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Embryonic–fetal erythroid characteristics of a human leukemic cell line

(K562 cell line/human globin mRNA and cDNA/molecular hybridization/i cell surface antigen/lactate dehydrogenase isoenzymes)

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ABSTRACT **We have studied a number of cell surface, enzyme, and protein markers in the human leukemic K562 cell line. We have confirmed previous observations that these cells accumulate human embryonic hemoglobins after exposure to hemin. In addition, our results demonstrated that these cells possess in their "uninduced" state i surface antigen, lactate dehydrogenase isoenzymes characteristic of embryonic or fetal erythroid cells, fetal and embryonic globin chains, and globin mRNAs. The levels of i antigen, embryonic globin chains, and embryonic globin mRNA increased substantially after exposure of the cells to hemin in suspension culture. In contrast, K562 cells lacked several surface, enzymatic, and functional properties typical of granulocytes, lymphocytes, monocytes, or adult erythroblasts, including HLA surface antigens, surface immunoglobulins, sheep erythrocyte rosetting, phagocytosis, terminal deoxynucleotidyl transferase, carbonic anhydrase, ABO and Rh blood groups, and adult hemoglobins. The K562 cell line therefore exhibits phenotypic properties of embryonic erythroid progenitor cells and a quantitative increase in the expression of some of these properties can be achieved by exposure of the cells to hemin.**

The K562 cell line is a Philadelphia chromosome-positive line derived from a pleural effusion of a patient with chronic granulocyte leukemia in terminal blast crisis (1). A number of recent observations suggest that suspension cultures of K562 cells may contain cells that can exhibit erythroid properties when exposed to appropriate *in vitro* culture conditions. The K562 line has been shown to produce glycophorin, the major sialoglycoprotein of the erythroid cell surface (2). At least two agents (hemin and sodium butyrate) known to "induce" the development of erythroid phenotypic features by mouse erythroleukemia cells (3) also promote hemoglobin accumulation in K562 cells: Andersson *et al.* (4) have shown that exposure to sodium butyrate leads to accumulation of hemoglobin detectable by benzidine staining and radioimmunoassay, and we (5) have demonstrated that K562 cells exposed to this agent during growth in semisolid culture systems give rise to benzidine-positive colonies resembling erythroid colonies derived from normal human erythroid stem cells (BFU-E). Rutherford *et al.* (6) have rigorously established that embryonic hemoglobins (Hb Portland and Hb Gower), as well as small quantities of fetal hemoglobin (Hb F) are produced by the K562 line after exposure to hemin in suspension culture. K562 cells thus appear to possess the capacity for at least partial erythroid differentiation. These cells are potentially valuable for studies of the dynamics of erythropoiesis and hemoglobin switching, and for analysis of expression of specific genes in neoplastic cells.

Lozzio *et al.* (7) have recently challenged the assumption that K562 cells are erythroid-like stem cells, on the basis of their observation that a K562 line exposed to hemin failed to exhibit morphologic characteristics of erythroid differentiation or to accumulate adult globins (α and β globin chains). In contrast, Andersson *et al.* (8) have reported that the same clone of K562 cells contains glycophorin. In order to characterize further the erythroid potential of K562 cells, we have examined uninduced and hemin-induced K562 cells for the presence of surface and enzymatic characteristics, as well as for the accumulation of globin and globin mRNA production. Our findings suggest that uninduced K562 cells possess detectable erythroid features whose phenotypic expression is enhanced by exposure to the inducing agent hemin. Moreover, the phenotype expressed is more characteristic of early embryonic or fetal, as opposed to adult, hematopoietic cells.

MATERIALS AND METHODS

Cell Line. K562 cells, clone 6, were a kind gift of C. G. Gahmberg; this culture has been shown to contain glycophorin on its surface (2), and was kindly provided to us by H. Furthmayr, Dept. of Pathology, Yale University School of Medicine. A second culture of K562 cells, designated in this report as "clone R," was the kind gift of T. R. Rutherford and J. B. Clegg; these cells have been shown to produce embryonic hemoglobin in response to hemin (6). Cells were maintained at 37°C in a humidified atmosphere in RPMI 1640 medium supplemented with 10% fetal calf serum. "Induction" was accomplished by adding a 10-fold or 100-fold concentrated stock of hemin to a final concentration of 0.05 mM. Hemin was prepared as described (9) and sterilized by filtration prior to addition to the cultures. Cell growth was continued for 4 or 6 days after the addition of hemin. Cells were then harvested by centrifugation, washed with 0.9% saline, and resuspended in the appropriate buffer for analysis. Exposure to hemin reduced cell viability, as assessed by trypan blue exclusion, by 10–35%.

