MINIREVIEW

Gene Therapy for Human Hemoglobinopathies (43665)

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The molecular defects in sickle cell disease and β-thalassemia have been well characterized and seem amenable to genetic correction (1). The development of effective genetic therapy could revolutionize treatment of the hemoglobinopathies. Before envisioning treating patients, methodologies will be required to ensure safe, efficient, and stable transfer of globin genes into hematopoietic stem cells and subsequent high-level gene expression in mature erythroid cells. This minireview will focus on the use of viral gene transfer vectors as potential therapeutic agents for the treatment of human hemoglobinopathies.

The thalassemias and clinically significant hemoglobinopathies are among the most common single gene disorders throughout the world. Patients with severe phenotypes rely on regular erythrocyte transfusions that can be associated with life-threatening iron overload despite intensive chelation (2). Long-term transfusion therapy may result in the development of anti-erythrocyte antibodies making subsequent transfusions difficult or, in some instances, impossible (3, 4). Allogeneic bone marrow transplantation has been performed with some success but is feasible in only a small percentage of affected patients (5, 6). Recent work has focused on the pharmacologic manipulation of fetal hemoglobin. Underpinning these efforts is the premise that increased γ-globin gene transcription and fetal hemoglobin synthesis leads to more effective erythropoiesis and/or decreased hemolysis in patients with β-thalassemia and sickle cell disease (7–9). These treatments are, however, potentially toxic with unknown long-term complications.

Globin Gene Organization

Hemoglobin is a tetrameric protein composed of two dimeric polypeptide units encoded by two different gene families on two separate chromosomes. The α-globin gene cluster, located on chromosome 16, includes the duplicated α genes (α_1, α_2) present in the fetal and adult stages of erythropoiesis and the embryonic ζ-gene. Located on chromosome 11 are the cluster of β-like genes including the two adult genes, δ and β, the two fetal genes, γ^A and γ^G, and the embryonic ε-gene (see Fig. 1). During normally developing erythropoiesis, six distinct hemoglobin species are present in the transition from intrauterine to adult life. Coordinated gene expression in the α- and β-gene clusters occurs at each site of erythropoiesis: the yolk sac of the embryo, liver of the fetus, and bone marrow postnatally. This process of coordinated expression, known as "hemoglobin switching," coincides with the change in hemoglobin phenotype. The β-like genes are activated and silenced in the 5′ to 3′ order of their transcriptional positions along chromosome 11.

Current switching models suggest competition between the individual β-like genes for regulatory elements defined within the distant DNase I hypersensitive sites known collectively as the locus control region (described below). A putative switching factor(s) is involved with either the silencing and/or activation of the β-gene cluster. Interaction of switching factors with β-like gene promoters may determine whether active gene transcription occurs. An example is the identification of a stage selector element in the human γ-

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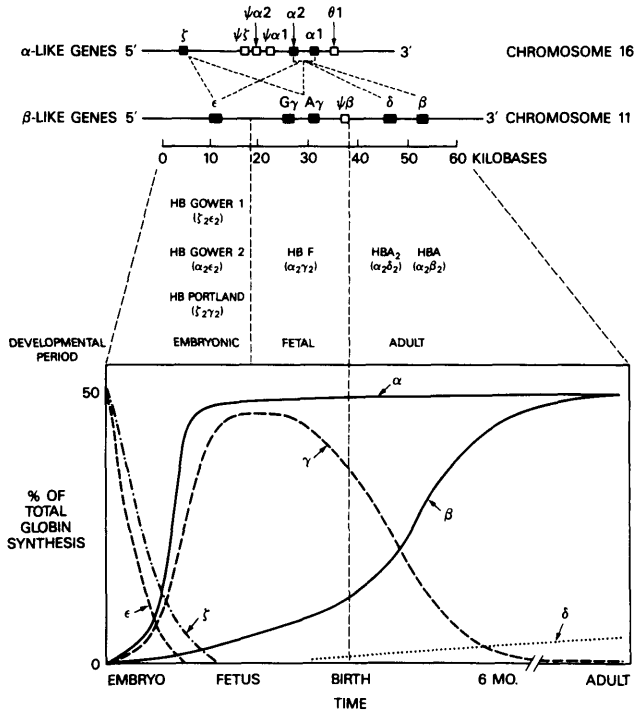

Figure 1. The spatial organization of the α- and β-globin gene clusters (top). Coordinated globin gene expression and hemoglobin switching (center) at each stage of erythrocyte development (bottom) (adapted from Ref. 87).

globin gene promoter and of the nuclear protein which binds to this element and enables the γ-gene to competitively silence the β-globin gene (10). Levels of the specific DNA-binding protein are higher in more developmentally immature cells in which γ-globin expression is elevated. Analysis of the α-like globin gene cluster suggests a similar type of regulation (11).

Regulation of Globin Gene Transcription

The expression of the individual globin genes is regulated at the level of gene transcription, as supported by measurement of globin transcriptional rates and by quantitation of globin mRNA from patients with thalassemia (12). In general, globin transcriptional regulation requires cis-acting DNA sequences located within the globin gene cluster and trans-acting factors which bind sequence-specific motifs within the cis-acting regulatory elements.

