sence of calcium, this site is apparently much less accessible. Calcium has no influence on the Gly-Pro-Arg (α chain type) site. The increase in the number of sites for Gly-Pro-Arg-Pro in the presence of calcium is attributable to its binding to the β chain type site. We had demonstrated through competitive binding studies that Gly-Pro-Arg-Pro binds weakly to the Gly-His-Arg-Pro binding site, in addition to its major binding at the two Gly-Pro-Arg type sites (*2*). Indeed, in the absence of calcium, the binding of Gly-Pro-Arg-Pro to the Gly-His-Arg-Pro binding site is so weak that only two Gly-Pro-Arg type binding sites are observed in a Scatchard plot. When calcium ions are present, the affinity of the Gly-His-Arg-Pro binding site for both Gly-His-Arg-Pro and Gly-Pro-Arg type peptides increases. As a consequence, the Scatchard plot extrapolates to more than two Gly-Pro-Arg-Pro binding sites.

The role of fibrinopeptide B release in fibrin polymerization has been the subject of considerable speculation. Because fibrinopeptide A is released by thrombin faster than fibrinopeptide B, it was long ago proposed that removal of fibrinopeptide A is responsible for an end-to-end polymerization producing intermediate polymers, while removal of fibrinopeptide B results in lateral aggregation of these intermediate polymers (*13*). Subsequently it was shown that lamprey fibrinogen could be clotted by bovine thrombin, whch removes only fibrinopeptide B, indicating an important function for fibrinopeptide B release in that species (*14*). Indeed, at low temperatures (< 25°C), even human fibrinogen can be clotted by the selective removal of fibrinopeptide B although clotting was not observed at 37°C unless fibrinopeptide A was also removed. Our finding that calcium increases the affinity of Gly-His-Arg-Pro by almost tenfold indicates that the true physiological circumstances surrounding fibrin formation require the presence of calcium ions. In the past much of the experimentation on the roles of fibrinopeptide release and fibrin formation have been conducted in the absence of calcium ions (*13–15*).

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Induction of Hemoglobin Accumulation in Human K562 Cells by Hemin Is Reversible

Abstract. *Twenty micromolar hemin causes no change in the rate of division of K562 cells but results in accumulation of 11 to 14 picograms of embryonic and fetal hemoglobins per cell. This effect is reversible, and hemoglobin induction in response to hemin, and loss of hemoglobin upon removal of hemin, can be cyclically repeated. The cells can be indefinitely subcultured in the presence of the inducer. Thus, the control of hemoglobin levels in K562 cells does not depend on irreversible differentiation.*

The mechanisms that control hemoglobin biosynthesis have been investigated in studies of animal models of hemoglobin "switching," erythroid cells in culture, and subcellular systems (*1*). During the last decade the murine Friend erythroleukemia cell line has been studied as a model system that can be induced by various chemicals to undergo erythroid differentiation and to accumulate hemoglobin (*2*). Analyses of cultured human erythroid cells have been restricted by their limited lifetime in vitro and stringent growth requirements. Recently, the K562 human cell line, originally isolated from the pleural effusion of a patient with chronic myelogenous leukemia (*3*) has been investigated as a potential human analog of the Friend cell. K562 cells can be induced by 50 to 100 μ*M* hemin to form embryonic and fetal hemoglobins (*4–6*). We have examined the growth characteristics and accumulation of hemoglobin in K562 cells grown with low concentrations of hemin. We report here that human K562 cells can be caused to accumulate human embryonic and fetal hemoglobins without irreversible erythroid differentiation or commitment.

