A Transgenic Mouse Model for DNA/RNA Gene Therapy of Human β Thalassemia

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Summary

The βIVS-2-654 C→T mutation accounts for approximately 20% of β thalassemia mutation in southern China; it causes aberrant RNA splicing and leads to β thalassemia. To provide an animal model for testing therapies for correcting splicing defects, we have produced two lines of transgenic mice with the human β thalassemia mutant gene. The transgenic mice carrying this mutant gene show the same aberrant splicing as their human counterparts and provide an animal model for testing therapies to correct splicing defects at either the RNA or DNA level.

Introduction

β thalassemia is an anemia of varying severity resulting from mutations that lead to a decrease in β globin subunits available. By now nearly one hundred β thalassemia mutations have been described (*Bunn et al., 1986; Kazazian et al., 1988; Kazazian, 1990*): the most frequent types are a point mutation occurring in an intron, which activates aberrant splicing sites. For example, in β IVS-2-654 the C→T transition at nucleotide 654 of intron 2 creates an additional 5' donor splice site at position 652 and activates an endogenous cryptic 3' acceptor site at position 579 (*Zhang et al., 1988)*. Spliced β IVS-2-654 mRNA retains nucleotides 580-652 of the second intron and, as a result, does not encode a functional β globin polypeptide. This particular splice mutation is frequent among patients in China and Thailand (*Kazazian et al., 1988; Cheng et al., 1984*), accounting for 20% of β thalassemia in some regions. Splice mutations occurring in an intron of an affected gene are uniquely suited to a new type of therapeutic approach. Because the coding sequences necessary for function are intact, the blocking of the aberrant splice sites with antisense oligonucleotides may enable formation of correctly spliced and functional mRNA. Sierakowska et al. (*1996*) have recently tested this idea and demonstrated that aberrant splicing caused by the β IVS-2-654 mutation can be partially corrected in tissue culture by antisense olionucleotides. However, testing this type of oligotherapy (or any other type of therapy) in vivo requires a suitable animal model. The design of the oligonucleotides, and their delivery to the target cells, must be optimized, and the efficacies of the treatment have to be evaluated – including the therapeutic range of mRNA production, the duration of their effect, and the possible occurrence of immunological responses. The model animal should carry the mutant globin gene in a form such that correction of the splicing mutation will yield normally functional mRNA. To achieve this goal, we have cloned the human β IVS-2-654 mutant gene and established transgenic mice of this mutant gene. Transgenic mice with this mutant gene show the same aberrant splicing as their human counterparts.

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Material and Methods

All described procedures were approved by the Institutional Animal Care and Use Committee and conducted in compliance with the Guide for the Care and Use of Laboratory Animals of China.

Cloning of the human thalassemia mutant gene

Peripheral blood was collected from a homozygous β IVS-2-654 patient. DNA was prepared by standard procedures. The intact β IVS-2-654 gene was amplified by PCR with primers corresponding to HUMHBB nts 62013-65327, based on the published DNA sequence of human β globin gene.

Cloning and verification of the recombinant plasmid

The pBGT-51 was used as a basal vector which was generously provided by Dr. James Ellis (Department of Genetics, Hospital for Sick Children, Toronto, Canada. jellis@sickkids.on.ca). It was constructed by introducing a 3.0 kb human β LCR fragment and a 4.2kb human β globin gene into the site EcoR V of the plasmid Litmus 38 (New England Biolabs). The β LCR contains three core elements HS4, HS3 and HS2. The β globin gene can be isolated by two-enzymes digestion of Acc65I and NdeI (TaKaRa). The β IVS-2-654 gene was cohesive-end-cloned into the vector between two cloning sides named as Acc65I and NdeI. The recombinant plasmid constructed was verified by enzyme-digestion and sequencing.

Purification of microinjected DNA and microinjection

Plasmid DNA was extracted. Target gene fragments were liberated from their plasmid backbones by digestion with EcoR V(TaKaRa). DNA fragments were recovered from 1.0% agarose gel slices using QIAguick Gel Extraction Kit (GIAGEN), and dissolved in injection buffer (buffer TE, pH 8.0). The general procedures for microinjection of the extraneous gene were described previously by Magram et al. (*1985*). Fertilized mouse eggs were flushed from the oviducts of superovulated KUNMING mice approximately 6-8 h following ovulation. Male pronuclei of the fertilized eggs were injected with 2 pl of DNA solution (approximately 3 ng/pl), and viable embryos were reimplanted in the oviducts of pseudopregnant mice as previously described (*Magram et al., 1985*). After birth, the animals were tested for the presence of the extraneous gene by PCR of tail samples taken after weaning at 3 weeks of age.