Regulation of the human β-globin gene cluster (ε, γ^G, γ^A, δ, β) is mediated via local cis-acting sequences including the globin promoters and enhancers 3′ of the γ- and β-genes. Initial efforts to define cis-acting elements responsible for globin gene expression in transgenic animals revealed that local sequences were not sufficient for normal globin expression. The observation that deletions upstream of the β-globin gene inactivated globin expression suggested that other regulatory elements were necessary. These distant regulatory elements that flank the β-globin cluster are associated with DNase I hypersensitive sites (HS), and inclusion of these sites modulates high level globin expression. These sites are collectively termed the locus control region (LCR). Four sites (5′ HS1-4) are located several kilobases 5′ to the ε-globin gene, and one site (3′ HS1) is mapped 3′ to the β-globin gene. The active elements of the LCR are encompassed within 300–400 base pairs of DNA found at each HS (13, 14). The HS2, 3, and 4, when linked to globin genes singly or in combination, substantially increase globin gene expression in transfected erythroleukemia cells or when introduced into transgenic animals (15).

Recently, several erythroid-specific enhancer sequences and trans-acting factors have been defined that appear to regulate globin gene transcription. One of the most powerful enhancer elements in the β-globin locus lies within the HS2 and is localized to tandem AP-1 binding sites (16). This element is required for high level γ-globin gene expression in stably transfected K562 cells. K562 cells provide a model for the study of globin gene regulation and have been used to define important cis-acting regulatory elements. The HS2 enhances by 150-fold transcriptional activity in hemin-induced K562 cells but is relatively inactive in nonerythroid cells. The trans-acting factor NF-E2 binds to the HS2 enhancer and is required for hemin-inducible activity of the enhancer (17). Transgenic animal experiments using only the HS2 site linked to a β-globin gene enabled the production of 25–50% levels of endogenous globin transcript (18). This factor has been characterized as a 45-kDa basic-leucine zipper DNA binding protein expressed in erythroid and megakaryocytic lineages (19).

The erythroid-specific transcription factor NFE-1 (GATA-1) binds to GATA consensus motifs found in several of the cis-acting elements. Experiments with transgenic mice indicate that GATA-1 may be important in the development and function of red blood cells (20, 21).

Globin Pathophysiology

The thalassemic syndromes are hereditary anemias which occur due to mutations that affect the synthesis of either α- or β-globin chains. The ratio of α- to β-chain synthesis is the major determinant of pathology. Excess of either globin chain can lead to the formation of aggregates or intracellular inclusions causing decreased red blood cell membrane deformity, ineffective erythropoiesis, and accelerated red cell destruction. Discussion of clinically relevant severe thalassemia syndromes is usually directed toward the β-thalassemias.

β-Thalassemia refers to inadequate β-globin chain synthesis encoded by a single β-globin gene on chromosome 11. Heterozygous individuals are characterized by a quantitative deficiency of β-globin production relative to α-globin. In homozygous patients with β-

thalassemia, deficient or absent β-globin gene synthesis causes the production of poorly hemoglobinized, defective erythrocytes resulting in hemolysis and severe anemia (22). Patients with severe disease require frequent red blood cell transfusions with attendant iron accumulation. The severity of this disease is modulated by increased γ-globin synthesis and increased fetal hemoglobin ($\alpha_2\gamma_2$, Hb F) or concomitant α-thalassemia.

In homozygous sickle cell anemia, the mutant hemoglobin (Hb S, $\alpha_2\beta_2^s$) is susceptible to polymerization resulting in altered erythrocyte rheological properties, vaso-occlusion, and multiorgan damage. The severity of sickle cell disease correlates with the degree of hemoglobin polymerization. Hb F has a sparing effect on polymerization and decreases the tendency of Hb S to precipitate within the erythroid cells.

In these disease states, the transfer and expression of either β- or γ-globin genes should be highly effective in correcting these genetic defects. Replacement with a functional β-globin gene could correct the defect in severe β-thalassemia, whereas gene insertion of a γ-globin gene could ameliorate the potential for polymerization in sickle cell disease. Studies of sickle cell and β-thalassemia patients reveal that mild or absent clinical manifestations occur in the presence of hemoglobin F levels of 20–40% (23, 24). Expression of a β-globin transgene at 10–20% of normal endogenous levels is adequate to achieve a dramatic phenotypic improvement in a thalassemic mouse model (25).

Gene Transfer

The following are the major requirements for globin gene transfer to be clinically applicable. (i) Transduced (introduced) globin gene expression must be at sufficient levels. (ii) Expression should be stably regulated and erythroid specific. (iii) The totipotent bone marrow stem cell should be transduced at high frequency and/or maintain a competitive advantage over nontransduced cells for its self-renewal. (iv) The introduction of foreign DNA into the target cell genome should have limited potential for insertional mutagenesis and/or endogenous gene disruption.

Ideally, replacement of a defective gene with the correct genetic sequence by targeting genes to specific sites within the genome would be an optimal method for genetic therapy. Homologous recombination into a β-globin locus has been achieved in cultured embryonic stem cells, albeit at a frequency too low to be of any current therapeutic value (26). However, as a result of recent advances in molecular biology, the introduction of new genetic material by nonhomologous recombination with gene insertion into a target cell genome now appears attainable.

Gene transduction is accomplished by a variety of techniques, including viral vectors, poly-lysine/DNA conjugates, and other physical techniques. Viral gene transfer vectors make use of the inherent efficiency of viruses to transfer and express their genetic information in mammalian cells (for review, see Ref. 27). As now envisioned, hematopoietic stem cells from an affected patient could be infected with an appropriate viral vector containing a correctly functioning globin gene. We will describe current work with retrovirus and the parvovirus, adeno-associated virus, as potential gene transfer vectors.