The K562 cells were grown in suspension in RPMI 1640 medium containing 10

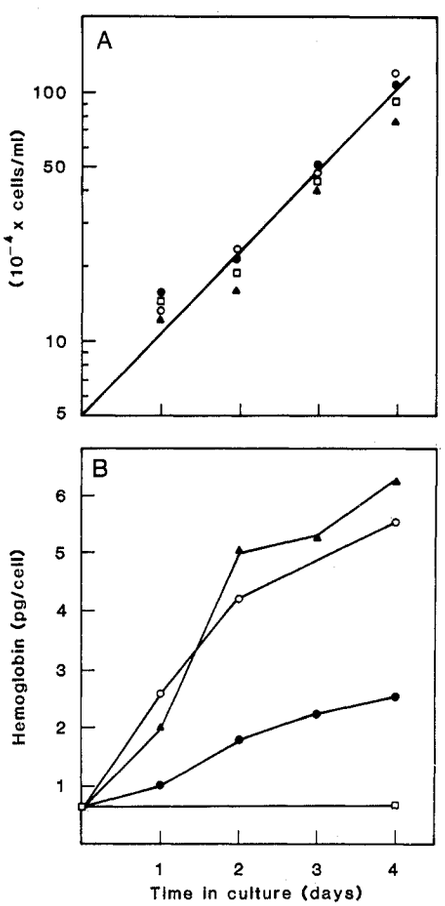

Fig. 1. (A) Cell counts from K562 cultures initiated at 5×10^4 cells per milliliter and treated with 10 μ*M* (●), 20 μ*M* (○), or 30 μ*M* (▲) hemin, or with no added hemin (□). A stock solution of 600 μ*M* hemin was prepared by adding 0.3 ml of 1*N* NaOH to 19.56 mg of hemin, and then adding 0.3 ml of 0.5*M* tris base, 2.5 ml of cell growth medium, and 0.35 ml of 1*N* HCl. The solution was sterilized by filtration. (B) Accumulation of hemoglobin in K562 cells induced with 10 μ*M* (●), 20 μ*M* (○), or 30 μ*M* (▲) hemin, or with no added hemin (□). Hemoglobin was measured spectrophotometrically in cell lysates as described (*7*).

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m*M* Hepes [4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid], 10 percent fetal calf serum, and antibiotics. The cells had a doubling time of about 24 hours. We found that the initial rates of cell growth were not affected by addition of hemin in the concentration range of 10 to 30 μ*M* (Fig. 1A). K562 cells synthesize hemoglobin in the uninduced state at levels of approximately 0.3 to 0.5 pg per cell as detected by spectrophotometric measurements of cell lysates (*7*). The extent of hemoglobin induction at these low concentrations of hemin is shown in Fig. 1B.

We have grown K562 cells in the continued presence of 20 μ*M* hemin for more than 6 months. Cultures were subdivided to maintain cell density between 5×10^4 and 1×10^6 cells per milliliter. The growth rates of hemin-treated and control cells were the same as that shown in Fig. 1A and were invariant over the course of several months. The hemin-treated cells accumulated hemoglobin until, after 14 days, they reached a steady-state level of between 11 and 14 pg per cell (Fig. 2). This plateau presumably reflects the combined effects of hemoglobin synthesis and degradation, and cell division. Cells removed from hemin-containing medium and resuspended in fresh medium showed diminishing levels of hemoglobin which, after 5 days, approached the control level. Reinduction of cells previously cultured with hemin, and subsequently without hemin, resulted in a pattern of hemoglobin accumulation identical to that of previously untreated controls (Fig. 2).

Several lines of evidence suggest that what we observed were characteristics of an entire cell population, not of a selected subset of cells. First, nearly all induced cells contained hemoglobin as shown by benzidine reactivity. At various times after induction, cells were washed three times in phosphate-buffered saline, suspended in benzidine (0.5 percent benzidine dihydrochloride, 0.3 percent H_2O_2 in 1*M* acetic acid) and examined microscopically. Uninduced cells, or cells treated with 20 μ*M* hemin for periods up to 1 day, showed only an occasional benzidine-positive cell. Uniform (> 90 percent) benzidine reactivity was observed after 3 to 4 days of induction, a time when cells contained, on the average, 5 pg of hemoglobin per cell. Fully induced cells varied in the intensity of their benzidine positivity. Second, cells could undergo at least four cycles of induction and release from induction (data not shown) with identical accumulation and loss of hemoglobin to that illustrated in Fig. 2. Third, neither the addition of hemin nor its removal altered the 24-hour cell doubling time.

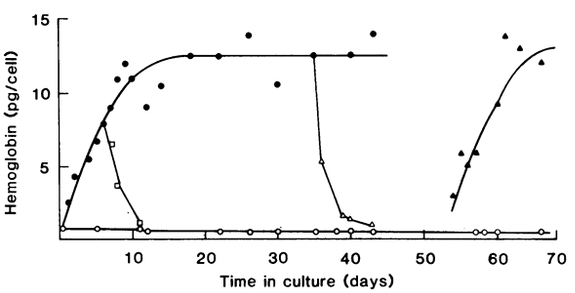

Fig. 2. Accumulation of hemoglobin in hemin-induced K562 cells. The K562 cells were grown in 20 μ*M* hemin (●) or without inducer (○). Portions of cells were transferred to medium without inducer after 6 days (□), and 35 days (△). Cells removed on day 35 to medium without inducer were reinduced with 20 μ*M* hemin (day 53) (▲). For each point, 1.5×10^7 cells were collected, washed three times with 0.15*M* NaCl, and lysed, and the hemoglobin in the lysates was measured spectroscopically (*7*). No free hemin was detected in the lysates under these conditions.

