

Recombinant human hemoglobins designed for gene therapy of sickle cell disease

(Hb S polymerization/anti-sickling Hb/transgenic mice)

STEVEN L. MCCUNE*, MICHAEL P. REILLY†, MATTHEW J. CHOMO†, TOSHIO ASAKURA†,
AND TIM M. TOWNES*‡

*Department of Biochemistry and Molecular Genetics, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL 35294; and
†Departments of Pediatrics and of Biochemistry and Biophysics, The Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, PA 19104

Communicated by Ernest Beutler, June 17, 1994

ABSTRACT Two human hemoglobins designed to inhibit the polymerization of sickle hemoglobin (Hb S; $\alpha_2\beta^S_2$) have been produced. Mutations that disrupt the ability of Hb S to form polymers were introduced into the normal human β -globin gene by site-specific mutagenesis. These mutations affect the axial and lateral contacts in the sickle fiber. The recombinant hemoglobin designated anti-sickling hemoglobin 1 (Hb AS1) contains the mutations $\beta 22$ glutamic acid to alanine and $\beta 80$ asparagine to lysine. Hb AS2 has the same $\beta 22$ glutamic acid to alanine mutation combined with $\beta 87$ threonine to glutamine. Human α - and β^{AS} -globin genes were separately fused downstream of β -globin locus control region sequences and these constructs were coinjected into fertilized mouse eggs. Transgenic mouse lines that synthesize high levels of each anti-sickling hemoglobin were established and anti-sickling hemoglobins were purified from hemolysates and characterized. Both AS hemoglobins bind oxygen cooperatively and the oxygen affinities of these molecules are in the normal range. Delay time experiments demonstrate that Hb AS2 is a potent inhibitor of Hb S polymerization; therefore, locus control region β^{AS2} -globin gene constructs may be suitable for future gene therapy of sickle cell disease.

Sickle cell disease results from an A to T transversion in the sixth codon of the human β -globin gene (1, 2). This mutation leads to the substitution of a nonpolar valine for a polar glutamic acid residue on the surface of the hemoglobin molecule. At low oxygen tensions, the $\beta 6$ valine interacts with a natural hydrophobic pocket on the surface of a second hemoglobin tetramer and initiates the polymerization of Hb S into long fibers composed of seven pairs of double strands (3-8). The formation of these fibers reduces the flexibility of erythrocytes and leads to the occlusion of small capillaries. Intracellular fiber formation also results in erythrocyte membrane damage and increased cell lysis. The ensuing disease is characterized by a chronic hemolytic anemia with episodes of severe pain and tissue damage that can result in stroke, kidney failure, heart disease, infection, and other complications (9).

The structure of the fiber that forms in sickle erythrocytes was derived from x-ray diffraction studies of Hb S crystals (10). Hb S tetramers are composed of two α -globin subunits (α_2) and two β^S -globin subunits (β^S_2) and form characteristic double strands. Interactions along the long axis of the fiber are termed axial contacts, whereas interactions along the sides of tetramers are lateral contacts (Fig. 1A) (9). The $\beta 6$ valine plays a critical role in the lateral contact by interacting with the hydrophobic residues $\beta 85$ phenylalanine and $\beta 88$ leucine (Fig. 1A). Hb A ($\alpha_2\beta_2$) has these same hydrophobic

residues and is readily incorporated into sickle fibers. An important axial contact is the interaction of the $\beta 22$ glutamic acid with the imidazole group of the $\alpha 20$ histidine on an adjacent tetramer (11) (Fig. 1A). The physiologic relevance of specific amino acids in the formation of the Hb S fiber has been demonstrated by experiments in which the polymerization of mixtures of Hb S and mutant hemoglobins was analyzed (12). These mixing experiments highlighted the particular importance of residues $\beta 22$ and $\beta 87$ in inhibiting Hb S polymerization. Hemoglobins F ($\alpha_2\gamma_2$) and A₂ ($\alpha_2\delta_2$) both differ from Hb A at positions corresponding to $\beta 22$ and $\beta 87$ and both significantly inhibit Hb S polymerization.

