Hemoglobin Switching in Humans Is Accompanied by Changes in the Ratio of the Transcription Factors, GATA-1 and SP1

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ABSTRACT

Background: Understanding the mechanism of developmental regulation of hemoglobin switching has scientific as well as clinical relevance because of the influence of fetal hemoglobin (HbF) production in adulthood on the clinical manifestation of thalassemia and sickle cell anemia. We have previously found that the normal developmental patterns of globin gene expression are recapitulated in an experimental system of primary cultures that support differentiation of erythroid progenitors. We further found that high activities of the transcriptional activators, GATA-1 and SP1, are associated with normal adult erythroid differentiation.

Materials and Methods: In the present work, we have studied the activities of GATA-1 and SP1 during differentiation of cultured erythroid progenitors derived from cord blood and from fetal livers, as well as from β^0-thalassemia patients.

Results: The results showed high GATA-1 binding ac-

tivity and very low SP1 activity in the fetal liver cultures. This pattern was in contrast to cultures derived from normal adult peripheral blood, in which both GATA-1 and SP1 activities were high. Cord blood cultures showed an additive combination of "adult" and "fetal" patterns. The progenitors derived from a β^0-thalassemia patient with high HbF production showed "fetal" pattern. On the other hand, in cultures of 2 β^0-thalassemia patients without high HbF, "adult" pattern was observed.

Conclusions: In the present work, we show that human fetal and adult erythroid progenitors are distinct in their transcription factors, and that the commitment to fetal or adult program occurs at a very early differentiation stage. Our studies also demonstrate that under anemic stress, recruitment of fetal progenitors may occur in adulthood.

INTRODUCTION

Elucidation of the mechanism underlying developmental regulation of hemoglobin switching has important medical implications. For example, elevated fetal hemoglobin (HbF) ($\alpha 2\gamma 2$) production in adult patients with sickle cell anemia or β-thalassemia ameliorates the disease symp-

toms, as the γ-globin chains may compensate for the reduction in β-globin chains. In some individuals this occurs due to γ-globin promoter mutations or to deletions in the β-globin gene cluster. In others, the mechanism is not understood.

In spite of intensive research, this mechanism has not been clarified, partly because of the unavailability of an adequate animal model, since the switch mechanism varies among different mammals. It has been suggested that normal adult erythroid cells go through a "fetal" stage during differentiation before maturing into cells producing adult hemoglobin ($\alpha 2\beta 2$) (1). The

clonal model, on the other hand, invokes distinct progenitors and separate pathways (2).

Our previous studies demonstrated that adult and neonatal patterns of globin gene expression are recapitulated in liquid cultures that support proliferation and differentiation of erythroid progenitors (3). The cultures are grown in two phases: first the early progenitors, burst forming units, erythroid, (BFUe), proliferate and differentiate into colony forming units, erythroid (CFUe). Further differentiation and maturation, including globin gene expression and hemoglobin biosynthesis, is erythropoietin (EPO) dependent (4). In our experiments (3), cultured adult progenitors, from peripheral blood, gave rise primarily to the β- and α-globin expressing cells, while progenitors of neonate cord blood (CBL) differentiated into cells that also synthesized high levels of γ-globin mRNA. Those experiments suggested that the fetal progenitors are distinct from adult progenitors and that CBL contains a mixed population of both. The peak of γ-globin gene expression usually preceded that of β, implying that fetal progenitors tend to differentiate faster than the adult ones.

We further found that the appearance of globin mRNA in adult cultures was preceded by a burst in the level of GATA-1, which was triggered by the addition of EPO (5). GATA-1 is a major transcriptional activator of erythroid differentiation (6,7), regulating a number of erythroid specific genes, including globin (8). We observed a parallel rise in the level of the ubiquitous factor, SP1, indicating that not only GATA-1, but also SP1 participates in adult erythroid maturation. Additional evidence for the role of SP1 in human erythroid differentiation comes from other studies. Analysis of binding of transcriptional factors at the β-globin locus control region (LCR) suggested that the combination of SP1 and GATA-1 plays a role in the high levels of globin gene expression (9). It was also found that GATA-1 and SP1 interact at the γ-globin promoter. Depending on their concentrations, this interaction led to synergistic transcriptional activation or to interference (10). Furthermore, it has been reported that the levels of GATA-1 and SP1 play an important role in the mechanism of globin switching in the chicken (11). Primary cells of the primitive erythroid lineage were found to contain 10-fold more of these two factors than did cells of the definitive erythroid lineage.

