Biomedical Science

Future Prospects for Treatment of Hemoglobinopathies

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LINDA HAWES CLEVER, MD
Editor

Strategies for the treatment of sickle cell anemia and β-thalassemia are founded on the knowledge that these disorders result from structural or functional defects in an adult gene for which an intact fetal counterpart exists. During the past decade, several pharmacologic agents have been investigated for their potential to ameliorate sickle cell anemia and β-thalassemia by increasing the synthesis of fetal hemoglobin in adults. Progress in understanding globin gene regulation is now being combined with advances in retrovirus-mediated gene transfer, and the once-distant goal of providing gene therapy for hemoglobinopathies is rapidly approaching reality.

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The quaternary structure of adult hemoglobin (Hb A) comprises four polypeptide subunits, two α-globin chains and two β-globin chains ($\alpha_2\beta_2$).[1] The α chains are encoded by a gene locus on chromosome 16, and the β chains arise from a similar locus on the short arm of chromosome 11 (Figure 1).[1] The β-globin gene locus contains a number of different genes encoding β-like gene products that are arrayed on the chromosome in a 5′ to 3′ manner that indicates the order of their expression during development. These include an embryonic globin gene (ε), duplicated fetal genes (γ), and adult genes (δ and β). During fetal life, γ-globin is the predominant β-like globin product, giving rise to a fetal hemoglobin (Hb F) containing two α and two γ chains ($\alpha_2\gamma_2$). Around the time of birth, γ-globin production declines precipitously and is replaced by the definitive β-globin gene product, with a corresponding production of adult hemoglobin (Hb A; Figure 2). As such, any structural or functional defects that affect the β-globin gene become clinically evident only on completion of the perinatal γ- to β-globin switch.[1]

During the past decade, a wealth of information concerning the regulation of hemoglobin switching has come to light, much of which is beyond the scope of this review. For our purposes it will be sufficient to note the following:

- Globin genes are regulated at the transcriptional level through the interaction of tissue-specific and ubiquitous transcriptional factors.[2]
- The mechanism of fetal-to-adult gene switching within the β-globin locus appears to involve competition between the γ and β genes for enhancer sequences clustered loosely in a region 6 to 22 kilobases "upstream" (5′) of the embryonic ε gene, designated the locus control region (LCR; see Figure 1)[3,4]; and
- The chronology of hemoglobin switching during gestation appears to be under the control of a developmental "clock" local to chromosome 11 because individual chromosomes isolated from fetal-stage erythroblasts (through hybridization with mouse erythroleukemia cells) undergo temporally correct switching in culture.[5]

Whereas the embryonic ε-globin gene is rendered transcriptionally inert early in gestation, γ-globin production persists at a low level following the fetal-to-adult switch.[1] In consequence, Hb F is found to comprise about 1% of the hemoglobin in normal adults. Its distribution, however, is restricted to a small subset (0.2% to 7%) of erythrocytes, designated F cells, where it accounts for roughly 15% to 20% of their intracellular hemoglobin. How exactly Hb F in adults comes to be sequestered heterocellularly is not known, but the available evidence can be explained under a model encompassing the kinetics of erythropoiesis.[6] The earliest identifiable erythroid progenitor is the burst-forming unit–erythroid (BFU-E). During normal erythroid maturation, BFU-Es give rise to erythropoietin-sensitive colony-forming progeny, the colony-forming unit–erythroid (CFU-E). After an indeterminate period of time, CFU-Es commit irrevers-

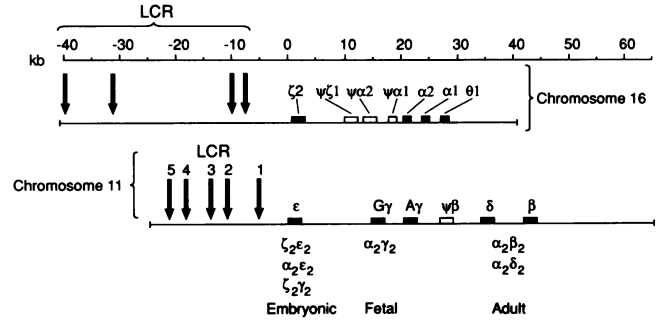

Figure 1.—The human globin genes are shown. Both β- **(top)** and α- **(bottom)** gene clusters contain developmentally controlled genes (see Figure 2). Several kilobases (kb) "upstream" (5′) of each gene cluster are sequences (locus control regions [LCR]) crucial for tissue-specific high-level expression. Important sequences are localized by the presence of DNA hypersensitive to digestion by nucleases, indicating an active chromatin conformation **(large arrows).** Similar hypersensitive sites are found in the promoter regions of active genes.