Amelioration of the clinical symptoms of sickle cell disease in patients with the concurrent condition of hereditary persistence of fetal hemoglobin suggests that a level of fetal hemoglobin comprising 20-25% of total cellular hemoglobin is clinically beneficial (13-15). Patients with this level of Hb F are essentially asymptomatic and gene therapy strategies directed at achieving this level of fetal hemoglobin expression have been suggested. However, Hb F levels of 20-25% may be difficult to obtain when genes are introduced into adult hematopoietic cells. Transgenic mouse experiments have demonstrated that locus control region (LCR) γ -globin constructs are expressed at least four times less efficiently than LCR β -globin constructs in adult erythroid tissue (16-18). Apparently, fetal-specific transcription factors are required for high-level expression of the γ -globin gene. Though it may be possible to drive expression of the coding region of the γ -globin gene using regulatory elements of the β -globin gene, a potential disadvantage of this approach is that the β -globin regulatory elements are present in three separate locations: promoter, second intron, and proximal 3' flanking region (19-23). Combining these three elements with the coding region of the γ -globin gene is not necessarily straightforward and might not provide a therapeutic level of expression. Since the β -globin gene is normally expressed at high levels in adult erythroid cells, the optimal construct for gene therapy would be a gene that combines the anti-sickling activity of Hb F or A₂ with the high transcriptional activity of the adult β -globin gene. In this paper, we demonstrate that the mutation of two amino acids is sufficient to provide the β -globin gene with the anti-sickling activity of Hb F or A₂.

METHODS AND MATERIALS

Construction and Microinjection of Anti-Sickling β -Globin Genes. Plasmids were constructed by standard procedures (24). Mutagenesis was performed using the Altered Sites system (Promega) (25). The mutagenic oligonucleotides were as follows: $\beta 22$, GTGAACGTGGATGCCGTTGGTGGT-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: LCR, locus control region; IEF, isoelectric focusing; OEC, oxygen equilibrium curve; BPG, 2,3-bisphosphoglycerate.
‡To whom reprint requests should be addressed.