In the present work, we analyzed the activities of GATA-1 and SP1 in primary cultures de-

rived from CBL, mid-gestation fetal livers, and adult β-thalassemia patients with high and low HbF production. Our results indicate that fetal and adult erythroid differentiation occurs along separate pathways.

MATERIALS AND METHODS

Erythroid Cultures

CBL samples were obtained from the Hadassah maternity ward. Liver tissues were derived from fetuses aborted at 20 weeks gestational age (because of a nonglobin genetic defect), obtained from the Department of Pathology, Hebrew University (Jerusalem, Israel). Peripheral blood samples of β-thalassemia patients were withdrawn prior to transfusions. All samples were obtained according to the Helsinki Committee guidelines.

The liver tissues were finely minced and dispersed by pipetting. The cultures were grown in two phases as previously described (3,4). The dispersed liver cells, or buffy coats of the blood samples, were cultured in the presence of conditioned medium from human bladder carcinoma cells for 7 days (from Day −7 to Day 0). The cells were then washed and recultured in medium supplemented with 1 U/ml recombinant EPO. Microscopic slides were prepared by cytospin, following lysis of the enucleated red blood cells with H_2O, and stained with May-Grunwald Giemsa. Hemoglobinized cells were identified by the benzidine peroxidase reaction (12).

RNA Analyses

RNA was purified from cytoplasmic miniextracts, prepared in parallel to nuclear extracts (13,14) from a total of 2×10^6 cells. Reverse transcription (RT) was performed with oligo(dT).

The primers for PCR were the following: for γ-globin, the 5' primer was ACAAGGCTACTAT CACAAGCCT (exon 1), and the 3' primer was CTTTATGGCATCTCCCAAGGAA (exon 2); for β-globin, the 5' primer was CACCTTTGCCA CACTGAGTGA (exon 2), and the 3' primer CCA GATGCTCAAGGCCCTTC (from the untranslated region 3' to exon 3). The amplification cycle was 92°C for 1 min, 60°C for 1 min, and 72°C for 1 min, repeated for 30 cycles. The reaction products were visualized by ethidium bromide staining following electrophoresis on 8% native acrylamide gels.

The PCR primers for the GATA-1 mRNA

analysis were GCCCATCTCTACCAAGGCCT and AGAAGGACACCACCCCTCCT, derived from the 3' end of the human GATA-1 mRNA, producing a 204 bp amplification product (5). The PCR primers for GATA-2 analysis were CCCTAAG CAGCGCAGCAAGAC and GATGAGTGGTCGG TTCTGGCC (15). The amplification cycle for both GATA-1 and GATA-2 was 92°C for 1 min, 62°C for 1 min, and 72°C for 1 min, repeated for 30 cycles.

Preparation of Nuclear Extracts and Gel Retardation Assays

Nuclear miniextracts (13) were prepared from a total of 2×10^6 cells for each time-point. Binding activities of GATA-1 and SP1 were assayed by the gel retardation assay as previously described (5). Oligonucleotides for the gel retardation assay were the following: for GATA-1, CGGGCAACT GATAAGGATTCCCA, derived from the promoter region of the mouse α-1 globin gene (16); and for SP1, CTTGGGGGCCCCTCCCCCACACTATCTCAAT, derived from the human γ-globin gene (this oligonucleotide contains a T to C transition at position -198, creating a strong SP1 binding site) (17). The specific 5' end-labeled oligonucleotides were annealed with an excess of the complementary oligonucleotide and repurified by polyacrylamide gel electrophoresis. Binding reactions were performed on equal amounts of nuclear proteins obtained from 1.2×10^5 cells. Competition experiments were performed by adding 100-fold molar excess of unlabeled competitor oligonucleotide. The DNA-protein complexes were then separated on 5% native polyacrylamide gels.

