Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN

BIOCHIMICA, BIOLOGIA MOLECOLARE E BIOTECNOLOGIE

CICLO XXI

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Strategies for the adult haemoglobin (HbA) production in β^0-thalassemia patients

Settore Scientifico Disciplinare BIO/10

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Anni 2006/2008

To Auro, Lena, Ramo and Enio…

…I'll see you again when the stars fall from the sky…

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ABSTRACT

Background: Le mutazioni nonsenso, dando origine ai codoni di stop UAA, UGA, UAG all'interno della sequenza codificante degli mRNA, promuovono la terminazione prematura della traduzione e causano circa il 30% delle malattie ereditarie, fra cui fibrosi cistica, distrofia muscolare di Duchenne, talassemia.

Attualmente, due sono gli approcci per la potenziale cura di malattie causate da mutazioni nonsenso: la terapia genica, intesa come introduzione di un gene esogeno, e il proseguimento della lettura da parte dei ribosomi (o tecnica read-through) indotto dagli aminoglicosidi, i quali diminuiscono l'accuratezza della fase di allungamento e riducono l'efficacia del complesso di terminazione della traduzione. Recenti pubblicazioni hanno descritto composti disegnati e prodotti per sopprimere la terminazione prematura della traduzione, inducendo il proseguimento della lettura da parte del ribosoma oltre codoni di stop prematuri, senza alterare il riconoscimento dei normali codoni di terminazione. Dall'altro lato, negli ultimi anni, numerosi gruppi di ricerca sono stati impegnati nello sviluppo di vettori lentivirali sicuri, cercando di eliminare effetti collaterali quali l'attivazione di oncogeni e il silenziamento del transgene.

Scopo della tesi: Il nostro obiettivo è stato quello di verificare gli effetti e l'utilità clinica nella β^0-talassemia della correzione a livello traduzionale delle mutazioni nonsenso tramite read-through e della terapia genica.

Metodi: Come prima cosa, siamo partiti dallo sviluppo di un modello cellulare della mutazione talassemica $\beta^0$39, utilizzabile per lo screening di un elevato numero di aminoglicosidi e molecole analoghe. Abbiamo prodotto due vettori lentivirali contenenti il gene β globinico wild type o $\beta^0$39 sotto il controllo di una regione di controllo LCR minimale e usato tali costrutti per la transduzione di cellule K562, successivamente subclonate, con lo scopo di ottenere numerosi cloni cellulari con un diverso numero di costrutti integrati. Questi cloni sono stati,

quindi, trattati con *geneticina* (*G418*) e altri aminoglicosidi e l'eventuale produzione di β globina analizzata tramite citofluorimetro.

In secondo luogo, abbiano effettuato lo screening di numerosi pazienti talassemici e isolato le cellule progenitrici eritroidi da quegli individui risultati omozigoti per la mutazione talassemica $\beta^0$39. Abbiamo, quindi, trattato tali cellule con la *geneticina* e analizzato l'eventuale produzione di β globina ed emoglobina adulta (HbA) tramite FACS e HPLC, rispettivamente.

Infine, abbiamo sottoposto i precursori eritroidi $\beta^0$39 alla terapia genica, utilizzando il vettore lentivirale esprimente il trascritto wild type della β globina, con lo scopo di verificare l'aumento della HbA tramite analisi con HPLC. Inoltre, trattando le cellule trasdotte con la *mitramicina*, abbiamo voluto verificare gli effetti combinati della terapia genica e dell'induzione dell'emoglobina fetale.

Risultati: Abbiamo ottenuto e caratterizzato 6 cloni contenenti il gene β globinico mutato $\beta^0$39 e sette contenenti il gene β globinico wild type, da usare come controllo. Abbiamo, quindi, scelto 2 cloni, uno βwt e uno $\beta^0$39, che mostravano un'espressione simile del gene globinico, e li abbiamo usati per testare svariati aminoglicosidi, per verificarne l'attività di read-through. La *geneticina* (*G418*) e la *gentamicina* hanno mostrato la maggiore capacità di indurre la correzione traduzionale della mutazione nonsenso, sebbene l'effetto prodotto dal *G418* sia stato maggiore di quello provocato dalla *gentamicina*. Un aumento significativo del numero di cellule contenenti β globina è stato, inoltre, rilevato in cellule progenitrici eritroidi $\beta^0$39 trattate con *G418*. Quest'ultimo risultato è stato confermato anche tramite HPLC, la quale ha mostrato un aumento nella concentrazione relativa di HbA rispetto alle emoglobine totali.

Infine, abbiamo ottenuto un forte incremento nella percentuale di HbA quando gli stessi precursori eritroidi sono stati sottoposti a terapia genica, tramite trasduzione con il vettore lentivirale contenente il gene β globinico wild type, ed un netto incremento di HbF è stato, inoltre, evidenziato dopo trattamento con *mitramicina*.

Conclusione: Entrambi gli approcci terapeutici per la cura delle β^0-thalassemie analizzati in questo lavoro, e cioè il proseguimento della lettura o read-through e la terapia genica, sebbene presentino ancora numerosi svantaggi, fra cui la tossicità degli aminoglicosidi e l'incertezza degli effetti dei vettori lentivirali sui geni endogeni, possono essere considerati tecniche con un elevato potenziale curativo. Naturalmente, nel caso della strategia read-through è augurabile l'identificazione di nuovi composti con un effetto terapeutico maggiore di quello degli aminoglicosidi, con minore tossicità e possibile somministrazione orale, mentre nel caso della terapia genica, ulteriori ricerche dovrebbero essere effettuate per eliminare i suoi ben noti svantaggi, fra cui in primo luogo la possibile attivazione di oncogeni.

ABSTRACT

Background: Nonsense mutations, giving rise to UAA, UGA, UAG stop codons within the coding region of mRNAs, promote premature translational termination and are the leading cause of about 30% of inherited diseases, including cystic fibrosis, Duchenne muscular dystrophy, thalassemia.

Currently, there are two approaches to directly overcome diseases caused by nonsense mutations: gene therapy, meaning introduction of an exogenous gene, and translational read-through induced by aminoglycosides, which decrease the accuracy of translation elongation and reduce the efficacy of the translation termination machinery. Interestingly, recent papers have described drugs designed and produced for suppressing premature translational termination, inducing a ribosomal read-through of premature but not normal termination codons. On the other hand, in recent years, many investigators have been studying the development of safe lentiviral vectors to avoid the side effects as activation of oncogenes and transcription silencing.

Aim: Our purpose was to verify the effects and clinical utility in β^0-thalassemia of translational read-through and gene therapy.

Methods: First, we started the development of a cellular model of the $\beta^0$39-thalassemia mutation that could be used for the screening of high numbers of aminoglycosides and analogous molecules. We produced two lentiviral vectors containing the βwt- or $\beta^0$39-thalassemia globin gene under a minimal LCR control region and used such constructs for the transduction of K562 cells, subsequently subcloned, with the purpose to obtain several K562 clones with different copy-number of the integrated constructs. These clones were then treated with *geneticin* (*G418*) and other aminoglycosides and the production of β-globin was analysed by FACS analysis.

Secondly, we screened several thalassemia patients and isolated erythroid progenitors from such individuals resulted homozygous for the $\beta^0$39 mutation. Then, we treated the cells with *geneticin* and analysed the production of β-globin and adult haemoglobin (HbA) by FACS and HPLC analysis, respectively.

Finally, we subjected the $\beta^0$39 erythroid precursor cells to gene therapy, through the use of the lentiviral vector expressing the βwt-globin mRNA, with the aim to verify the increase of HbA through HPLC analysis. Moreover, by treating the transduced cells with *mithramycin* we wanted to verify the combined effects of gene therapy and fetal haemoglobin (HbF) induction.

Main results: We obtained and characterized six clones carrying the $\beta^0$39-globin gene and seven clones carrying the βwt-globin gene, to be used as a control. We then chose two clones, a βwt and a $\beta^0$39, showing similar content of mRNA and used them to test various aminoglycosides to verify their read-through activity. *Geneticin* (*G418*) and *gentamicin* showed the highest capacity to read-through of nonsense mutation, although the effect of *G418* was found to be higher than that displayed by *gentamicin*. A significant increase in β-globin containing cells was also detected when $\beta^0$39 progenitor cells were treated with *G418*. This last result was confirmed by HPLC analysis, which showed an increase in the relative concentration of HbA compared to total Hbs.

Finally, we obtained a high increase in the proportion of HbA when the same progenitor cells were subjected to gene therapy, through transduction with the lentiviral vector containing the βwt-globin gene, and a clear increment of HbF was detected after treatment with *mithramycin*.

Conclusion: Both the therapeutic approaches for the cure of β^0-thalassemias analysed in this work, namely translational read-through and gene therapy, while still presenting many disadvantages, including toxicity of aminoglycosides and the uncertainty of the effects of lentiviral vectors on endogenous genes, can be considered techniques with a high curative potential. Naturally, in the case of translational read-through is hoped the identification of new compounds with a

therapeutic effect similar to but greater than that of aminoglycosides, with less toxicity and oral bioavailability, while in the case of gene therapy, further investigations should be made to eliminate its well known disadvantages, primarily including the possible activation of oncogenes.

GLOSSARY

Amp: ampicillin

ASL: anticodon stem-loop

ATM: ataxia-teleangiectasia

BFUe: burst-forming unit(s)

bp: base pair

BSA: bovine serum albumine

CFTR: cystic fibrosis transmembrane conductance regulator

CFUe: colony-forming unit(s)

CMV: cytomegalovirus

cPPT: central polypurine tract

Ct: threshold cycle

DMD: Duchenne's muscular dystrophy

D-MEM: Dulbecco's modified eagle medium

dNTP: triphosphate deoxyribonucleotide

DPBS: Dulbecco's phosphate buffered saline

EF: elongation factor(s)

E-MEM: essential minimum eagle medium

EPO: erythropoietin

FBS: fetal bovine serum

FSC: forward light scatter

GFP: green fluorescent protein

GTP: guanosine triphosphate

GVHD: graft versus host disease

HbF: fetal haemoglobin

HbA: adult haemoglobin

HbA_2: type 2 adult haemoglobin

HIV-1: human immunodeficiency virus

HPLC: high performance liquid chromatography

HS: hypersensitive site(s)

IF: initiation factor(s)

I-MDM: Iscove’s modified Dulbecco’s medium

HSC: hematopoietic stem cell(s)

LCR: locus control region

LTR: long terminal repeat

MCV: average globular volume

MCH: average globular content of Hb

MEL: murine erythroleukemia cells

MoAb: monoclonal antibody

mRNA: messenger RNA

NMD: nonsense mediated decay

nt: nucleotide

NTP: triphosphate ribonucleotide

PABPC1: major cytoplasmatic PABP (poly(A)-binding protein

PBS: phosphate buffered saline

PE: phycoerythrin

PEG: polyethylene glycol

PTC: premature termination codon

RCL: replication competent lentivirus

RF: release factor(s)

RGO: resistance globular osmotic

RNP: ribonucleoprotein

RPMI: Roswell park memorial institute medium

RRE: rev responsive element

rRNA: ribosomal RNA

DS: standard deviation

SDF: small DNA fragment(s)

SFHR: small fragment homologous replacement

SIN: self-inactivating transfer vector

S/MAR: scaffold/matrix attachment region

SMG1-7: suppressor with morphological effect on genitalia (trans-acting factor involved in NMD)

SSC: sideward light scatter

tRNA: transfer RNA

UPF1-3: upframeshift 1-3 (trans-acting factor involved in NMD)

VSV-G: G glycoprotein of vesicular stomatitis virus

WPRE: Woodchuck hepatitis virus post-transcriptional regulatory element

INTRODUCTION

Haemoglobin

The haemoglobin (Hb) (**figure 1a**) is a globular tetramer, with a molecular weight of 64,500 dalton, located within the red cells of vertebrates, where it has the function to transport oxygen from the lungs to the tissues. This tetramer is composed of two pairs of identical α and β subunits. The four subunits have the same tertiary folding and they each contain eight α helices, which are identified as A through H [Fronticelli et al, 1995]. These helices are connected to one another by short loop regions and arranged so to form a pocket where the active site of the group, essential to bind the heme group, is located (**figure 1a**). The α helices wrap up the central core in different directions, so that helices adjacent in sequence become distant in structure [Branden & Tooze, 2001].

The ability to bind oxygen of the haemoglobin depends on the presence of a prosthetic group called heme, which contains an iron ion in a large heterocyclic ring called porphyrin (**figure 1a**). When the oxygen binds to the iron ion of the deoxy-haemoglobin, it pushes this ion inside the heme, and since the ion is linked to an amino acid of the protein, a histidine, this also is pushed through the plan (**figure 1b**). This conformational change is transmitted to the peptidic skeleton with consequent change in the three-dimensional structure of the subunits and variations in the level of interactions with adjacent subunits, which include the destruction of salt bonds and the formation of new hydrogen bonds and hydrophobic interactions. All this contributes to the formation of a new quaternary structure. The variations in the interactions between the different subunits are transmitted from the surface to the heme group of a second subunit and cause a more facilitated access of the oxygen to the ion iron, leading, so, a greater affinity of the haemoglobin for a second molecule of oxygen.

A normal individual owns four different types of haemoglobin: embryonic Hb, the first to be produced during the haematopoiesis; HbF (fetal Hb) typical of the

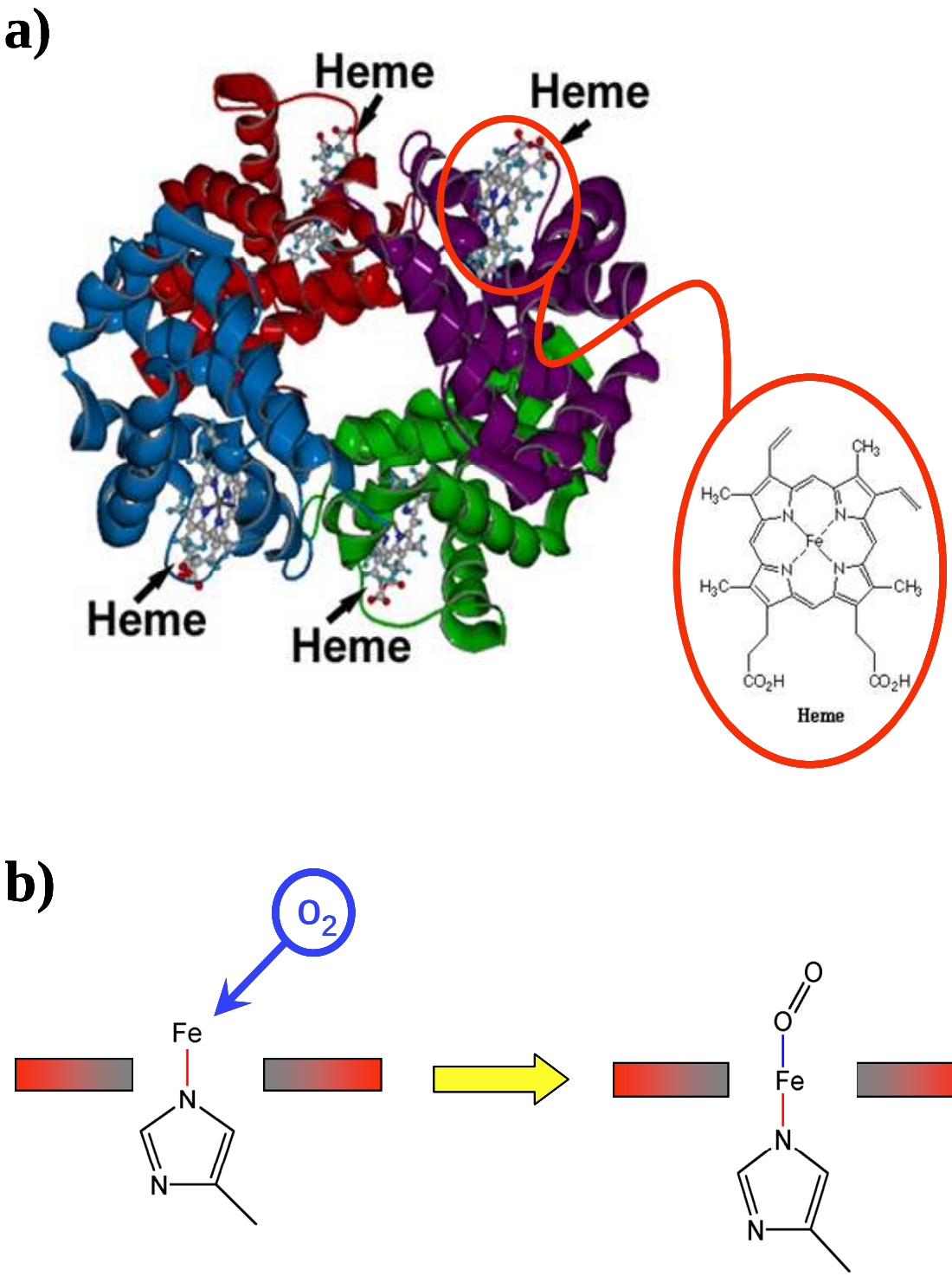


Figure 1 – Haemoglobin. a) Haemoglobin is a tetrameric globular protein, composed of two pairs of identical α and β subunits, having the same tertiary structure, consisting of eight α-helices connected to one another by short loop region and arranged so to form a pocket where the active site of the group, essential to link the heme group, is located. The ability to bind oxygen depends precisely on heme prosthetic group, which contains an iron ion in a large heterocyclic ring called porphyrin. **b**) When the oxygen binds to the iron ion of the deoxy-haemoglobin, it pushes the ion inside the heme plan and since the ion is bound to a histidine of the protein, this also is pushed through the plan, resulting in a conformational change of the entire subunit and variations in the level of interaction with the adjacent subunits. These variations are transmitted from the surface to the heme group of a second subunit, causing an easier access of the oxygen to the iron ion.

intrauterine life and present only in trace after birth; HbA (adult Hb), which is about the 98% of an adult subject haemoglobin; HbA_2, present only after birth and corresponding to the remaining 2%.

Globin clusters

The genes codifying for the α and β-like globin chains are put together in clusters (**figure 2**): the α-globin cluster is located on the short arm of chromosome 16, while the cluster β spans 80 Kb on the short arm of chromosome 11. In both clusters, the globin genes are arranged in the order in which they are expressed during development [Joy et al, 2000].

In the cluster β, upstream of the globin genes is the locus control region (LCR), a major regulatory element containing four erythroid-specific hypersensitive sites present at all stages of erythroid development (HS1–4) and a constitutive site (HS5) located further upstream. Two further HSs have been described by Routledge et al [2002]: HS6 and HS7, upstream of HS5.

The cluster β (**figure 2**) includes the embryonic block composed of ε gene, active until the 8th week of gestation; the fetal block with $^{G}\gamma$ and $^{A}\gamma$ genes, which produce globin chains that differ by one amino acid at position 136, namely glycine in $^{G}\gamma$ and alanine in $^{A}\gamma$, and are active from the 8th week of gestation until the 3rd month after birth; finally, the adult block with the δ and β genes producing globin chains different only for 10 amino acids including between the positions 22 and 115. These genes are activated gradually, as the activity of genes γ decreases (switch HbF – HbA) and expressed fully only after birth. The genes present in cluster β produce during the embryonic period the haemoglobins Gower I ($\zeta_2\varepsilon_2$), Portland ($\zeta_2\gamma_2$) and Gower II ($\alpha_2\varepsilon_2$); in the fetal period HbF ($\alpha_2\gamma_2$) and in the adult period HbA ($\alpha_2\beta_2$) and HbA_2 ($\alpha_2\delta_2$) [Bianco Silvestroni, 1998].

The cluster α (**figure 2**) includes an embryonic gene (ζ), a fetal-adult block consisted of the α_1 and α_2 genes, a few pseudo-genes ($\psi\xi$, $\psi\alpha_1$, $\psi\alpha_2$) and the θ gene, which is considered a pseudo-gene, because, while producing a mRNA, does not express any globin.

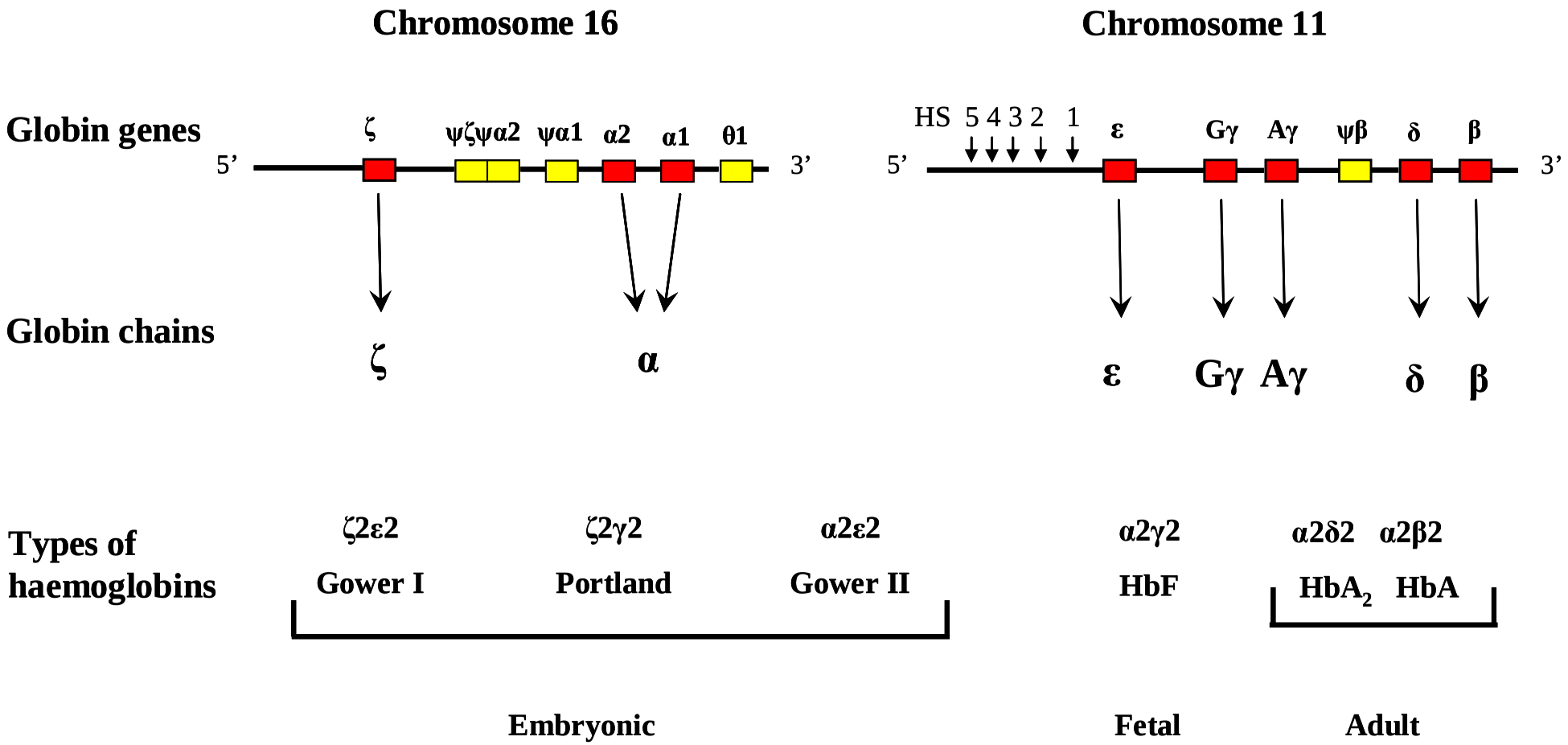


Figure 2 – Clusters of α and β globin genes with the globin chains and haemoglobin produced during each phase of development. α- and β-globin clusters are found on chromosomes 16 and 11 respectively, with genes that have a spatial order reflecting their temporal order. Highlighted in red are the genes that produce the globin chains, which are ζ, α, ε, β and γ. These form the tetramers of different haemoglobins, embryonic, fetal and adult. In yellow and with the letter ψ are indicated the pseudo-genes not expressed [Bianco Silvestroni, 1998].

Structure of the β-globin genes

The general structure of the β globin gene (**figure 3a**) is typical of other globin loci. The genomic sequence which encodes for 146 amino acids spans 1,600 bp, and are divided into three exons (coding regions) by two non-coding intervening sequences (introns or IVS). IVS1 interrupts the coding sequence within codon 30, and IVS2 between codons 104 and 105. Exon 2 encodes the residues involved in heme binding and αβ dimer formation while exons 1 and 3 encode the non-heme binding regions of the globin chain. Many of the amino acids involved in globin subunit interactions required for the Bohr effect and 2,3-DPG binding are found in exon 3. Conserved sequences important for gene function are found in the 5' promoter region, at the exon – intron junctions and in the 3' untranslated region (3'UTR) at the end of the mRNA sequences [Joy et al, 2000].

The promoter is an indispensable region for the efficient transcription of the gene. It includes several conserved sequences necessary for its efficient functioning (**figure 3a**), as recognized and bound by transcription factors. Among these are the sequence ATAA (or ATA-box), located about -30 nucleotides from the cap, the sequence CCAAT (or CAAT-box), located between -70 and -80 bp and the sequence CACCC, which is duplicated in a proximal and distal sequence, which show to exert equal influence on the transcription [Bianco Silvestroni, 1998].

The 5' untranslated region (5'UTR) occupies a region of 50 nucleotides between the very 5' terminus or 'cap' site of the β-globin mRNA and the initiation (ATG) codon. There are two prominently conserved sequences in the 5'UTR of the various globin genes (both α and β). One is the CTTCTG hexanucleotide found 8 through 13 nucleotides downstream from the cap site. The second conserved sequence is the CACCATG, in which the last three nucleotides form the initiation codon. The 3'UTR constitutes the region between the termination codon and the poly(A) tail (**figure 3a**). It is 132 nucleotides long with one conserved sequence, AATAAA, located 20 nucleotides upstream of the poly(A) tail. This consensus hexanucleotide serves as a signal for cleavage of the 3' end of the

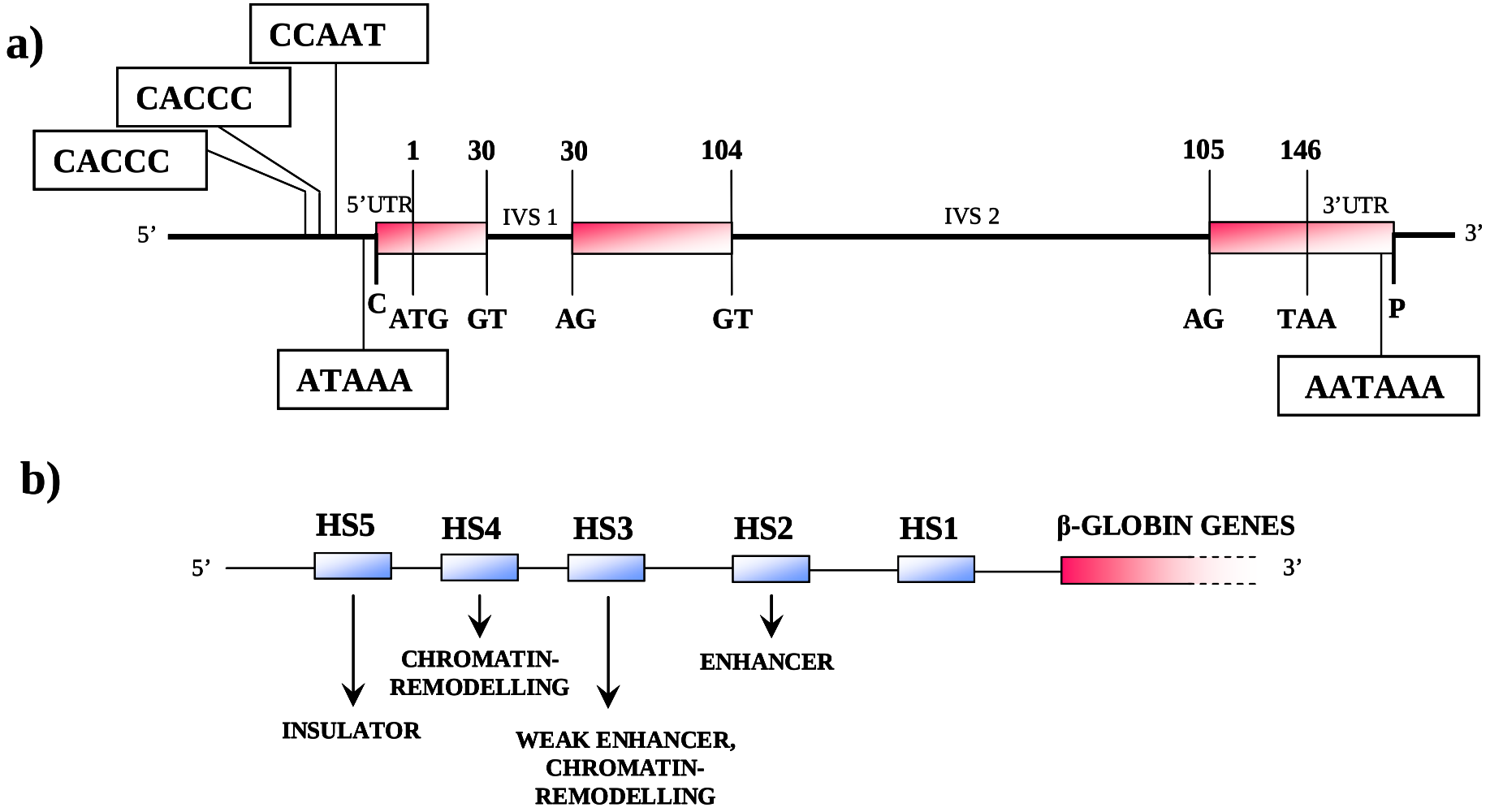


Figure 3 – β-globin gene and locus control region (LCR) structures. a) General structure of the β-globin gene with the exons (pink boxes), interrupted by the introns (IVS1 and 2), the 5' untranslated region (5'UTR), included between the cap site (C) and the initiation codon (ATG), the 3' untranslated region (3'UTR), containing the polyadenilation signal (AATAAA) and the promoter which contains different binding sites for transcription factors. **b**) General structure of the LCR region, located upstream of the globin gene (in pink). The hypersensitive sites (HS) are indicated with light blue boxes and their functions are reported below.

primary transcript and addition of the poly(A) tail which confers stability on the processed mRNA and enhances translation [Joy et al, 2000].

Locus control region (LCR)

The locus control regions (LCRs) have been identified in at least 36 loci of different species of mammals, including human, mouse, rat, rabbit and goat. As already mentioned, the LCR is physically defined by the presence of 5 HS (**figure 3b**), areas of nucleosomic destruction in which the DNA is susceptible to digestion by the DNase I. These areas make the region accessible to transcription and chromatin-remodeling factors [Harju et al, 2002].

It has been demonstrated, through the deletion of the LCR in mice and humans, that this region is necessary neither for the initiation and maintenance of an open chromatin structure, namely transcriptional active, nor for the transcription to baseline levels of various globin genes [Reik et al, 1998; Bender et al, 2000]. Bender et al [2000] proposed a model where the regulation of these two phenomena is attributed to several redundant factors, among which is also included the LCR, responsible only of the stimulation of transcription at high levels [Bender et al, 2000].

Individual HSs within the LCR appear to have different roles in chromatin remodeling and control of globin gene switching (**figure 3b**) [Harju et al, 2002]:

- HS1, located about 6 kb 5' to the ε-globin gene, may be dispensable for LCR function, since individuals with a deletion that encompasses this region show no sign of haematological defect [Hardison et al, 1997].
- HS2 is a general enhancer element that functions during all three developmental stages. Similar to other enhancers, HS2 encodes E-box sequences, which are binding sites for the basic helix-loop-helix family of transcription factor proteins, such as USF and Tal 1 (SCF). Binding of NF-E2 is directly correlated with HS2 enhancer function. However, HS2 does not display enhancer activity by itself in single-copy transgenes; it requires the presence of another LCR HS [Harju et al, 2002].

- HS3 is located upstream of HS2 and seems to have chromatin-opening function or chromatin-remodeling activity [Harju et al, 2002]. It causes high level expression of the β- or γ-globin genes after stable integration in MEL cells, but it does not enhance transient expression of these construct prior to integration. Such observations lead to assume that the principal effect of HS3 is on domain opening with at best a weak activity as a classical enhancer [Hardison et al, 1997].
- HS4 has no enhancer function by itself; perhaps it is required during development to achieve efficient domain opening by the LCR [Hardison et al, 1997].
- HS5 has always been considered as a constitutive hypersensitive site but Wai et al [2003] showed, on erythroid and not erythroid tissues of humans and mice, that it is, in fact, erythroid-specific. This site presents a unique function, namely that of insulator; in fact, it can insulate the β-globin gene from the enhancing effect of HS3 when it is positioned between the HS3 and the human β-globin gene [Li & Stomatoyannopoulos, 1994]. Insulator elements protect against the negative effects of neighboring heterochromatin and may serve as boundary elements that flank or demarcate an open, transcriptionally active chromatin domain. They may also block histone deacetylase activity [Harju et al, 2002] .

β-Thalassemia

The β-thalassemia is an autosomal recessive genetic disease, caused mainly by point mutations within and near the β globin gene. Nearly 200 different mutations have been described in patients with β-thalassemia and related disorders. Although most are small nucleotide substitutions within the cluster, deletions may also cause β-thalassemia. All the mutations result in either the absence of the synthesis of β-globin chains (β^0-thalassemia) or reduction in synthesis (β^+-thalassemia) [Olivieri, 1999]. The geographical distribution of these molecular defects is not uniform, because each ethnic population have common and rare alterations different from those of another population [Le Denmat et al, 1997].

Although β-thalassemia is considered, from a clinical point of view, as a recessive character, in fact, at haematological level, β-thalassaemic alleles behave

as dominant characters because it is well recognizable in heterozygous individuals the typical haematological phenotype of microcitemia [Bianco Silvestroni, 1998].

Classification of β-thalassemias

In β-thalassemias, it is possible to identify 4 different genotypes:

1) homozygosity with both alleles wild type.

2) heterozygosity with a wild type allele and one carrying the mutated β-globin gene: individuals with this genotype were called microcitemic and have the ability to transmit the mutated allele to their offspring. The sons of two microcitemic individuals, having the same mutation, have a 25% chance of being healthy homozygous, 50% being heterozygous, as parents, and 25% to be homozygous for the mutation.

3) heterozygosity for two different mutations of β-globin gene: this genotype correlates with thalassaemic phenotypes with various gravity, depending on the types of mutation present.

4) homozygosity for the same genetic defect: in most cases, the condition is the most severe thalassemia, called thalassemia major or Cooley's disease.

Because of the extensive number of mutations that can lead to a defective β-globin chain, it is possible to identify β-thalassaemic phenotypes of different gravity, based precisely on the type of defect present in the gene. Furthermore, depending on the fact that this defect could cause a reduction in the production of β-globin or its complete elimination, it is possible to subdivide the β-thalassemia in two large groups, β^+ and β^0, respectively.

Among the many β-thalassemia phenotypes, the most common are:

1) silent β-thalassemia: this term is used for a mild β-thalassemia condition which, in the heterozygote, is difficult to detect because the haematological parameters and the HbA_2 and HbF levels are essentially normal: only a slight imbalance in the *in vitro* chain synthesis can be observed [Gonzalez-Redondo et al, 1989]. These mutations have been usually identified by genetic and molecular analysis of families in which a proband was affected by thalassemia intermedia,

resulting from a compound heterozygous state for a typical β-thalassemia and silent β-thalassemia [Moi et al, 2004].

2) β-thalassemia trait (or microcitemia): it manifests itself in individuals heterozygous for a mutation in the β-globin gene. They have a mild anemia, decreased level of Hb, iperglobulia, reduced average globular volume (MCV), reduced average globular content of Hb (MCH), increased resistance globular osmotic (RGO) and abnormal erythrocyte morphology. The symptoms may, however, have different severity depending on the type of mutation [Bianco Silvestroni, 1998].

3) β-thalassemia intermedia: in a large number of patients with thalassemia intermedia, the reduced disease severity can be explained by the inheritance of milder β-thalassemia alleles that allow the production of a significant proportion of β-globin chains. A substantial number, however, have β^0-thalassaemia, and in such cases, the absence of β-globin chains is compensated by an inherent ability to produce fetal haemoglobin (HbF). Yet other thalassemic patients have inherited only one β-thalassemia allele. Most cases of unusually severe heterozygous β-thalassemia are due to the co-inheritance of extra α globin genes while others are due to the nature of the underlying β-thalassemia mutation itself. Hence, the underlying genotypes of β-thalassemia intermedia are extremely heterogeneous; the genetic basis can be the inheritance of one or two β-thalassemia alleles interacting with other genetic variables [Thein, 2004].

4) β-thalassemia major or Cooley's disease: it is the most serious form of β-thalassemia, characterized by a very small production of Hb or its total absence. It manifests itself in individuals homozygous for a β^0- or severe β^+-thalassemia allele, or in individuals heterozygous for two different β^0 and β^+ mutations. Patients with Cooley's disease have an anemia so severe that they undergo regular blood transfusions to survive [Thein, 2004].

Alterations of β-globin cluster

The alterations that may affect the β-globin gene are: deletions, defects of transcription, defects of mRNA maturation, defects of mRNA translation and defects that impair the stability of the β-globin chain.

Deletion of the β-globin gene: among genetic factors that give rise, if present in heterozygous, to β-microcitemias, deletions are quite rare. The existence of cases of microcitemia with a level of HbA_2 much higher than in normal β-thalassemia heterozygotes, often until 8 to 9%, was known even before the identification of the molecular defect. All these varieties have an apparent microcitemic haematological phenotype, with normal or slightly higher HbF and a ratio $\alpha/\beta>1$, and they are caused by extensive deletions that remove the β-globin gene but leave intact the δ-globin gene. Except a few rare exceptions, it is also deleted the region immediately upstream of β-globin gene that includes, at -200 bp, a binding site for NF-E2 protein. The increase in the proportion of HbA_2 is supported by the deletion of the 5' upstream of the β-globin gene [Bianco Silvestroni, 1998].

Defects of transcription: they are, in the great majority of cases, point mutations of the promoter. These mutations affect the various functional blocks of the region: the ATA box in which were found mutations in the nucleotides -28, -29, -30, -31; the proximal CACCC box with mutations in nucleotides -86, -87, -88, -90, -92; the distal CACCC box with the mutation -101 (C/T) [Bianco Silvestroni, 1998]. This last is associated, as those at nucleotides -90 and -92, with a phenotype of silent β-thalassemia [Gonzalez-Redondo et al, 1989].

All mutations in the promoter reduce, but never completely, the transcription of β-globin gene and therefore give explanation of the mild β-thalassemias and of some β-thalassemias with a silent or sub-silent phenotype [Bianco Silvestroni, 1998].

Defects in the mRNA maturation: these are divided in splicing defects and in cut and polyadenilation defects.

Splicing mutations: they are a very large and important group of mutations that cause about half of all β-thalassemias. The defects consist, usually, in point mutations that, with various mechanisms, alter the normal process of splicing.

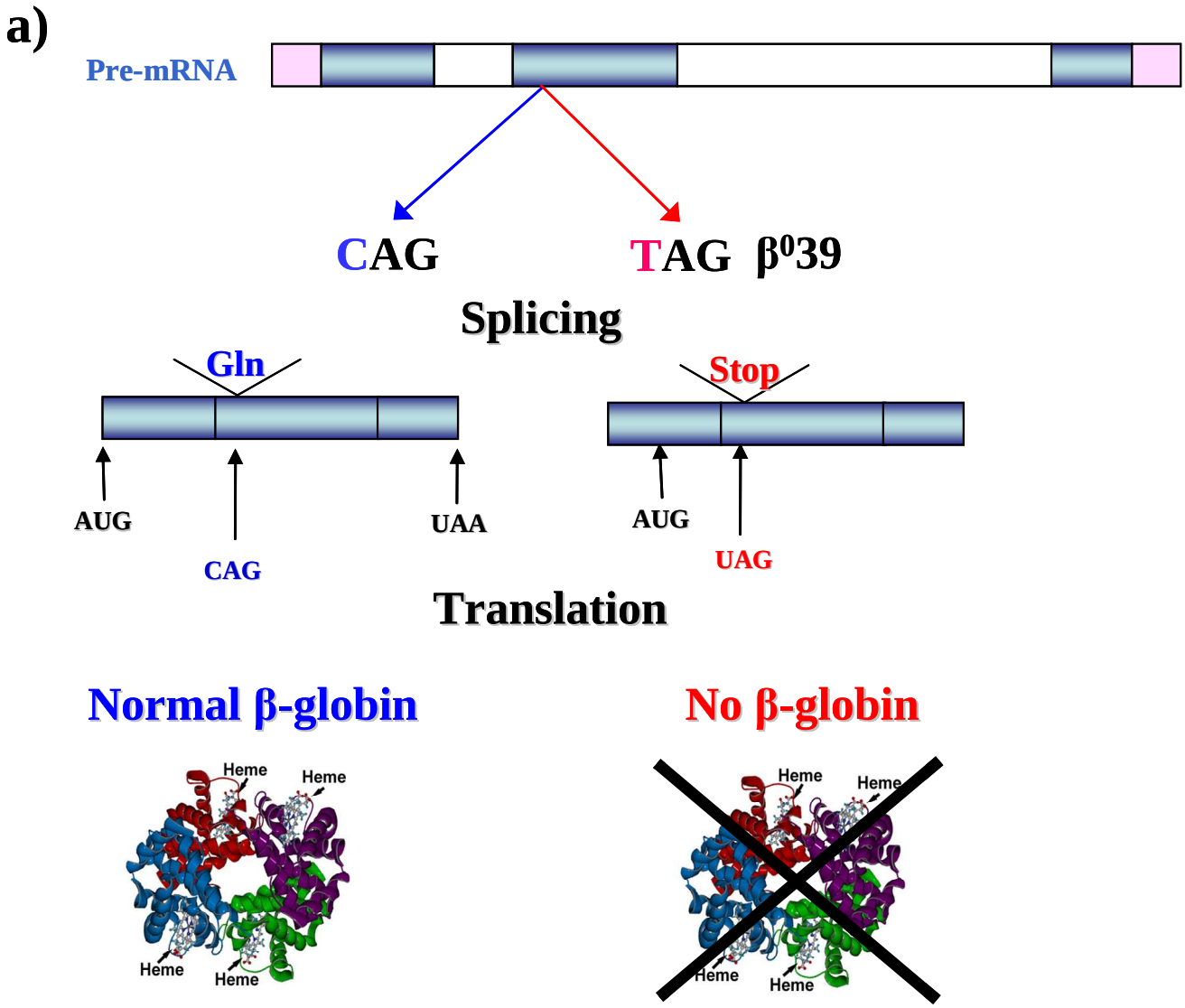
These mutations can occur either within an intron or within an exon. In the first case, they may cancel a regular site of splicing destroying the donor site GT or the acceptor site AG, reduce the efficiency of a splicing site altering its consensus sequence or create a new site of splicing, producing a new signal GT or AG in a cryptic site. When, however, mutations affecting the exons, the only thing that can happen is the creation of a new donor or acceptor site of splicing, thus producing an abnormal mRNA [Bianco Silvestroni, 1998].

Mutations in the cut and polyadenilation site of the pre-mRNA: if the AATAAA sequence is mutated, the cut and polyadenilation process occurs much more downstream (i.e. about 20 nt downstream the next AATAAA sequence). It produces, thus, mRNA much longer (several hundred nucleotides) and very unstable. The resulting phenotype, in heterozygous, is a mild β^+ microcitemia with a rate of HbA_2 little higher than the standard [Bianco Silvestroni, 1998].

Defects of mRNA translation: they constitute the third major group of mutations that cause β-thalassemia. The most numerous are those affecting exons and may be non-sense or frameshift [Bianco Silvestroni, 1998].

Non-sense mutations: they consists in the substitution of a base in a codon of the codifying DNA, with subsequent creation of a premature stop codon and, thus, translation termination. To this group belongs one of the most frequent mutations in our country, especially in Sardinia, the $\beta^0$39-thalassemia (**figure 4**). For instance, in $\beta^0$39-thalassemia the CAG (Gln) codon of the β-globin mRNA is mutated to the UAG stop codon [Trecartin et al, 1981; Piras et al, 2005], leading to premature translation termination and to mRNA destabilization through the well described nonsense-mediated mRNA decay (NMD) [Holbrook et al, 2004; Stalder & Mühlemann, 2008]. Other examples of stop mutation of the β-globin mRNA occurr at position 15 [Ahmed et al, 2001], 37 [Kornblit et al, 2005; Li et al, 2006] and 127 [Préhu et al, 2005] of the mRNA sequence.

Frameshift mutations: they consist of deletions or additions of one or a few nucleotides, which cause a shift in the reading frame of the mRNA (frameshift) and, therefore, a more or less early termination of the translation. If the shift begins in a codon far away from the termination one, the chain, as well as having an altered amino acid sequence, is usually much shorter than the normal. If,



b)

TAG

GAGAAGACTC TTGGGTTTCT GATAGGCACT GACTCTCTCT GCCTATTGGT
CTATTTTCCC ACCCTTAGGC TGCTGGTGGT CTACCCTTGG ACCCAGAGGT
TCTTTGAGTC CTTTGGGGAT CTGTCCACTC CTGATGCTGT TATGGGCAAC
CCTAAGGTGA AGGCTCATGG CAAGAAAGTG CTCGGTGCCT TTAGTGATGG
CCTGGCTCAC CTGGACAACC TCAAGGGCAC CTTTGCCACA CTGAGTGAGC
TGCACTGTGA CAAGCTGCAC GTGGATCCTG AGAACTTCAG GGTGAGTCTA
TGGGACCCTT GATGTTTTCT TTCCCCTTCT TTTCTATGGT TAAGTTCATG

Figure 4 – β⁰39 mutation. a) Schematic reppresentation of the maturation and translation of βwt and β⁰39 transcripts: in the presence of the mutation β-globin chains are not produced. **b**) Sequence of the second exon (in blue) of the human β-globin gene, with the codon 39 (in red) and the mutation CD 39 C/T (β⁰39).

however, the mutation is located in a region closer to the end of the translation, it is very likely that this will continue beyond the normal stop codon, to meet a new termination codon, producing a stretched globin chain [Bianco Silvestroni, 1998].

Mutations that cause serious instability of the β-globin chain: these defects are frameshift or non-sense mutations or deletions of a few nucleotides, for the most part in the 3rd exon. They give rise to a β-globin chain stretched or, more rarely, truncated but always highly unstable, which does not have the tendency to form tetramers and then falls rapidly, so that the abnormal Hb is not present in the circulation. It is distinctive, hence, the data that the synthesis ratio between α and β chains is highly unbalanced, while that between α and β mRNA is normal. Because of the instability of the β-globin chain, large quantities of both β-globin and α-globin chains remain free [Bianco Silvestroni, 1998].

Physiopathology of β-thalassemia

The pathophysiology of β-thalassemia relates to a quantifiable deficiency of functional β-globin chains, which leads to an imbalanced globin chain production and an excess of α-globin chains [Thein, 2004].

In β-thalassemia, the synthesis of normal α-globin chains from the unaffected α-globin genes continues as normal, resulting in the accumulation within the erythroid precursors of excess unmatched α-globin. The free α-globin chains are not able to form viable tetramers and instead precipitate in red cell precursors in the bone marrow forming inclusion bodies. These α-chain inclusions can be demonstrated by both light and electron microscopy in erythroid precursors in the bone marrow as well as in peripheral red cells following splenectomy. They are responsible for the extensive intramedullary destruction of the erythroid precursors and hence the ineffective erythropoiesis that underlies all β-thalassemias [Thein, 2005].

Erythrokinetic and morphologic studies showed that particularly in the β-thalassemias the major cause of anemia was death of erythroid precursors in the bone marrow and other sites of extramedullary erythropoiesis. The cause of this ineffective erythropoiesis was unknown until it was showed, that β-thalassemia

major erythroid precursors, but not myeloid precursors, underwent accelerated apoptosis, as detected by an increase in DNA "laddering," a sign of enhanced nucleosomal cleavage. Quantitative studies subsequently showed that erythroid apoptosis in β-thalassemia major was increased about four times above normal. In combined erythrokinetic and marrow analyses of patients with moderate to severe forms of α- and β-thalassemia, it was shown that there was a fairly tight correlation between ineffective erythropoiesis and marrow erythroid apoptosis. This lead to the proposal that the mechanism of ineffective erythropoiesis in the thalassemias is enhanced apoptosis.

The level of apoptosis achieved in the bone marrow of β-thalassemia patients seems to depend, at least in part, on their level of erythroid expansion. Some hypothesis were formulated, trying to explain this dependence: the most accredited one says that when a cell line is forced by physiologic or pathologic mechanisms to increase its proliferative rate, the chances for errors increase, leading to aberrations that are detected by the affected cells, which then turn on their apoptotic programs [Centis et al, 2000].

