5. L. B. Brattsten and C. F. Wilkinson, *Pestic. Biochem. Physiol.* **3**, 393 (1973).
6. R. I. Krieger and C. F. Wilkinson, *Biochem. Pharmacol.* **18**, 1403 (1969).
7. Agway Research and Technical Center, Ithaca, N.Y., supplied the following insecticide solvents: Solvesso xylene [boiling point (b.p.), initial, 139°C], Mentor 28 (b.p., initial, 266°C), HAN (b.p., initial, 210°C) (Humble Oil & Refining Co.); Amsco-Solv E-98 (b.p., initial, 194°C) and Amsco odorless spray base (AMSCO Division, Union Oil Co.); Jayflex diiso-octylphthalate (b.p., midrange, 230°C) and Benzoflex 9-88 (b.p. 232°C) (Velsicol Chemical Corp.); 60-, 70-, and 100-second spray oils (b.p., midrange, 208°, 227°, and 246°C) (Sun Oil Co.); and Hess odorless spray base. AMOCO Chemicals Corp., Chicago, Ill., supplied samples of Panasol AN-2 and AN-2K.
8. R. J. Kuhr, *J. Agr. Food Chem.* **18**, 1023 (1970); H. W. Dorough and J. E. Casida, *ibid.* **12**, 294 (1964).
9. R. G. Helgesen, personal communication.
10. L. B. Brattsten, unpublished observation.
11. Supported in part by grant ES-00400 from the Public Health Service and grant RF 69073 from the Rockefeller Foundation. We thank M. M. Root for technical assistance.

10 May 1976; revised 17 January 1977

Stability of the Individual Globin Genes During Erythroid Differentiation

Abstract. *The genes for sheep β^A, β^C, and γ globin were all present in DNA from erythroid cells which synthesized only β^C globin. Similarly, selective excision of nonexpressed genes was shown not to occur during human erythroid differentiation. In contrast, evolutionary deletion of the β^C gene accounts for the inability of many sheep to make this globin.*

Many species have several globin genes which are differentially expressed during ontogeny (*1*). For example, in humans the expression of the gene for γ globin results in the production of hemoglobin F (HbF) ($\alpha_2\gamma_2$) during gestation. Synthesis of γ globin decreases to low levels after birth, when expression of the β globin gene results in the synthesis of hemoglobin A (HbA) ($\alpha_2\beta_2$). An analogous perinatal change from HbF to HbA occurs in sheep. In addition, sheep having the gene for β^A globin exhibit a second change, which occurs during anemia or other erythropoietic stress (*2*); the synthesis of β^A globin ceases and β^C globin is produced, resulting in the appearance of hemoglobin C (HbC) ($\alpha_2\beta_2{}^C$). The change from HbA to HbC is reversible; for example, after termination of erythropoietic stress, the β^A globin gene is again expressed and β^C globin synthesis stops.

Kabat (*3*) proposed that selective intrastrand excision of particular globin genes might occur early during erythropoiesis and be the mechanism whereby terminally differentiated cells become committed to synthesize the individual globins. Erythropoiesis is a continual process, whereby undifferentiated but committed precursor cells enter the erythron and actively synthesize hemoglobin for only a few days before maturing into circulating red cells (*4*). We have obtained experimental evidence which suggests that sheep erythroid cells become irreversibly committed to the synthesis of β^A or β^C globin (or a mixture of the two) during the transition from the undifferentiated precursors to recognizable erythroid cells (*5*). Kabat's model predicts that erythroid cells from those sheep homozygous for β^A globin might, during severe erythropoietic stress, contain the β^C gene but lack the γ gene or, possibly, both γ and β^A genes. Similarly, an adult human making only β globin would lack the gene for γ globin in his erythroid cells. The γ and β genes are closely linked in humans (*6*) and probably are also in other species. Thus, the formation of intrastrand loops by base pairing between homologous DNA sequences adjacent to each gene is a reasonable possibility and would provide a site for nuclease excision of particular genes. Nonetheless, this interesting hypothesis has never been tested directly. To do so we used complementary DNA's (cDNA's) specific for the nucleic acid sequences of the individual human and sheep globins as hybridization probes to determine the complement of genes present in differentiated erythroid cells.