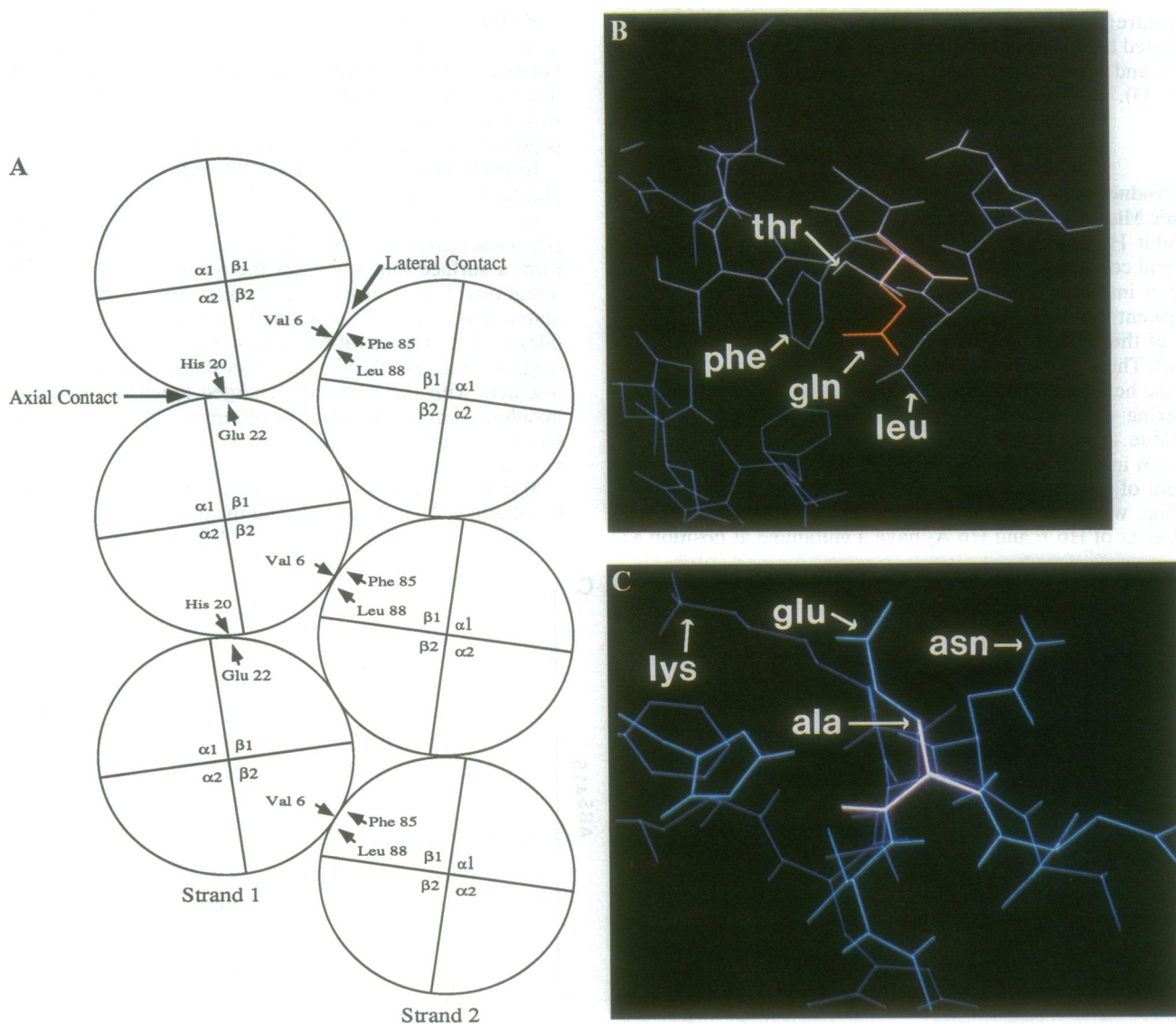


FIG. 1. Structure of human Hb S fibers. (A) Double-stranded sickle fiber. Two types of contacts occur between Hb S tetramers incorporated into fibers. Contacts along the long axis of the fiber are termed axial contacts, whereas contacts along the sides of tetramers are lateral contacts. The $\beta 6$ valine plays a critical role in the lateral contact by interacting with the hydrophobic residues $\beta 85$ phenylalanine and $\beta 88$ leucine. An important axial contact is the interaction of the $\beta 22$ glutamic acid with the imidazole group of the $\alpha 20$ histidine on an adjacent tetramer. (B) Lateral contact in the sickle fiber. This contact forms when the $\beta 6$ valine of Hb S interacts with a hydrophobic pocket on an adjacent tetramer. This hydrophobic pocket consists primarily of the residues $\beta 85$ phenylalanine (phe) and $\beta 88$ leucine (leu). These two residues are essential for correct positioning of the heme moiety and cannot be mutated. However, a threonine (thr) residue at position 87 can be replaced by a glutamine (gln), shown in red. The longer side chain of the glutamine prevents the $\beta 6$ valine from interacting with the hydrophobic pocket. (C) Axial contact in the sickle fiber. The side chains of the amino acids $\beta 17$ lysine (lys), $\beta 19$ asparagine (asn), and $\beta 22$ glutamic acid (glu) project to form a surface that stabilizes the axial contact. In both anti-sickling hemoglobins, the $\beta 22$ glutamic acid is replaced by an alanine residue (ala). This residue fails to interact with the positively charged histidine from the neighboring tetramer and thus disrupts the axial contact. The new alanine residue is shown in light blue.

GAG; $\beta 80$, GCTCACCTGGACAAGCTCAAGGGCACC; $\beta 87$, GGCACCTTTGCCAGCTGAGTGAGCTG. Fragment preparation and microinjection were as described (26). Transgenic animals expressing high levels of human hemoglobin were identified by isoelectric focusing (IEF) of hemolysates. IEF was performed using the isothermal controlled electrophoresis system (Fisher) with precast agarose IEF gels (Isolab).

Analysis and Purification of Recombinant Human Hemoglobins. Initial analysis of hemoglobin tetramers was performed by anion-exchange high-performance liquid chromatography (HPLC) utilizing a Synchropak AN 300 (4.6 mm \times 25 mm) column (SynChrome, Lafayette, IN) (27). Preparative IEF was performed on 4% acrylamide gels with 2% Pharmalyte (Pharmacia) (pH 6.7–7.7). Bands of hemoglobin were sliced

from the gel and eluted in 0.1 M potassium phosphate buffer (pH 7.0) (28). Mouse and human globins were separated by reversed-phase HPLC using a Series 4500i HPLC system (Dionex). Approximately 25–30 μ g of hemoglobin was injected into a C₄ reversed-phase (4.6 mm \times 250 mm) column (Vydac, Hesperia, CA) and eluted with a linear gradient of acetonitrile and 0.3% trifluoroacetic acid (29, 30).

Functional Analysis of Recombinant Human Hemoglobins. Oxygen equilibrium curves (OECs) were measured with a Hemox analyzer (TCS Medical Products, Southampton, PA) as described (31). The OECs were determined in 0.1 M potassium phosphate buffer (pH 7.0) at 20°C with a hemoglobin concentration of 25 μ M. Kinetics of polymerization were determined in 1.8 M potassium phosphate buffer as described (32). Polymerization was initiated using the tem-

perature jump method in which the temperature of deoxygenated hemoglobin solutions is rapidly changed from 0°C to 30°C and aggregation is monitored turbidimetrically at 700 nm (32, 33).