Retroviral Vectors

Retroviruses contain a single-stranded RNA genome that, upon entry into a cell, is converted into a double-stranded DNA before its integration into the host cell chromosome. Early interest in these viruses stemmed from their ability to induce tumors by insertional mutagenesis. The integrated provirus, containing its own powerful transcriptional elements, is thought to activate nearby proto-oncogenes or inactivate tumor suppressor genes. To avoid the possibility of wild-type retroviral infection, packaging cell lines are used to produce replication-defective recombinant retroviral particles. Vector and packaging cell strategies have been well documented (27).

Globin Retroviral Vectors. Transfer of genomic human β-globin sequences using retroviral vectors has evolved with both the development of improved packaging cell lines and greater understanding of globin gene regulation. Initial experiments utilized a 3.0-kb fragment of genomic β-globin into an ecotropic vector in both orientations (28). A neomycin resistance gene in those constructs was employed to rapidly screen for high titer producer clones subsequently used to generate infectious recombinant retroviral virions. The marker also facilitated isolation of target cells transduced by the retrovirus. Only the reverse orientation construct was functional and sufficient for proviral integration and subsequent viral production. Individual clones contained a single transferred proviral copy. Human β-globin transcripts were detected and the level of expression increased in dimethyl sulfoxide-induced MEL clones. Dimethyl sulfoxide, hemin, and other agents have been used in several human and murine leukemic cell lines to induce β-globin cluster gene expression. Furthermore, negligible globin expression was detected in 3T3 fibroblasts, indicating that the construct carrying a β-globin promoter contributed to erythroid-specific expression. The level of expression (compared with endogenous murine β-globin expression) was approximately 0.01%. Similar experiments using an amphotropic vector containing a neomycin resistance gene with a γ-β globin hybrid were used to infect MEL cells with human β-globin expression at 10% of the endogenous induced expression (29). In both instances, viral titer was low and the provirus rearranged in some of the clones tested. Constructs containing portions of the

5′ untranslated region and intron 2 in the reverse orientation interfered with generation of full-length transcripts and yielded low titer recombinant virus (30). The 5′ region could be removed, but the intron 2 was required for expression. Reverse orientation globin constructs may contain polyA termination signals, possibly accounting for abbreviated transcription and low titer virus generation.

Examination of retrovirally transduced β-globin in human hematopoietic cells demonstrated gene transfer and expression in erythroid colonies (burst-forming unit, erythroid) (31). Expression reached 5% of the endogenous β-globin in several pooled colonies using RNase protection assay. Expression was determined by using a 6-base pair insertion ("marking") at the 5′ untranslated region to allow detection of the transferred gene. Infection frequency was low (0.04%) and attributed to the low viral titer (5×10^4 colony-forming units/ml). A truncated β-globin minigene (lacking introns) was also tested in MEL cells but expressed at undetectable levels regardless of the orientation.

The first *in vivo* experiment described a recombinant retroviral construct encoding a human β-globin gene that was used to infect murine hematopoietic cells and reconstitute transplanted mice (32). Expression was limited primarily to the erythroid lineage and varied from 0.4% to 4.0% of the endogenous mouse β-globin mRNA level. The proviral copy number per cell ranged from 0.02 to 0.40 copies/cell, found in all lineages. Long-term human β-globin gene expression was detected in transplanted animals at 4–9 months. The infection rate was low (18 of 104 animals reconstituted with infected bone marrow), which indicated that the marrow infection conditions needed to be optimized (increased viral titer, enrichment of pluripotent cells, and induction of quiescent stem cell cycling).

Confirmatory experiments in several laboratories demonstrated β-globin retroviral transfer in murine hematopoietic cells (33, 34). Long-term expression in all lineages from secondary recipient animals indicated that pluripotent stem cells rather than committed progenitor cells were infected. Co-culturing conditions for bone marrow target cells with recombinant retrovirus improved, largely through the inclusion of hematopoietic growth factors to shorten G_0 and promote entry into cell cycle required for retroviral replication and integration (35). Despite improved transduction frequency in pluripotent bone marrow cells, globin expression still ranged from 1% to 5% of the endogenous level.

The recently discovered LCR regulatory elements flanking the β-globin gene cluster suggested a new approach to the design of retroviral vectors (36). Individual LCR fragments were included within a marked β-globin/neomycin gene cassette and used to generate recombinant amphotropic virions infectious for MEL cells (37). One construct incorporating an HS2 fragment resulted in high level expression in a few clones (three), but with extreme expression variability (10–310%). The viral titers were 10^4–10^5 colony-forming units/ml. Subsequently, investigators from several laboratories have been unable to generate LCR-globin producer lines of sufficient titer that do not exhibit proviral rearrangement or deletion. A recent report describes the use of a 36-base pair sequence encompassing the NFE-2 binding site within the HS2 region linked to human β-globin (38). The level of β-globin expression increased marginally from 6.0% to 12.0% with the addition of the enhancer element. Viral titers were again low, and introduction of multiple copies of the 36-base pair fragment promoted gross proviral rearrangement.

Generation of an ecotropic retrovirus containing an LCR cassette with truncated HS 4, 3, 2, and 1 sites linked to a human β-globin yielded 60–70% expression in MEL cells compared with endogenous murine globin expression. Transfer into murine hematopoietic progenitors and subsequent transplantation into lethally irradiated recipients resulted in human β-globin expression. These experiments suggest that inclusion of LCR elements may support high level β-globin gene expression in murine hematopoietic stem cells; however, significant rearrangement of the provirus occurred and the vectors employed yield low recombinant viral titers (39).

Many of the alternative viral vectors currently available either do not integrate into host cells at high frequency, are not easily rescuable from the integrated state, are limited in their host range, or include other viral genes, thereby creating a need for the development of a safe and efficient viral vector system. We feel that the human DNA virus, adeno-associated virus, offers a promising alternative to the currently utilized vectors.

