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*Erythropoiesis*

**Review Article**

# Slow and Steady Wins The Race? Progress in the Development of Vectors for Gene Therapy of β-Thalassemia and Sickle Cell Disease

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**The cloning of the human β-globin genes more than 20 years ago led to predictions that β-thalassemia and sickle cell disease would be among the first monogenic diseases to be successfully treated by gene replacement therapy. However, despite the worldwide enrollment of more than 3,000 patients in approved gene transfer protocols, none have involved therapy for these diseases. This has been due to several technical hurdles that need to be overcome before gene replacement therapy for β-thalassemia and sickle cell disease can become practical. These problems include inefficient transduction of hematopoietic stem cells and an inability to achieve consistent, long-term, high-level expression of transferred β-like globin genes with current gene transfer vectors. In this review we highlight the relationships between understanding the fundamental mechanisms of β-globin gene locus function and basic vector biology and the development of strategies for β-globin gene replacement therapy. Despite slow initial progress in this field, recent advances in a variety of critical areas provide hope that clinical trials may not be far away.**

*Keywords*: Gene therapy, β-thalassemia, sickle cell disease, β-globin, retrovirus, lentivirus, chromatin structure

## INTRODUCTION

Mutations of the α- and β-globin gene loci comprise the most prevalent group of inherited single gene diseases. It has been estimated that seven percent of the world's population are heterozygous carriers of clinically significant mutations affecting the expression of the globin genes or the function of their gene products [1]. Mutations within the genes of the β-globin locus give rise to β-thalassemia and the β-hemoglobinopathies, including sickle cell disease (SCD). While the high prevalence of these mutations appears to have arisen because they lessen the morbidity of malarial infection in the heterozygous state (reviewed in [2]), they continue to result in early death and chronic debilitating disease in the homozygous state [1]. The underlying pathophysiologies of these diseases involve destruction of erythrocytes due to abnormal polymerization

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of hemoglobin molecules containing the sickle $\beta$-chain (sickle cell disease) and $\alpha$-chain imbalance ($\beta$-thalassemia) [3,4]. Because erythrocytes are derived from the hematopoietic stem cell (HSC), transfer of a functional human adult ($\beta$) or fetal ($\gamma$) globin gene to a patient's stem cells could cure or lessen the severity of these diseases. $\gamma$-globin gene transfer is particularly attractive for SCD since the $\gamma$-globin protein does not participate in the sickling reaction and thus confers greater protection than the $\beta$-globin protein [5]. A major rationale for using $\beta$-thalassemia and SCD as models for the development of gene therapy strategies is that less than full reconstitution of normal $\beta$- or $\gamma$-globin gene expression is likely to produce significant clinical benefit as co-inheritance of mutations that confer hereditary persistence of fetal hemoglobin (HPFH) have an attenuated phenotype [6–8]. A second rationale for $\beta$-globin replacement therapy is that significant clinical benefits have been achieved using the pharmacologic agents 5-azacytidine, hydroxyurea and sodium butyrate as inducers of $\gamma$-globin synthesis (reviewed in [9,10]). Gene replacement therapy offers significant advantages over these pharmacologic strategies in that a single treatment could permanently restore $\beta$-globin (or $\gamma$-globin) protein production and both known and potential side-effects of long-term drug therapy could be avoided [9]. Successful gene transfer therapy would also offer a potentially safer, less expensive and more widely applicable curative therapy than allogeneic bone marrow transplantation [11,12].

The $\beta$-globin genes were among the first human genes to be cloned [13–15]. This led to speculation that genetic diseases involving these genes would be among the first diseases to be treated using gene therapy. However, ten years after the approval of the first human gene transfer protocol [16] and after the treatment of more than three thousand patients worldwide, no $\beta$-thalassemia or SCD patient has been treated in an approved gene therapy trial [16,17]. This disappointing lack of progress has largely been due to several technical hurdles which must be overcome before gene therapy can be applied to these diseases. These challenges include developing more efficient methods for the stable transfer of genetic material into the genomic DNA of hematopoietic stem cells and achieving long-term, high-level expression from integrated $\beta$- or $\gamma$-globin genes.

