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Embryonic erythroid differentiation in the human leukemic cell line K562

(ζ thalassemia/embryonic hemoglobins)

TIM RUTHERFORD, J. B. CLEGG, D. R. HIGGS, R. W. JONES, J. THOMPSON, AND D. J. WEATHERALL

University of Oxford, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, England

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ABSTRACT **K562 human leukemia cells synthesize embryonic hemoglobins after culture in the presence of hemin. We have rigorously identified these hemoglobins by globin chain analysis and peptide mapping. No adult hemoglobin could be detected, and β-globin synthesis was less than 2 ppm of total protein synthesis. Persistent embryonic globin gene expression is known to occur as a consequence of globin gene deletions. However, restriction endonuclease mapping showed that the globin gene complexes in K562 cells are indistinguishable from normal. Hemin increased the rate of embryonic globin synthesis. The pattern of hemoglobin synthesis proved to be stable when cells from different laboratories were compared. One line, however, synthesized large amounts of Hb X and very little Hb Portland in response to hemin. Hb X has been previously detected in human embryos; we show here that it has the composition $\varepsilon_2\gamma_2$ and is diagnostic of imbalanced chain synthesis or "ζ thalassemia."**

We have identified several agents that induce hemoglobin synthesis in K562 cells. Different inducers induced different patterns of embryonic hemoglobin synthesis but never any adult hemoglobin synthesis.

One of the limitations to the study of the factors regulating the changes in human hemoglobin production that occur between embryonic, fetal, and adult life (1) has been the lack of a self-sustaining cell line in which human hemoglobin genes are expressed. Recently, Andersson *et al.* (2, 3) reported that the human cell line K562, derived from a patient who had chronic myeloid leukemia in terminal blast cell crisis (4), has certain erythroid characteristics. In a preliminary report (5), we have shown that these cells can be induced to produce embryonic and fetal but not adult hemoglobin. In this paper, we report an analysis of the potential of the K562 cell line as a model for studying hemoglobin differentiation.

Lozzio *et al.*, who established the K562 cell line in culture (4) have questioned the erythroid nature of K562 cells (6). If the pattern of globin synthesis we observed is the consequence of abnormal and unstable gene expression in a leukemic cell line or the result of gene rearrangement in culture, the utility of these cells for the study of the normal control of hemoglobin synthesis would be slight.

We found that the cells available to us showed a stable pattern of erythroid differentiation in response to hemin and that embryonic hemoglobins were synthesized but adult (β) globin synthesis was strictly repressed. This is the converse of the situation in normal adult erythroid tissue but closely resembles the state of the normal embryo. Restriction endonuclease analysis of the globin genes has failed to detect any gene deletion or rearrangements, and we thus propose that the presence of embryonic and the absense of adult globin synthesis is the consequence of epigenetic control analogous to that in normal embryonic erythroid cells. Nevertheless, by selection of variant cell lines and the choice of hemoglobin inducer, the varieties of hemoglobin produced can be manipulated *in vitro*.

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MATERIALS AND METHODS

Cells and Cell Culture. K562 cell inocula were obtained from different laboratories (see below). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum. For induction, cells were cultured for 6 days in the presence of 0.05 mM hemin or 1.25 mM butyric acid as described (7). Cells were harvested by low-speed centrifugation and washed three times with ice-cold phosphate-buffered saline. The packed cell pellet was suspended in an equal volume of distilled water, and the cells were lysed by three cycles of freezing and thawing. Stroma was removed by centrifugation at 500 × *g* for 15 min and 140,000 × *g* for 45 min.

Analysis of Hemoglobins. Hemoglobin contents of cell lysates were determined from the visible absorbtion spectrum (7). Hemoglobins were separated by starch gel electrophoresis in Tris/EDTA/boric acid (pH 8.6) and stained with benzidine. Cellulose acetate and preparative starch block electrophoresis were carried out as described (8).

Determination of Globin Synthesis. Cells were pelleted by low-speed centrifugation and resuspended at a concentration of 0.5×10^6 cells per ml in medium lacking leucine and supplemented with 10% dialyzed fetal calf serum. After 10 min at 37°C [^{3}H]leucine (Amersham) was added to a concentration of 20 μCi/ml (1 Ci = 3.7×10^{10} becquerels). Incubation was continued at 37°C for 30 min, after which the cultures were chilled on ice and lysates were prepared as above.