Polymerase chain reaction

New-borne mice were obtained by natural delivery or by Caesarean birth. Tail samples were obtained from 3-to-4-week-old mice. Genomic DNA was extracted by standard procedures. Transgenic founder mice were screened by PCR. The primers used for PCR screen were: (a) 5'-GTG TAC ACA TAT TGA CCA AA-3'; and (b) 5'-AGC ACA CAG ACC AGC ACG TT-3' which correspond to HUMHBB nts (64238-64639). The PCR mixture, containing 2µl of primer (a) and (b) each, 5µl deoxyribonucleotide triphosphates each, 5µl reaction buffer 2U Taq polymerase (TaKaRa) and 2µl DNA template, had its total volume adjusted to 50µl by adding double distilled water. Then the mixture was overlaid with 50µl mineral oil and subjected to amplification for 30 cycles. Each cycle consisted of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds. Positive and negative controls were set up in the PCR identification. The PCR products was separated on a 1.0% agarose gel and photographed.

The transgenic mouse colony

A monogamous mating system was used, leaving stud and dam together even through pregnancy and nursing. The first generation (F1) was obtained from crossing founder mice with normal KUNMING mice (either by crossing transgenic male mouse to normal female mouse; or transgenic female mouse to normal male mouse) to produce littermates of two genotypes: transgenic mouse, non-transgenic mouse. The animals of the second

generation (F2) resulted from brother-sister F1 mating (total of 17 pairs), either by intercrossing transgenic male mouse to non-transgenic female mouse or transgenic female mouse to non-transgenic male mouse. This mating system was applied to regulate the number of transgenic animals used for future research purposes. Pups were weaned at 21 days of age. All animals were group-housed in shoe box cages, with iron tops under climate-controlled conditions on a 12-h light/dark cycle, fed and provided with tap water *ad libitum*.

Reverse transcriptase-polymerase chain reaction
Peripheral blood was sampled from mouse tail-veins in micro tubes previously rinsed with sterile acid citrate dextrose. Total RNA was prepared using Fast RNA isolation Micro Kit (for RT-PCR) as described by the manufacturer. Total RNA was reverse-transcribed to obtain the cDNA that was going to be amplified. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on cDNA with AMV enzyme(TaKaRa) for 28 cycles of 95°C/1 minute, 65°C/30 seconds and 72°C/45 seconds. The primers used to determine aberrant splicing of the human β globin pre-mRNA were: (a) 5'-GGA CCC AGA GGT TCT TTG AGT CC-3'; and (b) 5'-GCA CAC AGA CCA GCA CGT TGC CC-3', which correspond respectively to nucleotides 21-43 of the second exon and nucleotides 28-6 of the third exon. PCR was also performed with the above same system except for adding 1 ∝l cDNA to the PCR mixture. A 450bp fragment of β-actin mRNA was also amplified by RT-PCR as the internal control. The PCR products were identified on a 1.0% agarose gel and photographed.

Analysis of hemoglobin
Peripheral blood was sampled from mouse tail-veins and analyzed using the Vivant hemoglobin analysator (BIO-RAD company). Peripheral blood was prepared as described by the manufacturer and the result was calculated by computer. We set up a negative control using normal KUNMING mice and the results were compared with their human counterparts.

Clinical observation of transgenic mice
We observed the transgenic animals' mental status, dietary status and activity every day, and recorded if their appearance was abnormal.

Results
The human β IVS-2-654 gene amplified was a 3314bp fragment. The recombinant plasmid constructed was 9.2kb. Digested with Bcg I (TaKaRa), the recombinant plasmid decomposed into two fragments of 7.2kb and 2.0kb. This result demonstrated that the β IVS-2-654 gene in the recombinant plasmid was in the direction of 3' to 5', which was the direction of the β LCR in the plasmid. Compared with the sequence in GenBank, the sequence obtained by sequencing showed that there was a C→T mutation in the second intron of the β IVS-2-654 gene. The 6.4kb fragments containing the 3.0kb β LCR and the 3.4kb β IVS-2-654 gene were recovered from their plasmid backbones.
Of 33 mice which were initially PCR screened for the presence of the extraneous gene with primers specific to the human β IVS-2-654 gene, two mice were positive for integration, one male and one female. The PCR product was 400bp long. Sequencing result showed that there was a C→T mutation in the genome of the founder mice. We obtained two lines from two founder mice, the data of integration are shown in Table 1 and 2.