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ABBREVIATIONS USED IN TEXT

BFU-E = burst-forming unit–erythroid
CFU-E = colony-forming unit–erythroid
Hb = hemoglobin
LCR = locus control region

ibly to the terminal maturation pathway, and globin synthesis accelerates dramatically. An intriguing feature of erythropoiesis in the adult is the retention by many early erythroid-lineage cells (BFU-Es and CFU-Es) of what appears to be an immature globin "program."[6] During normal terminal maturation with its increasing globin synthesis, a distinct intracellular transition from fetal to adult globin production ensues. F cells in the adult presumably derive from progenitor cells

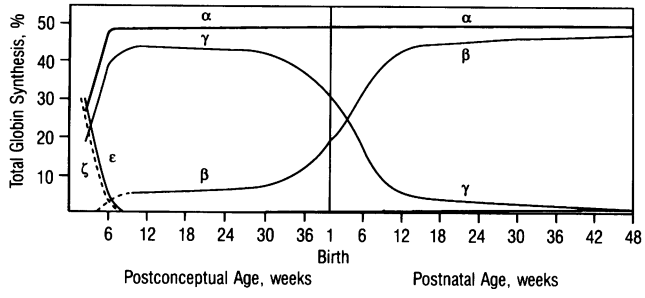

Figure 2.—Globin gene expression is shown during development. Embryonic ζ and ε genes are extinguished early in gestation. α-Globin expression remains roughly constant following the disappearance of ζ, whereas γ- and β-globin genes are expressed in a more reciprocal pattern.

that enter terminal maturation prematurely and fail to inactivate completely their primitive globin programs. F-cell numbers increase in response to erythropoietic stress such as that induced by acute anemia or following bone marrow transplantation or the application of cytotoxic drugs.

Inherited Disorders Affecting Hemoglobin

Sickle cell anemia and β-thalassemia are inherited disorders of hemoglobin structure and synthesis, respectively, that have their origins in mutations affecting the β-globin gene locus. Each year, about 160,000 persons worldwide are born destined to have clinical disease associated with these disorders. Many more persons inherit mutations affecting the α-globin gene locus (α-thalassemia),[7] but their condition is usually clinically mild and will not be discussed in this review.

In sickle cell anemia, a point mutation in the DNA sequence of the β-globin gene yields a variant polypeptide containing valine at position 6 in place of the wildtype glutamic acid.[1] The resulting globin protein (designated β^s) complexes with another of its kind and two α globins to form a hemoglobin variant (Hb S) with anomalous physical properties. In its deoxy form, Hb S undergoes spontaneous polymerization; the mechanics of polymer elongation involve a generation of oblique forces on the cell membrane with consequent severe distortions in red cell morphology (yielding ultimately the characteristic "sickle" shape), membrane damage, and a substantial loss of plasticity. On deoxygenation during traversal of the microvasculature, Hb S-containing cells undergo progressive stiffening, which may precipitate the occlusive crises that underlie the clinical manifestations of this disease. In addition to episodic pain crises, patients with sickle cell disease suffer from an acute extravascular hemolysis of Hb S-containing cells by the mononuclear phagocytic system. No effective conventional therapies for sickle cell anemia exist, and treatment of this disorder is confined to the management of acute pain crises and the consequences of end-organ damage.

β-Thalassemia is characterized by deficient or absent production of β-globin chains.[8] This condition may arise from various mutations including lesions that alter grossly the structure of the β-globin gene or remove it altogether; mutations in the β-globin promoter that substantially inhibit transcriptional activity; and point mutations in the β gene that have an adverse effect on subsequent messenger RNA processing (through the activation of cryptic splice or donor junctions) or on translation (frameshift mutations).[9] An impairment of β-gene function results in a considerable decline in total hemoglobin production, and accumulated excess α-globin chains form intracellular precipitate aggregates that promote cell lysis and destruction by host-defense mechanisms.[8] The vast majority of these cells are vanquished before they exit from the marrow compartment, resulting in a markedly ineffective erythropoiesis and a severe anemia; those that make their way into the peripheral blood display profound microcytosis and other red cell deformities. In compensation for this condition, erythropoiesis expands dramatically, resulting in a hyperplastic marrow with adverse consequences for developing bones.