Intensities of the shifted bands were determined by phospho-imaging of the gels using the Bio-Imaging Analyzer, BAS1000, FUJIX.

RESULTS

GATA-1 and SP1 Activities in Erythroid Progenitors Derived from CBL

To gain an insight into the mechanism of hemoglobin switching, we examined the factors GATA-1 and SP1 in differentiating progenitors derived from CBL. Samples of CBL were grown in the two-phase liquid culture system (4). In the first phase, mononuclear cells were cultured with conditioned medium containing hemapoietic growth factors not including EPO. EPO was

added at the beginning of the second phase. Samples were taken at different time points, and nuclear extracts were prepared. We found that, similar to the adult cultures, high levels of GATA-1 binding activity were present on Days 7–12 (Fig. 1a and b). However, while the level of SP1 in the adult cultures rose in parallel to that of GATA-1 (5) (see also Fig. 3d), in the CBL culture SP1 was very low on Days 7 and 9, and increased substantially, to adult level, only on Day 12. These observations are consistent with the notion that CBL contains a mixture of adult and fetal progenitors. We concluded that the high GATA-1 and SP1 (seen on Day 12) characterize the adult progenitors present in the culture, which appear to differentiate more slowly than the fetal progenitors. We attributed the high GATA-1/low SP1 activities seen on Days 7–9 to the differentiating fetal cells. Therefore, the results implied that the fetal erythroid differentiation program is associated with high GATA-1 activity and low SP1.

Differentiation of Fetal Liver Progenitors in Liquid Culture

To obtain more direct evidence we proceeded to investigate differentiating erythroid cultures derived from midgestation human fetal livers, as a source of pure fetal progenitors. Livers were obtained from aborted fetuses (Week 19–20 gestational age) and single-cell suspensions were prepared. Those included a large proportion of nucleated, hemoglobinized erythroid cells (orthochromatic normoblasts). The cells were plated with conditioned medium (Day -7). Three days later (Day -4) a small number of nonhemoglobinized erythroid cells (pronormoblasts and basophilic normoblasts) appeared. Those were presumably cells that were beyond the CFUe stage, and already EPO-induced, when placed in culture. During the first phase, the number of the nucleated erythroid cells decreased dramatically, due to enucleation of the orthochromatic normoblasts and cell death. The early erythroblasts, which were visible on Day -4, did not give rise to hemoglobinized cells, since they probably died in the absence of EPO. After EPO addition (Day 0) significant erythroid proliferation and differentiation ensued. The differentiation of a representative culture after EPO addition is shown in Fig. 2a.

Globin mRNA was analyzed by the RT-PCR procedure. As seen in Fig. 2b, cells from the first phase contained both β- and γ-mRNA (Days -4
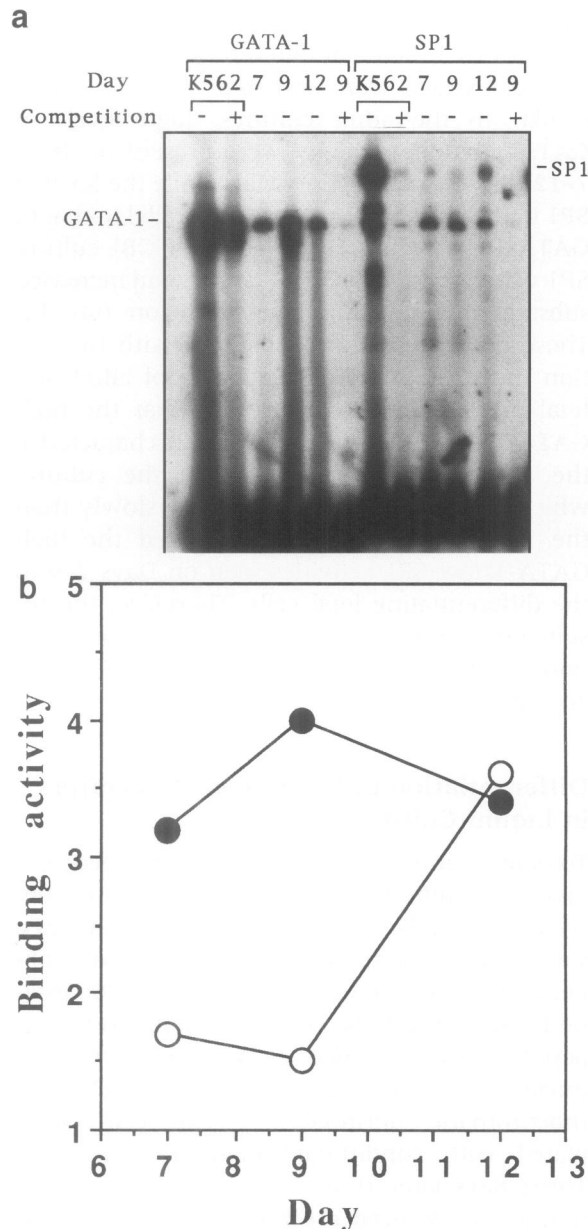
FIG. 1.   Analysis of transcription factors GATA-1 and SP1 in CBL culture, second phase