Adeno-Associated Virus

Adeno-associated virus (AAV) is a defective member of the parvovirus family. The AAV genome is encapsidated as a single-stranded DNA molecule of plus or minus polarity (40, 41). Strands of both polarities are packaged, but in separate virus particles (42) and both strands are infectious (43). The single-stranded DNA genome of the human virus AAV-2 is 4675 base pairs in length (44) and is flanked by inverted terminal repeated sequences of 145 base pairs each (45). The first 125 nucleotides form a palindromic sequence that can form a T-shaped hairpin structure and can exist in either of two orientations (designated flip or flop). This unique structure has led to the suggestion (46) that AAV may replicate according to a model first proposed by Cavalier-Smith (47) in which the terminal hairpin of AAV is used as a primer for the initiation of DNA replication. The AAV sequences that are required

in cis for packaging, integration/rescue, and replication of viral DNA appear to be located within a 191-base pair (bp) sequence that includes the terminal repeat sequences (48, 49).

The viral DNA sequence displays two major open reading frames, one in the left half and the other in the right half of the conventional AAV map (43). At least three regions which, when mutated, give rise to phenotypically distinct viruses have been identified in the AAV genome (50). The rep region, which occupies the conventional left half of the genome, encodes one or more proteins that are required for DNA replication and for rescue from the recombinant plasmid. The cap and lip regions appear to encode for AAV capsid proteins; mutants within these regions are capable of DNA replication but do not produce virus (50). AAV contains three transcriptional promoters, p5, p19, and p40 (45, 51–53).

AAV-2 can be propagated as a lytic virus or maintained as a provirus, integrated into host cell DNA (54). In a lytic infection, efficient replication requires coinfection with either adenovirus (55, 56) or herpes simplex virus (57)—hence the classification of AAV as a "defective" virus. When no helper virus is available, AAV can persist in the host cell genomic DNA as an integrated provirus (58, 59). Virus integration appears to have no apparent effect on cell growth or morphology (60, 61). Studies of the physical structure of integrated AAV genomes (59, 62) suggest that viral insertion into the host chromosome is usually in a tandem head to tail orientation and occurs within the AAV terminal repeated sequence. Integrated AAV genomes are stable, persisting in tissue culture for greater than 100 passages (59). Although AAV is a human virus, its host range for lytic growth is unusually broad. Virtually every mammalian cell line evaluated (including a variety of human, simian, canine, bovine, and rodent cell lines) can be productively infected with AAV, provided that an appropriate helper virus is used (i.e., canine adenovirus in canine cells) (54). These same cells are also capable of establishing an AAV latent infection in the absence of helper.

Despite the wide range of susceptible cell types, no disease has been associated with AAV in either human or animal populations (63), even though exposure is commonplace. Anti-AAV antibodies have been found frequently in humans and monkeys. Estimates suggest that about 70–80% of infants acquire antibodies to AAV types 1, 2, and 3 within the first decade; more than 50% of adults have been found to maintain detectable anti-AAV antibodies. AAV has been isolated from fecal, ocular, and respiratory specimens during acute adenovirus infections, but not during other illnesses (64).

Infectious AAV Clone. We initially cloned intact duplex AAV DNA into the bacterial plasmid pBR322 (65) and found that the AAV genome could be rescued from the recombinant plasmid by transfection of the plasmid DNA into human cells with adenovirus 5 as helper. The efficiency of rescue from the plasmid was sufficiently high to produce yields of AAV DNA comparable to those observed after transfection with equal amounts of purified virion DNA. The AAV sequences in the recombinant plasmid could be modified, and then "shuttled" into eukaryotic cells by transfection. In the presence of helper adenovirus (Ad), the AAV genome was found to be rescued free of any plasmid DNA sequences and replicated to produce infectious AAV particles (65–68). This developed an approach for mutant construction (67) that enabled us and others to explore viral gene function (43, 69), and to identify the cis-acting sequences needed for AAV rescue, replication, packaging, and integration (49).

AAV has been tested as a viral vector system to express a variety of genes in eukaryotic cells. Hermonat and Muzyczka (69) produced a recombinant AAV (rAAV) viral stock in which the neomycin resistance gene (neo) was substituted for the AAV capsid region and observed rAAV transduction of neomycin resistance into murine and human cell lines. The stable integrated viral vector could be rescued to produce replicating rAAV sequences after superinfection with Ad and wild-type AAV. Tratschin *et al.* (70) created an rAAV that was found to express the chloramphenicol acetyltransferase gene in human cells under the AAV p40 promoter. LaFace *et al.* (71) observed gene transfer into hematopoietic progenitor cells using an AAV vector. Wondisford *et al.* (72) cotransfected cells with two different recombinant AAV vectors, each encoding a subunit of human thyrotropin, and observed expression of biologically active thyrotropin.

The desirable size of inserted non-AAV or foreign DNA is limited to that which permits packaging of the rAAV vector into virions, and this depends on the size of retained AAV sequences. Tratschin *et al.* (70) constructed an AAV/chloramphenicol acetyltransferase genome that was 3% (approximately 150 nucleotides) longer than the wild-type AAV genome, and found that it could be packaged into virions. An AAV genome too large to be packaged resulted from insertion of a 1.1-kbp fragment of bacteriophage-λ into a nonessential region of AAV (R. J. Samulski and T. Shenk, unpublished). Thus, the total size of the rAAV to be packaged into virions should be about 4800–5000 nucleotides in length.

