MINIREVIEW

Recombinant Hemoglobins as Blood Substitutes: A Biotechnology Perspective

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Abstract. **Human hemoglobin can be produced in microbial and mammalian organisms using many different expression systems. It is anticipated that recombinant hemoglobins (or globin genes) will have many applications including as an infectious agent-free, inexpensive raw material for a red blood cell substitute, as a vehicle for expression or delivery of other biomolecules, and in gene therapy of inherited hemoglobinopathies. Recombinant expression, combined with site-directed mutagenesis, is facilitating the modification of the functional properties of hemoglobin. Although a functional hemoglobin molecule can be derived using many of the known expression systems, the choice of the production system for manufacturing depends on the scale, acceptable cost, and the associated environmental impact of the various processes. While the efficient bacterial production systems yield a modified, "surrogate" hemoglobin, the transgenic animal-derived product is virtually identical to the human erythrocyte-derived hemoglobin. Both the microbial fermentation, and the mammalian transgenic systems can be geared to produce the enormous quantities of hemoglobin expected to be required to meet the anticipated demand for a successful blood substitute in the future.** [P.S.E.B.M. 1995, Vol 208]

Hemoglobin is one of the most abundant animal proteins on earth. The major purpose of hemoglobin is to bind and release oxygen, a function that is of vital interest to all aerobic organisms. Hemoglobin expression in mammals is restricted to the erythrocytes, where it is found at an intracellular concentration exceeding 300 g/l, which is equivalent to 5 m*M* (1). The molecular mechanisms responsible for the superefficient hemoglobin expression system are now understood in sufficient detail to permit its reconstruction in experimental systems (2, 3). Because of its abundance, and due to the renewable nature of red blood cells (RBCs), the supply of human hemoglobin is virtually endless. This feature has been effectively exploited for the benefit of millions of surgical, trauma, and anemic patients requiring blood transfusions every year (4). However, the cost of collection, testing, and storage of human blood has escalated alarmingly in the past decade. In recent years, the concept of a hemoglobin-based oxygen carrying solution, commonly known as a "blood substitute," has attracted the attention of scientists and the general public, mainly due to the numerous shortcomings of allogeneic transfusion (5–7). Some of these problems (namely, erratic supply of certain types of blood, viral and parasitic contamination, short shelf-life, and toxicity due to leukocytes) could be eliminated by choosing a recombinant, nonhuman means of producing human hemoglobin (8).

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Hemoglobins have been highly conserved through evolution, and the nonglobin part of this molecule, the ubiquitous prosthetic group protoporphyrin IX (heme), is common to heme-containing proteins of diverse species of animals and microbes. This feature greatly simplifies the production of recombinant hemoglobin, since only the exogenous globin genes need be expressed. The heterologous globins can then be combined with the endogenously produced heme to synthesize functional hemoglobin. The evolutionary conservation of function of hemoglobin also has favorable consequences for expression of recombinant hemoglobin in mammals, since the recombinant hemoglobin can supplement, or substitute for, the function of the endogenous protein. Therefore, the only requirements for recombinant production of hemoglobin are the synthesis of soluble α and β globins, the presence of adequate amounts of heme, and a means for the recovery and purification of the assembled hemoglobin.

It is obvious that in addition to native hemoglobins, any number of mutants, or "variants" of hemoglobin (or "designer" hemoglobins) can be produced in recombinant systems (9). Indeed, the initial motivation for recombinant production of hemoglobin was to aid in the understanding of the structure-function relationship by analyzing the effect of various changes in the primary structure of globins (10). These studies can also lead to the design of an improved hemoglobin molecule suited for use as a blood substitute or for other applications. Other uses of recombinant hemoglobins include their application towards gene therapy of hemoglobinopathies, as a vehicle for the production of nonglobin proteins and as carriers for the delivery of drugs.