(a) Autoradiogram of gel retardation assay. The numbers on top represent days in the second phase, after EPO addition. K562, an erythroleukemia cell line serving as a positive control; competition, binding in the presence of 100-fold molar excess of unlabeled competitor oligonucleotide. The lower band in the SP1 retardation assay, which comigrates with the GATA-1-shifted band, is probably due to nonspecific binding (17). (b) Quantitative analysis of the autoradiograms. ●, GATA-1 binding activity; ○, SP1 binding activity.

and 0), suggesting that they included both fetal-type and adult-type cells. The adult-type cells in the fetal liver were presumably derived from the circulating fetal blood. At this gestational age, approximately 10% of the hemoglobin in the fetal blood is adult hemoglobin (normal β/γ-chain synthesis ratio is ~10% [18]). In the second phase, the cells contained only γ-globin mRNA (Fig. 2c, Days 5 and 8), indicating that those cells were derived from pure fetal progenitors. The 343 bp band corresponds to the unspliced γ-RNA (or DNA).

Analyses of GATA-1 and SP1 Activities in the Fetal Liver Cultures

Nuclear proteins were extracted and assayed for the levels of GATA-1 and SP1 by the gel retardation assay using end-labeled double-stranded oligonucleotides containing either binding site. The results obtained from the fetal liver culture shown in Fig. 2 are presented in Fig. 3a and b, and summarized graphically in Fig. 3c. Two additional fetal liver cultures gave similar results (not shown). EPO was added on Day 0. This was followed by an increase in the level of GATA-1, with a peak on Day 6, while the level of SP1 remained low throughout the culture period, in contrast to adult cultures, in which SP1 activity was significantly higher and its kinetics paralleled that of GATA-1 (compare Fig. 3c and d). For comparison, binding activity of GATA-1 and SP1 of a normal adult-derived culture grown, harvested and analyzed in parallel to the fetal liver culture is shown in Lanes 14–15 (Days 5 and 7) in Fig. 3a and b. In the fetal liver cells harvested on Day −7, when the culture first started, the levels of both transcription factors were high, consistent with the presence of adult-type β-globin expressing cells. The levels of both transcription factors decreased as those (presumably BM-derived) cells matured.

Before replating the cells in the second phase (Day 0), the culture was divided, and part was grown without EPO. Neither GATA-1 nor SP1 activities were detected in the absence of EPO throughout the culture period (Lanes 16–19, Fig. 3a and b). Those nuclear extracts were positive for the ubiquitous transcription factor OTF-1 (data not shown), which served as an internal control for nuclear protein extraction. These results corroborate that GATA-1 binding activity is EPO dependent.