Table 1. Characteristics of transgenic line from female founder

Generation	Total mice	Transgenic mice	Integration frequency	Transcription ratio (based on the transgenic mice)
F1	38	6	16%	80%
F2	44	10	23%	76%

Table 2. Characteristics of transgenic line from male founder

Generation	Total mice	Trans-genic mice	Integration frequency	Transcrip-tion ratio (based on the trans-genic mice)
F1	43	11	26%	76%
F2	57	21	37%	75%

The result of RT-PCR using the RNA from the transgenic mice demonstrated the existence of a 303bp fragment. No amplification was observed with RNA from normal KUNMING mice (data not shown). Overall our data establish unequivocally that the human β IVS-2-654 gene is transcribed in the transgenic mice and that all of the corresponding processed mRNA is 73-bp larger than that expected from normal processing.

The peak value difference between the normal hemoglobin and the β thalassemia heterozygote is obvious in humans. But the result of hemoglobin analysis indicated that, the hemoglobin type, distribution and the total quantity in transgenic mice are similar to that of a normal mouse. The results indicated that transgenic mice, even though the exogenous gene had transcripted to a certain extent, the resulting protein levels are too low to detected.

The clinical observation of transgenic mice showed that the mental status, dietary status and activity of transgenic animals are similar to that of a normal mouse. All analysis conducted above indicated that the transgenic mouse does not present the typical clinical symptom of β thalassemia.

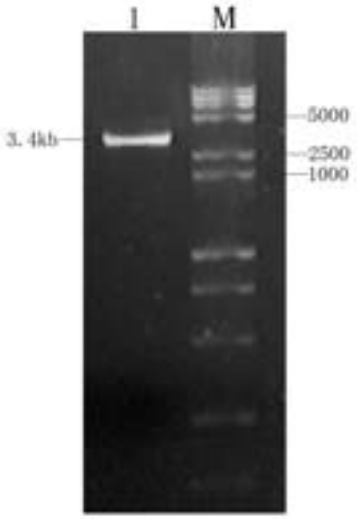

Figure 1. The results of PCR
M: DL2000+15000 Marker
Lane **1**:βIVS-2-654PCR product

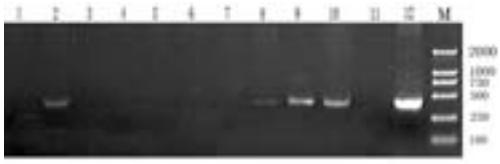

Figure 2. The results of PCR
M: DL2000 Marker
Lane 12: Positive control
Lane 11: Negative control
Lane 2, 9, 10: Positive result
Lane 1, 3, 4, 5, 6, 7, 8: Negative result

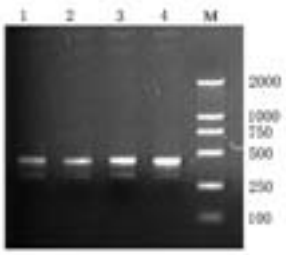

Figure 3. The results of reverse transscription PCR amplified transgenic mice.
M: DL2000 Marker
Lane1, 2, 3, 4: Positive result

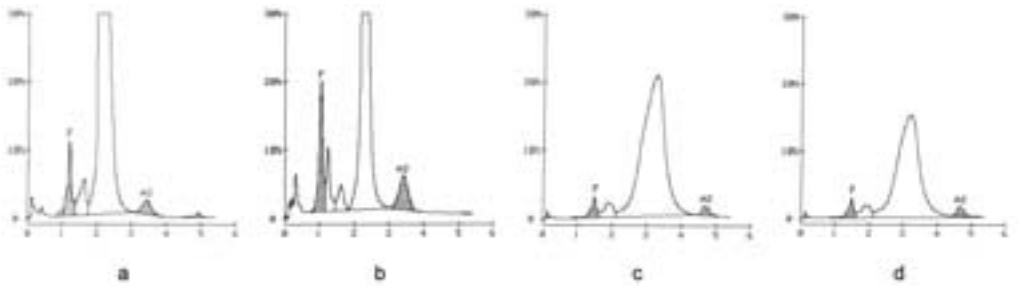

Figure 4. The results of hemoglobin analysis of humanβIVS-2-654 globin gene transgenic mice.
a. Normal human hemoglobin; **b.** Heterozygous patient of thalassemia(βIVS-2-654, M/N) hemoglobin;
c. Normal mice hemoglobin; **d.** Transgenic positive mice hemoglobin.