Production Systems

The salient features of expression systems successfully employed for the production of hemoglobin are summarized in Table I. Hemoglobin produced in all of these systems is functional in binding oxygen. Indeed, published reports indicate that at least a major fraction of the expressed protein is functionally indistinguishable from human hemoglobin. Unique features of each system that impact the structure, function, or abundance of hemoglobin are detailed below.

Microbial Production. The first successful production of recombinant hemoglobin was achieved in *Escherichia coli* by the expression of a globin cDNA fused to the coding region of a bacteriophage repressor protein (11). The fusion was designed so as to allow the recovery of the globin by enzymatic digestion of the fusion protein *in vitro*. The purified (insoluble) globin, which is identical in primary structure to the globin derived from human blood, is solubilized and then reconstituted with heme to produce a functional protein. Variations of the original procedure, employing other fusion partners and different gene-regulatory sequences, have been described (12, 13). In all of these systems very high level expression of the fusion protein is achieved, resulting in the protein coming out of solution and forming "inclusion bodies" inside the mi-

Table I. Expression Systems for the Production of Recombinant Hemoglobin[a]

Organism	Features	Disadvantages
E. coli (fusion protein)	First method, simple Insoluble globins 10%–20% expression	Reconstitution expensive
E. coli (co-expression)	Soluble hemoglobin Engineering rapid 2%–10% expression	Modified N terminus Expensive purification
E. coli (MAP[b] co-expression)	Soluble hemoglobin Authentic protein 2%–10% expression	Heterogeneity Expensive purification
Yeast	Soluble hemoglobin 1%–3% expression	Low-level expression Misfolding[c]
Insect cells	Insoluble globin 5%–10% expression	Expensive cell-culture No heme incorporation[d]
Mammals[e] (transgenic)	Made in erythrocytes Authentic hemoglobin 5%–50% expression	Hybrid hemoglobins Animal husbandry issues
Cell-culture	Transfected stem cells Gene therapy uses	Expensive Scale up problems

[a] See test for citations.
[b] Methionine aminopeptidase.
[c] Misfolding reported in one study (Ref. 23).
[d] Only β globin expressed at high level.
[e] Mice and pigs.

crobial cell. The insoluble protein can be readily purified for further processing. The major disadvantages of this system are the time and expense involved in refolding the globin chains and incorporating heme. Other inevitable consequences of overexpression of proteins in *E. coli* include unintended deamidation, acetylation, and the incorporation of amino acid analogs such as norleucine (14). For these reasons, the fusion protein expression technology is not suitable for large scale production of hemoglobin.

Attempts to produce globins individually (not as a fusion protein) encountered two problems. First, because of the different N-terminal processing of proteins in *E. coli,* the expressed globins retained an extra N-terminal methionine and were consequently both structurally and functionally altered (15, 16). The α and β globins were not produced at equal levels, presumably due to differences in their rates of transcription or translation. Both problems were solved ingeniously by a tour de force in genetic engineering (17). By taking advantage of the extensive literature on the structure of hemoglobins, variant α and β globins were designed and co-expressed from a single plasmid. Two changes were introduced in the globin chains: substitution of the first amino acid found in the mature globins (valine) with methionine and engineering of a β108 Asn→Lys mutation. The latter mutation, present in the naturally existing $Hb^{Presbyterian}$ variant, caused the lowering of the oxygen affinity of hemoglobin (18). In this bacterial expression system, the globin chains were efficiently synthesized and incorporated bacterial heme. The resultant hemoglobin was soluble, and when purified was found to be functionally similar to $Hb^{Presbyterian}$. Another feat of protein engineering resulted in the formation of a "linked" tetrameric hemoglobin (19). This was accomplished by co-expressing genes encoding a mutant β globin and a modified α globin (where two α globins are linked in tandem to form a di-α globin chain). The modified hemoglobin was in fact a trimer, but was functionally similar to the natural tetramer as far as oxygen binding was concerned. However, covalent linkage between the two α globins stabilized this molecule and prevented dissociation into αβ dimers, which is observed upon dilution of native hemoglobin. This recombinant hemoglobin has a prolonged circulatory half-life in animals and is currently being tested in the clinic (19).