So far, most gene therapy approaches to the hemoglobinopathies have utilized retroviral vectors. This gene transfer system has several advantages including the ability to efficiently transduce a variety of cell types, the ability to stably integrate the therapeutic gene into host genomic DNA, a size capacity able to accommodate $\beta$-globin regulatory and coding sequences and a proven clinical safety record [18,19]. Overall, retroviral vectors have been the most commonly used vector for gene transfer protocols in general and for protocols targeting bone marrow stem cells in particular [16,17]. Unfortunately, retroviral vectors also have shortcomings including relatively low level transduction efficiency of hematopoietic stem cells. Recent studies have shown that this is partly due to low level expression of appropriate viral receptors on the surface of hematopoietic stem cells [20]. Commonly used retroviral vectors, which are based on the Moloney Murine Leukemia Virus (MMLV) and related members of the same retroviral species, are also unable to efficiently integrate into the genomic DNA of non-cycling target cells. Because of these problems, expression of transferred genes in human peripheral blood cells remains too low to achieve therapeutic benefit [21,22]. New strategies designed to overcome these problems include up-regulation of retroviral receptors on hematopoietic stem cells [23], induction of stem cell cycling with hematopoietic growth factors and drugs [24,25], pseudo-typing of retroviral vector envelope proteins [26], and the recent use of lentiviral vectors, which are retroviruses which do not require cell cycling for stable integration into hematopoietic stem cells [27]. Another novel strategy involves the transfer, not of the $\beta$- or $\gamma$-globin genes themselves, but of genes coding for regulatory proteins which up-regulate expression

of the γ-globin genes. The stage selector protein described by Jane and colleagues is an example of such a protein which could participate in the reactivation of the fetal globin genes [28,29].

The second major obstacle to successful gene therapy for the β-hemoglobinopathies is the inability to obtain consistent long-term, high-level expression of integrated therapeutic genes. The importance of this concept was emphasized in the report of the Panel to Assess the NIH Investment in Research on Gene Therapy which noted that "... *very little research effort is focused on the mechanisms that govern maintenance or shutoff of gene expression following gene delivery in gene therapy experiments*" [30]. The report urged the NIH "... *to give high priority to basic research to elucidate how recipient cells and particularly stem cells, handle and express foreign DNA sequences.*" [30]. This concern is especially relevant to gene therapy for β-thalassemia and the hemoglobinopathies where life-long expression from the integrated therapeutic gene is a goal.

Clearly, the normal β-globin genes are capable of long-term, high-level expression. This has provided a major rationale for studying normal β-globin locus structure and function to define the mechanisms by which appropriate expression of the endogenous genes is achieved. By incorporating globin gene regulatory elements (such as promoters and enhancers) into gene therapy vectors, it has been hoped that similar expression of transferred genes could be attained. However, after twenty years of investigation into the regulation of β-globin gene expression and the application of the results of this research to the development of β-globin gene transfer vectors, expression levels adequate to begin human trials have yet to be achieved.

In this review we focus on how understanding the basic science of gene regulation in general, and of the β-globin locus in particular, has driven the development of β-globin gene replacement vectors. We begin by discussing the role chromatin structure plays in the regulation of genes integrated into genomic DNA, as understanding this process appears to be important to developing vectors capable of high-level expression. We then review current information on the regulation of the genes of the β-globin locus. Next we discuss the development of β-globin gene transfer vectors. Because retroviruses have been the principle model for the development of gene replacement strategies for the β-globin related diseases, we have primarily focused on this system. Finally, we discuss how novel insights into the chromatin structure of the β-globin locus are leading to the development of a new generation of retroviral vectors specifically designed to address the problems of inconsistent and low-level gene expression.