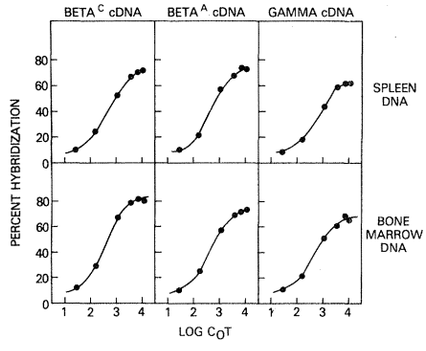

Fig. 1. Annealing of sheep bone marrow and spleen DNA to β^C, β^A, and γ cDNA's. Each hybridization reaction contained 2 mg of DNA and 0.2 ng of one of the cDNA's. Each probe was labeled with ^{3}H-labeled deoxycytidine triphosphate to give a specific activity of approximately 15,000 count/min per nanogram. The specific activities of all of the probes were identical. The reaction volume was 132 μl. Six individual portions (22 μl) were sealed in capillary tubes, incubated at 95°C for 5 minutes, and then placed in a constant temperature bath at 58°C. The capillary tubes were removed at times ranging from 10 minutes to 66 hours, and the contents were expelled into 0.5 ml of 0.05*M* phosphate buffer and stored in liquid nitrogen. At the completion of the hybridization period, each individual reaction was analyzed by batch elution of the single- and double-stranded fractions from hydroxyapatite, as described (*13*).

Messenger RNA's (mRNA's) were prepared from reticulocytes of anemic adult and normal fetal sheep. Full-length cDNA's containing sequences for α and one of the non-α globins (either β^A, β^B, β^C, or γ) were synthesized with RNA-dependent DNA polymerase (*7*). The non-α globin cDNA's were then purified by thermal denaturation of heterologous nucleic acid duplexes followed by chromatography on hydroxyapatite (*8*). For example, HbB cDNA (α,β^B) was annealed to HbF mRNA (α,γ) at 50°C, a permissive temperature that allows formation of γ-β^B duplexes. The temperature was then raised to 68°C, a temperature above the melting temperature of the heterologous β-γ duplexes but below the melting temperature of the homologous α-α duplexes. The single-stranded β^B cDNA was separated from α cDNA–α mRNA duplexes by batch chromatography on hydroxyapatite. Using these methods, we have prepared cDNA's that are specific for each of the sheep globins and are contaminated with only 5 to 15 percent α globin cDNA. Human β globin cDNA was prepared as described with mRNA from reticulocytes of a patient with α thalassemia (Hb H disease) (*9*). A cDNA specific for human γ globin sequences was obtained by using fetal reticulocyte mRNA (α,β,γ) to prepare mixed cDNA. The γ cDNA sequences were recovered by hybridization of this mixed probe to adult reticulocyte mRNA (α,β), followed by recovery of the single-stranded γ cDNA by hydroxyapatite chromatography (*10*).

We initially tested Kabat's gene excision hypothesis using DNA from the bone marrow of an anemic sheep. The animal was homozygous for HbA ($\alpha_2\beta_2{}^A$). After 15 days of phenylhydrazine injection, it was severely anemic (hematocrit, 10 to 12 percent), and mRNA prepared from its reticulocytes directed the synthesis only of β^C and α globin when translated in Xenopus oocytes (*11*). Because of anemia the ratio of myeloid to erythroid cells (M : E) in the

bone marrow was reduced to 0.1:1 to 0.2:1 (normal is 3:1 to 4:1). Thus at least 80 to 90 percent of the bone marrow DNA was derived from differentiated erythroid cells. We have previously performed mixing experiments with mouse and human DNA and found that a four- to fivefold reduction in the concentration of particular genes is easily detectable by hybridization analysis (*12*). DNA was extracted as described (*13*) both from the marrow and the spleen. The spleen contained less than 3 percent erythroid cells. Samples of these DNA's were annealed to cDNA's specific for β^C, β^A, and γ globin in 50 percent formamide at 58°C, a temperature only 2° to 4° below the melting temperature of perfect DNA-DNA duplexes. Under these stringent conditions the annealing kinetics of the three probes were identical with both DNA samples (Fig. 1). We concluded therefore that the β^A and γ globin genes were present in the erythroid cells of this animal.