RESULTS AND DISCUSSION

Production of Recombinant Human Hemoglobin in Transgenic Mice. Two recombinant hemoglobins were designed to inhibit Hb S polymerization. A mutation that disrupts the lateral contact is shown in Fig. 1B. The $\beta 6$ valine residue of Hb S interacts with a natural hydrophobic pocket on the adjacent tetramer. This hydrophobic pocket consists primarily of the residues $\beta 85$ phenylalanine (phe) and $\beta 88$ leucine (leu). These two residues are essential for correct positioning of the heme moiety and cannot be mutated without severely altering oxygen affinity (11). However, a threonine (thr) residue at position 87 can be replaced by glutamine (gln) shown in red, and our supposition was that the longer side chain of glutamine might prevent the $\beta 6$ valine from interacting with the hydrophobic pocket. Both the γ and δ subunits of Hb F and Hb A₂ have a glutamine at position 87

and this change may account at least partially for their anti-sickling properties. Hb AS2 also contains a glutamine at position 87 together with an alanine at position 22. Hb AS1 has the same $\beta 22$ alanine, and asparagine at $\beta 80$ is replaced by lysine. This $\beta 80$ lysine significantly inhibits sickling when present as a single site mutation in Hb A (34).

In both anti-sickling hemoglobins glutamic acid was replaced by alanine at position $\beta 22$ to disrupt the axial contact (Fig. 1C). The side chains of the amino acids $\beta 17$ lysine (lys), $\beta 19$ asparagine (asn), and $\beta 22$ glutamic acid (glu) project to form a surface that stabilizes the axial contact (11). While mutations at residues $\beta 17$ or $\beta 19$ impair hemoglobin function, amino acid $\beta 22$ can be mutated from glutamic acid to alanine (ala) without an alteration in hemoglobin function (9). The negative charge of the glutamic acid side chain at this position interacts with the positively charged imidazole group of a histidine from the neighboring tetramer. The shorter nonpolar alanine side chain fails to stabilize this interaction. The new alanine residue is shown in light blue.

Mutations were introduced into the human β -globin gene by site-directed mutagenesis and the mutant sequences were inserted downstream of a 22-kb DNA fragment containing the