The hemoglobins synthesized by hemin-induced K562 cells have been reported to be predominantly the embryonic hemoglobins Gower I ($\zeta_2\epsilon_2$) and Portland ($\zeta_2\gamma_2$), and fetal hemoglobin ($\alpha_2\gamma_2$) as identified by starch gel (*4*) and polyacrylamide gel (*6*) electrophoresis. To characterize the hemoglobins synthesized by K562 induced by low concentrations of hemin we studied them by preparative isoelectric focusing and identified the types of hemoglobin by their chain composition as determined by electrophoresis in a gel containing acetic acid, urea, and Triton X-100 (*5*) (Fig. 3). Despite some overlap in bands of hemoglobins excised from isoelectric focusing gels, hemoglobins Gower I (region IV) and Portland (region I) were detected, along with lesser amounts of fetal hemoglobin (region II). Isoelectric focusing beyond the time periods indicated in the legend to Fig. 3 resulted in the separation of the band corresponding to hemoglobin Portland into three or four separate bands, each with the chain composition $\zeta_2\gamma_2$. We have not characterized the differences among these bands responsible for their separation.

When Friend cells are induced with chemicals such as dimethylsulfoxide, hexamethylbisacetamide, or sodium butyrate they undergo erythroid differentiation that has features in common with normal erythropoiesis, including commitment to terminal differentiation (*2, 8*). Globin messenger RNA (mRNA) and hemoglobin accumulation are events that accompany or follow such commitment to differentiation (*8*). However, hemin is capable of inducing Friend cells to accumulate globin mRNA and hemoglobin (*9*) but does not lead to terminal differentiation (*10*).

Hemoglobin accumulation (*11*) has been induced in various K562 clones by hemin, sodium butyrate, actinomycin D, and hydroxyurea (*4–6, 12*). Furthermore, hemin stimulation at concentrations of

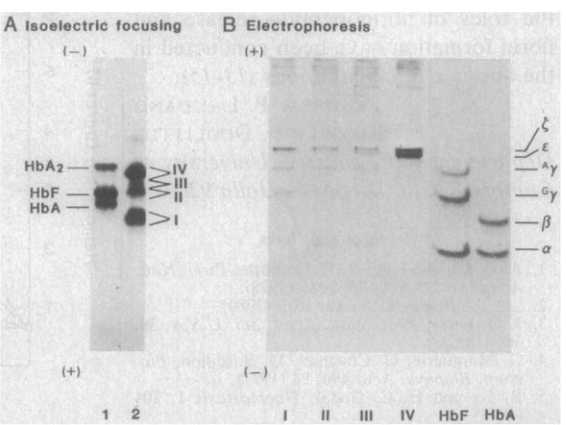

Fig. 3. (A) Isoelectric focusing pattern of lysates of K562 cells. (Lane 1) Mixture of purified hemoglobins A_2, F, and A. (Lane 2) Lysate of K562 cells grown in 30 μ*M* hemin for 6 days. Cells were washed three times with 0.15*M* NaCl and lysed by three cycles of freezing and thawing in 0.1*M* KCN (a volume approximately equal to that of the cell pellet). The focusing gel was prepared as described (*14*) except the ampholines used were *p*H 3.5 to 10 (0.9 ml) and *p*H 7 to 9 (0.9 ml) and bisacrylylcystamine (Bio-Rad) was used as the cross-linking agent instead of bisacrylamide as described in the manufacturer's literature. Focusing was for 30 minutes at 5 W and 30 minutes at 10 W with a 5-minute prefocus. The gel was stained with benzidine. (B) Regions denoted I to IV in lane 2 of the gel in (A) were cut from an adjacent lane of the gel containing an identical sample that had not been stained, and dissolved in 10 μl of β-mercaptoethanol. These samples were diluted in buffer (*5*) to separate the globin polypeptides, run in a gel containing acetic acid, urea, and Triton X-100 (*5*), and stained with Coomassie blue. Purified hemoglobins A and F were dissolved in the same buffer and run as controls. From left to right, region I; region II; region III; region IV; hemoglobin F; and hemoglobin A. The identification of the ζ and ε bands, and Aγ and Gγ bands is made by analogy to previously published data (*5*).

50 to 100 μM leads to accumulation of globin mRNA (*6*), increased activity of heme synthetic enzymes (*13*), and increased expression of cell surface and cytoplasmic markers of embryonic and fetal erythroid cells (*6*). We found that the embryonic and fetal hemoglobin accumulation that results from induction of K562 cells with low concentrations of hemin is reversible and is not accompanied by terminal differentiation.