Another solution to the N-terminal processing problem has been devised (20). The expression of methionyl aminopeptidase in *E. coli* permits the removal of the amino-terminal methionine from recombinant proteins produced in these modified cells. Hemoglobin produced in such bacteria have the correct primary sequence. Unfortunately the processing is often incomplete and pools of fully processed and unprocessed recombinant hemoglobins are generated. The close similarity of the two hemoglobin isoforms makes their separation difficult and presumably expensive.

Soluble and functional hemoglobin has also been produced in yeast by the co-expression of α and β globin genes (21, 22). In this system the amino-terminal processing is similar to that seen in the erythrocytes, and therefore the recombinant protein is identical to human hemoglobin at the N-termini. In one study, two different sub-types of the recombinant protein were isolated, each with similar primary structure but differing in the secondary and tertiary structure (23). As a consequence of misfolding, the modified hemoglobin had a higher affinity for oxygen and was unsuitable for the intended application. Another fraction of the assembled protein has been found to contain a different form of heme, sulf-heme, and is also altered in function (24). In general, the level of expression of hemoglobin in yeast appears to be lower than in *E. coli* (25). This fact, combined with the heterogeneity described above, suggests that yeast-derived hemoglobin is likely to be more expensive than the bacterial product.

Microbial expression systems have several advantages. All aspects of microbial engineering, growth (fermentation), downstream processing, and monitoring have been thoroughly studied and understood (26). Although the many metric tons of hemoglobin required for a blood substitute application will require a manufacturing process of unprecedented scale and expense, it is not unreasonable to expect that it can be achieved. However, because a therapeutic dose of hemoglobin is likely to be in hundreds of grams (to be administered intravenously) extremely high-grade purification must be achieved to ensure that the hemoglobin is free of endotoxins and other toxic microbial and process contaminants.

Insect Cells. Insect cells infected with Baculovirus produce large amounts of viral proteins. The viral gene expression machinery can be commandeered to produce recombinant proteins (27). Although correctly folded multimeric proteins such as immunoglobulins have been produced in this system (28), successful synthesis of a functional hemoglobin has not yet been described. The α and β globin genes have been expressed individually where, as expected, denatured globin chains accumulated in the insect cells without the incorporation of heme, even though heme is present in these cells (29). In this system the α globin gene was poorly expressed and this deficiency was traced to the 5′ untranslated region (UTR) of the mRNA. Replacement of the α globin gene 5′ UTR with the corresponding sequences from the β globin gene resulted in an increase in α globin expression. With appropriate modification of gene design and cell growth conditions, it should be possible to produce

functional hemoglobin in this system. When whole larvae, instead of insect cells in culture, are used, reasonably high-level expression can be anticipated (30). However, it is questionable if unmodified hemoglobin can be consistently and economically obtained using this method of expression.

Mammalian Systems. In theory, it is possible to introduce globin genes into hematopoietic stem cells and to obtain hemoglobin expression in the cells of the erythropoietic lineage derived from differentiation and propagation of the gene-altered stem cells (31). Currently, individual steps in this scheme are feasible, but it would require several leaps in technology development as well as exorbitant expense to carry out the whole process leading to the recovery of hemoglobin. This method, however, provides a tantalizing option for the future, particularly for gene therapy applications (see below).