Patients with homozygous β-thalassemia require periodic transfusions to maintain vitality, but the onset of iron overload ensures mortality in most by the third decade of life. Current approaches to transfusion therapy involve the coadministration of iron chelators such as deferoxamine, which results in prolonged life expectancy.

Persistence of Fetal Hemoglobin in the Adult Ameliorates Sickle Cell Anemia and β-Thalassemia

Several lines of evidence point to the conclusion that increasing cellular levels of Hb F should prove clinically beneficial to sufferers of sickle cell anemia and β-thalassemia. A number of mutations have been characterized that underlie the hereditary persistence of fetal hemoglobin in adulthood. Persons showing high levels of fetal hemoglobin (10% to 40% in heterozygotes; 100% in homozygotes) are clinically asymptomatic and show normal exercise tolerance.[1]

Studies of compound heterozygotes for sickle cell disease and the hereditary persistence of fetal hemoglobin have revealed that levels of Hb F exceeding 20% are associated with a mild or benign clinical course. Other reports indicate that protection from severe end-organ damage may occur with Hb F values in the range of 10%, and the results of a recent study involving several thousand sickle cell patients observed over a period of years indicate that even small increases in fetal hemoglobin levels can have important long-term effects on disease morbidity.[10]

The role of fetal hemoglobin in sickle erythrocytes has been studied extensively.[11] Because the rate of Hb S polymerization depends strongly on its intracellular concentration, increasing the proportion of nonsickle hemoglobins within a particular cell through the increased production of fetal hemoglobin ($\alpha_2\gamma_2$) and a mixed tetramer containing one β^s chain and one γ chain ($\alpha_2\gamma\beta^s$) will shift reaction kinetics away from polymer formation. A more important finding is that Hb F has a profound direct inhibitory effect on polymer formation, and mixed tetramers containing a γ chain do not

participate in polymer formation. With respect to polymerization potential, the effect thus brought about directly by Hb F can be expressed in terms of a decline in Hb S concentration, such that a 10% increase in intracellular fetal hemoglobin has a "sparing" effect equivalent to a 10% decline in hemoglobin concentration. Because the kinetics of polymerization vary with the 30th power of deoxy-Hb S concentration, even modest changes in this index can have a major influence on polymer formation.

In the case of β-thalassemia, any increase in the production of non-α-globin genes will result in a more effective erythropoiesis.[8] Substantial amounts, however, of fetal hemoglobin tetramers would be required before clinical effects could be expected to be alleviated. Thalassemia in patients who have inherited a mutation for the hereditary persistence of fetal hemoglobin generally remains clinically benign.

Therapeutic Strategies for Hemoglobinopathies

Although sickle cell anemia and β-thalassemia have a common origin in mutations affecting the β-globin gene, their pathophysiologies dictate a fundamentally different approach to the treatment of each disorder. In β-thalassemia, total β-like globin synthesis is deficient, and hence notable production of any non-α-globins will prove beneficial. This can be achieved either through the activation of high levels of fetal hemoglobin synthesis in an adult or through the production of adult hemoglobin (Hb A) potentiated by the presence of a high-expressing retrovirally introduced β-globin gene.

In sickle cell disease, globin production is adequate; the function of an optimal therapeutic agent would therefore be directed toward the inhibition of Hb S polymerization. The retroviral introduction of a normal β-globin gene would counter the detrimental effects of Hb S only indirectly through a reduction of the intracellular fraction of Hb S. Unlike Hb F, however, Hb A copolymerizes to a limited extent with Hb S, and mixed tetramers ($\alpha_2\beta^s\beta$) enter readily into elongating polymers. The greater sparing effect conferred by Hb F thus dictates that a retrovirally transduced γ-globin gene would prove to be a far more optimal therapy for sickle cell disease and would have the advantage of requiring relatively low levels of cellular expression to achieve a beneficial functional outcome.