Surface, Functional, and Enzymatic Assays. K562 cells were examined before and after hemin induction for HLA surface antigens, lymphocyte surface and enzymatic markers, phagocytosis, erythrocyte surface antigens, and lactate dehydrogenase (LDH), using established methods described in *Results* and table legends (cf. refs. 9–14). Terminal deoxyribonucleotidyl transferase assays were kindly performed by H. S. Allaudeen, Dept. of Pharmacology, Yale University School of Medicine, using published methods (15).

Analysis of Globin and Hemoglobin. Cells were harvested by centrifugation, washed, and lysed in a solution of 150 mM NaCl/50 mM Tris·HCl, pH 8.0/3 mM $MgCl_2$/0.5% Triton X-100. In some cases, protein was concentrated by pressure

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Abbreviation: LDH, lactate dehydrogenase.

Table 1. Surface and functional markers absent from K562 cells

Assay	Principal cell type associated with marker	Result	
		Induced	Uninduced
Surface immunoglobulin	B lymphocytes	Absent	Absent
"E-rosetting"	T lymphocytes	Absent	Absent
Terminal transferase	Pre-T lymphoblasts	Absent	Absent
Latex particle phagocytosis	Monocytes, macrophages	Absent	Absent
HLA antigens	Multiple cell types	Absent	Absent

Assays for surface immunoglobulins, E-rosetting with sheep erythrocytes, and phagocytosis were performed exactly as described by Dwyer (13). Terminal transferase screening was performed exactly as described by Shaw *et al.* (15). K562 cells (clone 6) were analyzed before and after 4 days of induction with 0.05 mM hemin. For each assay, appropriate positive and negative control cells were analyzed simultaneously with K562 cells and yielded the appropriate positive or negative results. HLA antigen activity was assessed by a sensitive complement-mediated micro-cytotoxicity assay (11, 12). Appropriate control cells gave positive results (data not shown).

dialysis prior to analysis. Hemoglobin was detected by electrophoresis on acrylamide gels in Tris/borate buffer at pH 8.6 and staining with benzidine in nitroprusside solution, using methods described in detail previously (6, 16). Globin chains were analyzed on acetic acid/urea/Triton X-100/acrylamide gels, using the method of Rovera *et al.* (17), as modified by Alter (18). Protein bands were detected by staining with 1% Coomassie blue; radioactive protein from mRNA translation assays was detected by autoradiography of dried gels.

Isolation and Analysis of mRNA. Purification of mRNA, translation of mRNA in a wheat germ mRNA-dependent cell-free protein-synthesizing system, synthesis of cDNAs by reverse transcriptase from the mRNA templates, and molecular hybridization analysis of the cDNAs and mRNAs were performed, using the methods we have described in detail elsewhere (19, 20).

Globin mRNA species were detected by gel blotting, using the methods described by Wahl *et al.* (21) and Alwine *et al.* (22). Electrophoretic separation of RNAs was performed on 2.5% agarose gels in 10 mM sodium phosphate buffer at pH 7.0 prior to transfer of the RNA bands to activated cellulose paper and hybridization to cloned human, α, β, and γ globin cDNA probes (23), which had been rendered radioactive by nick-translation in the presence of [^{32}P]dCTP, using established methods (24). After hybridization overnight at 37–42°C in the buffer described by Alwine *et al.* (22), the strips were washed initially in a solution of 50% (wt/vol) formamide/0.75 M NaCl/0.075 M sodium citrate/50 mM sodium phosphate, pH 7.0, then washed a final time in 0.30 M NaCl/0.030 M sodium citrate/0.1% sodium dodecyl sulfate prior to drying and autoradiography.

RESULTS

The results of a survey of clone 6 cells before and after induction with hemin for surface, functional, and enzymatic properties are shown in Table 1. Surface characteristics of B and T lymphoid cells were absent, as was latex particle phagocytosis, a property of monocytes and macrophages. These characteristics remained undetectable after induction with hemin.