Cost-effective mammalian-cell expression of recombinant hemoglobin is possible today by means of transgenic technology (32). A transgenic organism carries additional genetic information in its genome (33). These added genes, the transgenes, are typically introduced into the genome when the organism is unicellular (i.e., in the single-celled zygote). As the cells divide and the embryo develops into a fetus, all cells inherit the transgene. Therefore, in the ideal newborn transgenic animal, the transgene is present in all tissues. The expression of the transgene can be regulated by the choice of genetic control elements such as promoters, enhancers, and locus control regions (LCRs). Major advances in the understanding of gene regulation, especially the role of super-control elements, have contributed to the design of expression vectors which direct predictable levels and patterns of expression in transgenic animals. The ability of regulating gene expression in transgenic animals has opened the doors to the application of this technology to the production of human hemoglobin in transgenic animals (34). Human hemoglobin can be produced in transgenic mice by co-expressing human α and β globin genes linked to the human β globin LCR (35). These studies, in turn, have encouraged the use of this system for the commercial production of hemoglobin in transgenic pigs as a starting material for a cell-free "blood substitute" (36). At present, pigs producing human hemoglobin at levels up to 32 g/l have been developed (Fig. 1). Hemoglobin expression in these pigs is stable and is faithfully transmitted to the next generation (37).

Because of the similarity in the transgenic and the natural erythrocyte production systems, the recombinant hemoglobin is virtually indistinguishable from the human-derived protein (38). In particular, no unwanted modifications or structural heterogeneity have been seen in the transgenic hemoglobin derived from mice or pigs. Erythrocytes exist in a sterile environment of the circulatory system and can be easily isolated for the recovery of hemoglobin. The choice of pigs as transgenic "hosts" has several advantages. Large quantities of blood can be harvested from pigs under aseptic conditions and a herd of more than 100,000 transgenic pigs, starting from a single transgenic boar (a male pig), can be developed in less than 5 years. Since hemoglobin comprises >95% of the soluble proteins in these cells, purification of the recombinant protein is fairly simple. A unique feature of the transgenic hemoglobin production is the presence of inter-species hybrid hemoglobins in the erythrocyte (Fig. 1). Human hemoglobin produced in the pigs must be separated from pig and hybrid hemoglobins as well as from other red blood cell constituents. This has been achieved by simple ion-exchange chromatography (Fig. 2). Consistency of the biochemical properties of transgenic swine-derived human hemoglobin over several generations of pigs has been established (Table II).

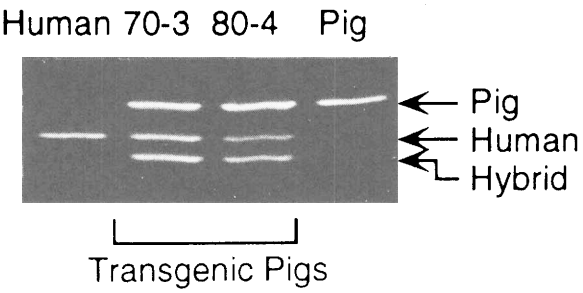

Figure 1. Isoelectric focussing (IEF) pattern showing separation of human, pig, and an inter-species hybrid (human α/pig β) hemoglobins found in the hemolysates of transgenic swine (70-3 and 80-4) expressing human globin genes. Human and pig hemolysates are shown as controls.

Transgenic production of pharmaceutical proteins is still in its infancy and many production issues remain to be resolved (39). For hemoglobin production, new technologies of breeding, blood collection, and processing need to be developed. The inter-species hemoglobin hybrids share many physical and chemical properties with human and animal hemoglobin, a feature which makes it harder to separate them from the recombinant human hemoglobin. The production of hybrid hemoglobin may be advantageous for delineation of the architectural elements of intersubunit surfaces of hemoglobin. These studies may prove useful for the design of more stable hemoglobins for various applications. The production of human and hybrid hemoglobins is not without physiological consequence for the host animal, since the total amount of hemoglobin in the RBCs of transgenic animals remains almost unchanged as compared to the nontransgenic animals (40). Therefore, it is beneficial for the efficient production of recombinant hemoglobins, if the exogenous hemoglobins are functional in the transgenic RBCs in a manner similar to the endogenous animal