Pharmacologic Stimulation of Hemoglobin F Synthesis

To date, several pharmacologic agents have shown a capacity for meaningfully increasing Hb F synthesis in both humans and nonhuman primates.[12] These include cell-cycle-specific cytotoxic compounds, nucleoside analogues, hematopoietic growth factors, and butyric acid derivatives. Four drugs have been subjected to clinical trials of varying scope: the cytosine analogue 5-azacytidine; hydroxyurea, a well-characterized cytostatic agent that blocks DNA replication through the inhibition of the ribonucleoside diphosphate reductase enzyme complex; erythropoietin, the principal growth factor of the erythroid lineage; and sodium butyrate. Furthermore, synergistic activity has been observed when certain combinations of these agents are used.

The application of the aforementioned agents with the aim of stimulating fetal hemoglobin synthesis in patients suffering from hemoglobinopathies has recently been reviewed in detail.[12] Patients with sickle cell disease to whom single courses of 5-azacytidine were administered as intravenous infusions have increased their genetically determined baseline levels of Hb F fourfold to sevenfold.[13-16] Observed rises in Hb F levels have been paralleled by decreases in the synthesis of Hb S, and absolute fetal hemoglobin concentrations in the range of 6% to 18% have been recorded. Similar gains have been achieved using single-course treatments in patients with β-thalassemia, with consequent transient improvements in the quality of erythropoiesis.[17-20] As in the case of patients with sickle cell anemia, increases in Hb F were accompanied by decreases in the synthesis of adult hemoglobin, thus compromising the final hemoglobin concentration and hence overall therapeutic efficacy.

The ability of hydroxyurea to produce increases in Hb F in sickle cell patients has been documented by studies in nearly 80 patients. The daily administration of hydroxyurea in doses that do not give rise to meaningful myelosuppression (10 to 30 mg per kg per day) has produced increased Hb F levels in nearly every case, with most patients achieving peak absolute Hb F levels of 5% to 15%, and some patients exceeding 20%.[21-23] In addition to its effect on Hb F, hydroxyurea therapy produces changes in the mean cell volume and the mean corpuscular hemoglobin concentration that result in substantial reductions in circulating dense and irreversibly sickled cells; the effect of these changes on the rheologic properties of erythrocytes should complement the effect of Hb F on disease morbidity. There is preliminary evidence that patients with sickle cell anemia on long-term hydroxyurea therapy have a decreased incidence of painful crises.[23]

A handful of sickle cell patients treated with recombinant human erythropoietin alone (1,000 to 2,000 U) have responded with modest increases in fetal hemoglobin synthesis that appear to be dependent on the coadministration of iron supplements.[24,25] These gains, however, combined with the potentially detrimental effects of an erythropoietin-induced erythrocytosis in sickle cell disease, all but ensure that this drug will not be used by itself as a therapeutic agent for hemoglobinopathies. Recent studies in patients with sickle cell disease, however, have shown that administering erythropoietin in combination with hydroxyurea therapy using an alternating dose schedule (4 days of hydroxyurea, 3 days of erythropoietin) can increase considerably the Hb F response elicited by hydroxyurea alone, producing twofold or greater augmentations in some cases.[26] If the magnitude of these increases proves to be a consistent phenomenon, the additional expense of treatment with recombinant erythropoietin will prove more than justified, particularly if its synergy with hydroxyurea can be translated into further reductions in crisis frequency.

Administering sodium butyrate to nonhuman primates has produced strong Hb F responses.[27,28] Moreover, this compound and other butyrate analogues have proved capable of inducing fetal gene activity in cultured populations of erythroid progenitors from patients with thalassemia and those with sickle cell disease.[29] The induction of Hb F in vivo has recently been shown in a few patients with sickle cell disease or thalassemia.[30]

The use of the aforementioned agents has been motivated by observations bearing on factors that affect globin gene regulation during development and erythroid maturation. For example, 5-azacytidine was first used specifically for its effect on DNA methylation. In normal chromatin, DNA is methylated; the pattern of methylation is not random but correlates to some degree with gene activity. This correlation