The lack of β-globin chains, in β^0 homozygous patients, and the consequent ineffective erythropoiesis begin a series of events leading to the onset of several clinical manifestations of the disease (**figure 5**). The first and most serious manifestation is the anemia, determined primarily by the total absence of HbA. In erythroid precursors are present, since the early states of development, inclusion bodies formed by the precipitation of α chains, aggregates of ferritin, mitochondria loaded with iron, both due to the absence of haemoglobin, and accumulation of glycogen. As already explained, most of erythroblasts faces death in bone marrow, but a small part of them, containing HbF, enters to the general circulation. They are, however, severely damaged, restricted and little elastic and, once in the spleen, are unable to cross the splenic sinus and are therefore eliminated by macrophages. Few cells, insufficient to compensate the anemic state, less affected because containing a fair share of HbF, manage to cross the spleen without being destroyed but only undergoing the removal of some precipitates (pitting) and finally destroyed in the following steps. The average life of red cells of patients

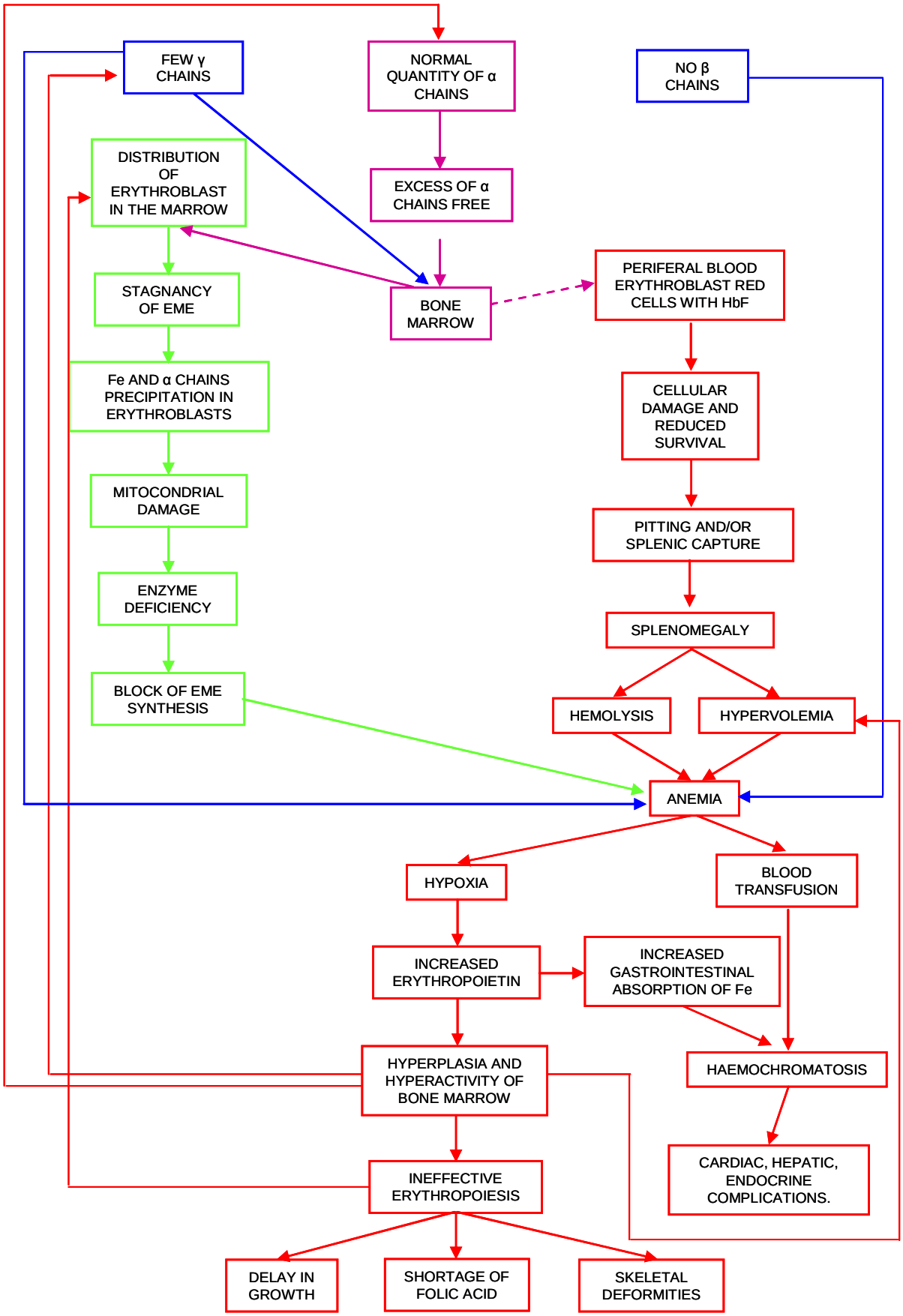


Figure 5 – General physiopathology of β^0-thalassemias.

with thalassemia major which come into circulation is, therefore, very short: the destruction is premature and the hyperhemolysis results in sub-icterus, increase of indirect bilirubin and urobilin. Moreover, the high work of removal done by the spleen leads to one of the predominant features of β-thalassemia patients and that is splenomegaly (**figure 5**) [Bianco Silvestroni, 1998].

A direct effect of anemia is the increased production of erythropoietin, which leads to intense proliferation and expansion of bone marrow with the resulting skeletal deformities. Another important consequence of anemia is the iron loading in hearth, liver and spleen that results from the increased gastrointestinal absorption and the blood transfusions [Thein, 2004]. These iron deposits are responsible for

many complications that accompany the disease. Among these may include osteopenia and osteoporosis, depending also on anemia and growth of bone marrow, heart complications, which are the leading cause of death in β-thalassemia, endocrine disorders, diabetes and liver cirrhosis, limit situation, rarely reached even when, in addition to deposits of iron, the liver is infected with hepatitis C virus, recruited via blood transfusion.

Therapies

It is possible to divide the therapies for β-thalassemia into two principal groups: the maintenance and definitive therapies.

Maintenance therapies

The traditional therapies that have contributed over the last 35 years to eliminate, or nearly, manifestations of the disease and to determine the extension and improvement of quality of life of patients are blood transfusions and iron chelation therapy.

Blood transfusions: in patients with β-thalassemia major, this therapy is not only an alternate remedy that is applied to eliminate or reduce an anemic state, but is also a therapy that acts on the main clinical manifestations of disease by

changing their pathogenesis. Practiced under the current protocols, the therapy eliminates dangerous fluctuations of haemoglobin level and allows the patient to have regular growth and development, good living conditions, normal resistance to infection. It determines also a drastic reduction in intestinal absorption of iron that already with an average level of haemoglobin of 9 g/dl is only 10% higher than the normal. Finally, this therapy produces another and even more important effect, namely a reduction of bone marrow activity, because it reduces the production of erythropoietin and consequently the enormous expansion and hyperactivity of bone marrow and the biggest manifestations that this involves. Another effect that complements the block of bone marrow is the cessation or marked reduction of the ineffective erythropoiesis, with a sharp decline in production of altered erythroblasts and precipitated α chains. The consequence of the entry in the circulation of a very small number of altered erythroblasts and red blood cells, intended to be seized in the spleen, is a slower increase in the volume of this organ with a delayed onset of hypersplenism [Bianoc Silvestroni, 1998].

Thanks to new techniques for early detection, the start time of transfusion therapy in patients with severe thalassemia major is usually in the second half or towards the end of the first year of life. Once started, the transfusion must be such as to ensure a level of haemoglobin pre-transfusion of approximately 9 – 10 g/dl, and this requires the patient to be transfused every 2 – 3 weeks with an average of 500 grams of blood, which was previously deprived of all leukocytes, through centrifugation, sedimentation, filtration and washing, to avoid feverish reactions. The washing with physiological solution can be repeated a second time in the case of patients particularly susceptible to plasma proteins, such as cytokines.

Two risks are associated with transfusion therapy: 1) the use of blood containing viruses such as hepatitis B and C viruses, leading to onset of infection and, in the specific case, of hepatitis; 2) assumption of large amounts of iron, which, added to that taken by gastrointestinal adsorption, leads to the formation of large deposits in several organs with the consequences of which has already spoken.

Iron chelation therapy: the first iron chelator introduced in the treatment of the β-thalassemia major is *deferoxamine* (Desferal®), already used since the 1960s but

applied with methods and doses truly effective only by the end of the 1970s. This is a hexadentate agent that effectively chelates iron, masking all six coordination sites so that the complex cannot generate ROS. Deferoxamine does not enter cells, so the iron is removed from transferrin or non-transferrin-bound sources of iron in blood and bile. The chelated iron is excreted equally in urine and stool. Deferoxamine must be given parenterally, either by bolus plus intravenous continuous infusion or via prolonged subcutaneous infusion. For best results the infusion needs to be continued for 8 – 12 hours using a constant infusion pump and must be carried out 250 nights per year in order to prevent the iron buildup that occurs in a patient getting RBC transfusions every three weeks. The high cost of the drug, pump, and infusion materials, as well as the side effects, limit its acceptability and utility [Schrier and Angelucci, 2005].

In recent years, new iron chelators, orally active, were developed. Among these drugs, the most used is deferiprone (Ferriprox®). As a small, lipophilic bidentate molecule, deferiprone would theoretically have the potential to be more effective than deferoxamine in removing intracellular iron from some tissues [Piga et al, 2003]. The available data appear to demonstrate that deferiprone is an effective oral iron chelator able to reduce iron overload and to maintain a safe body iron level [Galanello, 2007]. Moreover, a comparative study of the effects of the two chelators (deferiprone and deferoxamine) on survival and cardiac complications, showed greater activity of deferiprone on deposits of iron in the heart, thus improving the conditions of the organ. This action was attributed both to the ability of this molecule to enter into the cells and to its longer half-life and the opportunity to make an uninterrupted therapy (seven days a week), with more frequent doses (three per day) than that achieved with deferoxamine [Piga et al, 2003].

Several are the side effects of these two drugs: in the case of deferoxamine, prolonged use can lead to chronic toxicity, with onset of cataracts, damage to the retina, optic atrophy, ototoxicity and cartilage dysplasia [Merson and Olivieri, 2002]; while deferiprone can cause, even in the early stages of use, neutropenia, agranulocytosis and arthralgia [Schrier and Angelucci, 2005].

Due to the different distribution of deferoxamine and deferiprone in the cell, a combination therapy was proposed, which could bring several benefits through a synergistic action of the two drugs. In fact, the deferiprone could act transferring the iron from the deposits inside the cell to molecules of deferoxamine in the extracellular compartment, with the function to excrete the iron in urine and stool [Ding et al, 2002].

A new orally active iron chelator is actually been testing in clinical trials: the deferasirox (ICL670, Exjade®) is a tridentate iron chelator with a relative long circulating half-life of 8 to 16 hours and documented cellular permeability [Walter et al, 2008]. Preclinical studies have demonstrated that ICL670 is a highly potent and selective chelator, and clinical evaluation in patients with iron overload have demonstrated an efficacy and safety profile compatible with long-term use. Six-month preliminary data from a 1-year comparative study of thalassaemic patients with transfusional iron overload suggest that a single daily dose of ICL670 20 mg/kg is as effective as deferoxamine in the removal of iron from body stores [Cappellini, 2005].

As for the future perspectives of maintenance treatment, in addition to research and testing of new oral chelators, investigators have focused their attention to substances that can stimulate the production of HbF in order to reduce the annual requirement of blood transfusions of thalassemia patients, thus reducing the iron contribution. Among the different molecules so far tested in clinical trials, are included hydroxyurea (HU) [Bradai et al, 2003] and derivatives of butyrate [Reich et al, 2000]. In the case of HU, a general increase in the level of Hb was noticed in all patients in the study, while in the case of derivative of butyrate, only two patients over 8 showed a marked improvement. In the latter case, it was suggested that the stimulating action of the substance may depend in some way on a genetic factor responsible for a particular predisposition to the production of HbF and on the serum levels of erythropoietin present in patients prior to the administration of derivative. Because of the side effects and the low activity of HU and derivatives of butyrate, many other molecules capable of inducing the production of HbF are under study, among these, are included cytosine arabinoside [Cortesi et al, 1999],

mithramycin [Bianchi et al, 1999; Fibach et al, 2003], rapamycin [Mischiati et al, 2004; Fibach et al, 2006].

Definitive therapies

This group includes all the therapeutic techniques aimed to resolve the disease, including bone marrow and umbilical cord blood cells transplantation.

Bone marrow transplantation (BMT): the first successful BMT for thalassaemia major was performed in 1982 and now over 1500 transplants have been performed worldwide with the most experience from Pesaro, Italy [Lawson et al, 2003]. Just in Pesaro, in 1989, following analysis of potential negative factors such as low quality of chelation therapy before the transplantation, the marked hepatomegaly and portal fibrosis, were established three classes of risk, in which splitting transplant patients, and, according to them, several protocols of action were developed [Lucarelli et al, 1998].

Naturally, the essential prerequisite to make bone marrow transplantation is the availability of a compatible donor. Only after the identification of the donor, the patient is assigned to one of the categories of risk and is therefore subjected to ablation of the hematopoietic and immune systems. Once this deletion has taken place, the patient is ready to be transplanted by infusion of bone marrow into a peripheral vein [Lucarelli et al, 2002].

The complications that can arise early after the transplant are different: the foremost one is the rejection of the transplant itself, accompanied by the recover of thalassemia conditions and dependency on blood transfusions. Another danger is the disease known as *graft versus host disease* (GVHD), which can occur in acute and chronic form. In both cases, major events take place at the level of immune system and epithelial cells of skin, liver and intestines, with complications that can lead to death [Robbins, 1997].

Regarding the long-term complications, they include all the consequences of the years prior to transplantation, in which the patient has undergone a transfusion and chelation therapy, including 1) the accumulation of iron that lead to infertility, disturbance of growth and alteration of the endocrine system; 2) chronic hepatitis,

mainly due to infection by the hepatitis C virus and 3) liver fibrosis [Lucarelli et al, 2002].

Unfortunately, in most cases, even after the transplantation it is necessary to subject the ex-thalassemic patients to chelation therapy to eliminate all the iron overloads formed in the previous period. To do this, the preferred therapeutic technique is the phlebotomy, which consists in the removal of 6 ml/kg of blood every 14 days and in the administration of autologous plasma or saline solution to restore the normal blood volume. Those patients who, for various reasons, can not be subjected to this treatment, are treated with subcutaneous infusions of deferoxamine [Lucarelli et al, 2002].

Umbilical cord blood cells transplantation: it is not a technique widely used in the treatment of β-thalassemia major, but has many advantages over bone marrow transplantation, including a lower incidence of GVHD and increased survival. In a study where 44 patients undergoing a umbilical cord blood transplantation in different institutions were analyzed, none of them went to meet death, which confirms the high reliability of the technique. It should, however, noted that in all cases the period of post-transplantation analysis was of short duration, but the prognosis remains, however, favourable for the total absence of chronic GVHD, which difficulty occurs after two years [Locatelli at al, 2003].

The positive results achieved, until now, in the cure of thalassemia with umbilical cord blood transplantation should encourage the use of this technique in individuals suffering from haemoglobinopathies and thus stimulate the donation of umbilical cord from women at the end of pregnancy.

Gene therapy

In recent years, various approaches have been proposed for the treatment of haemoglobinopathies, especially β-thalassemia and sickle cell anemia, based on gene transfert, gene targeting or correction of mRNA processing.

The *gene transfer* into hematopoietic stem cells is a technique widely studied, which has already proved effective in animal models. Since the patient's own cells

are used, the need for HLA-identical donor and immunological complications associated with bone marrow transplantation are removed.

This gene strategy has 4 goals: 1) the transfer of a single gene in specific hematopoietic stem cells, 2) the endogenous expression of the transgene at high levels, 3) the maintenance of that expression over time and 4) the use of not pathogenic vectors. Many of the available techniques are not in accordance with all four of these expectations. For example, non-viral vectors, which may be in the form of naked DNA, cationic lipid binding DNA through electrostatic interactions or particles of condensed DNA, have all proved ineffective for in vivo transfection; in addition, the gene transfer results in transient expression of genes of interest. Increased efficiency can be achieved through the use of adenoviral vectors, but, again, the expression is not permanent because of the immune response against cells that express low levels of viral proteins. Instead, the vectors derived from onco-retrovirus, such as the Moloney murine leukemia virus, have proved suitable for gene therapy, thanks to their ability to integrate the gene of interest into the genome of target cells without, however, any viral gene transfer [Stathopulos, 2003].

Recombinant onco-retroviruses were the first viral vectors used to transfer the human β-globin gene in mouse hemaopoietic stem cells (HSCs). Early experimentation with vectors harboring the β-globin gene resulted in tissue-specific but low and variable human β-globin expression in bone marrow chimeras, usually varying between 0 and 2% of endogenous mouse β^{major} RNA levels. Initial efforts to incorporate locus control region (LCR) subfragments into onco-retroviral vectors resulted in low titers, low expression, or unstable vectors prone to sequence rearrangements. Incorporation of the core elements of HS2, HS3, and HS4 of the human β-globin LCR significantly increased expression levels in murine erythroleukemia (MEL) cells, but failed to abolish positional variability of expression. This finding suggested that a minimal LCR comprising juxtaposed core elements did not provide full LCR function, but rather acted like an erythroid-specific enhancer [Sadelain et al, 2005; Lisowski & Sadelain, 2008].

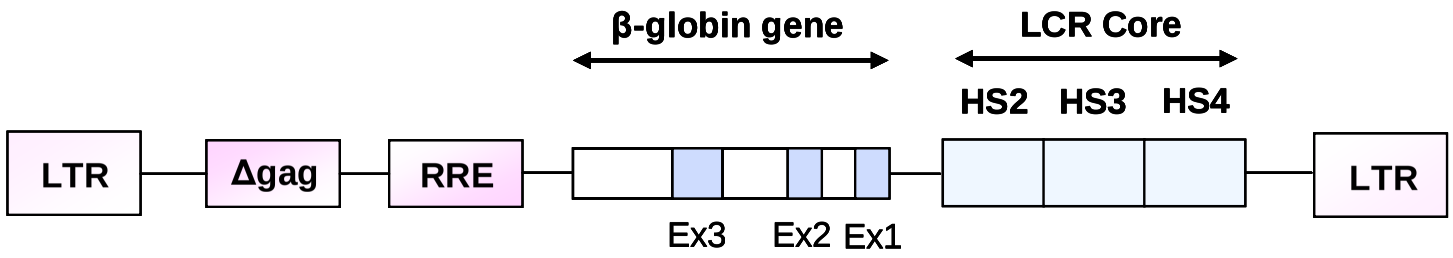
As a result of these continuing failures, some investigators have thought of using more extensive fragments containing the sites HS2, HS3 and HS4.

Unfortunately, incorporation of larger HS spanning elements into onco-retroviral vectors is problematic due to the inability of the vector to incorporate large quantities of genetic material in a stable manner. Another major limitation of onco-retroviral vectors is the obligate requirement of these vectors to transduce cells that divide shortly after infection because the vector RNA cannot migrate into the nucleus due to the presence of a nuclear membrane, and thus, must wait for mitosis to make the transition. Since most HSCs are in a quiescent state, they must be induced to divide in order to achieve higher transduction efficiencies and overall expression levels. The problem with stimulating quiescent hematopoietic stem cells is that they tend to lose long-term repopulating capacities when treated with cell division stimulants such as cytokines [Stathopulos, 2003].

In view of such difficulties, most investigators began exploring alternative vector systems, as lentiviral vectors, and alternative transcriptional control elements. Lentiviral vectors are replication-defective retroviral particles containing lentiviral core proteins and enzymes, which are pseudotyped with a heterologous retroviral envelope or equivalent. Lentiviral vectors derived from HIV-1 and other lentiviruses have elicited great interest for their ability to transduce non-dividing cells. While onco-retroviral vectors are restricted to cells proceeding through mitosis, the preintegration complex of lentiviral vectors can translocate to the nucleus and successfully integrate in the absence of cell division. Lentiviral vectors can transduce a broad spectrum of target cells, including neurons, retinal photoreceptors, dendritic cells, macrophages, hepatocytes, and HSCs. Another fundamental attribute of lentiviral vectors is their relative genomic stability and their packaging capacity [Sadelain et al, 2005; Lisowski & Sadelain, 2008].

With the use of lentiviral vectors, some investigators has managed to obtain a new construct containing the β-globin gene, the sequences of the promoter and proximal enhancers and a region comprising the LCR elements, including the sites HS2, HS3 and HS4. Studies comparing this vector, called TNS9 (**figure 6**), with an equal contains, however, only minimal LCR sequence (RNS1) have

RNS1:



TNS9:

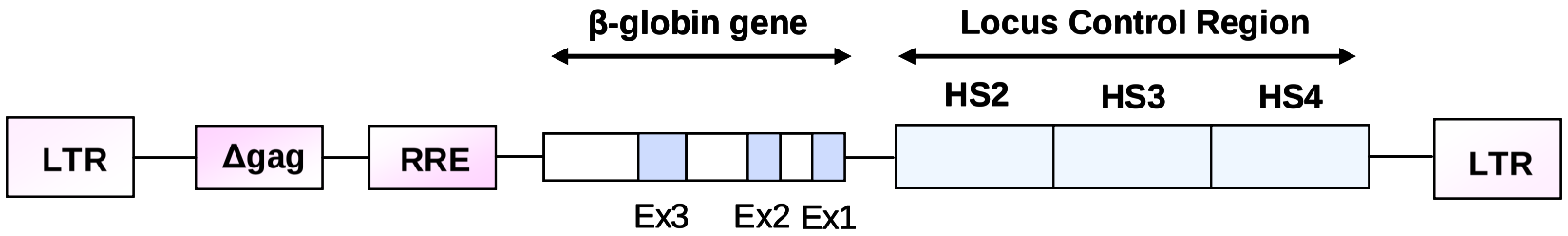


Figure 6 – RNS1 e TNS9 vectors. Both lentiviral vectors encode the human β-globin gene. In RNS1, the promoter starts at position -265 and the 1.0 kb LCR consists of the core elements of HS2, HS3 and HS4. In TNS9, an extended promoter and the 3' human β-globin enhancer are included, along with large genomic segments flaking the core elements (3.2 kb LCR) [Sadelain, 2002].

demonstrated, for TNS9, a dramatic increase in expression of the transgene and less dependence from the insertional position [Sadelain, 2002].

This dependence is due to the presence in the genome of regions with high, low and no expression that affect the transcription not only of the contained endogenous genes but also of possible inserted transgenes (**figure 7**). The transcriptional activity of these regions depends on the chromatin structure, which can be opened or closed, and that is transmitted to the new inserted genetic sequence, unless that contains elements that prevent such influence. Just to avoid at all to get different effects depending on the position of the vector insertion, some investigators have been looking for possible new regulatory sequences to add to the construct. The element that has proved most effective is the *insulator* cHS-4 from the chicken β-globin LCR, thanks to its ability to isolate the coding sequence by the influences of the surrounding chromatin [Emery et al, 2002].

Moreover, the insulators have the additional aim to avoid the insertional oncogenesis, depending on the random integration of foreign genetic material, whether of viral or non-viral origin. In a mouse study where myeloid leukemia was shown to be caused by insertional oncogenesis, the transgene was a truncated, but not fully disabled form of the human low-affinity nerve growth factor receptor. The risk of insertional oncogenesis has also been established in humans, in the context of gene therapy for X-linked severe combined immunodeficiency disease (SCID-X1).

This therapy was remarkably successful in 10 of 11 treated patients, but two patients developed a lymphoid leukemia that could be linked to the integration of the retroviral vector in or near the *LMO-2* oncogene. In both instances, the *LMO-2* oncogene, which is normally silent in T lymphocytes, was transcribed as a direct consequence of the neighboring vector insertion [Sadelain et al, 2005; Lisowski & Sadelain, 2008].

Using the TNS9 vector, the Sadelain's group have first demonstrated efficient gene transfer in bone marrow hematopoietic stem cells, tissue-specific transgene expression and long-term correction in a mouse model of β-thalassemia intermedia. Subsequently, they have produced a mouse model for the most severe form of β-thalassemia, the Cooley anemia, demonstrating that mice die as a

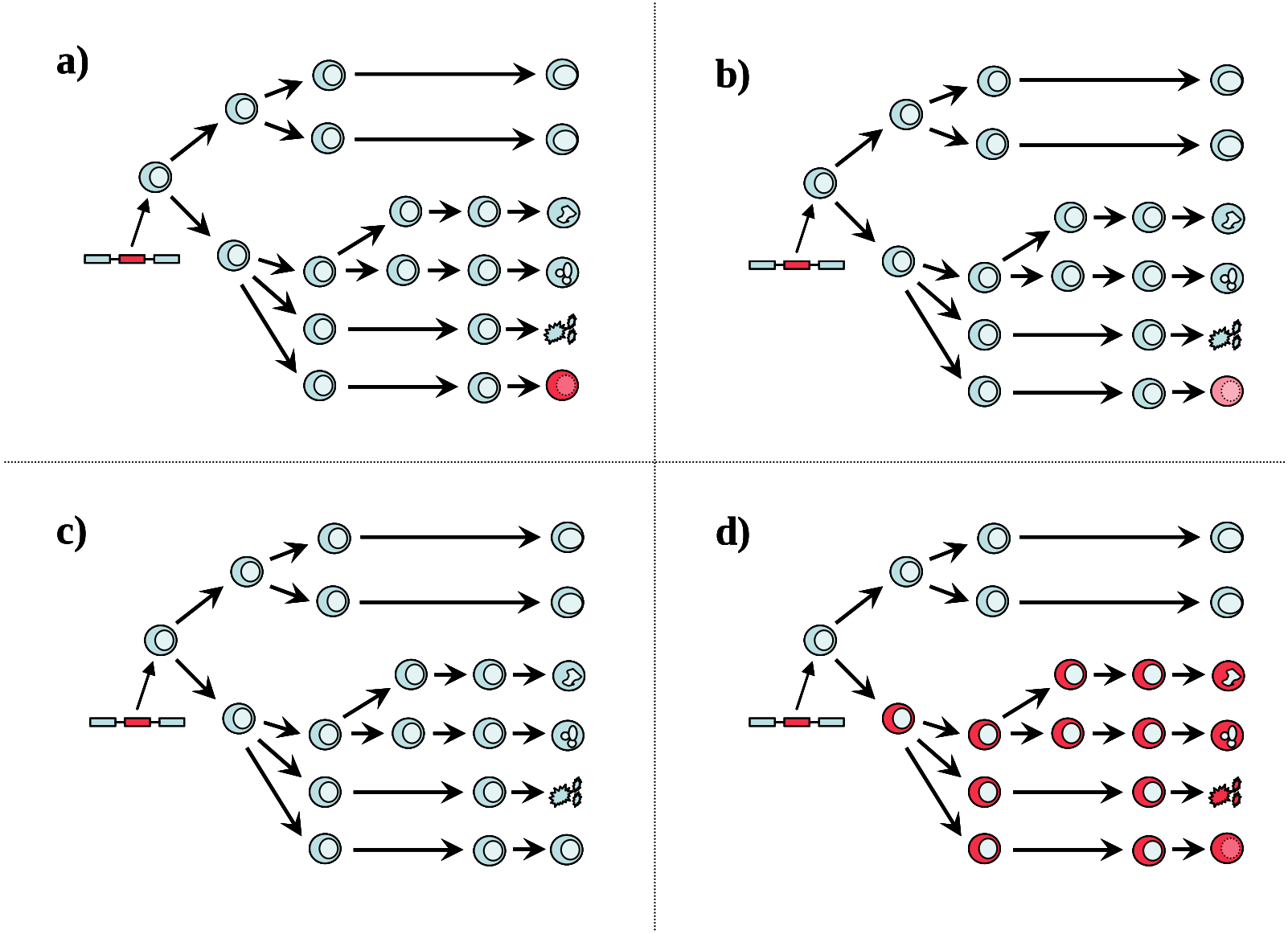


Figure 7 – Position effects hamper regulated trasgene expression. Each quadrant represents a possible pattern of expression following vector integration at random: a) high-level, erythroid-specific expression; b) low-level, erythroid-specific expression; c) trasgene silencing; d) dysregulated expression [Sadelain, 2002].

consequence of extreme ineffective erythropoiesis and that they can be rescued and cured by lentivirus-mediated transfer of the human β-globin gene [May et al, 2002; Rivella et al, 2003].

In a more recent study, Malik et al [2005] show that a lentiviral vector that would express the human β-globin gene under control of its regulatory elements and be flanked with chromatin insulator elements upon integration into host hematopoietic cells results in (1) high-level transduction of bone marrow progenitor cells from four patients with transfusion-dependent thalassemia major, (2) complete phenotypic and functional correction of the *in vitro* model of human thalassemia erythropoiesis, (3) levels of β-globin similar to that derived from normal bone marrow progenitors, and (4) effective human erythropoiesis with circulating β-globin-producing human erythroid cells in genetically corrected xenografts, at levels comparable to normal bone marrow xenograft controls.

Unfortunately, the uncertainties about possible side effects in the long term remain numerous and, for this reasons, further studies are required, aimed at the development of new vectors and new cellular and animal models.

The oligonucleotide-based *gene targeting strategies*, able to correct point mutations, include those that use triplex-forming oligonucleotides, RNA/DNA hybrid oligonucleotides (chimeraplasty) and small DNA fragments (SDFs), which are used in the small fragments homologous replacement (SFHR) strategy. The SDFs used in SFHR are composed of either single-stranded (ssDNA) or double-stranded DNA (dsDNA), contain non-coding sequences, and are essentially homologous to target loci. These SDFs effect homologous exchange between incoming SDF sequences and endogenous (genomic or episomal) sequences, ultimately resulting in phenotypic changes (**figure 8**) [Gruenert et al, 2003].

Gene targeting is the only procedure that can produce predefined alterations in the genome of eukaryotic cells. As such, gene targeting is an attractive approach to gene therapy of genetic diseases because: (1) it can lead to the accurate correction of the defect in the target locus of interest; (2) it can correct both recessive and dominant mutations, unlike gene augmentation, which is restricted to recessive defects; (3) it is not restricted by the size of the mutated gene since only the damaged portion need be considered; gene augmentation requires delivery of the

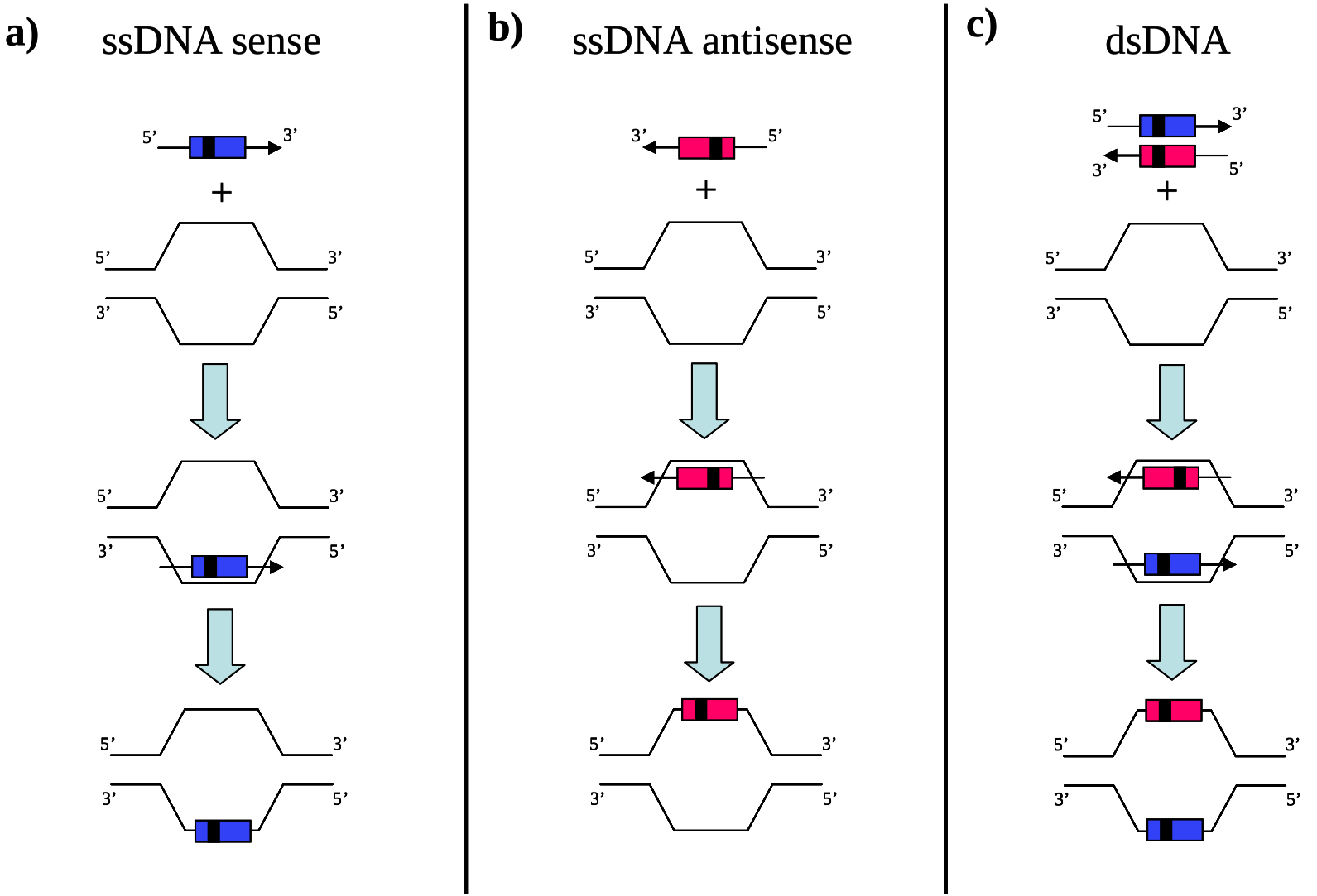


Figure 8 – Schematic representation of different SDF configurations possible in SFHR-mediated modification of a target sequence. The SDf can interact with the target sequence as a) sense and/or b) antisense ssDNA, or as c) dsDNA [Gruenert et al, 2003].

entire coding region which may be impractical for large genes; (4) actual correction of the faulty gene would permanently reverse the genetic disorder by restoring production of the normal protein product; the corrected gene would be under the control of its own regulatory sequences, thereby avoiding the potential problems of non-physiological gene expression and long-term gene inactivation frequently associated with gene augmentation procedures; and (5) it avoids the risk, associated with many gene augmentation approaches, of a harmful mutation caused by the chance integration of the therapeutic DNA at a sensitive genomic site such as an oncogene [Yanez and Porter, 1998].

A potential drawback of gene correction by gene targeting is that it requires previous knowledge about the location and type of mutation to be corrected, and may require the use of different therapeutic DNA for different patients with the same disease. Also, although it can be a high fidelity procedure, mutations associated with gene targeting have been observed. The main handicap, however, lies in the inefficiency of gene targeting which typically ranges from 10^{-5} to 10^{-7} targeted cells per transfected cell [Yanez and Porter, 1998].

In a recent study, a gene targeting approach, based on the introduction of small DNA fragments (SDF) into erythroid progenitor cells, was applied to specifically modify the β-globin gene sequence at codon 39. The strategy was first tested in normal individuals by delivering mutant SDF that were able to produce the $\beta^0$39 (C→T) mutation. Secondly, wild-type SDF were electroporated into target cells of $\beta^0$39-thalassemia patients to correct the endogenous mutation. In the first case, with the use of mutated SDF, the efficiency of small-fragment homologous replacement (SFHR)-mediated modification was approximately 2.4%, while, in the case of thalassemic cells treated with wild type SDF, targeted correction was detected in eight of ten different experiments [Colosimo et al, 2007].

Unlike the SFHR strategy, the chimeraplasty involves the use of RNA/DNA oligonucleotides that, once entering the cell, appear to stimulate the mismatch repair machinery resulting in the correction of the defect. These chimeric oligonucleotides have been shown to actually be effective both in correcting a point mutation and in the insertion of a single base in the genome of mammalian cells, both in vitro and in vivo, and were able to correct a thalassemic mutation

introduced in MEL cells. But, although the chimeraplasty is an attractive strategy for gene therapy, many questions are still unanswered and it will, therefore, necessary to deepen the understanding of this approach through further research [Li et al, 2001].

The last type of gene therapy to be considered is the *antisense strategy*, which allow the correction of mRNA processing and can be used only with a particular group of mutations, the splicing mutations.

In various antisense mechanisms developed over the years, the oligonucleotides are used to modulate the transfer of information from gene to protein, altering the intermediate metabolism of RNA. Depending on the type of oligonucleotide used and the target sequence recognized by it, it is possible to produce two different results: silencing a gene or modifying the mRNA processing. When the aim is to inhibit gene expression it is necessary to use methods that lead to activation of the RNase H or to the arrest of translation.

A key point of the metabolism of mRNA molecules is the excision of introns. Splicing reactions are sequence-specific and require the concerted action of many proteins, which form the splicesoma. Antisense oligonucleotides, which bind to regions required for the splicing can prevent the binding of necessary factors or physically prevent the cutting reactions. This implies, therefore, inhibition of production of mature mRNA [Crook, 1999]. In this perspective, many investigators thought to use oligonucleotides to correct the effect of mutations that cause aberrant splicing, restoring the normal splicing and the function of the defective gene. Requests for compounds to be used to change the splicing are different from those for the oligonucleotides used in silencing. In particular, they should not activate the RNase H, as this would destroy the target pre-mRNA before being spliced, and should be able to compete efficiently with the splicing factors for access to primary messenger, which is located inside the nucleus of the cell [Sazani and Kole, 2003].

The early studies, in which antisense oligonucleotides were used to modulate splicing, were carried out by Kole and colleagues, who have successfully used these molecules to correct the aberrant splicing of pre-mRNA of β-globin, due to thalassemic mutations [Dominski and Kole, 1993].

The antisense nucleotides can also be used to regulate the alternative splicing, stimulating or inhibiting the production of a protein variant [Mercatante et al, 2001] and to stimulate the *exon skipping*, in which the oligonucleotides can be used to promote a process of alternative splicing which avoids the incorporation of a defective exon in a mature mRNA [De Angelis et al, 2002].

Lentiviral vectors

Over the years, several types of vectors have been used in gene therapy, to introduce the exogenous gene into the target cells, among these vectors the lentiviral ones have proven to be of particular interest. Lentiviral vectors, in fact, have characteristics that make them particularly suitable for use in gene therapy, including their ability to permanently integrate into the genome of cells *in vitro* and *in vivo*, allowing an expression stable over time [Naldini et al., 1996].

Lentiviral vectors derive from lentiviruses, which, being a subfamily of retroviruses, are RNA viruses that replicate through a DNA intermediate. All retroviruses encode *gag*, *pol*, and *env* genes. The protein products of these genes are unique to each type of retrovirus, but they do share basic common features. In infected cells, the proviral genome is transcribed into a single precursor mRNA from the viral promoter located in the 5' (upstream) long terminal repeat (LTR), with the 3' (downstream) LTR containing transcription termination and polyA signals. The *gag* gene encodes the viral core proteins, whereas the *pol* gene encodes the viral replication enzymes. These two genes are initially expressed as a Gag–Pol fusion polyprotein. The viral protease self-cleaves the Gag–Pol precursor and further processes the Pol protein to individual protease (for cleaving viral precursor proteins into their mature forms), reverse transcriptase (for replicating viral nucleic) and integrase (for integrating the viral genome into chromosomal DNA). The *env* gene encodes the viral envelope glycoprotein, which is cleaved by cellular proteases to the external envelope glycoprotein and the transmembrane protein. [Buchschacher and Wong-Staal, 2000].

In addition to the structural *gag*, *pol*, and *env* genes common to all retroviruses, *human immunodeficiecy virus (HIV)* contains two regulatory genes, *tat* and *rev*,

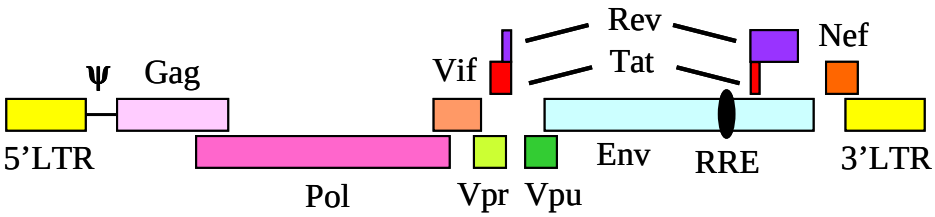
essential for viral replication, and four accessory genes, *vif*, *vpr*, *vpu*, and *nef*, that are not crucial for viral growth *in vitro* but are critical for *in vivo* replication and pathogenesis (**figure 9a**) [Dull et al, 1998].

Infection (**figure 9b**) begins when a virion enters a susceptible target cell through the specific interactions between the envelope glycoprotein and the cellular receptors. Viral-cell membrane fusion and virion internalization (by direct fusion or endocytosis) result in release of the virus core into the cell cytoplasm. Viral RNA is reverse transcribed into double-stranded DNA and transported to the cell nucleus. Viral DNA is permanently integrated (hence referred to as the *provirus*) into chromosomal DNA, where it is replicated during the cell cycle, just as cellular genes are, and subsequently passed to daughter cells. This feature makes retroviral vectors useful for permanently introducing foreign genes into cells. Proviral DNA is transcribed into RNA and transported to the cytoplasm, where it can be translated into viral proteins. Viral precursor structural proteins and replication enzymes assemble with viral RNA to form new virion cores, which obtain the viral envelope glycoprotein as they bud from the cellular membrane. Further processing of the precursor core proteins result in the formation of mature, infectious progeny virus particles [Buchschacher and Wong-Staal, 2000].

From their first application, the lentiviral vectors have been strongly developed in design, in biosafety and in their ability of transgene expression into target cells. The design of viral vectors is based on the separation of *cis*-acting sequences required for the transfer of the viral genome to target cells from the *trans*-acting sequences encoding the viral protein. Vectors particles are assembled by viral proteins expressed *in trans* from construct(s) devoid of most viral *cis*-acting sequence (packaging constructs). The viral *cis*-acting sequences are linked to an expression cassette for the transgene (transfer vector construct) [Vigna and Naldini, 2000].

Over the years, three different generations of lentiviral vectors based on HIV-1 were developed. In these vectors was, first, gradually reduced the number of viral genes present in order to make defective replication in target cells, secondly, the remaining genes were separated each other and transferred in many different constructs.

a)



b)

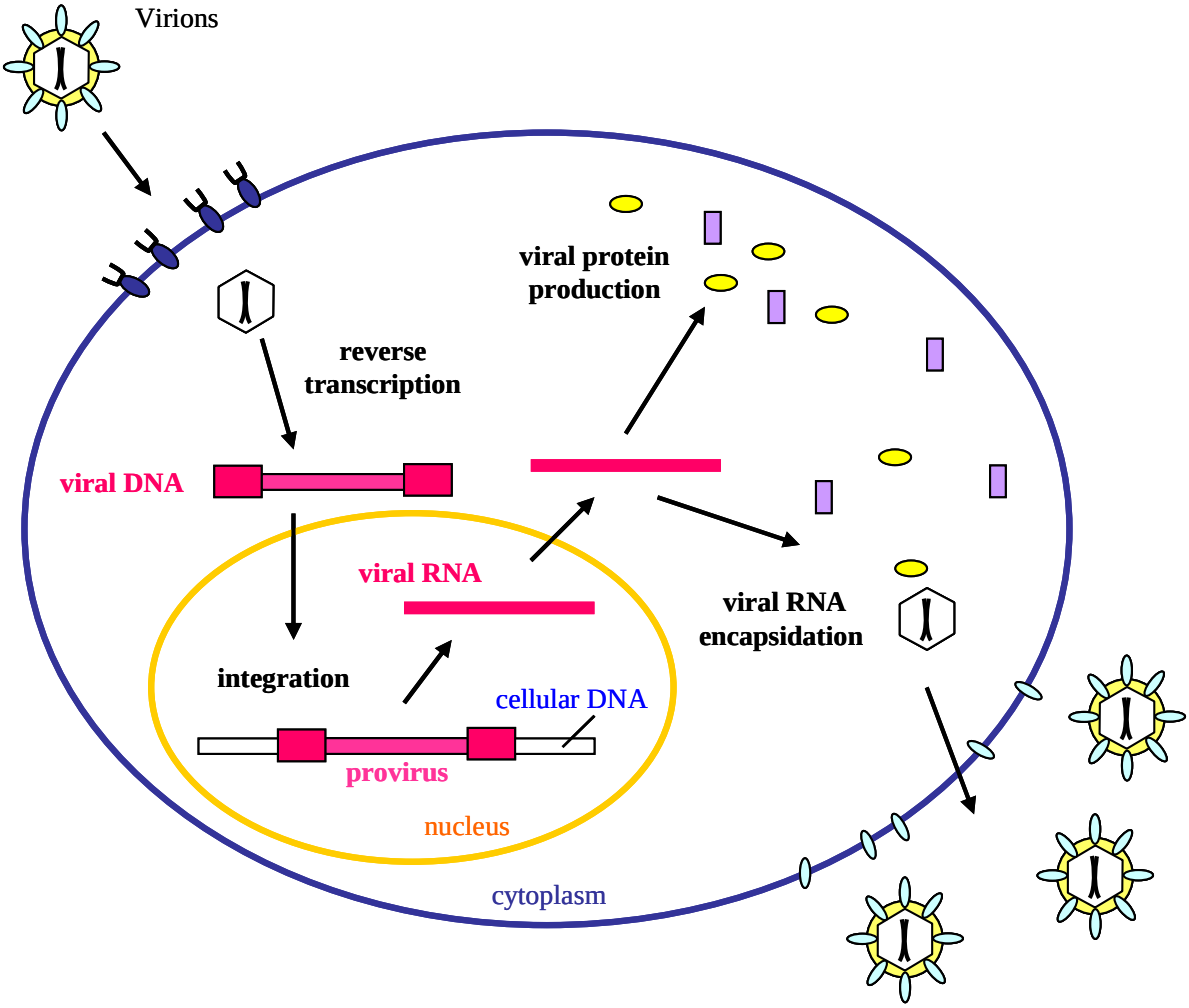


Figure 9 – Retroviruses. a) Schematic representation of the *HIV-1* genome. There are indicated: two *Long Terminal Repeat* (5' e 3' LTR), *packaging* signal **ψ** and the structural, regulatory and accessory genes. **b**) Retroviral replication cycle. The infection begins when the virus envelope glycoprotein recognizes specific receptor located on the cell surface, which mediate the internalization of the core into the target cell; after reverse transcription of the viral RNA, the provirus integrates into the cellular genome, with a subsequent intense translational activity. Then, the viral RNA passes into the cytoplasm, where a part is translated to produce viral proteins while a part associates with viral proteins to form new core particles [Buchschacher and Wong-Staal, 2000].

Due to safety concerns, *first generation lentiviral vectors* for gene transfer employed a three-plasmid expression system and generated a pseudotyped vector through transient transfection of packaging cells. The first generation system included a packaging construct that had a deleted *env* fragment (1.4 kb) and *vpu* gene, a partially deleted packaging sequence (DC) and the replacement of the 5' and 3' long terminal repeat (LTR) regions responsible for the regulation of viral gene expression, with a strong cytomegalovirus (CMV) promoter and polyadenylation signal of the insulin gene, respectively. A second plasmid encoding an alternative envelope protein, the G glycoprotein of vesicular stomatitis virus (VSV-G), was controlled by the CMV promoter. The VSV-G envelope protein is advantageous due to its higher stability that allows concentration by ultracentrifugation and since VSV-G pseudotyped viral particles show broad host cell infectivity. Finally, a third plasmid encoding the expression cassette for the transgene contained all the *cis*-acting sequences required for encapsidation, reverse transcription, and integration. The transgene plasmid also contained the CMV promoter, intact 5' as well as 3' LTRs, the portion of the *gag* gene containing the packaging sequence (C) and a sequence known as the *rev* responsive element (RRE) that is involved in the control of splicing. By using a multiple plasmid expression system for the construction of the vector and minimizing the number of overlapping sequences between plasmids, the possibility of producing a replication competent lentivirus (RCL) by recombination was greatly reduced [Stathopulos, 2003].

Second generation HIV-1-derived vectors improved on biosafety by deleting HIV-1 genes not required for generating functional vector particles. Second generation vectors used multiple plasmids as described for first generation; however, the packaging plasmid had the *vif*, *vpr*, *vpu*, and *nef* genes, as well as the entire *env* gene deleted. In total, five of the nine genes found in wild-type HIV-1 were deleted in these second generation vectors [Stathopulos, 2003].