## CHROMATIN STRUCTURE AND GENE EXPRESSION

Within the nucleus of eukaryotic cells genomic DNA is packaged by specific proteins in a highly structured fashion [31]. This combination of proteins and nuclear DNA is termed chromatin. The structure of chromatin is highly dynamic and plays an important role in the regulation of normal gene expression (reviewed in [32]) and expression resulting from gene therapy vectors integrated into nuclear chromatin [33]. The first level of packaging involves the wrapping of DNA about disc-shaped, multi-protein structures called nucleosomes. The nucleosomes themselves are comprised of four related proteins termed histones. Each nucleosome packages approximately 200 bp of DNA which is wrapped twice around the structure. Regions of DNA which contain actively expressed genes (such as the β-globin locus in erythroid cells) are loosely packaged and the nucleosomes of the region assume a beads-on-a-string arrangement. This is associated with the formation of enzymatically modified histone proteins which become hyperacetylated. Regions of DNA which do not contain actively expressed genes (such as the β-globin gene locus in non-erythroid cells) are much more compacted. The

nucleosomes in these regions are thought to form tightly condensed helical arrays called solenoids or 30 nanometer fibers [31]. Inactive areas of chromatin are also characterized by DNA methylation and hypoacetylation of histone proteins (reviewed in [34]). Higher levels of packaging also occur in inactive areas but are much less well defined. Experimentally, differences in chromatin structure are often assessed by their sensitivity to digestion by nucleases, which are enzymes capable of digesting DNA. DNase I is a nuclease commonly used in chromatin studies. Tightly compacted, inactive areas of chromatin are highly resistant to DNase I digestion, while transcriptionally active areas of chromatin are more sensitive. Transcriptional regulatory elements such as promoters and enhancers are often hypersensitive to nuclease digestion due to the displacement or disruption of nucleosomes within these areas exposing DNA to the nuclease. These regions of locally altered chromatin structure are termed nuclease or DNase I hypersensitive sites or "HSs".

Chromatin structure is relevant to the development of gene transfer vectors because the expression of a stably integrated therapeutic gene is likely to depend on the surrounding chromatin structure. If the gene is integrated into a compacted, transcriptionally-inactive region of the genome it is unlikely to be expressed. This phenomenon is termed "position-dependent expression" as expression is dependent on the site of integration. Position-independent expression or, stated in a different way, consistent expression is an important goal in the development of gene transfer vectors. Furthermore, it has been shown that integrated genes which initially express at high levels may be subject to transcriptional silencing over time [35–37]. This is thought to result from a closing down of the chromatin structure around the integrated gene. As is discussed below, it would be advantageous to be able to include elements within gene therapy vectors which are able to independently open and maintain surrounding domains of active chromatin structure regardless of their site of integration within the genome.

## REGULATION OF HUMAN β-GLOBIN GENE EXPRESSION

Perhaps because the human β-globin genes were among the first mammalian genes to be cloned, the human β-globin gene locus has served as a premiere model for studying the regulation of complex mammalian loci. As shown in Figure 1, the genes of the locus are arranged in their order of developmental expression, from the embryonic ε-globin gene to the fetal γ-globin genes and the adult δ-and β-globin genes [2]. Early studies of the locus identified promoters and both intronic and downstream enhancers of β-globin locus gene expression (Figure 1B). In early gene transfer experiments, these elements were able to direct erythroid specific expression of the genes but expression levels in both transgenic mice and tissue culture cell lines were very low and highly variable [38,39]. Clearly, important elements for gene regulation were missing.