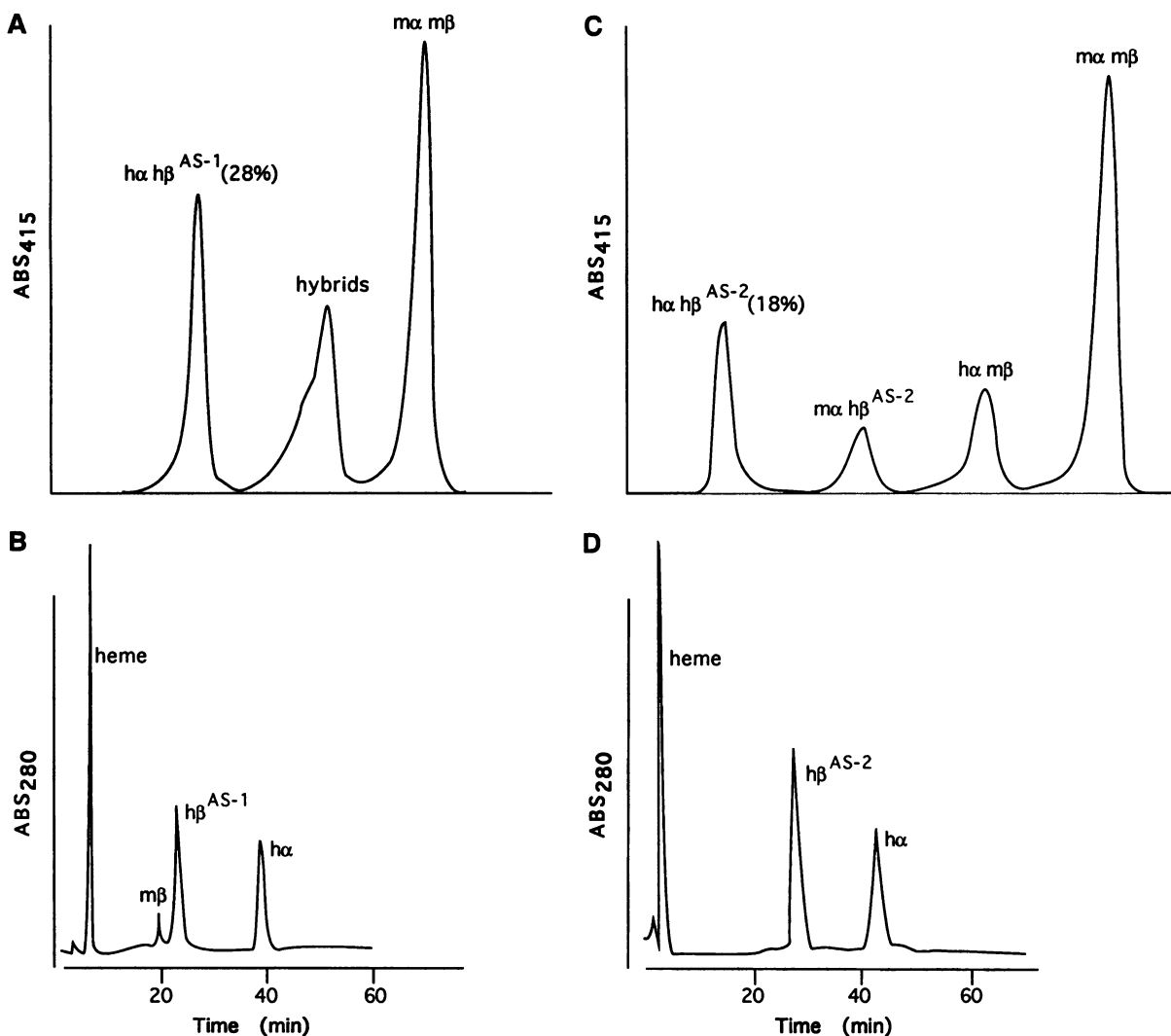


FIG. 2. Chromatograms of hemolysates and purified anti-sickling hemoglobins. (A) Chromatograph of hemolysate obtained from transgenic mice expressing Hb AS1. Hemoglobins were separated by nondenaturing HPLC. Twenty-eight percent of the hemoglobin in erythrocytes of these animals is recombinant human $\alpha\beta^{AS1}$. (B) Denaturing HPLC of $\alpha\beta^{AS1}$ purified from the hemolysate shown in A. Purification was performed by preparative IEF. Approximately 10% of the β -globin chains were of murine origin. (C) Chromatograph of hemolysate obtained from transgenic mice expressing Hb AS2. Hemoglobins were separated by nondenaturing HPLC. Eighteen percent of the hemoglobin in the erythrocytes of these animals is recombinant human $\alpha\beta^{AS2}$. (D) Denaturing HPLC of $\alpha\beta^{AS2}$ purified from the hemolysate shown in C. Purification was performed by preparative IEF. ABS, absorbance.