Third generation lentiviral vectors (**figure 10a**) were introduced by Dull et al. [1998]. The lentiviral vector described in their paper was packaged by three nonoverlapping expression constructs, two expressing HIV proteins and the third expressing the envelope of a different virus. Moreover, all HIV sequences known

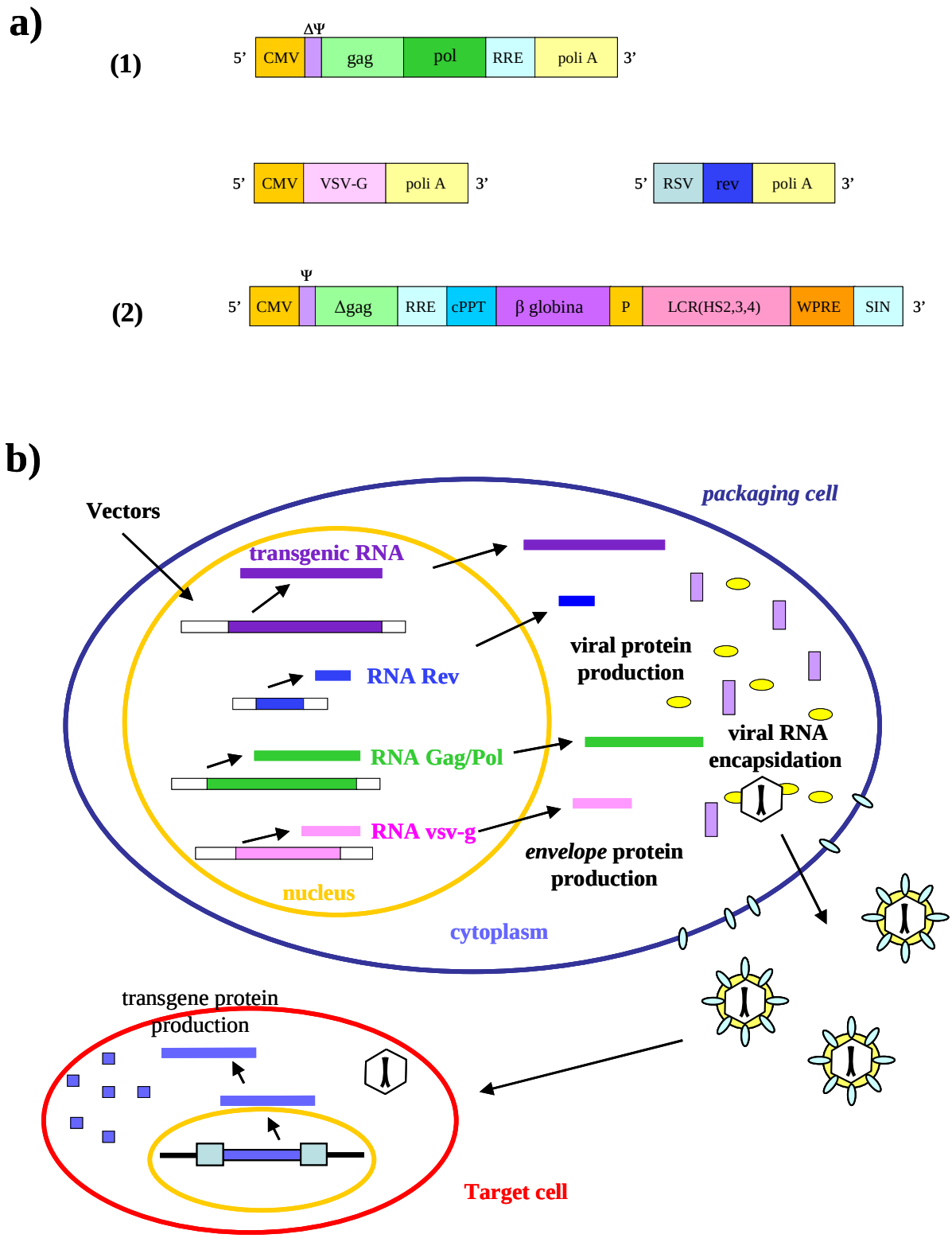


Fig. 10 – Lentiviral vectors. a) Example of third generation lentiviral vectors for the gene therapy of haemoglobinopathies. Three *helper* vectors (**1**) carrying the gene sequences of *Gag* and *Pol*, *Rev* and the *VSV-G* protein. A *SIN* (*Self-INactivation) vector* (**2**) containing the transgene (β-globin) under the control of the promoter P, together with the *in cis* elements: cPPT, WPRE, β-globin locus control region LCR. It also present the packaging sequence, Ψ (Δ= partial). **b**) Mechanism of production of the lentiviral vector. The construct carrying the exogenous gene and the *helper* vectors are used for the transfection of *packaging* cells, where there are the transgene transcription and the sythesis of the virus-specific proteins: the transgenic RNA is then incapsidated to generate viral particles carrying the desired genetic information. These particles, release into the supernatant, are finally used to transduce the target cells, where the integration in the genome and the expression of the exogenous gene take place, while the virus is not longer capable of replication [Buchschacher and Wong-Staal, 2000].

to be required for encapsidation and reverse transcription were absent from these constructs, with the exception of the portion of the *gag* gene that contributes to the stem-loop structure of the HIV-1 packaging motif. Secondly, they deleted another factor crucial for HIV replication, the *tat* gene. Its product is one of the most powerful known transcriptional activators and plays a pivotal role in the exceedingly high replication rates that characterize HIV-induced disease. They finally described a core packaging system split in two separated nonoverlapping expression constructs, one for the *gag* and *pol* reading frames optimized for Rev-dependent expression and the other for the *rev* cDNA. This third-generation packaging system matched the performance of its predecessors in terms of both yield and transducing efficiency. However, it increased significantly the predicted biosafety of the vector.

The biosafety was also improved generating *self-inactivating transfer vectors (SIN).* These constructs duplicate, upstream in the vector, a major deletion in the U3 region of the LTR when transduced into target cells. As this region contains the viral enhancer and promoter sequences, its deletion results in the transcriptional inactivation of the LTR. Self-inactivating lentiviral vectors could be produced with infectious titers and levels of transgene expression driven by an internal promoter similar to vectors carrying wild type LTRs. Concerning biosafety, a self-inactivating vector diminishes the risk of oncogene activation by promoter insertion and alleviates substantially the risk of vector mobilization and recombination with wild type virus [Vigna and Naldini, 2000; Miyoshi et al, 1998; Iwakuma et al, 1999].

To increase the transduction efficiency and the expression of transgenes delivered by retroviral vectors, some sequences were added to the viral construct:

- the *Woodchuck hepatitis virus Post-transcriptional Regulatory Element (WPRE),* which stimulate the expression of transgene in a way not depending on the cell type or on the species. Its effect is not influenced by the cycling status of the transduced cells but the WPRE is only functional when present within a transcript in the sense orientation. This element increases the level of nuclear transcripts, without influencing the RNA half-life. Moreover, the WPRE may facilitate another step in RNA processing, directing RNAs that would normally be

degraded within the nucleus to be efficiently expressed. This processing could be facilitated at the level of 3' cleavage and polyadenilation. The WPRE could also function to facilitate the generation of RNA-protein complexes which would protect newly synthesized transcripts from degradation in the nucleus [Zufferey et al, 1999].

- The *central polypurine tract (cPPT)*, a *cis*-acting sequence that is located in the *pol* gene of the wild type HIV-1. This element facilitates the nuclear transport of the pre-integration complex through the nuclear membrane of quiescent cells. The introduction of the cPPT sequence in the transfer vector backbone strongly increased the total amount of genome integrated into the DNA of target cells [Follenzi et al, 2000; Vigna and Naldini, 2000; Stathopulos, 2003].

Another strategy to regulate transgene silencing relies on the addition of positive regulatory elements to the expression cassette, such as locus control regions (LCRs), chromatin insulators or matrix attachment sites:

- The discovery of *DNaseI hypersensitive sites (HS)* far upstream of the human *β*-globin cluster has improved the prospects for gene therapy for human haemoglobinopathies. As mentioned above, these HS sites are nucleosome-free regions of open chromatin that are highly accessible to *trans*-acting factors. Moreover, this *cis*-acting DNA element, termed *β*-LCR, has been reported to confer position-independent and copy number-dependent expression. This region, which is 20 kb long, has been reduced to shorter forms of a few hundred base pairs long that still confer the silencing modulation activity. The insertion of the *β*-LCR sequence within HIV-1 lentiviral vectors has been useful for increasing erythroid-specific synthesis of the *β*-globin protein in transgenic mice [Delenda, 2004].

- Several chromatin insulator boundary elements have also been described to protect expression cassettes from position effects. These include the HS4 core from the chicken *β*-globin LCR [Delenda, 2004].

- The insertion of a *scaffold or matrix attachment region (S/MAR)* from the immunoglobulin-kappa gene into HIV-1-based SIN transfer vectors has significantly increased the liver and hepatocyte transduction efficiency. Moreover, the human interferon-*β* S/MAR element inserted in the same kind of lentiviral

vector also resulted in an increase in the duration time of transgene expression in human HSCs [Delenda, 2004].

Naturally, the four lentiviral constructs produced in the third generation must be assembled in virus particles able to infect the target cells. The production of lentiviral particles is carried out through co-transfection of packanging cells with the different constructs (**figure 10b**). The use of these cells ensures many advantages, such as an increase in the titer and a further reduction of the possibility of producing wild type viruses [Buchschacher & Wong-Staal, 2000]. Among the different cell lines used for this purpose are included cells of human embryonic kidney 293T [Stathopulos, 2003].

Models for the study of β-thalassemia

The experimental models can reproduce, in the limit of the type of model, the clinical and molecular conditions of human β-thalassemia and their use can lead to the discovery of therapeutic strategies aimed to treat the disease.

In addition to the *in vitro* models, which reproduce a molecular mechanism modified by a pathological alteration, were developed more complex models *ex vivo* and *in vivo*.

The *ex vivo* models of β-thalassemia include different types of cell lines that can be modified through the introduction of constructs containing the β-globin gene, wild type or mutated. Among these cells, of particular importance are the human (K562) and mouse (MEL) erythroleukemic lines.

The erythroid K562 cell line was isolated and characterized by Lozzio and Lozzio [1975] from a pleural effusion of a patient with chronic myeloid leukemia (CML) in the terminal stage. K562 cells possess all the genes of β-globin locus, as the β gene, but this is not expressed, due to trans-active effects or to the presence of negative regulatory factors or to the lack of positive regulatory factors for the transcription of the gene [Young et al, 1985]. These cells do not produce adult haemoglobin and so they do not show some important pathological aspects of β-thalassemic cells, for example, the excess α chains [Gambari and Fibach, 2007]. For this reason, they are widely used for the production of *ex vivo* models with

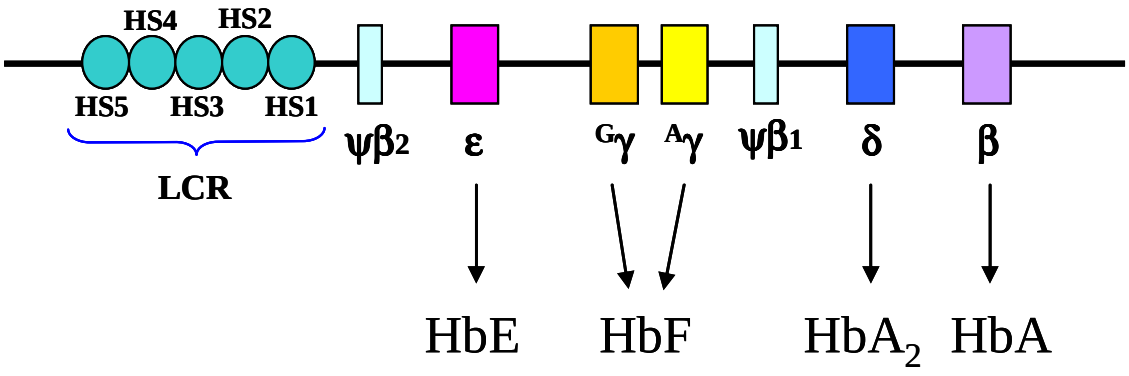
various purposes: 1) the study of the molecular mechanisms that regulate the expression of globin genes [Young et al, 1985; Donovan-Peluso et al, 1987; Donovan-Peluso et al, 1991], 2) the study of gene expression after induction of the cellular differentiation [Plonczynski et al, 1999]; 3) screening of new compounds that induce cellular differentiation [Bianchi et al, 1999, Bianchi et al, 2001], 4) development of approaches for gene transfer of human genes [Young et al, 1984], 5) the production of reporter systems to monitor the activities of the globin genes [Vadolas et al, 2002].

The erythroid MEL (Mouse ErythroLeukemia or FLC) cell line, of murine origin, derives from the transformation of erythroid cells with the Friend virus, which arrested them at the proerythrobastic stage [Friend et al, 1971]. These cells are used for different types of studies, from the cell differentiation to the regulation of globin genes [Chae and Kim, 2002]. They can also be used for the study of mutations in the human β-globin gene, following its introduction into the cell and subsequent integration into cellular genome. In this regard, Ho et al [1999] produced an expression system for *ex vivo* analysis of β-globin gene mutations, based on the transfection of MEL cells with β-globin gene wild type and mutated. After being validated, the system was used to examine the effects and mechanisms of action of two mutations in the β-globin gene 5' not translated region (5'UTR).

For the study of diseases, as well as *ex vivo* models, are of particular importance *in vivo* models, because they reproduce as far as possible the disease in study. It is essential, however, choose the animal most appropriate for the type of disease and the study phase of this, on which to develop clinical protocols.

With regard to β-thalassemia, investigators have generated several animal models based on mice, which present an α-globin cluster, located on chromosome 11, similar to the human one and, in contrast, a β cluster, on chromosome 7, quite different (**figure 11**). The murine β cluster includes four functional genes under the control of the LCR region. Of these, bh1 and ε^{y} are the globin genes of the embryonic phase, while b1 (β^{major}) and b2 (β^{minor}) the globin genes of the adult stage, responsible of the production of 80% and 20% β-globin chains of adult haemoglobin, respectively [Whitelaw et al, 1990; Shehee et al, 1993]. The

a)



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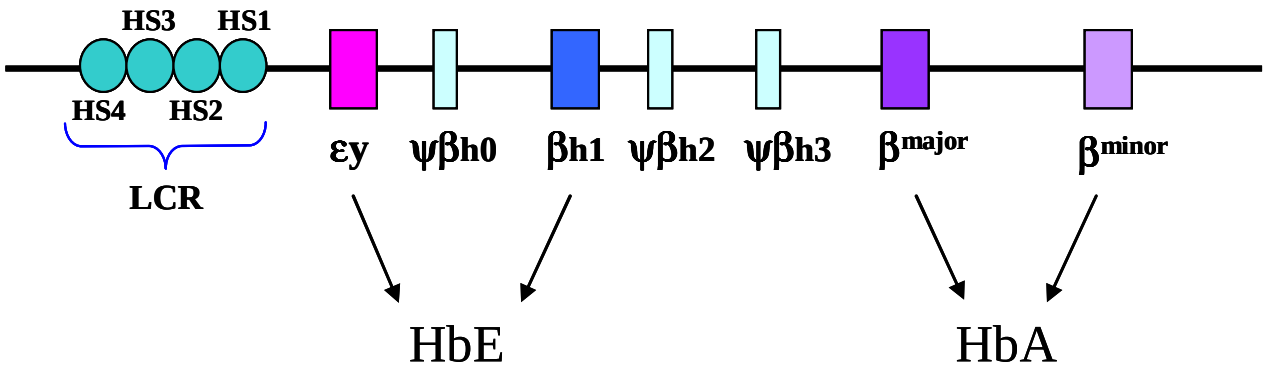


Figure 11 – Comparison between human and murine β-like clusters. a) Schematic representation of the human β cluster, located on the chromosome 11. There are indicated: the structural genes ε, $^{G}\gamma$, $^{A}\gamma$, δ and β, the pseudogenes ψβ2 and ψβ1, and the locus control region (LCR), containing the hypersensitive sites (HS1, HS2, HS3, HS4 and HS5). **b)** Schematic representation of the murine β cluster, located on the chromosome 7. There are indicated: the structural genes εy, βh1, β^{major} e β^{minor}, the pseudogenes ψβh0, ψβh2, ψβh3, and the locus control region (LCR), containing the hypersensitive sites (HS1, HS2, HS3 and HS4). In figure are, therefore, reported different types of haemoglobin, produced according to the β-like globin encoded by the different genes. It is possible to note the lack of fetal haemoglobin in mouse. HbE, embryonic haemoglobin; HbF, fetal haemoglobin; HbA, adult haemoglobin; HbA_2, type 2 adult haemoglobin.

different composition of the cluster β, missing a γ-globin gene comparable to the human one, initially brought considerable difficulty in developing mouse thalassemic models. This genetic limit has, in fact, allowed the production of a few models of murine β-thalassemia intermedia, as the complete absence of expression of β genes leads to prenatal death.

The first mouse model produced, called *th1*, [Skow et al., 1983] had a spontaneous deletion of 3.3 kb of DNA, including the β^{major} gene and upstream regulatory sequences, including promoter. The heterozygous subjects had a clinical situation of thalassemia intermedia and analysis of the synthesis of β-globin chains revealed a production of 95% in heterozygous and of 75% in homozygous mice. This event is explained by an increase in the synthesis of β^{minor} chains to compensate for the total absence of β^{major} chains. This compensatory increase is due to a competitive advantage of β^{minor} mRNA compared to α mRNA during the start of protein synthesis [Curcio et al., 1986].

The second mouse model, *th2*, [Shehee et al., 1993] was generated by producing a break in the β^{major} gene through the insertion of an exon of the *neo* gene (which confers resistance to the antibiotic G418) and of the relative promoter, made by homologous recombination in embryonic stem cells. This process does not remove the promoter of β^{major} gene and, therefore, the β^{minor} gene is to have two promoters that compete with its for the LCR region, with consequent reduction in its transcription. In addition, the β^{minor} transcript faces strong competition for the translational factors from the mRNAs of the β^{major} and neo genes, non-coding, however, for functional β-globin chains. For these reasons, homozygous subjects faced a prenatal death, while the survival in heterozygous was 60% of cases with severe anemia, due to thalassemia intermedia.

The third mouse model, *th3*, generated simultaneously in two different laboratories, by Ciavatta et al [1995] through homologous recombination, and by Yang et al [1995] through gene targeting, results from deletion, in embryonic stem cells, of β^{major} and β^{minor} genes. Homozygous subjects did not survive birth, while heterozygous ones had a clinical phenotype of thalassemia intermedia. This animal model of β^{0} heterozygous thalassemia can be used to test gene therapy, to study

the pathophysiology of the disease and to develop treatments that can improve the condition of individuals with β-thalassemia.

The fourth mouse model, *th4*, [Lewis et al., 1998] was the first animal model resulting from a human splicing mutation. It was produced by gene targeting, replacing β^{major} and β^{minor} genes with a single copy of human β-globin gene containing a mutation that causes a splicing defect and that leads to a condition of β^0-thalassemia. The resulting homozygous subject died in prenatal stage, while the heterozygote presents β-thalassemia intermediate, caused by aberrant splicing as in the human counterpart. This animal model can be used to develop therapies to correct defective splicing, as, for example, the use of antisense oligonucleotides.

The lack of murine models which reproduce β-thalassemia major has long been a limit to the characterization of critical physiopathological events, for the detection of biological mechanisms and the valuation of genetic and pharmacological treatments in this disease [Breda and Rivella, 2003]. For this reason, a mouse model of adult β^0-thalassemia major was created through the introduction, in animals subjected to myeloablation, of fetal liver cells, taken from an homozygous embryo of the *th3* mouse model, which is devoid of β^{major} and β^{minor} genes. Already after a few weeks, the mice showed a severe anemia and succumbed within 60 days because of the ineffective erythropoiesis [Rivella et al, 2003].

Among the various possible genetic treatments, an approach used by Breda and Rivella [2003] was the use of lentiviral vectors, arising from dTNS9 vector, and coding for the human β-globin gene. The developed mouse model, in the absence of endogenous β-globin gene, proved to be a valuable tool for quantification of the globin transgene expression and production of human β chains obtained in vivo. It was also demonstrated that the gene transfer through these vectors is a valid and applicable approach to study haematopoietic lethal phenotypes in animal models.

Because there is a significant difference between mouse and human gene sequences and it is of vital importance to develop therapeutic strategies that targeted the human sequences, Jamsai et al [2004] thought to produce transgenic mice by microinjection of extended fragments of human genomic DNA in fertilized oocytes. From these assumptions, they began to develop humanized

murine models, which, expressing the human gene locus, are more sophisticated and better simulate the disease than the earlier models.

Jamsai et al [2004] generated a humanized mouse model, breeding a transgenic mice containing the human β-globin locus, carrying a β^0 mutation in the β-globin gene, with a heterozygous mouse th3. From this crossbred, it was therefore possible to isolate a transgenic heterozygous mouse, which may express, in addition to normal β^{major} and β^{minor} genes, present in heterozygosity, the γ-globin gene during fetal development and the β-globin gene containing the mutation in the adult stage, a condition that leads him to have a β-thalassemia intermedia. This model can be used to study the corrections of DNA mutations in hematopoietic stem cells and the identification of new inducers of fetal haemoglobin.

The same group [Vadolas et al, 2006] also produced a humanized mouse model containing in emizygosity the β-globin locus with a human β^+ mutation that causes a defect in splicing. The synthesis of human β-globin chains is comparable to that of a human β-thalassemic patient with the same mutation. In fact, comparing the model containing the mutated transgene with one containing the wild type transgene, it is possible to note that the presence of the mutation results in a decrease of 90% in the synthesis of human β-globin chains, difference attributable to an aberrant splicing. This animal model is an excellent *in vivo* system for the screening of antisense oligonucleotides usable to correct splicing mutations.

Cultures of erythroid progenitors

The hematopoietic pluripotent stem cell undergoes a series of developmental changes that commit it to a specific cell lineage. The first erythroid-committed progenitors are defined functionally as burst-forming units (BFUe). Following proliferation and differentiation, these cells give rise to colony-forming units (CFUe) which bear on their surface the receptors for erythropoietin. These progenitor cells continue to proliferate and differentiate, eventually becoming mature red blood cells containing haemoglobin with oxygen-carrying capability. Erythroid cell maturation, known as erythropoiesis, is mediated by a combination

of regulatory proteins acting in concert to direct the development of progenitor cells into mature erythrocytes.

Erythropoiesis has been studied extensively as a model of gene regulation. During human ontogeny, erythropoiesis begins in the embryonic yolk sac (primitive erythropoiesis), transfers to the fetal liver, and then to the bone marrow before birth (definitive erythropoiesis). As the site of erythropoiesis changes, the globin genes being expressed also change, progressing from embryonic to fetal and finally to adult. Thus, globin gene regulation serves both as a model for developmental gene regulation during ontogeny and for the molecular mechanism directing cellular differentiation [Pope et al, 2000].

As already mentioned, erythroid cell lines and transgenic animals have been used extensively as models to study β-thalassemia and to develop new therapeutic strategies. In addition, primary cultures of erythroid cells can be readily established from most normal individual and patients and their growth *in vitro* represents more closely than cell lines the *in vivo* situation. The cells can be cultured either in semi-solid medium where they develop into discrete colonies, or in liquid medium where they grow as single cells or clusters in suspension. In both systems erythropoietin (EPO) is essential for full development of Hb-containing erythroid cells [Fibach, 2003].

Semi-solid cultures

In the cloning method, suspensions of single hematopoietic cells derived from the bone marrow, peripheral blood or other sources such as fetal liver, neonatal cord blood or adult spleen, are dispersed in semi-solid media usually containing methyl cellulose or plasma clot. Colonies start to appear after 3 – 4 day incubation, and they reach their final size and haemoglobinization after 1 – 2 weeks. Each colony represents a clone derived from one erythroid-committed progenitor. Based on the final size of the colonies and the time required for their haemoglobinization various type of progenitors can be distinguished; the late erythroid colony-forming units (CFUe) reach final size and haemoglobinization after 1 week and then disappear, while the early erythroid burst-forming units (BFUe) develop after 2

weeks. Erythroid colonies can be distinguishes from other (myeloid) colonies by their red colour or by their positive reaction with heme-specific reagents.

The fact that in this culture system the cells are immobilized in semi-solid medium results in several disadvantages. The cell yield per colony and the total cells per culture are low making it technically difficult to carry out biochemical, molecular and immunological characterizations of the developing cells. It is a one-step continuous culture and addition of the tested agents during the culture is difficult. In addition, the proportion of HbF produced in colonies grown in semi-solid medium is significantly higher than that produced *in vivo* by the donor of the cells [Fibach, 2003].

The two-phase liquid cultures

The liquid culture procedure overcomes several of these obstacles. It is possible to obtain large cultures of relatively pure and synchronized erythroid cell population and compounds can be added on different days when the culture consists of cells at specific stages of maturation. This culture procedure is separated into two phases: in phase I, peripheral blood mononuclear cells were cultured for 1 week in the presence of a combination of growth factors, but not EPO, and cyclosporine A, able to suppress the lymphocyte activation and proliferation (**figure 12a**). The vast majority of cells in these cultures are small lymphocytes and only a minute fraction of the cell population are various hemopoietic progenitors. Moreover, during the phase I the early erythroid-committed progenitors (BFUe), present originally in the peripheral blood, proliferate and differentiate into the more mature, CFUe-like, progenitors. In phase II (**figure 12a**), following exposure to EPO, the latter cells proliferate and mature within 2 weeks into Hb-containing orthochromatic normoblasts. Shown in **figure 12b** are cytospin preparations of cells at various stages of phase II. Early in the phase II (days 0 – 5) lymphocytes continue to present the most abundant cell type. Proerythroblasts begin to be discernible on days 4 – 5 as large, round, smooth cells which, following staining with Wright-Giemsa, demonstrate deep blue cytoplasm and a central large nucleus with abundant chromatin and 2 – 3

Liquid culture system

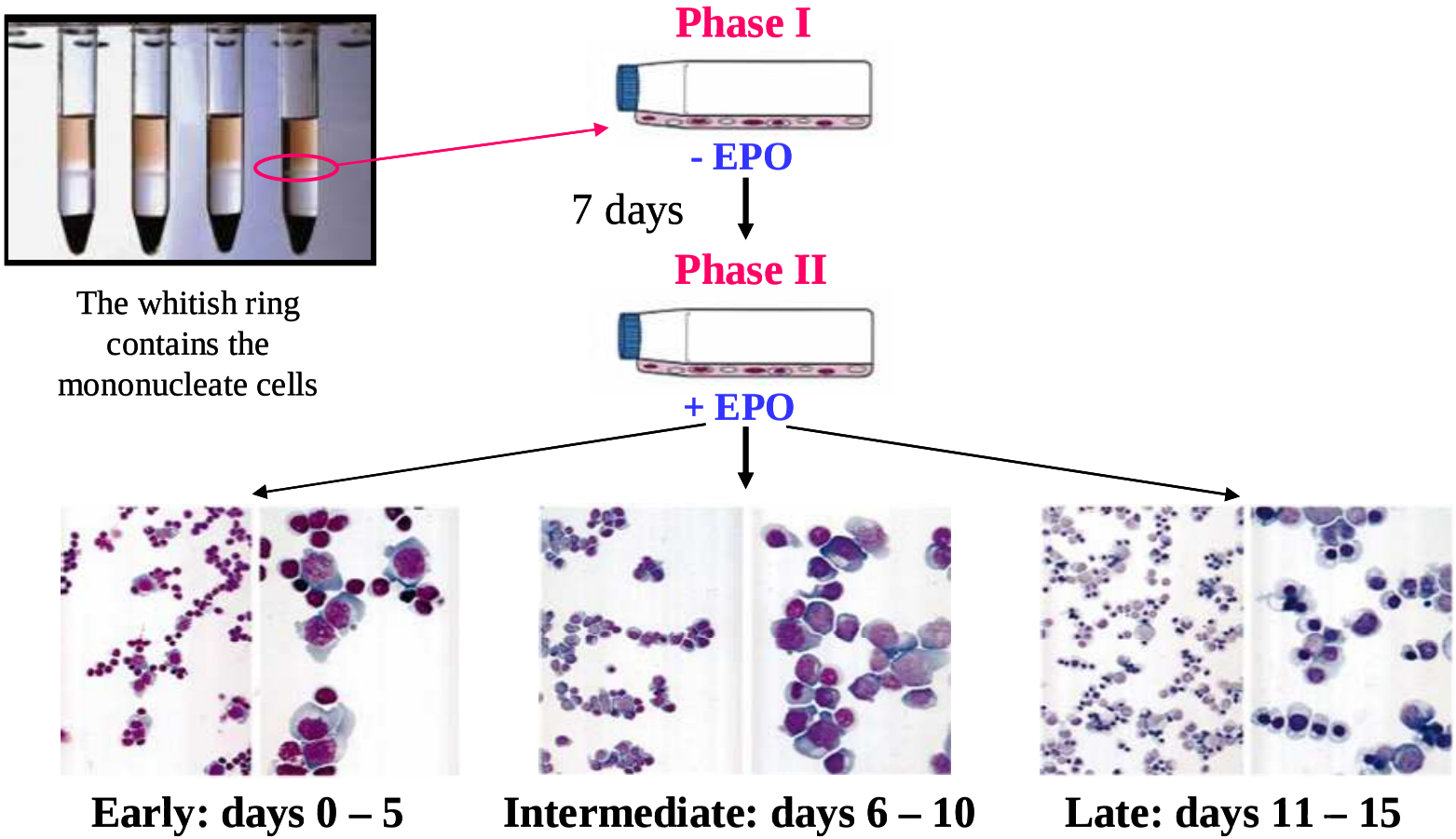


Figure 12 – Two-phase liquid culture method used in culturing human erythroid precursor cells. a) Mononuclear cells are isolated from human peripheral blood and cultured for 7 days in phase I medium containing cyclosporine A and growth factors but not erythropoietin (EPO). The cells are then washed and put into phase II medium containing EPO. During the following 14 days, cells continue to proliferate and mature into Hb-producing normoblasts. **b)** Cells taken from Phase II culture on days 0, 4, 6, 10, 13 and 15 (x400 or x1000) stained with a modified Wright stain.

prominent nucleoli. As these cells multiply they form clusters. During the intermediate phase (days 6 – 10) lymphocytes gradually give way to an increasing proportion of proerythroblasts and basophilic normoblasts, which when indisturbed form aggregates consisting of hundreds of cells. In late phase II (days 11 – 15), erythroid cells continue to proliferate and mature into polychromatic and orthochromatic Hb-containing normoblasts. Within the intermediate and late phases, the absolute number and proportion of small lymphocytes diminished significantly.

Globin expression in the maturing erythrocyte in the two-phase system recapitulates expression patterns which occur in the fetal to adult haemoglobin switch during ontogeny. This is seen in the reciprocal relationship between γ- and β-globin gene expression in which γ-globin peaks in early phase II then declines as β-globin increases and then peaks in late phase II. As expected, α-globin also reaches maximum expression when total haemoglobin increases in the late stage [Pope et al, 2000].

Pope et al [2000] analysed also the expression of the transcription factors Sp1, GATA-1 and EKLF, essential for the primitive and definitive erythropoiesis. They observed that Sp1 is expressed at maximum levels in the early stage of phase II. It then declines through the intermediate stage to moderately low levels where it is maintained, an expression pattern which is consistent with its key role in early developmental viability. GATA-1 is expressed at high levels in both the early and intermediate stages of phase II and then declines as the cells reach maturity during the late stage. This pattern of expression supports the crucial role of GATA-1 in the maturation of the primitive erythroblast and in the gene expression as the cells continue to develop. The erythoid-specific transcription factor EKLF also reaches peak expression during the intermediate stage of phase II preceding a significant rise in β-globin expression.

Since the erythroid cells in phase II are grown in suspension, samples of cells can be withdrawn at any time without disturbing the cultures and assayed for morphology, size, number, cell viability and apoptosis, cell cycle or expression of surface antigens. The Hb content of the developing erythroid cells can be measured by a variety of techniques, such as the alkaline denaturation and

benzedine staining and high-performance liquid chromatography (HPLC), cation-exchange HPLC for haemoglobins and reverse-phase HPLC for globin chains. Using the HPLC techniques, Hb is measurable in culture as early as 5 days in phase II. The mean cellular Hb concentration of erythroid cells are calculated from the values of the HPLC determinations divided by the number of benzedine-positive cells. Moreover, the distribution of the erythroid cell population with respect to intracellular content of HbA or HbF can be analysed by flow cytometry using monoclonal antibodies directed specifically against HbA or HbF.

This system has been used to study the effects of several compounds on the HbF production. For studying their potential to enhance HbF production, compounds can be added to phase I, phase II or both. Non-toxic drugs such as cytokines and hemin may be added to the cultures at any time. With cytotoxic drugs, such as hudroxyurea and 5-azacytidine, because of their cyto-toxic/static effects, they are usually added on day 4 – 8 of phase II [Fibach, 2003].

Among the several compounds tested on healthy human and thalassemic-cultured erythroid precursor cells, mithramycin [Fibach et al, 2003], rapamycin [Mischiati et al, 2004], angelicin [Lampronti et al, 2003] and everolimus [Zuccato et al, 2007] showed to be potent HbF inducers.

Separation of haemoglobins by high-performance liquid chromatography (HPLC)

Human haemoglobin abnormalities have been studied for many years, particularly since 1949 when the HbS was discovered in sickle cell anemia. Most of the hundreds of variants, discovered during the years, are the result of single base substitution in the DNA of either the $^{G}\gamma$-, $^{A}\gamma$-, δ-, β-, α-globin genes, but other variants with hybrid chains, extended chains, chains with deletions, etc., have also been discovered.

Numerous procedure have been used to identify and/or characterize the normal and abnormal Hbs. Most common are elctrophoretic methods, isoelectric focusing (IEF) techniques, and macro-chromatography procedures. Since the early ’80s, high-performance liquid chromatography (HPLC) has influenced all aspects of

analytical chemistry and it is therefore not surprising that different types of HPLC have also been applied to the study of the haemoglobinopathies [Huisman, 1987].

Separation of haemoglobins by anion-exchange HPLC

The anion exchanger SynChropak AX 300 was introduced in early ’80s for the separation of normal and variant Hb types. Separation of the Hbs A and F is important; however, the elution times of HbF and the minor HbA_1 are about the same, which prevents the quantification of HbF in adult red cell lysates with a low HbF percentage. The Hbs A_2 and E also cannot be separated. Identification and quantification of adult Hb abnormalities in cord blood samples are possible, particularly for the β chain variants HbS and HbC. HbD-Los Angeles and HbS, however, can only be incompletely separated by this procedure although a slower gradient will improve the separation of these variants. The method is particularly useful for the quantification of Hb Kenya which is composed of α chains combined with $^{A}\gamma\beta$ hybrid chains [Huisman, 1987].

Separation of haemoglobins by cation-exchange HPLC

Huisman’s group applied cation-exchange HPLC using many different blood samples, taken from patients with different pathologies in order to identify the elution time proper of each haemoglobin, so as to identify its position on the chromatogram obtained from the analysis.

Separation obtained using blood samples collected from severely affected, uncontrolled, diabetic patient shows large quantities of HbA_{1c} (glycosylated HbA) and HbA_{1d} (normally not observed or present in small quantities; this fraction is HbA with glucose attached to more than one amino acid residue, including some of the α chains). HbA_2 elutes after HbA_0 and is completely separated from the major Hb, thus allowing its quantification. HbE does not separate from HbA_2, while the minor Hbs elute in front of the major HbE component behind two HbF zones, i.e. the major HbF_0 and its acetylated derivative HbF_1.

Using samples from patients with homozygous HbS or HbC conditions, it was possible to verify that the elution characteristics of these β chain variants readily allow the quantification of HbF (F_0+F_1) and HbA_2 in one chromatographic experiment. **Figure 13a** illustrates the positions of various β chain abnormal Hbs in this type of chromatography. It is interesting to note that variants with identical substitution (Glu→Lys) but in different position of the β chain (C: β6; E: β26; O-Arab: β121) elute at completely different elution time, indicating that the chromatographic properties of these variants are greatly influenced by the location of this Glu→Lys substitution in the β chain.

The original cation-exchange HPLC methodology was most useful for the separation of abnormal Hbs in blood from newborn babies. The major advantage of this method is the fast elution of HbF (F_0+F_1) well separated from HbA and the most common β chain variants. Their elution is preceded by that of Hb Bart's (or γ_4); the presence of a notable quantity of this unusual variant is indicative for the presence of an α chain deficiency or α-thalassemia.

The excellent separation of HbF and HbA makes cation-exchange HPLC the method of choice to analyse and quantitate Hbs F, A and A_2 in patients with homozygous β^0- or β^+- or $(\delta\beta)^0$-thalassemia. **Figure 13b** gives two examples [Huisman, 1987].

Over the years, HPLC has been extensively used for the prenatal and postnatal diagnoses of thalassemia and haemoglobinopathies [Eastman et al, 1996; Fucharoen et al, 1998] and the screening of haemoglobin variants [Ou and Rognerud, 1993; Clarke and Higgins, 2000]. Moreover, this procedure has been used for the quantification of cellular Hb content in samples of erythroid precursor cells treated with HbF inducers [Fibach et al, 1993; Fibach et al, 1995; Lampronti et al, 2003; Mischiati et al, 2004].

Nonsense-mediated mRNA decay

Eukaryotic gene expression involves an intricate chain of complex biochemical reactions, starting with the synthesis of mRNA, followed by the production of encoded proteins, and ending with the degradation of both the mRNA and the

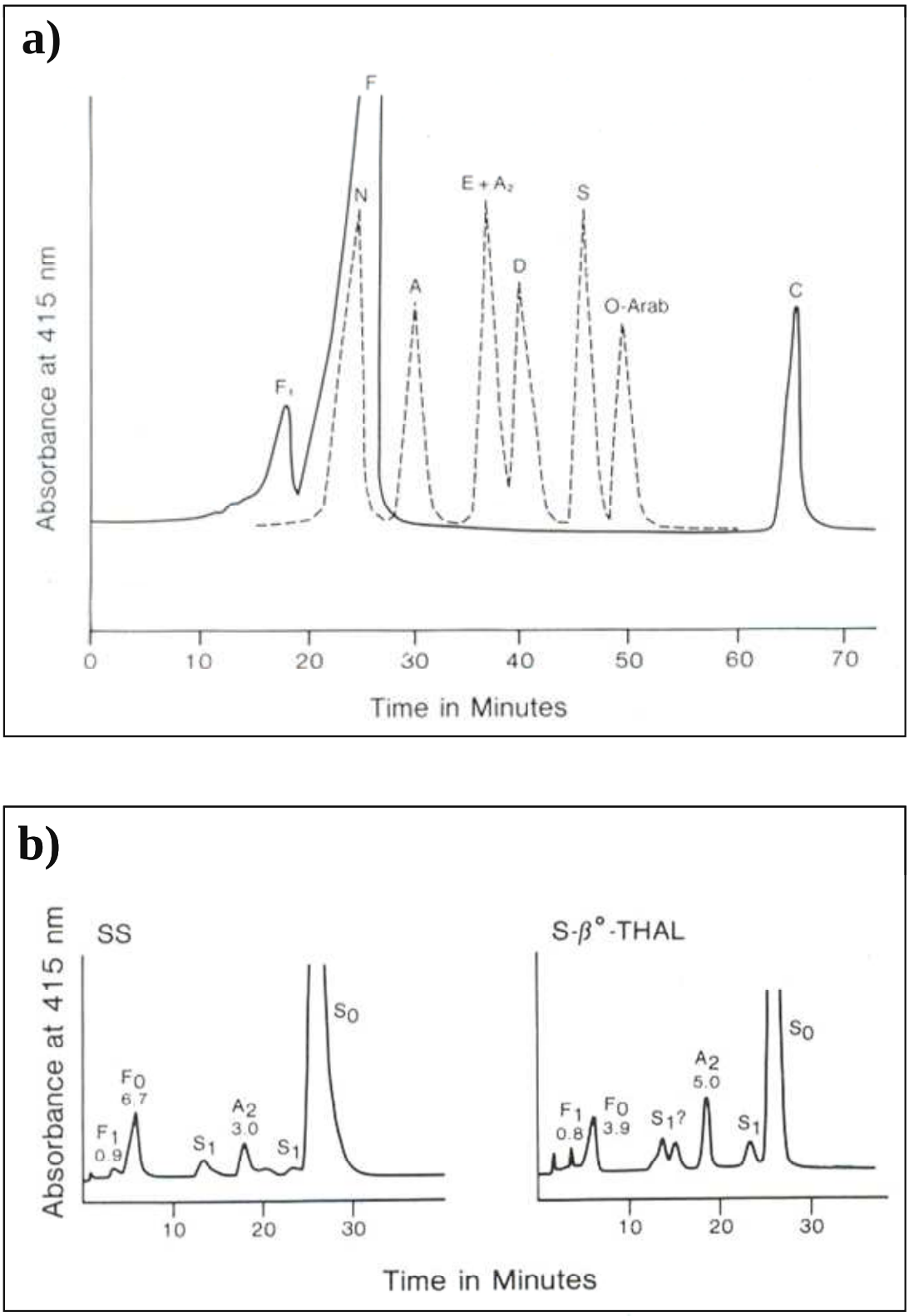


Figure 13 – HPLC chromatograms. a) Positions of different adult Hb types in chromatograms of red cell lysates from newborn babies. **b**) Separation of Hb types by HPLC cation-exchange methodology. This procedure adequately separates HbA, HbA2 and HbS and allows the differentiation between the two conditions listed.

protein. Tight control of and high accuracy within these processes are absolutely required to prevent inappropriate gene expression and to ensure cell survival, and cells have therefore evolved mechanisms to control many steps along the chain. One of the best-studied quality control mechanisms is nonsense-mediated mRNA decay (NMD). NMD was initially described as a mechanism for recognizing and degrading faulty transcripts harbouring a premature translation-termination codon (PTC); such nonsense transcripts would otherwise result in the production of C-terminally truncated proteins with potentially dominant-negative effects. PTCs can arise either from mutations at the DNA level (e.g. nonsense mutations, frame-shifting deletions and insertions) or from altered splicing signals that induce production of alternatively spliced mRNA isoforms with truncated reading frames. Over the last five years, it has become clear that NMD not only degrades faulty transcripts but also regulates the steady-state level of many physiological mRNAs involved in a variety of different cellular processes, such as DNA repair, the cell cycle, and metabolism. Genome-wide screens in budding yeast, Drosophila and human cells have revealed that NMD regulates expression of about 3 – 10% of the transcriptome. Furthermore, it has been estimated that about 30% of the known disease-associated mutations in humans generate a PTC-containing (PTC+) mRNA, and in many of these cases NMD influences the severity of the clinical manifestations caused by the mutation [Stalder and Mühlemann, 2008].

Trans-acting factors involved in NMD

The NMD core factors Upf1p, Upf2p and Upf3p (for ‘‘upframeshift 1-3’’) were initially identified in genetic screens in yeast, and SMG1-7 (for ‘‘suppressor with morphological effect on genitalia’’) were found to be NMD effectors in *C. elegans*. Sequence alignments revealed that SMG2 is homologous to Upf1p, SMG3 to Upf2p, and SMG4 to Upf3p, and that SMG1–7 are present in all higher eukaryotes analyzed to date, with the exception of *D. melanogaster*, which contains no clear homolog for SMG7. Additional NMD factors are very likely to exist, although they remain to be discovered. Given that they are conserved from yeast to humans, UPF1, UPF2 and UPF3 are believed to function at the heart of

NMD. UPF1 is the most highly conserved NMD factor, and elucidating its structure and function will provide the key to understanding the mechanism of NMD. UPF1 interacts with the eukaryotic release factors eRF1 and eRF3, it binds to UPF2 through its cysteine and histidine rich (CH-rich) region near the N-terminus, and it interacts with SMG1, SMG5, SMG6 and SMG7. UPF1 exhibits RNA binding, RNA-dependent ATP hydrolysis, and 5'-to-3' ATP-dependent RNA helicase activities, and inhibition of any of these activities suppresses NMD. It has been shown *in vitro* that UPF1 dissociates from RNA after addition of ATP. Interaction with eRF1 and eRF3 strongly reduces the ATPase and RNA binding activities of UPF1. Vice versa, RNA binding stimulates the ATPase activity and leads to a dissociation of UPF1 from eRF1 and eRF3 [Stalder and Mühlemann, 2008].

Mechanism of NMD

Actually, there are three different models, which try to elucidate the exact mechanism of NMD.

1. the first, widely accepted, model (**figure 14**) assumes that in mammalian NMD, an intron apparently functions as second signal, besides the premature termination codon (PTC), for triggering NMD by leaving a "mark" on the mRNA at the exon-exon junction as a consequence of the splicing event. This mark known as the exon-junction complex (EJC) enables the NMD RNA surveillance pathway to differentiate between PTCs and normal stop condons present in the last exon ensuring the degradation only of transcripts containing nonsense codons that are followed by an intron. Evidence from a number of studies suggests that during the initial rounds of translation, referred as the "pioneer round", the translating ribosome pauses at the PTC. Release factors eRF1 and eRF3, which associate with the termination ribosome, recruit UPF1, which subsequently makes contact with UPF2 and UPF3, which are bound to EJC. Formation of this complete "surveillance complex" containing UPF1-3 is believed to initiate degradation by NMD. In normal transcripts, in which the termination codon occurs in the final exon, all EJCs are displaced by the translating ribosome. Thus, the complete

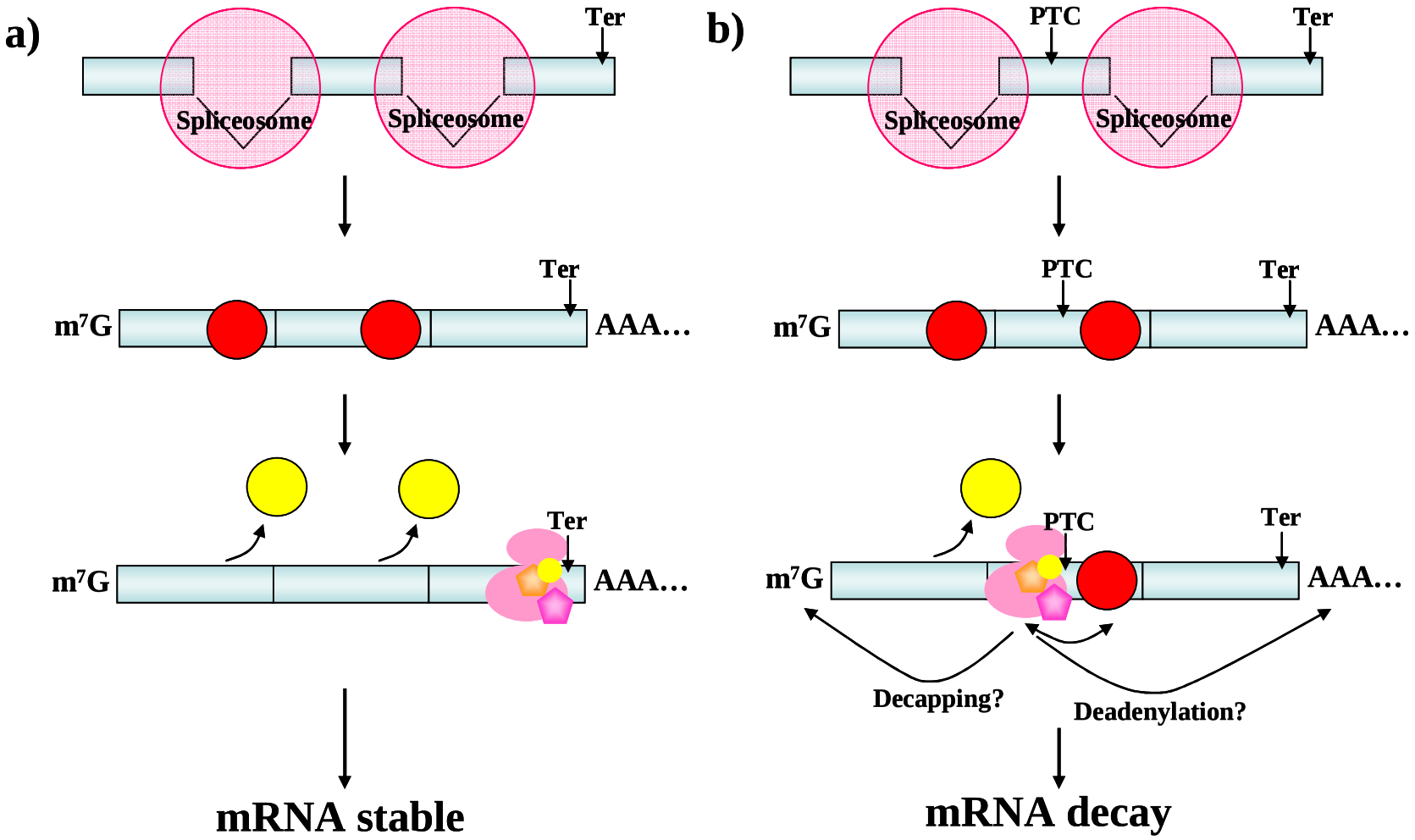


Fig. 14 – Simplified first model of NMD. During splicing in the nucleus, a protein complex (EJC) is deposited 5' of exon-exon junctions. Upon transport through the nuclear pore, the EJC (red) is remodeled, several proteins are released and others persist and accompany the mRNP into the polysomes. During translation of a PTC-free mRNA (a), the EJCs (yellow) are stripped off the mRNA by the translating ribosome (pink). This first round of translation validates the mRNA as error-free and, hence, it remains stable. If the translating ribosome encounters a PTC upstream of at least one EJC (b), however, NMD is triggered [Holbrook et al, 2004].

surveillance complex cannot form during translation termination because of the absence of UPF2 and UPF3, thus NMD is avoided. The mammalian NMD surveillance system cannot distinguish PTCs in the penultimate exon that are located less than about 55 bp from the final intron. The “55bp-rule” for the 3’end of the penultimate exon is believed to reflect the location of the EJC on spliced mRNAs; a translocating ribosome would displace an EJC upstream of the last exon-exon junction before the ribosome could recognize a stop codon located less than about 55 bp from the exon-exon junction. As a consequence, the inability to differentiate nonsense codon mutations in the last and 3’end of the penultimate exon from the normal termination codon can result in the stable translation of mRNAs that contains PTCs located within this “protected” region. Thus, mRNA with non-sense codons present in such positions can escape the NMD pathway. Such an “escape from NMD surveillance” may cause expression of large amounts of aberrant truncated proteins with potential dominant-negative or gain-of-function effects in cells [Khajavi et al, 2006].

2. The second model is called “the *faux* 3’UTR model” and suggests that proper termination of translation and normal rates of mRNA decay are likely to require interactions between a terminating ribosome and a specific ribonucleoprotein (RNP) structure or set of factors localized on 3’ to the stop codon. Failure to terminate in this manner seems to slow ribosome release and facilitate reinitiation. The latter event must only be a symptom of aberrant termination, not a cause of NMD, because elimination of reinitiation site AUG codons does not alter the decay rate of nonsense-containing mRNAs. However, the failure to release a terminating ribosome effectively might be a stimulus for NMD, possibly promoted by the ribosome failure to recycle in cis and/or by the recruitment of UPF1P to aberrant termination event or to a bound negative regulatory factor [Amrani et al, 2004].

