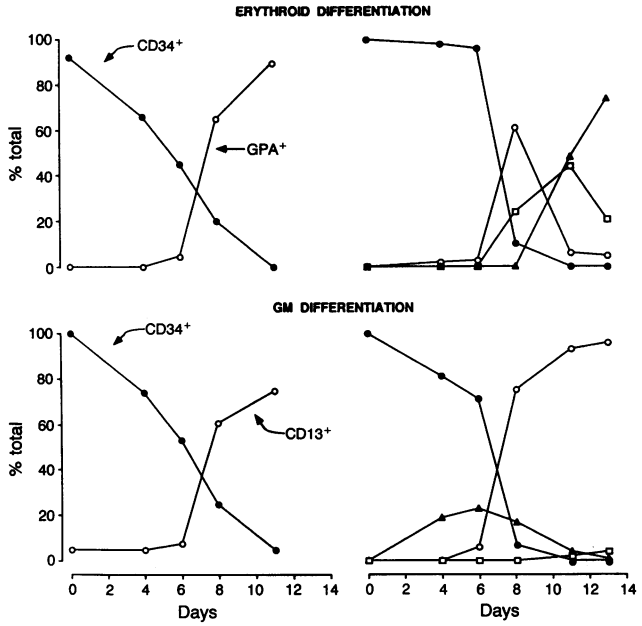

FIG. 4. (*Left*) Gradual decline of CD34$^+$ (●) and appearance of lineage-specific markers (○) on progenitors grown in erythroid (*Upper*) or GM (*Lower*) liquid culture system as outlined in Fig. 2 legend. The appearance of differentiated erythroid or GM cells in the erythroid or GM culture system was monitored with an anti-glycophorin A (GPA) (Ortho Diagnostics, Raritan, NJ) or anti-CD13 (Becton Dickinson) mAb, respectively. (*Right*) Morphologic analysis of progenitors differentiating in liquid erythroid (*Upper*) or GM (*Lower*) culture. Cells were harvested at the indicated days, smeared on glass slides by Cytospin centrifugation, and stained with May–Grümwald/Giemsa reagents. For erythroid cultures: ●, blasts; ○, proerythroblasts and basophilic erythroblasts; □, polychromophilic erythroblasts; ▲, orthochromophilic erythroblasts. For GM cultures: ●, blasts; ▲, immature GM cells; ○, neutrophils and eosinophils; □, monocytes. Mast cells and megakaryocytes were not observed in either the erythroid or the GM culture system.

These highly undifferentiated progenitors (CFU-GEMM, BFU-E, CFU-GM) are largely quiescent, give rise to large colonies in clonogenic culture, and consist of a population that is ≈90% CD34$^+$, 80–90% HLA-DR$^+$, <10% CD45RO$^+$, and <10% CD33$^+$ (Fig. 1). In contrast, marrow progenitors are mostly CD34$^+$33$^+$: the small CD34$^+$33$^-$ population includes the earliest progenitors and stem cells (27). Furthermore, the purified progenitors comprise a small population of multipotent progenitors with self-renewal capacity—i.e., with stem-cell properties (19). Indeed, adult blood mononuclear or CD34$^+$ cells can reconstitute the hematolymphopoietic system when transplanted in patients (28, 29) or monkeys (20), respectively.

Kinetics of Pure Progenitors' Proliferation and Differentiation. The pure progenitors, triggered into cycling by HGFs in FBS-free liquid suspension culture, undergo extensive proliferation coupled with a wave of gradual and synchronized differentiation, which takes place selectively along either the GM pathway (upon treatment with high doses of IL-3 and GM-CSF) or the erythroid pathway (upon addition of low amounts of IL-3 and GM-CSF and saturating levels of Epo) (representative results in Figs. 2–4).

In the first week of culture the progenitors show both a gradual decrease of their proliferative potential (as indicated by the progressive decline of the size of the progenitor-generated colonies; Fig. 2 *Bottom*) and a homogeneous shift of their membrane phenotype (as indicated by double labeling experiments with CD34/33 and CD34/45RO; Fig. 3). This indicates that they undergo a wave of gradual and synchronized differentiation.