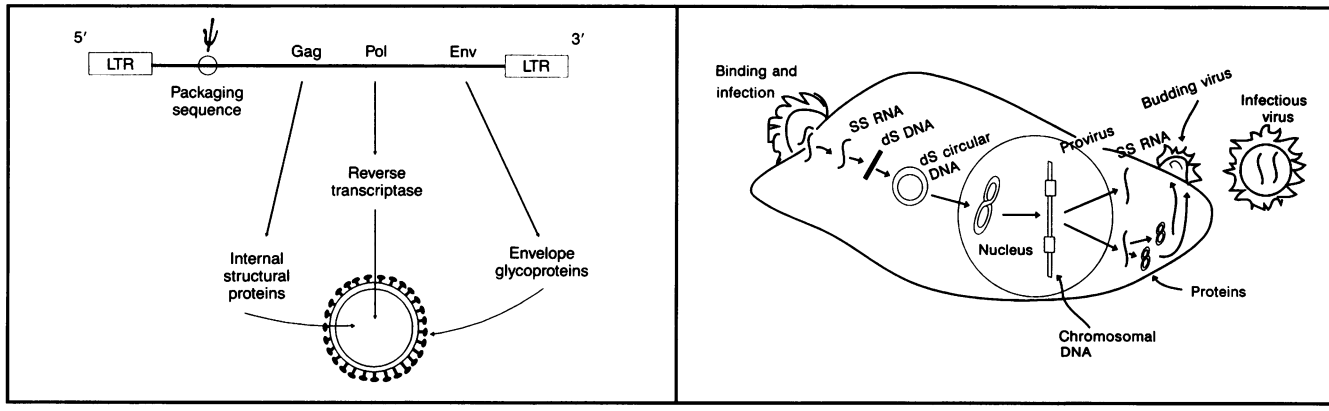

Figure 3.—Left, retroviral structure is shown with, **Right,** retroviral life cycle. Removing the ψ packaging signal interrupts the retroviral life cycle, abrogating its ability to assemble infective virions while retaining replication competence (see also Figure 4). Note that retroviral integration into the host genome can occur only during the S phase. LTR = long terminal repeat

was first observed in the β-globin cluster, where it was discovered that the γ-globin genes were demethylated in fetal-stage erythroblasts (where they express at a high level) but heavily methylated in adult-stage erythroblasts (where they are virtually quiescent).[31] These observations suggested immediately that γ-gene reactivation in the adult might be actualized, or at least potentiated, by the application of 5-azacytidine, a cytosine analogue known to effect a global decline in genomic DNA methylation status after being incorporated into replicating DNA, presumably through the interactive inactivation of DNA methyltransferase.

The observation that Hb F synthesis was increased during conditions of accelerated or altered erythropoietic kinetics, combined with the knowledge that the high mitotic rate of maturing erythroblasts made them particularly susceptible to the action of cell-cycle–specific cytotoxic drugs, raised the possibility that a proper application of such agents might be capable of manipulating erythroid kinetics in such a way as to produce a sustained Hb F response.[6] To this end, a number of such agents have been applied in studies using animals and in acute-phase trials, of which only hydroxyurea (because of its low toxicity and ease of administration) has been studied on a long-term basis. Recombinant human erythropoietin was initially administered in an effort to alter erythroid kinetics without cytostatic effects.

The application of sodium butyrate was prompted by the discovery that butyric acid metabolites were present in abnormally high levels in both diabetic women and their newborn children, the latter of whom had considerably delayed fetal-to-adult hemoglobin switching.[32] Subsequently, sodium butyrate was shown in a developing ovine model to be capable of retarding or suppressing completely the switch to adult hemoglobin production.[33]

The mechanisms by which these and other agents produce their effect on Hb F remain the subject of considerable debate. Although largely amibiguous with respect to any particular agent, the evidence to date appears to argue for a mechanism involving manipulation, kinetic or otherwise, of the cellular or maturational clock of hemoglobin switching.

Gene Therapy for Hemoglobinopathies

Human and animal retroviruses are RNA-based organisms that encode a reverse transcriptase activity, which in turn potentiates host genome integration through an intermediate DNA provirus. Retroviruses are propagated by host DNA replication, transcription, and translation machinery that provide for the packaging of viral RNA in a protein coat and its subsequent encapsulation and "budding" in a phospholipid exosome derived from the host plasma membrane (Figure 3).