3. Recently, Stalder and Mühlemann [2008] proposed a new “unified” model for PTC recognition (**figure 15**). This model essentially extends the *faux* 3’UTR model to all species and proposes that, during mammalian NMD, downstream EJCs act as an evolutionary adaptation to efficiently recognize nonsense mRNAs produced by extensive alternative splicing. Their laboratory showed that the

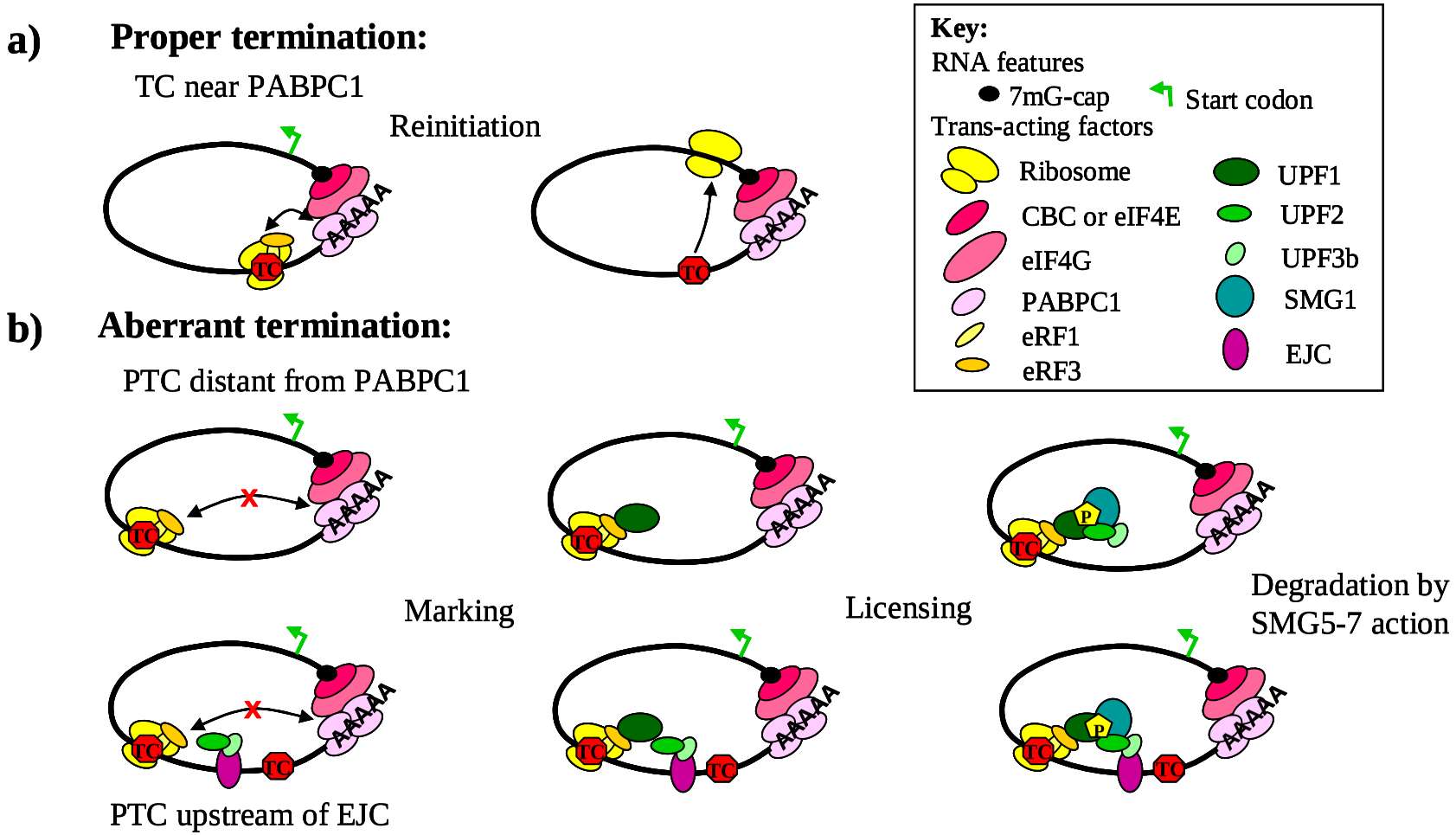


Figure 15 – Third model for NMD. a) The mRNP is thought to form a closed-loop structure through the interaction of cap-bound eIF4E or CBC (for "cap-binding complex") with eIF4G, which in turn interact with the poly(A) tail-bound PABPC1. When the ribosome terminates at a PTC in the vicinity of the poly(A) tail, a PABPC1-mediated signal promotes proper termination of translation, resulting in efficient reinitiation of the ribosome at the 5' end of the mRNA and the production of a stable mRNP. **b**) If the ribosome terminates at a PTC that it is too far away from the poly(A) tail for it receive the PABPC1-mediated translation-termination-promoting signal, UPF1 binds to the stalled ribosome instead, thereby marking this PTC as a premature. Subsequently, UPF2 and UPF3b interact with UPF1, promoting SMG1-mediated phosphorylation of UPF1. This licensing step commits the mRNA to rapid degradation by as yet unknown pathways that involve the binding of SMG5-7 to the phosphorylated UPF1 (upper part). An EJC downstream of a PTC functions as an NMD enhancer by shortening the time window between UPF1 binding and its phosphorylation by locally concentrating UPF2 and UPF3b (lower part) [Stalder and Mühlemann, 2008].

physical distance, rather than the number of nucleotides, between a PTC and the poly(A) tail is a crucial determinant in defining a PTC as premature. It was shown that UPF1 and PABPC1, the major cytoplasmic PABP (poly(A)-binding protein), compete for the interaction with eRF3 in human cells in vitro. The balance of this competition enables the cell to distinguish between correct and aberrant translation termination. Thus, if a ribosome stalls at a PTC that is too far away from the termination-promoting environment (i.e. distant from PABPC1), resulting in slow termination kinetics, the balance between the two antagonizing signals is tilted toward UPF1 binding (‘marking’ within **figure 15b**). Notably, binding of UPF1 to the stalled ribosome in this context is EJC-independent. Although UPF1 has been found to preferentially associate with PTC-containing mRNA in *C. elegans*, some association with PTC-free mRNA was also observed. This suggests that simple binding of UPF1 to a terminating ribosome is not sufficient to elicit degradation, but rather a ‘second signal’ is required. The second signal might be the binding of UPF2 and UPF3 to UPF1, an event that is important for the SMG1-mediated phosphorylation of UPF1 in higher eukaryotes, and which stimulates the RNA helicase and ATPase activities of UPF1. As a consequence of ATP hydrolysis, binding of UPF1 to the RNA might be facilitated. ATPase-defective UPF1 mutants show enriched co-immunoprecipitation with UPF2 and UPF3b in mammalian cells. In summary, Stalder and Mühlemann [2008] postulate that the phosphorylation of UPF1 and the stimulation of its ATP hydrolysis and helicase activity represent a ‘point of no return’ in the NMD pathway, and they define this as the ‘licensing step’ (**figure 15b**). Contrary to the popular model for mammalian NMD, several studies have demonstrated that PTCs can trigger NMD in the absence of an EJC further downstream on the mRNA. However, it is apparent that the extent of mRNA downregulation in these examples of EJC-independent NMD is lower than in corresponding examples of NMD of transcripts with EJCs in the 3’UTR. Consistent with the idea that EJCs have an important role in NMD, knockdown of EJC core-factors in mammalian cells reduced the downregulation of many NMD reporter mRNAs. In the light of this, Stalder and Mühlemann [2008] propose that, in mammals, the EJC has evolved as a specialized second signal to enhance mammalian NMD. This unified NMD model provides a

mechanistic explanation for the NMD-enhancing function of EJCs located downstream of a PTC. As part of such a 3'UTR-bound EJC, UPF2 and UPF3 are ideally positioned for immediate interaction with ribosome-bound UPF1 and SMG1. As a consequence, the time window between the binding of UPF1 to the terminating ribosome (the marking step) and its SMG1- mediated phosphorylation (the licensing step) would be shortened, and thus the competition between PABP and UPF1 for binding to the stalled ribosome would tilt toward NMD (**figure 15b**). They hypothesize that, in mammals, under the evolutionary pressure to efficiently recognize and eliminate the large number of nonsense mRNAs produced by extensive alternative pre-mRNA splicing, the EJC as a spatial mark of previous splicing events has been incorporated into the mechanism of PTC recognition as an enhancer.

Degradation of PTC-containing transcripts

Once aberrant mRNA is targeted for decay by PTC-recognition, its degradation will be mediated by several different enzyme complexes involved in different NMD pathways. Generally, most aberrant mRNA degradation is initiated from 3' poly(A) by deadenylase complex. Subsequently, there are two pathways involved in rapid mRNA decay. In one pathway, deadenylation triggers decapping, and this exposes the mRNA body for digestion by the major cytoplasmic 5'-3' exonuclease XRN1. decay of mRNA through this pathway is though to occur in specialised cytoplasmic bodies or mRNA decay foci (also known as P-bodies or GW-bodies) that are enriched in XRN1, the decapping enzymes and decapping co-activators. Besides 5'-3' mRNA decay pathway, recent work in yeast shows that there is an alternative, decapping-independent NMD pathway, involving deadenylation and subsequent 3'-5' exonucleolytic decay [Li et al, 2006].

The role of NMD in regulation of normal gene expression

Probably, the primary role of the NMD pathway is to regulate the stability and abundance of physiological transcripts. In all eukaryotes, there is a low but

significant level of incompletely spliced pre-mRNA that encode in-frame premature termination codons and would therefore be substrates for the NMD pathway. Aberrant mRNAs resulting from other natural sources of error, including faulty transcription or splicing, would also be subject to the quality control imposed by this surveillance system. Another class of transcripts expected to be a target of NMD comprises those that contain upstream open reading frames (uORFs) in their 5'UTR. These uORFs may be interpreted to be prematurely terminated by the scanning machinery, resulting in rapid decay. In theory, there is a number of other physiological variants of gene structure that may elicit scrutiny by the nonsense surveillance pathway. For example, the growing class of non-coding RNAs may serve as substrate for NMD. Moreover, a small but significant number of mammalian genes contain an intron within the 3'UTR. In theory, the corresponding transcripts would be subject to rapid decay if a sufficient distance were present between the *bona fide* termination codon and the splice donor [Frischmeyer and Dietz, 1999].

Further contribution from NMD to physiological gene expression are evident in the maturation of the immune system. In B and T cells, the immunoglobulin and T-cell receptor genes, respectively, are rearranged extensively, introducing PTCs in two-thirds of cases, and transcripts from these genes are degraded by NMD. Presumably, at the level of the entire B- and T-cell population, permitting expression of only successfully rearranged genes results in a large number of fully functional variant immunoglobulin or T-cell receptor protein, greatly increasing the ability of the immune system to respond to a variety of antigens [Holbrook et al, 2004].

NMD-mediated protection against hereditary disorders

The medical significance of NMD in this respect was first appreciated in β-thalassemia, and this disorder demonstrates the protective effects of NMD against the production of faulty proteins. In the common recessive form of β-thalassemia caused by NMD-sensitive PTC mutations, defective β-globin mRNA is degraded by NMD and, therefore, synthesis of truncated β-globins is limited. In contrast to

homozygotes, heterozygotes with these mutations generally synthesise enough β-globin from the remaining allele to support near-normal haemoglobin levels and are therefore healthy. Rare NMD-insensitive last-exon PTC mutations, however, give rise to truncated, nonfunctional β-globin that overwhelms the cellular proteolytic system and causes toxic precipitation of insoluble globin chains. This stark contrast between asymptomatic heterozygotes with NMD-competent mutations and affected heterozygotes with NMD-incompetent mutations shows that, for PTC mutations in the β-globin gene, NMD protects most heterozygous carriers from dominant disease [Holbrook et al, 2004].

NMD in acquired genetic conditions

Mutations in tumor-suppressor genes are common steps in the development and progression of cancer. NMD degrades transcripts containing PTCs from the gene BRCA1 and reduces abundance of mRNAs containing PTCs from the TP53 and Wilson tumor loci. Studies indicate that if abnormal transcript containing PTCs were not degraded by NMD, clinically recessive tumor-suppressor mutations could result in the synthesis of truncated, dominant oncoproteins. NMD may thus protect heterozygous carriers of tumor-supressor gene containing PTC mutations from developing cancer, at least for as long as the other tumor-suppressor allele remains intact [Holbrook et al, 2004].

Protein synthesis (Translation)

The translation is the process by which the nucleotide sequence of a mRNA is used to sort and combine the amino acids in a polypeptide chain. In eukaryotic cells, protein synthesis occurs in the cytoplasm, where there are three types of RNA molecules with different functions, but that cooperate together: 1) messenger RNA (mRNA) that carries the genetic information copied from the DNA in the form of three-base code “words”, each of which specifies a particular amino acid. 2) Transfer RNA (tRNA) which is the key to deciphering the code word in mRNA. Each type of amino acid has its own type of tRNA, which binds and

carries it to the growing end of a polypeptide chain if the next code word on mRNA calls for it. The correct tRNA with its attached amino acid is selected at each step because each specific tRNA molecule contains a three-base sequence that can base-pair with its complementary code word in the mRNA. 3) Ribosomal RNA (rRNA) which is associated with a set of proteins to form ribosomes. These complex structures, which physically move along an mRNA molecule, catalyze the assembly of amino acid into protein chains. They also bind tRNAs and various accessory molecules necessary for protein synthesis. Ribosomes are composed of a large and small subunit, each of which contains its own rRNA molecule or molecules.

A ribosome is composed of several different ribosomal RNA (rRNA) molecules and more that 50 proteins, organized into a large subunit (60S) and a small subunit (40S). The proteins in the two subunits differ, as do the molecules of rRNA. The small ribosomal subunit contains a single rRNA molecule, referred to as *small rRNA* (18S); the large subunit contains a molecule of *large rRNA* (28S) and one molecule each of two much small rRNAs. These rRNAs interact with mRNA and tRNAs in each phase of the translation, and the proteins contained in the ribosome bring them together to carry out the biochemical events leading to formation of proteins on ribosomes.

The complex process of translating mRNA into protein can be divided into three stages: initiation, elongation and termination.

The AUG codon for methionine functions as the start codon in the vast majority of mRNA. A critical aspect of initiation is to begin protein synthesis at the start codon, thereby setting the stage for the correct in-frame translation of the entire mRNA. Both prokaryotes and eukaryotes contain two different methionine tRNAs: $tRNA_i^{Met}$ can initiate protein synthesis and $tRNA^{Met}$ can incorporate methionine only into a growing protein chain. The same aminoacyl-tRNA synthetase (MetRS) charges both tRNAs with methionine, but only $tRNA_i^{Met}$ can bind at the appropriate site on the small ribosomal subunit to begin synthesis of a protein chain.

Initiation of protein synthesis in eukaryotic cells begins with formation of a *preinitiation complex* prior to mRNA binding. Two eukaryotic factors, eIF3 and

eIF6, serve to keep the ribosomal subunits apart after they have previously finished synthesizing a protein chain so that they can participate again in starting a new chain (**figure 16**). To assemble a eukaryotic preinitiation complex, an active "ternary complex" of eIF2 bound to a GFP molecule and Met-tRNA$_i^{Met}$ associates with a small ribosomal subunit complexed with two other factors, eIF3 and eIF1A, which stabilize binding of the ternary complex (**figure 16-1**).

Most eukaryotic mRNAs have a single start site near the 5' capped end of the mRNA. Interaction of the small ribosomal subunit with the methylated 5'-cap structure present in all eukaryotic mRNAs requires a set of proteins collectively called eIF4 (**figure 16-2**). After the methylated cap structure is recognized by eIF4F, any secondary structure at the 5' end is removed by an associated helicase activity. The bound *initiation complex*, which contains the eIF3 group of proteins, then probably slides along the mRNA, most often stopping at the first AUG. However, selection of the initiating AUG is facilitated by specific surrounding sequences called *Kozak sequences*. At this point, the hydrolysis of GTP in eIF2-GTP provides the energy for the releasing of all factors of the initiation complex (**figure 16-3**). Finally, the factor eIF5 assists union of the 40S complex with the 60S subunit (**figure 16-4**).

The correctly positioned eukaryotic 80S ribosome – Met-tRNA$_i^{Met}$ complex is now ready to begin the task of stepwise addition of amino acids by the in-frame translation of the mRNA. As is the case of initiation, a set of special proteins, termed elongation factors (EFs), are required to carry out the process. The key steps in elongation are entry of each succeeding aminoacyl-tRNA, formation of a peptide bond, and the movement, or translation, of the ribosome with respect to the mRNA.

The initiating Met-tRNA$_i^{Met}$ is bound at the *P site* and base-paired with the AUG start codon, while the second aminoayl-tRNA is brought into the ribosome as a ternary complex in association with an EF1 α-GTP and becomes bound to the *A site* on the ribosome (**figure 16-5**). If the anticodon of the incoming aminoacyl-tRNA corretly matches the second codon of the mRNA, a tight binding ensues at the A site. If the second codon does not match the incoming aminoacyl-tRNA, it diffuses away. The choice of the correct aminoacyl-tRNA and its tight binding at

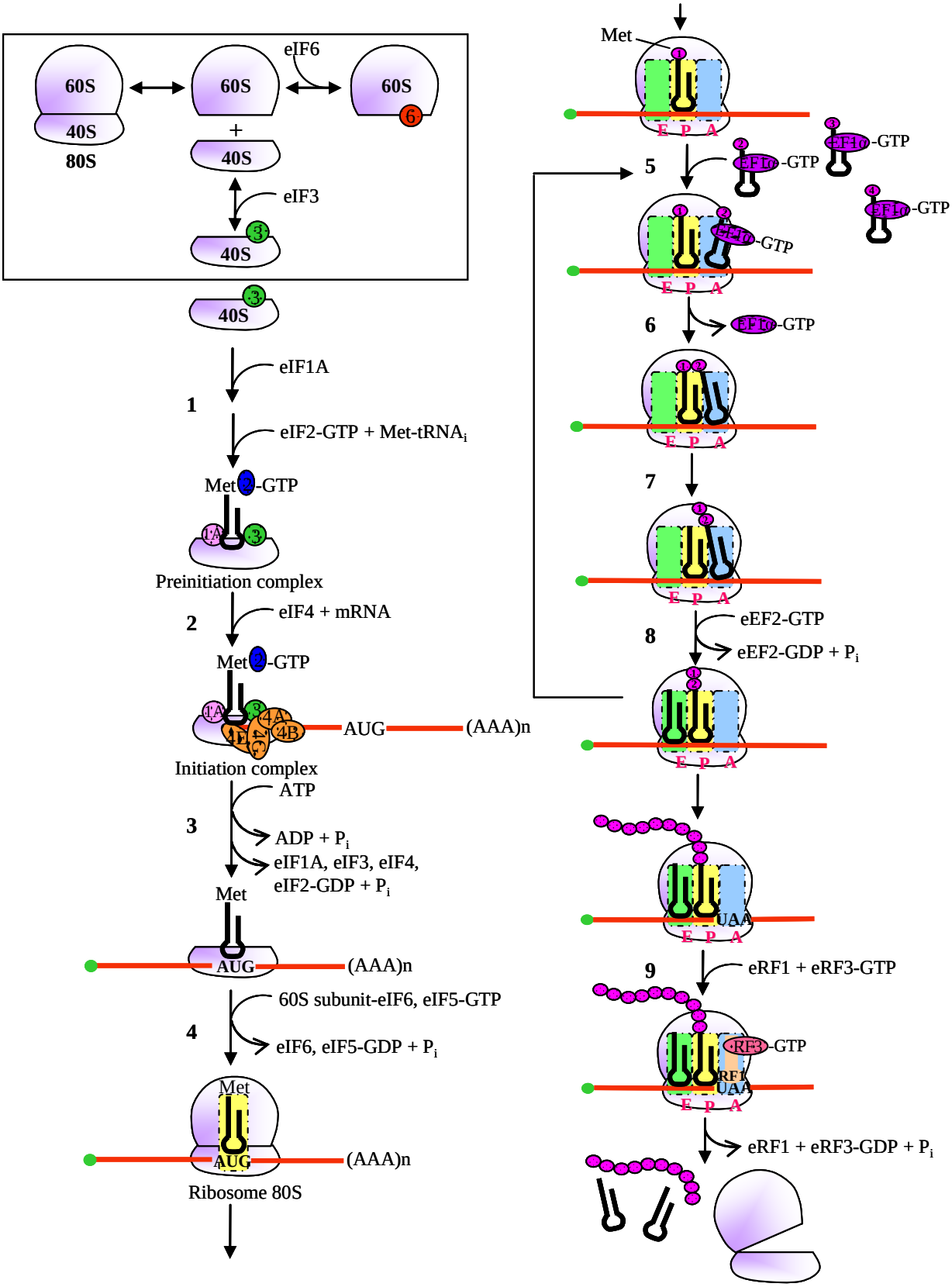


Figure 16 – Protein synthesis. At the end of the translation 40S and 60S subunits are separated and linked to the initiation factors eIF3 and eIF6. (1-2) Sequential addition of eIF2-GTP factor and eIF4 complex to the 40S-eIF3 complex, to form the initiation complex. 3) Flow of the initiation complex on the mRNA and positioning of this, through the binding of the Met-tRNA$_i$ to the start codon. 4) Formation of the ribosome 80S through association of the major and minor subunits, due to the movement of eIF6 by eIF5-GTP. 5) A complex carrying the second amino acid (aa$_2$) binds to the A site of ribosome, containing Met-tRNA$_i$ in the P site. 6) Conformational change of ribosome, inducing hydrolysis of elongation factor. 7) The 28S rRNA catalyzes the formation of the peptide bond between Met$_i$ and aa$_2$. 8) GTP-hydrolisis of the EF2 induces a subsequent conformational change of ribosome, which involves its translocation of a codon along the mRNA and displacement of the deacylated Met-tRNAi to the E site and the tRNA bound to the peptide to the P site. 9) The release factor eRF1 binds to the A site and recognizes termination codon and works in conjunction with the factor eRF3-GTP in the cutting of the protein chain from the peptidil-tRNA [Lodish, 2000].

the A site requires energy that is supplied by hydrolysis of the EF1 α-GTP complex (**figure 16-6**).

With the initiating Met-$tRNA_i^{Met}$ at the P site and the second aminoacyl-tRNA tightly bound at the A site, the α amino group of the second amino acid reacts with the activated methionine on the initiator tRNA, forming a peptide bond. This critical *peptidyltransferase reaction* actually effects the transfer of the growing peptide chain on the peptidyl-tRNA at the P site to the activated amino acid on the incoming aminoacyl-tRNA (**figure 16-7**).

During the process of peptide synthesis and tRNA site changes, the ribosome is moved along the mRNA a distance equal to one codon with the addition of each amino acid. This translocation step is catalyzed by EF2-GTP, which is hydrolyzed to provide the required energy. After peptide linkage $tRNA_i^{Met}$, now without its activated methionine, is moved to an *exit (E) site* on the ribosome and is soon discharged (**figure 16-8**). Concurrently, another ternary complex, carrying the next amino acid to be added, enters the ribosome, and the cycle continues.

The final phase of protein synthesis, like initiation and elongation, requires highly specific molecular signals that decide the fate of the mRNA-ribosome-tRNA-peptidyl complex. Two types of specific termination (release) factors are involved in this process. Eukaryotic eRF1, whose shape is though to be similar to that of tRNAs, apparently acts by recognizing the stop codons. Like some of the initiation factors and the elongation factors, the release factor eRF3 is a GTP-binding protein and acts in concert with the codon-recognizing factor to promote cleavage of the peptidyl-tRNA, thus releasing the completed protein chain (**figure 16-9**) [Lodish, 2000].

Translational read-through

Translational read-through is a strategy with the purpose to suppress the effects of a premature nonsense codon by modifying gene expression. This may be achieved by two different mechanisms. First, by decreasing the accuracy of translation elongation; second, by reducing the efficacy of the translation termination machinery. In the past few decades it has been realized that

aminoglycoside antibiotics can decrease the fidelity of the eukaryotic elongation machinery. Consequently, this drug group may hold a valuable potential in the pharmacogenetic therapy of nonsense mutation related genetic disorders.

Aminoglycosides inhibit prokaryotic protein synthesis at appropriate ("therapeutic") concentrations. However, when applied at microbiologically sub-lethal levels some may induce incorporation of the wrong amino acid (mis-incorporation) at a sense codon or the insertion of an amino acid at a stop codon (leading to translational read-through) [Kellermayer, 2006]. Similar effects, namely errors in the translation of polyU mRNAs, were demonstrated by Wilhelm et al [1978 a, b] in eukaryotes and in human cells but at concentrations 10 – 15 times higher than the typical therapeutical antibacterial concentrations. The usefulness of aminoglycosides as antibacterial agents lies in the ability to bind with high affinity adenine 1408 present on the minor rRNA in prokaryotes and with low affinity the guanine in the same position in eukaryotes. Precisely for this reason, to have an effect on eukaryotic cells, the needed concentration of aminoglycoside is greater [Recht et al, 1999].

Mechanism of action of aminoglycosides

During protein synthesis the ribosome catalyses the sequential addition of amino acids to a growing polypeptide chain, using mRNA as a template and aminoacylated tRNAs (aa-tRNAs) as substrates. Correct base pairing between the three bases of the codon on mRNA and those of the anticodon of the cognate aa-tRNA dictates the sequence of the polypeptide chain. Discrimination against noncognate tRNA, which generally has two or three mismatches in the base pairing, can be accounted for by the difference in the free energy of base pairing to the codon compared with cognate tRNA. For near-cognate tRNA, which usually involves a single mismatch, the free-energy difference in base pairing compared with cognate tRNA would predict an error rate that is one to two orders of magnitude higher than the actual error rate of protein synthesis, and it has long been recognised that the ribosome must improve the accuracy of protein synthesis by discriminating against near-cognate tRNAs. This discrimination involves the

30S subunit, which binds the mRNA and the anticodon stem-loop (ASL) of tRNA [Ogle et al, 2001].

At the beginning of the elongation cycle, which involves the addiction of a new amino acid to a growing polypeptide chain, the aa-tRNA is presented to the ribosome as a ternary complex with elongation factor 1A (EF1A) and guanosine triphosphate (GTP). The selection of cognate tRNA is believed to occur in two stages, an initial recognition step and a proofreading step, that are separated by the irreversible hydrolysis of GTP by EF1A. In this scheme, the discrimination energy inherent in codon-anticodon base pairing is exploited twice to achieve the necessary accuracy. Experiments suggest that the binding of cognate rather than near-cognate tRNA results in higher rates of both GTP hydrolysis by EF1A and accommodation, a process in which the acceptor arm of the aa-tRNA swings into the peptidyl transferase site after its release from EF1A. In both steps, the higher rate is proposed to be the result of structural changes in the ribosome induced by cognate tRNA. In the context of proofreading mechanisms alone, it is unclear whether additional structural discrimination by the ribosome, over that inherent in the energetics of codon-anticodon base pairing, is actually required in decoding. The prospect of a direct involvement of the ribosome in the recognition of the codon-anticod duplex was revived when it was shown the error-inducing antibiotic *paromomycin* changes the conformation of a 16S rRNA fragment of the decoding centre, in prokaryotes (**figure 17a**); the affected residues were stringly implicated in tRNA binding. Furthermore, kinetic experiments have shown that both GTP hydrolysis and accommodation (peptide bond formation) are accelerated for cognate relative to near-cognate ternary complex. This implies that the energy derived from the binding of a cognate aa-tRNA anticodon induces conformational changes in the ribosome, and is used to drive the irreversible chemical steps on the tRNA selection pathway [Ogle et al, 2001; Ogle et al, 2003].

The binding of mRNA and cognate tRNA in the A site induces A1492 and A1493, two universally conserved bases, to flip out of the internal loop of helix 44. This binding also causes the conserved base G530 to switch from the *syn* conformation present in the native structure to an *anti* conformation. In their new conformations, A1492 and A1493 interact, respectively, with the first and second

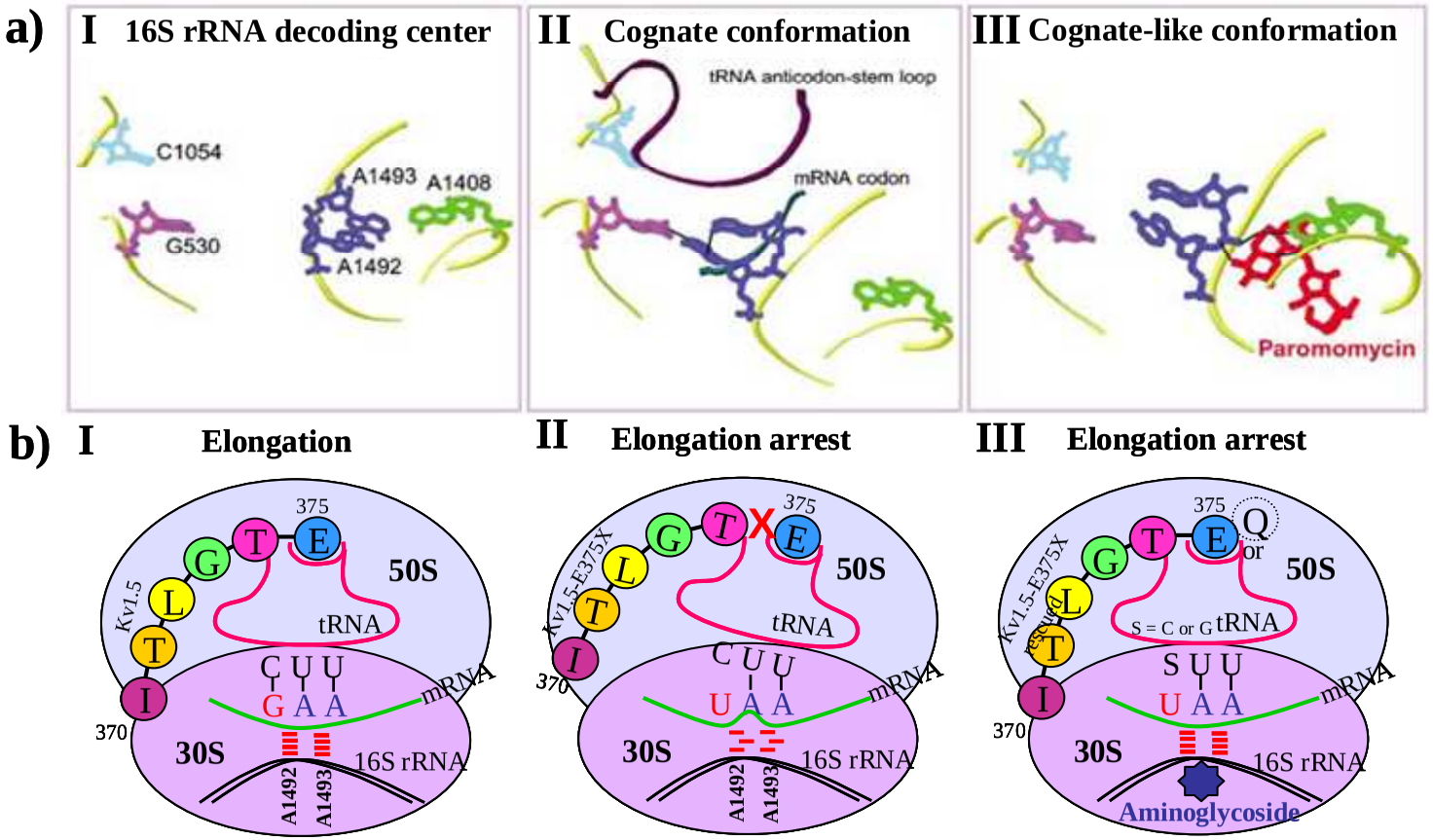


Figure 17 – Aminoglycoside interaction with ribosomal protein translation. a) Interaction of paromomycin with 16S rRNA of the 30S ribosome subunit. **I)** In the absence of the antibiotic, the conserved 16S rRNA (yellow strand) nucleotides A1492 and A1493 are stacked in the interior of the helix 44. **II)** Binding of mRNA codon and cognate tRNA causes conformational change of A1492, A1493 and G530 to accomodate energetically favorable interactions of ribosome components, tRNA and mRNA. **III)** Paromomycin binding to the interior of helix 44 induces a local conformational change in nucleotide A1492 and A1493, flipping them out. These changes facilitate binding of near-cognate tRNA. Black lines represent H-bonds. **b)** Aminoglycoside induced read-through of the premature E375X stop codon in Kv1.5. **I)** Normally, tRNA carrying glutamic acid (E) matches the mRNA codon to process Kv1.5 polypeptide elongation. Matching of the mRNA codon to the proper tRNA anticodon results in conformational alignment of A1492 and A1493 in the ribosomal decoding centre (red dashes) and polypeptide chain elongation. **II)** E375X (UAA codon in mRNA) mutation prevents codon-anticodon pairing and excludes the possibility of the A1492 and A1493 alignment in the ribosomal decoding centre (red dashes) terminating protein translation. **III)** Aminoglycoside binding to 16S rRNA induces conformational alignment in the ribosomal decoding centre despite codon-anticodon mismacth. In the presence of aminoglycosides, the UAA codon may be paired with CUU or GUU tRNA anticodon promoting polypeptide chain elongation with glutamate or glutamine [Zingman et al, 2007].

base pairs of the codon-anticodon helix, whereas G530 interacts with both the second position of the anticodon and the third position of the codon. The result of these induced changes is that the first two base pairs of the codon-anticodon helix are closely monitored by the ribosome in a way that would be able to discriminate between Watson-Crick base pairing and mismatches, whereas the environment of the third, or “wobble”, position appears to be suited for accommodating other base-pairing geometries [Ogle et al, 2001].

When the ribosomal base A1492 interacts with a mismatch between the first position of the codon and the anticodon of near-cognate tRNA (**figure 17b**), the wobble geometry displaces the base of the codon into the minor groove so that it can no longer form hydrogen bonds with A1492, but at the same time there is no room for a water molecule to solvate the polar groups. This amounts to a large desolvation penalty, which can reduce the affinity of near-cognate tRNA anticodon ASL fragments to the ribosome by more than three orders of magnitude. The principles and extent of discrimination against mismatch at the second position are similar [Ogle et al, 2003].

The changes induced in the 30S subunit by cognate tRNA are energetically favourable because various compensating interactions can be made by the ribosome with the codon-anticodon helix but only when the first two base pair are canonical Watson-Crick base pairs. In the case of near-cognate tRNA, fewer of these interactions would be possible, so the induced changes would normally be less favourable. By paying part of the energetic cost of these induced changes, especially in displacing A1492 and A1493 from their internal loop in helix 44, amimoglycoside facilitates the binding of near-cognate tRNA, along with the full changes induced as a result. This result is consistent with the finding that *paromomycin* accelerates precisely those steps (GTP hydrolysis and accommodation) for near-cognate tRNA that are normally slower as compared with cognate tRNA and agrees with earlier proposals that *paromomycin* works by inducing structural changes in the decoding site, rather than by making direct interactions with mRNA or tRNA [Ogle et al, 2001].

Aminoglycoside antibiotics approach the clinic

Aminoglycosides have been shown to suppress premature translation termination at nonsense codons with an efficacy varying from 1% to 25% in human cell lines, largely depending on the context of the stop mutations. Consequently, an increased amount of full size, functional protein may be produced as a result of aminoglycoside therapy, even if only in low percentage. This effect may be significant, however, especially in recessive disorders resulting from nonsense mutations, where protein expression approaches zero [Kellermayer, 2006].

In 1985, Burke and Mogg were the first to demonstrate that aminoglycosides *geneticin (G418)* and *paromomycin* could partially restore the synthesis of a protein codifying by a mutated gene, in an animal cell culture.

Subsequently, various experiments on the suppression of disease-causing premature stop codon mutations in cystic fibrosis transmembrane conductance regulator (CFTR) were carried out. Howard et al [1996] observed a dose-dependent increase in the expression of full-length CFTR from the R553X mRNA, produced by HeLa cells transfected with the mutated cDNA, as a function of *G418* concentration, indicating that *G418* stimulates read-through of the R553X mutation. Quantification showed that the amount of the full-length CFTR produced was as much as 25% of the level of protein expression obtained from the wild-type CFTR cDNA. Even more full-length CFTR (35% of wild-type) was observed in the cells transfected with the CFTR G542X cDNA. The same group found that IB3-1 cells, a bronchial epithelial cell line carrying the ΔF508 mutation on one chromosome and the W1282X premature stop mutation on the other, regained cAMP-activated chloride conductance following growth for 18 hours in the presence of either *G418* or *gentamicin* [Bedwell et al, 1997]. To address whether aminoglycosides can suppress a *CFTR* premature stop mutation in an animal model, they constructed a mouse model with a null mutation in endogenous *CFTR* locus (*Cftr-/-*) that also expressed a human CFTR-G452X cDNA under control of the intestinal fatty acid binding protein promoter. They then investigated whether the daily administration of the aminoglycoside

antibiotics *gentamicin* or *tobramycin* could restore the expression of a detectable level of CFTR protein. Immunofluorescence staining of intestinal tissues from *Cftr–/– hCFTR*-G542X mice revealed that *gentamicin* treatment resulted in the appearance of hCFTR protein at the apical surface of the glands of treated mice. Weaker staining was also observed in the intestinal glands following *tobramycin* treatment [Du et al, 2002].

An important concern for the clinical use of aminoglycosides is the side effects associated with their use, which include kidney damage and hearing loss. Because of their potential toxicity, administration protocols have been developed that maintain aminoglycoside serum concentrations within a clinically acceptable range of recommended peak and trough levels. In the previous study, Du et al showed that *gentamicin* suppressed the *hCFTR*-G542X nonsense mutation *in vivo*, although peak serum concentrations used were well above the levels allowed during the recommended clinical use of these compounds. Thus, it was not clear whether an effective level of suppression could be accomplished at concentrations within a safe clinical range. For this reason, they examined whether *gentamicin* and *amikacin* could suppress the G542X mutation when administered at doses within the recommended therapeutic range of serum peak an trough levels. They obtained evidence that both compounds could suppress the *hCFTR*-G542X mutation in the *Cftr-/-* mouse model and partially restore functional CFTR expression when administrated at doses that produced serum levels in the recommended range. However, their results indicate that *amikacin* suppressed the *hCFTR*-G542X mutation *in vivo* much more effectively than *gentamicin* under these conditions, suggesting that it may represent a better choice for the suppression of the *hCFTR*-G542X stop mutation [Du et al, 2006].

Moreover, Wilschanski et al [2003] performed a topic treatment, by the application of *gentamicin* to the nasal epithelium of patients with cystic fibrosis. This restored CFTR function in about 90% of patients who had stop mutations in *CFTR*. After *gentamicin* treatment, the electrophysiological abnormalities caused by the *CFTR* defect resolved in 21 percent of the patients and chloride or sodium transport was restored in 68 percent. *Gentamicin* seems to be more effective in

patients who are homozygous for stop mutations than in those who are heterozygous.

Positive results also came from aminoglycoside treatment of Duchenne's muscular dystrophy (DMD). The group of Barton-Davis [1999] tested the *in vivo* effects of gentamicin on the *mdx* mouse, the animal model for this disease which has a stop codon in the dystrophin gene. The muscle cells taken from treated *mdx* mice showed a restoration of dystrophic protein with an increase of 10% - 20%. Howard et al [2004] tested *gentamicin*, *amikacin*, *tobramycin* and *paromomycin* for the ability to induce stop codon read-through of eight premature termination codons, shown to be causative for Duchenne's or Becker's muscular dystrophy. They showed variable aminoglycoside suppression with induced read-through levels between approximately 1 and 10% depending on the sequence context and aminoglycoside utilised. Despite these encouraging results, two clinical trials of *gentamicin* treatment in DMD have observed no changes in muscle strength or increased dystrophin expression in post-treatment muscle biopsies. These conflicting studies must be interpreted cautiously, because our understanding of the molecular mechanisms associated with *gentamicin* treatment is incomplete. For example, the efficiency of termination and aminoglycoside suppression are determined by the stop codon and the surrounding sequence context, but the rules that govern these context effects are poorly understood. In addition, *gentamicin* consists of three major components, *gentamicin* C1, C2, and C1a. Variations in the amount of each isoform do not substantially influence antibacterial activity but may result in differing levels of stop codon read-through.

Given the initial positive results, investigators have tried to use these antibiotics on other types of genetic disorders caused by nonsense mutations. Lai et al. [2004] proposed a therapeutic approach for ataxia-teleangiectasia, an autosomal recessive neuro-degenerative disorder with onset in early childhood, treating 13 lymphoblastoid cell lines with primary premature termination codon mutations, with different types of aminoglycosides (*G418, gentamicin, tobramycin* and *paromomycin*). They obtained a restoration of the ATM gene using *G418* and *gentamicin*.

Pinotti et al [2006], through functional assays and a fluorescence-based approach, investigated the spontaneous and aminoglycoside-mediated read-through of nonsense mutations in FVII. Results indicated that treatment with the aminoglycosides *G418* or *gentamicin* induces a significant increase in extracellular FVII protein levels. However, the specific activity of FVII variants resulted in being remarkably reduced as compared with wild-type FVII, thus indicating the synthesis of dysfunctional FVII molecules.

Finally, Keeling et al [2001] tested gentamicin activity on the restoration of the level of protein expression in Hurley disease. They found that *gentamicin* treatment was capable of restoring 2.5 – 3% of normal α-L-iduronidase activity in cultured Hurley fibroblasts.

A major limitation of the read-through technique is that the aminoglycoside action is not gene-specific and can act on correct termination codons, that are not derived from nonsense mutations. The proteins generated would, therefore, have altered functions and, if not having a proper quaternary structure, they can produce formation of toxic aggregates. These proteins may also interact with protein complexes in important cellular pathways damaging functions of the cell [Kaufman, 1999].

Context-dependent suppression of termination codons

Translation termination is normally a highly efficient process. Although the mis-incorporation of an amino acid at a stop codon (a process termed suppression) normally occurs at a frequency of only 10^{-4} in intact cells, the overall efficiency of this process can be influenced by a number of variables. Not only do different stop codons appear to facilitate the termination process with different efficiencies, but the efficiency of translation termination can also be influenced by the local sequence context surrounding the stop codon. A statistical analysis of the context surrounding the stop codons at the end of genes in several organisms revealed a bias toward certain nucleotides on either side of the termination codon. In all species examined, the strongest bias was found at the base immediately following the stop codon. This led to the proposal that the actual translation termination

signal may consist of a tetranucleotide sequence. The importance of nucleotides immediately upstream and downstream of the stop codon in determining the efficiency of translation termination is clearly established in prokaryotes. Studies of the context influence on the efficiency of translation termination in yeast cells indicated that upstream and downstream sequences act together to determine the overall efficiency of translation termination. In particular, the fourth base was found to be an important determinant for the efficiency of termination in yeast, but this effect varied as a function of the stop codon that was present. The importance of the local sequence context in determining the efficiency of translation termination in higher eukaryotes is less well understood [Manuvakhova et al, 2000].

Manuvakhova et al [2000] wanted to determine whether the sequence context can also influence the ability of aminoglycosides to suppress stop codons in mammals. To do this, they constructed a reporter system that allowed them to accurately monitor the efficiency of translation termination in different sequence contexts using a rabbit reticulocyte translation system.

One of the most surprising findings of their study was the complex effects observed in the relative level of read-through when only the fourth base of the tetranucleotide termination signal was changed. In assays with the UGAN series of constructs, they found that C in position 4 always led to the most read-through. This trend was observed in the basal read-through of the *in vitro* translation system, and in read-through induced by members of the 4,6 or 4,5 disubstituted classes of aminoglycosides. In most cases, read-through was lowest when U was present in the fourth position of the UGAN constructs. The only exception to this trend was with *lividomycin*, where A and G in position 4 allowed the lowest level of readthrough. When assays were done with the UAGN constructs, they found that the 4,6 disubstituted class of aminoglycosides was again similar to the uninduced level of read-through, with U mediating the most read-through followed by C. Nucleotides A or G induced the least read-through with these compounds. However, this trend was not maintained when they examined the level of read-through induced by the 4,5 disubstituted classes of aminoglycosides in the UAGN constructs. Finally, a distinct trend in the rank order of suppression

was mediated by almost every aminoglycoside in the UAAN constructs. These results again indicate that different aminoglycosides influence the decoding site (and consequently, the proofreading step that discriminates between cognate and near-cognate aminoacyl-tRNAs within the A site) in subtly different but important ways. They conclude from these results that a simple pattern of suppression that can be attributed solely to the fourth position of the tetranucleotide signal does not exist.

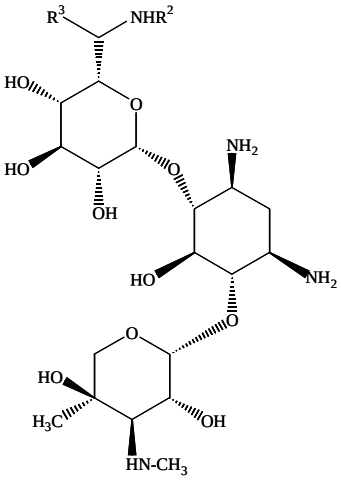
Their results also demonstrate that the sequence context beyond the tetranucleotide termination signal can influence the level of read-through induced by several aminoglycosides.

Finally, it is important to note that other translation factors present in the complete translation system may also influence the overall efficiency of translation termination. For example, one or more components of the surveillance complex (which includes Upf1p, Upf2p, and Upf3p) may mediate important effects on termination, because the loss of any of these proteins results in a nonsense suppressor phenotype [Manuvakhova et al, 2000].

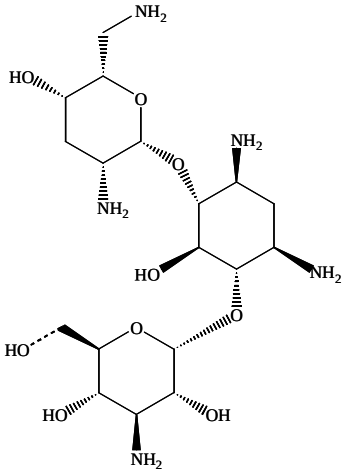
Aminoglycosides and analogous

As a class of antibiotics, aminoglycosides have a backbone structure consisting of an aminocyclitol ring saturated with amine and hydroxyl substitutions. In the majority of clinically useful aminoglycosides, this aminocyclitol moiety is streptamine or 2-deoxystreptamine. *Streptomycin*, possessing a streptidine molecule, is the only exception. The aminocyclitol nucleus is connected through glycosidic linkages to various amino sugars (aminoglycosides). The aminoglycosides can be conveniently divided into three structural types based on the position of their glycosidic linkages. These structural types include the *4,6-disubstituted 2-deoxystreptamines*, containing most of the clinically useful aminoglycosides such as *gentamicin*, *tobramycin*, *amikacin*, *kanamycin* and *netilmicin*, the *4,5-disubstituted 2-deoxystreptamines* (*neomycin* and *paromomycin*), and *others* (*streptomycin* and *spectinomycin*) (**figure 18**). Spectinomycin, although often considered an aminoglycoside, does not contain an

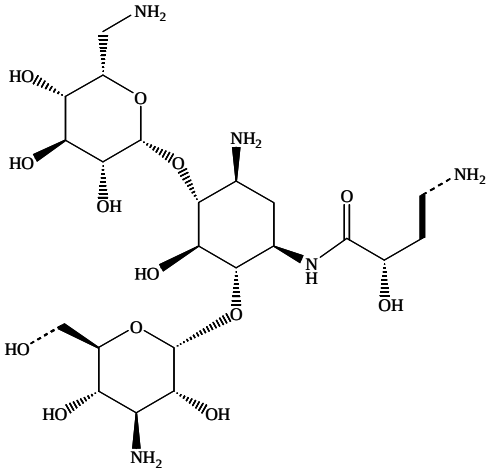
a) 4,6-Disubstituted 2-deoxystreptamines



Gentamicin

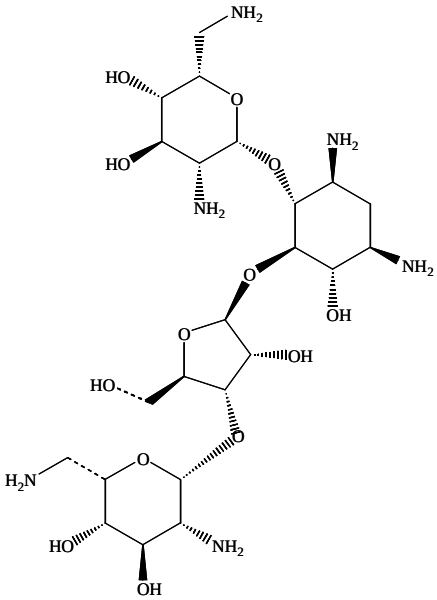


Tobramycin

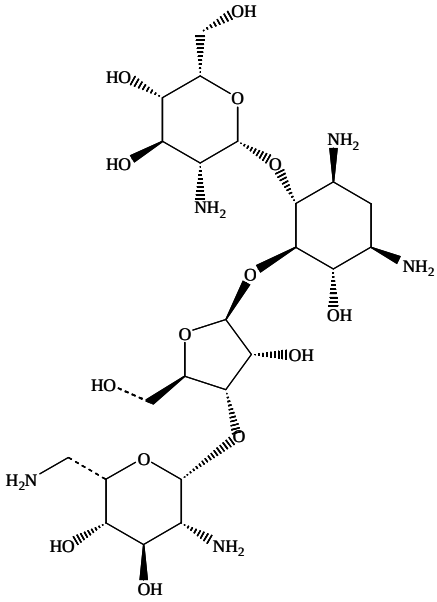


Amikacin

b) 4,5-Disubstituted 2-deoxystreptamines

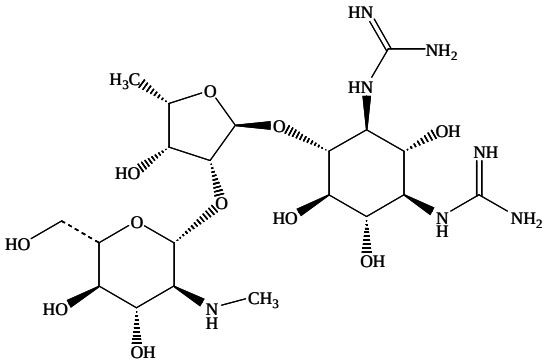


Neomycin

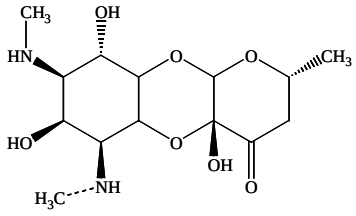


Paromomycin

c) Others



Streptomycin



Spectinomycin

Figure 18 – Representative structures of clinically useful aminoglycosides [Jana and Deb, 2006].

amino sugar. Thus, several investigators have suggested that the term aminocyclitol be used to describe this entire group of agents rather than the less precise term aminoglycoside. The aminoglycoside structure is important in understanding their chemical properties. These are basic, strongly polar compounds that are positively charged (cationic). They are highly soluble in water, relatively insoluble in lipids, and have enhanced antimicrobial activity in alkaline rather than acidic environments. As a result, aminoglycosides are minimally absorbed from the gut and penetrate the blood brain barrier poorly. The cationic nature of the aminoglycosides contributes to their antimicrobial activity. Because of their positive charge, they are able to bind negatively charged lipopolysaccharide of the bacterial cell wall and a variety of intracellular and cell membrane anionic molecules such as DNA, RNA, and phospholipids. Unfortunately, their positive charge at physiological pH also contributes to their toxicities, e.g., nephrotoxicity, ototoxicity, and neuromuscular blockade. Aminoglycosides are metabolically stable compounds that are excreted unchanged in the urine.