Labeled proteins were separated by $NaDodSO_4$ gel electrophoresis (9) in a 5–15% polyacrylamide gel. Gels were cut into 1-mm slices by using a Mickle gel slicer, and each slice was digested with hydrogen peroxide and counted in scintillator 299 (Packard).

Individual globin chains were separated by polyacrylamide gel electrophoresis in the presence of acetic acid, urea, and Triton X-100 (10). Unlabeled proteins were detected by staining with Coomassie brilliant blue R. Labeled material was detected by fluorography using EN^{3}HANCE (NEN).

Adult and fetal globin chains were separated by CM-cellulose chromatography (11); embryonic globin chains are not eluted under these conditions. In some experiments, hemoglobin samples to which human umbilical-cord-blood lysate was added as carrier were first purified by gel filtration on Sephadex G-100.

Peptide Analysis. Tryptic peptide maps were prepared by standard techniques (11). Ninhydrin-positive spots were eluted with 6 M HCl for amino acid analysis.

Restriction Endonuclease Analysis. DNA from K562 cells was prepared by phenol/chloroform extraction (12), and 15- to 20-μg samples were digested with 15–20 units of restriction endonucleases (see legend to Fig. 3) in the appropriate buffers

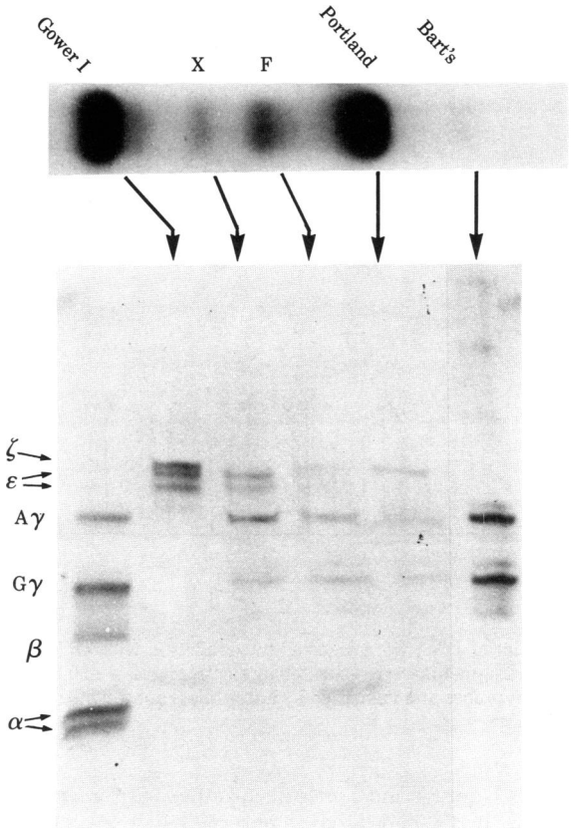

FIG. 1. Chain composition of K562 cell hemoglobins. Cells were grown for 6 days in the presence of 0.05 mM hemin, washed, and lysed, and the hemoglobins were purified by gel filtration on Sephadex G-100 and electrophoresis on cellulose acetate strips. The components of each hemoglobin were then separated by polyacrylamide gel electrophoresis in the presence of acetic acid/urea/Triton X-100. At top, pattern of hemoglobin synthesis and tentative identification of each hemoglobin. Below, globin-chain compositions that show exact correspondence to predicted—*viz.* Hb Gower I ($\zeta_2\varepsilon_2$) (15), Hb F ($\alpha_2\gamma_2$), Hb Portland ($\zeta_2\gamma_2$) (16), and Hb Bart's (γ_4); Hb F is slightly contaminated with Hb Portland. Chains were identified by comparison with samples from adult blood and cord blood from an infant with Hb Bart's hydrops fetalis syndrome whose blood contained ζ globin chains. Hb X appears to contain only ε and γ chains.

(as specified by the supplier) at 37°C for approximately 24 hr. The digested DNA was subjected to electrophoresis on 0.8% agarose gel, transferred to nitrocellulose filters, hybridized with a ^{32}P-labeled probe for 2–3 days, and then washed under stringent conditions and autoradiographed for 1–7 days (13).

α, β, and γ probes were prepared by nick translation of the α plasmid JW101, the β plasmid JW102, and the γ plasmid JW151 (gifts of B. G. Forget) by a modification of the method of Maniatis *et al.* (14). ζ specific probe was prepared and nick translated as described (13).