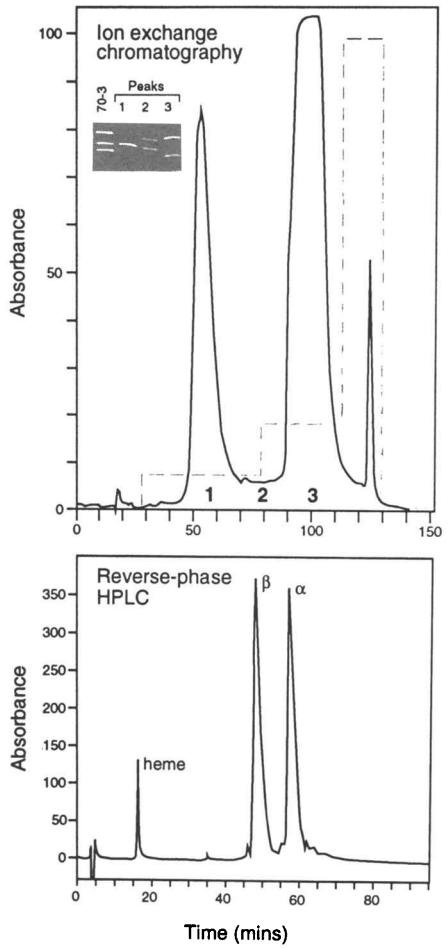

Figure 2. Purification of human hemoglobin from transgenic pig hemolysate. (Top) An anion exchange chromatogram showing separation of human, hybrid, and pig hemoglobins eluted using a step gradient. The inset shows IEF patterns of transgenic hemolysate (lane marked 70-3), and hemoglobins contained in three peaks shown in the chromatogram. Peak 1 contains pure human hemoglobin. (Bottom) Reversed phase (RP)-HPLC separation of heme and transgenic α and β globin chains contained in human hemoglobin of Peak 1.

hemoglobin. Fortunately, this is indeed the case for the pig, since pig and human hemoglobins are structurally as well as functionally similar.[2] It has been suggested that the total substitution of pig hemoglobin with human hemoglobin in transgenic swine may one day be possible (41). A significant encumbrance in the transgenic pig production system, as compared with microbial expression systems, is the length of time required to obtain the recombinant protein. Even with the short gestation span and high fecundity of pigs, it may take more than 1 year to develop a transgenic producer line. In contrast, large-scale microbial fermentation can be more rapidly scaled up and production strains can be changed more quickly. Transgenic mice provide a more expedient alternative mammalian expression system for producing and testing hemoglobin variants. A line of transgenic mice can be established in approximately 3 months.

[2] Katz DS, White SP, Huang W, Kumar R, Christanson DW. Structure determination of porcine hemoglobin at 2.8 Å resolution. *Submitted to J Mol Biol.*

Table II. Stability of Human Globins Produced by Transgenic Swine

Sample[a]	Generation	Molecular mass (Dalton)	
		α globin	β globin
Swine			
38-4	G0	15126.32	15867.19
2-149	G1	15126.45	15866.92
2-149-x	G2	15126.36	15867.01
Human	—	15126.43	15867.27

[a] Globins separated by RP-HPLC; molecular mass determined by electrospray mass spectrometry.

Applications of Recombinant Hemoglobins

"Blood Substitute." The development of hemoglobin solutions as a substitute for RBCs is a laudable and long sought after goal in hematology (42). The long and disappointing history of development of hemoglobin based oxygen carriers (HBOCs), characterized with many missteps and a lack of sustained research and development efforts, has been discussed in detail elsewhere (43). Briefly, the successful use of HBOCs has been hampered by numerous toxicities associated with hemoglobin or impurities contained in these solutions. At present, the well-recognized side effects of hemoglobin solutions include nephrotoxicity due to renal filtration of hemoglobin dimers, vascular hypertension presumably mediated by a nitric oxide-dependent mechanism, and syndromes associated with endotoxin and other inflammation-inducing impurities. Several approaches to the stabilization of hemoglobin tetramer or polymers have been described (Fig. 3). Recombinant technology has already permitted the design of a