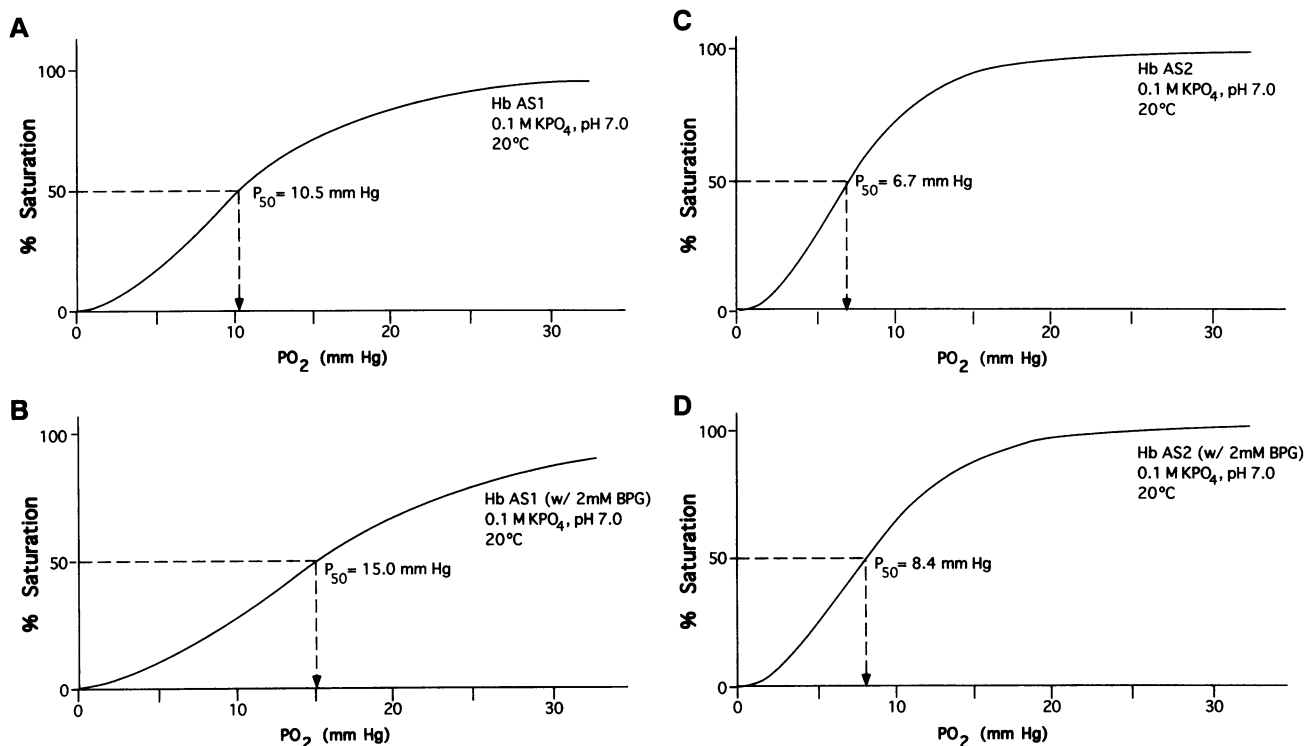


FIG. 3. OECs for purified human anti-sickling hemoglobins. (A) OEC for Hb AS1 at pH 7.0 in 0.1 M potassium phosphate buffer at 20°C. (B) OEC for Hb AS1 under the same conditions as in A with the addition of 2 mM 2,3-bisphosphoglycerate (BPG). (C) OEC for Hb AS2 at pH 7.0 in 0.1 M potassium phosphate buffer at 20°C. (D) OEC for Hb AS2 under the same conditions as in C with the addition of 2 mM BPG.

DNase hypersensitive sites 1–5 (5' HS 1–5) of the β -globin LCR (26). These constructs were injected into fertilized mouse eggs, and blood from mice that developed was analyzed by IEF to identify transgenic animals that synthesize high levels of human hemoglobin (26, 28). Transgenic lines were established and hemolysates obtained from several animals were analyzed by anion-exchange HPLC to quantitate the amounts of human, mouse, and hybrid hemoglobins (27). Fig. 2 A and C demonstrate that 28% of total hemoglobin was Hb AS1 in one $\alpha\beta^{AS1}$ transgenic line and 18% of total hemoglobin was Hb AS2 in one $\alpha\beta^{AS2}$ transgenic line. Hemoglobins AS1 and AS2 were isolated by preparative IEF (28) and the purity of the human hemoglobins was assessed by denaturing reverse-phase HPLC, which separates the α - and β -globin subunits (29, 30). Fig. 2 B and D demonstrate that Hb AS1 was $\approx 90\%$ pure and Hb AS2 was purified to homogeneity.

Functional Analysis of Recombinant Human Hemoglobins. The OECs for purified Hb AS1 and Hb AS2 are illustrated in Fig. 3 A and C (31). These sigmoidally shaped curves demonstrate the normal cooperativity of oxygen binding (Fig. 3 A and C). The P_{50} value, which measures the partial pressure of oxygen at which hemoglobin is half-saturated, was determined for Hb AS1 and Hb AS2 and compared with Hb A and Hb F (Table 1). The P_{50} for Hb AS1 is elevated slightly and this hemoglobin responds normally to the allosteric effector BPG—that is, oxygen affinity is decreased in

the presence of 2 mM BPG (Fig. 3B). The P_{50} for Hb AS2 is slightly lower than normal (6.7 mmHg) but BPG raises this value to 8.4 mmHg (Fig. 3D). The oxygen affinity of Hb AS2 is functionally equivalent to Hb F in the presence of 2 mM BPG and, therefore, Hb AS2 should adequately bind and deliver oxygen *in vivo*.

Inhibition of Hb S Polymerization by Recombinant Human Hemoglobins. The ability of hemoglobins AS1 and AS2 to disrupt Hb S polymerization was analyzed by delay time measurements (32). Briefly, Hb S (100%) or mixtures of Hb S (75%) and Hb A, AS1, AS2, or F (25%) were deoxygenated, and polymerization as a function of time was measured spectrophotometrically as the temperature of the hemoglobin solution was raised from 0°C to 30°C (32, 33). Fig. 4A demonstrates that HbS polymerizes relatively rapidly and that Hb A, AS1, AS2, and F delay Hb S polymerization to different extents. Hb AS1 inhibits Hb S polymerization more efficiently than Hb A; however, Hb AS1 inhibits much less effectively than Hb F, which is known to inhibit sickling *in vivo* at a 3:1 ratio (13–15). Finally, Hb AS2 inhibits Hb S polymerization at approximately the same level as Hb F. This finding strongly suggests that Hb AS2 will inhibit HbS polymerization *in vivo* if expression of AS2 at a level of 25% of total hemoglobin can be achieved.