As mentioned above, several AAV vector systems have been designed that contain a recombinant plasmid capable of being packaged into AAV particles. The recombinant virus generated then functions as a vector for stable maintenance or expression of a gene or a DNA sequence in eukaryotic cells when under control of an AAV or SV40 transcriptional promoter. However,

a common problem encountered in all these AAV vector systems has been the inability to produce recombinant virus stocks free of helper AAV virus. Various methods have been used in attempts to decrease the percentage of contaminating helper virus (73). This problem has been a major drawback in the use of AAV as a prevalent viral vector. Our recent work, however, has succeeded in generating high titer viral stocks that are free of helper virus.

AAV Vectors. We have recently developed a method for producing substantially helper-free stocks of rAAV that can be used to efficiently and stably transduce foreign genes into host cells or organisms (49). Our present method for producing recombinant stocks is directed toward producing a viral expression vector system with improved efficiency, applicability, definition, and safety relative to viral vector systems currently utilized. The method utilizes a two-component system comprised of functionally, but not structurally, related rAAV genomes, one of which contains a segment of foreign DNA (the vector) but lacks DNA sequences necessary for viral replication, and the other (the helper AAV) which provides those viral functions not encoded by the vector but which cannot itself be incorporated into virions. Importantly, the vector and the helper DNA are sufficiently nonhomologous so as to preclude homologous recombination events that could generate wild-type AAV. Along with this development of the vector, we have conducted a study characterizing natural AAV integration. In this study, we have encountered the unexpected observation that wild-type AAV utilizes site-specific integration when establishing viral latency (see below).

Production of the AAV Vector System. We have constructed an infectious adeno-associated viral genome that contains two *Xba*I cleavage sites flanking the viral coding domain (43) (Fig. 2); these restriction enzyme cleavage sites were created to allow nonviral sequences to be inserted between the cis-acting terminal repeats of AAV (49). The AAV helper plasmid termed pAAV/Ad contains adenovirus type 5 terminal sequences (107 bp) in place of the normal AAV termini. This helper cannot be packaged into AAV virions, since it lacks the terminal cis-acting domain required for this function. The AAV terminal sequences were originally substituted with adenovirus terminal sequences in pAAV/Ad so as to transcriptionally enhance AAV gene expression (74). This hybrid plasmid did not contain the Ad packaging sequences (75) and therefore could not be packaged into Ad virions either.

The presence of the adenovirus termini substantially enhanced the expression of AAV-specific proteins from pAAV/Ad DNA when compared with pAAV/no TR (DNA which contained neither adenovirus nor AAV terminal sequences). When the helper plasmid pAAV/Ad and a vector pAAV/NEO were co-transfected into human cells in the presence of adenovirus, rescue and replication of the AAV/NEO sequences were detected. The vector viruses generated from this complementation contain only the terminal 191 nucleotides of the viral chromosome, demonstrating that all cis-acting AAV functions required for replication and virion production are located within that region. Recombinant viral DNA carrying a drug resistance marker gene were integrated into the cellular genome. These transduced genes could not be excised and replicated when the cells were subsequently infected with adenovirus, suggesting another level of safety (49). Thus, the AAV termini (145 bp) are sufficient for integration of the AAV chromosome into a host cell genome. No AAV gene expression is required to establish a latent infection using this vector, and up to 70% of the cells can be transduced.

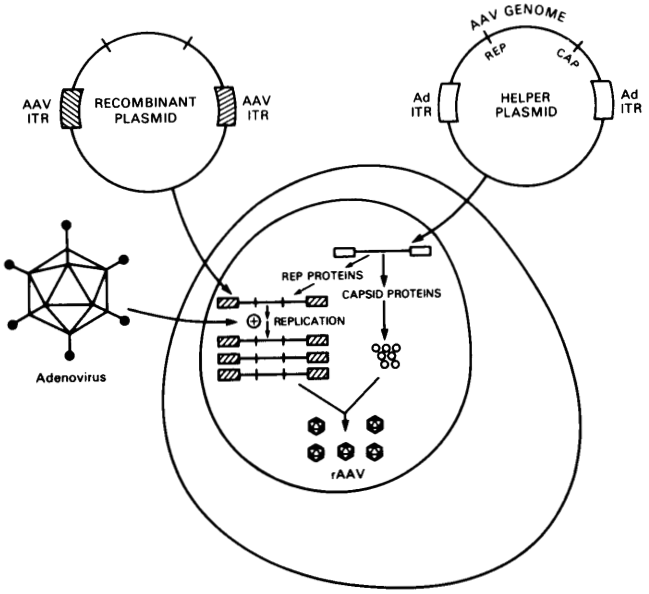

Figure 2. Generation of helper-free recombinant adeno-associated virions. A producer cell line is co-transfected with "helper" and recombinant (rAAV) plasmids. Helper plasmid generates rep and cap protein synthesis required for rAAV replication and encapsidation. Adenovirus infection provides necessary replication and packaging functions (see text for details).

Site-Specific Integration. The proviral integration form of wild-type AAV is a unique feature of this virus. Initial studies characterizing the AAV proviral state using restriction digestion and Southern blotting of genomic DNA demonstrated that the proviral DNA was covalently linked to cellular DNA in tandem concatamers (59, 76). These results have been confirmed and extended by the development of a protein-DNA binding enrichment technique used to isolate AAV proviral DNA from latent human cell lines (77). The strategy for retrieving AAV-cellular junctions involved a protein filter binding procedure that relied upon the interaction between λ repressor and its operator sequences. An infectious recombinant clone was used to

generate an AAV-λ hybrid that contained the λ operator site. The recombinant virus was used to establish latently infected AAV-λ cell lines. Genomic DNA isolated from the latent cell lines digested with restriction enzymes were mixed with purified λ repressor protein and passed over a membrane filter. Southern hybridization analysis of the retained fragments showed a physical linkage between AAV proviral DNA and cellular sequences. Nucleotide comparison of clonal cellular sequences demonstrated viral-cellular junction rearrangements involving deletion of portions of the terminal repeats. An unrearranged preintegration junction cellular sequence used as probe confirmed the sequence location at chromosome 19. Polymerase chain reaction amplification using AAV and junction-specific primers generated viral/junction breakpoints that lay within a 100-bp sequence on chromosome 19. *In situ* analysis of latently infected cell chromosomes using AAV-specific probes further demonstrated that viral DNA integrated into only one locus. Both single and multiple copy number insertion patterns were located within this integration region.