The experiments demonstrate that high SP1 activity is not involved in late fetal erythroid
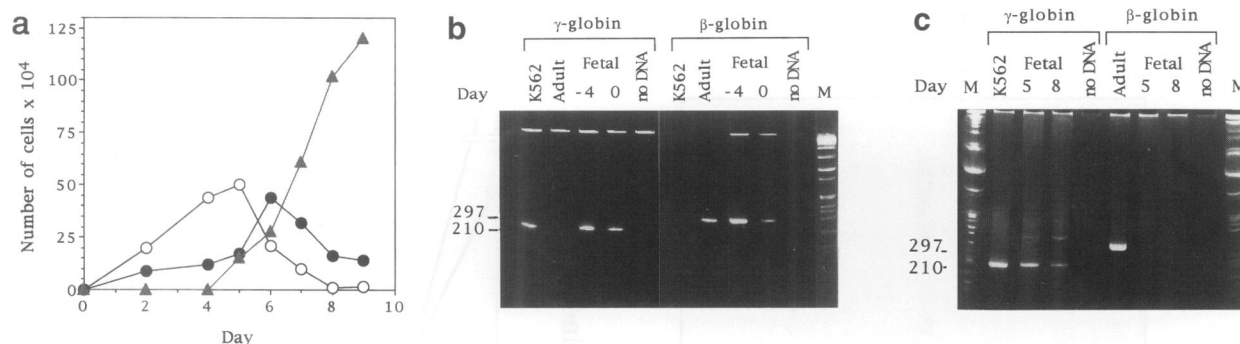
FIG. 2. Differentiation of cultured erythroid progenitors derived from fetal liver

(a) Morphological studies, second phase. The graphs represent the number of cells per 2×10^6 nucleated cells harvested at each time point after the addition of EPO on Day 0. O, nonhemoglobinized erythroid cells (pronormoblasts and basophilic normoblasts); ●, Partially hemoglobinized erythroid cells (polychromatophilic normoblasts); ▲, Fully hemoglobinized cells (orthochromatic normoblasts). (b and c) RNA analyses of first and second phase of the fetal liver culture, respectively. Total cytoplasmic RNA was harvested and aliquots of the same RNA samples were analyzed by RT-PCR for either γ-globin mRNA (210 bp amplification product) or for β-globin mRNA (297 bp). The numbers represent days in culture. K562 RNA served as a positive control for γ-mRNA; adult culture-derived RNA, purified from a sample harvested on Day 5, served as a positive control for β-mRNA. M, DNA size marker.

differentiation. We, therefore, concluded that fetal progenitors are distinct from adult progenitors not only in their globin mRNA species but also at the level of transcription factors.

The transcription factor GATA-2 has the same binding specificity as GATA-1. It has been demonstrated, by gene targeting experiments in mouse embryonic stem cells, that GATA-2 plays a critical role in early hematopoiesis, particularly of the adult type (19). We tested the fetal culture for presence of GATA-2 RNA by RT-PCR (15). Some GATA-2 mRNA was observed during the first phase of the culture, supporting the suggested role of GATA-2. After EPO addition, only GATA-1 and no GATA-2 mRNA was detected (data not shown).

Recruitment of Fetal Progenitors in Adulthood

Some forms of high HbF production in adults are associated with anemic stress, and are thus expressed only in patients with β-thalassemia or sickle cell anemia but not in their parents or carrier siblings. A possible mechanism is the recruitment of fetal cells, induced by the anemic stress. The ability to distinguish between fetal and adult erythroid cells at the level of transcription factor allows the testing of this hypothesis.

We analyzed GATA-1 and SP1 in erythroid cultures derived from peripheral blood of three β^0-thalassemia patients, carrying β^0 mutations.

