Hemoglobin Ontogenesis: Test of the Gene Excision Hypothesis

Abstract. *The gene excision hypothesis of hemoglobin ontogenesis was tested in persons with HbSC disease, with the use of monospecific fluorescent antibodies for the identification of hemoglobins S, C, and F in individual erythrocytes. The results are incompatible with the prediction that only one gamma- or beta-globin gene may be active in any single chromosome and provide further evidence for incomplete repression of gamma-globin genes lying* cis *to active beta-globin genes.*

The production of a series of hemoglobin chains other than α chains is characteristic of normal human development. The ϵ chains of embryonic hemoglobins are supplanted by the γ chains of fetal hemoglobin (HbF) which, in turn, are almost wholly replaced by the β and, to a far lesser extent, the δ chains of the adult HbA and HbA_2. A similar series, including embryonic, fetal, and adult hemoglobin, has been observed among members of several mammalian genera, notably the bovids *Capra*, *Ovis*, and *Bos*, while many other mammals (for example, *Equus*, *Canis*, *Felis*, *Sus*, *Cavia*, *Mus*, and *Rattus*) appear to synthesize only embryonic and adult hemoglobins (*1*). Thus, although the number and diversity of age-specific components varies among genera, a regulating mechanism whereby the synthesis of one or more hemoglobin components is gradually replaced by the synthesis of others seems characteristic of all mammals examined to date. The nature of this mechanism remains unknown.

Kabat (*2*) has attempted to account for the genetic basis of antibody diversity and the regulation of hemoglobin gene activity during development by proposing a mechanism based on gene excisions. In this scheme, a promoter region (site of attachment of RNA polymerase) is followed in sequence by the ϵ-, $^{G}\gamma$-, $^{A}\gamma$-, and β- and δ-globin genes; all but the β and δ genes are separated by terminator and operator sites, and hence only the gene adjacent to the promoter is active at any given time. In early embryos, stem cells produce erythrocytes containing only embryonic hemoglobins. During development of the embryo, occasional intrachromosomal crossing over with looping-out excision of promoter-proximal structural genes would occur independently in either of the two homologous chromosomes that bear the non-α gene cluster, leading to successive populations of hemopoietic stem cells in whose chromosomes different non-α genes would come to lie next to the promoter site. Thus, younger clones might produce erythrocytes with only ϵ chains, slightly older ones might produce cells with ϵ and $^{G}\gamma$ chains, or $^{G}\gamma$ and/or $^{A}\gamma$ chains, and still older ones might give rise to red cells with *cis* ($^{A}\gamma$) and *trans* ($\beta + \delta$) chains, until the oldest clones, capable of producing only mature cells with $\beta + \delta$ chains, predominate. The gene excision hypothesis predicts, then, that no more than two non-α genes (excluding the δ genes) can be expressed in a single erythrocyte, and this is the point upon which it is most readily subject to direct testing (*3*).

Gene excision would be supported if it were shown that persons heterozygous for two abnormal alleles at the β locus, who also happen to synthesize HbF, have no red cells containing two abnormal hemoglobins as well as HbF, since the presence of two different β chains and one γ chain in an individual cell would require that γ and β genes on a single chromosome be active. Since antibodies against normal and mutant hemoglobins can be produced, purified, conjugated with a fluorochrome, and used to identify single cells that contain the hemoglobins against which the antibodies