Different classes of aminoglycoside antibiotics bind to different sites on the rRNA, depending on the structural complementarity between the two. For example, neomycin, paromomycin, gentamicin and kanamycin are believed to bind to the A site on the 16S rRNA in *E. coli* in a similar fashion and were shown to protect bases A1408 and G1494 in chemical footprinting experiments. Four bases, A1408, A1492, A1493, and G1494, in the rRNA A site interact with tRNA, although with different affinities. The binding of the aminoglycosides to the A site in the decoding region (i.e., the site of codon and anticodon recognition) interferes with the accurate recognition of cognate tRNA by rRNA during translation. These interactions are also thought to interfere with the translocation of tRNA from the A site to the peptidyl– tRNA site (P site) [Jana and Deb, 2006].

Fourmy et al. [1998] provided structural evidence on the mode of interactions of paromomycin, a representative aminoglycoside of the neomycin class, with a 27-nucleotide RNA template that was designed to mimic the A site region of the 16S rRNA in *E. coli*. Specific interactions were observed between aminoglycoside chemical groups important for antibiotic activity and conserved nucleotides in the

RNA. Common chemical groups among A site binding aminoglycosides on rings I and II of paromomycin make sequence specific contacts with the A site RNA. Rings III and IV contact RNA non-specifically by electrostatic interactions with the phosphodiester backbone. The structure suggests that rings I and II are essential for specific binding of aminoglycosides to rRNA and that the contribution of rings III and IV to the specificity of binding is minor. This prediction is supported by experimental data: neamine, which lacks rings III and IV, binds to 16 S rRNA and causes miscoding. This suggests that the conserved rings, I and II, are the minimum motif for specific binding of aminoglycosides to the A site of the ribosome.

The 4,6-disubstituted geneticin (G418), a gentamycin-related aminoglycoside, is used in medicine as an anti-parasitic agent, although with some limitations due to its toxicity to human ear and kidney cells. In molecular biology, geneticin is routinely employed as a selection marker in eukaryotic cells transfected with a gene coding for an enzyme that inactivates the aminoglycoside. It contains three rings that are functionalised by hydroxyl, ammonium amd methyl groups. The two sugar rings constituting the neamine part common to most of the aminoglycosides bind to the A site. The essential hydrogen bonds involving ring I (to A1408) and ring II (to the phosphate oxygen atoms of the bulged adenine bases 1492 and 1493 and to G1494) are conserved and additional contacts are observed from ring III (phosphate oxygen atoms of G1405 and U1406) [Vicens and Westhof, 2003].

Finally, it is important to point out which are the advantages and disadvantages of aminoglicosydes. Advantages consists in: rapid bacterial action, relatively low cost, chemical stability, no allergic reaction and synergistic action with other antibiotic. On the contrary, disadvantages consist in: inactivity against anaerobes, narrow therapeutic index, toxicities (nephrotoxicity, ototoxicity) and lack of oral absorption [Jana and Deb, 2006].

Given the significant disadvantages of the aminoglycosides, several research groups started projects aimed at developing molecules able to promote read-through and tested the molecules on *in vitro* and *in vivo* model systems. For instance, in a recent paper by Welch et al. [2007], two high-throughput screens (comprising 800,000 low molecular weight compounds) were performed to

identify compounds that promoted UGA nonsense suppression. Chemical scaffolds were identified and optimized through extensive medicinal chemistry efforts. These analyses identified PTC124 (3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid; C15H9FN2O3) as a candidate for further development.

PTC124 showed to promote the suppression of premature termination codons in primary muscle cell cultures from DMD patients, in *mdx* mice and in a *CF* mouse model that expressed a human *CFTR*-G542X transgene in a *Cftr-/-* background, leading to a significant restoration of dystrophin and CFTR expression and function [Welch et al, 2007; Du et al, 2007].

Moreover, PTC124 showed to be able to promote read-through of premature termination without affecting normal termination, even at drug exposure levels substantially greater than the values achieving maximal activity [Welch et al, 2007].

This selectivity of PTC124, its well characterized activity profile, oral bioavailability and pharmacological properties indicate that this drug may have broad clinical potential for the treatment of a large group of genetic disorders with limited or no therapeutic options.

Mithramycin

Mithramycin is an aureolic acid-type polyketide described for the first time in 1950s and produced by several actinomycetes, like Streptomyces argillaceus, Streptomyces plicatus, Streptomyces tanashiensis and Streptomyces atroolivaceus.

Initially, the members of the aurelic acid family were isolated due to their antibiotic activity against Gram-positive bacteria. However, they are not active against Gram-negative bacteria due to permeability problems. Their main pharmacological interest resides in their antitumor activity. The members of this family interact with the DNA helix minor groove in regions with high GC content and in a nonintercalative way. This binding is carried out by complexes of dimers together with a Mg^{2+} ion. During these interactions, several H-bonds are created among the aglycon hydroxyl groups and the guanine amino protons.

The interaction with the double helix causes a DNA-dependent inhibition on RNA synthesis, which gives this family of compounds a strong antitumor activity against a variety of cancer cell lines. Based on this antitumor activity, mithramycin has found clinical application in the treatment of Paget's disease and testicular carcinoma. In addition, its hypocalcemic effects has been used to manage hypercalcemia in patients with malignancy-associated bone lesions [Remsing et al, 2003; Lombó et al, 2006].

The specificity for GC-rich regions along the DNA makes these compounds good inhibitors of specific promoter regions, preventing the binding of regulatory proteins. This effect has been described for the *c-myc*, *c-Ha-ras*, *c-myb* and *MDR1* genes. Mithramycin binds at the C-fos-depending Sp1 regulatory regions, and therefore, it prevents transcription due to this transcriptional factor, generating a global inhibition mechanism [Lombó et al, 2006].

In 1999, Bianchi et al reported that mithramycin (MTH) is a potent inducer of erythroid differentiation in K562 cells. Differentiation was found to be associated with increase in the synthesis of γ-globin mRNA and production of mostly Hb Portland. In addition, the same group evaluated the effects of MTH on HbF production in healthy human and thalassemic-cultured erythroid precursor cells. In several independent experiments, using peripheral blood from different healthy donors, they reproducibly found an increase in HbF production. This increase was found to be consistently higher than that induced by hydroxyurea (HU), a potent inducer of HbF both *in vitro* and *in vivo*. These data were fully in agreement with quantitative RT-PCR analysis, showing a preferential increase of γ-globin mRNA accumulation in MTH-treated erythroid precursors in comparison to α-globin and β-globin mRNA. In addition, the data demonstrate that MTH is a powerful inducer of HbF production in erythroid progenitors from β-thalassemia patients. Unlike HU, the effect of MTH was not associated with inhibition of cell growth [Fibach et al, 2003].

AIM OF THE THESIS

The β-thalassemia is an autosomal recessive disease characterized by a reduced or absent synthesis of β-globin chains, caused by the presence of mutations within or near the β-globin gene. Among these is the C → T substitution at the first base of codon 39 in the second exon of the gene. Such mutation, known as $\beta^0$39, belongs to the nonsense mutations, which lead to the formation of stop codons, causing the premature termination of translation. The β-thalassemia caused by the presence in homozygosity of this mutation shows a very serious phenotype, because the synthesis of β-globin chains is totally absent.

Actually, there are two approaches to directly overcome diseases caused by nonsense mutations: gene therapy, meaning introduction of an exogenous gene into diseased cells, and translational read-through induced by aminoglycosides, which decrease the accuracy of translational elongation and reduce the efficacy of the translation termination machinery.

Our purpose was to study and verify the effects and clinical utility of such approaches.

In this respect, we intended to develop third generation lentiviral vectors containing the βwt- or $\beta^0$39-globin gene, the first useable in gene therapy and both to produce cellular models, suitable to study and develop new therapeutic strategies targeted to the correction of this mutation, in particular aminoglycosides activity. Therefore, we aimed to produce such cellular models using K562 human erythroid cell line and utilize them to verify the translational correction activity on $\beta^0$39 nonsense mutation of several aminoglycosides. Whether identified active compounds, we proposed to use them to correct the mutation in erythroid precursors cells isolated from $\beta^0$39-thalassemia patients, whose *in vitro* growth represents more closely than cell lines the *in vivo* situation.

Finally, we intended to use the $\beta^0$39 erythroid progenitors to test out the possible utilize of the lentiviral vector producing βwt-globin mRNA for gene

therapy of β^0-thalassemias and the combined effects of gene therapy and fetal haemoglobin induction by treatment with *mithramycin*, a potent HbF inducer.

MATERIALS AND METHODS

Synthetic oligonucleotides

The oligonucleotides used as primers in the chain polimerization and sequencing reactions were synthesized by Sigma-Genosys (Cambridge, UK), while those used for the Real Time quantitative PCR were purchased from Applied Biosystems (Applera Italia, Monza, Italy). All the oligonucleotides were designed using the software Primer Express™, version 2.2 (Perkin-Elmer Applied Biosystems) and the sequences are reported on **table 1 and 2**.

Restriction enzymes

The restriction endonucleases used in the cloning experiments were commercial: **table 3** shows the sequence recognised, concentration, buffer, incubation temperature and company from which the enzymes were bought.

Plasmids and vectors

Plasmids, lentiviral and helper vectors used in the cloning experiments were kindly provided by Prof. Stefano Rivella (Weill Medical College of Cornell University, New York). All the maps were created using the following softwares: *BioEdit version 7.0.0* (Tom Hall Copyright © 1997-2004) and *Plasmapper version 2.0.*

Instead, for the restriction analysis we used *pDRAW32 1.0* (Revision 1.1.86, by Kjeld Olesen, Acaclone software) and *RestrictionMapper* (www.restrictionmapper.org).

Aminoglycosides

The aminoglycosides used for the treatment of K562 cell clones and erythroid precursors were bought from GIBCO (Invitrogen-Life Technologies, Carlsbad, CA, USA) (*G418*) and Sigma (St Louis, MO, USA) (*gentamicin*, *streptomycin*, *neomycin*, *tobramycin*, *paromomycin*, *kanamycin*, *amikacin*). All of them were resuspended in sterile water, sterilized by filtration with acetate cellulose filters having pore diameter of 0.22 μm, divided in aliquots and stored at – 20°C.

Cloning

Enzymatic digestion

For each digestion, 15 μg of DNA were incubated with 5 U/μg restriction enzyme and 1X buffer, chosen depending on the endonuclease used. The final reaction volume was at least ten times higher than the enzyme volume. The incubation was performed at the optimum temperature for the restriction enzyme (usually 37°C), for about 16 hours or less, depending on the requested result. The digestion products were visualised through an electrophoretic analysis on 0.8% agarose gel, using one of the following molecular weight markers: *1kb DNA Ladder (10,000, 8,000, 6,000, 5,000, 4,000, 3,500, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500, 250 bp)* (MBI Fermentas, Maryland, USA) or *pUC Mix Marker 8 (1,116, 883, 692, 501, 489, 404, 331, 242, 190, 147, 111, 110, 67 bp)* (MBI Fermentas). The enzyme was inactivated by incubation at 65°C for 20 minutes.

Electrophoresis on agarose gel

To prepare the agarose gel for the electrophoretic analysis, the required amount, depending on the gel percentage, of the agarose powder was dissolved in 100 ml of 1X TAE buffer (0.04 M Tris-Acetato, 0.001 M EDTA pH 8), boiling until the agarose melt completely. The solution were cooled and 0.5 μg/ml ethidium bromide was added. The gel was done solidify for about 30 minutes in a

clean casting plate with an appropriate comb and then immersed into 1X TAE buffer. 1 μl of loading buffer (0.25% Orange G, 50% glycerol in TE) was added to each sample before being loaded. On both the sides of the gel a molecular weight marker was added. 1 μl of *1kb DNA Ladder (10,000, 8,000, 6,000, 5,000, 4,000, 3,500, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500, 250 bp)* (MBI Fermentas) or *pUC Mix Marker 8 (1,116, 883, 692, 501, 489, 404, 331, 242, 190, 147, 111, 110, 67 bp)* (MBI Fermentas) was up to the loading volume with water. The electrophoresis was performed at 80 Volt. The DNA was visualised using UV rays and then photographed with a Polaroid camera.

Purification of vectors and inserts by QIAquick® Gel Extraction Kit

After the digestion reaction, the DNA fragments, corresponding to vector and insert, were extracted from the agarose gel and purified with the *Qiaquick Gell Extraction Kit* (QIAGEN, Milan, Italy).

This technique is based on the use of a column containing a silica-gel membrane with selective binding properties. Special buffers are optimized for efficient recovery of DNA and removal of contaminants.

After electrophoretic separation, the DNA fragment was excised from the agarose gel. The gel slice was weighted and 3 volumes of *Buffer QG* to 1 volume of gel was added. An incubation was performed at 50°C for 10 minutes, mixing every 2 – 3 minutes to help to dissolve the gel. After the gel slice was dissolved completely, 1 gel volume of isopropanol was added to the sample, which was applied to the *QIAQuick* column, which was centrifuged for 1 minute at the maximum speed. The eluate was discarded and 0.5 ml of *Buffer QG* was added to the column, to eliminate all the agarose traces. The column was centrifuged once again and then washed using 0.75 ml of *Buffer PE*, incubating at room temperature for 5 minute and centrifuging at 13,000 rpm for 1 minute. Another centrifugation allowed the elimination of all washing solution. To elute DNA, 50 μl of *Buffer EB* (10 mM Tris-HCl pH 8.5) were added to the center of the *QIAQuick* membrane and an incubation was performed at room temperature for a range of time, from 1

to 15 minutes, depending on the fragments size. The column was finally centrifuged at the maximum speed for 1 minute and the DNA was collected.

Spectrophotometric quantification

The spectrophotometric quantification was performed by using a single ray UV spectrophotometer *SmartSpec^{TM} Plus* (BIORAD, Hercules, California, USA). Each quantification required from 0.5 to 1 μl of genomic DNA, in order to determine an optical density at 260 nm ($OD_{260\ nm}$) of 0.1 to 1.0: after deducting the value of optical density of the blank, the concentration of the extracted DNA was calculated considering that 1 $OD_{260\ nm}$ corresponds to the concentration of 50 ng/μl. For each sample was also measured the optical density at 280 nm to determine the possible contamination of protein molecules, taking into account that the ratio OD_{260nm}/OD_{280nm} of the pure DNA is between 1.8 and 2.0.

Ligation

The ligation reaction was carried out among the fragments of DNA corresponding to the vector and insert, previously purified and quantified. We used from 50 ng to 130 ng of vector and a quantity of insert so as to have a molecular ratio vector:insert, usually equal to 1:4 – 1:8.

The reaction was set up in a final volume of 10 μl: first, vector, insert and ultrapure water were incubated at 65°C for 5 minutes to stretch DNA molecules which have to be ligated; then, it was added a solution containing 1 μl of 10X *Ligation Buffer* (400 nM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP) and 5 U of *T4 DNA Ligase* (MBI Fermentas). The reaction was finally incubated at 22°C for 1 hour and at 16°C for 1 night.

The enzyme was inactivated through incubation at 65°C for 10 minutes and the reaction was stored at -80°C.

Preparation of ultra-competent bacteria

The bacteria of the *E. coli* strains *JM109, XL1-Blue* and *TOP10* were made ultra-competent through the rubidium chloride method: the bacteria were inoculated into 2 ml of LB Medium (Luria Bertani Medium: 10 g/l bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl) and kept at 37°C with vigorous shaking for 1 night. The day after, they were diluted 100 times with *Psi broth* (5g/l yeast extract, 20 g bacto-tryptone, 5 g/l $MgSO_4$ pH 7.6), allowed to proliferate to an optical density, measured at 550 nm, corresponding to 0.48 OD_{550nm} and incubated on ice for 15 minutes. The bacteria were collected by centrifugation at 5,000 rpm for 5 minutes at 4°C, resuspended with 80 ml (0.4 volumes compared to the initial culture) of *TfbI* (30 mM CH_3COOK, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% v/v glycerol, pH 5.8) and incubated on ice for 15 minutes. They were then re-collected, resuspended in 8 ml (0.04 volumes compared to the initial culture) of *TfbII* (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% v/v glycerol, pH 6.5), divided in aliquots of 500 µl, incubated on ice for 15 minutes, frozen immediately in liquid nitrogen and stored at -80°C.

Trasformation

The ultra-competent bacteria were thawed slowly on ice, then an aliquot of 120 µl was placed in a tube of polypropylene, pre-cooled on ice, and 50% of the plasmid obtained by the ligation reaction was added, stirring gently. After an incubation on ice for 4 hours, the reaction was subjected to thermal shock to allow the penetration of the plasmids inside the cells: first, it was incubated at 42°C for 45 seconds and immediately on ice for 2 minutes. Then, after adding 1 ml of LB Medium, pre-equilibrated at room temperature, there was a 1 hour incubation at 37°C with slow shaking, in order to develop the antibiotic resistance. The bacteria were, finally, plated on Petri dishes containing semi-solid medium (LB Medium added to 15 g/l bacto-agar), in the presence of antibiotic (100 µg/ml ampicillin), and incubated at 37°C for 1 night. The recombinant bacteria give rise to white

colonies, while those that have not incorporated the plasmid can not survive because they lack the resistance to the ampicillin.

Rapid method for analysing the size of the plasmid (Rapid Disruption)

The technique allows the qualitative analysis of the size of a recombinant plasmid, starting from the bacterial clone, through electrophoretic analysis.

In the presence of single bacterial colonies of about 1 mm in diameter, using a sterile stick a small portion of each colony was transferred to a new Petri dish added with the antibiotic (*Master Plate*): the dish was kept in incubator at 37°C for 1 night and then stored at 4°C. About 1 quarter of each colony to be analysed was taken from the *Master Plate* and transferred into vials containing 12.5 µl of a 10 mm EDTA pH 8 solution; after adding 12.5 µl of lysis solution (0.2 N NaOH, 0.5% SDS, 20% sucrose), an incubation was carried out at 70°C for 5 minutes. To the lysate were added 0.375 µl of KCl and 0.75 of bromophenol blue 0.4%, it was then incubated on ice for 5 minutes and centrifuged at 12,000 rpm for 3 minutes at 4°C to remove bacteria debris. To analyse the size of the plasmid contained in the supernatant, an aliquot of this was subjected to electrophoretic migration in 0.8% agarose gel. The bacterial colony containing the correct plasmid was finally recovered from the *Master Plate*.

Heat lysis of bacteric clones

In order to rapidly obtain a plasmid template to be amplified by PCR, the bacteric clone was washed twice, then resuspended in 20 µl of ultrapure water, lysed at 96°C for 6 minutes and immediately chilled on ice. After centrifugation at maximum speed for 20 minutes at 4°C, the incorporated plasmid was collected in the supernatant.

Polymerization chain reaction (PCR)

The polymerization chain reaction amplifies a specific sequence of DNA, allowing to use the sample for several types of analysis, including sequencing.

In the case of the amplification of a plasmid DNA incorporated into a bacterial clone, the template can be made up of 2 µl of the supernatant obtained by the heat lysis of the colony of interest.

Each reaction was carried out in a final volume of 100 µl, in the presence of 1X buffer (10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 33 µM dNTPs, 0.25 µM forward and reverse primers and ultra-pure water.

Every reaction was subjected to a denaturation of 5 minutes at the temperature of 96°C, then 2 U of *DyNAzyme™ II DNA Polymerase* (Finnzymes, Espoo, Finland) was added; 30 – 40 cycle of amplification were performed, each of which was characterized by: 30 seconds at 95°C to denature the DNA molecules; 20 seconds at a temperature of 1 or 2 degree below the *melting* temperature (T_m) of primers, to facilitate the annealing to the filaments of DNA; a final phase of elongation of the DNA chains at 72°C for a time variable depending on the size of the amplificate, considering that polymerase can include about 1,000 bp/minute. The reactions were finally maintained at a temperature of 72°C for additional 10 minutes to complete the process of elongation.

The obtained PCR products were analysed by electrophoresis on agarose gel at a different percentage depending on the length of the amplificate. Those to be allocated to sequencing were also purified from the primers and free nucleotides using filtrating membrane *Microcon®* (Millipore Corporation, Billerica, MA, USA).

Plasmid purification

Once identified, the bacterial clone containing the plasmid of interest was subjected to purification by two different techniques: *FlexiPrep Kit* (Pharmacia Biotech, GE Healthcare Europe, Milan, Italy), which uses the resin *Sephaglas™ FP* for the isolation of the plasmid DNA and the removal of contaminants through

different steps of centrifugation, used to purify small quantity of plasmid, and three different QIAGEN kits, which differ in the amount of plasmid that can purify: *QIAGEN Plasmid Purification Mini Kit, QIAGEN Plasmid Purification Midi Kit, QIAGEN EndoFree® Plasmid Purification Maxi Kit*. Each of these kits requires the use of a column containing an ion exchange resin.

MINIprep con FlexiPrep Kit

A recombinant bacterial colony was inoculated into 6 ml of LB Medium containing the appropriated antibiotic and incubated for approximately 16 hour with vigorous shaking. The plasmid purification was performed with 5 ml of bacterial cells. The bacteria were first collected by centrifugation at 14,000 rpm for 30 seconds, washed with 1 ml of STE (10 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA) and finally resuspended with 400 µl of *Solution I* (100 mM Tris-HCl pH 7.5, 10 mM EDTA, 400 µg/ml RNase I). There were then added 400 µl of *Solution II* (1 M NaOH, 5.3% w/v SDS), which, after mixing by inverting the tube 6 times, allowed the lysis of bacteria; the solution was finally neutralized by the addition of 400 µl of *Solution III* (3 M potassium, 5 M acetate) and the subsequent mixing by inverting consecutively for 6 times. A centrifugation at 12,000 rpm permitted the separation of chromosomal DNA, proteins and cellular debris from the plasmid DNA, contained in the supernatant: to this last were, then, added 0.7 volumes of isopropanol and an incubation at room temperature for 10 minute was performed to allow the precipitation of plasmid. After a centrifugation at 12,000 rpm for 10 minutes at 4°C, the plasmid collected was treated with 300 µl of *Sephaglass* microresin (*Sephaglas FP* in 7 M guanidine-HCl, 50 mM Tris-HCl pH 7.5, 10 mM CDTA solution), mixed for 1 minute to dissolve the pellet, centrifuged at 14,000 rpm for 15 seconds, washed first with 400 µl of *Wash Buffer* (20 mM Tris-HCl pH 7.5, 2 mM CDTA, 200 mM NaCl, 60% v/v ethanol), then with 600 µl of 70% ethanol and, finally, allowed to air dry for 10 minutes. The elution of the plasmid DNA from the resin was performed in *TE* pH8 (*Tris-EDTA*: 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8), incubating at room temperature for 5 minutes and, then, centrifuging at 12,000 rpm for 1 minute to obtain a clear supernatant.

Finally, the plasmid was analysed through electrophoresis in 0.8% agarose gel, quantified and stored at -80°C.

MINIprep with QIAGEN Plasmid Purification Mini Kit

A recombinant bacterial colony was inoculated into 20 ml of LB Medium containing the appropriated antibiotic and incubated for approximately 16 hour with vigorous shaking.

The plasmid purification was performed using the *QIAGEN Plasmid Purification Mini Kit* and all the bacterial suspension. The bacteria were first collected by centrifugation at 3,500 rpm for 30 minutes at 4°C, washed with 1 ml of *STE* and re-centrifuged at 8,000 rpm for 15 minutes at 4°C; the bacterial precipitate was, then, resuspended in 300 µl of *Buffer P1* (50 mM Tris-HCl pH 8, 10 mM EDTA, 100 µg/ml RNase A). There were subsequently added 300 µl of the lysis *Buffer P2* (200 mM NaOH, 1% w/v SDS) and, after mixing by inverting the tube 4 times, an incubation at room temperature for 5 minutes was performed; finally, 300 µl of *Buffer P3* (3 M potassium acetate pH 5.5) pre-chilled at 4°C were added to neutralize the lysis: the solution was mixed 4 times by inverting the tube and incubated on ice for 15 minutes. The plasmid DNA was separated from chromosomal DNA, proteins and cellular debris through centrifugation at 12,000 rpm for 30 minutes at 4°C: the supernatant containing the plasmid DNA was re-centrifuged at 12,000 rpm for 15 minutes at 4°C, then the plasmid was purified using a chromatographic column *QIAGEN Tip-20*, on ion exchange resin. Three different buffers were loaded on the column: 1 ml of *Buffer QBT* (750 mM NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol, 0.15% v/v Triton X-100) to equilibrate, 4 ml of *Buffer QC* (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol) for the 2 subsequent washing and 800 µl of *Buffer QF* (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% v/v isopropanol) to elute. The plasmid DNA was precipitated from the elution buffer adding 0.7 volumes of room-temperature isopropanol, mixing vigorously and centrifuging at 12,000 rpm for 30 minutes at 4°C; after removing the supernatant, the precipitate was washed with 1 ml of

room-temperature 70% ethanol, centrifuged at 12,000 rpm for 10 minutes at 4°C, resuspended with 40 µl of *TE* pH 8 and incubated at 65°C for 5 minutes.

Finally, the plasmid was analysed through electrophoresis in 0.8% agarose gel, quantified and stored at -80°C.

MIDIPrep with QIAGEN Plasmid Purification Midi Kit

A recombinant bacterial colony was inoculated into 200 ml of LB Medium containing the appropriated antibiotic and incubated for approximately 16 hour with vigorous shaking.

The plasmid purification was performed using the *QIAGEN Plasmid Purification Midi Kit* and 100 ml of the bacterial suspension. The bacteria were first collected by centrifugation at 10,000 rpm for 10 minutes at 4°C, washed with 32 ml of *STE* and re-centrifuged at 10,000 rpm for 15 minutes at 4°C; the bacterial precipitate was, then, resuspended in 4 ml of *Buffer P1* (50 mM Tris-HCl pH 8, 10 mM EDTA, 100 µg/ml RNase A). There were subsequently added 4 ml of the lysis *Buffer P2* (200 mM NaOH, 1% w/v SDS) and, after mixing by inverting the tube 4 times, an incubation at room temperature for 5 minutes was performed; finally, 4 ml of *Buffer P3* (3 M potassium acetate pH 5.5) pre-chilled at 4°C were added to neutralize the lysis: the solution was mixed 4 times by inverting the tube and incubated on ice for 15 minutes. The plasmid DNA was separated from chromosomal DNA, proteins and cellular debris through centrifugation at 12,000 rpm for 30 minutes at 4°C: the supernatant containing the plasmid DNA was re-centrifuged at 12,000 rpm for 15 minutes at 4°C, then the plasmid was purified using a chromatographic column *QIAGEN Tip-100,* on ion exchange resin. Three different buffers were loaded on the column: 4 ml of *Buffer QBT* (750 mM NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol, 0.15% v/v Triton X-100) to equilibrate the column, 20 ml of *Buffer QC* (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol) for the 2 subsequent washing and 5 ml of *Buffer QF* (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% v/v isopropanol) to elute. The plasmid DNA was precipitated from the elution buffer adding 0.7 volumes of room temperature isopropanol, mixing vigorously and centrifuging at 12,000 rpm for 30 minutes at

4°C; after removing the supernatant, the precipitate was washed with 3 ml of room-temperature 70% ethanol, centrifuged at 12,000 rpm for 10 minutes at 4°C, resuspended with 200 µl of *TE* pH 8 and incubated at 65°C for 5 minutes.

Finally, the plasmid was analysed through electrophoresis in 0.8% agarose gel, quantified and stored at -80°C.

MAXIPrep with QIAGEN EndoFree® Plasmid Purification Maxi Kit

A recombinant bacterial colony was inoculated into 250 ml of LB Medium containing the appropriated antibiotic and incubated for approximately 16 hour with vigorous shaking.

The plasmid purification was performed using the *QIAGEN EndoFree® Plasmid Purification Midi Kit* and all the bacterial suspension. The bacteria were first collected by centrifugation at 10,000 rpm for 10 minutes at 4°C, washed with 32 ml of *STE* and re-centrifuged at 10,000 rpm for 15 minutes at 4°C; the bacterial precipitate was, then, resuspended in 10 ml of *Buffer P1* (50 mM Tris-HCl pH 8, 10 mM EDTA, 100 µg/ml RNase A). There were subsequently added 10 ml of the lysis *Buffer P2* (200 mM NaOH, 1% w/v SDS) and, after mixing by inverting the tube 4 times, an incubation at room temperature for 5 minutes was performed; finally, 10 ml of *Buffer P3* (3 M potassium acetate pH 5.5) pre-chilled at 4°C were added to neutralize the lysis: the solution was mixed 4 times by inverting the tube and incubated on ice for 15 minutes. The plasmid DNA was separated from chromosomal DNA, proteins, cellular debris and SDS pouring the lysate into the barrel of a *QIAfilter MAXI Cartridge*, incubating at room temperature for 10 minutes, inserting the plunger into the *QIAfilter MAXI Cartridge* and filtering the cell lysate. 2.5 ml of Buffer ER were then added to the filtered lysate, mixed by inverting approximately 10 times and incubated on ice for 30 minutes. After, a chromatographic column *QIAGEN Tip-500*, on ion exchange resin, was equilibrated with 10 ml of *Buffer QBT* (750 mM NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol, 0.15% v/v Triton X-100) and the filtered lysate was applied to the resin, which was washed twice with 30 ml of *Buffer QC* (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol). The plasmid DNA was then eluted with

15 ml of *Buffer QN* (1.6 M NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol) and precipitated from the elution buffer adding 0.7 volumes of room-temperature isopropanol, mixing vigorously and centrifuging at 12,000 rpm for 30 minutes at 4°C; after removing the supernatant, the precipitate was washed with 5 ml of endotoxin-free room-temperature 70% ethanol, centrifuged at 12,000 rpm for 10 minutes at 4°C, air-dried for 5 – 10 minutes and resuspended with 200 µl of endotoxin-free *TE* pH 8.

Finally, the plasmid was analysed through electrophoresis in 0.8% agarose gel, quantified and stored at -80°C.

Microcon® YM-100

The polymerization chain reaction products of over 100 bp, to be used for sequencing, were purified using *Microcon® YM-100* (Millipore Corporation), a filtrating membrane with molecular weight limit. On each filter was loaded all the PCR reaction and a centrifuge at 2,400 rpm for 15 minutes was performed. The filter was, then, washed with 300 µl of ultra-pure water and centrifuged at 2,400 rpm for 15 minutes. To elute the DNA, 25 µl of ultra-pure water were loaded on the membrane, which was then left in constant agitation at 140 rpm for 15 minutes. The filter was then placed upside down in a new vial and subsequently centrifuged at 2,400 rpm for 5 minutes. The eluate was finally stored at -80°C.

DNA sequencing

Each reaction was set up in a final volume of 20 µl, using 10 – 100 ng of PCR products, 3.2 pmoles of primers at the concentration of 50 ng/µl and sterile water up to the volume of 12 µl; were then added 8 µl of *Terminator Ready Reaction Mix* of *ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit*, v 2.0 (Perkin-Elmer, Applied Biosystems), containing the dideoxyribonucleotides labelled with four different fluorochromes, the enzyme *AmpliTaq® DNA Polymerase* and salts ($MgCl_2$, Tris-HCl pH 9.0). Each samples was subjected to 45 thermal cycles: 10 seconds at 96°C, for the denaturation of the DNA, 5 seconds at

a temperature slightly lower than the *melting* temperature (T_m) of primer, for the annealing, 3 minutes at a temperature equal to that of annealing, for the elongation.

The unincorporated dideoxyribonucleotides were eliminate from the obtained products through the use of a 96-well plate *Multiscreen*^{TM} (Millipore Corporation) loaded with resin *Sephadex G-50 Superfine*. The reactions were freeze-dried and resuspended in 2.5 µl of loading buffer (83% formamide deionized, 4 mM EDTA pH 8, 8.3 mg/ml dextran blue), collected by centrifugation, denatured at 96°C for 3 minutes and chilled on ice.

After loading on a sequencing gel (6 M urea, 4% acrylamide *PAGE-PLUS*™ *Concentrate*, TBE 1X, 0.06% ammonium persulfate, 0.07% TEMED), the samples were, finally, subjected to electrophoresis, through *ABI PRISM*^{TM}*377 DNA Sequencer* (Perkin-Elmer, Applied Biosystems), using *TBE 1X* (*Tris-borate*: 89 mM Tris, 89 mM boric acid, 2 mM $Na_2EDTA{\cdot}2H_2O$) as running buffer.

For the sequence analysis was used the software *Sequencing Analysis 3.3* (Perkin-Elmer, Applied Biosystems).

Purification with *Sephadex*^{TM} *G-50 Superfine*

The products of PCR reactions to be used for sequencing were subjected to purification using a 96-well plate *Multiscreen*^{TM} (Millipore Corporation) containing the resin *Sephadex*^{TM} *G-50 Superfine* (Amersham Biosciences, GE Healthcare). After hydration, two hours long, of the resin contained into the wells to be used, the plate was centrifuged at 2,705 rpm for 6 minutes at room temperature, to remove the water used for the hydration. The sequence reactions were then loaded at the centre of the resin of the hydrated wells and the plate was again centrifuged at 2,705 rpm for 6 minutes at room temperature.

Cell cultures

The human leukemia K562 cells [Lozzio and Lozzio, 1975] were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640, SIGMA)

supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaillé, France), 2 mM L-Glutamine (CAMBREX – Biowhittaker Europe, Milan, Italy), 100 units/mL penicillin and 100 mg/mL streptomycin (Pen-Strep, CAMBREX – Biowhittaker).

The 293T cell line, human embryonic cells of kidney transformed with the T antigen of the SV40 virus, and HeLa cell line, tumor cells of the human cervix, were respectively cultured in Dulbecco's Modified Eagle Medium (D-MEM, CAMBREX) and Minimum Essential Medium Eagle (E-MEM, CAMBREX) supplemented with 10% fetal bovine serum (FBS) (Biowest), 2 mM L-Glutamine (CAMBREX – Biowhittaker), 100 units/mL penicillin and 100 mg/mL streptomycin (CAMBREX – Biowhittaker). These two cell lines, which grow in adhesion, need before splitting to be washed with DPBS (Dulbecco's Phosphate Buffered Saline, CAMBREX) and detached from the support through treatment with trypsin (trypsin 0.05%, EDTA 0.02%) (EuroClone, Siziano, Italy). During the experiment of transfection or transduction was used Iscove's Modified Dulbecco's Medium (I-MDM, CAMBREX) supplemented with 10% fetal bovine serum (FBS, Biowest), 2 mM L-Glutamine (CAMBREX – Biowhittaker), 100 units/mL penicillin and 100 mg/mL streptomycin (CAMBREX – Biowhittaker).

All the cells were cultured at 37°C in humidified atmosphere of 5% CO_2/air.

Virus Production

Viral stocks were generated by transfection of *pCCL.βwt.PGW* or *pCCL.β^{0}39.PGW* and the helper vectors *pMD2.VSVG* (coding the envelope protein VSV-G), *pMDLpg.RRE* (coding the proteins GAG and POL) and *pRSV.REV* (coding the protein REV) into 293T cells [Dull et al, 1998]. The 293T cells (5 x 10^6) were plated in 10-cm diameter cell culture dishes 24 hours before transfection in Dulbecco's Modified Eagle Medium (D-MEM, CAMBREX – Biowhittaker) with 10% Fetal Bovine Serum (FBS, Biowest), 2 mM L-Glutamine (CAMBREX – Biowhittaker), 100 U/mL penicillin and 100 µg/ml streptomycin (Pen-Strep, CAMBREX – Biowhittaker) in humified atmosphere of 5% CO_2/air. The culture medium was changed with Iscove's Modified Dulbecco's Medium (I-MDM)

(CAMBREX – Biowhittaker) 2 hours before transfection. A total of 25.5 μg DNA was used for transfection of one dish: 3 μg of the envelope plasmid *pMD2.VSVG*, 5 μg and 2.5 μg of the two packaging plasmid, *pMDLpg.RRE* and *pRSV.REV* respectively, 15 μg of the lentiviral vector. A 0.1 times TE (10 mM Tris, pH 8.0, plus 1 mM EDTA) and H_2O solution (2 times to 1 respectively) was added to the plasmid up to a final volume of 450 μl. The precipitate was, subsequently, formed by adding 50 μl of 2.5 mM $CaCl_2$ solution, mixing well and adding dropwise 500 μl of 2 times HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_2, pH 7.12) while vortexing, so as to allow the formation of small-size precipitates of calcium phosphate. The solution was immediately added dropwise to the cells. The medium was replaced after 16 hours, so as to remove the residual $CaPO_4$ salts. The viral supernatant was collected after another 24 hours, replaced, and again collected after 24 hours. Each time, the supernatant was filtered through 0.2-μm pore size cellulose acetate filters, divided in aliquots and stored at -80°C.

Virus titration

The viral titre of the lentiviral particles produced was determined by infection of HeLa cells. The HeLa cells (5×10^4) were plated in three wells of a 6-well plate (9.5 cm^2 each well) 24 hours before transduction in 1 ml of E-MEM supplemented with 10% Fetal Bovine Serum (FBS, Biowest), 2 mM L-Glutamine (CAMBREX – Biowhittaker), 100 U/mL penicillin and 100 μg/ml streptomycin (Pen-Strep, CAMBREX – Biowhittaker) in humified atmosphere of 5% CO_2/air. The aliquot of the viral solution was thawed at room temperature, while in the wells were removed the following volumes of medium: 500 μl from the first well and 100 μl from the second and the third. 500 μl of viral solution were then taken and added to the first well. After mixing well, 100 μl of medium were transferred from the first to the second well, so as to obtain a 1:10 dilution of the viral solution (corresponding to 50 μl). The same operation was carried out from the second to the third well, so as to achieve a further 1:10 dilution. To facilitate the transduction, 10 μl of 100X polybrene (800 μg/μl, Chemicon International, Millipore Corporation) were added to each well. The cells were then cultured at

37°C in humidified atmosphere of 5% CO_2/air for 16 hours, after which the medium was changed to eliminate the residual viral particles.

To determined the viral titre, the cells were subjected to FACScan analysis, to detect the percentage of GFP (Green Fluorescent Protein) – positive cells, and to quantitative real time PCR, to detect the copy-number of viral genome inserted into the cellular genome [Sastry et al, 2002].

Preparation of cells for FACS analysis

Hela cells were washed with DPBS (Dulbecco's Phosphate Buffered Saline), detached using 1 ml of trypsin, which was blocked adding 2 ml of FBS (Fetal Bovine Serum, Biowest), and centrifuged at 1,200 rpm for 5 minutes at 4°C. Half of the collected cells was transferred to a tube for cytometry, adding 2 ml of DPBS to run a wash. After centrifugation at 1,500 rpm for 5 minutes at 4°C, the supernatant was removed and the cellular pellet was resuspended with 500 µl of DPBS. Samples were kept on ice until the analysis of the fluorescence that was made with FACScan (flow-activated cell sorting, Becton-Dickinson, San Jose, CA) using the *Cell Quest Pro* software (Becton Dickinson).

FACS analysis

The FACS analysis of the Hela cells led to the setting of some parameters of the instrument. Channels to be used for data collection and their parameters were then set up: *FSC* (*Forward Light Scatter*) and *SSC* (*Sideward Light Scatter*), which collect data from the physical and morphological characteristics of the cells through phenomena of diffraction, reflection and refraction of light, and *FL1* (*FL-1 Height*), which receives data on green fluorescence emitted by the GFP expressed by the lentiviral genome. It was then set the maximum number of cells to be counted by the instrument, 20,000 cells. Finally, using the untransduced HeLa cells, lacking fluorescence, we set the parameters of diagrams obtained from the analysis, cytogram and histogram. First, we made a *Gate*, namely we selected within the heterogeneous population a homogeneous subpopulation, requiring to

the instrument the analysis only of that subpopulation. In the second, instead, we imposed that the peak formed by cells were placed entirely on the left of the value of fluorescence of 10^1, used as a threshold value.

Genomic DNA extraction

The cells were centrifuged at 2,000 rpm for 5 minutes at 4°C, then the pellet was washed with DPBS and, after a second centrifugation, resuspended in 400 µl of solution A (10 mM Tris-HCl pH 8.00, 400 mM NaCl, 2 mM EDTA). There were then added 20 µl of 10% SDS (sodium dodecyl phosphate) and 20 µl of proteinase K solution (10 mg / ml proteinase K, 2 mM CaCl2, 50 mM Tris, H2O, glycerol). After 2 hours of incubation at 56°C, 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1), calculated in relation to the initial volume, was added and the tube was inverted consecutively for 5 minutes and centrifuged at 12,000 rpm. Once collected the aqueous phase, extraction with phenol was repeated for better purifying the DNA. To the aqueous phase, obtained by the second extraction, was then added an equal volume of chloroform-isoamyl alcohol (24:1), the tube was again inverted consecutively for 5 minutes, and finally centrifuged at 12,000 rpm for 5 minutes. Once collected the aqueous phase, its volume was measured and 2.5 volumes of cold 96% ethanol were added. After centrifugation at 12,000 rpm for 5 minutes at 4°C, the pellet was resuspended in 50 µl of TE 1X (pH 8.00, filtered). To facilitate the re-suspension of genomic DNA, all samples were kept at 56°C for about 2 hours and then stored at 4°C.

Quantitative real time PCR assay

Quantitative PCR reactions were performed using 100 ng or 200 ng of genomic DNA and a specific probe for the β-globin gene. As reference genes, there were used $^{A}\gamma$-globin gene in the case of HeLa cells and K562 clones, and UBC gene or C21orf104 sequence in the case of erythroid precursor cells. The reaction mix, having a final reaction volume of 25 µl, contained: the pair of primers forward *PFβ* and reverse *PRβ* for β-globin gene, the probe *Pβ*, labelled in 5' with the

chromogenic molecule reporter FAM (6-carboxy -fluorescein) and in 3' with the quencher group TAMRA (6-carboxy-N, N, N', N'-tetramethyl-Rhodamine) (**table 2**) and the *TaqMan Universal PCR Master Mix* 1X (APPLERA Italy, Applied Biosystems). That mix contains the triphosphate deoxyribonucleotides (dNTPs), where dUTP replaces dTTP; 1 mM MgCl2; the chromophore "Rox", which serves as a reference for the normalization of data by the instrument in order to cancel any volume errors made during the operation by the operator; the enzyme *AmpliTaq Gold DNA polymerase*; the enzyme *AmpErase uracil-N glicosilase*, which degrades sequences containing uracile in place of thymine, leaving intact the original filament of template. This enzyme acts in the first phase of the reaction (when the temperature is 50°C) removing all the contaminant molecule, which may be present in the plate or tips; at a temperature of 95°C it is irreversibly inactivated.

On the same samples of HeLa and K562 cell clones, we carried out amplification reactions of the $^{A}\gamma$-globin gene, used as reference gene, with the specific forward *PFγ* and reverse *PRγ* primers and probe *Pγ* labelled in 5' with the chromogenic molecule *reporter* FAM (6-carboxy -fluorescein) and in 3' with the *quencher* group TAMRA (6-carboxy-N, N, N', N'-tetramethyl-Rhodamine) (**table 2**). The γ-globin gene represents a good reference because of its location in the same chromosome, within the same gene cluster, of the β-globin gene. In the case of erythroid precursor cells, on the same samples analyzed with the β-globin probe, we carried out amplification reactions of the UBC and C21orf104 sequences, used as reference genes, using the UBC and C21orf104 kits for allelic quantification (Applied Biosystems), so as to be able to choose the best reference gene for each experiment based on the slope of the standard curves.

All reactions were arranged in duplicate on 96-well optical plastic plates *Microamp Optical* (APPLERA Italy, Applied Biosystems, Monza, Italy), and for each probe used a negative control was performed, represented by a reaction in the absence of genomic DNA; moreover, we carried out reactions for 5 points of the standard, DNA of not infected Hela cells, at scalar concentrations, to build two curves of calibration, one for the β-globin gene and one for the γ-globin gene. The amplification was then performed using the thermal cycler *ABI Prism™ 7700*

(APPLERA Italy, Applied Biosystems), using the program *Sequence Detector v 1.7* (APPLERA Italy, Applied Biosystems). Two first cycles (50°C for 2 minutes and 95 ° C for 10 minutes) were necessary to activate the 5' – 3' exonuclease function of the polymerase; the next 40 cycles consist of a phase of denaturation at 95°C for 15 seconds and a phase at 60°C for 1 minute, in which take place both the annealing of primers and probe and the extension of the filament of DNA.

The change in the fluorescence emitted by the quencher group during the amplification is minimal compared to that of the reporter group, which is why it is used as internal reference, in order to obtain automatic normalization of the reporter group. The system is based on the following mathematical relationship:

$\Delta Rn = (Rn+) - (Rn-)$

where (Rn+) is the ratio between the emission of quencher and that of reporter, calculated at each cycle of amplification, and (Rn-) represents the ratio between the two emissions before the start of the PCR reaction.

Considering the different values of fluorescence ΔRn at each cycle, it is possible to get a spectrogram, which shows the number of cycles on the x-axis and the calculated ΔRn (Normalized Reporter) value on the y-axis. From the spectrogram it is possible to derive the value of the cycle threshold, called Ct, which represents the cycle at which you can record the first appreciable increase in the intensity of emitted fluorescence, not covered by the background signal. The value of threshold is chosen by the operator and corresponds to a specific ΔRn value to which all the samples are compared and on the basis of which it is possible to derive their respective values of Ct. To minimize the experimental error, it is necessary to consider a reference gene into the system, i.e. the γ-globin gene. The fold of β-globin transgenes was expressed as $2^{-\Delta\Delta CT}$, where ΔΔCT represents the subtraction of the ΔCT obtained from untransduced cells from the ΔCT derived from infected cells. ΔCT is the difference between the amplification cycle equivalent to a significant fluorescence level (CT) obtained by the amplification of β-globin gene and the corresponding amplification cycle of the reference gene. This equation can be applied to have a relative quantification of

our templates if the slopes of β-globin and reference genes amplifications are comparable.

Production of stable transgenic cell lines

K562 transduction

Cells were infected with the thawed viral supernatant at various multiplicities of infection (MOIs: 0.5 – 2). The cells (1 x 10^5) were plated, two hours before the infection, in a 9.5-cm^2 well with 50% RPMI (Sigma), 50% I-MDM (CAMBREX – Biowhittaker) supplemented with 10% FBS (Biowest), 2 mM glutamine (CAMBREX – Biowhittaker), 100 U/ml of penicillin and 100 μg/mL of streptomycin (Pen-Strep, CAMBREX – Biowhittaker). In order to facilitate the cell infection, 10 μl of the 800 μg/μl transduction agent polybrene (Chemicon International, Millipore Corporation) was added to the K562 cells plated, which were subsequently cultured in a 5% CO_2 incubator. The medium was change after about 16 hours, so as to eliminate the remained viral particles.