Discussion

In this research, we have cloned the human β IVS-2-654 gene and have established transgenic mice carrying this mutant gene. The C→T mutation at nucleotide 654 of intron 2 in the human β globin gene is one of the gene mutations causing aberrant splicing. Because of the mutation, an additional 5' donor splice site at position 652 is created and an endogenous cryptic 3' acceptor site at position 579 is activated. The mutant pre-mRNA is spliced in an abnormal manner, and the spliced mRNA retains nucleotides 580-652 of the second intron. Specific primers to human β globin exons 2 and 3 were used in RT-PCR to analyse the RNA from the peripheral blood cells of the transgenic mice. A 303bp fragment could be detected. The fragment corresponds to the aberrantly spliced mRNA. The results demonstrated that the human β IVS-2-654 gene was transcribed in the transgenic mice.

In the transgenic mice, we did not detect the expression product of the exogenous gene successfully by using Vivant hemoglobin analysator; notwithstanding it had been transcribed. When fragments of DNA containing one of the human β -like globin genes are randomly integrated into the genome of transgenic mice, the result is generally very low level expression of the transgene in a small percentage of transgenic lines (*Magram et al., 1985; Townes et al., 1985; Magram et al., 1989; Behringer et al., 1987; Kollias et al., 1987; Tuan et al., 1984; Grosveld et al., 1987)*. However, these transgenes are appropriately expressed at the correct developmental stage and specifically in red blood cells, suggesting that the tissue and development-specific information required for targeted expression lies within or near the genes themselves. Grosveld and colleagues *(Ryan et al., 1989*) indeed showed that linkage of this upstream region to globin genes in transgenic mice greatly increased the percentage of mice that expressed the transgene, and also increased the level of output of the linked globin genes at each integration site, so that transgenes demonstrated integration site-independent, copy number-dependent expression. We obtained by PCR the human β IVS-2-654 gene, which included the promoter, exons, introns, and the poly(A) signal. The β IVS-2-654 gene was cloned into the pBGT-51 to replace the normal human β globin gene in the plasmid. With the help of the β LCR, we hope to make the β IVS-2-654 gene, expressing in an erythroid-specific, integration site-independent, and high level manner, in transgenic mice. However, more recent studies have suggested that linkage of single hypersensitive sites to globin genes does not eliminate integration site-specific variation (*Curtin et al., 1989; Fraser et al., 1990; Talbot et al., 1990; Caterina et al., 1991; Liu et al., 1992; Ellis et al., 1993; Caterina et al., 1994; Robertson et al., 1995*). Collectively, these studies have shown that the variation in output seen among different transgenic animals can largely be explained by different integration sites in the transgenic animals (*Walters*

et al., 1995). These studies complement work that has been performed in tissue culture cells, where others have observed that LCR elements seem to act by increasing the probability that an individual integration event will be transcriptionally productive, not by increasing the output of the promoter linked to the LCR element (*Moon et al., 1990; Moreau et al., 1981; Weintraub et al., 1988; Skow et al., 1983*). Our experimental results have provided strong evidence for this theory in vivo. With the help of β LCR, the transgene has transcribed in the transgenic mice; but we cannot detect its output. This phenomenon can be explained by the random integration site of the β IVS-2-654 gene. And the experimental result also told us that linkage of the LCR element does not eliminate integration site-specific variation and increase the output of the β IVS-2-654 gene.

All analysis conducted above indicated that the transgenic mouse does not present the typical clinical symptom of β thalassemia. We note that the transgenic mouse itself bears normal β globin gene. Therefore the introduced extraneous gene, which even if it can produce pathological β globin to a certain level, is still unable to cause the transgenic mouse to present the characteristic β thalassemia clinical symptoms.

Four types of mouse models for β thalassemia have been described. In the first model, which is a naturally occurring deletion, one of the two mouse adult β globin genes, β major, is deleted *(Shehee et al., 1993)*. About 60% of mice homozygous for this deletion survive to adulthood. Heterozygotes show very mild thalassemia. The second model for β thalassemia was created by insertional disruption by gene targeting of the mouse adult β major globin gene (*Ciavatta et al., 1995*). Mice homozygous for this mutation do not survive past a few hours after birth. The heterozygotes are anemic and have features of thalassemia similar to those found in human β thalassemia intermedia. Other two models were produced by complete deletions of the murine adult β globin genes, β major and β minor (*Ciavatta et al., 1995;* Yang *et al., 1995)*. The phenotypes of the heterozygotes for these two models are equivalent and include microcytic anemia and splenomegaly. The homozygotes die immediately after birth.