The minimal elements required for AAV integration are currently being delineated. AAV vectors containing only the inverted terminal repeat integrate at high frequency, suggesting the importance of the inverted terminal repeat for integration. This also indicates that AAV integration relies on host cellular enzymes. Furthermore, work in our laboratory demonstrated the lack of site-specific integration with recombinant vectors containing only AAV termini (78). Taken together, this would imply that viral *trans*-acting factors are required for site-specific integration. Since limited amplification of the AAV genome is required for integration, the AAV *rep* proteins described previously are likely candidates for this function.

Since this initial observation, we have documented site-specific integration in a number of cell types (human T cells, colon, lung, and monkey kidney cells). Our preliminary characterization of this chromosomal region has revealed that (i) this sequence is highly conserved in primates, (ii) this chromosomal sequence appears not to be expressed in either latent or nonlatent HeLa cells, and (iii) the integration site maps to the Q arm of chromosome 19. Extensive characterization of this chromosomal region will be essential in understanding regulation of rAAV transduced genes. For this reason, we have recently isolated two overlapping cosmid clones that hybridize to the chromosome 19 integration sequence (N. Epstein and R. J. Samulski, unpublished results). Further analysis of this region should be illuminating regarding both the integration mechanism and the potential of targeted gene delivery.

AAV Vectors Expressing Globin Genes. During our characterization of AAV vectors for targeted integration, we initiated a study to test this minimum AAV vector for the efficient transduction and expression of globin gene sequences in the erythroid cell line K562. We constructed an rAAV vector containing the human γ^A-globin gene, marked with a 6 nt deletion in the 5′ untranslated region to allow its transcript to be distinguished from native γ-globin transcripts (Fig. 3). The globin gene was linked to a 400-nucleotide DNA fragment containing LCR site 2, and a bacterial neomycin resistance gene used for selection. Site 2 alone has been shown to confer high level globin gene expression in erythroleukemic K562 cells (see Regulation of Globin Gene Transcription) and when treated with hemin, these cells can be induced to differentiate and increase expression of ε- and γ-globin genes (79).

We used the packaging strategy describe above (Production of the AAV Vector System) to generate AAV/globin hybrid virus. Recombinant virus was titered using human fibroblast target cells in the presence of geneticin (HeLa and/or Detroit 6) (78). Unconcentrated titers ranged from 10^4 to 10^5 neomycin-resistant colonies/ml. Southern analysis of low molecular weight DNA revealed no detectable wild-type particles using this protocol.

The erythroid cell line K562 was then infected with the recombinant virus and neomycin-resistant colonies were obtained (78). A polyclonal population of 30 isolated clones as well as individual clones were chosen for further study. First we characterized the configura-

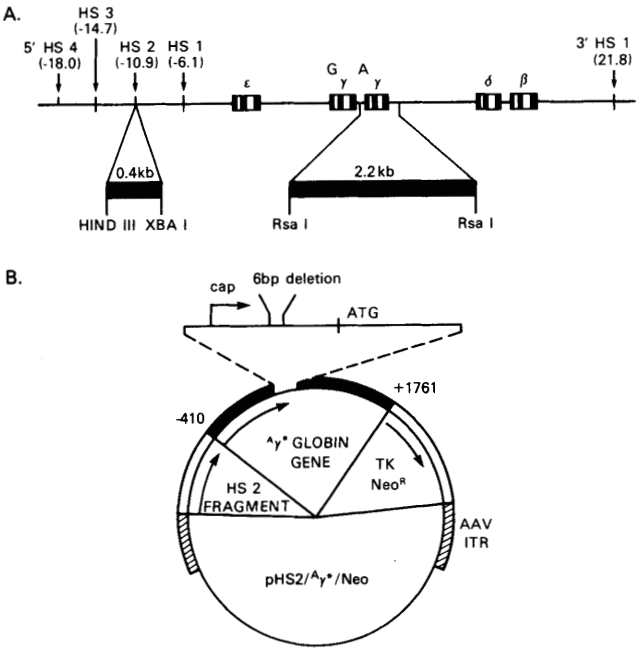

Figure 3. Construction of the rAAV/HS2/γ^{A*}/Neo^R-globin plasmid. (A) Schematic representation of the human β-globin cluster. The five functional genes are indicated. Arrows indicate the locations of the major DNase 1 sites. The HS2 fragment and the γ^A-globin gene used in the vector are shown. (B) HS2 fragment, marked γ^{A*} gene (with the 6 bp deletion) and the Neo^R gene cassette subcloned into the recombinant AAV backbone.

tion of the integrated AAV/globin genome. Digestion of genomic DNA with *Pvu*II demonstrated an expected 1.2-kb globin fragment which corresponded to a single, unrearranged proviral copy per cell. The K562 cell line, which is triploid at chromosome 11 (location of the globin cluster) by karyotype analysis (17), served as a control when estimating the AAV/globin copy number of individual clones. Individual clones digested and characterized as above revealed one to two copies of the transduced gene per cell. No rearrangement of the transduced gene was detected in either individual or pooled clones demonstrating the stability of the transduced gene.