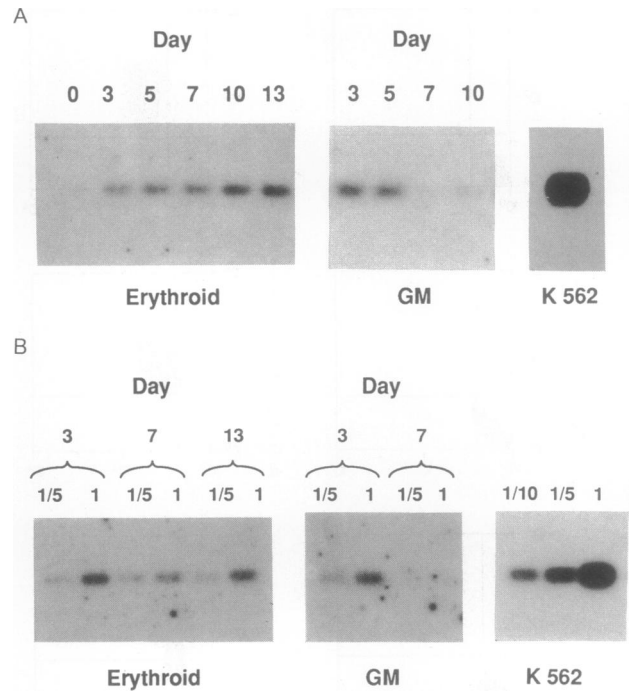

FIG. 5. (*A*) GATA-1 mRNA expression in pure (step IV) progenitors and their progeny grown in the erythroid or GM differentiation liquid culture system, evaluated by RT–PCR. K562, human erythroleukemia cell line. (*B*) Dose–response of GATA-1 mRNA expression in step IV progenitors and their progeny grown in the erythroid or GM differentiation liquid culture system. Serial dilutions (1:10, 1:5, 1:1) of each sample were amplified.

In the second week of culture, progressive expression of specific markers of differentiated erythroid or GM precursors (e.g., glycophorin A and CD13, respectively) is observed, as well as the converse decline of the frequency of CD34$^+$ cells to undetectable levels (Fig. 4 *Left*). Similarly, cell morphology shows a gradual wave of differentiation selectively along the erythroid or GM pathway to terminal differentiation (Fig. 4 *Right*). The *in vitro* differentiated cells exhibit virtually normal morphologic features (data not shown).

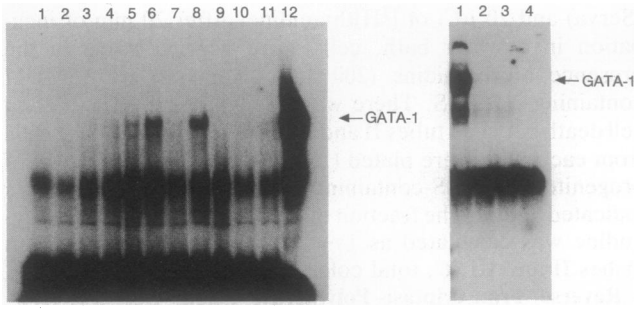

FIG. 6. GATA-1 in step III purified progenitors and their progeny grown in the erythroid or GM differentiation liquid culture system, evaluated by gel mobility-shift analysis with a probe derived from the promoter of the Epo receptor (nucleotides −61 to −28) and encompassing the region of the GATA site. (*Left*) Lanes: 1, day 0; 2, erythroid culture day 1; 3, GM culture day 1; 4, erythroid day 3; 5, GM day 3; 6, erythroid day 7; 7, GM day 7; 8, erythroid day 13; 9, GM day 13; 10 and 11, negative controls; 12, K562 positive control. (*Right*) Gel shift assay using extract from day 3 cultures. Lanes: 1, K562 positive control; 2, erythroid culture day 3; 3, GM culture day 3; 4, negative control. The purification of the step III progenitors was 39% (*Left*) and 27% (*Right*). GATA-1 was not detected in CD34$^-$ cells purified from step III progenitors and grown for 3 days in the GM culture system (data not shown).