As shown in Table 2, clone 6 cells also lack ABO and Rh blood group antigens, but possess i antigen activity, a feature characteristic of fetal erythrocytes. Uninduced K562 cells were weakly reactive with anti-i antiserum but, after exposure to 0.05 mM hemin for 4 days, the cells exhibited a strong increase in i antigen activity. The level of i antigen observed was comparable to that obtained in parallel assays with cord blood erythrocytes and the same antiserum. As shown in Table 2, our anti-i antiserum reacted only with cord, and not with adult human, erythrocytes.

LDH isoenzymes were analyzed by electrophoresis of concentrated cell lysates on Cellogel, followed by specific enzymatic staining, as described by Ruddle and Nichols (14). Human fetal liver erythroblasts, from a 16-week-old abortus, yielded a symmetrical pattern with five LDH isoenzyme bands indicating approximately equal production of LDH A and LDH B subunits, whereas adult erythrocytes showed three rapidly migrating anodal bands demonstrating a greater proportion of LDH B than LDH A subunits. Both uninduced and hemin-induced K562 cells were observed to give a five-banded symmetrical pattern virtually identical to that of fetal liver erythroblasts (data not shown). Hemin induction had no effect on the intensity or relative expression of the LDH isoenzymes.

Analysis of uninduced and induced K562 cells for the presence of hemoglobin was accomplished by benzidine staining of proteins separated by acrylamide gel electrophoresis in a Tris/borate/EDTA system. As shown in Fig. 1, the positions of authentic hemoglobin markers were analyzed in parallel lanes; the positions of Hb A ($\alpha_2\beta_2$), Hb F ($\alpha_2\gamma_2$), and Hb A_2 ($\alpha_2\delta_2$) are shown in lanes 1 and 2. The positions of the embryonic hemoglobins, Hb Barts (γ_4) and Hb Portland ($\zeta_2\gamma_2$), were verified by analysis of an erythrocyte lysate from a fetus with homozygous α-thalassemia (hydrops fetalis with Hb Barts) (lane 7); the position shown for Hbs Gower ($\zeta_2\epsilon_2$ and $\alpha_2\epsilon_2$) is similar to that obtained by Rutherford *et al.* (6). Both clones of uninduced K562 cells contained low levels of hemoglobin, consisting predominantly of Hb Gower and Hb Portland (lane 3) or of Hb Gower alone (lane 4). A considerable increase in hemoglobin accumulation was observed after hemin induction with both clone 6 and clone R cells (lanes 5 and 6). Moreover, distinct differences in the types of hemoglobin accumulating were observed: clone 6 cells (lane 5) contained Hb Gower, Hb Port-

Table 2. i erythrocyte surface antigen on K562 cells

Cell type	i antigen titer
Uninduced K562	1:4
Hemin-induced K562	1:256
Adult group O erythrocytes	Absent undiluted
Cord blood group O erythrocytes	1:256

A 3–5% suspension of K562 cells, clone 6, before and after 4 days of hemin induction, was suspended in 0.9% NaCl after packed cells had been washed twice in 0.9% NaCl. Anti-i antiserum was kindly provided by Marie Crookston. Serial dilutions of anti-i in 0.9% NaCl were incubated with the cells for 30 min at 4°C. The mixtures were centrifuged in a Dade Immunofuge for 15 sec, and titration scores were determined macroscopically. Standard blood bank techniques (10) were used to screen for the following antigens, all of which were absent from induced and uninduced clone 6 cells but present in high titer on appropriate test erythrocytes: A, B, C, c, E, e, Fy^a, Jk^a, M, N, and P_1.