By manipulating retroviruses with recombinant DNA technology, exogenous DNA fragments can be inserted into the viral genome.[34] Through the selective retention of retroviral sequences responsible for the reverse transcription process and efficient encapsidation, as well as appropriate viral transcription signals located in the long terminal repeats of most retroviruses, it has become possible to engineer replication-incompetent viruses that retain their infective potential (Figure 4). Such manipulations can result in the generation of a novel species that functions effectively as a vehicle for the transfer of desired DNA sequences into the genome of a target cell population susceptible to infection by the virus. Gene therapy aims to harness the power of retrovirus-

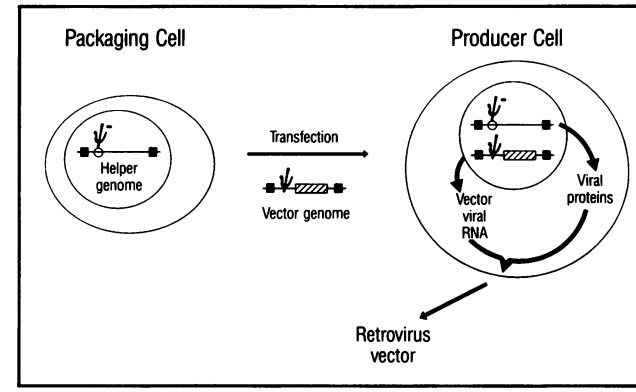

Figure 4.—The production of infective particles containing retroviral vectors is shown. Helper cells infected with ψ^- viruses are cotransfected with packaging-competent but replication-incompetent retroviruses serving as vectors for the transfer of exogenous DNA sequences. The coculture of amphotropic and ecotropic packaging cell lines results in superinfection and the production of high virus titers ("ping-pong" strategy).

mediated gene transfer to correct genetic diseases by introducing nondefective gene copies or other genes that may aid in retarding the pathogenesis of a particular disease process.

Assuming the availability of suitable retroviral vectors, current gene therapy protocols for hemoglobinopathies involve autologous bone marrow transplantation. Marrow is removed from a patient, cultured in media containing hema-

topoietic growth factors necessary for the proliferation of progenitor cells, subjected to infection by retroviral particles harvested from the supernatant of a producer cell line, and reintroduced into the patient, who has undergone irradiation in the intervening period (Figure 5).

Several groups have reported successful gene transfer into murine and nonhuman primate hematopoietic stem cells, although obstacles remain to be surmounted before this technology becomes feasible for application in humans. Principal difficulties include the low efficiency of gene transfer into target cells and the low level of expression of exogenous DNA sequences once transfer has been achieved.

Inefficient Gene Transfer

The long-term efficacy of gene therapy in the treatment of hemoglobinopathies is contingent on successful gene transfer into totipotent hematopoietic stem cells. Because these cells make up only a minute fraction of marrow progenitors,

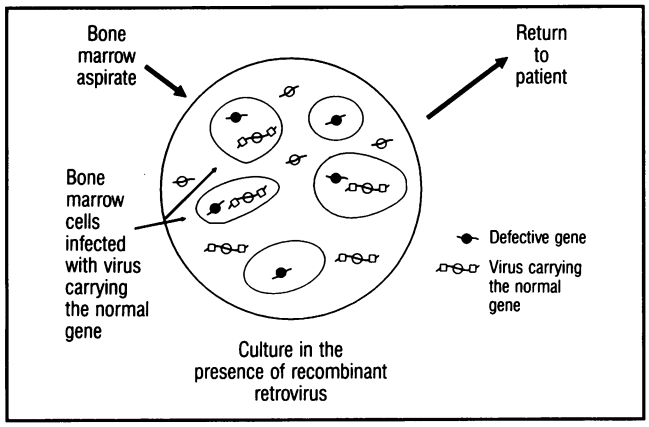

Figure 5.—The diagram illustrates gene therapy for hemoglobinopathies. Bone marrow aspirate containing hematopoietic stem cells is cultured in media replete with infective retrovirus vectors and a complement of hematopoietic growth factors. The latter increases the proportion of stem cells undergoing DNA synthesis and hence increases the infective efficiency of the virus. Infected cells are subsequently returned to the patient by intravenous infusion, whereupon they circulate, lodging once again in the marrow compartment.

the efficiency of gene transfer is correspondingly low. As such, high titers of infectious virions need to be achieved in producer cell lines. Furthermore, because viral integration into a host genome is dependent on DNA replication, the efficiency of gene transfer is compromised severely because stem cells are presumed to be largely quiescent.