These usually have severe clinical presentation because of the complete absence of β-globin chains. Patient A (Fig. 4a), homozygous for the β-globin mutation IVS2-1, has a mild form of thalassemia (intermedia) owing to very high HbF production (γ/α chain synthesis ratio 0.42 at the age of 27 years). His high HbF production in adulthood is not due to γ-globin promoter mutations but to an unidentified factor that functions in the presence of anemic stress (20). As seen in Fig. 4a, his cultured erythroid cells present a "fetal" pattern (high GATA-1/low SP1 levels), supporting the model of persistent recruitment of fetal progenitors in adulthood. Additional evidence comes from other parameters associated with fetal erythropoiesis: high titer of i antigen, an elevated level of Gγ (42%) and extensive (40%) γ-globin DNA hypomethylation (in his erythroid cells) (21). On the other hand, his niece (Patient B, Fig. 4b), who is also homozygous for the same β-globin mutation, has a severe clinical form of thalassemia (major) without high HbF (γ/α chain synthesis ratio 0.24 at the age of 2 years). Because of the low number of cells, only two time points were analyzed. Her culture differentiated rapidly, and the samples represent the later stages of erythroid differentiation. Nevertheless, it can be seen that she presents "adult" pattern of transcription factors (significant SP1 activity, which parallels GATA-1). Likewise, an unrelated patient (Patient C, Fig. 4c) with a β-globin genotype IVS2-1/FS44 and low
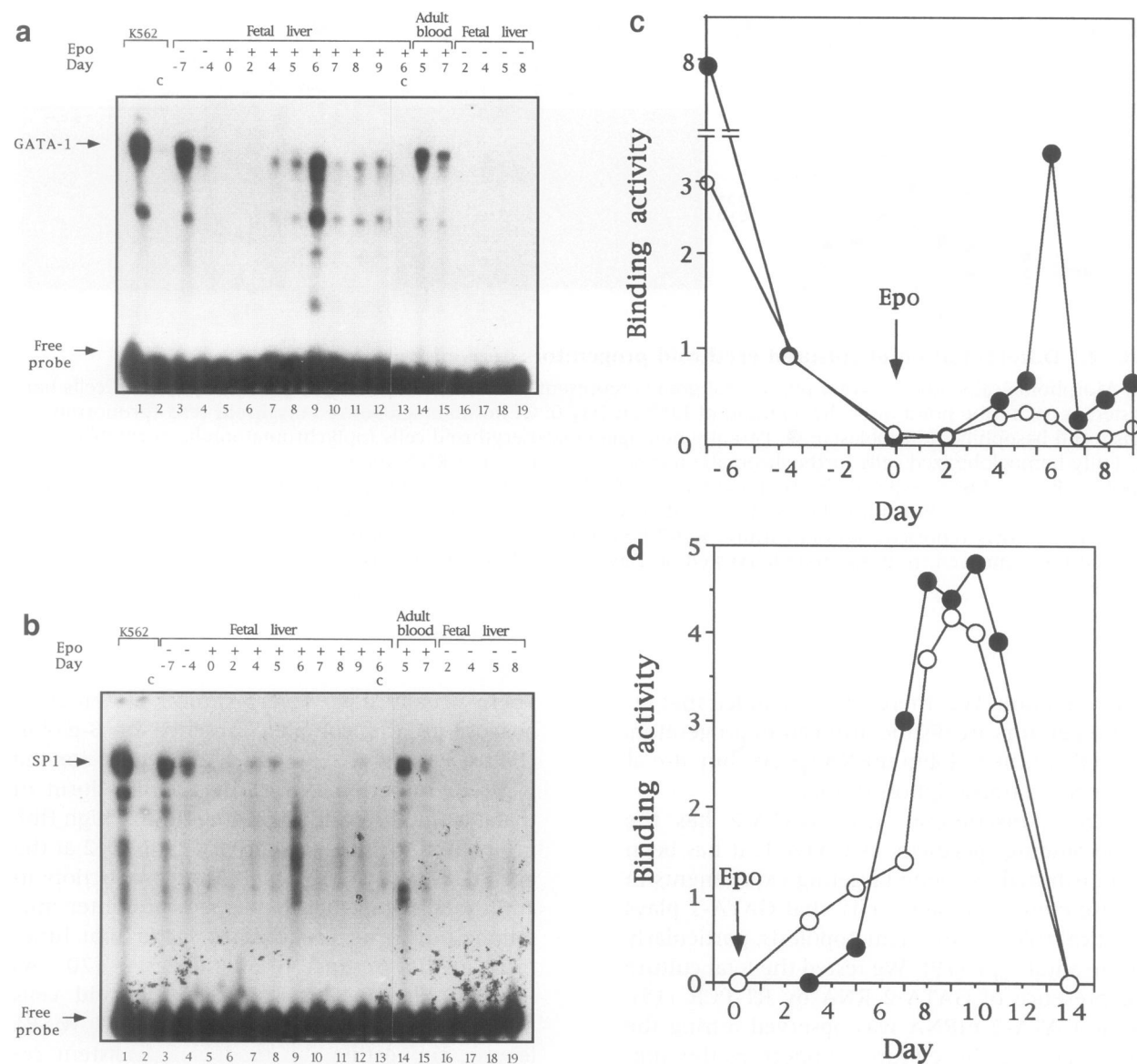
FIG. 3. Transcription factors GATA-1 and SP1 in differentiating fetal erythroid cells