RESULTS

Embryonic Hemoglobin Synthesis in K562 Cells. K562 cells grew vigorously in culture with a doubling time of about 24 hr. On addition of 0.05 mM hemin, the doubling time increased to about 36 hr, but there was no growth arrest in the culture and cell viability and cloning efficiency were not reduced. After 6 days of growth, an average of 3.2 pg of hemoglobin was accumulated per cell.

Starch gel electrophoresis of the K562 hemoglobin showed that it consisted mainly of embryonic hemoglobins (Fig. 1). The individual hemoglobins were purified by gel filtration on Sephadex G-100 and electrophoresis on cellulose acetate strips. The globin chain composition of each hemoglobin was analyzed by Triton/urea gel electrophoresis (see Fig. 1, bottom); the compositions directly confirmed the electrophoretic identification of the hemoglobins. Of particular note was the absence of α and β chains from the Hb Portland sample, suggesting that there was no Hb A hidden in the tail of the Portland band.

Absence of β-Chain Synthesis. Cells grown for 6 days in the presence of hemin were labeled for 30 min with [^{3}H]leucine. The labeled hemoglobins were purified by gel filtration, and the constituent globin chains were separated by CM-cellulose chromatography. Labeled α and γ chains were seen, but no labeled β-globin could be detected. To search for minute traces of β-globin synthesis, the labeled hemoglobins (after gel filtration) were further purified by chromatography on DEAE-cellulose. Labeled product comigrating with unlabeled Hb A carrier was analyzed by CM-cellulose chromatography; there was no peak of labeled β-globin chains, although we estimate that β-chains labeled at a level of 2 ppm of total leucine incorporation would have been detected.

DNA Arrangement in the Globin Gene Regions. The DNA of K562 cells was digested with a series of restriction endonucleases. The digests were separated by agarose gel electrophoresis, and the separated fragments were transferred to nitrocellulose filters and hybridized to ^{32}P-labeled cloned α, ζ, β, and γ gene probes. The patterns of DNA bands obtained were indistinguishable from those of normal human DNA. Although only single restriction enzyme digests were used, it is clear that no major deletions or rearrangements have occurred in the α–ζ or ε–γ–ζ–β gene complexes (Fig. 2).

Globin Chain Synthesis. K562 cells grown in the presence or absence of hemin were labeled for 30 min with [^{3}H]leucine, and the labeled proteins were separated by NaDodSO$_4$ gel electrophoresis. Incorporation of [^{3}H]leucine into globin was 3.8% of total incorporation in uninduced cells but 14% of total incorporation in cells grown for 6 days in the presence of hemin.

The labeled proteins were separated on acid/urea/Triton X gels. Bands corresponding to ζ, ε, $^A\gamma$, $^G\gamma$, and α chains were detected by fluorography in lysates of both induced and uninduced cells (Fig. 3).

Comparison of Hemoglobin Synthesis in Different K562 Inocula. Our cells derive from an inoculum of cells sent to G. Klein in 1975 (19). We have compared a number of K562 cultures derived from that inoculum but maintained since then in different laboratories with a sample that had been kept frozen since 1975. Of 10 lines studied, all showed essentially the same pattern of hemoglobin synthesis as the uncloned cultures. Thus, the pattern of hemoglobin synthesis in K562 cells in response to hemin is stable on prolonged culture.

Nevertheless differences were found in the pattern of hemoglobin synthesis in one subline, that designated K562(S). After induction with hemin, the hemoglobin was analyzed by starch gel electrophoresis (Fig. 4). The most prominent band migrated in the position of Hb X. Bands corresponding to Hb Gower I and Hb Bart's were also present but the Hb Portland band was almost undetectable. This pattern was consistently obtained and confirms the results of Cioe *et al.* (20).