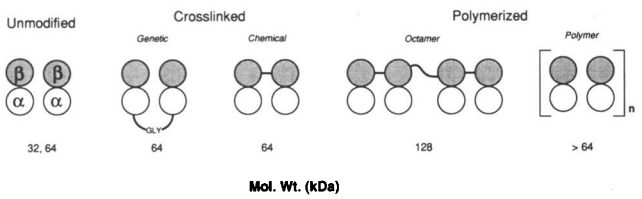

Figure 3. Structure of stabilized hemoglobins. The unmodified acellular hemoglobin exists as an unstable tetramer that can be easily dissociated into dimers. Covalent linking of the α globin chains by means of a Gly linker introduced by genetic modification, and chemical cross-linking across the two dimers have been used to produce stabilized nondissociable tetramers. The unmodified hemoglobin or the cross-linked tetramer can be chemically polymerized to derive functional octamer or higher molecular weight polymers of hemoglobin.

nondissociable tetrameric hemoglobin that has a prolonged circulatory half-life and no discernible renal injury in animals (44). In early human safety studies, when administered in small quantities, this hemoglobin did not cause renal toxicity, supporting the hypothesis that filtration of hemoglobin dimers through the kidney was responsible for the nephrotoxicity of unmodified hemoglobin (45). However, these trials were not without all adverse reactions. Several side effects, including myalgia, fever, mild hypertension, and gastrointestinal problems were reported in patients receiving different doses of the modified hemoglobin. Some of these conditions could be attributed to endotoxin or other impurities and can be eliminated by more rigorous purification of the hemoglobin (46). Other effects, such as transient hypertension, have also been reported in clinical trials using hemoglobin tetramers generated by chemical cross-linking of nonrecombinant hemoglobin and are believed to be related to the nitric oxide-scavenging effect of hemoglobin (47). It is not clear whether or not the transient hypertension observed in these trials is of any clinical significance, as different levels of increase in blood pressure have been reported in various studies (IBC Conference on Blood Substitutes, Philadelphia, September 1993). In contrast, Phase I clinical experience with a polymerized (nonrecombinant) hemoglobin preparation suggests that these solutions may not have any hypertensive effect (46). If transient hypertension proves to be an unacceptable toxic effect, these clinical results may raise questions on the suitability of a stabilized tetramer for use as a blood substitute. Consequently, efforts are underway to develop recombinant means of producing polymerized hemoglobin. In the first approach, directed mutagenesis has been used to engineer hemoglobin variants that can be polymerized *in vitro* by chemical cross-linking or by controlled oxidation of sulfhydryl groups to form disulfide bridges (48, 49). Total synthesis of polymeric hemoglobin can be achieved *in vivo,* but at a considerably lower efficiency. Therefore, it is likely that for clinical testing and commercialization, a recombinantly synthesized hemoglobin (or its suitably designed variant) will be modified *in vitro* to produce an effective polymerized HBOC.