The free probe and the shifted bands are indicated by the arrows. The day of harvest and EPO addition are shown above. The lanes are numbered below. (a) GATA-1 binding activity. (b) SP1 binding activity. Lanes 1 and 2, nuclear extracts of K562 cells as a positive control; Lanes 2 and 13 (designated c), cold competition—binding in the presence of 100-fold molar excess of unlabeled oligonucleotide; Lanes 3–13, fetal liver cultures, first and second phase; Lanes 14 and 15, binding activity of nuclear extracts of a normal adult-derived culture grown, harvested and analyzed in parallel to the fetal liver culture; Lanes 16–19, nuclear extracts of a parallel culture of the same liver, grown in the second phase in the absence of EPO. (c) Quantification of the autoradiograms (shown in a and b). (d) GATA-1 and SP1 binding activities of a normal adult-derived culture. ●, GATA-1 binding activity; ○, SP1 binding activity.

HbF (γ/α chain synthesis ratio of 0.24) also shows "adult" pattern. It appears that not all β-thalassemia patients are capable of significant recruitment of fetal progenitors. Further studies are needed to clarify the mechanism underlying this phenomenon.

DISCUSSION

Our studies demonstrate that erythroid progenitors derived from fetal liver have a genetic program that is distinct from that of adult-derived cells. In response to EPO, the fetal erythroblasts
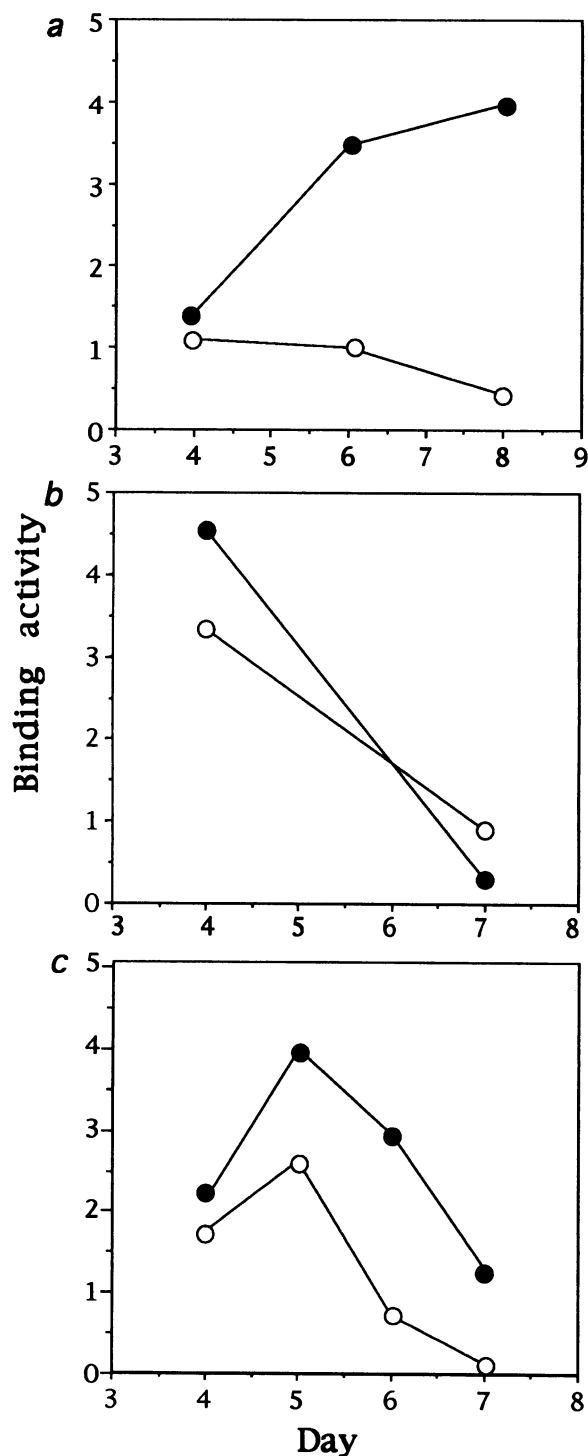
FIG. 4. GATA-1 and SP1 activities in erythroid cultures derived from β^0-thalassemia patients