Cell cloning

After having determined the cell concentration through an automatic cell counter (SEAC h5-m), subsequent dilutions were executed using RPMI (Sigma) supplemented with 20% FBS (Biowest), 2 mM glutamine (CAMBREX – Biowhittaker), 100 U/ml of penicillin and 100 μg/mL of streptomycin (Pen-Strep, CAMBREX – Biowhittaker), so as to obtain a final concentration of 4 cells/ml. The cell suspension was aliquoted in a 96-well plate, 100 μl of solution/well, in order to obtain individual clones. The plate was then placed at 37°C in humidified atmosphere of 5% CO_2/air for about 2 hours and then carefully checked at the optical microscope, to identify which well had a single cell.

Isolation of transgenic clones

After making the cells expand for about a week, from each isolated clone was obtained a single colony, whose components are all equal to the mother cell. To identify colonies in which all the cells were able to emit green fluorescence, due to the GFP, whose gene is located on the lentiviral vector, they were analyzed by a fluorescence microscope. Once grown, it was possible to transfer the cells from the 96-well plate to a 24-well plate, and so up to a flask.

DNA extraction from blood

The DNA extraction from blood was performed using the *QIAGEN Blood & Cell Culture DNA Mini Kit* (QIAGEN) and 1 ml of whole blood. First, it was necessary to determine the leukocyte concentration which should not be more than 1×10^7 leukocyte per ml. However, no more than 5×10^6 should be used for the isolation. 1 volume of ice-cold *Buffer C1* (1.28 M sucrose, 40 mM Tris-CL pH 7.5, 20 mM $MgCl_2$, 4% Triton X-100) and 3 volumes of ice-cold distilled water were then added to the blood, so as to lysate the blood red cells and the leukocyte membrane. After mixing by inverting the tube several times, until the suspension becomes translucent, an incubation on ice for 10 minutes was performed. The suspension was then centrifuged at 1.300 g for 15 minute at 4°C and, after discarding the supernatant, the pelleted nuclei were re-suspended using 0.25 ml of ice-cold *Buffer C1* and 0.75 ml of ice-cold distilled. The suspension was again centrifuged at 1.300 g for 15 minute at 4°C. This wash step removes all residual cell debris and haemoglobin from the nuclear pellet. The wash was repeated when the pellet was not white. The pellet was completely re-suspended with 1 ml of *Buffer G2* (800 mM guanidine HCl, 30 mM Tris-Cl pH 8.0, 30 mM EDTA ph 8.0, 5% Tween-20, 0.5% Triton X-100) by vortexing for 10 – 30 seconds at maximum speed and 25 µl of *QIAGEN Protease* were added. The suspension was then incubated at 50°C for 60 minutes. After equilibrating a chromatographic column *QIAGEN Genomic-tip 20/G*, on ion exchange, with 2 ml of *Buffer QBT* (750 mM NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol, 0.15% v/v Triton X-100), the

sample was vortexed at maximum speed and applied to the equilibrated *QIAGEN Genomic-tip*, which was then washed three times with 1 ml of *Buffer QC* (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol). The DNA was eluted twice with 1 ml of *Buffer QF* (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% v/v isopropanol) and precipitated by adding 1.4 ml of room-temperature isopropanol, mixing and centrifuging at > 5,000 g for 20 minutes at 4°C. After carefully removing the supernatant, 1 ml of cold 70% ethanol was added and the sample vortexed briefly and centrifuged at > 5,000 g for 10 minutes at 4°C. The supernatant was then removed and the pellet was air-dried for 5 – 10 minutes and re-suspended with 30 – 50 μl of TE pH 8.0. Finally, the DNA was dissolved at 55°C for 1 – 2 hours.

Cultures of erythroid precursor cells isolated from the blood of healthy subjects and patients with β-thalassemia

The *in vitro* culture of stem cells from peripheral blood is carried out in two phases in liquid medium, which include different steps. The starting point is the buffy-coat, the corpuscular part of the blood, resulting from a pocket of blood collected from a healthy donor after informed consent. The buffy-coats had a volume of 35 ml that were diluted 1:2 with 1x PBS (Phosphate Buffered Saline) at room temperature. The 1X PBS was prepared by dilution with distilled H_2O from 10X PBS, which consists of a solution of 2 M NaCl, 27 mM KCl, 0.1 M Na_2PO4, 18 mM KH_2PO_4, in distilled H_2O, sterilized by filtration through 0.2-μm pore size cellulose acetate filters, and stored at 4°C. The diluted sample was divided into 40 ml aliquots and subjected to a centrifugation for density gradient on *Lympholyte-H* (Nycograde™ polysucrose 400 and sodium diatrizoate, Celbio, Milan, Italy): this created a gradient of dextran and other substances to facilitate the separation of corpuscular parts of the blood. The centrifugation created four separated layers, that from top to bottom are: serum, a whitish ring containing lymphocytes, fibroblasts, macrophages and erythroid precursors, a cloudy part containing *Lympholyte* with not separated cells and a red fund formed by red blood cells. The whitish ring is taken off, subjected to several washes with 1X PBS and transferred

to the phase I medium, as follows: α-MEM medium (α-minimal essential medium, Sigma-Aldrich), prepared from a powder and diluted with water; a solution of PEN-STREP (50 U/litre penicillin and 50 mg/litre streptomycin, Sigma-Aldrich); 10% FCS (Fetal Calf Serum, Gibco, BRL, Life Technologies); 10% conditioned medium (CM), obtained from cell cultures of bladder cancer (cells 5637), rich in haematopoietic growth factors except the erythropoietin (EPO) and separated from the cells themselves by filtration; 1 μg/ml cyclosporine A (Sigma-Aldrich), prepared from cyclosporine diluted in absolute ethanol and 1X PBS in 1:1 ratio. The culture was then incubated at 37°C in humidified atmosphere of 5% CO_2/air. It is important to check every day the cell viability and the absence of contamination.

After 5 – 7 days of culture in phase I medium, the not adherent cells were recovered, washed and resuspended with fresh medium of phase II, which includes: α-MEM; 30% FCS; 10% deionized bovine serum albumin (BSA, Sigma-Aldrich) dissolved in α-MEM; 0.01 mM β-mercaptoethanol (β-ME, Sigma-Aldrich) prepared from a 100 mM solution diluted with sterile H_2O; 0.001 mM dexamethasone (Sigma-Aldrich), prepared from a 6.4 mM solution diluted in sterile methanol (this compound is able to stimulate the erythroid line); 2 mM L-Glutamine (Sigma-Aldrich); 1 U/ml human erythropoietin (EPO) (Tebu-bio, Magenta, MI, Italy); 10 ng/ml Stem Cell Factor (SCF, PeproTech EC Ltd, London, England), dissolved in 10 mM acetic acid. Some of these components (BSA, β-ME, dexamethasone, L-Glutamine) were sterilized using 0.2-μm pore size filters and stored in darkness at -20°C. The EPO and SCF, being protein factors, must be stored at -80°C to prevent degradation. The incubation in this phase lasted from 4 to 6 days. It is important to check every day the cells viability, the absence of contamination, but especially the formation of groups or "clones" of cells in the supernatant. Only if there are such cell agglomerations of pro-erythrocytes, it is possible to continue with treatments. Sometimes there may be few and/or small cell agglomerations after the usual 4 – 6 days of phase II, as it has a slower growth, in which case it is possible to extend the phase II for another 4 – 5 days to get a sufficient proliferation of colonies and then continue with

treatment. After each stage it is necessary to count the cells using the *Coulter Counter Z1* (Coulter Electronics Limited, Luton, Beds, England).

The volume of blood that can be taken from β-thalassemia patients is much lower than that can be taken from normal individuals (for ethical reasons and depending on the fact that these are individuals with a haematological disease). In fact, these individuals already have low levels of haemoglobin because of their disease and a sample of blood can only worsen the state of anemia of these people. Thus, in general, we use the minimum amount of blood needed to prepare 20 ml of culture, this amount may be sufficient for the high frequency of erythroid precursors contained, a situation that is backed and driven by the lack of haemoglobin. This cell culture can produce a maximum of 10^8 cells.

Usually, 20 ml of whole blood were collected and brought up to 35 ml with 1x PBS. The diluted sample was subjected to centrifugation for gradient density on Lympholyte-H. From this point forward, the method was the same as the culture of erythroid precursors derived from the blood of healthy subjects.

Transduction of erythroid precursor cells

After having determined the amount of virus to be used for the transduction of erythroid precursor cells with a MOI equal to 0.3, based on the viral titre, all the progenitors were put in a 1.5 ml tube with 1 – 1.5 ml of α-MEM (Sigma-Aldrich) without FCS but supplemented with EPO and 800 ng/μl polybrene so as to have final concentrations equal to 1 U/ml and 10 mg/ml, respectively.

An aliquot of viral particles were then thawed at room temperature for 30 minutes, mixing once in a while, and the volume decided for the infection was transferred in the tube containing the cells, along with 15 μl of polybrene 100X (800 μg/μl). The cells were then incubated at 37°C in humidified atmosphere of 5% CO_2/air for 6 hours, mixing well every 30 minutes. The infected erythroid progenitors were then plated at 10^6 cells/ml. The medium was change after about 16 hours, so as to eliminate the remained viral particles. After 5 days of incubation the cells were collected and analysed.

Treatment with *mithramycin*

Treatments with *mithramycin* (Sigma-Aldrich) were carried out by adding the appropriate drug concentrations to the transduced erythroid precursors, seeded at 10^6 cells/ml, and incubating at 37°C in humidified atmosphere of 5% CO_2/air for 5 days.

Inhibition curve

To determine the concentrations of aminoglycosides to use in the treatment of clones and erythroid precursors, we performed the curve of toxicity of each aminoglycosides so as to derive the IC50, namely the concentration that causes an inhibition of cell growth equal to 50%. We plated 6 x 10^4 wt3 βwt-globin and m5 $\beta^0$39-globin K562 clones cells in 9.5-cm^2 wells with 2 ml RPMI (Sigma) with 10% FBS (Biowest) and treated with increasing concentrations of aminoglycosides. After 72 hours, the cells in each well were counted using the Bürker chamber (Vetrotecnica, Padova, Italy). The data obtained were then reported in a graph with the number of cells on the y-axis and the concentrations of aminoglycosides on the x-axis.

Treatment with aminoglycosides

Treatments with aminoglycoside antibiotics were carried out by adding the appropriate drug concentrations to the K562 clones cells and to the erythroid precursors, seeded at 30,000 cells/ml and at 10^6 cells/ml respectively, and incubating at 37°C in humidified atmosphere of 5% CO_2/air for 3 days.

RNA extraction

The total cellular RNA was extracted by *TRIZOL® Reagent* (Gibco - Invitrogen-Life Technologies). All reagents and materials used were RNase – free. The cells (5 – 10 x 10^6) were centrifuged at 1,200 rpm for 5 minutes at 4°C, then

the pellet obtained was washed with 1X DPBS, re-centrifuged and then resuspended with 1 ml *TRIZOL® Reagent* (Gibco - Invitrogen-Life Technologies). After incubating for 5 minutes at room temperature, 200 µl of chloroform were added, stirring well, and incubating on ice for 5 minutes. The samples were then centrifuged at 12,000 rpm for 10 minutes at 4°C. To the collected aqueous phase an equal volume of isopropanol was added. The samples were then placed at -80°C for at least 1 hour. The RNA precipitate was centrifuged at 12,000 rpm for 20 minutes at 4°C, washed with 500 µl of cold 70% ethanol, re-centrifuged, freeze-dried and resuspended in 20 µl of water RNase – free.

The extracted RNA was analyzed by electrophoresis on 0.8% agarose gel, before the treatment with DNase I and the reaction of RT-PCR.

Treatment with DNase I

Treatment with DNase I was made starting from the total RNA extracted from the cells before reverse transcription, through the use of the *RQ1 RNase – free DNase kit* (Promega).
The reaction was carried out in a final volume of 10 µl, in the presence of 2 µg RNA, 1 µl of *RQ1 DNase 10X Reaction Buffer* (400 mM Tris-HCl pH 8.0, 100 mM MgSO4, 10 mM CaCl2) and 2 U of *RQ1 RNase – free DNase*; the reaction was, therefore, incubated at 37 ° C for 30 minutes.

Finally, after putting the reaction on ice, the enzyme was inactivated by the addition of 1 µl of *RQ1 DNase Stop Solution* (20 mM EGTA pH 8.0) and incubated at 65°C for 10 minutes.

Reverse transcription reaction

The reaction of reverse transcription was made with the *ImProm-II™ Reverse Transcription System kit* (Promega). Half of RNA treated with DNase I, equivalent to 1 µg, was subjected to reverse transcription, using Oligo$(dT)_{15}$ or random primers, 0.5 µg per reaction. The RNA and primers were incubated at 70°C for 5 minutes and then immediately cooled to 4°C. We, then, added the reaction

solution, prepared considering a final volume of 20 µl and progressively adding the following reagents: 5X *ImProm-II™ Reaction Buffer*, 25 mM MgCl2, 10 mM dNTPs mix, 1U/µl of *Recombinant RNasin® Ribonuclease inhibitor* and l µl of *Improm-II™ Reverse Transcriptase*. The reaction of reverse transcription was then performed: the sample was incubated for 5 minutes at 25°C, to allow the annealing of the reverse transcriptase to the RNA, for 60 minutes at 42°C, to allow the extension, and at 70°C for 15 minute to inactivate the enzyme.

Non-quantitative real time PCR for the characterization of the β-globin transcript

Through the use of the *Custom TaqMan® SNP genotyping Assays* kit (Applied Biosystems), it is possible to discriminate between two transcripts that differ in only one base, such as the βwt and the $\beta^0$39 transcripts.

The kit consists of a *MIX 40X* containing two primers, *PR* and *PF*, and two *TaqMan® MGB probes*, the *Pβ⁰39* labelled with the chromophore FAM (6-carboxy-fluorescein) and *Pβwt* labelled with the chromophore VIC (compound under secret patent) (**table 2**).

The reactions, carried out in duplicate for each sample, were arranged with 50 ng of cDNA, 0.625 µl of *40X Assay Mix* and 12.5 µl of *1X TaqMan® Universal PCR Master Mix* and brought to a final volume of 25 µl with ultrapure water. The reactions were placed in a plastic optical 96-well plate *MicroAmp Optical* (Applera Italia, Applied Biosystems) and were carried out using the *ABI Prism Thermal cycler™ 7700* (Applera Italia, Applied Biosystems) and the program *Sequence Detector v 1.7* (Applera Italia, Applied Biosystems). First, two cycles (50°C for 2 minutes and 95°C for 10 minutes) were made to activate the exonuclease 5' – 3' function of the polymerase; the next 40 cycles consisted of a step of denaturing at 95°C for 15 seconds and a step at 60°C for 1 minutes, in which both the annealing of primers and probe and the extension of the filament of DNA take place.

Intracellular labelling technique

This method was carried out with the *BD Cytofix/Cytoperm™ Kit* (BD Biosciences Pharmingen), which allows the cell permeabilization and the antibody binding to the proteins inside the cell. Since the labelled K562 clones cells were used for the analysis of the fluorescence using both the FACS and the fluorescence microscope, 1.5 x 10^6 cells were centrifuged at 1,800 rpm for 3 minutes at 4°C. After removing the supernatant, the pellet was washed with 500 µl of cold 1X PBS (Phosphate-Buffered Saline), re-centrifuged and then resuspended in 500 µl of *BD Cytofyx-Cytoperm solution* and incubated for 20 minutes at 4°C. After centrifugation at 1,800 rpm for 3 minutes at 4°C, the pellet was washed with 500 µl of cold 1X PBS (Phosphate-Buffered Saline), incubated for 3 minutes at room temperature and then centrifuged at 1,800 rpm for 3 minutes at 4°C. After making a further wash, the cells were resuspended with 300 µl of solution 1X PBS – 1% BSA (bovine serum albumin, Sigma) and left in incubation 1 hour at room temperature in darkness. After that, the sample was centrifuged at 1,800 rpm for 3 minutes at 4°C and the pellet resuspended with 30 µl of the solution of the monoclonal antibody *βglobin-PE* (PE-Phycoerythrin, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:10 in 1X PBS-1% BSA and incubated overnight at 4°C in darkness. After incubation, the sample was centrifuged at 1,800 rpm for 3 minutes at 4°C, washed with 500 µl of cold 1X PBS re-centrifuged and the pellet resuspended with 30 µl of cold 1X PBS.

Immunohistochemistry assay

Part of the labelled cells (1/3) was transferred on a section of a chamber slide (CultureSlide, FALCON, Becton Dickinson). Each section of this slide had been previously treated with 30 µl of Poly-L-Lysine diluted 1:10, dried and washed with ultrapure water. In the section with the cells, 200 µl of 4% formalin were added, incubating for 25 minutes at room temperature. The fixed cells were finally washed for three times with 100 µl of cold 1X PBS. Each wash was followed by 5 minutes incubation. After dried, the slide was covered with a micro cover glass,

fixed with a few drops of glycerol and displayed by a fluorescence microscope (Olympus BX60, Olympus Italia, Segrate (MI), Italy). It was possible to take pictures of cells through the camera mounted on microscope (Nikon digital Sight DS-2mV, Nikon Instruments, Calenzano (FI), Italy), subjected to certain exposures of fluorescent light with the help of special optical filters.

In the case of treatment of erythroid precursors, analysed only by FACS, we labelled 5 x 10^5 to 7.5 x 10^5 cells and then all the volumes of the reagents were halved.

Flow cytometry (FACS) assay

The 2/3 of the volume of each sample labelled using the *Cytofix/Cytoperm™ kit* were transferred to a tube for FACS analysis and there were added 500 μl of *Stainning Buffer* (DPBS, 1% FBS). The FACS analysis of these cells was performed by FACScan (flow-activated cell sorting, Becton-Dickinson) using the *Cell Quest Pro* software (Becton-Dickinson).

The analysis of K562 cells by the flow cytometer, as previously done for the Hela cells in the calculation of the viral titre, requires setting some basic parameters to obtain significant data for the experiment. Channels that were chosen for data collection were: *FSC* (*Forward Light Scatter*) and SSC (*Sideward Light Scatter*), which collect data from the physical and morphological characteristics of cells, *FL1* (*FL-1 Height*), which receives data on green fluorescence emitted by GFP, *FL2* (*FL-2 Height*), which receives data on fluorescent red - orange emitted by antibody *βglobin-PE* against β globin. After setting the number of cells to count (10,000 – 30,000), using cells that do not express green and red fluorescent, we have worked on histograms of green (No. cells - GFP) and red (No. cells - βglobin-PE) fluorescent in a way that the peak, representing cells, was beyond the fluorescence threshold of 10^1. A *Gate* was done on the morphological cytogram trying to exclude all the died or dying cells, in order to assess the effect of the compounds only on living cells. In addition, to analyze cells emitting two different fluorescences at the same time, the green and red, it was necessary to perform an electronic compensation on *FL1* and *FL2*

channels. This compensation consists in the subtraction from each channel of the interference created by the too wide wavelength emitted by the fluorochrome, using only cells that express either green fluorescent or red fluorescence.

In the case of erythroid precursors, it was not necessary to make compensation, because they emitted only the red fluorescence of the *βglobin-PE* antibody. Channels that were chosen in this case were: *FSC* (*Forward Light Scatter*), *SSC* (*Sideward Light Scatter*) and *FL2* (*FL-2 Height*).

Quantitative real time PCR of the β-globin transcript

Depending on the type of treated cells, the quantitative PCR reactions were prepared using different primers and probes. The *Custom TaqMan® SNP Genotyping assays* (Applied Biosystems) kit was used in the case of K562 clones, as the two probes (*Pβwt* and $P\beta^0 39$) are able to discriminate between βwt and $\beta^0$39-globin transcripts. On the contrary, in the case of erythroid precursors were used pair of forward and reverse primers and probe for β-globin mRNA (*PFβ-PRβ* and *Pβ*), α-globin mRNA (*PFα-PRα* and *Pα*) and γ-globin mRNA (*PFγ-PRγ* and *Pγ*). All the probes were labelled in 5' with the chromogenic molecule *reporter* FAM (6-carboxyfluorescein) and in 3' with the *quencher* TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine) (**table 2**). In the case of the erythroid progenitors it was possible to use a generic probe for β-globin mRNA sequence because these cells express only the mutated transcript and it was therefore not necessary to discriminate between the two transcripts.

The reaction mix, with a final volume of 25 μl, contained, as well as primers and probes, the *1X TaqMan Universal PCR Master Mix* (Applera Italia, Applied Biosystems). On the same samples, amplification reactions of a reference gene were carried out. As reference gene we used either GAPDH or r18S. The specific primers and probe, labelled in 5' with the chromogenic molecule VIC and in 3' with TAMRA, are contained in the *GAPDH kit* (Applera Italia) and *r18S kit* (Applera Italia), respectively.

All quantitative PCR reactions were performed in duplicate using 20 – 50 ng of cDNA and for each probe used we made a negative control, represented by a

reaction in the absence of cDNA; moreover, we carried out reactions for 5 points of the standard, cDNA of not treated cells, at scalar concentrations, to build two curves of calibration, one for the β-globin gene and one for the reference gene. The amplification was carried out using the *ABI Prism 7700 Sequence Detector*, which consists of a thermal cycler, *ABI Prism 7700*, in which the reagents are placed in optical plastic 96-well plate *MicroAmp Optical* (Applera Italia), a computer and a software, the *Sequence Detector Application Program version 1.7*, which manages the instrumentation and analysis of data. Initially, two cycles (50°C for 2 minutes and 95°C for 10 minutes) were made to activate the exonuclease 5' – 3' function of the polymerase; the next 40 cycles consisted of a step of denaturing at 95°C for 15 seconds and a step at 60°C for 1 minutes, in which both the annealing of primers and probe and the extension of the filament of DNA take place.

To minimize the experimental error, the quantification of cDNA from different samples is more reliable if it is considered a reference gene internal into the system. Through this strategy it is possible to have a "relative" quantification, that is based on the difference between the levels of expression of a target gene in different samples evaluated compared with a reference gene, which must equally be expressed in all the analyzed samples. Usually, the genes used as reference are: the gene for β-actin, the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the β2-microglobulin, or the 18S rRNA.

The analysis of the Real time PCR was already described for viral titration.

HPLC analysis for the quantification of haemoglobins in cultured erythroid precursor cells

The relative (%) and absolute (pg/cell) concentration of HbA (adult haemoglobin) compared to total haemoglobins was determined by separating and quantifying the haemoglobins using HPLC. The instrument used for the analysis is a HPLC *Beckman Coulter System Gold 126 Solvent-166 Detector Module* (Beckman Coulter, Milan, Italy), set at a wavelength of 415 nm to selectively detect the haemoglobins present in pellets of cells harvested from cultured

erythroid precursors, the eleventh day of phase II, and lysated before chromatography. The cells were collected by centrifugation at 1,600 rpm for 5 minutes at 4°C, washed with PBS and resuspended with a volume of water dependent on the quality of the cell pellet (colour and cell number). The cellular suspension was then strongly vortex, incubated for 15 minutes on ice and centrifuged at 14,000 rpm for 5 minutes at room temperature. The supernatant was finally collected and used for the analysis.

To separate the haemoglobins a cation – exchange column *Syncropak CCM 103/25* was used, eluding with a gradient of sodium acetate present in the mobile phase, which consist of aqueous BisTris-KCN-buffers.

To exactly identify the retention times, commercial standards were used: purified HbA (adult haemoglobin, Sigma-Aldrich) and HbF (fetal haemoglobin) present in the samples of haemoglobins AFSA2 (Alpha Wassermann Italy) or in the standard of haemoglobins AF (Analytical Control Systems, Inc. USA).

RESULTS

PRODUCTION OF AN EXPERIMENTAL MODEL OF β^0-THALASSEMIA

The $\beta^0$39 mutation is characterized by the substitution C → T in the first base of the codon 39, contained in the second exon of the β-globin gene. Such substitution causes the creation of a premature translation termination codon, leading to the inhibition of the synthesis of β-globin chains. Since this mutation is of particular importance both for the severity of the disease that occurs and for its wide distribution and frequency in the Mediterranean countries, we decided to develop an experimental model of β-thalassemia that could be used to test new therapeutic strategies aimed at its correction. We also decided to produce cellular lines expressing βwt-globin gene at various levels to be used as experimental control.

In **figure 19** is presented the schematic representation of the strategy used to produce the cellular model. The first step of this strategy was the production of two lentiviral vector containing the βwt- or $\beta^0$39-globin gene under the control of its promoter and the locus control region (LCR). The obtained vectors were then used to create lentiviral particles, essential for the infection of human erythroid K562 cells. The infected cells were then subjected to cell cloning by limiting dilution in order to isolate cell clones containing at least one copy of the vector using a fluorescence microscope. Such clones were also characterized by real time PCR and FACS analysis.

Production of the *pCCL.$\beta^0$39.PGW* and *pCCL.βwt.PGW* lentiviral vectors

To create a cellular model with an expression of the βwt- or $\beta^0$39-globin gene stable over time, we decided to insert these genes into a third generation lentiviral vector. Vectors of this type, in fact, have a number of elements that make the

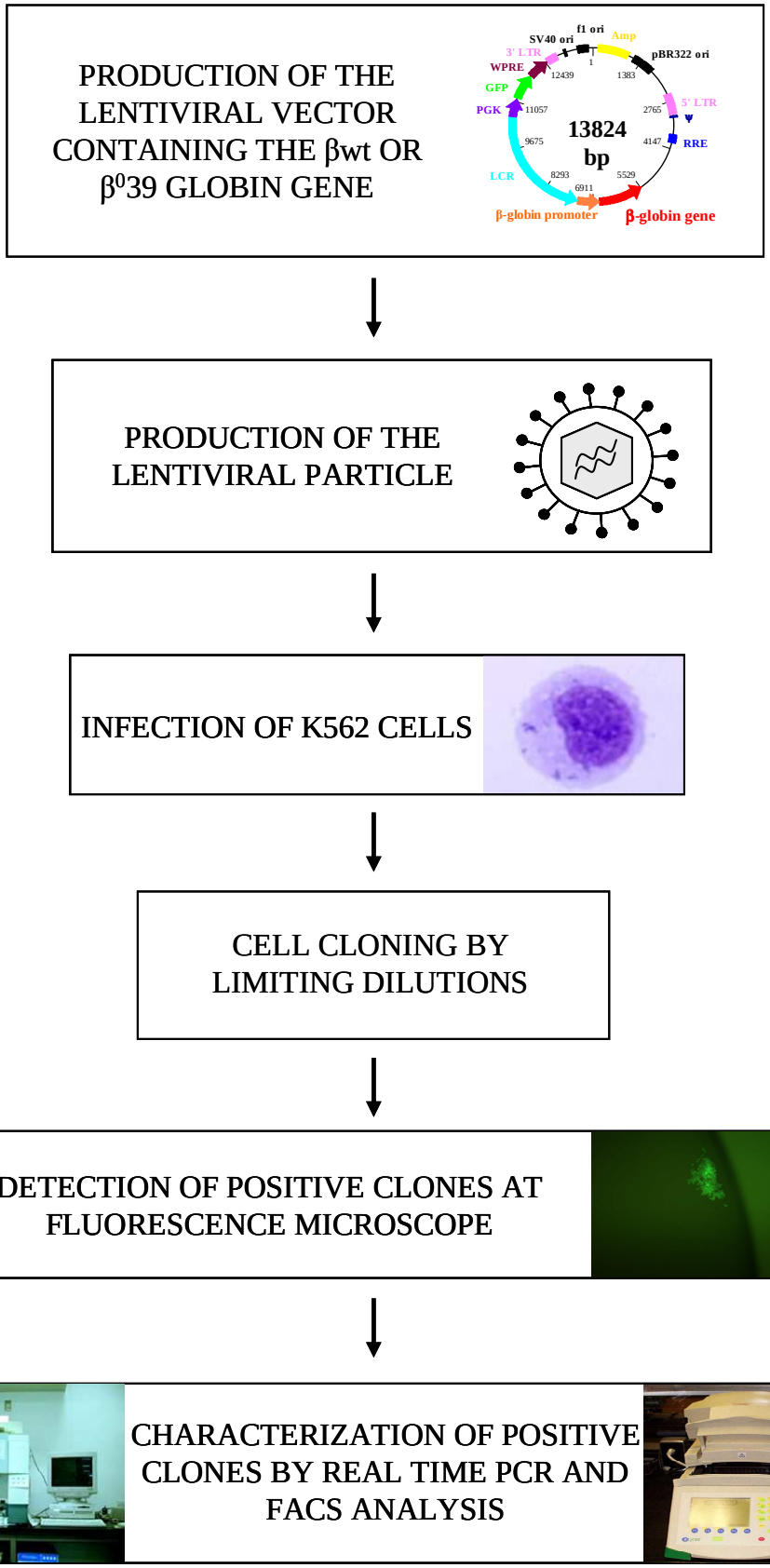


Figure 19 – Schematic representation of the experimental strategy used to produce the β-thalassemia *ex vivo* model. The β-globin $\beta^0$39 gene was introduced into a lentiviral vector, subsequently utilized to produce viral particles with the function to infect K562 cells. The transduced K562 cells were, then, subjected to cellular cloning by limiting dilution in order to isolate clones, in which at least a copy of the vector was integrated. Such clones were detected at the fluorescence microscope and, finally, characterized through quantitative real time PCR and FACS analysis.

integration of the transgene into the cellular genome safe and efficient: two modified viral *LTR* (*Long Terminal Repeat*), which are important for the reverse transcription, integration and gene expression; the ψ packaging sequence required for the incapsidation of genomic RNA, which has to be transferred; the trans-active element REV-*RRE* (*Rev Responsive Element*), which is required for processing and transport of viral RNA outside the nuclear compartment [Stathopulos, 2003].

In **figure 20** is schematically presented the experimental strategy for the production of the lentiviral vector containing the $\beta^0$39-globin gene. The first phase of this strategy included the use of the third generation lentiviral vector *dTNS9 MluI(-),* 11,459 bp long (**figure 20a**), supplied by Prof. Stefano Rivella (Weill Medical College of Cornell University, New York). This vector contains a βwt-globin gene, deleted of part of the second intron, under the control of its promoter and a small β locus LCR, including however large areas of hypersensitive sites HS2 (840 bp) , HS3 (1308 bp) and HS4 (1069 bp) [May et al, 2000].

The first step was made to remove a sequence of about 3 kilobases (kb), containing restriction sites that could interfere with the following digestions. We therefore digested the vector *dTNS9 MluI(-)* with *PmeI* and *SmaI* endonucleases (**table 3**), generating two fragments with blunt ends. The digestion products were purified by phenol-chloroform extraction, precipitation and *Microcon® YM-100* (Millipore Corporation) and subjected to ligation reaction. Such reaction was set up in the absence of PEG (polyethylene glycol), which has the function to increase the viscosity of the solution, and in a more diluted way so as to facilitate intramolecular ligation of the vector (8,431 bp), containing the resistance to the ampicillin, and to completely remove the unwanted sequence of 3,027 bp. The reaction was, therefore, set up using 130 ng of vector and 5 U of *T4 DNA ligase* (MBI Fermentas) by incubation at 22°C over night. The ligation products were finally used for the transformation of ultra-competent bacteria JM109, using Petri plates added with ampicillin, required for the selection of clones containing the vector.

Colonies grown in the plate were analysed by Rapid Disruption, enabling the identification of the construct of interest through its molecular weight. The vector

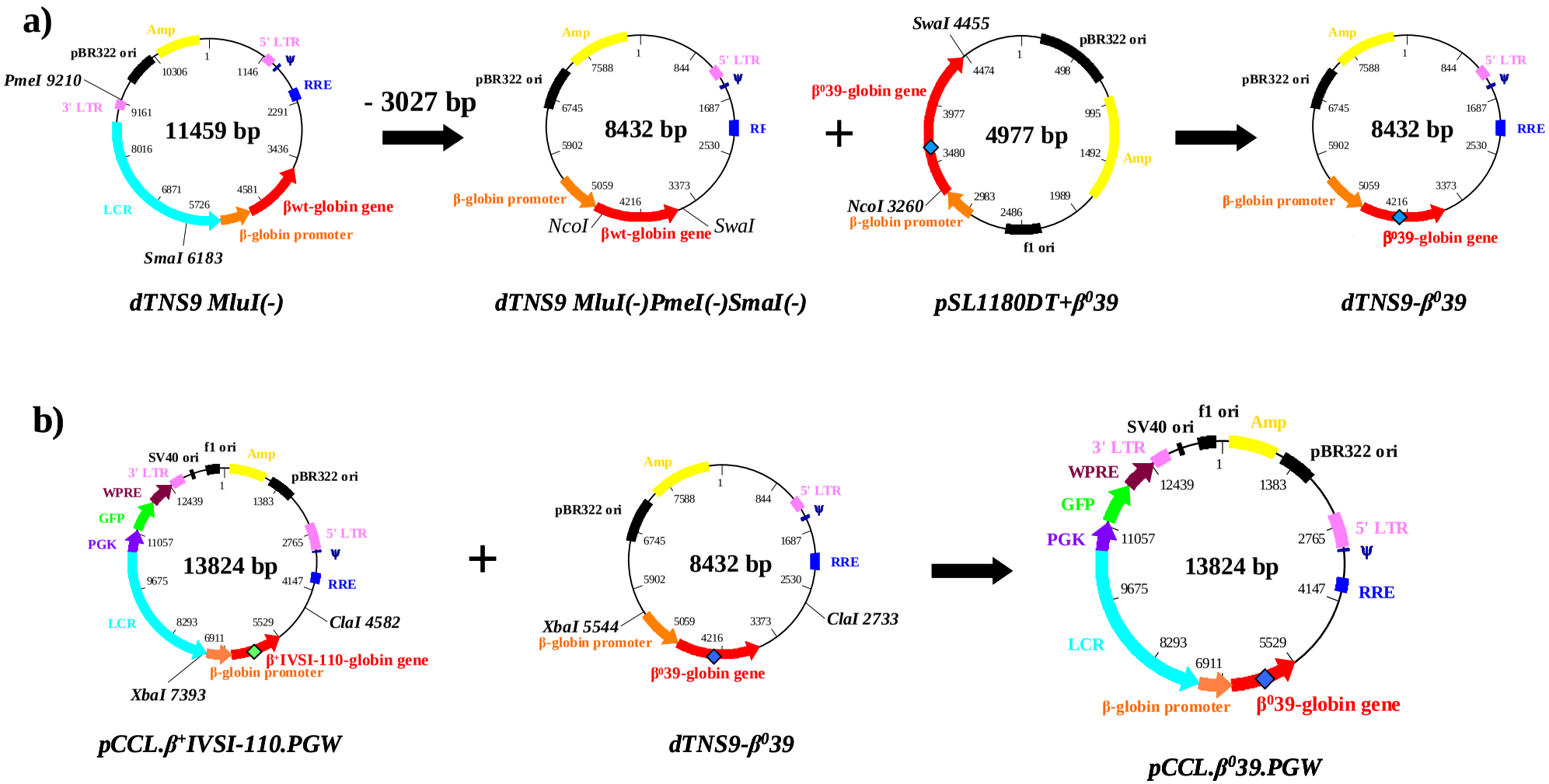


Figure 20 – Cloning strategy followed to produce the *pCCl.β⁰39.PGW* lentiviral construct. a) The *dTNS9 MluI(-)* vector was deleted of a 3 kb region, in order to eliminate a restriction site for the enzyme *SwaI*: it was then obtained the construct *dTNS9 Mlu(-)Pme(-)SmaI(-)*, containing the βwt-globin gene. Such gene was subsequently replaced with the β⁰39-globin gene, coming from the expression plasmid *pSL1180DT+β⁰39*, previously subjected to a site-directed mutagenesis, with the subsequent production of the *dTNS9-β⁰39* vector. **b**) The β⁰39-globin gene, contained in *dTNS9-β⁰39* vector was transferred into the *pCCL.β⁺IVSI-110.PGW* lentiviral vector, replacing the β⁺IVSI-110-globin gene, thus obtaining the *pCCL.β⁰39.PGW* vector.

was then purified using *FlexiPrep kit* (Pharmacia Biotech) and its identity was confirmed by a subsequent restriction reaction, which produced, following the digestion with *NcoI* and *SwaI* (**table 3**), 2 expected fragments, 7240 bp and 1191 bp long (data not shown). In this way we produced the vector *dTNS9 MluI(-)PmeI(-)SmaI(-).*

The second step of the cloning strategy (**figure 20a**) was the replacement of the βwt-globin gene present in *dTNS9 MluI(-)PmeI(-)SmaI(-)* vector, with the $\beta^0$39-globin gene, contained in *pSL1180DT+$\beta^0$39* vector, produced by site-directed mutagenesis of the *pSL1180DT+βwt* construct, kindly supplied by Prof. Stefano Rivella (Weill Medical College of Cornell University, New York). It was therefore necessary to digest both vectors with the restriction enzymes *NcoI* and *SwaI* (**table 3**), resulting in production of fragments with blunt and sticky ends. Such fragments were separated by electrophoresis on agarose gel and purified with *QIAquick Gel Extraction Kit* (QIAGEN). This method involves the use of columns containing a silica membrane able to bind the DNA, which is then purified from contaminants and eluted.

The fragments were then subjected to a ligation reaction, set up using 5 U of *T4 DNA ligase* (MBI Fermentas), 100 ng of vector and a molecular ratio vector:insert equal to 1:6; was then performed an incubation at 22°C for 1 hour and at 16°C over night. The ligation products were then used for the transformation of a different strain of *E. Coli*: the *XL1-blue*, because the expected vector is fairly large (**figure 20a**) and these bacteria should be able to host it more easily. The grown colonies were again analysed by Rapid Disruption, thus identifying the vector of interest by analysis of molecular weight, which was later confirmed, after isolation of the vector by *QIAGEN Plasmid Purification Mini Kit* (QIAGEN), by restriction analysis using the *ClaI* and *XbaI* enzymes (**figure 21a**).

To confirm the presence of the β-globin gene within the vector, the relative bacterial clone was subjected to heat lysis and the resulting supernatant was used as a template for a PCR reaction, using the *BGF1* and *BGR1* primers (**Table 1**), specific for the human β-globin gene. The existence of the PCR product, identified by electrophoresis on agarose gel, demonstrated the presence of the gene in the

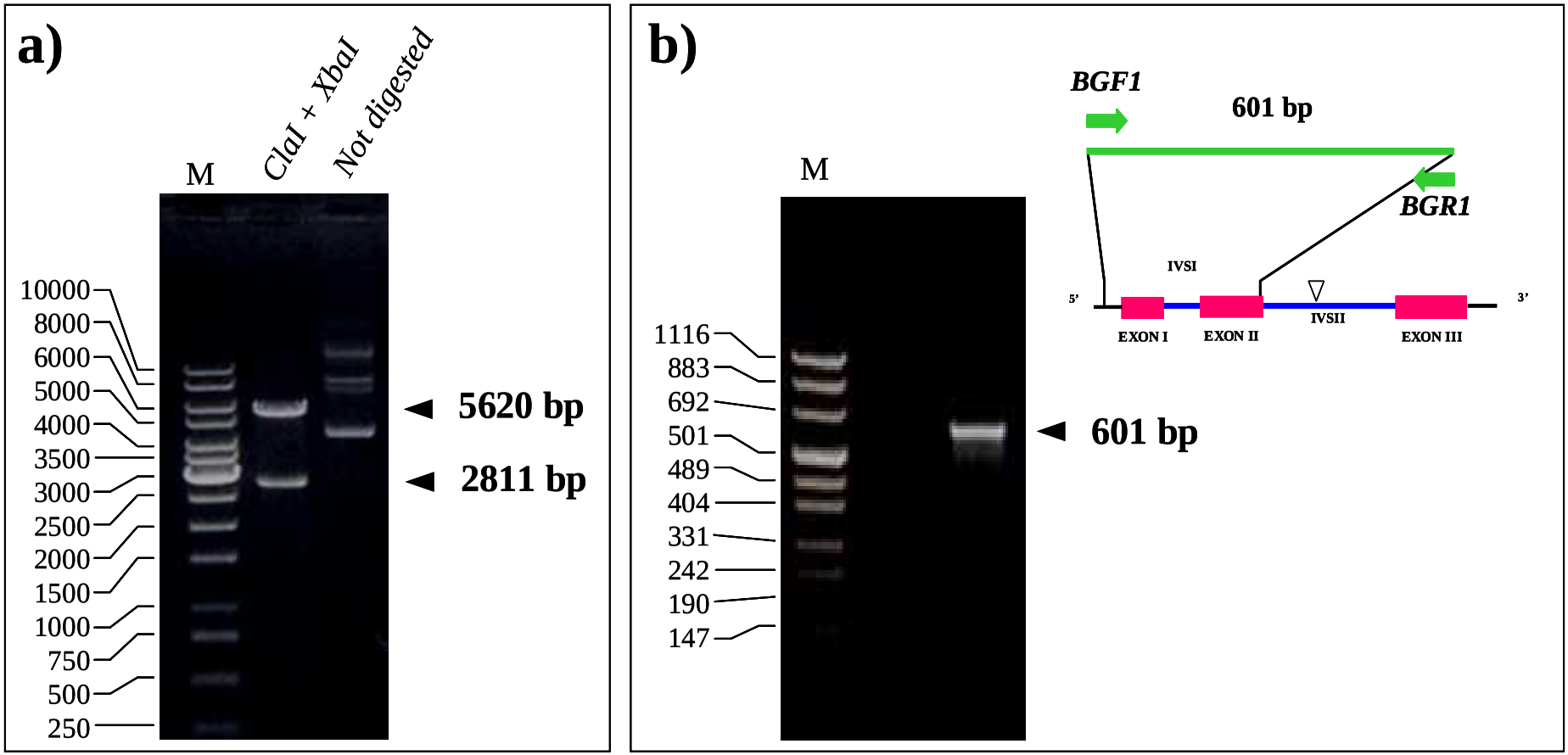


Figure 21 – Characterization of the *dTNS9-β⁰39* vector. a) electrophoretic analysis of the fragments obtained by digestion of *dTNS9-β⁰39* vector with *ClaI* and *XbaI* enzymes. M, molecular weight marker 1kb DNA Ladder (MBI Fermentas). **b**) electrophoretic analysis of the PCR product obtained by amplification of the exon 1 and 2 of the β-globin gene. M, molecular weight marker pUC Mix Marker 8 (MBI Fermentas).

vector (**figure 21b**). In that way we, therefore, obtained the vector *dTNS9-β^{0}39*, 8,432 bp long.

The third step of the cloning (**figure 20b**) provides for the replacement of β-globin gene with the mutation β$^+$IVSI-110, present in the third generation lentiviral vector *pCCL.β$^+$IVSI-110.PGW* supplied by Prof. Stefano Rivella (Weill Medical College of Cornell University, New York), with the β^{0}39-globin gene carried by *dTNS9-β^{0}39* vector.

So, we performed the enzymatic digestion of *pCCL.β$^+$IVSI-110.PGW* and *dTNS9-β^{0}39* with *ClaI* and *XbaI* restriction enzymes (**table 3**). The obtained fragments owned sticky ends and those of interest, the vector (10,996 bp) and the insert (2,811 bp), were purified from agarose gel using *QIAquick Gel Extraction Kit* (QIAGEN). The ligation reaction was finally carried out using 50 ng of vector, a molecular ratio vector:insert equal to 1:4 – 1:8 and incubating over night at 16°C. The product of ligation was then used to transform ultra-competent *TOP10* bacteria, optimal for large vectors, using again ampicillin for the clones selection. Also in this case, clones of interest were identified through analysis of the molecular weight of the internalized construct after Rapid Disruption.

The recombinant clones were then subjected to heat lysis and used as template for PCR reaction with *BGF1* and *BGR1* primers. The identification of the desired PCR product by electrophoresis confirmed the presence of the β-globin gene in the vector (data not shown). The obtained vector, called *pCCL.β^{0}39.PGW*, was finally purified by *QIAGEN Plasmid Purification Midi Kit* (QIAGEN).

The third generation lentiviral vector containing the βwt-globin gene was produced through only the third step of the cloning strategy. The β$^+$IVSI-110-globin gene present in the *pCCL.β$^+$IVSI-110.PGW* vector was replaced with the βwt-globin gene, collected from the *dTNS9 Mlu(-)Pme(-)SmaI(-)* vector, by digestion of both the constructs by *ClaI* and *XbaI* endonucleases and ligation of the desired fragments of 10,996 bp for the vector and 2,811 bp for the insert, purified from agarose gel using *QIAquick Gel Extraction Kit* (QIAGEN). Also in this case we used the ligation product to transform ultra-competent *TOP10* bacteria and the grown colonies were analysed by Rapid Disruption, so as to identify the internalized vector through analysis of the molecular weight. The

presence of the vector in the selected clones was confirmed though heat lysis and PCR reaction with *BGF1* and *BGR1* primers. The *pCCL.βwt.PGW* vector was finally purified by *QIAGEN Plasmid Purification Midi Kit* (QIAGEN).

At this point, the *pCCL.βwt.PGW* and *pCCL.β^{0}39.PGW* vectors were used as templates for PCR reactions, performed using two primers designed by us on the vector, upstream (*LV1F*) and downstream (*LV1R*) of the insert (**table 1**), and purified with *Microcon® YM-100* (Millipore Corporation). The PCR products were then used as templates for sequencing the insert present in the vectors. The fragments obtained in the sequence reaction were then purified using *Multiscreen*™ (Millipore Corporation), separated by electrophoresis on polyacrylamide gel and analysed using the sequencer *ABI PRISM*™ *377 DNA Sequencer* (Perkin-Elmer Applied Biosystems). **Table 1** lists all the primers used, which made it possible to sequence entirely the insert, thus, highlighting the lack of anomalies in the nucleotide sequence, except the expected β^{0}39 mutation in *pCCL.β^{0}39.PGW* vector. The portion of electropherogram shown in **figure 22** was obtained by sequencing the *LV1F-LV1R* PCR product with *BGR1* reverse primer (**table 1**), using the *pCCL.β^{0}39.PGW* vector as template.

The sequencing is a very important step in the cloning strategy for several reasons. It is essential: 1) to check and determine the presence of the β^{0}39 mutation in the *pCCL.β^{0}39.PGW* vector, as may exist the possibility that a mutation occurs spontaneously; 2) to verify that the ends of the insert present in both the vectors are intact; 3) to verify the absence of any other type of mutation in both the *pCCL.βwt.PGW* and *pCCL.β^{0}39.PGW* vectors.

With this strategy, we have thus obtained two third generation lentiviral vectors, *pCCL.βwt.PGW* and *pCCL.β^{0}39.PGW* (13,824 bp long) (**figure 20b**), which possess as well as the insert with the β-globin gene and its promoter: 1) functionally important sequences of hypersensitive sites of β-globin cluster LCR; 2) two modified *LTR* (deletion in the U3 sequence of the 3' LTR); 3) ψ packaging signal; 4) *RRE* sequence; 5) GFP reporter gene under the control of the promoter of phosphoglicerate kinase (PGK), useful for identifying transduced cells; 6) *WPRE* sequence, introduced to increase the levels of GFP expression; 7)

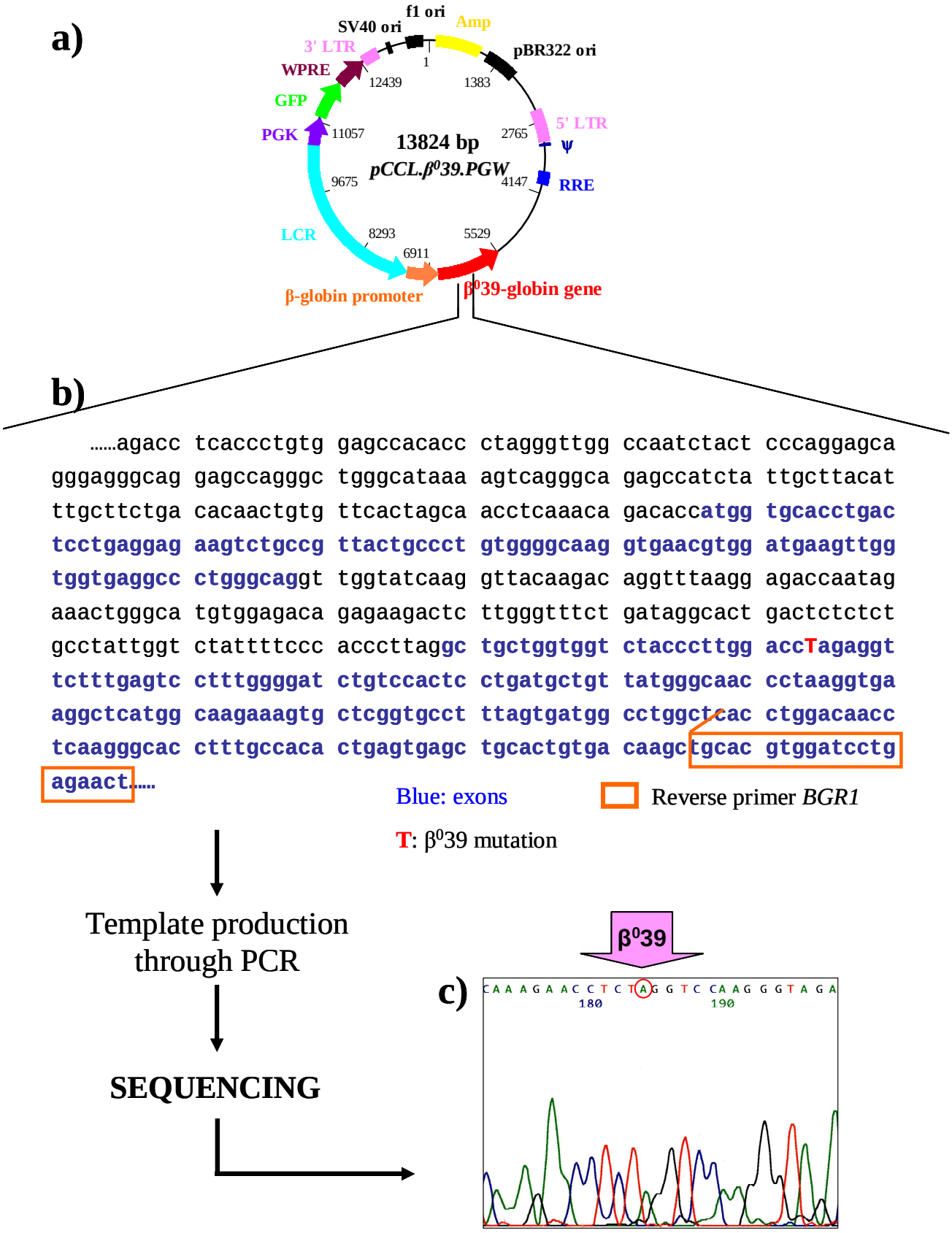


Figure 22 – Sequencing of the second exon of the β-globin gene contained into the *pCCL.β^{0}39.PGW* vector. a) Map of the *pCCL.β^{0}39.PGW* vector. **b)** Sequence of the region of β^{0}39-globin gene corresponding to 1st and 2nd exons. Box highlights the sequence of the reverse primer used for the sequencing. **c)** Electropherogram, obtained by the sequencing performed using the *BGR1* reverse primer, of the region containing the β^{0}39 (C→T) mutation, highlighted by the arrow.

insulators, useful to isolate the vector from the surrounding chromatin when integrated.