We next tested the gene excisional hypothesis using DNA extracted from human erythroid cells. Bone marrow samples obtained from two patients with sickle cell anemia were pooled. Each patient had only 1 to 2 percent HbF in his circulating blood, and, because of the hemolytic anemia, the bone marrow was composed predominantly of erythroid cells (ratio of M to E, 0.2:1). Annealing of DNA extracted from these cells to human γ and β cDNA's was compared to results obtained with DNA extracted from normal human spleen. Both the bone marrow and spleen DNA contained the β and γ genes (data not shown). Thus the γ globin gene was present in differentiated adult human erythroid cells despite its lack of expression.

In sheep, HbC is synthesized during erythropoietic stress only in those animals which have the gene for β^A globin. Sheep homozygous for another adult hemoglobin, HbB ($\alpha_2\beta_2^B$), do not exhibit the synthesis of HbC ($\alpha_2\beta_2^C$) even when made anemic. The β^B and β^A globin genes are alleles. Animals heterozygous for the β^A and β^B genes produce HbA and HbB under normal conditions. During anemia HbA is replaced with HbC but HbB synthesis continues.

DNA prepared from the spleen of an animal homozygous for HbB ($\alpha_2\beta_2^B$) allowed us to test the specificity of our hybridization conditions. At 58°C, 2° to 4° below the melting temperature of perfect DNA-DNA duplexes, the β^B globin cDNA annealed to DNA from this animal as expected. However, the amount of the β^C probe incorporated into hybrid

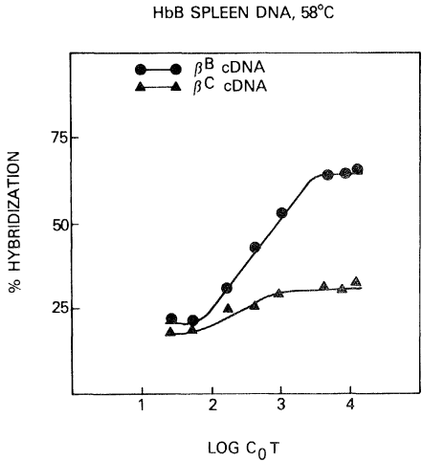

Fig. 2. Annealing of DNA from the spleen of a sheep homozygous for HbB to β^B and β^C cDNA. Two-milligram samples of DNA were hybridized to 0.2 ng of the specific probe, as described in the legend to Fig. 1.

was no greater than that anticipated from the small amount of alpha cDNA that contaminated this preparation (Fig. 2), implying the absence of the β^C gene in this DNA. The sequence differences among the β^A, β^C, and γ globins are equal to or greater than the difference in sequence between the β^B and β^C globins, and the divergence of the human γ and β globin genes is considerably greater (*8*). Therefore, we are confident that at 58°C each of the probes we have used anneals only to the gene sequence to which it corresponds.

Our results suggest that animals homozygous for β^B globin are unable to make HbC because they lack the β^C globin gene. Evolution of the β^C globin gene apparently occurred before the divergence of the several caprine species (goat, moufflon, and aoudad) that share this gene with sheep (*14*). The fact that most sheep in the western world are homozygous for β^B globin suggests that HbC in this species has lost whatever selective advantage it once conferred.

We have demonstrated that nonexpressed globin genes are retained during terminal erythroid differentiation. Earlier studies have shown that translational control does not account for commitment of erythroid cells to production of a specific globin (*8, 15*). Thus γ globin mRNA is not present in the adult erythroid cells that make only HbA, and β globin mRNA is not present in fetal erythroid cells making only HbF. It will be important, however, to examine the relative amounts of mRNA sequences in both nuclei and cytoplasm obtained from cell populations actively making two types of globin. To date, these studies have not been done. Thus, it remains possible that modulation of the final amount of specific mRNA in the cytoplasm is either achieved at the level of gene transcription or during RNA processing in the nucleus.

EDWARD BENZ, JR., PATRICIA TURNER
JANE BARKER, ARTHUR NIENHUIS
Section on Clinical Hematology, Molecular Hematology Branch, National Heart, Lung, and Blood Institute, Bethesda, Maryland