We also examined two cell hybrids formed between K562 cells and Epstein–Barr virus-transformed human lymphocytes. These hybrids, generated by Klein *et al.* (21), show the dominance of the erythroid characteristics and repression of most of the characteristics of the lymphoid parent. After induction with hemin, the hemoglobins of the hybrid cells were separated by starch gel electrophoresis (see Fig. 4). The cell hybrid PUTKO

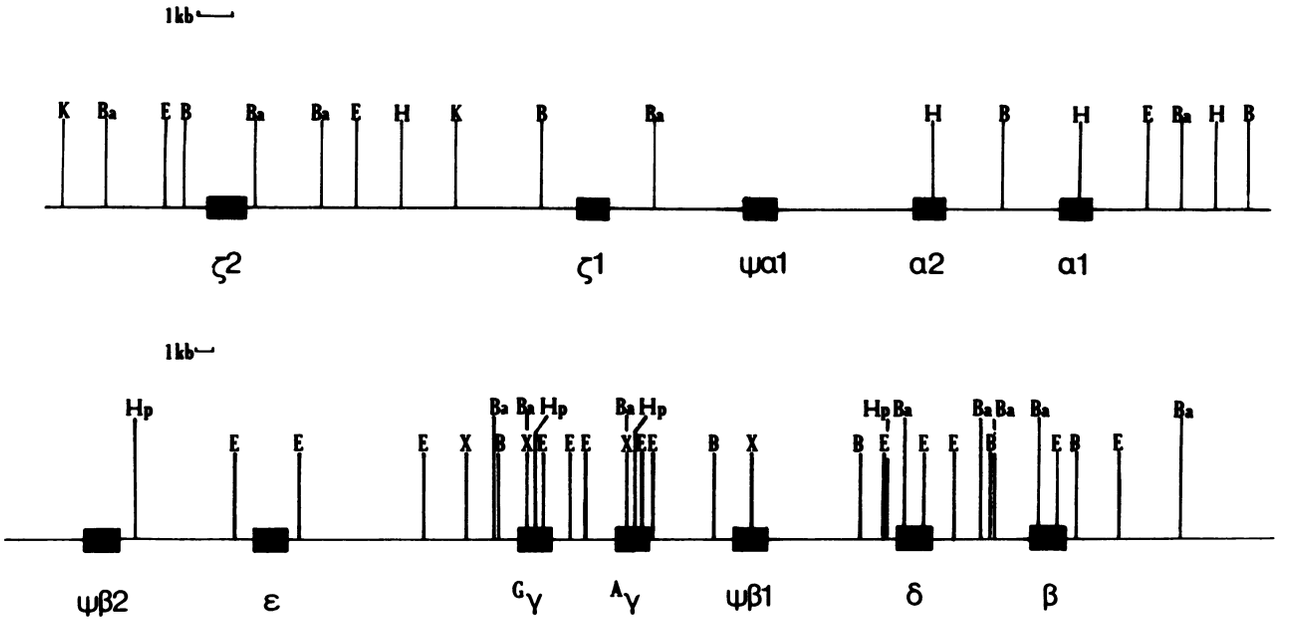

FIG. 2. Restriction endonuclease map of K562 cell globin gene complexes. Samples of DNA were digested to completion with endonucleases *Bam*HI (Ba), *Bgl* II (B), *Kpn* I (K), *Eco*RI (E), *Hind*III (H), *Hpa* I (Hp), and *Xba* (X). DNA fragments were separated by electrophoresis on 0.8% agarose gels, transferred to nitrocellulose filters, hybridized to ^{32}P-labeled globin-specific DNA probes and identified by autoradiography. This map agrees with refs. 17 and 18. kb, kilobase.

showed a pattern similar to that of K562(S), including a very prominent Hb X band.

Composition of Hb X. According to Pataryas and Stamatoyannopoulos (22), the composition of Hb X has not been reported. Therefore, we examined the chain composition of purified hemoglobin X from K562(S) and our original K562 line by Triton/urea gel electrophoresis. Hb X appeared to contain ε and γ chains, but no ζ, α or β chains.

Relatively large amounts of Hb X were purified by gel filtration and starch block electrophoresis. After digestion with trypsin, peptides having the amino acid compositions of ε and γ specific peptides ε1, 2, 3, 5, and 10 and γ3, 10, and 11 were identified, but no peptides having α or ζ chain-like compositions were found. The peptide pairs ε9 and γ9, ε8–9 and γ8–9 could not be separated under our conditions but had compositions corresponding to equimolar mixtures of ε and γ chains.

Effects of Other Inducers. Cells were cultured in the presence of compounds known to induce the differentiation of Friend cells (23) (Table 1). Butyric acid induced hemoglobin synthesis in K562(S) but not in our original line, and hydroxyurea and actinomycin D induced hemoglobin synthesis in both lines. Unlike Hoffman *et al.* (24), we could not detect any effect of erythropoietin.