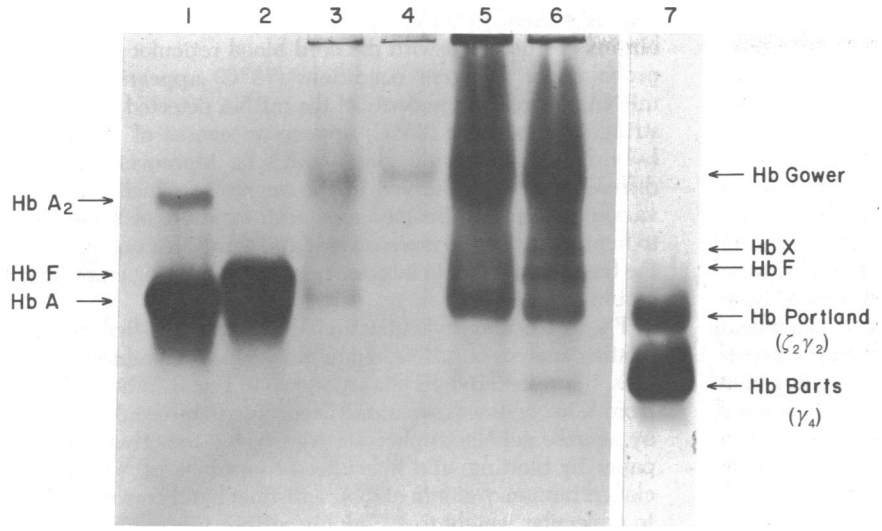

FIG. 1. Hemoglobin accumulation in K562 cells. Total cellular protein from uninduced and hemin-induced K562 cells, adult erythrocytes, and cord blood erythrocytes was applied to acrylamide gels in Tris/borate/EDTA buffer (pH 8.6), subjected to electrophoresis, and stained with benzidine (6, 16). Positions of known hemoglobin markers are indicated. Position of Hb Gower was assigned by comparison to published data (6). Lane 1, adult erythrocyte lysate (Hb A and Hb A_2); lane 2, cord blood lysate (Hb F and Hb A); lane 3, lysate from uninduced clone 6 K562 cells; lane 4, lysate from uninduced clone R K562 cells; lane 5, lysate from clone 6 K562 cells after 6 days of hemin exposure; lane 6, lysate from clone R K562 cells after 6 days of hemin exposure; lane 7 (from a separate gel), erythrocyte lysate from a 24-week-old fetus with homozygous α-thalassemia (hydrops fetalis with Hb Barts), stained with amido black and showing the relative positions of Hb Portland and Hb Barts.

land, and small amounts of Hb F. Clone R cells, in contrast (lane 6), also contained small but readily detectable amounts of Hb Barts (γ_4) and Hb X (possibly ϵ_4). In addition, Hb F accumulation was more apparent in clone R cells (lane 6).

Hemin induction of clone R cells appears to be associated with imbalance between the synthesis of α-like (ζ and α) and non-α (ϵ and γ) globin, as observed by Rutherford *et al.* (6), whereas such an imbalance was much less apparent in the hemoglobin synthesized by clone 6. We have observed that the extent of overall hemoglobin synthesis and the degree of globin chain imbalance can be quite variable even within the same cell clone, and they probably depend on other factors such as the amount of fetal calf serum used and the cell density during culture. Indeed, cells grown to a high cell density in the absence of hemin demonstrated small degrees of enhanced hemoglobin accumulation (data not shown). We also observed embryonic hemoglobin accumulation that increased in response to hemin in a third clone of K562 cells (data not shown).

The data shown in Fig. 1 suggest that embryonic (ϵ) and fetal (γ), but not adult (β and δ), globins are expressed in K562 cells. We have verified this hypothesis by globin chain analysis of protein isolated from induced and uninduced clone R cells, using acrylamide gel electrophoresis in the presence of acid/urea/Triton (Fig. 2A). Although the protein patterns are complex, major bands that increase upon hemin induction are seen in the positions of embryonic, $^{A}\gamma$, and $^{G}\gamma$ globin chains. No bands exactly comigrating with β globin are seen. Unfortunately, α globin chain analysis in this study was complicated