The delay times determined in Fig. 4A were all measured at a concentration of 60 mg/dl. Fig. 4B illustrates the results of similar experiments performed at variable concentrations of total hemoglobin. The ratio of Hb S to Hb A, AS1, AS2, or F in all of these experiments was 3:1. In this figure the logarithm of the reciprocal of the delay time and the logarithm of hemoglobin concentration are plotted. As reported by Hofrichter *et al.* (3), an empirical relationship between delay time (t_d) and hemoglobin concentration can be described by the following equation: $1/t_d = \gamma S^n$, where $S = [\text{Hb}]/[\text{total}]/[\text{Hb}]_{\text{soluble}}$, and γ is an experimental constant. The n value is related to the size of nuclei formed during polymerization. The n values of the data shown in Fig. 4B are between 2 and

Table 1. P_{50} values for recombinant and naturally occurring human hemoglobins

Sample	P_{50} , mmHg	
	Without BPG	With BPG
Hb AS1	10.5	15.0
Hb AS2	6.7	8.4
Hb A	8.7	13.3
Hb F	8.8	10.0

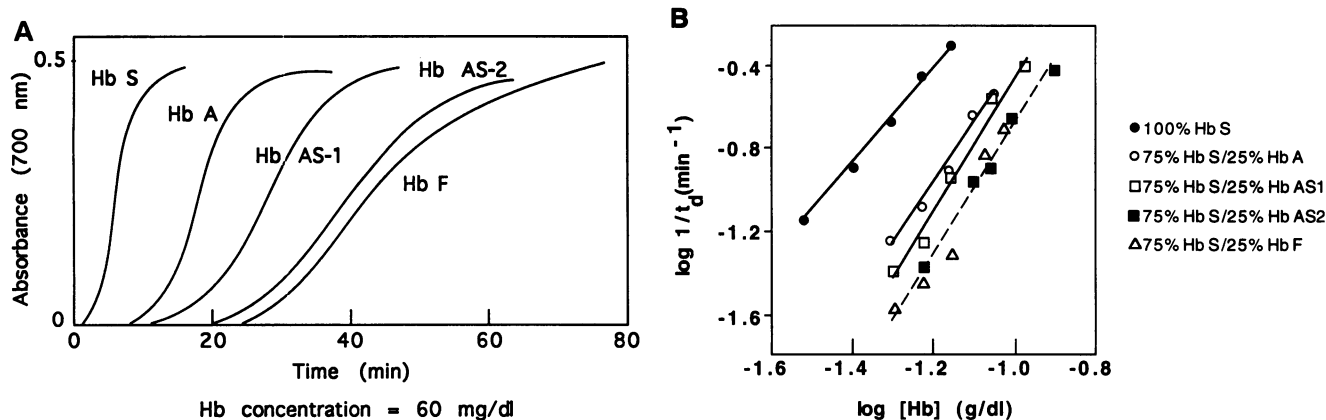


FIG. 4. Polymerization delay times for deoxygenated mixtures of human hemoglobins. (A) Delay times for hemoglobin mixtures containing 100% Hb S or 75% Hb S together with 25% Hb A, Hb AS1, Hb AS2, or Hb F. Curves were determined at a hemoglobin concentration of 60 mg/dl using the temperature jump method (18). The delay time is an indication of the ability of a hemoglobin to disrupt the polymerization of Hb S. (B) Delay time vs. hemoglobin concentration. Hemoglobin concentration ranged from 30 mg/dl to 125 mg/dl. The progression of the plots from left to right demonstrates the increased Hb concentrations that are required for polymerization to occur in the presence of the various non-S hemoglobins.

3, which agree well with those shown previously in high phosphate buffer (32). At higher concentrations of hemoglobin, the delay times for Hb AS2 and Hb F overlap, indicating that Hb AS2 and Hb F have virtually identical antipolymerization activity.

The results described above demonstrate that the genetic modification of two surface amino acids in Hb A produces a unique human hemoglobin (Hb AS2) that inhibits Hb S polymerization as effectively as Hb F. As discussed above, the β -globin LCR enhances β -globin gene expression much more effectively than γ -globin gene expression in adult erythroid cells. Therefore β^{AS2} , which is a β -globin gene with the antipolymerization properties of γ -globin, may be the molecule of choice for future genetic therapy of sickle cell disease. Further modifications of this anti-sickling β -globin gene may provide a hemoglobin that surpasses Hb F in its ability to disrupt the polymerization of Hb S.

We thank M. Carson and S. Ealick for computer graphics assistance and Josef Prchal for critical review of the manuscript. Transgenic animals were produced by the University of Alabama at Birmingham Transgenic Animal Core Facility. This work was supported in part by Grants HL 43508, HL 35559, and HL 38632 from the National Institutes of Health and by a grant from the Mizuno Fund. S.L.M. is supported by the University of Alabama at Birmingham Medical Scientist Training Program and by a March of Dimes Predoctoral Research Training Fellowship.