RT–PCR Analysis of GATA-1 mRNA in Differentiating Pure Progenitors. We utilized this *in vitro* model to evaluate the expression of GATA-1 mRNA by RT–PCR analysis in progenitors differentiating along the erythroid or GM pathway (representative results in Fig. 5). The GATA-1 gene is barely expressed in the starting population of quiescent progenitor/stem cells (Fig. 5). Addition of IL-3 and GM-CSF induces cell proliferation (Fig. 2), which is coupled to initiation of GATA-1 expression (Fig. 5*A*). Further, the initial level of GATA-1 mRNA (Fig. 5*A*) may be directly related to the cycling activity (Fig. 2). In the erythroid differentiation pathway GATA-1 mRNA expression is maintained and gradually enhanced through the terminal stages of maturation (Fig. 5*A*). Differentiation along the GM pathway is initially associated with GATA-1 expression, which is then abruptly down-regulated at the level of late progenitors/early differentiated precursors (Fig. 5*A*). It must be emphasized that both mast cells and megakaryocytes are absent from the GM culture experiments (Fig. 4). Further, a series of controls including dose–response curves (Fig. 5*B*) ensured a semiquantitative evaluation of GATA-1 mRNA by RT–PCR (see also *Materials and Methods*).

Gel Shift Analysis of GATA-1 Protein in Differentiating Pure Progenitors. We performed gel shift assays using a GATA target site derived from the Epo receptor gene promoter (15). Nuclear proteins were prepared from ≈30% purified hematopoietic progenitors, grown in the erythroid or GM culture system. GATA-1 present in K562 cell extract forms a specific complex on the Epo receptor probe (ref. 15; Fig. 6 *Left*, lane 12). This complex is not detected when extract from the purified, quiescent progenitors is used (Fig. 6 *Left*, lane 1) but is initially detected in the differentiated progenitors after 3 days in erythroid or GM culture (Fig. 6 *Right*, lanes 2 and 3). However, at day 7 of culture, the complex is evident only in the erythroid culture (Fig. 6 *Left*, lanes 6 and 7) and then progressively increases during erythroid maturation up to day 13 (Fig. 6 *Left*, lane 8), with a 30-fold increase throughout erythroid differentiation. Thus, complex formation in differentiating progenitors correlates with GATA-1 mRNA detected by RT–PCR analysis.

Concluding Remarks. Our findings reveal that the regulation of the GATA-1 gene in normal hematopoietic progenitors is complex and involves mechanisms that efficiently activate, maintain, and selectively abrogate expression. Initiation of GATA-1 expression in early progenitor cells is temporally related to the onset of cycling activity triggered by IL-3/GM-CSF. This suggests that activation of the genetic program underlying proliferation of stem/progenitor cells induces the switching on of GATA-1 transcription and translation. Maintenance of expression in developing erythroid cells is likely to be mediated in part through positive autoregulation (14). The abrupt down-regulation of GATA-1 expression in GM development implies mechanism(s) operating in late GM progenitors to curtail autoregulation or actively extinguish expression. Though GM progenitors appear to express GATA-1, their development to terminal cells proceeds in the absence of GATA-1 (13). Hence, the initial domain of GATA-1 expression extends beyond that of the mature lineages in which it is thought to serve as a transcriptional regulator (30). This pattern is reminiscent of the widespread distribution of the myogenic factor MyoD in mesoderm prior to sustained expression in developing muscle (31).

Our studies are compatible with a two-step model for the expression of GATA-1 in early hematopoiesis, which involves (*i*) initially a switch on, linked to initiation of stem/progenitor-cell cycling, and (*ii*) subsequently, increasing expression along the erythroid differentiation pathway contrasted with abrupt down-regulation in the GM lineage at a relatively advanced stage of differentiation. If cessation of GATA-1 expression is obligatory for maturation of GM progenitors, on/off switches of lineage-restricted transactivators may underlie the binary fate decisions of hematopoietic progenitor cells.

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