As mentioned above, preliminary data from our laboratory indicates that AAV transducing vectors containing only the viral terminal repeats do not target to chromosome 19 (see Site-Specific Integration). We also probed genomic digests of the globin recombinants with chromosome 19-specific probe to further characterize the provirus integration in K562 cells. As expected, unlike wild-type AAV, which utilizes targeted integration, the AAV/globin viruses appeared to have integrated randomly.

RNase protection assays demonstrated both basal (uninduced) and hemin-induced expression of the marked globin gene. Endogenous and transduced γ^A globin transcripts were identified as predicted bands of 145 nt and 117 nt, respectively. As shown in Figure 4, a strong signal at 145 nt represented transcription from the endogenous γ-globin genes present in K562 cell line. The probe measured both γ^A and γ^G endogenous transcripts. Assuming that all six endogenous copies of globin were expressed, we measured uninduced expression of the marked gene to be 70% that of a single endogenous gene which, with hemin induction, rose to 90%. Several non-erythroid tissue culture lines were examined for evidence of γ-globin transcripts. A small (1–5% of rAAV/K562 signal) but detectable signal was found in Detroit 6 and HeLa cells but not in T lymphoid CEM cells.

To further verify these results, messenger RNA expression was analyzed using the polymerase chain reaction (PCR). We determined previously the transcriptional start site of the marked Ag globin gene in transfected K562 cells by a primer extension; PCR primers were designed accordingly. Cytoplasmic RNA isolated from either single or pooled K562 clones was reverse transcribed and amplified using primers that anneal to both endogenous and marked globin genes. The 6 nt deletion enabled resolution of these transcripts on 8% polyacrylamide-urea gels. Both uninduced and hemin-induced cells were analyzed, with mock-transduced K562 cells serving as a control. The marked transcript was detected in all clones tested.

Quantitation of RNA message by PCR was comparable to that determined by RNase protection and

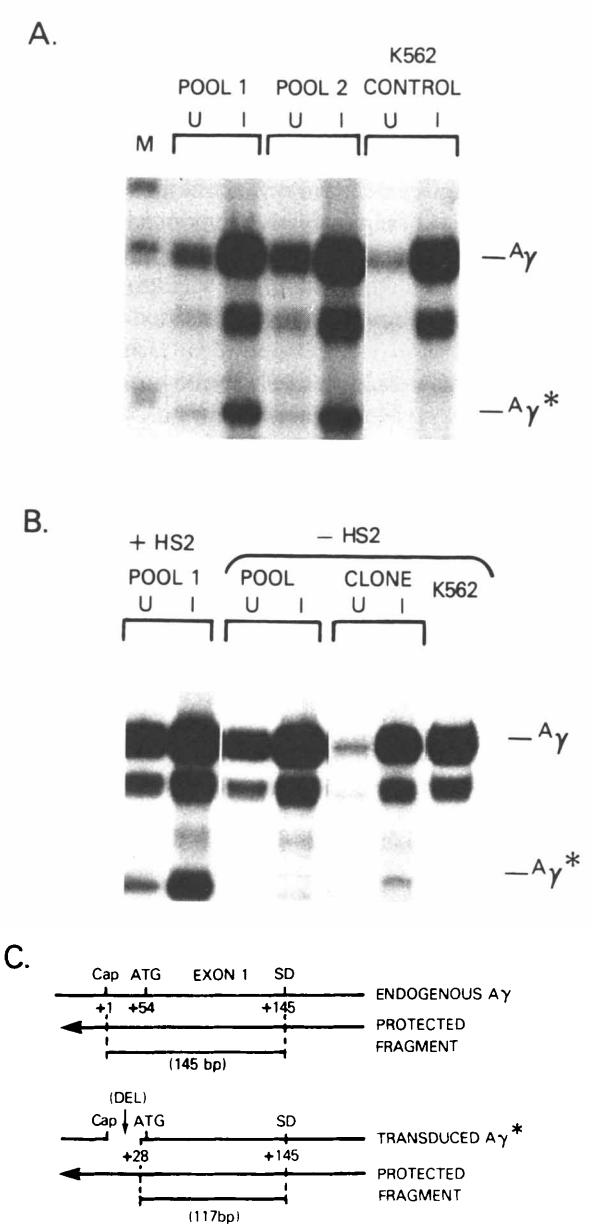

Figure 4. RNase protection assay of RNA extracted from K562 cells infected with (A) rAAV/HS2/γ^{A*}/Neo^R plasmid or (B) rAAV/γ^{A*}/Neo^R. (C) The expected RNA-protected fragments from the endogenous (γ^A) and transduced (γ^{A*}) genes are shown. U, uniduced cells; I, hemin-induced cells. Refer to Ref. 78 for details.

confirmed high level expression of the transduced Ag gene, including induction by hemin. S1 primer extension experiments confirmed that the correct globin start site was used in the proviral state. To rule out the possibility that the regulated globin expression we observed with this construct was due to globin regulatory sequences (LCR2) and not viral sequences or chromosomal positioning, we constructed an AAV/globin hybrid virus identical to the one described above minus the LCR2 site. Similar characterization of these transduced genes indicated a marked reduction in globin expression as expected.