- Ingram, V. M. (1956) *Nature (London)* **178**, 792-794.
- Ingram, V. M. (1957) *Nature (London)* **180**, 326-328.
- Hofrichter, J., Ross, P. D. & Eaton, W. A. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4864-4868.
- Wishner, B. C., Ward, K. B., Lattman, E. E. & Love, W. E. (1975) *J. Mol. Biol.* **98**, 179-194.
- Crepeau, R. H., Dykes, G., Garrell, R. & Edelstein, S. J. (1978) *Nature (London)* **274**, 616-617.
- Dykes, G. W., Crepeau, R. H. & Edelstein, S. J. (1979) *J. Mol. Biol.* **130**, 451-472.
- Padlan, E. A. & Love, W. E. (1985) *J. Biol. Chem.* **260**, 8280-8291.
- Eaton, W. A. & Hofrichter, J. (1987) *Blood* **70**, 1245-1266.
- Bunn, H. F. & Forget, B. G. (1986) *Hemoglobin: Molecular, Genetic, and Clinical Aspects* (Saunders, Philadelphia).
- Edelstein, S. J. (1981) *J. Mol. Biol.* **150**, 557-575.
- Dickerson, R. E. & Geis, I. (1983) *Hemoglobin: Structure, Function, Evolution, and Pathology* (Benjamin/Cummings, Menlo Park, CA).
- Nagel, R. L., Bookchin, R. M., Johnson, J., Labie, D., Wajcman, H., Isaac-Sodeye, W. A., Honig, G. R., Schiliro, G., Crookston, J. H. & Matsumoto, K. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 670-672.
- Altay, C., Schroeder, W. A. & Huisman, T. H. J. (1977) *Am. J. Hematol.* **3**, 1-14.
- Brittenham, G., Lozoff, B., Harris, J. W., Mayson, S. M., Miller, A. & Huisman, T. H. J. (1979) *Am. J. Hematol.* **6**, 107-123.
- Wood, W. G., Pembrey, M. E., Serjeant, G. R., Perrine, R. P. & Weatherall, D. J. (1980) *Br. J. Hematol.* **45**, 431-445.
- Enver, T., Ebens, A. J., Forrester, W. C. & Stamatoyannopoulos, G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7033-7037.
- Behringer, R. R., Ryan, T. M., Palmiter, R. D., Brinster, R. L. & Townes, T. M. (1990) *Genes Dev.* **4**, 380-389.
- Dillon, N. & Grosveld, F. (1991) *Nature (London)* **350**, 252-254.
- Behringer, R. R., Hammer, R. E., Brinster, R. L., Palmiter, R. D. & Townes, T. M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7056-7060.
- Kollias, G., Hurst, J., deBoer, E. & Grosveld, F. (1987) *Nucleic Acids Res.* **15**, 5739-5747.
- Trudel, M., Magram, J., Bruckner, L. & Costantini, F. (1987) *Mol. Cell. Biol.* **7**, 4024-4029.
- Antoniou, M., deBoer, E., Habets, G. & Grosveld, F. (1988) *EMBO J.* **7**, 377-384.
- deBoer, E., Antoniou, M., Mignotte, V., Wall, L. & Grosveld, F. (1988) *EMBO J.* **7**, 4203-4212.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, New York), 2nd Ed.
- Lewis, M. K. & Thompson, D. V. (1990) *Nucleic Acids Res.* **18**, 3439-3443.
- Ryan, T. M., Townes, T. M., Reilly, M. P., Asakura, T., Palmiter, R. D., Brinster, R. L. & Behringer, R. R. (1990) *Science* **247**, 566-568.
- Ip, C. Y. & Asakura, T. (1986) *Anal. Biochem.* **156**, 348-353.
- Behringer, R. R., Ryan, T. M., Reilly, M. P., Asakura, T., Palmiter, R. D., Brinster, R. L. & Townes, T. M. (1989) *Science* **245**, 971-973.
- Shelton, J. B., Shelton, J. R. & Schroeder, W. A. (1984) *J. Liq. Chromatogr.* **7**, 1969-1977.
- Adachi, K. & Asakura, T. (1987) *J. Chromatogr.* **419**, 303-307.
- Asakura, T. & Reilly, M. P. (1986) in *Oxygen Transport in Red Blood Cells*, ed. Nicolau, C. (Pergamon, New York).
- Adachi, K. & Asakura, T. (1979) *J. Biol. Chem.* **254**, 7765-7771.
- Adachi, K., Segal, R. & Asakura, T. (1980) *J. Biol. Chem.* **255**, 7595-7603.
- Nagel, R. L., Johnson, J., Bookchin, R. M., Garel, M. C., Rosa, J., Schiliro, G., Wajcman, H., Labie, D., Moo-Penn, W. & Castro, O. (1980) *Nature (London)* **283**, 832-834.