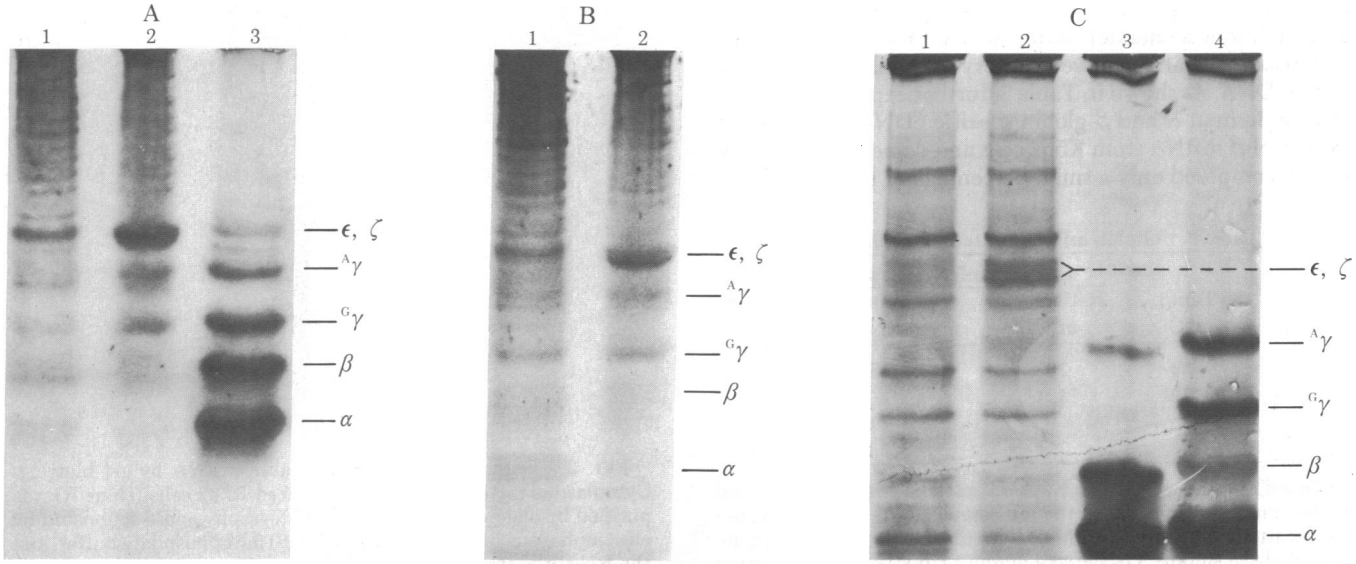

FIG. 2. Globin chain synthesis and globin mRNA translation in K562 cells (clone R). Proteins from cell lysates and cell-free translation reaction mixtures were fractionated by electrophoresis in slab gels of acrylamide (12%) in the presence of acetic acid/urea/Triton X-100 (17, 18). The gels were stained with Coomassie blue, then dried on filter paper for autoradiography of proteins labeled with [^{35}S]methionine. (*A*) Intact cell globin. Gel stained with Coomassie blue. Lane 1, lysate from uninduced K562 cells; lane 2, lysate from K562 cells exposed to hemin for 6 days; lane 3, mixture of erythrocyte lysates from blood of an adult, a newborn infant, and a fetus with homozygous α-thalassemia (hydrops fetalis with Hb Barts) to provide markers for the various globin chains. Although only ζ embryonic globin chains are present in this mixture, the ϵ embryonic globin chain is known to migrate closely with the ζ chain in this system (25). The faint band between the embryonic and $^{A}\gamma$ globin chain probably represents carbonic anhydrase. (*B*) Intact cell globin synthesis. Autoradiograph of same gel as *A*. Proteins were labeled by prior incubation of intact cells with [^{35}S]methionine for 45 min. (*C*) Autoradiograph of mRNA-directed cell-free protein synthesis in the presence of [^{35}S]methionine in wheat germ lysates. Lane 1, mRNA of uninduced K562 cells; lane 2, mRNA of K562 cells exposed to hemin for 4 days; lane 3, globin mRNA from blood of adults with sickle cell anemia; lane 4, globin mRNA isolated from cord blood of newborn infant.

by the presence of comigrating bands and probably some loss of α chains.

In addition to steady-state levels of globin, we also analyzed synthesis of globin (during 45-min incubations with [^{35}S]methionine) by uninduced cells, in order to determine whether enhanced globin accumulation resulted from actual increase in globin chain synthesis or merely from improved formation of hemoglobin tetramers from preformed globin chains after addition of exogenous heme. As shown in Fig. 2*B*, increased amounts of newly synthesized embryonic globin chains were observed after hemin exposure. Similarly, mRNA isolated from uninduced and induced K562 cells and translated in a wheat germ cell-free protein-synthesizing system in the presence of [^{35}S]methionine showed increased synthesis of embryonic globin chains in the case of mRNA isolated from K562 cells exposed to hemin for 4 days (Fig. 2*C*). In intact cell and cell-free protein synthetic studies (Fig. 2 *B* and *C*), the syntheses of γ globin chains do not appear to be significantly different.