Production and titration of lentiviral particles containing the βwt- or $\beta^0$39-globin transgene

In **figure 23** is schematically presented the strategy for the production of viral particles beginning from our *pCCL.βwt.PGW* and *pCCL.$\beta^0$39.PGW* vectors. This strategy involves the use of three other constructs, called helper vectors, which, once inserted into packaging cells together with the lentiviral vector, allow it to carry out a replication cycle, resulting in production of viral particles and release of such particles outside the cell [Dull et al, 1998]. These helper vectors, kindly supplied by Prof. Stefano Rivella (Weill Medical College of Cornell University, New York), are essential because they provide structural and regulatory elements, absent in the pCCL.βwt.PGW and pCCL.$\beta^0$39.PGW vectors and without which they can not be expressed. The capside, which allows to enclose and protect the lentiviral genome, is formed by the G glycoprotein of vesicular stomatitis virus (VSV-G), expressed by *pMD2.VSVG* construct (5824 bp), and the protein encoded by the *gag* structural gene present in *pMDLpg.RRE* vector (8895 bp), which also contains the *pol* gene, encoding enzymes necessary for the viral cycle; all these genes are under the control of the strong CMV promoter. The *pMDLpg.RRE* construct carries also a small sequence of the *RRE* regulator, which binds the REV protein expressed by the *pRSV.REV* vector (4174 bp) under the action of the Lac promoter, with the function to mediate the transport of the lentiviral genome into the cytoplasm.

For the production of viral particles, we used as packaging cells the 293T cell line. Such cells are tumor cells of human embryonic kidney that grow in adhesion. Because of their reduced ability to adhere, their treatment requires caution.

The day before the transfection of 293T cells, we plated 4.5 x 10^6 cells in 10 cm-diameter plates, using 10 ml of D-MEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum), with the aim to have at the time of transfection, the cells well adhered to the support and in number of 9 x 10^6,

taking into account that their population doubling time is about 24 hours. Two hours before the transfection, we changed medium of the plated cells and replaced it with I-MDM (Iscove's Modified Dulbecco's Medium) supplemented with 10% FBS, necessary for the trasfection process. The transfection of 293T cells was, therefore, carried out preparing, initially, a mixture with the three helper vectors, the lentiviral vector, *pCCL.βwt.PGW* or *pCCL.$\beta^0$39.PGW*, and calcium chloride ($CaCl_2$). A 2X HBS (HEPES-buffered saline) solution was then added and the consequent reaction with $CaCl_2$ determined the production of calcium phosphate salts ($CaPO_4$). Immediately after the addition of HBS, the solution was added dropwise to the cells, then incubated at 37°C in humidified atmosphere of 5% CO_2/air for about 16 hours. At the end of the incubation, we changed the medium to eliminate residual $CaPO_4$ salts. Through perforation of the cellular membrane performed by the salts of calcium phosphate during the 16 hours of incubation, constructs penetrate inside the cell and the lentiviral vector, which owns the two *LTR* sequences, integrates into the cell genome. When, through the transcriptional machinery of the cell, the lentiviral genome is transcribed, thanks to the presence of structural and regulatory proteins expressed by the other viral constructs, it is incapsidated inside the protein structure of the virus, forming a viral particle. After 24 hours, the medium of transfected 293T cells, containing the viral particles produced, was collected, filtered and then placed at -80°C. Fresh medium was then added to the cells and finally collected and filtered after another 24 hours.

The viral particles, identified with the day of the medium collection, then day 1 and day 2, own a viral titre, namely a particular transducing effect. Each of these particles is a unit of infection, that is the smallest amount of virus capable of infecting a target cell [Zhang et al., 2004].

Figure 23 shows the strategy for the determination of the viral titre, the first phase of which consists in the infection of Hela cells, a tumor cell line of human cervix, with scalar concentrations of the lentiviral particles solution. Of particular importance for the determination of the titre is the fact that each freezing - thawing cycle makes the lentiviral particles half their transducing effect. Precisely for this

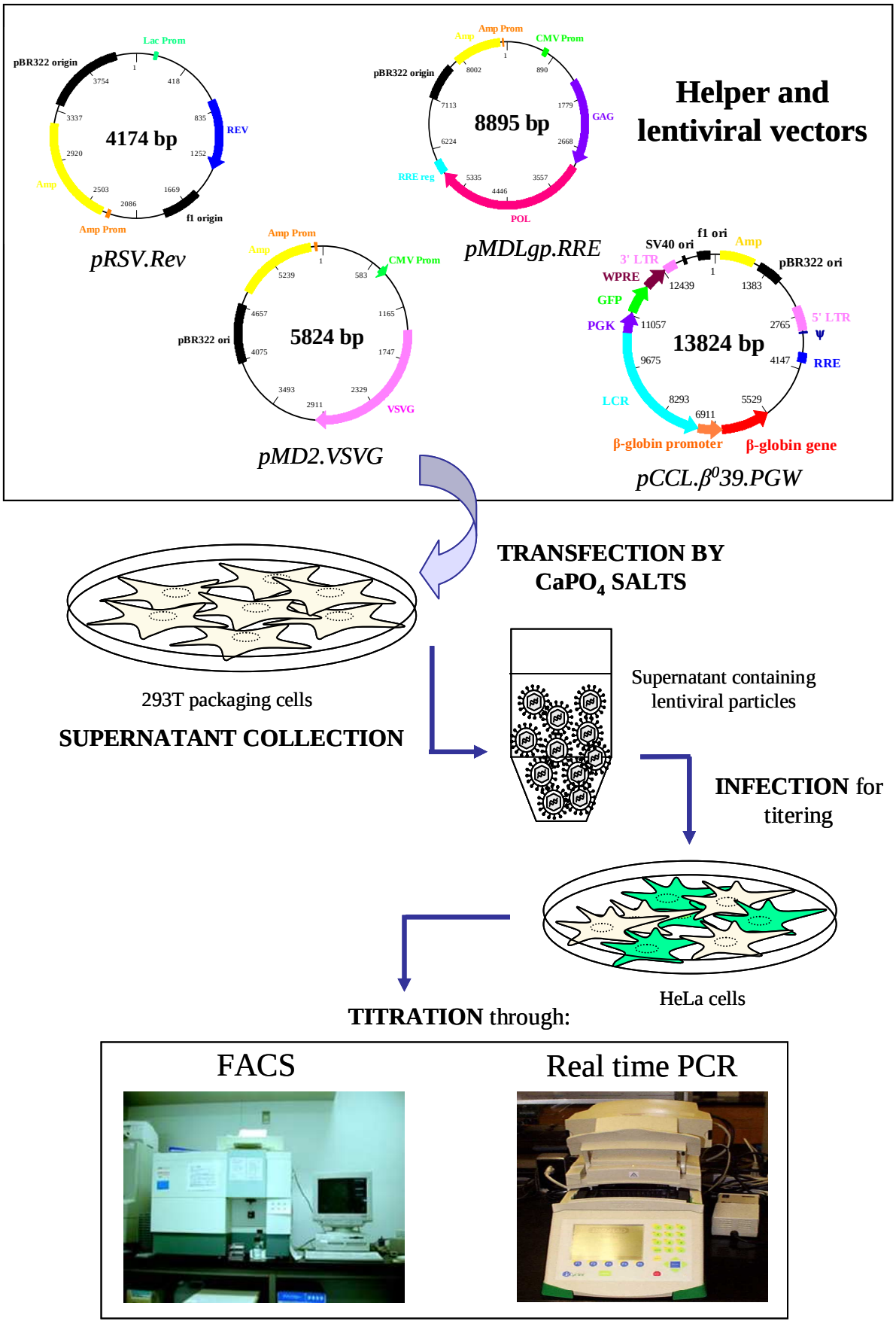


Figure 23 – Schematic representation of experimental strategy for the production and titration of lentiviral particles containing the β-globin transgene. The lentiviral (*pCCL.βwt.PGW* or *pCCl.β*^{0}*39.PGW*) and helper vectors were used to transfect, through calcium phosphate salts, 293T packaging cells, which, 24 hours after the transfection, began to release defective viral particles in the supernatant. Such particles were, therefore, collected and titered through infection of HeLa cells. Titration was performed through both flow cytometry (FACS), which detected the percentage of GFP-positive cells, and quantitative real time PCR, which allowed to calculate the exact number of vectors per cell.

reason, all the produced aliquots of viral particles were frozen at -80°C immediately after their filtration. One of these aliquots was then thawed to perform the titration, so that the calculated titre was the same as in the other aliquots still frozen. A comparison between different types of titration is reported in Sastry et al [2002].

To determine the titre of the day 1 viral solution, we plated Hela cells, 24 hours before infection, in three wells at a concentration of 5 x 10^4 cells/well in 1 ml of E-MEM (Minimum Essential Medium Eagle) supplemented with 10% FBS. Because also these cells have a population doubling time of about 24 hours, at the time of infection, they have certainly reached the optimal concentration of 1 x 10^5 cells/well. As for the transfection, two hours before the infection the medium was replaced with I-MDM. So, after 24 hours from the plating, we added to the Hela cells the lentiviral solution, doing a serial dilution in the three wells, so that the particles present in the second and third well were, respectively, diluted 1:10 and 1:100 compared to those in the first (i.e. we inoculated 500 μl, 50 μl and 5 μl of lentiviral solution). In order to significantly increase the efficiency of infection, we added to each sample a trasducing agent, the 100X polybrene (800 μg/ml, CHEMICON). The plate was then incubated at 37°C in humidified atmosphere of 5% CO_2/air for about 16 hours, after which, we changed the medium of the cells, to remove residual viral particles.

After about 72 hours from the infection, the cells were washed, detached and centrifuged. Half of each sample was then transferred to a tube for flow cytometer (FACS) analysis, leading up to 500 μl with 1X DPBS (Dulbecco's Phosphate Buffered Saline), while the other half was plated in a new well and incubated for another 72 hours, in order to obtain a sufficient number of cells from which we could extract the genomic DNA.

Use of the flow cytometer (FACS) for the calculation of the viral titre (*GFP titre*)

The transduced Hela cells, having integrated the viral genome of *pCCL.βwt.PGW* or *pCCL.$\beta^0$39.PGW*, express the GFP protein, which is detected

when excited with fluorescent light. This feature allows us to analyze the infected cells with the flow cytometer or FACS (flow-activated cell sorting) (FACScan, Becton-Dickinson) using the *Cell Quest Pro* software (Becton-Dickinson), and to determine the GFP titre obtained from the proportion of cell fluorescence detected by the cytometer.

Flow cytometer

In **figure 24** is schematically presented the principle underlying the flow cytometer. The monodisperse cell suspension, contained in the tube for FACS, is driven by a laminar flow in a fluidic system to the point of measurement, through a flow chamber, in which occur the gradual dilution and the cells alignment in a single row, maintained in this position for the presence of an isotonic saline solution that surrounds them, in order to avoid the formation of cell clusters.

Arrived at the point of measurement, the cells are hit by a light beam from a source of excitement, the argon ion laser, centred on a wavelength of 488 nm (blue). The cell emits signals related to its physical and morphological characteristics, based on phenomena of diffraction (*Forward Light Scatter*) and of reflection and refraction (*Sideward Light Scatter*). The combination of these two signals permits to create a diagram of dispersion or cytogram, where it is possible to visualize different cell populations based on their physical characteristics.

If a fluorochrome is present in the cells, as in our case, when excited by light beam it emits a signal corresponding to a determined wavelength. This signal is collected by lenses, selected by beam separator mirrors, optical filters and semitransparent mirrors (dichroic) and sent to the appropriate sensor (photomultiplier or photodiode), which measures the amplitude. The fluorescence signals are measured as pulses of amplitude proportional to the number of fluorochrome molecules present on or inside the cell.

The amplified signal from each sensor are digitalized, associated with each other and sent to an analyzer – elaborator that provides the data presentation and their statistical definition. Every single value of a parameter constitutes an event,

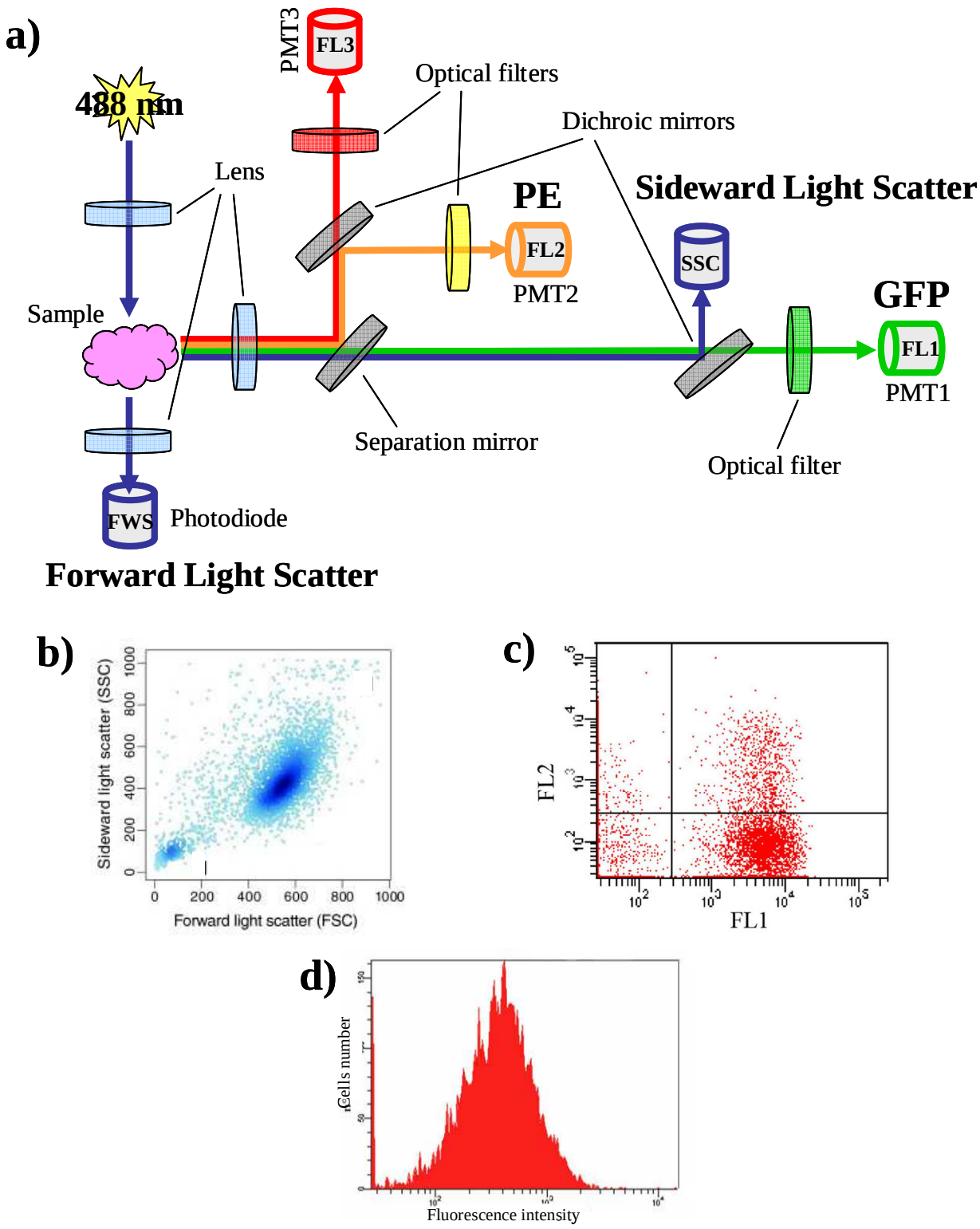


Figure 24 – Schematic representation of the operation and analysis of flow cytometer. a) the cellular suspention put inside the cytometer is hit by a light beam emitted from an argon ions laser at a wavelenght of 488 nm. The cell hit by the light beam emits a signal corresponding to a particular wavelenght, which is collected by lens, selected by beam separation mirrors and optical filters and sent to the relative sensors (PMT and photodiodes), which misure its proportionality: *FSC* (*Forward Light Scatter*) based on the phenomenon of diffraction and related to the size of the cell; *SSC* (*Sideward Light Scatter*) based on the phenomena of reflection and refraction and related to the morphology of the cell; *FL1, FL2, FL3* related to the green, red-orange and red fluorescences, respectively. The combination of the signals detected by *FSC* and *SSC* permits to create a diagram of dispersion or cytogram (**b**), where it is possible to recognize different cellular populations, based on their physical characteristics. **c**) Example of cytogram of cellular dipersion based on the fluorescence emitted by the cells labelled with fluorochromes, in this case for the green, *FL1*, and red-orange fluorescence, *FL2*. **d**) The analysis of the fluorescence is carried out by a histogram showing the detected fluorescence intensity on the x-axis, along with the number of beaming cells on the y-axis: each cellular population generates a peak with gaussian distribution, quantifying by the parameter of median.

the set of events, relative to the measured data, which accumulate in the channels, are represented through a distribution diagram or histogram [Brando, 1990]

Calculation of the viral titre (*GFP titre*)

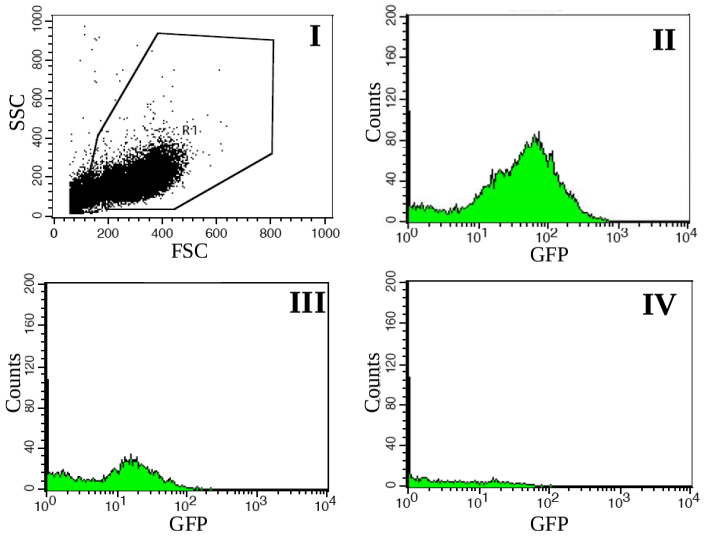
The first step of the FACS analysis of the transduced Hela cells was the configuration of channels and their parameters, through which data are collected: *FSC* (*Forward Light Scatter*) and *SSC* (*Sideward Light Scatter*), that collect data of the physical and morphological characteristics of cells, and *FL1* (*FL-1 Height*), which receives data on the green fluorescence emitted by GFP. In addition, we set the maximum number of cells, called events, which should be counted by the instrument (20,000 cells).

To complete the configuration, we analysed untransduced Hela cells, in order to act on the obtained cytogram and distribution diagram (histogram), so as to set additional parameters. In the *FSC* - *SSC* cytogram in **figure 25**, it is possible to view a cloud that corresponds to the cell distribution. Such cloud is not entirely uniform due to the presence of dead cells, identifiable because positioned near the convergence of the axes of the graph. We, therefore, performed an electronic selection of the living cells to be analysed, separating them from dead ones, an option called *gate*, establishing a "gate" that enables entry to the analysis system only of events that have values within the set limits [Brando, 1990]. In the histograms (Counts - GFP) of untransduced Hela cells (data not shown), the parameters are set so that the peak formed by cells was placed totally on the left of the value of fluorescence equal to 10^1, which is usually used as threshold.

Finished the parameters configuration, we analysed the HeLa cells infected with *pCCL.βwt.PGW* and *pCCL.β^{0}39.PGW* viral particles: from the obtained histograms (**figure 25a,c**) we derived the proportion of GFP - positive cells (**figure 25b,d**) of each sample. These percentages were found to be much lower than expectations, which predicted: about 100% for cells transduced with the undiluted virus, indicating a saturation of the system, about 30% for Hela infected with 1:10 dilution and about 2 – 3% for cells transduced with the virus diluted

pCCL.βwt.PGW

a)

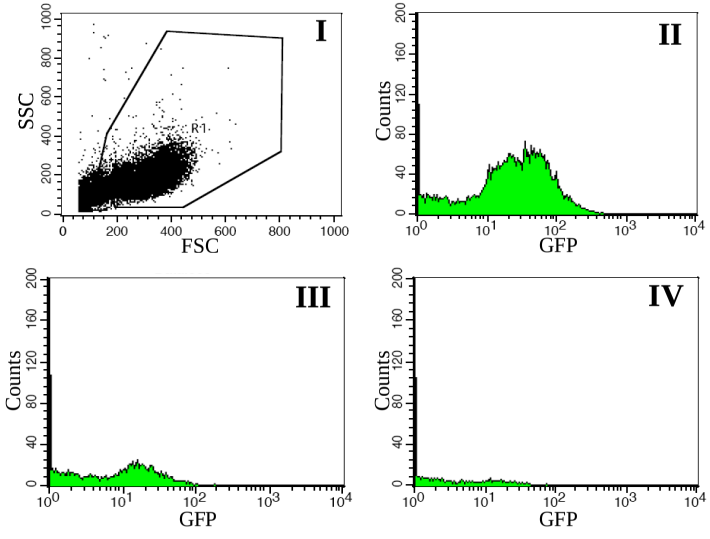


b)

SAMPLE	GFP-POSITIVE CELLS
HeLa + viral supernatant not diluted	74.32 %
HeLa + viral supernatant diluted 1:10	14.68%
HeLa + viral supernatant diluted 1:100	1,39%

pCCL.β^{0}39.PGW

c)



d)

SAMPLE	GFP-POSITIVE CELLS
HeLa + viral supernatant not diluted	59.86 %
HeLa + viral supernatant diluted 1:10	9.48%
HeLa + viral supernatant diluted 1:100	0.99%

Figure 25 – Titration of the produced lentiviruses by flow cytometry (FACS). In the figure (**a,c**) are reported the diagram of the distribution of untransduced HeLa cells based on their morphological characteristics (**I**) and the histograms of the cells transduced with *pCCL.βwt.PGW* (**a**) and *pCCL.β^{0}39.PGW* (**c**) viruses, obtained following the analysis of the fluorescence emitted by the cells infected with decreasing volumes of viral supernatant: not diluted (500 µl (**II**)), diluted 1:10 (50 µl (**III**)), diluted 1:100 (5 µl (**IV**)). The diagrams (**I**) present on the x-axis the *FSC* (*Forward Light Scatter*) parameter and on the y-axis the *SSC* (*Sideward Light Scatter*) parameter, while the histograms show on the x-axis the green fluorescence intensity (*FL-1 Height*), emitted by the GFP, and on the y-axis the number of cells beaming fluorescence (*counts*). *R1* (**I**) indicates the area of graph containing the cells chosen for the analysis. **b,d**) In tables are reported the proportions of fluorescent cells, obtained by the analysis of the histograms, taking into consideration that they are perceived as GFP-positive when emit fluorescence intensity of more than 10^1.

1:100. The percentage of fluorescent cells obtained with FACS is an essential factor to calculate the viral *GFP titre*, which is determined by the formula:

$$\text{GFP titre} = (F \times Co/V) \times D$$

where F is the percentage of fluorescent cells; Co, the total number of infected cells; V, the total volume in which it was performed the infection; D, the dilution factor of the virus. The unit of measurement of the viral titre is TU/ml, namely transducing units per ml [Sastry et al., 2002].

This formula expected to use, for calculating the viral titre, the data relative to the sample with the proportion of fluorescent cells that comes closest to 30%. This is because it is plausible to think that when only 3 out of 10 cells are GFP-positive, with a good chance they were infected by a single virus, namely a single transducing unit. The titre calculated using these assumptions is approaching the real titre of the viral particles. The choice between our samples was difficult because we got the percentages (**figure 25b,d**) significantly lower than expected. In conclusion, our preference fell on the samples 2 (*pCCL.βwt.PGW*: 14.68%; *pCCL.β^{0}39.PGW*: 9.48%), as the first and third were too differed from the ideal proportion of 30%. The *GFP titres* were calculated as 3×10^5 and 1.8×10^5 TU/ml for the *pCCL.βwt.PGW* and *pCCL.β^{0}39.PGW* viral particles respectively, relatively low viral titres that allows to explain the low percentage of the obtained GFP-positive cells.

A major limitation of *GFP titre* is not to consider two important situations: 1) not always an infected cell expresses the GFP protein at levels that exceed the FACS threshold of detection, and 2) even assuming that with a percentage of fluorescent cells equal to 30%, in most cases, a single infection happened, this does not exclude the possibility of multiple infections.

Use of the quantitative real time PCR assay for the calculation of the viral titre (*DNA titre*)

After further 72 hours of incubation, the Hela cells infected with scalar quantities of *pCCL.βwt.PGW* and *pCCL.β^{0}39.PGW* viral particles were lysated and their DNA extracted by phenol - chloroform extraction and 70% ethanol precipitation. This DNA was then quantified with a spectrophotometer and used as a template for the reaction of quantitative real time PCR, so as to determine the number of vectors integrated into the genome of the different cell samples. Essential data for being able to calculate the viral *DNA titre*.

Calculation of the viral titre (*DNA titre*)

Since for calculating the viral *DNA titre* it is needed to quantify the number of *pCCL.βwt.PGW* or *pCCL.β^{0}39.PGW* vectors integrated into Hela cells, used for the titration, it was necessary to design a pair of primers and an oligonucleotidic probe both for the β-globin gene, present in the vectors, and for a gene to be used as reference. The choice of reference gene is arbitrary, with the only condition that it must be present in two copies in the genome, one for each allele, as it must be used as a reference to determine the increase in the number of β-globin genes in transduced cells. Making a proper normalization of the experiment is essential as cell populations may be in different replication stages, and because proliferating cells contain a double set of genetic information in comparison with non-proliferating cells, comparing two populations without first having standardized data on a known gene leads to wrong conclusions [Huggett et al., 2005]. In our case, having decided to use the γ-globin gene for standardization, we had to design a probe that can discriminate ^{A}γ gene from ^{G}γ gene. **Table 2** shows the sequences of primers and TAMRA probes used in experiments. Their design was carried out using the *Primer Express^{TM} version 2.0* software.

The reactions of real time PCR were prepared using the *TaqMan® Universal PCR Master Mix 1X* (Applied Biosystems), containing the DNA polymerase, dNTPs and saline buffer, the *PFβ* and *PRβ* primers and *Pβ* probe for the

amplification reactions of β-globin gene and *PFγ* and *PRγ* primers and *Pγ* probe for the amplification reactions of γ-globin gene. The step of annealing - elongation was done at 60°C. To normalize the data obtained, we decided to amplify, in the same conditions of samples, scalar quantities of a control genomic DNA, extracted from untransduced Hela, used as standard.

The graphs obtained show, in the x-axis the amplification cycles and in the y-axis the values of fluorescence (ΔRn) (data not shown). After establishing a threshold value of fluorescence (0.034) we were unable to get the threshold cycle (Ct) of each sample obtained both with the *Pβ* probe and with the *Pγ* probe. The Cts obtained with the DNA from HeLa cells infected with *pCCL.βwt.PGW* and *pCCL.β^{0}39.PGW* are shown in **figure 26a** and **figure 26c**, respectively.

We used the Cts obtained with the DNA from untransduced Hela (standard) to set up two different calibration curves, for the β-globin and γ-globin genes, subsequently used for the normalization of the samples (data not shown). The results were then analysed and the final data are reported in histograms of **figure 26** (**b and d**), where it is possible to estimate the fold of β-globin compared to γ-globin genes. As expected, samples 1 (Hela infected with the undiluted lentiviral solution) present the largest increases of β-globin respect to γ-globin genes, while samples 2 (Hela infected with the lentiviral solution diluted 1:10) and samples 3 (Hela infected with the lentiviral solution diluted 1:100) show smaller increases. These data reflect the number of vectors in each cell, taking into account that the fold of β-globin compared to γ-globin genes in untransduced Hela cells is equal to 1.

Using these data, we calculated the viral *DNA titre*, according to the formula:

$$\text{DNA titre} = (F \times Co/V) \times D$$

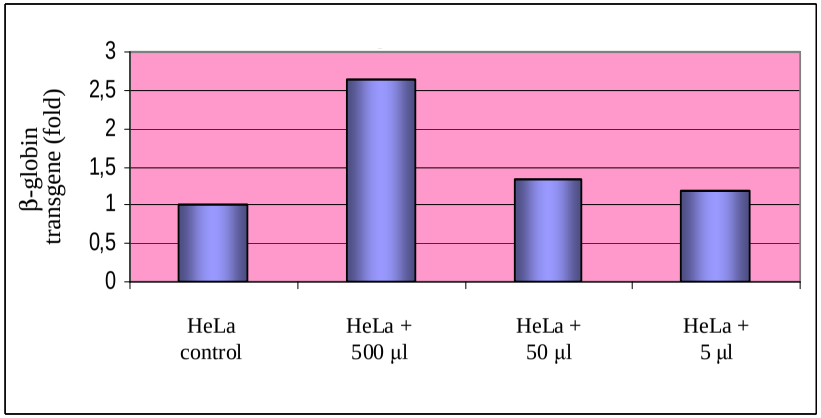
where F is the subtraction of the fold of β-globin gene of untransduced HeLa cells from the same fold of the chosen sample; Co, the total number of infected cells; V, the total volume of infection; D, the dilution factor of the virus. Even in this case the unit is TU/ml [Sastry et al., 2002].

pCCL.βwt.PGW

a)

SAMPLE	Ct beta	Ct gamma
Standard 50 ng	23.262	23.248
Standard 100 ng	22.024	22.201
Standard 150 ng	21.470	21.612
Standard 200 ng	21.042	21.256
Standard 300 ng	20.287	20.778
HeLa + virus not diluted	20.884	22.464
HeLa + virus diluted 1:10	21.632	22.219
HeLa + virus diluted 1:100	21.362	21.793

b)



pCCL.β⁰39.PGW

c)

SAMPLE	Ct beta	Ct gamma
Standard 50 ng	22.628	23.047
Standard 100 ng	21.697	21.996
Standard 150 ng	21.193	21.252
Standard 200 ng	20.722	20.720
Standard 300 ng	19.967	20.263
HeLa + virus not diluted	20.324	21.314
HeLa + virus diluted 1:10	21.217	21.565
HeLa + virus diluted 1:100	21.175	21.457

d)

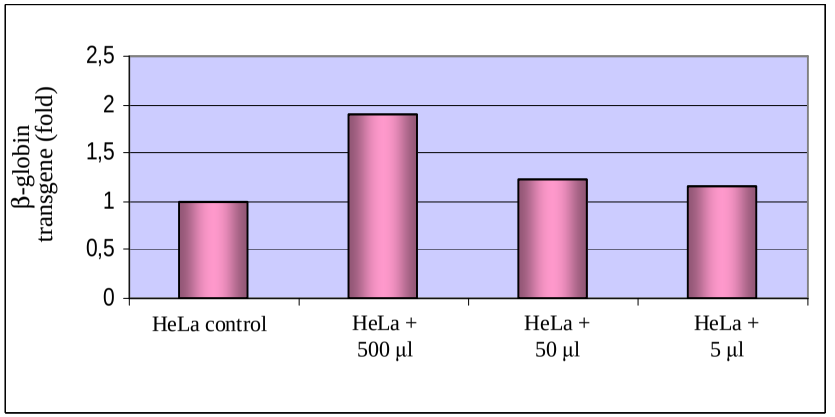


Figure 26 – Titration of the produced *pCCL.βwt.PGW* and *pCCL.β⁰39.PGW* lentiviruses by quantitative real time PCR. a,c) In tables are reported threshold cycles (Ct) obtained by amplification of genomic DNAs isolated from HeLa cells untransduced (standard) and transduced with scalar quantities of *pCCL.βwt.PGW* (**a**) or *pCCL.β⁰39.PGW* (**b**) lentiviral supernatant, using the probes *Pβ* and *Pγ*. The fold β (**b,d**) permits to quantify the number of *pCCL.βwt.PGW* (**b**) and *pCCL.β⁰39.PGW* (**d**) vectors integrated into the cellular genome, and is expressed as 2-ΔΔCt, where ΔΔCt represents the substraction of the ΔCt obtained by amplification of genomic DNA from untransduced HeLa cells from the ΔCt derived from HeLa infected with lentiviruses.

Of course, even for the calculation of *DNA titre* it is necessary to choose only one of the samples. Our choice fell on samples 2, obtaining viral titres of 6 x 10^5 and 4.5 x 10^5 TU/ml for the *pCCL.βwt.PGW* and *pCCL.$\beta^0$39.PGW* viral particles respectively, so as to make a more accurate comparison with the *GFP titres* previously calculated, because the titre of a lentiviral vector varies according to the conditions of the infective process: the volume of inoculum, the number of target cells, cell type and viability/susceptibility, vector exposure time for uptake and vector half life. Furthermore, the VSV-G envelope binds to its target in cell membranes which are known to be phospholipids, such as phosphatidylcholine (PC) and phosphatidylserine (PS), (the receptors for VSV-G). PC is the most abundant membrane phospholipid while PS domains are present in much smaller quantity but bind more strongly and fuse faster with the VSV-G protein. This issue is probably one of the most overlooked variables in vector transduction. Membrane phospholipid movement is highly dynamic. Its biosynthesis and degradation are very much dependent on cell type and positions in the cell cycle and/or metabolic activity. Also, the rate of degradation is rapid in G1, slows drastically during S phase, and picks up the pace again as cells exit mitosis and re-enters G1, which suggests that the cell cycle phase may be an important variable for VSV-G protein coated lentiviral transduction. This can affect the success of infection and, therefore, the viral titre. The variation of these parameters can result in a difference in the viral titre in different aliquots of the same viral stock, hence the viral titre is not a value that exactly indicates how many infectious units per ml were added to the cells [Zhang et al., 2004].

Comparison between *GFP titre* and *DNA titre*

Comparing the calculated viral titres,

	GFP titre	***DNA titre***
pCCL.βwt.PGW	3 x 10^5 TU/ml	6 x 10^5 TU/ml
pCCL.$\beta^0$39.PGW	1.8 x 10^5 TU/ml	4 x 10^5 TU/ml

it is possible to note how the *DNA titres* have a value far greater than the *GFP titre*. This may depend on the limits of the FACS analysis previously exposed, which may cause an underestimation of the viral titre. This conclusion was also confirmed by the work of Sastry et al [2002], who noted in their experiments that the viral titre obtained by real time PCR was up to ten times higher than that calculated by FACS and also more accurate and reliable, because not underestimated. For this reason, we decided, for future infections, to consider only the *DNA titre*.

Transduction of K562 cells

As far as studies on erythropoiesis, the K562 cell line [Lozzio and Lozzio, 1975] is well known as a useful experimental model system to study the expression of embryo-fetal globin genes, as well as their modulation [Rutherford et al, 1979; Rutherford et al, 1981; Osti et al, 1997; Bianchi et al, 2000; Bianchi et al, 2001]. Interestingly, especially for reaching the aims of our study, K562 cells express the β-globin gene at very low levels both in their uninduced state as well as after erythroid differentiation stimulated by a variety of chemical inducers, such as cytosine arabinoside [Cortesi et al, 1999], mithramycin [Bianchi et al, 1999; Fibach et al, 2003], rapamycin [Mischiati et al, 2004; Fibach et al, 2006].

In deciding the volume of viral solution to be used for transducing the human erythroid K562 cell line, we based our decision on MOI (multiplicity of infection), a parameter used to predict the viral infectivity in a population of target cells and therefore assume how many events of gene transfer can take place. This parameter is based on the viral titre, precisely the *DNA titre*, and the concentration of cells to be infected, according to the ratio [Zhang et al., 2004]:

$$MOI = (TU/ml)/(cells/ml)$$

Usually, the MOI is decided arbitrarily by the researcher, according to the experiment to be performed. In our case, we chose to infect cells with different MOIs, from 0.5 to 2, because the objective was to obtain cell lines incorporating a number of vectors relatively low but variable.

So, we carried out various infections of K562 cells using the *pCCL.βwt.PGW* and *pCCL.$\beta^0$39.PGW* viral particles, which have a *DNA titre* of 6 x 10^5 and 4 x 10^5 TU/ml, respectively. For each of these infections were plated 10^5 cells in 50% of RPMI (Roswell Park Memorial Institute) and 50% of I-MDM media added with 10% FBS. After two hours, we added the volume of viral solution calculated depending on the chosen MOI and the 100X polybrene (800 μg/ml, CHEMICON), necessary to facilitate the transduction. After about 16 hours, we changed the medium of the cells, with the purpose to remove all residual viral particles and because the K562 cells grow better in RPMI medium.

For the following 7 days, the transduced K562 cells were incubated at 37°C in humidified atmosphere of 5% CO_2/air in order to recover from trauma caused by viral infection and therefore be subjected to cell cloning. This was done by limiting dilutions of the cell culture to obtain the final concentration of 4 cells/ml. This suspension was then divided into 96 wells, 100 μl/well, so as to have 4 cells per 10 wells. In fact, to be relatively safe to isolate almost entirely single clones it would recommend to aliquot a cell suspension with a concentration of 2 cells/ml, but, as with low MOIs the proportion of infected cells may be very limited, with a concentration of 2 cells/ml we could not isolate almost any positive clone.

Once the cloned cells were expanded, they were analysed by fluorescence microscope to identify, through the green fluorescence emitted by GFP, the infected clonal lines. In all, we isolated seven βwt-globin K562 cell clones and six $\beta^0$39-globin K562 cell clones, whose images are shown in **figures 27 and 28**, respectively. It's easy to note that the emitted fluorescence intensity is different depending on the clone, certainly due to different numbers of integrated vectors.

βwt-globin K562 cell clones

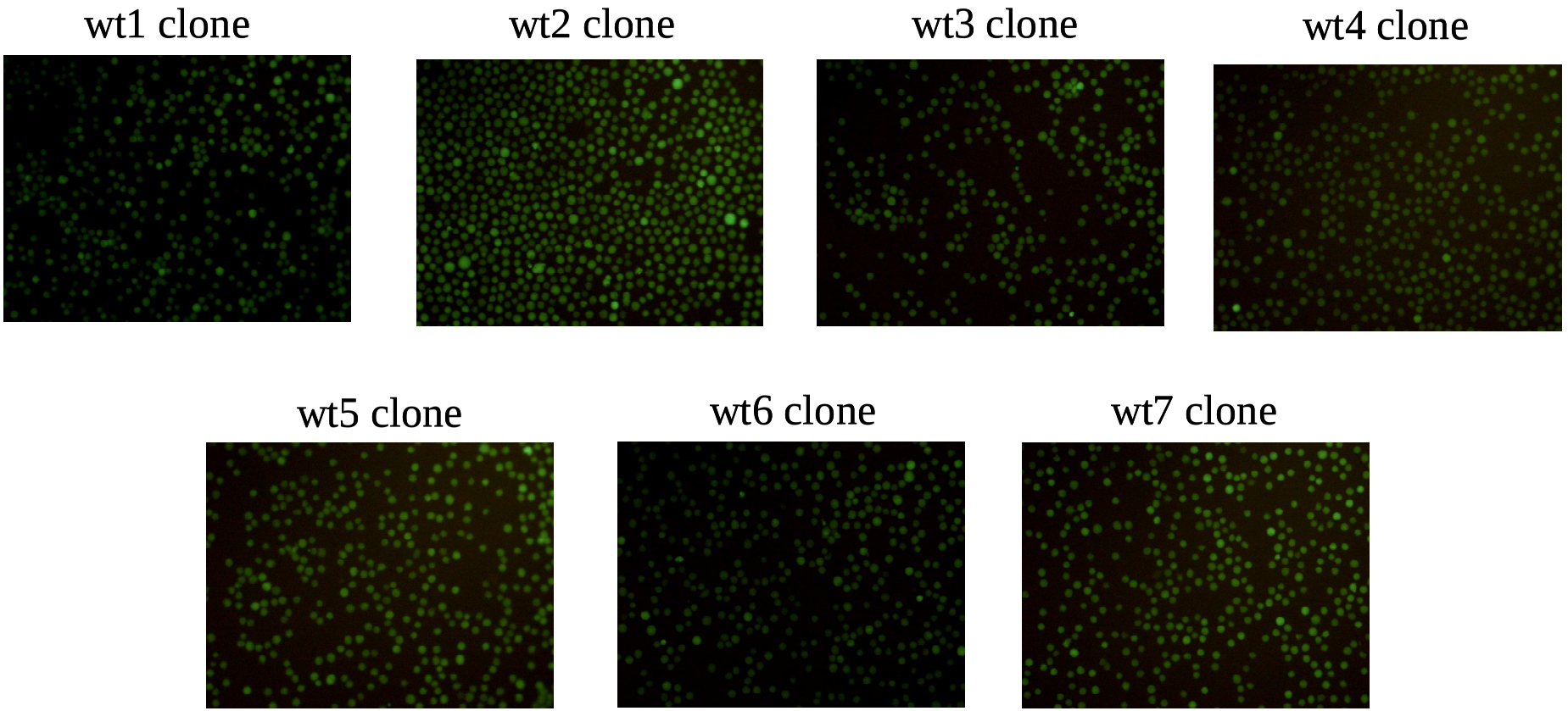


Figure 27 – βwt-globin K562 cell clones containing βwt-globin transgenes. These clones were obtained through transduction of K562 with the *pCCL.βwt.PGW* lentiviruses and subsequent cellular cloning for limiting dilutions. The infections were carried out at various multiplicities of infection (MOIs: 0.5 – 2), in order to obtain cells with various numbers of integrated vectors. Positive clones were selected at the fluorescence microscope, depending on the intensity of the fluorescence emitted.

β⁰39-globin K562 cell clones

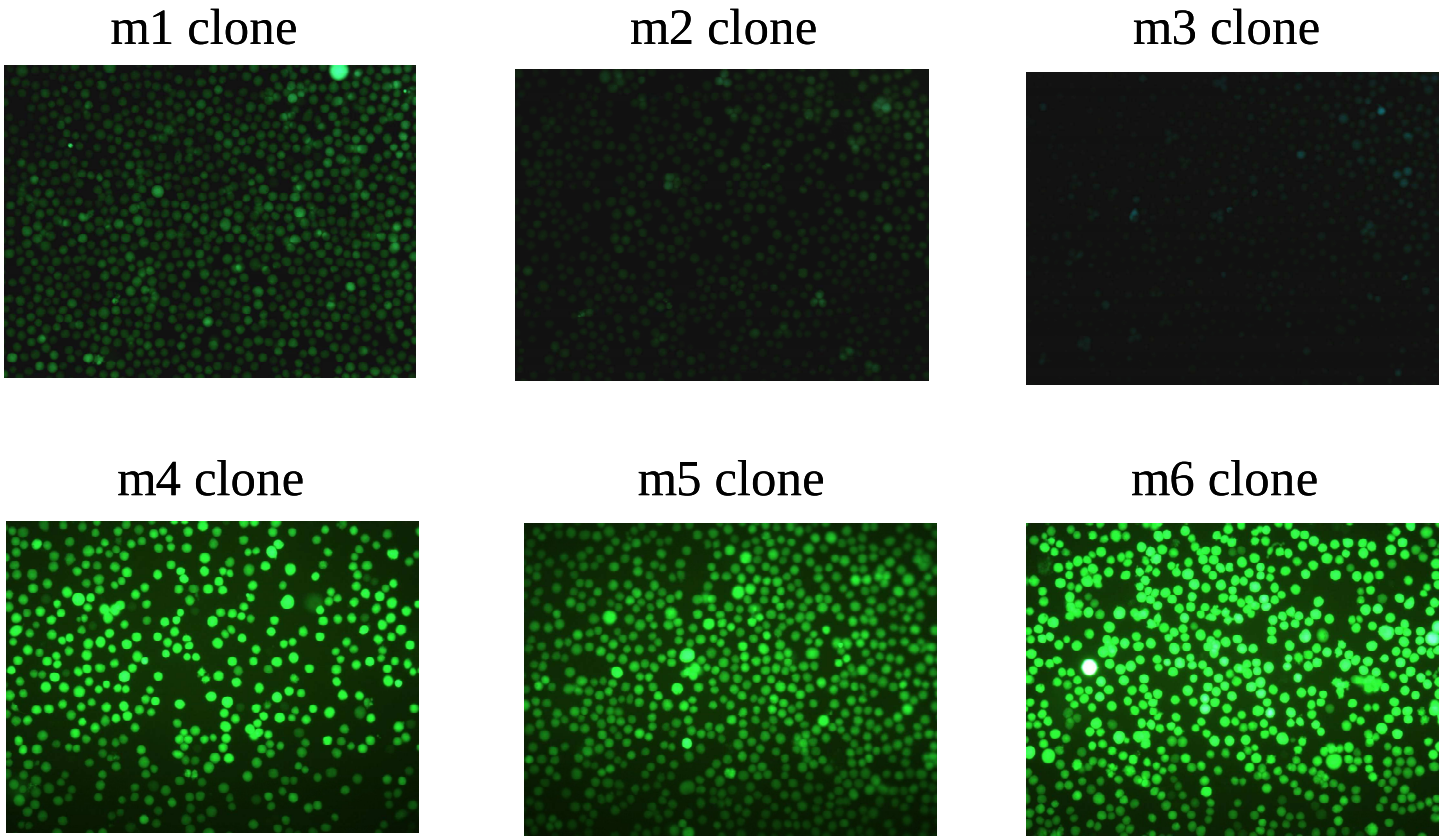


Figure 28 – β⁰39-globin K562 cell clones containing β⁰39-globin transgenes. These clones were obtained through transduction of K562 with the *pCCL.β⁰39.PGW* lentiviruses and subsequent cellular cloning for limiting dilutions. The infections were carried out at various multiplicities of infection (MOIs: 0.5 – 2), in order to obtain cells with various numbers of integrated vectors. Positive clones were selected at the fluorescence microscope, depending on the intensity of the fluorescence emitted.

Characterization of K562 cell clones

Once obtained the βwt-globin and $\beta^0$39-globin K562 cell clones it was necessary to characterize them in terms of the genome and transcriptosome, in order to determine not only the number of integrated vectors, but also the presence of the expected β-globin transcript, βwt and $\beta^0$39 respectively, essential for an *ex vivo* model of β-thalassemia, to be used to test new therapeutic strategies aimed at correcting nonsense mutations.

Analysis of integration and GFP fluorescence of the βwt-globin and $\beta^0$39-globin K562 cell clone

The first step was to extract the genomic DNA of βwt-globin and $\beta^0$39-globin K562 cell clones by cell lysis, phenol – chloroform extraction and 70% ethanol precipitation. Once quantified using the spectrophotometer, the DNA was used as a template in quantitative real time PCR reactions in order to determine the fold of β-globin transgenes integrated into the cellular genome. This type of analysis has two main purposes: 1) relating the fold of β-globin transgenes present in a single cell to the MOI used for the infection; 2) choosing the clone suitable for the type of experiment to be carried out, as the greater or lesser expression of β-globin gene changes the experimental condition.

The performed real time PCR assay faithfully reflects the reactions made for the determination of *DNA titre*, because it is always based on the recognition of β-globin genes in genomic DNA. In this case, the choice of γ-globin gene as reference gene is of great importance because of the karyotype of the K562 cell line. In fact, such cells do not present a normal diploid karyotype, as they derived from a pleural effusion of a patient with chronic myelogenous leukemia (CML), which is a clonal malignant disorder of the pluripotent hematopoietic stem cell, presenting complex translocation, with three, four or five chromosomes involved. So, in K562 cells most chromosomes are in three or more copies, making very difficult to identify a correct and suitable reference gene [Gribble et al, 2000; Naumann et al, 2001]. γ-globin gene is a good choice because of its location on

the chromosome 11 close to the β-globin gene. In that way, we were absolutely sure that the ratio between β- and γ-globin genes was always equal to 1 in untransduced K562 cells. Unfortunately, it is probable that there exist different sublines of K562 with slightly different karyotype. Precisely because of this, not knowing the exact number of chromosomes 11 in our original K562 cell line, we were not able to determine the exact number of integrated vectors, but we limited ourselves to determine the fold of β-globin transgenes integrated into the genome of each clone.