These results represent the first viral-based introduction in which correct levels and regulation of γ-globin gene expression were achieved in an erythroid-derived cell line. High level, regulated globin expression was obtained when using a construct containing the LCR site 2. The LCR/globin construct efficiently integrated into the genome without rearrangement in all clones studied. (Recent extensive data from our laboratory suggests approximately 10–20% rearrangement.) Moreover, the messenger RNA expression of the transduced gene was comparable to endogenous γ-globin levels. The correct globin start site was utilized in the transduced gene, and tissue-specific expression (which was hemin inducible) was maintained.

The rAAV model can also be used to study the effects of specific mutations in the regulatory HS2 element on globin transcription (80). Specific mutations within the HS2 region of the rAAV/γ^A/globin vector described in Figure 3B were constructed, and neomycin-resistant clones containing the rAAV genome present in single copy were evaluated. Mutations within the NFE-2 binding motif resulted in a marked reduction of hemin-induced expression of the transduced globin gene when compared with expression from constructs containing the native HS2. In contrast, another set of mutations in the GATA-1 motif, which prevent binding of GATA-1, had no effect on basal and hemin-induced expression from the transduced globin gene. Other analyses of HS function rely on gene transfer methods which result in multiple copy integrants, and cooperative interaction between tandem gene copies are difficult to exclude. In this respect, the rAAV model is superior in that over half of the evaluable clones containing the native and mutant HS2 element had a single unrearranged copy of the rAAV genome. This single copy rAAV transduction model may also useful for evaluating other regulatory elements and their effects on the transcription of genes linked *in cis*.

This suggests that recombinant AAV vectors can be used effectively for the transfer of globin genes into human cells. Previous work has demonstrated that AAV vectors are capable of transducing a selectable marker into murine hematopoietic progenitor cells (71). Moreover, we have generated and tested an AAV vector carrying B19 viral coding sequences for infection in erythroid progenitor cells from human bone marrow (81). B19 is an autonomously replicating parvovirus shown to be the etiologic agent of various clinical disorders in humans (hydrops fetalis, polyarthralgia syndrome, and transient aplastic crises associated with various hemolytic anemias). This parvovirus has so far been shown to replicate only in erythroid progenitor cells in human bone marrow. Using the recombinant AAV vector carrying the B19 coding sequences, rAAV stocks demonstrated that the AAV-based vector was capable of infecting human bone marrow cells (81). Furthermore, the recombinant B19-AAV hybrids inhibited erythroid hematopoietic colony formation, indicating the expression of B19 genes.

We have recently demonstrated transduction of both rAAV and wild-type AAV human pluripotent cells (S. Goodman, unpublished results). rAAV carrying a β-galactosidase gene were capable of infecting $CD34^+$ selected bone marrow cells, as assessed by DNA PCR analysis of progenitor colonies derived from $CD34^+$ cells grown in methylcellulose. Incubation of wild-type AAV with $CD34^+$ selected cells demonstrated site-specific integration on chromosome 19q. rAAV vectors containing LCR-globin cassettes are currently being tested to transduce human and primate $CD34^+$ selected bone marrow cells. Gene transfer with rAAV into animal tissue has not yet been demonstrated.

Safety Issues

The safety of retroviral gene transduction has been reevaluated in murine and primate models. Most retroviral vector systems employ components of the Molony murine leukemia virus, known to induce T cell leukemia and lymphomas in mice (82). Initial investigations suggested no apparent pathologic affects of murine amphotropic replication-competent virus in primates (83, 84). However, a bone marrow transplantation/gene therapy experiment in primates using a high titer recombinant retrovirus contaminated with a moderate titer of wild-type retrovirus induced a rapidly progressive T cell lymphoma in three of 10 animals tested (85). Over 50–100 copies of the wild-type replication-competent provirus were detected in the tumor DNA, implicating viral insertional mutagenesis as the pathogenic mechanism. The use of newer retroviral packaging systems to reduce or eliminate wild-type retrovirus is an absolute necessity for human use (86).

One of the salient features of the AAV system is the lack of any demonstrable pathology to the host cell. As mentioned previously, no epidemiologic evidence currently exists linking AAV to human disease. The potential toxicity of rAAV in animals is unknown. Wild-type adenovirus, required for the generation of rAAV, is capable of causing disease in immunocompromised hosts. rAAV packaging systems will need to be modified to eliminate adenoviral contamination.

Summary

Gene transfer of human globin genes into human pluripotent stem cells via viral vectors may soon be realized. The high level of globin gene expression believed to be required for the treatment of severe hemoglobinopathies necessitated the inclusion of cis-acting sequences (LCR). Retroviral vectors containing the LCR elements are prone to rearrangement, low titer, and poor expression. Inclusion of a "minilocus" containing four HS sites linked to a globin gene resulted in

higher expression in transplanted mice, but rearrangement of the provirus still occurs, and it is unclear what significance these experiments have with regard to human marrow stem cell transduction.

Recombinant AAV is among the newest of genetic transfer vectors. This once obscure virus possesses unique properties that distinguish it from all other vectors. Its major advantage is the lack of pathogenicity in humans. Wild-type AAV has the unusual ability to selectively integrate into the mammalian genome at a specific region, thus reducing the concern for genomic disruption and insertional mutagenesis. The ability of AAV to carry regulatory elements without interference from the viral template may enable greater control of transferred gene expression. Disadvantages currently include the inferior packaging systems which yield low numbers of recombinant virions which are contaminated with wild-type adenovirus. The small AAV genome that can be packaged (~5 kb) rules out its use for transfer of larger genes. Recombinant AAV viruses do not appear to demonstrate the same site-specific genomic integration as wild-type viruses. Elucidation of the mechanism of site-specific integration should prove useful in the development of safe vectors for gene transfer as well as provide insight into the nature of DNA recombination in humans.

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