Hemin, therefore, caused accumulation of hemoglobin in K562 cells without apparent disturbance of cell growth or loss of proliferative capacity. This clearly dissociates increases in intracellular hemoglobin content from other events considered central to erythroid differentiation. Whether this indicates some specific effect of hemin as an inducer, or some attribute of K562 cells which prevents them from undergoing terminal differentiation, is not known. The results suggest that mechanisms controlling hemoglobin metabolism may be found that are independent of irreversible erythroid maturation.

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Outward Currents in Developing *Drosophila* Flight Muscle

Abstract. *The development of two different voltage-sensitive potassium channels was studied in* Drosophila *flight muscle by voltage clamp techniques. Early in development active channels are not present in the membrane. The first channels to appear are the A current channels, which carry a fast, rapidly inactivating potassium current. The channels for delayed rectification appear later. Channels carrying inward current also appear only after the A current channels. During development, the A current may be easily studied in isolation from other currents and thus provides a desirable system for studying the genetic determinants of this current.*

Investigation of adult *Drosophila* flight muscle (*1*) has revealed two distinct outward currents, a fast transient current, which we refer to as the A current, because of its similarity to the molluscan A current (*2*), and a slower current similar to the well-known delayed rectifier (*3*). We now report that the two currents mature at different times during pupal development.

After the observation was made in *Mollusca* that a single cell can have two or more different types of potassium channels (*2*), this phenomenon was reported in many different animal taxa, including mammals (*4*). Little is known,

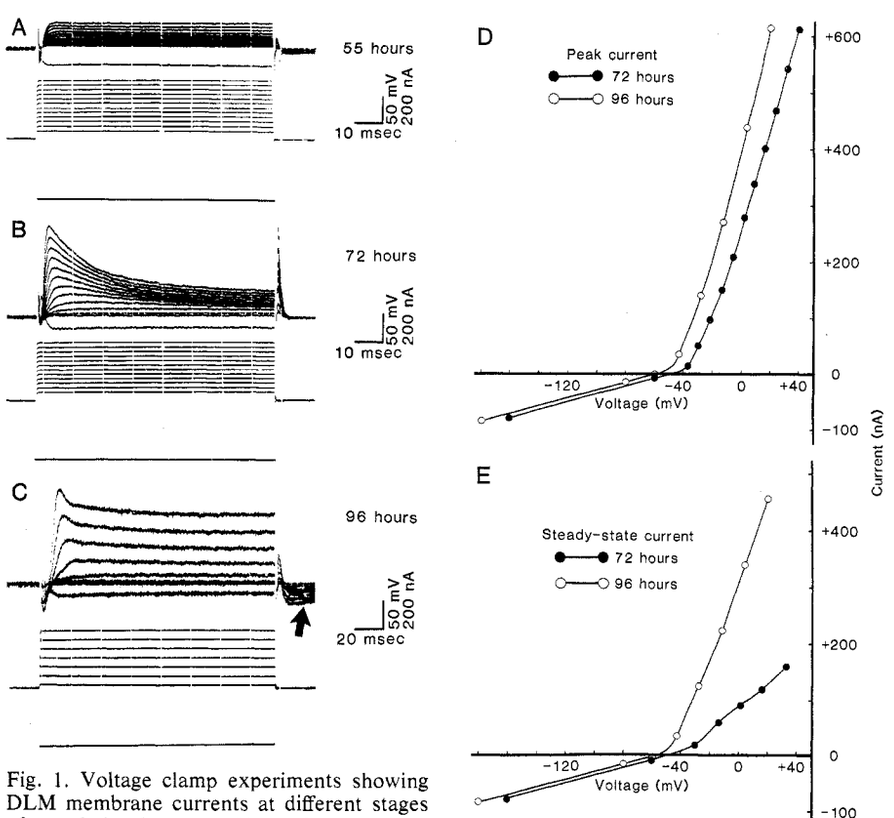

Fig. 1. Voltage clamp experiments showing DLM membrane currents at different stages of pupal development. (A to C) Hours are timed from puparium formation; current is shown in the upper traces, voltage in the lower traces. Outward current is in the upward direction. Holding potentials in (A) and (B), −60 mV; in (C), −80 mV. Because of the high capacitance of these fibers (approximately 0.02 μF) and limitations of the voltage clamp equipment, accurate current readings cannot be made until approximately 3 msec after the command pulse is initiated. The initial rising slope of current is therefore due to charging time rather than to the opening of a current channel. See text for explanation of arrow. (D and E) Current-voltage relationships for DLM fibers; the early peak current component at 72 and 96 hours is shown in (D), and the steady-state current at 72 and 96 hours is shown in (E). Current in (E) is measured 200 msec after command pulses are applied.

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