(a) Patient A has thalassemia intermedia due to elevated HbF production. (b) Patient B is the niece of Patient A. She is transfusion dependent β-thalassemia major. (c) An unrelated β-thalassemia major patient. ●, GATA-1 binding activity; ○, SP1 binding activity.

express a high level of GATA-1 but only a low level of SP1, in contrast to adult-derived cells, in which both transcription factors rise in parallel. Moreover, we observed good correlation between the patterns of these transcription factors and the RNA species present. High GATA-1 and high SP1 correlated with β-globin mRNA expression, as was seen both in the first phase of the fetal liver cultures and in the later stage of the CBL culture (Fig. 1, Day 12). On the other hand, high GATA-1 and low SP1 were associated with only γ-globin mRNA expression, as observed in the second phase of the fetal liver cultures.

Our experiments further indicate that the fetal and adult progenitors retain their identities when present together in the same environment in the CBL cultures. Thus, commitment to the adult versus the fetal program is determined at a very early stage of differentiation, probably pre-BFUe, before the progenitors are placed in culture. It is possible that the fate of the progenitor is determined by effector cells present in the respective erythropoietic organ (liver versus bone marrow).

Hereditary persistence of fetal hemoglobin (HPFH) in adulthood may be induced by several mechanisms. These include mutations in *cis* to the γ-globin genes (promoter point mutations and large deletions in the β-globin gene cluster) as well as unlinked genetic factors (22 and references cited therein). In addition, HbF production may be induced by anemic stress. The pattern of transcription factors in the differentiating erythroid cells of Patient A, with the high HbF, provides strong evidence for the activation of fetal progenitors. However, the analyses of erythroid cultures of the other patients indicate that this is not a general phenomenon. We have previously found that the genetic factor that allows high HbF production in Patient A is unlinked to the β-globin gene cluster (20).

The level of HbF production in patients with sickle cell anemia and β-thalassemia is of high clinical significance, since γ-globin peptides may alleviate the disease symptoms. Several agents, such as hydroxyurea and butyrate derivatives, have been shown to increase γ-globin gene expression in adult patients. HbF and F-reticulocyte levels in sickle cell anemia patients were found to increase significantly 48–72 hr after the administration of hydroxyurea began (23). Similarly, γ-globin synthesis followed the administration of butyrate closely (24). These findings suggest that the function of these drugs was exerted on cells that were already well on the maturation path-

way, after the point of commitment to the adult program. Furthermore, experiments in erythroid cultures (grown similarly to those studied here) showed that both hydroxyurea and butyrate derivatives significantly elevated γ-globin expression when added to the cultures 4 days after EPO (25,26), beyond the CFUe stage. We suggest that the activation of HbF by these agents is not via recruitment of fetal progenitors, but by a fundamentally different mechanism. We hypothesize that the adult progenitors are capable of increased HbF production when appropriately triggered. In the adult progenitor, the complete β-globin gene domain has an "open" chromatin configuration due to the activity of the LCR (27,28). We postulate that this "open" chromatin state, in the absence of specific repressors (such as the ε-globin gene silencer [29]), allows γ-globin gene expression to be turned on in response to nonspecific, general gene activators. Further studies are needed to clarify this issue, which has important therapeutic implications.

ACKNOWLEDGMENTS

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