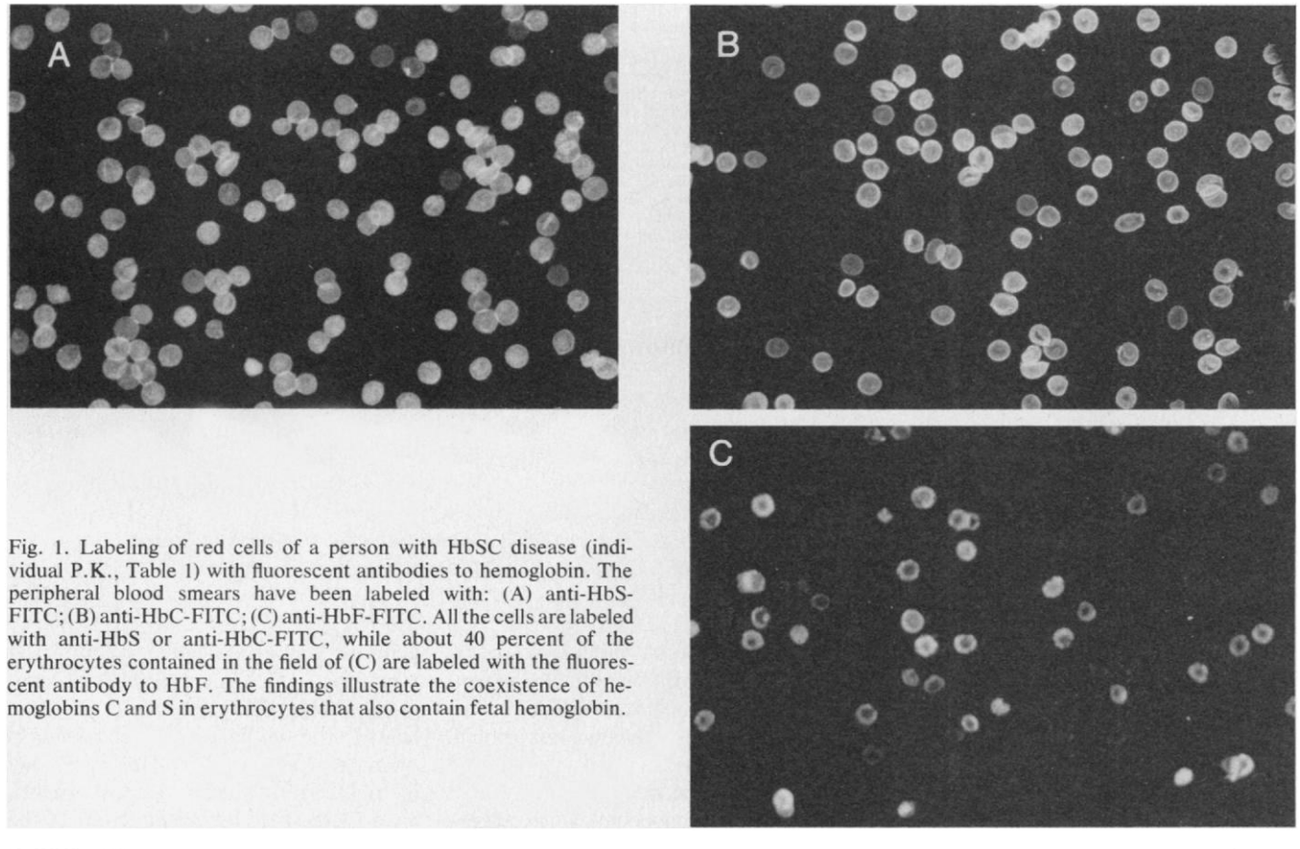

Fig. 1. Labeling of red cells of a person with HbSC disease (individual P.K., Table 1) with fluorescent antibodies to hemoglobin. The peripheral blood smears have been labeled with: (A) anti-HbS-FITC; (B) anti-HbC-FITC; (C) anti-HbF-FITC. All the cells are labeled with anti-HbS or anti-HbC-FITC, while about 40 percent of the erythrocytes contained in the field of (C) are labeled with the fluorescent antibody to HbF. The findings illustrate the coexistence of hemoglobins C and S in erythrocytes that also contain fetal hemoglobin.

Table 1. Proportion of cells containing hemoglobins S, C, and F in individuals heterozygous for both HbS and HbC alleles (HbSC disease).

Person	HbF percent	Percentage* of erythrocytes labeled by fluorescent		
		Anti-HbF	Anti-HbS†	Anti-HbC†
J. M.	4.0	18.9	100.6	100.3
R. L.	0.7	4.1	99.1	100.5
J. L.	1.2	5.3	99.7	99.4
S. W.	2.5	16.1	99.4	100.1
D. W.	1.4	4.4	100.3	99.7
P. K.	6.9	35.2	99.6	99.6
M. D.	2.9	12.9	100.9	100.2
W. F.	0.7	2.3	100.5	99.1

*Estimated by counting an average of 5000 (6). †Deviations from 100.0 reflect the expected counting error.

were raised (*4*), the hypothesis can be tested by immunochemical methods for the identification of specific hemoglobins in single erythrocytes. We tested the hypothesis in eight HbS/HbC heterozygotes. In these individuals, variable proportions of red cells contain HbF (F cells) (*5*). Blood smears were reacted with antibody to HbS, antibody to HbC, or antibody to HbF, each conjugated to fluorescein isothiocyanate (FITC) (*4*). Examination of the labeled preparations under white and fluorescent light indicated that all red cells from the eight compound heterozygotes were labeled with FITC-conjugated antibodies to HbS (anti-HbS-FITC) (Fig. 1A); similarly, all red cells were labeled with FITC-conjugated antibodies to HbC (anti-HbC-FITC) (Fig. 1B). Labeling with anti-HbF-FITC (Fig. 1C) disclosed from 2.3 to 35.2 percent HbF-containing red cells (F cells) in the eight subjects (Table 1). Double labeling experiments utilizing anti-HbF-FITC and tetramethyl rhodamine isothiocyanate–conjugated antibodies to HbS or antibodies to HbC demonstrated coexistence of HbS and HbC in the red cells containing HbF. These results thus show that both HbS and HbC are present in the F cells, and that the cells containing fetal hemoglobin derive from nucleated precursors that carry a minimum of three (that is, γ, β^S, β^C) active non-α chain genes. Obviously, at least one γ-chain gene and the β-chain gene that is in *cis* position to it can be synchronously active.