The four types of mouse models described above, who possess the β thalassemia clinical situation, is due to their endogenous β globin gene having defected naturally or anthropogenically. Those mouse models are not ideal animal models for β thalassemia gene therapy research in vivo, because the target of gene therapy is hominal β IVS-2-654 mutant gene and /or its aberrant splicing, but not rodent ones. In additon, those β thalassemia mouse models have weak bionergy, readily die and one hard to breed.

The present mouse model for β thalassemia is a transgenic mouse carrying a human gene with β IVS-2-654 splice mutation and the nomal mouse β globin gene. The exogenous gene is transcribed in the transgenic mouse but can not cause the β thalassemia symptoms. So the transgenic mouse has strong bionergy and productivity, and can form a stationary genetic clone in a short time.

Thus, the present transgenic mouse provides an animal model of β IVS-2-654 thalassemia resulting from a splicing mutation. In addition, unlike mouse models for thalassemia caused by complete inactivation or deletion of genes in which direct gene therapy requires the addition of a functional gene, the transgenic animal can be treated in ways designed to correct the aberrant splicing at both the RNA and DNA level. Even a small increase in the production of correctly spliced mRNA should be detectable without the need to kill the animals by testing for human β globin mRNA in their circulating RBC or reticulocytes. The present mouse will therefore provide an animal model in which the antisense and other types of gene therapy can be tested in vivo.

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References

Behringer RR, RE Hammer, RL Brinster, RD Palmiter & TM Townes: Two 3' sequences direct adult erythroid-specific expression of human beta-globin genes in transgenic mice. Proc Natl Acad Sci U S A. 1987,*84*,7056-7060.

Bunn HF & BG Forget: Hemoglobin: Molecular, Genetic, and Clinical Aspects. Philadelphia, PA, Saunders, 1986

Caterina JJ, TM Ryan, KM Pawlik, RD Palmiter, RL Brinster, RR Behringer & TM Townes: Human beta-globin locus control region: analysis of the 5' DNase I hypersensitive site HS 2 in transgenic mice. Proc Natl Acad Sci U S A. 1991, *88*,1626-1630.

Caterina JJ, DJ Ciavatta, D Donze, RR Behringer & TM Townes: Multiple elements in human beta-globin locus control region 5' HS 2 are involved in enhancer activity and position-independent, transgene expression. Nucleic Acids Res. 1994,*22*,1006-1011.

Chada K, J Magram, K Raphael, G Radice, E Lacy & F Costantini: Specific expression of a foreign beta-globin gene in erythroid cells of transgenic mice. Nature. 1985, *314*,377-380.

Cheng TC, SH Orkin, SE Antonarakis, MJ Potter, JP Sexton, AF Markham, PJ Giardina, A Li & HH Kazazian Jr: beta-Thalassemia in Chinese: use of in vivo RNA analysis and oligonucleotide hybridization in systematic characterization of molecular defects. Proc Natl Acad Sci U S A. 1984, *81*,2821-2825.

Ciavatta DJ, TM Ryan, SC Farmer & TM Townes: Mouse model of human beta zero thalassemia: targeted deletion of the mouse beta maj- and beta min-globin genes in embryonic stem cells. Proc Natl Acad Sci U S A. 1995,*92*,9259-9263.

Curtin PT, DP Liu W Liu, JC Chang & YW Kan: Human beta-globin gene expression in transgenic mice is enhanced by a distant DNase I hypersensitive site. Proc Natl Acad Sci U S A. 1989, *86*, 7082-7086.

Ellis J, D Talbot, N Dillon & F Grosveld: Synthetic human beta-globin 5'HS2 constructs function as locus control regions only in multicopy transgene concatamers. EMBO J. 1993, *12*,127-134.

Fraser P, J Hurst, P Collis & F Grosveld: DNaseI hypersensitive sites 1, 2 and 3 of the human beta-globin dominant control region direct position-independent expression. Nucleic Acids Res. 1990, *18*, 3503-3508.