Globin gene expression in K562 cells was analyzed more quantitatively by molecular hybridization of poly(A)-containing mRNAs isolated by oligo(dT)-cellulose chromatography from uninduced K562 cells and cells exposed to hemin for 4 days. As shown in Table 3, mRNAs from both induced and uninduced cells hybridized efficiently to a globin cDNA probe prepared from mRNA of cord blood reticulocytes, and known to contain cDNA copies of α, β, and γ mRNA. Under moderately stringent conditions (65°C, 0.2 M sodium phosphate, pH 6.8), mRNA from induced K562 cells contained approximately 3 times as much globin mRNA as did mRNA from uninduced cells. These mRNAs saturated 80% of the cDNA probe (data not shown). Under these conditions, cross-hybridization between globin cDNA and mRNA sequences specific for embryonic and γ or α globin genes occurs (our unpublished data). When the same experiment was conducted at 78°C, complete saturation of the globin cDNA probe was observed (data not shown), but the apparent globin mRNA content was reduced nearly to 40% suggesting that a major portion of the mRNA detected under moderately stringent conditions was the result of cross-hybridization between embryonic globin mRNA and γ or α cDNA. As shown in Table 3, further experiments using purified human α and β globin specific cDNAs verified the absence of β mRNA from K562 cells and demonstrated that α mRNA composed only a small percentage of the total globin mRNA present in these cells. Thus, the majority of the globin mRNA detected with the cord blood reticulocyte cDNA probe under stringent conditions (78°C) appears to be γ mRNA, whereas the majority of the mRNA detected under less stringent conditions (65°C) appears to consist of the cross-hybridizing embryonic globin mRNAs. Moreover, distinct differences were observed in the degree to which levels of various globin mRNAs increased after exposure of K562 cells to hemin: little or no increase in α and γ mRNAs occurred, in the face of a significant increase in cross-hybridizing embryonic globin mRNAs.

Table 3. Globin mRNA content of K562 cells

cDNA	Temp., °C	ng globin mRNA/μg total mRNA: Reticulocyte mRNA	Uninduced mRNA	Induced mRNA
$\alpha+\beta+\gamma$	65	166	2.9	8.0
$\alpha+\beta+\gamma$	78	121	1.7	3.2
α	78	65	1.0	0.76
β	78	14.2	<0.1	<0.1

Oligo(dT)-cellulose-purified K562 cell mRNAs were incubated with known and fixed amounts of the indicated cDNAs at the indicated temperatures for 40 hr in 0.2 M sodium phosphate, pH 6.8/0.5% sodium dodecyl sulfate. The percent of input DNA bound into hybrids was then determined as described (19, 20). Globin mRNA content was calculated on the basis of the nanograms of the RNA sample required to saturate a known amount (0.1–0.15 ng) of the cDNA. With $\alpha+\beta+\gamma$ cDNA (cDNA prepared from cord blood reticulocyte mRNA) (column 1), cross-hybridization between embryonic and γ globin specific sequences occurs at 65°C (line 1). At 78°C (line 2) no cross-hybridization occurs, and only γ cDNA–γ mRNA hybridization contributes to analysis of the K562 RNA because of the minute amounts of α (line 3) and β (line 4) mRNAs present in the sample. Properties of the purified α and β cDNAs (lines 3 and 4) have been described (19, 20).

The molecular hybridization data were verified by gel blotting analysis of mRNAs from induced and uninduced K562 cells. Representative results are shown in Fig. 3. When mRNA from K562 cells was separated according to molecular weight by agarose gel electrophoresis, transferred to activated filter paper by blotting, and hybridized to a probe of ^{32}P-labeled cloned human γ globin cDNA, a distinct band corresponding in molecular weight to 10S globin mRNA was observed (Fig. 3). Uninduced K562 cell mRNA also yielded a faint band of hybridization in this position (data not shown). Similar results were obtained when K562 cell mRNA was analyzed in an identical fashion, utilizing cloned cDNA probes specific for α, ζ, and ϵ globin genes (data not shown). In contrast, no hybridization of the mRNA from these cells was observed when the gel blots were incubated with a ^{32}P-labeled probe of cloned β globin cDNA (data not shown). In all cases, the mRNA from