References and Notes

1. D. J. Weatherall, in *Congenital Disorders of Erythropoieses*, D. J. Weatherall, Ed. (Elsevier, Amsterdam, 1976), p. 307; H. H. Kazazian, Jr., *Semin. Hematol.* **11**, 525 (1974); R. A. Rifkind, L. N. Cantor, M. Cooper, J. Levy, G. M. Maniatis, A. Bank, P. A. Marks, *Ann. N.Y. Acad. Sci.* **241**, 113 (1974); H. Kitchen and I. Brett, *ibid.*, p. 653.
2. E. M. Tucker, *Biol. Rev. Cambridge Philos. Soc.* **46**, 341 (1971); T. H. J. Huisman, J. P. Lewis, M. H. Blunt, H. R. Adams, A. Miller, A. M. Dozy, E. M. Bond, *Pediatr. Res.* **3**, 189 (1969); T. F. Thurman, S. H. Boyer, E. F. Crossby, M. K. Shepard, A. N. Noyes, F. Stohlman, *Blood* **36**, 589 (1970); A. W. Nienhuis, N. A. Elson, J. E. Barker, W. F. Anderson, *Ann. N.Y. Acad. Sci.* **241**, 566 (1974).
3. D. Kabat, *Science* **175**, 134 (1972).
4. R. Schofield and L. G. Lajtha, in *Congenital Disorders of Erythropoiesis*, D. J. Weatherall, Ed. (Elsevier, Amsterdam, 1976), p. 3; R. A. Rifkind and P. A. Marks, *Blood Cells* **1**, 417 (1975).
5. A. W. Nienhuis and H. F. Bunn, *Science* **185**, 946 (1974); J. E. Barker, W. F. Anderson, A. W. Nienhuis, *J. Cell Biol.* **64**, 515 (19 ➡ J. E. Barker, J. E. Pierce, A. W. Nienhuis, *ibid.* **71**, 715 (1976).
6. A. W. Nienhuis and W. F. Anderson, *Clin. Haematol.* **3**, 437 (1974); E. J. Benz, Jr., and B. G. Forget, *Prog. Hematol.* **9**, 107 (1976).
7. I. M. Verma, G. F. Temple, H. Fan, D. Baltimore, *Nature (London) New Biol.* **235**, 163 (1972); D. L. Kacian, S. Spiegelman, A. Bank, M. Terada, S. Metafora, L. Dow, P. A. Marks, *ibid.*, p. 167; J. Ross, H. Aviv, E. Scolnick, P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 264 (1972); G. B. Weiss, G. N. Wilson, A. W. Steggles, W. F. Anderson, *J. Biol. Chem.* **251**, 3425 (1976).
8. E. J. Benz, Jr., C. E. Geist, A. W. Steggles, J. E. Barker, A. W. Nienhuis, *J. Biol. Chem.* **252**, 1908 (1977).
9. R. Velez, J. Kantor, D. Picciano, W. F. Anderson, A. Nienhuis, *ibid.* **250**, 3193 (1975); A. Deisseroth, R. Velez, A. W. Nienhuis, *Science* **191**, 1262 (1976).
10. W. G. Lanyon, S. Ottolengi, R. Williamson, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 258 (19 ➡ Y. W. Kan, J. P. Holland, A. M. Dozy, N. E. Varmus, *ibid.*, p. 5140.
11. J. Gurdon, C. Lane, N. Woodland, G. Marbaix, *Nature (London)* **233**, 177 (1971); L. Chan, P. O. Kohler, B. W. O'Malley, *J. Clin. Invest.* **57**, 576 (1976).
12. A. Deisseroth and A. W. Nienhuis, *In Vitro* **12**, 734 (1976).
13. J. Marmur, *J. Mol. Biol.* **3**, 208 (1961); A. Deisseroth, R. Velez, R. Burk, J. Minna, W. F. Anderson, A. Nienhuis, *Som. Cell Genet.* **2**, 373 (1976); D. J. Brenner, G. R. Fanning, A. V. Rake, K. E. Johnson, *Anal. Biochem.* **28**, 447 (1969).
14. T. H. J. Huisman, *Ann. N.Y. Acad. Sci.* **241**, 549 (1974).
15. A. W. Nienhuis and W. F. Anderson. *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2184 (19 ➡ W. G. Lanyon, S. Ottolengi, R. Williamson, *ibid.* **72**, 258 (1975).
16. We thank Dr. W. F. Anderson for support and encouragement, C. E. Geist and B. Kefauver for technical assistance, and Dr. J. E. Pierce and L. Stewart for assistance in the care of the animals. We are also grateful to Dr. J. W. Beard who, through the Offices of Program Resources and Logistics, Viral Oncology Branch, National Cancer Institute, provided the RNA-dependent DNA polymerase used in this work.

16 November 1976; revised 28 December 1976