Grosveld F, GB van Assendelft, DR Greaves, G Kollias: Position-independent, high-level expression of the human beta-globin gene in transgenic mice. Cell. 1987,*51*,975-985.

Kazazian HH Jr, & CD Boehm: Molecular basis and prenatal diagnosis of beta-thalassemia. Blood. 1988,*72*,1107-1116.

Kazazian HH Jr, CE Dowling, CD Boehm, TC Warren, EP Economou, J Katz & SE Antonarakis: Gene defects in beta-thalassemia and their prenatal diagnosis. Ann N Y Acad Sci. 1990, *612*, 1-14.

Kollias G, J Hurst, E deBoer & F Grosveld: The human beta-globin gene contains a downstream developmental specific enhancer. Nucleic Acids Res. 1987, *15*, 5739-5747.

Liu D, JC Chang, P Moi P, W Liu, YW Kan & PT Curtin: Dissection of the enhancer activity of beta-globin 5' DNase I-hypersensitive site 2 in transgenic mice. Proc Natl Acad Sci U S A. 1992, *89*,3899-3903.

Magram J, K Chada & F Costantini: Developmental regulation of a cloned adult beta-globin gene in transgenic mice. Nature. 1985, *315*, 338-340.

Magram J, K Niederreither & F Costantini: Beta-globin enhancers target expression of a heterologous gene to erythroid tissues of transgenic mice. Mol Cell Biol. 1989, *9*, 4581-4584.

Moon AM & TJ Ley: Conservation of the primary structure, organization, and function of the

human and mouse beta-globin locus-activating regions. Proc Natl Acad Sci U S A. 1990, *87*, 7693-7697.

Moreau P, R Hen, B Wasylyk, R Everett, MP Gaub & P Chambon: The SV40 72 base repair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucleic Acids Res. 1981, *9*, 6047-6068.

Robertson G, D Garrick, W Wu, M Kearns, D Martin & E Whitelaw: Position-dependent variegation of globin transgene expression in mice. Proc Natl Acad Sci U S A. 1995, *92*, 5371-5375.

Ryan TM, RR Behringer, NC Martin, TM Townes, RD Palmiter & RL Brinster: A single erythroid-specific DNase I super-hypersensitive site activates high levels of human beta-globin gene expression in transgenic mice. Genes Dev. 1989, *3*, 314-323.

Shehee WR, P Oliver & O Smithies: Lethal thalassemia after insertional disruption of the mouse major adult beta-globin gene. Proc Natl Acad Sci U S A. 1993, 90, 3177-3181.

Sierakowska H, MJ Sambade, S Agrawal & R Kole: Repair of thalassemic human beta-globin mRNA in mammalian cells by antisense oligonucleotides. Proc Natl Acad Sci U S A. 1996, *93*, 12840-12844.

Skow LC, BA Burkhart, FM JohnsonRA Popp, DM Popp, SZ Goldberg, WF Anderson, LB Barnett & SE Lewis: A mouse model for beta-thalassemia. Cell. 1983, *34*, 1043-1052.

Talbot D, S Philipsen, P Fraser & F Grosveld: Detailed analysis of the site 3 region of the human beta-globin dominant control region. EMBO J. 1990, *9*, 2169-2177.

Townes TM, JB Lingrel, HY Chen, RL Brinster & RD Palmiter: Erythroid-specific expression of human beta-globin genes in transgenic mice. EMBO J. 1985, *4*, 1715-1723.

Tuan D & IM London: Mapping of DNase I-hypersensitive sites in the upstream DNA of human embryonic epsilon-globin gene in K562 leukemia cells. Proc Natl Acad Sci U S A. 1984, *81*, 2718-2722.

Walters MC, S Fiering, J Eidemiller, W Magis, M Groudine & DI Martin: Enhancers increase the probability but not the level of gene expression. Proc Natl Acad Sci U S A. 1995, *92*, 7125-7129.

Weintraub H: Formation of stable transcription complexes as assayed by analysis of individual templates. Proc Natl Acad Sci U S A. 1988, *85*, 5819-5823.

Yang B, S Kirby, J Lewis, PJ Detloff, N Maeda & O Smithies: A mouse model for beta 0-thalassemia.Proc Natl Acad Sci U S A. 1995, 92, 11608-11612.

Zhang JZ, SP Cai, X He, HX Lin, HJ Lin, ZG Huang, FF Chehab & YW Kan: Molecular basis of beta thalassemia in south China. Strategy for DNA analysis. Hum Genet. 1988, *78*, 37-40.