Preliminary strategies for overcoming these obstacles have been developed.[35] To increase the production of viral particles by conventional producer cell lines, a "ping-pong" coculture technique has been used wherein ecotropic and amphotropic producer cell lines are combined within the same culture medium, with the result that both cell types become superinfected (>10 copies of provirus per genome) by one another. From this mixture, a cell line producing roughly 10^9—versus $< 10^6$ for conventional producer lines—replication-competent virus particles per milliliter can be isolated. Viral supernatants derived from such cell lines have enabled highly efficient gene transfer into stem cells capable of long-term, multilineage repopulation following irradiation in both small and large animal models.[35]

Because retroviruses employ host DNA replication machinery to effect integration, target cells must be actively replicating their DNA at the time of infection for stable gene transfer to take place. A number of hematopoietic growth factors have demonstrated a capacity to stimulate the proliferation of multipotent hematopoietic progenitors in vitro, but no one factor appears capable of effecting widespread recruitment of this primitive cell type from quiescence. A systematic examination of several growth factors in both cultures of murine progenitors and lethally irradiated mice has revealed that interleukin 3, when used in combination with interleukin 6, mobilizes stem-cell proliferation and substantially enhances the efficiency of retrovirus-mediated gene transfer.[36] These results should prove extensible to large animal and human models.

Deficient Expression of Exogenous DNA

For effective gene therapy, most retrovirally transduced genes must be expressed at levels approximating those of endogenous genes. This is particularly relevant for sickle cell anemia, where the expression of a transduced globin gene would have to be of such a level as to overcome the deleterious effects of the highly expressed chromosomal β^s gene.

Encapsulation and packaging of retroviral vectors by producer cell lines place severe restrictions on the size of exogenous DNA fragments that can be carried by the vector. This restriction, often referred to as the packaging limit, necessitates that the sequence information required for the tissue-specific, high-level expression of a particular introduced gene fragment be delineated to determine the most efficient combination of enhancer sequences. Most genes contain within their promoter regions transcription information that is actualized by transcriptional factors in the nuclear environment. Transfection studies, however, have shown that promoter sequences alone are generally insufficient to direct the high-level expression of their associated gene. Such expression requires the presence of appropriate enhancer elements, but even when they are present problems remain because enhancers are themselves subject to dampening effects that vary according to their position in the genome. Apparently the chromatin in large regions of the genome is "inactive" in certain tissue types, and exogenous DNA sequences that have integrated into such regions remain transcriptionally inert. Because retroviral integration occurs at essentially random locations throughout the host genome, the expression of DNA sequences introduced into mammalian tissues will be subject to these "position effects." Because of the inherently low efficiency of gene transfer, optimal enhancer elements for gene therapy will need to ensure a position-independent, high-level expression of linked genes while maintaining tissue-specificity. With respect to the β-globin gene, such an element has been delineated.[37]

The high-level expression of globin genes in erythroid tissues is made possible by sequence information contained within the locus control region.[38] When linked to a β-globin gene, the LCR is also able to overcome position effects. Insufficient data exist for this conclusion to be extended to the γ genes. Systematic deletions have pinpointed the precise location of powerful enhancer elements within the LCR that function optimally in conjunction with a linked β-globin gene. In its native state on the chromosome, the LCR occupies roughly 20 kb of DNA.[37] Using the aforementioned deletion data, recombinant constructs spanning only 2 to 3 kb have been assembled; these constructs can direct the expression of a linked β-globin gene in either erythroleukemic cell

lines or transgenic mice to a level approaching that obtained with linkage to the wildtype LCR.[37,39]

Unfortunately, even such reduced constructs are too large to be incorporated, along with linked genes, into properly functioning retroviral vectors. These fragments, however, comprise several enhancer sequences, two of which, when used individually, are capable of delivering roughly half the activity of the entire LCR, thus reducing the size of an efficient β-globin enhancer to only a few hundred base pairs.[40] Such minimal enhancer sequences, when combined with a linked β-globin gene (including its flanking 3′ and 5′ sequences that have been shown to be important for correct expression) are well within the packaging limits of most retroviral vectors. The long terminal repeats of a number of retroviruses are known to contain "silencer" elements, which may abrogate the potency of small recombinant enhancers. Such silencer elements, however, are not expected to have the power to suppress enhancer elements on the scale of the LCR or its fully active recombinant derivatives.

If retroviral packaging limits continue to present difficulties, a more detailed delineation of enhancer elements within the LCR will be needed. The interaction of LCR elements with linked globin genes has thus far been studied primarily in the context of the β gene and is therefore of great value in the construction vectors aimed at gene therapy for thalassemia. For reasons outlined earlier, however, ideal therapy for sickle cell anemia will require the introduction of a γ-globin gene. As such, further information concerning the interaction of this gene with sequences in the LCR may be needed.

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