The hemoglobins induced were examined by starch gel electrophoresis. In the presence of hemin, K562(S) cells synthesized predominantly Hb X and Hb Gower I. In response to butyric acid, less Hb X but considerably more Hb Portland was synthesized. [This confirms the results of Cioe *et al.* (20).] Hydroxyurea induced the predominant synthesis of Hb Gower I.

In our original K562 line, hydroxyurea induced both Hb Gower I and Hb Portland synthesis. In addition to Hbs X and F, an unidentified band migrating more slowly than Hb X was also seen. We do not yet know whether this is an authentic hemoglobin. No Hb A bands were observed.

DISCUSSION

We have shown that K562 leukemia cell lines can be induced to synthesize hemoglobin. The hemoglobins induced by hemin—i.e., Hbs Gower I and Portland, together with trace amounts of Hbs F, Bart's, and X—were observed in approximately the proportions otherwise found only in very early human embryos (15). Our structural studies suggest that Hb X has the composition $\varepsilon_2\gamma_2$. Thus, it appears that the induced K562 cell, like the normal human embryo, produces a slight excess of non-α chains.

The abnormal persistent production of embryonic (ζ) chains is known to occur in association with the deletion of the α genes in Hb Bart's hydrops fetalis syndrome (25). However, by restriction endonuclease mapping, we have shown that there were no substantial deletions or rearrangements in the globin gene regions of the K562 cell DNA. In particular, in these cells, the β genes were present and intact but not expressed, whereas the embryonic globin genes are present but not expressed in normal adult erythroid cells. We therefore propose that globin gene expression in K562 cells is controlled by epigenetic mechanisms similar to those operating in normal embryonic erythroblasts.

We have shown that Hb X has the composition ($\varepsilon_2\gamma_2$). Like Hb Bart's (γ_4) and Hb H (β_4), Hb X is composed of non-α-like chains only. Thus, the predominant synthesis of Hb X by K562(S) cells in response to hemin suggests a marked imbalance in the ratio of synthesis of non-α-like (ε and γ) to synthesis of α-like (ζ and α) chains in this line. The deficiency in synthesis of ζ chains can be seen in Fig. 3. Thus, the cells might be described as having "ζ thalassemia." We do not yet know the molecular basis of the defect in ζ-chain synthesis in this cell line. Karyotype analysis has shown that it is not due to loss of copies of chromosome 16, which carry the α and ζ globin genes (26). Traces of ζ synthesis can be seen in K562(S) cells treated with hemin, and the lower relative proportion of Hb X in cells induced with butyric acid or hydroxyurea suggests a more balanced ratio of non-α-like to α-like globin-chain synthesis.

It is not clear why leukemic cells from an adult should synthesize predominantly embryonic hemoglobin. If the transformation event that gave rise to the leukemia took place while the cells were still programmed for embryonic erythropoiesis, the preleukemic cells must have lain dormant for decades. If cells

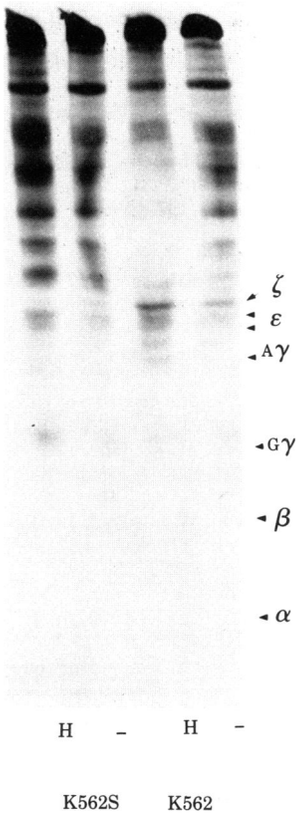

FIG. 3. Induction of globin synthesis. Cells grown for 6 days in the presence or absence of 0.05 mM hemin were labeled for 30 min with [^{3}H]leucine. Lysates were analyzed by Triton/urea gel electrophoresis, and labeled proteins were detected by fluorography. Bands were identified by comparison with stained gels (see legend to Fig. 1).