It seems thus that globin gene excision cannot account for the regulation of fetal hemoglobin synthesis and for the patterns of ontogenesis of human hemoglobins; more likely, a situation like that predicted by a "leaky model" (*2*) of hemoglobin regulation prevails during the course of human development; that is, during the transition from, for example, fetal to adult hemoglobin synthesis, the repression of γ-chain synthesis is not necessarily complete in any given maturing cell. Were the repression of γ-chain synthesis to remain incomplete in later life, the "turning on" of γ genes might be a conceivable approach to therapy for the patient with sickle-cell anemia or homozygous β thalassemia; this possibility would be denied were the expression of γ- and β-globin gene activity regulated by gene excision through intracistronic crossing over.

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2. D. Kabat, *Science* **175**, 134 (1972).
3. ———, *Ann. N.Y. Acad. Sci.* **241**, 119 (1974). Kabat subjected this prediction to testing using cord blood from HbA/HbS heterozygotes. The methods he used for identification of HbF, HbS, and adult hemoglobin (HbA or HbS) in single cells were based on differences in solubilities of these hemoglobins. The proportions of cells staining for hemoglobin after application of each method were in close agreement with those expected by the mutually exclusive model; however, the lack of sensitivity and specificity of the techniques employed render the results inconclusive.
4. Antibodies against HbF [W. G. Wood, G. Stamatoyannopoulos, G. Lim, P. E. Nute, *Blood* **46**, 671 (1975)], HbS [Th. Papayannopoulou, T. C. McGuire, G. Lim, E. Garzel, P. E. Nute, G. Stamatoyannopoulos, *Br. J. Haematol.* **34**, 25 (1976)], and HbC have been prepared and purified to monospecificity by affinity chromatography. Purification of anti-HbS included extensive absorption against Sepharose HbC, while anti-HbC was extensively absorbed against Sepharose-HbS. Each of these antibodies, when conjugated with FITC and applied to fixed smears of blood, permits the visualization only of cells containing the corresponding antigen. Artificial mixtures consisting of 10 percent SC red cells and 90 percent AS red cells were prepared and labeled with anti-HbC-FITC; only 10 percent of the cells were labeled with the antibodies, indicating the complete absence of cross-reactivity of the antibodies to HbC with HbS. Identical results were obtained when artificial mixtures of 10 percent SC cells and 90 percent AC cells were labeled with anti-HbS–FITC.
5. S. H. Boyer, T. K. Belding, L. Margolet, A. N. Noyes, *Science* **188**, 361 (1975); W. G. Wood, G. Stamatoyannopoulos, G. Lim, P. E. Nute, *Blood* **46**, 671 (1975); S. H. Boyer, T. K. Belding, L. Margolet, A. N. Noyes, P. J. Burke, W. R. Bell, *Johns Hopkins Med. J.* **137**, 105 (1971).
6. To obtain quantitative data, the total number of cells and the number of fluorescent cells were counted in several fields of each of the preparations labeled with anti-HbS-FITC, anti-HbC-FITC, or anti-HbF-FITC. The cells of each microscopic field were first counted under white light to determine the total number of cells per field; next, these cells were counted under fluorescent light to determine the number labeled by each type of anti-hemoglobin antibody. Approximately 5000 cells from each subject were counted, and all contained hemoglobins S and C; in addition, the proportion of the cells indicated in Table 1 contained fetal hemoglobin.
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Sickle Hemoglobin Aggregation: A New Class of Inhibitors

Abstract. *A number of tri- and tetrapeptides have been found to inhibit the aggregation and gelation of deoxygenated sickle cell hemoglobin. These inhibitors have hydrophobic phenylalanine residues at one end and hydrogen bonding lysine or arginine side chains at the other end. The backbone is not very specific. The inhibitors do not modify the oxygen carrying properties of hemoglobin. When the inhibitor and sickle hemoglobin are put inside reconstituted cells, the erythrocytes do not sickle upon deoxygenation. Compounds of this type may develop into useful agents in the therapy of sickle cell anemia.*

The substitution of valine for aspartic acid in the sixth position of the β chain of human hemoglobin is responsible for the aggregation of deoxygenated hemoglobin molecules in the disease sickle cell anemia (*1*). In the deoxygenated state, the hemoglobin molecules form aggregates or gels of elongated microtubular structures which have considerable rigidity within the erythrocyte (*2*). This leads to the sickling of the cells which is ultimately responsible for the pathology of sickle cell anemia. Recent studies have focused on the architecture of the