Many other properties of hemoglobin are also amenable to recombinant modification. Studies are underway to identify the amino acid replacements required to convert human hemoglobin to a 2,3 Diphospho Glycerate (DPG)-independent, chloride-regulated oxygen carrier, similar to bovine and feline hemoglobins (50, 51). If successful, these experiments may provide yet another substrate for producing a hemoglobin solution for resuscitation. Apart from tetramer disassociation, the major cause for the short shelf-life and half-life of hemoglobin is the oxidation and loss of heme from hemoglobin (52). The Fe^{3+} (ferric) form of hemoglobin does not bind oxygen. In the erythrocytes, an enzymatic reduction process is used to recycle the ferric form of hemoglobin to the active ferrous (Fe^{2+}) form (53). In the free circulation, this recycling system is inoperative and the oxidized hemoglobin is effectively lost. The rate of oxidation of hemoglobin is influenced by the oxygen affinity of hemoglobin (54). A site-directed mutagenesis and bacterial expression approach is being tested to identify variants of myoglobin and hemoglobin that have retarded rates of oxidation in solution (55). Other attempts to modify the rate of oxidation of hemoglobin include mutations designed to alter the confirmation of the heme pocket (56). Since hemoglobin binds both oxygen and nitric oxide through the heme groups, it is not clear if these mutants or any other variants will have the ability to discriminate between these two ligands. Therefore, the rationale for the mutagenic or direct chemical modifications of hemoglobin designed to reduce or eliminate the binding of ligands other than oxygen and carbon dioxide is yet to be defined. However, it is interesting to note that cross-linked tetramers obtained by different chemical procedures have variable rate of generating free radicals (57). An indirect approach to solving this problem is based on some evidence that hemoglobin binds nitric oxide mainly in the extravascular space (58). Hence, modifications of hemoglobin, such as polymerization, which retard its extravasation may have the additional benefit of reducing some of the side effects of HBOCs observed in the clinical trials mentioned above. Another approach to preventing extravasation and increasing the circulatory half-life of hemoglobin is the modification of the surface charge (59). Negatively charged molecules are retained longer in the circulation. Such modified forms can be generated by mutagenesis or by chemical reaction. Other structural and functional properties of hemoglobin amenable to mutagenic analysis include the allosteric mechanisms (60, 61), dimer stability (62), and hybrid formation.[3]

Gene Therapy. Structural, regulatory, or metabolic disorders of globin genes and heme biosynthesis are the basis of many common human pathological conditions such as anemias and thalassemias (63). Some geographical areas and certain ethnic populations exhibit very high rates of genetic defects in globin genes. Recently, advances in molecular genetics and gene transfer have spurred interest in the development of vectors and modalities for gene therapy of these diseases (64). Exogenous expression of globin genes deficient in thalassemic individuals is a rational

[3] Roy RP, Nagel RL, Acharya AS. Inter-species hybrids of mouse and human hemoglobins: Molecular aspects of the low oxygen affinity of mouse hemoglobin.

approach for somatic cell therapy. In sickle cell anemia, the expression of γ or δ globins will have a sparing effect on the polymerization of the mutant β^s hemoglobin. Previous work, using chemical reactivation of fetal hemoglobin genes, have proven the efficacy of this approach (65, 66). All currently known chemical approaches are generally nonspecific and may have unintended global effects on gene regulation. Genetic approaches are likely to be more selective and hence will have fewer dangerous side effects. The depth of understanding of the regulation of globin genes and the available arsenal of vehicles (67) for the delivery of desired genes portends well for the success of the proposed trials.

Other Uses. Many creative and useful applications of recombinant hemoglobins are being developed. Some have proposed the use of tetrameric hemoglobin solutions as hypertensive agents for therapy of shock (which is believed to be mediated by nitric oxide) in trauma or burn victims (68). If successful, this application will benefit from what is currently perceived as a major deficiency of HBOCs! Another footnote to the use of hemoglobin for intravenous therapy is the design of variants that can be linked to other pharmaceutical agents, such as drugs and hormones (69). These drugs can be tethered to the surface of hemoglobin and distributed almost everywhere in the body. Such conjugates can extend the physiological half-life of labile drugs. Oxygen carrying hemoglobin solutions can be used for storage and perfusion of organs and cellular implants (70). Additionally, cultured or transplanted cells, such as those of pancreatic islets, may survive and function for longer periods *in vivo* if they are endowed with a source of oxygen by the expression of exogenous hemoglobin or myoglobin (71).