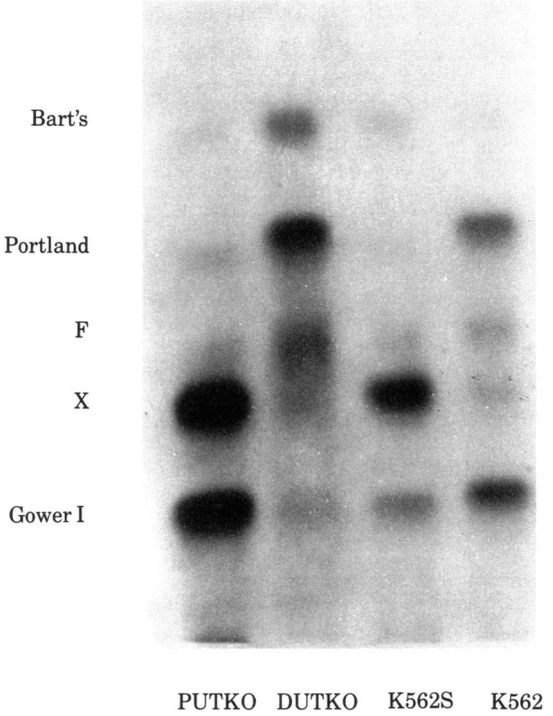

FIG. 4. Variant hemoglobin patterns in K562 cell lines and cell hybrids. Cells were grown for 6 days in the presence of 0.05 mM hemin, and lysates were prepared. The hemoglobins were separated by starch gel electrophoresis in Tris/EDTA/boric acid (pH 8.6) and stained with benzidine.

programmed for embryonic erythropoiesis survive in the marrow of normal adults but are unable to respond to the adult bone marrow microenvironment, it is possible that such lines are more susceptible to leukemogenesis than normal stem cells, as has been suggested as the basis for the fetal erythropoiesis that occurs in juvenile myeloid leukemia (27). On the other hand, there is recent evidence of a relationship between fetal hemoglobin synthesis in erythroid colonies for normal adult erythrocyte progenitors and the degree of maturity of the precursors from which they are derived (28). If this theory is extrapolated from fetal to embryonic hemoglobin synthesis, then the K562 cells must have been derived from an extremely immature progenitor.

Doubt has been raised about the relationship of the various K562 cell lines to the original parental line established by Lozzio *et al.*, who report that their K562 cell line shows no erythroid characteristics (6). However, under the experimental conditions that they report, they would not have detected embryonic hemoglobin production. It has been reported recently that, in a significant proportion of cases classified morphologically as undifferentiated myeloid leukemia, glycophorin was present on the surface of the blast cells (29). As myeloid leukemias frequently originate with a pluripotent stem cell (30), it may be misleading to regard myeloid as synonymous with granulocytic in this context.

Whatever the nature of the parental K562 cell line, the cells used in our studies show clear erythroid properties. Although they do not have the morphological appearance of erythroblasts, they synthesize glycophorin and spectrin (2, 3), heme, globin chains, and ferritin (unpublished data), and they carry the fetal specific antigen i (31). This cannot be dismissed as aberrant gene expression in a leukemic cell line but rather represents a biochemically erythroid phenotype. These cells thus provide a unique source of material for investigating the molecular biology of factors regulating the expression of the embryonic and adult globin genes. Although the pattern of hemoglobin synthesis can be manipulated in these cells by the use of different inducers, we have never observed the synthesis of adult hemoglobin by them. If means can be found to activate the adult globin

Table 1. Inducibility of K562 cells by Friend cell inducers

	Inducibility	
	K562	K562(S)
Hemin	+ve	+ve
Dimethylsulfoxide	−ve	−ve
N-Methylacetamide	−ve	−ve
N-Methylpyrrolidone	−ve	−ve
Hexamethylenebisacetamide	−ve	−ve
Hypoxanthine	−ve	−ve
n-Butyric acid	−ve	+ve
Hydroxyurea	+ve	+ve
Erythropoietin (2 units/ml)	−ve	−ve

Cells were cultured for up to 10 days with doses of the potential inducer up to and including cytostatic (or cytotoxic) doses. Cells were spun down at different times and the color of the cell pellet was compared by eye with that of control cells. Although only semiquantitative, this method is as sensitive and reliable as any other. All positive effects were detectable by 6 days of culture. Optimum doses for induction were 0.05 mM hemin and hydroxyurea at 10 μg/ml for K562 and 0.05 mM hemin, 1.25 mM *n*-butyric acid, and hydroxyurea at 15 μg/ml for K562(S), +ve, negative; −ve, positive.

genes, then K562 cells could also be used to study the cell biology of hemoglobin switching. Given the limitation that the K562 is a neoplastic cell, it has great potential as a model system for analyzing the regulation of hemoglobin differentiation.

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