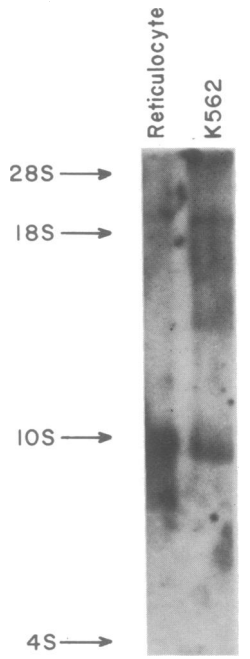

FIG. 3. Detection of K562 cell globin mRNA by gel blotting. Cytoplasmic mRNA from hemin-induced K562 cells (clone R) was purified by oligo(dT)-cellulose chromatography and fractionated by electrophoresis in 2.5% agarose gels in 10 mM phosphate buffer, and the RNA was transferred (blotted) to activated filter paper by the method of Stark (21, 22). The RNA on the paper was hybridized to human γ globin cDNA probe labeled with [^{32}P]dCTP to a specific activity of 10^8 dpm/μg by nick-translation of plasmid JW151 (23); autoradiography was for 48 hr at −20°C. Lane 1, 0.6 μg of adult reticulocyte mRNA; lane 2, 2 μg of clone R mRNA. Positions of 28S, 18S, and 10S RNAs detected by ethidium bromide staining of the reticulocyte RNA (lane 1) prior to blotting are indicated. High molecular weight bands visible in the autoradiographs probably represent mRNA aggregates that persits in this nondenaturing electrophoresis system. The results obtained with uninduced K562 cells and control HL60 cells and with other cDNA probes are described in the text.

hemin-exposed cells yielded stronger bands than the mRNA from control K562 cells, although the degree of difference in signal intensity was variable, and strict specificity of α versus ζ and γ versus ϵ cDNA hybridization was not observed under the conditions of filter hybridization that we utilized. Nevertheless, no similar bands of hybridization were observed, under the same conditions, with mRNA isolated from a human myeloid leukemia cell line (HL60) (data not shown).

DISCUSSION

Our results confirm the findings of Rutherford *et al.* (6) on the accumulation of embryonic hemoglobins in K562 cells after exposure to hemin. In addition, we have demonstrated a similar phenomenon in two additional separate isolates of K562 cells. The potential for hemoglobin synthesis in K562 cells is therefore not isolated to the clone of cells initially studied by Rutherford *et al.* (6). Differences in the relative amounts of the various hemoglobins accumulating after hemin exposure were also observed between different K562 cell isolates and with different culture conditions.

In addition, we have demonstrated increased accumulation of steady-state levels and of newly synthesized embryonic globin chains in K562 cells after hemin exposure. The observed increase in embryonic hemoglobins in these cells is therefore not simply explained by the association of exogenous heme with preformed globin chains, although our results do not rule out the possibility that heme is stabilizing globin chains being synthesized at a constitutive level in these cells. Our analysis of K562 cell mRNA by cell-free translation and molecular hybridization with authentic globin cDNA also demonstrated that the steady-state level of embryonic globin mRNAs is increased in these cells after exposure to hemin. These results do not necessarily indicate increased globin gene transcription; hemin could also theoretically be acting at the level of globin mRNA stabilization. It is of interest, however, that both our studies of globin chain synthesis and of globin mRNA accumulation suggest a preferential effect of hemin on embryonic globin chain and mRNA accumulation over that observed for the α and γ globin gene products.

Although unequivocal demonstration of embryonic (and other) globin chains and mRNA in K562 cells must ultimately rely on amino acid and nucleotide sequence analysis of the appropriate products, we do have additional experimental results that support the conclusions listed above concerning the presence of fetal and embryonic globin mRNA in K562 cells. cDNA synthesized from K562 cell mRNA was cloned in bacterial plasmids by recombinant DNA technology. A number of recombinants were obtained that hybridized to globin cDNA, and restriction endonuclease analysis of some of these individual cloned cDNAs yielded results consistent with those expected for human α, ζ, or γ globin cDNAs (our unpublished results).

Finally, our studies of cell surface and enzymatic properties of K562 cells provide additional information to the hemoglobin and globin analyses that support the conclusion that this cell line contains immature erythroid precursor cells with a fetal or embryonic program of protein synthesis as evidenced by i antigen reactivity and LDH isoenzyme pattern.

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