The abundance of hemoglobin in the erythrocytes and the capability of high-level expression of exogenous globins in transgenic animals have led to the development of hemoglobin fusion proteins designed for the production of small peptides (72). The fusion hemoglobin contains the desired peptide as an extension at the carboxyl end of human α or β globins (Fig. 4). A protein cleavage site is engineered interspaced between the carboxyl end of globin chains and the amino terminal residue of the desired peptide. The peptide is recovered after cleavage of the fusion protein with enzymes or chemical reagents that recognize this cleavage signal as their substrate. Due to the selective compartmentalization of hemoglobin (95% of soluble proteins) into the erythrocytes, purification of the fusion hemoglobin is easy and efficient. Peptides of up to 26 residues have been produced in mice. In this system, the sterile nature of blood allows for the recovery of endotoxin-free peptides for therapeutic applications at a low cost.

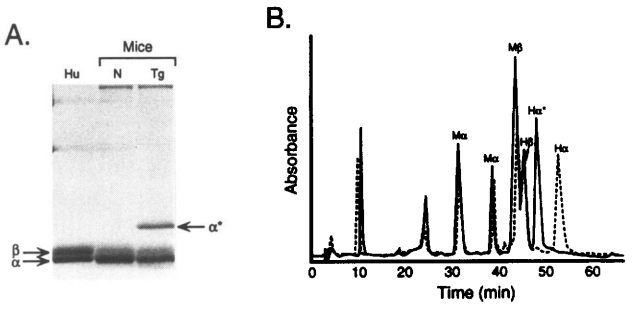

Figure 4. Production of peptides as fusion with α globin in transgenic erythrocytes. (A) An SDS-PAGE showing separation of α and β globins and fusion α globin (α*) present in the human (hu), mouse (N), and transgenic mouse (T) hemolysates. (B) Comparison of RP-HPLC patterns of hemolysates of transgenic mice producing either human hemoglobin (dotted line) or hemoglobin containing a fusion peptide (solid line). Absorbance was monitored at 210 nm. The identity of peaks corresponding to each globin and the fusion globin (Hα*) are marked.

Epilogue

In the first decade (1984–1993) of their existence, recombinant hemoglobins have been extensively studied for many different applications. Undoubtedly the most important use of recombinant hemoglobin will be in the development of HBOCs. In the next several years, the design of large-scale production systems and the optimization of the physicochemical properties of hemoglobin should be completed. Manufacturing of hemoglobin poses many challenges for biotechnology. It has been pointed out that production of commercially viable hemoglobin will require a process that is orders of magnitude larger in scale, and yields a product which is 10 times greater in purity at a significantly lower cost, compared with all existing recombinant protein production technologies (73). Currently, recombinant human insulin is produced in large quantities. Because of the small dose (milligrams) and high price (estimated to be thousands of dollars per gram), insulin, a successful product, can be manufactured using conventional technology. A therapeutic dose of HBOCs is likely to be at least 100 g, necessitating both an economical process of production and a high degree of purity. If a successful HBOC product were to supplant only 10% of the demand (in the United States, approximately 1.5 million units) for RBC transfusion, at least 35,000 kg of recombinant hemoglobin will be required annually (based on 35 g/unit of HBOC). Moreover, the environmental impact of effluents generated by the recombinant production of such a magnitude will demand creative solutions to the use of water and other reagents in the process. It is noteworthy that the anticipated demand for hemoglobin can be met with by a herd of fewer than 100,000 transgenic pigs. Since greater than 60 million pigs are raised annually in the United States for agricultural purposes, clearly the "transgenic farm" poses no in-

surmountable problems in animal breeding or waste disposal. Whether or not any of the recombinant technologies lead to the widespread clinical use of HBOCs depends largely on the results of ongoing and anticipated clinical trials. Ancillary factors such as the regulatory environment, health care economics, and the status of allogeneic blood transfusion will make a significant impact upon the success, failure, or further protracted delays in the development of blood substitutes, as well as other clinically useful applications of hemoglobins.

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