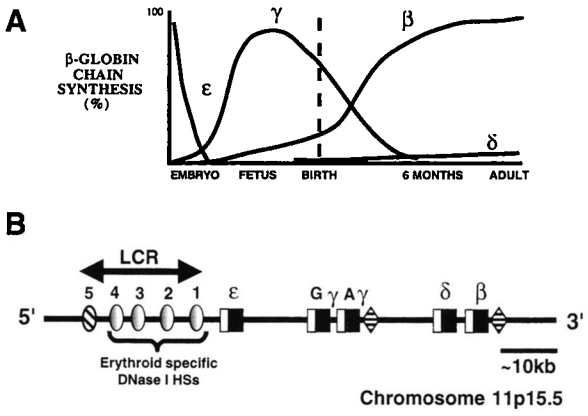


FIGURE 1 The human β-globin gene locus. A) Expression of the β-globin genes is developmentally regulated. B) Organization of the locus. Globin genes are depicted as black boxes, gene promoters as white boxes and local enhancers as diamonds. The β-globin locus control region (LCR) is comprised of five domains of altered chromatin structure termed DNase 1 HSs. Four of these HSs are erythroid specific.

The description of the β-globin locus control region (LCR) in 1987 apparently identified these critical elements (Figure 1B). The LCR is comprised of five DNase I HSs, four of which (5′ HS 1–4) are erythroid specific and are able to direct consistent, high-level, position-independent expression of linked globin genes in transgenic mice [40,41]. The functional activities of the LCR are primarily mediated by specific core sequences found near the centers of each HS domain [42–52]. Each of the 200–400 bp HS core elements contain evolutionarily conserved binding sites for factors primarily expressed in hematopoietic cells, such as NF-E2, GATA-1, SSP, and EKLF, as well as more ubiquitous factors such as Sp1, YY1, and CDP (reviewed in [53]). On a chromatin structural level these nuclease sensitive regions are characterized by highly specific nucleosome positioning including regions where nucleosomes are disrupted or displaced [54,55]. The active elements of the LCR HSs closely correspond to the regions of nucleosome displacement [47,48,52,54,55]. In transgenic mice containing multiple copies of linked transgenes and individual LCR elements, HS2, HS3, and HS4 are each able to confer position independent expression [56]. However, only when all of these LCR elements are present as full-length, or smaller "mini" or "micro" LCRs is near-normal expression of the transgene consistently obtained [41,51,57]. In single copy transgenic mice HS3 is uniquely able to confer position-independent expression [58]. A well characterized, classical enhancer element is present within the core region of HS2 [59,60]. This enhancer activity requires binding sites for the hematopoietic transcription factor NF-E2 [52,61]. The other LCR HSs do not contain significant enhancer activity. While relatively little research has been performed on HSs 1 and 5, HS1 may be important for consistent expression and for mediating interactions between the LCR and the β-globin promoter [62] and, as discussed below, HS5 may function as a chromatin structure insulator [63].

Despite intense investigation the mechanisms by which the elements of the LCR are able to influence expression of the genes of the human β-globin locus remain unclear. Most current models propose the formation of a holocomplex in which the core elements of the LCR functionally interact. This holocomplex is then envisioned to form a three dimensional looping structure that is able to interact with the promoters of the globin genes (reviewed in [53,64]).

## DEVELOPMENT OF β-GLOBIN GENE TRANSFER VECTORS

### Retroviral Transfer Vectors

Since the development of the first gene transfer trial more than ten years ago, retroviral vectors have been the most frequently used method for stable integration of genes into target cell genomes [17]. Retroviruses are enveloped RNA viruses which enter cells through interactions with specific receptors on the cell surface [65]. The presence of these receptors on target cells is an important determinant of the virus's ability to transduce a specific cell type. The infecting virus particle carries a single-strand RNA genome which is converted into a double-strand DNA provirus by the virally-encoded reverse transcriptase within the infected cell. The viral integrase, in cooperation with cellular proteins, then mediates incorporation of the DNA provirus into the genomic DNA of the target cell [66].

The retroviral system has been adapted to serve as a safe and effective method for stably transferring potentially therapeutic genes into the genomic DNA of target cells (Figure 2). The retroviral gene transfer vector is commonly constructed within the context of a bacterial plasmid. The vector contains the viral long terminal repeats (LTRs) and packaging signal ($\psi$) as well as the gene or genes to be transferred. Because the retroviral structural genes *gag*, *pol* and *env* are not contained within the vector, it is unable to direct the formation of infectious

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