Even in this case, we obtained, for each sample, as result of the real time PCR assay, a graph with the amplification curves of both the β- and γ-globin genes. These curves and the Cts, determined following the choice of the threshold (0.034), of the βwt-globin and $\beta^0$39-globin K562 cell clones are shown in **figure 29 and 30**, respectively. From these profiles is immediately evident that, in all the samples, the β-globin gene is present in a number of copies greater than the γ-globin gene, because its amplification curve always takes fewer cycles to reach the threshold. It was, however, by analysis of data and determination of the fold of β-globin compared to γ-globin gene (**figure 31**) that we could say that these clones actually contained at least one copy of the vector.

Once determined the fold of the β-globin transgenes integrated in each clones, we decided to verify if there was a correlation between this parameter and the emitted GFP fluorescence intensity. With this purpose, we analysed each clone by flow cytometer (FACS), following the same configuration procedure used for the titration of the lentiviral particles, but using original K562 cells instead of untransduced HeLa cells to set the *FSC*, *SSC* and *FL1* parameters. From the histogram (counts - GFP), we obtained the median of the GFP fluorescence emitted by each clone, shown in **figure 31**. We decided to consider the median, instead of the arithmetic mean, because it is the value that corresponds to the middle item in a ranked list of all measurements. It's robust because it does not necessarily move in response to small numbers of outliers, or to skewing of the tails of a distribution, whereas the mean is tugged by both.

From the histogram in **figure 31** it is possible to note that the two parameters, fold of β-globin transgenes and median of GFP fluorescence, have similar trends,

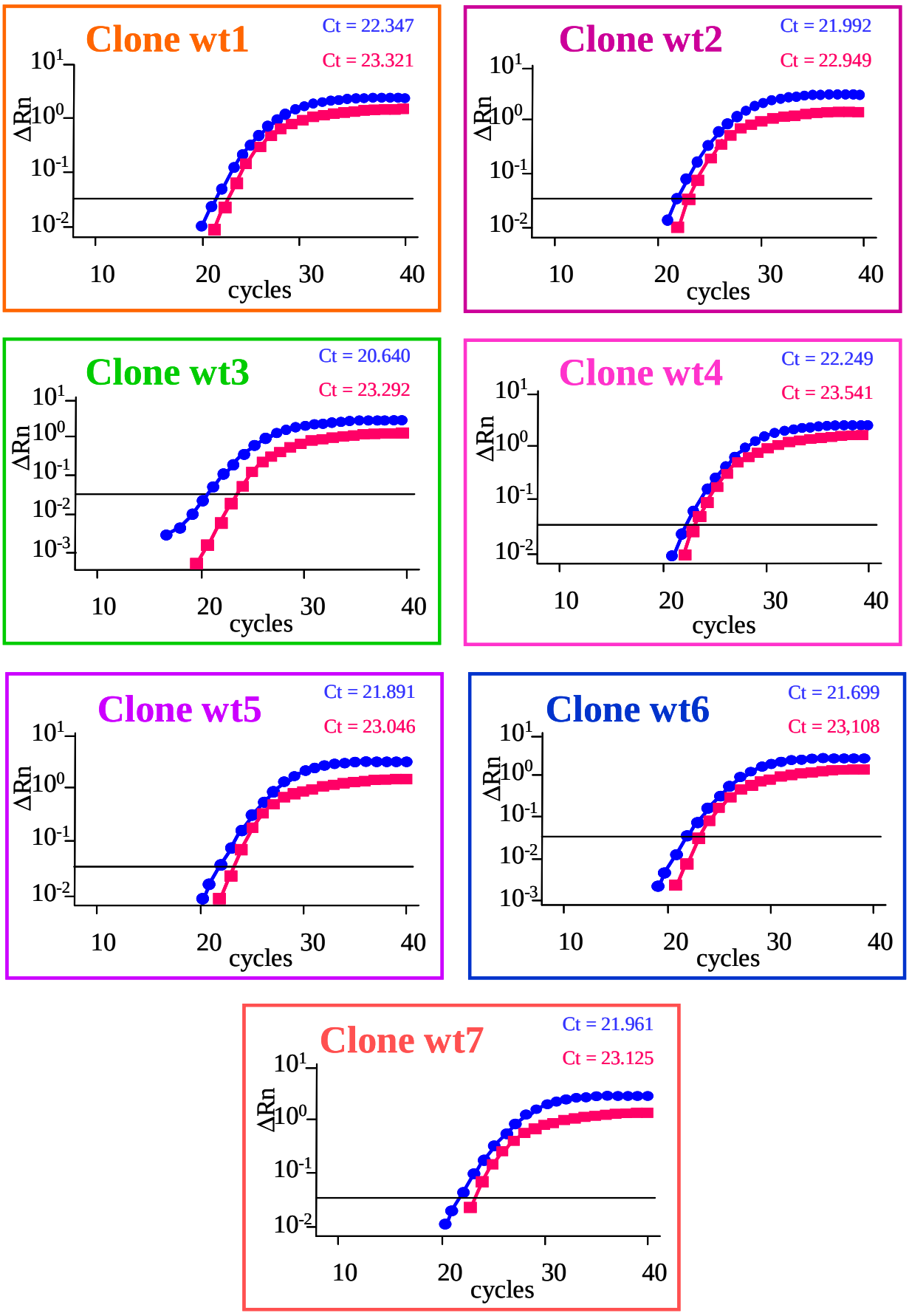


Figure 29 – Real time PCR analysis of β-globin gene using genomic DNA from βwt-globin K562 cell clones. The representative profiles show the amplification of β- (blue circles) and γ-globin (pink squares) genes. For each clones both the threshold cycles (Ct), obtained with the *Pβ* and *Pγ* probes, are reported.

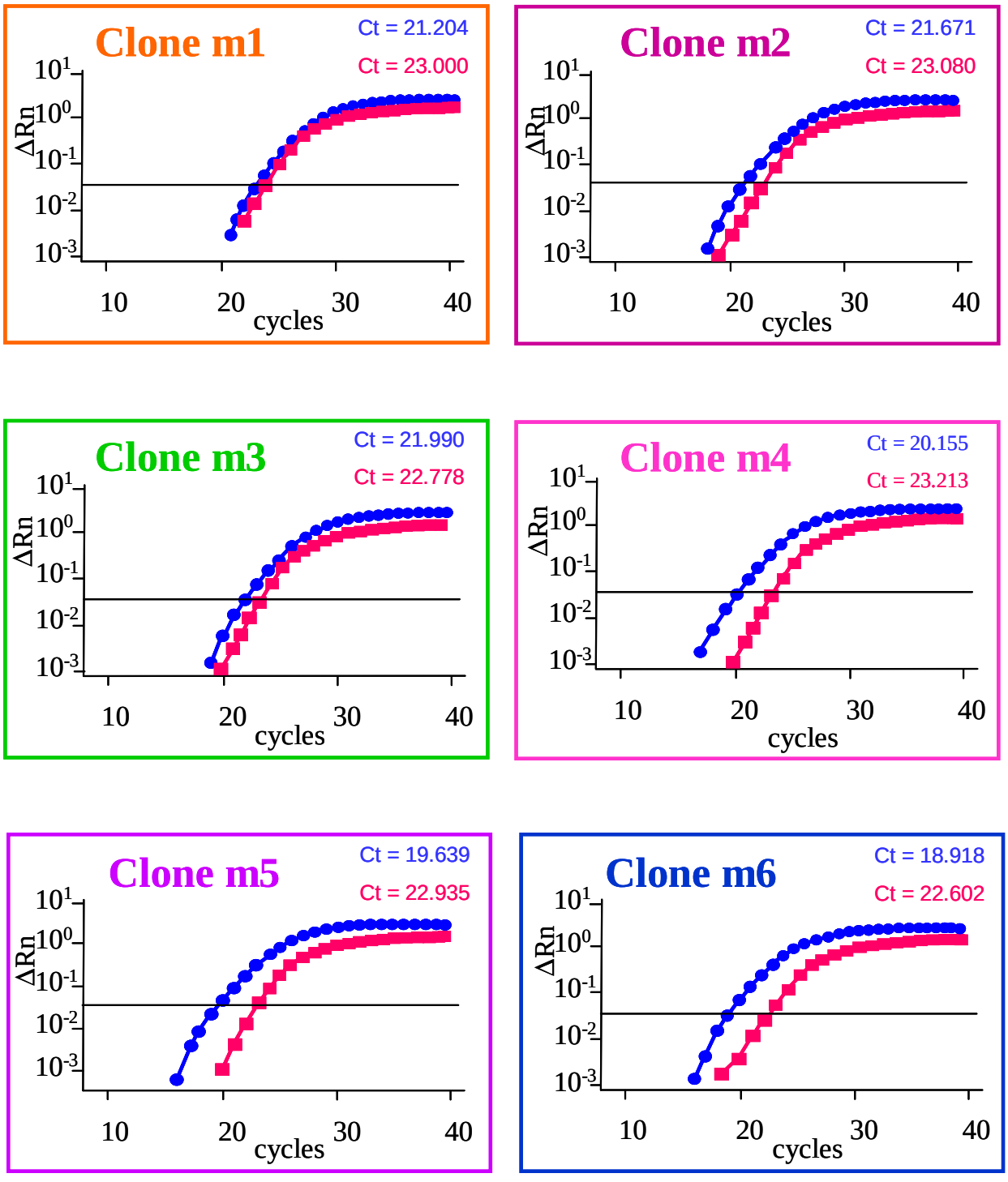


Figure 30 – Real time PCR analysis of β-globin gene using genomic DNA from β^{0}39-globin K562 cell clones. The representative profiles show the amplification of β- (blue circles) and γ-globin (pink squares) genes. For each clones both the threshold cycles (Ct), obtained with the *Pβ* and *Pγ* probes, are reported.

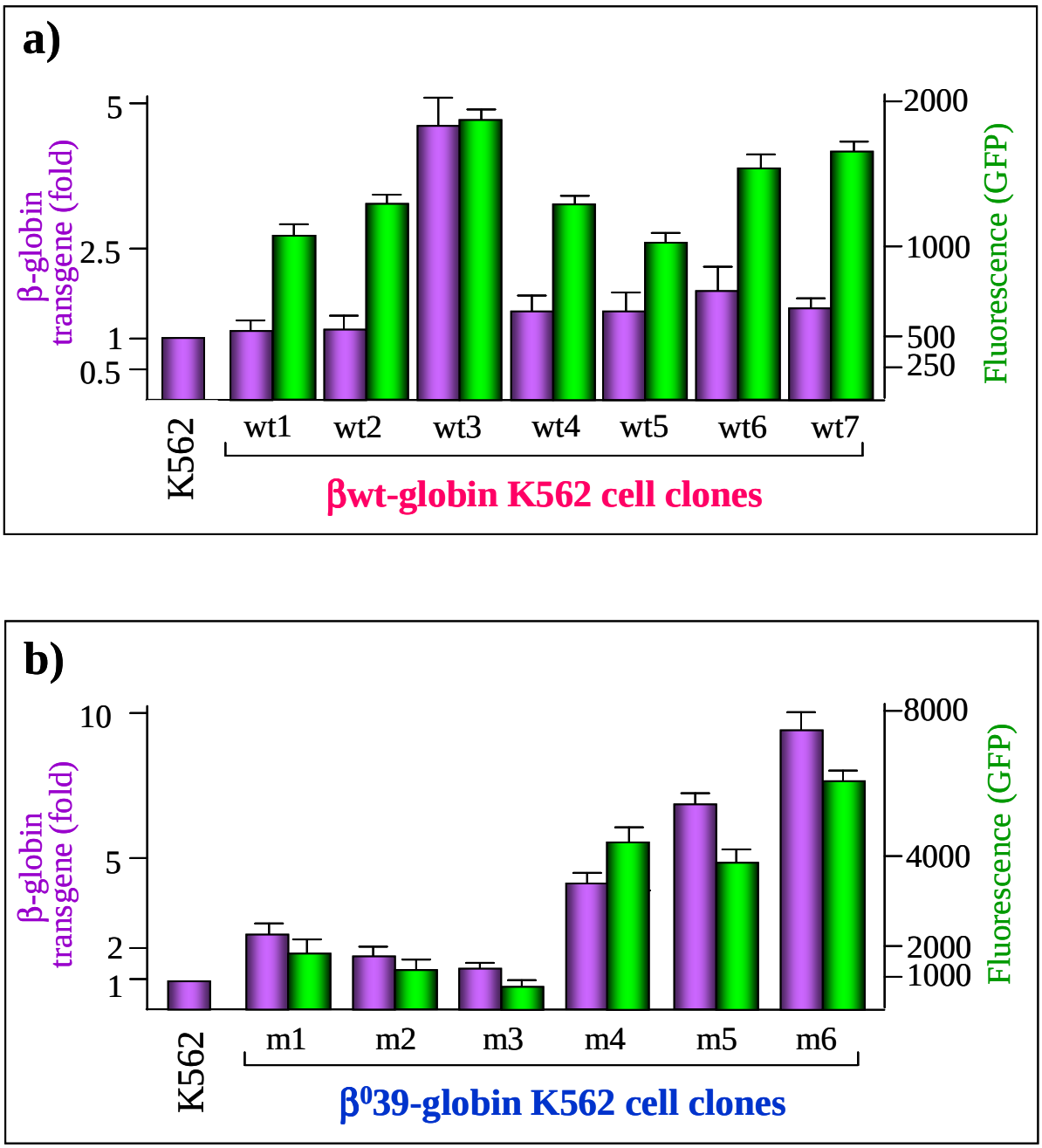


Figure 31 – Analysis of integration and GFP fluorescence of the βwt-globin and β^{0}39-globin K562 cell clones. In the histograms are reported both the fold of β-globin transgenes (violet boxes) integrated into the genomic DNA of the βwt-globin (**a**) and β^{0}39-globin (**b**) K562 clones respect to the original K562 cells and the median of GFP fluorescence (green boxes) emitted by each clone. The data represent the averages ± SD of three indipendent determinations. In order to determine the integration efficiency of the βwt-globin and β^{0}39-globin genes by real time PCR, the obtained Ct values were compared to those obtained using primers amplifying γ-globin gene sequences.

indeed clones with greater fold of β-globin genes present a higher median of fluorescence, unless a few cases. This result is expected because the GFP gene in the vector is under the control of the constitutive PGK promoter, which is less affected by the state of the chromatin surrounding the site of insertion.

Detection of the βwt- and $\beta^0$39-globin transcripts by non-quantitative real time RT-PCR assay

From each K562 cell clone, RNA was extracted by *Trizol*, precipitated with 70% ethanol and quantified using a spectrophotometer. The RNA was then reverse transcribed, after treatment with DNase to remove any residual genomic DNA. The reverse transcription reaction was performed using 1μg of templates and *oligodT* primers, specific for the poly-(A) tail of the RNA messengers. After an incubation for 5 minutes, we added a mixture containing the enzymes: *Recombinant RNasin® Ribonuclease inhibitor* and *Improm-II™ reverse transcriptase*. After the necessary thermal steps of annealing and elongation, we obtained the cDNA of all K562 cell clones, to be used in non-quantitative real time PCR, using probes able to discriminate between βwt and $\beta^0$39 transcripts.

Since designing probes able to discriminate two sequences differing by only one nucleotide is particularly complicated, we decided to use for our purpose a Genotyping kit designed by Applied Biosystems for the mutation $\beta^0$39. It consists of a *40X MIX* containing the forward *PF* and reverse *PR* primers and two TaqMan® MGB probes, the *Pβ⁰39* labelled in 5' with the fluorochrome FAM and the *Pβwt* labelled in 5' with VIC. As these fluorochromes have a different wavelength of emission and as the probes have different nucleotide sequences and specificity, they may act in the same sample and be recognized together. Usually, this kind of kit is used to detect the absence or presence in heterozygosity or homozygosity of a mutation in the genomic DNA. Our intention, however, was to use as template the cDNA from the clonal lines, so trying to obtain greater specificity, we carried out experiments at different annealing – elongation temperatures, up to 65°C, and with various amounts of template, from 20 ng to 100 ng. At the end, given the results obtained in the tests, we decided to perform

the reactions with 50 ng of cDNA at a temperature of annealing –elongation of 60°C.

In **figure 32** are presented the profiles derived from the non-quantitative real time RT-PCR analysis of the $\beta^0$39-globin K562 cell clones. It is easy to notice the presence of amplification curves obtained with both *P$\beta^0$39* and *Pβwt* probe in all samples. This result implies the existence in these clones of two β-globin transcripts, wild type and mutated, the first due to the copies of the endogenous β-globin gene, the second due to the transgenes. Taking into consideration the Ct obtained by the various curves and comparing the two curves of each sample, it is possible to note that the $\beta^0$39-globin transcript is far more represented than the βwt, because the relative amplification curve employs a fewer number of cycles to reach the threshold.

Of course, also the transcripts of βwt-globin K562 cell clones were analysed using the Genotyping kit. In this case, we obtained anyway the amplification curve with the *P$\beta^0$39* probe (data not shown), but it was totally atypical and with a Ct higher than 30. This result suggests the existence of a possible cross-hybridization of the mutated probe with the wild type transcript but it does not preclude the use of the kit, because the amplification curves and Cts obtained with the mRNA from $\beta^0$39-globin K562 clones are significantly different from those obtained with the βwt-globin K562 clones. So, the cross-hybridization can be consider negligible.

Quantification of the βwt- and $\beta^0$39-globin transcripts by quantitative real time RT-PCR assay

Once identified the β-globin transcript produced by all the K562 cell clones, we felt necessary to quantify it so as to be able to choose clones to be used as $\beta^0$39-thalassemia cellular model and control model, based on the content of β-globin mRNA. In fact, it is more reliable to compare clones accumulating similar amounts of β-globin sequences, especially when the aim is to test therapeutic strategies able to alter the mRNA processing.

In this respect, we performed the real time RT-PCR analysis on the RNA from logarithmically growing cells as already explained, using the Genotyping kit so as

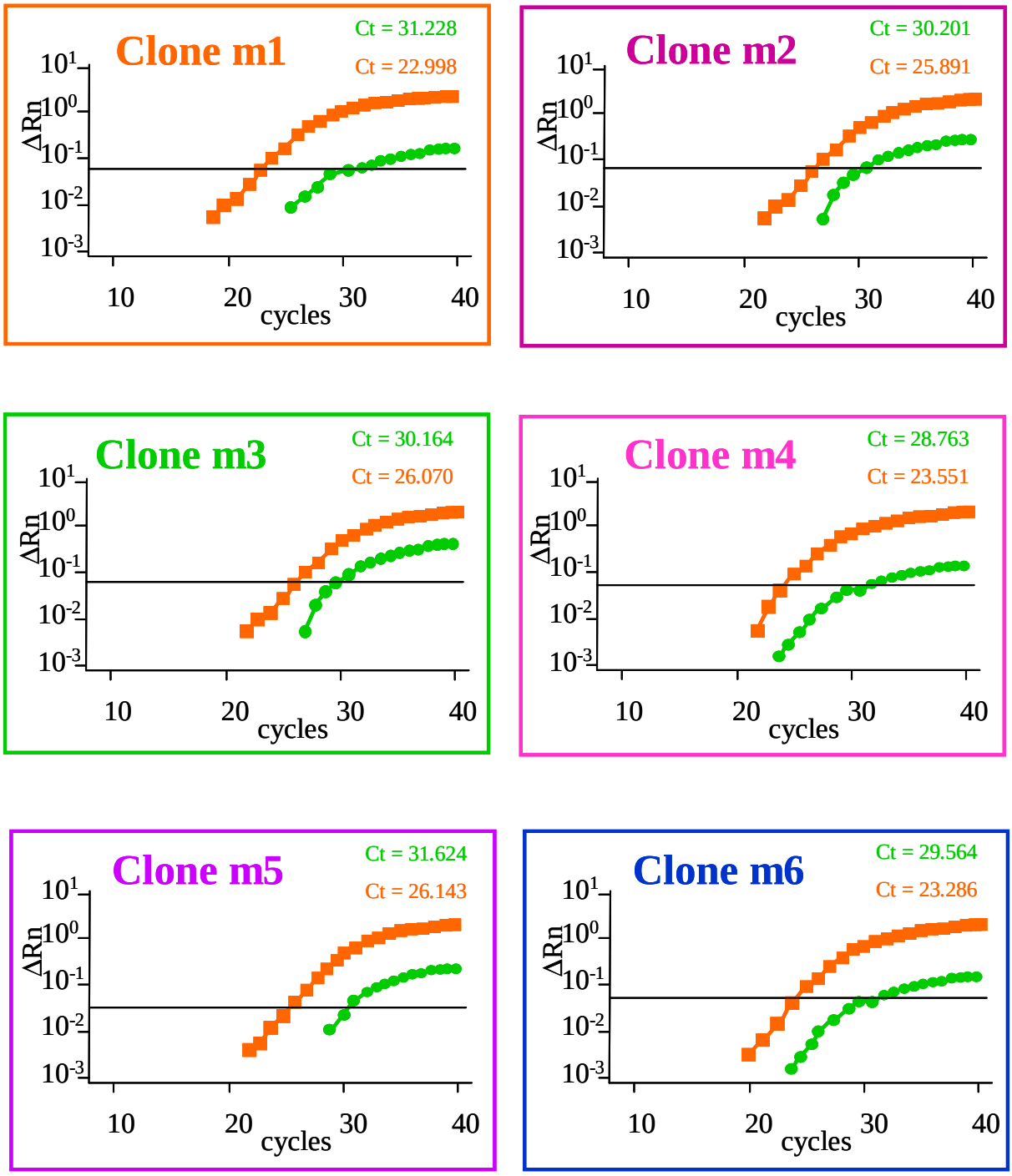


Figure 32 – Non-quantitative real time RT-PCR analysis of β-globin mRNA from β^{0}39-globin K562 cell clones. The representative profiles show the amplification of the cDNAs obtained by reverse transcription of mRNAs isolated from β^{0}39-globin K562 clones. The assay was performed using the *Genotyping Kit* designed for the β^{0}39 mutation and containing the probes *Pβwt* (green circles) and *Pβ^{0}39* (orange squares), labelled with *FAM* and *VIC*, respectively.

to discriminate between the two β-globin transcripts, mainly in the case of the $\beta^0$39-globin K562 cell clones. For the quantification it was necessary to identify a standard sample to be used as reference so as to analyse all the clones in comparison to it. Because we could not use the original K562 cells as standard, due to absence of $\beta^0$39 mRNA, we decided to use the βwt-globin and $\beta^0$39-globin K562 cell clones with the lowest fold of β-globin transgenes (wt1 and m3) as standard for the quantification of the β-globin transcripts in the βwt-globin and $\beta^0$39-globin K562 cell clones respectively.

Although real time RT-PCR is widely used to quantify biologically relevant changes in mRNA levels, there remain a number of problems associated with its use. These include the inherent variability of RNA, variability of extraction protocols that may co-purify inhibitors and different reverse transcription and PCR efficiencies. Consequently, it is important that an accurate method of normalisation is chosen to control for this error. Normalising to a reference gene is a simple and popular method for internally controlling for error in real time RT-PCR. This strategy targets RNAs encoded by genes, which have been collectively called housekeeping genes and benefits from the fact that all the steps required to obtain the final PCR measurement are controlled for. The procedure is simplified as both the gene of interest and reference gene are measured using real time RT-PCR. The most commonly used reference genes include β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18S ribosomal RNA [Huggett et al, 2005]. In this case, we chose the GAPDH, of which we had a commercial kit designed for real time PCR (from Applied Biosystems).

The cDNA obtained from the RNA isolated from each clone was then amplified using the Genotyping kit and GAPDH kit to detect the β-globin and GAPDH transcript respectively.

The results gave clear evidence that within the βwt-globin K562 cell clones, high expression (relative to wt1) was detected in wt6 and low expression in wt3, wt5 and wt7 clones (**figure 33a**); on the other hand, within the $\beta^0$39-globin K562 clones, the β-globin gene was highly expressed (in comparison to m3) in m1, m4,

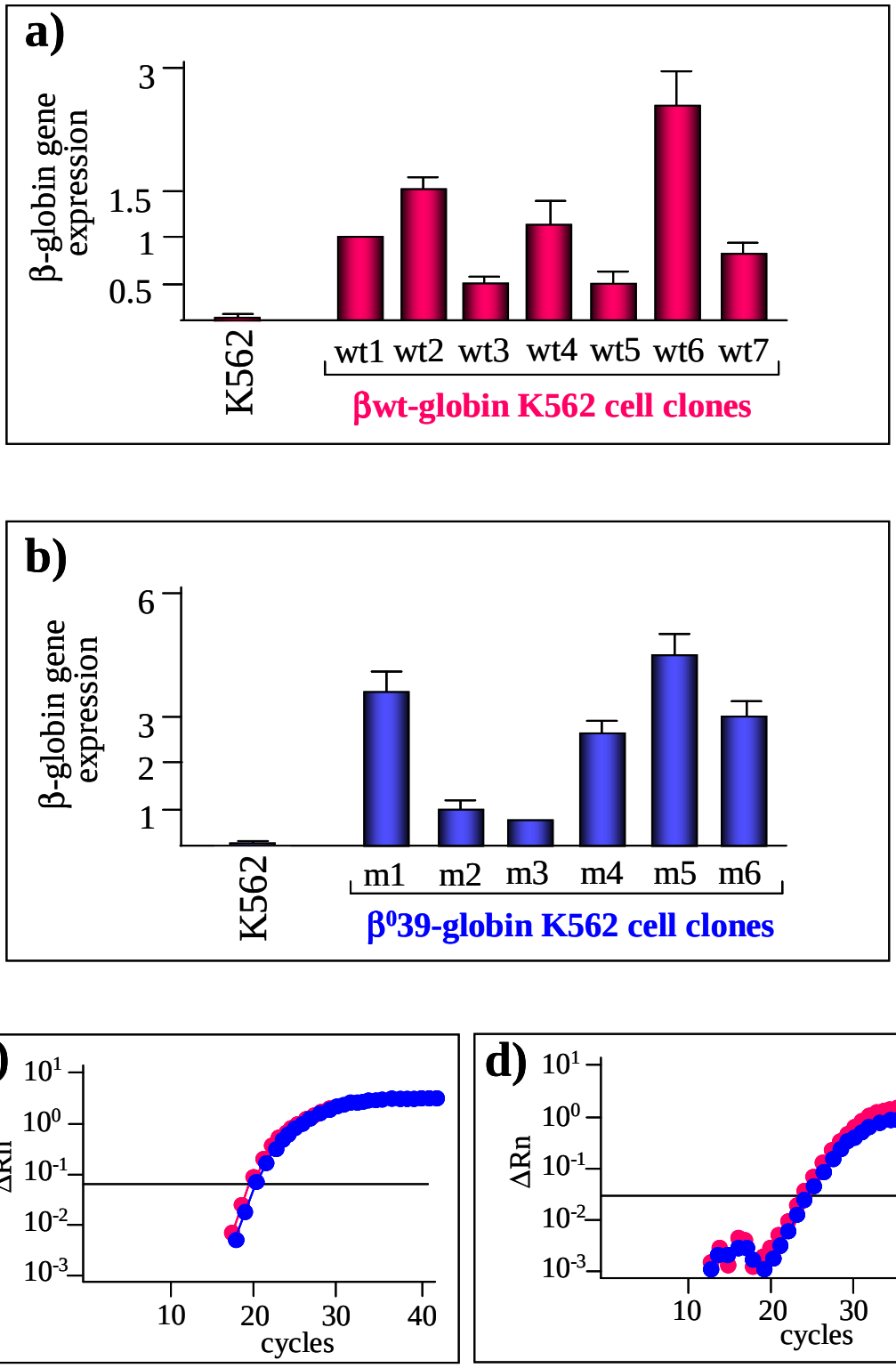


Figure 33 – Quantitative real time RT-PCR analysis of β-globin mRNA from K562 cell clones. a,b) Histograms showing the β-globin mRNA content in βwt-globin (**a**) and $\beta^0$39-globin (**b**) K562 clones (average ± SD o three indipendent determinations). **c,d)** Representative profiles of quantitative RT-PCR analysis performed on RNA isolated from wt3 βwt-globin (pink circles) and m5 $\beta^0$39-globin (blue circles) K562 clones, using primers amplifying GAPDH (**c**) and β-globin (**d**) mRNA sequences. Two different probes were used for the β-globin mRNA: *Pβwt* and *Pβ⁰39*, contained in the *Genotyping Kit* and labelled with *FAM* and *VIC*, respectively.

m5 and m6 K562 cell clones and poorly expressed in m2 K562 clone (**figure 33b**). Despite the fact that no silencing effect was noted, no clear relationship is evident between levels of gene expression and number of integration units. This is however expected, since transcription might depend also on the site of integration. Moreover, in $\beta^0$39-globin K562 cell clones the accumulation of β-globin mRNA sequences is affected by the known effect of nonsense mediated mRNA decay (NMD), which can significantly reduce but not totally eliminate the nonsense $\beta^0$39 transcript [Holbrook et al, 2004; Stalder and Mühlemann, 2008]. After comparative analysis of the RT-PCR data, clones wt3 (βwt-globin K562 cells) and m5 ($\beta^0$39-globin K562 cells) were selected for the experiments employing possible corrections with aminoglycosides, due to the fact that, among the βwt-globin and $\beta^0$39-globin K562 cell clone sets, they express similar, although not identical levels of β-globin mRNA molecules, as depicted in **figure 33 (c and d)**. Interestingly, clone m5 displays higher β-globin gene integration units and GFP production in respect to clone wt3 suggesting, as expected, NMD affecting $\beta^0$39-globin mRNA, although in this case the large number of mutated transgenes present into the cells may induce the saturation of the process, leading to the accumulation of a significant amount of nonsense transcripts. The differential expression of β-globin mRNA between m5 and wt3 are maintained when the culture conditions were changed from expansion to differentiation, by treating cells with cytosine arabinoside (data not shown).

USE OF THE EXPERIMENTAL MODEL SYSTEM TO CHARACTERIZE THE READ-THROUGH ACTIVITY OF AMINOGLYCOSIDES

As already mentioned, the $\beta^0$39-thalassemia mutation is a nonsense mutation. In fact, the replacement C → T at the first base of codon 39 of the β-globin gene sequence causes the development of a premature translation termination codon, resulting in inhibition of β-globin protein synthesis. Given the proven read-through ability of aminoglycosides and their application as possible therapeutic

compounds for genetic diseases caused by nonsense mutations [Howard et al, 1996; Bedwell et al, 1997; Barton-Davis et al, 1999; Du et al, 2002; Howard et al, 2003; Lai et al., 2004; Pinotti et al., 2006], we decided to test the action of the most used aminoglycosides on our experimental model. The aim was to see if the read-through activity demonstrated by these compounds, and thus their power to correct the nonsense mutations, was also confirmed in β-thalassemia. If that happens, it would have production of β-globin chain, which could lead to the formation of adult haemoglobin (Hb A).

GENETICIN

From the literature it came out that the aminoglycosides more effective in the translational read-through, for the treatment of genetic diseases, are *geneticin* or *G418* [Howard et al, 1996; Bedwell et al, 1997, Lai et al, 2004; Pinotti et al, 2006] and *gentamicin* [Barton-Davis et al, 1999; Keeling et al, 2001; Du et al, 2002; Wilshanski et al, 2003; Howard et al, 2004]. Given the ready availability of *geneticin*, as a first experiment of treatment with aminoglycosides on our model, we decided to use precisely the G418. Once confirmed the practicality of this method to correct the $\beta^0$39-globin mutation, we decided to broaden the spectrum of aminoglycosides, in order to identify the compound most suitable for the β-globin gene.

Evaluation of the toxicity of *geneticin* (*G418*)

Before we could start the treatment, it was obviously necessary to check the geneticin toxicity and, therefore, its action on cell growth of wt3 βwt-globin and m5 $\beta^0$39-globin K562 cell clones, with the aim of identifying a range of concentrations at which we could use the *G418* without causing serious cell damage. In this regard, we carried out a curve of growth inhibition with the wt3 and m5 clones: the cells were plated at a concentration of 3×10^4 cells/ml in 2 ml of culture medium, for a total 6×10^4 cells per well, in 12 wells, and treated with

scalar concentrations of geneticin from 0 to 1 μg/μl. After 3 days was then calculated the total number of cells in each treated sample.

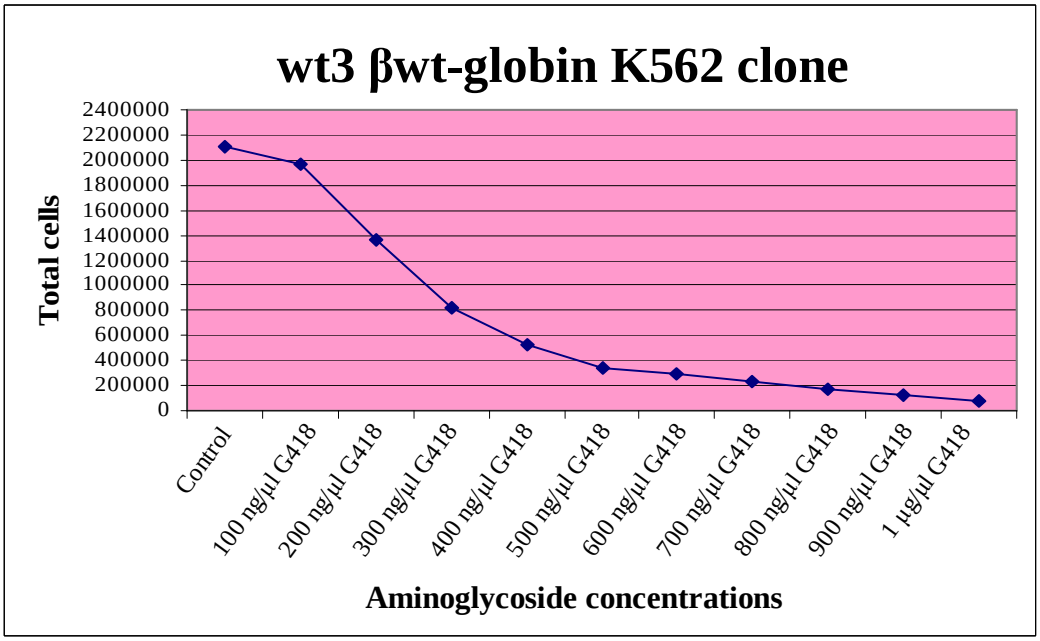
The obtained data were included in two graphs, one for each clone, along with the *geneticin* concentrations, resulting in curves of cell growth inhibition which are shown in **figure 34**. From the curves we were able to determine that the geneticin concentration determining 50% cell growth inhibition (IC50) was similar for both the clones and about 200 ng/μl. We therefore decided to treat the cells with the following concentrations of aminoglycoside: 100 ng/μl, 200 ng/μl and 400 ng/μl, so as to have a larger view of the *G418* activity and identify the optimal concentration for the treatment.

Evaluation of the effects of *geneticin* on original K562 cells

Before starting the treatment of our cellular models with *G418*, it was necessary to determine whether it could have an induction effect on the expression of β-globin gene in original K562 cell line, from which we started to get our clones. We plated 3 x 10^4 cells/ml in 4 ml of RPMI supplemented with 10% FBS for a total of 1.2 x 10^5 cells, in 2 wells. One of the two samples were then treated with 400 ng/μl *geneticin*, while the second was kept as control. Both were then incubated at 37°C in humidified atmosphere of 5% CO_2/air for 72 hours. The choice of the *geneticin* concentration was due to the fact that 400 ng/μl is the highest concentration among those chosen for the treatment of wt3 and m5 clones and is the concentration that was proven to be more effective [Pinotti et al, 2006].

After incubation for 72 hours, we collected from each well 1.5 x 10^6 cells which were labelled with the monoclonal antibody (MoAb) against the β-globin chain, to enable the detection of its production by immunohistochemistry and FACS analysis. To this purpose, we used the *BD Cytofix/Citoperm™ Kit* (BD Biosciences Pharmingen).

Figure 35 summarizes the basic concepts of the intracellular labelling technique, which includes three steps: 1) permeabilization of the cell membrane; 2) treatment with an inhibitor of aspecific binding sites; 3) incubation of the permeabilized cells with an antibody specific for the desired protein. The labelled



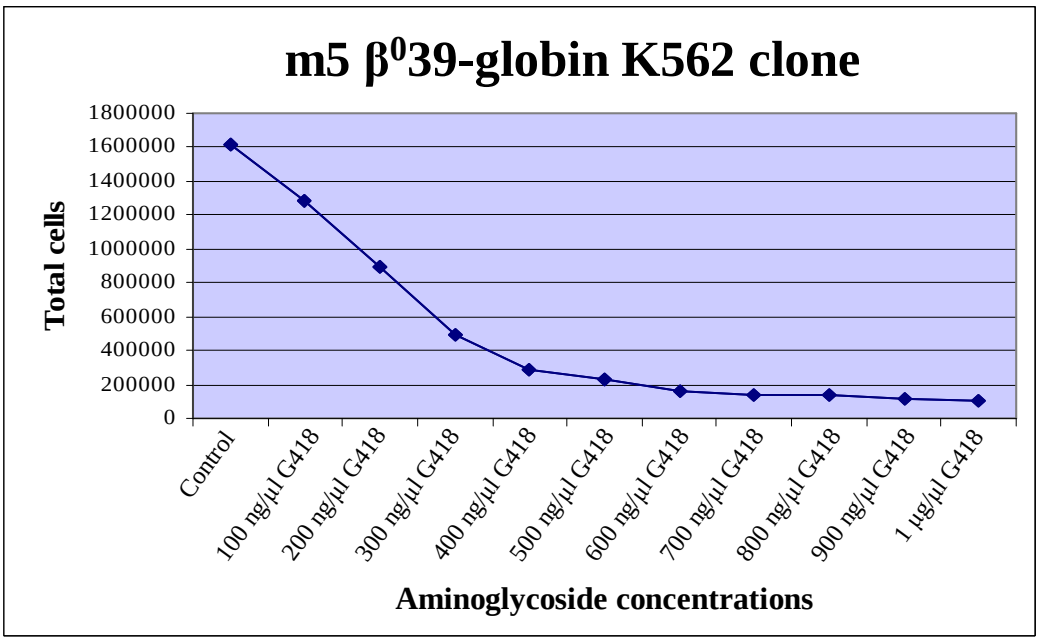


Figure 34 – Inhibition effects of *geneticin* (*G418*) on βwt-globin wt3 and β⁰39-globin m5 K562 cell clones. The cells were incubated for 3 days at increasing concentrations of *G418* and, then, counted using the Bürker chamber. On the x-axis are reported the concentrations of geneticin while on the y-axis the total cells counted.

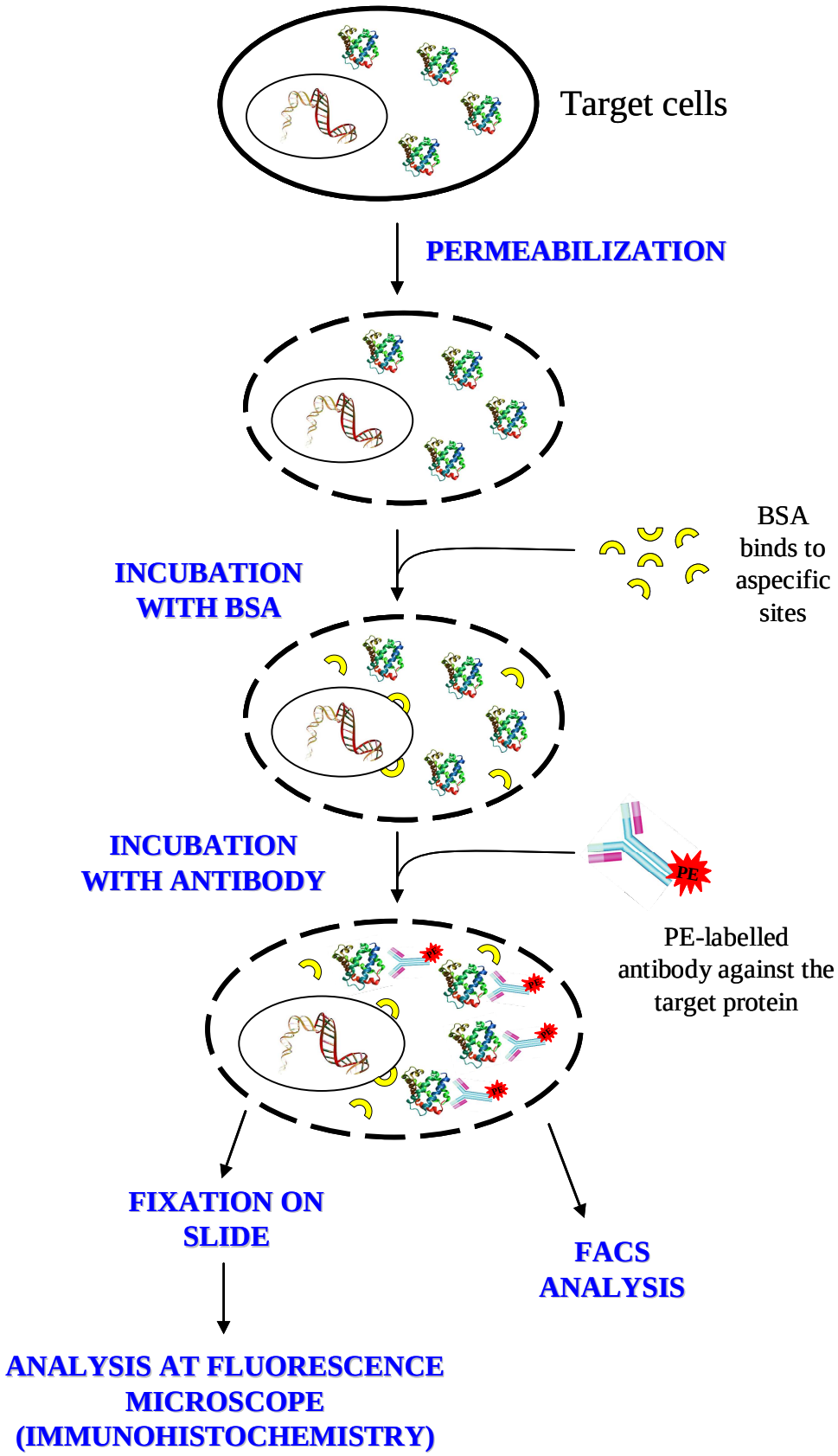


Figure 35 – Schematic representation of the intracellular labelling technique. To detect a cytosolic, rather than a surface, protein, it is necessary, first, to permeabilize the target cells. Such cells were, later, treated with BSA to inhibit the aspecific binding sites and, then, incubated with the fluorescent monoclonal antibody (MoAb) specific for the protein to be detected. The MoAb is able to enter into the cell, recognize its target site and bind to it. Subsequent washes eliminate the excess of MoAb not bound. The fluorophore bound to the MoAb makes the positive cells fluorescent, which can be detected by both fluorescence microscope, after fixation on a slide, and flow cytometer (FACS).

cells can be either fixed on a slide and analysed by a fluorescence microscope or analysed by FACS, so as to have quantitative data about the proportion of fluorescent cells and the intensity of the emitted fluorescence.

The cells were initially permeabilized with 500 μl of *BD Cytofyx-Citoperm solution*, then subjected to incubation first with 300 μl of 1X PBS (phosphate buffered saline) 1% BSA (bovine serum albumin) solution, to inactivate the aspecific sites which could bind to the antibody, and then with the *βglobin-PE* (PE, phycoerythrin) (Santa Cruz Biotechnology) MoAb, specific for the recognition of β-globin chain, diluted 1:10 with PBS - 1% BSA. *βglobin-PE* is a monoclonal antibody labeled with the fluorochrome phycoerythrin (PE), which, when excited with light of wavelength equal to 488 nm, emits red fluorescence at 580 nm.

The BSA, besides inactivating aspecific binding sites, neutralizes the electrostatic charge of the tube wall and prevents it attracts the antibody molecules. In the absence of BSA, in fact, the MoAb gradually adheres to the wall, stratifying and resulting in gradual reduction of its concentration. So, when it is necessary to make dilutions of the antibody, it is convenient to restore the concentration of BSA.

After incubation with the antibody, we divided the cells of the two samples so as to be fixed on a slide and analyzed by fluorescence microscope (1/3 of total cells) and at the same time analyzed by FACS (2/3 of total cells).

FACS analysis

The FACS analysis was performed by FACScan (Becton-Dickinson), using the *Cell Quest Pro* software (Becton-Dickinson), whose principle has already been widely described and summarized in **figure 24**.

As usual, the first step was to set the channels of the instrument with its parameters: *FSC* and *SSC*, which elaborate the data of physical and morphological characteristics of the cells, and *FL2* (*FL-2 Height*), a channel able to analyse data on the red - orange fluorescent. Original K562 cells labelled with the *βglobin-PE* MoAb can be fluorescent only if they contain β-globin chains, as the antibody is

specific for this protein, furthermore these cells do not have, unlike our clones, the GFP reporter gene and then they are not able to emit the green fluorescence. For this reason, it was not necessary to configure the *FL1* parameter.

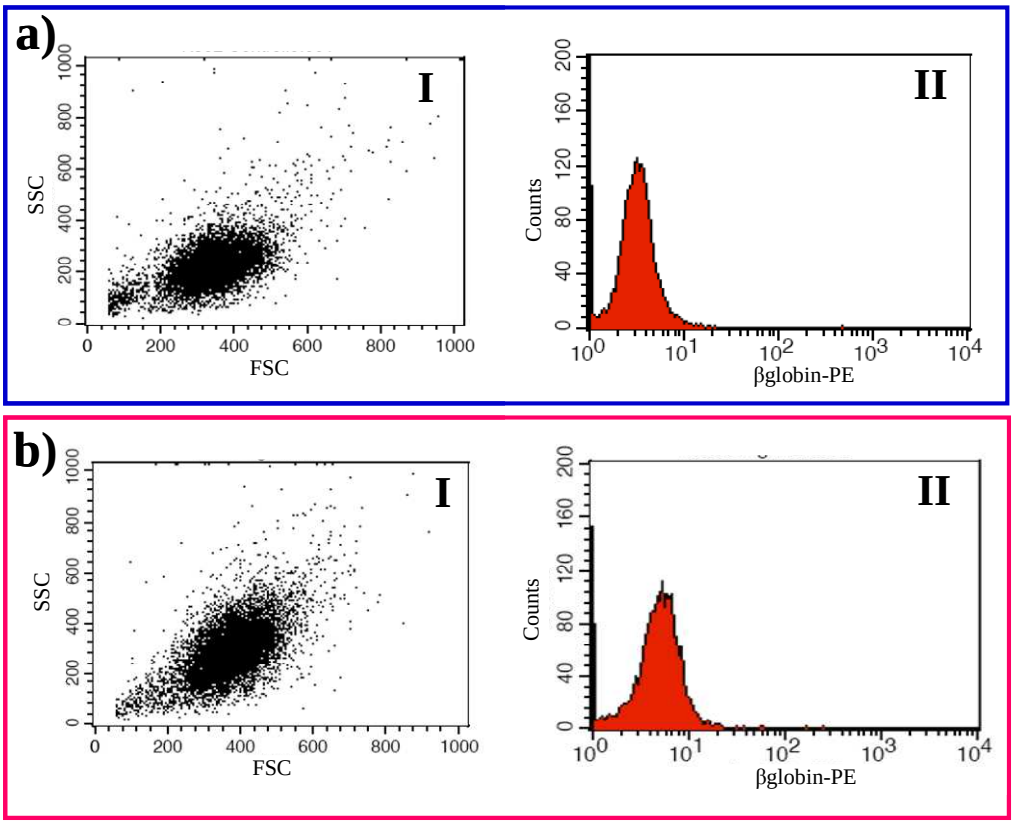
Figure 36 shows the diagrams obtained from the FACS analysis of the two samples: K562 untreated (**a**) and treated with 400 ng/μl *G418* (**b**). It may be noted in addition to the *FSC - SSC* cytogram (**I**), where the cells are distributed based on their morphology, a *βglobin-PE* histogram (**II**), with on the x-axis the red fluorescence and on the y-axis the number of emitting cells. By comparing the two *FSC - SSC* cytograms, it is possible to note a slight movement of the cloud of treated K562 cells (**b**) to greater values of *SSC*. This shift indicates an increased granularity of cytoplasm and is certainly due to the toxic effect of the G418 on cells. Precisely because of the presence of a different cellular distribution between the two samples, due to the aminoglycoside action, we decided not to draw any gate in the cytogram in order to analyze the totality of the cells.

From the histograms for the red fluorescence (**IV**), it is possible to notice a slight shift of the cells treated with G418 beyond the fluorescence threshold of 10^1. The magnitude of that shift is shown in the table (**figure 36c**): the control K562 had a proportion of red fluorescent cells equal to 0.31%, as expected because of the low expression of β-globin gene in these cells, while in K562 treated with *geneticin* this percentage increased to 2.09%. Similarly, the median of the intensity, a value that indicates the midpoint of the distribution of fluorescent cells, changed from 3.13, in control cells, to 4.96 in the treated sample.

The fact that the control sample presented a weak fluorescence, maybe due to spontaneous fluorescence background, is normal and is the limiting factor of the resolving power of the instrument [Brando, 1990].

Immunohistochemistry analysis

In parallel with the FACS analysis, we proceeded with the fixation of the labelled cells on a slide so as to be analyzed using a fluorescence microscope to verify the presence or absence of β-globin chains. The used slide was previously



c)

SAMPLE	PERCENTAGE of red-fluorescent cells	MEDIAN of the fluorescence intensity
K562 control	0.31	3.13
K562 + 400 ng/µl G418	2.09	4.96

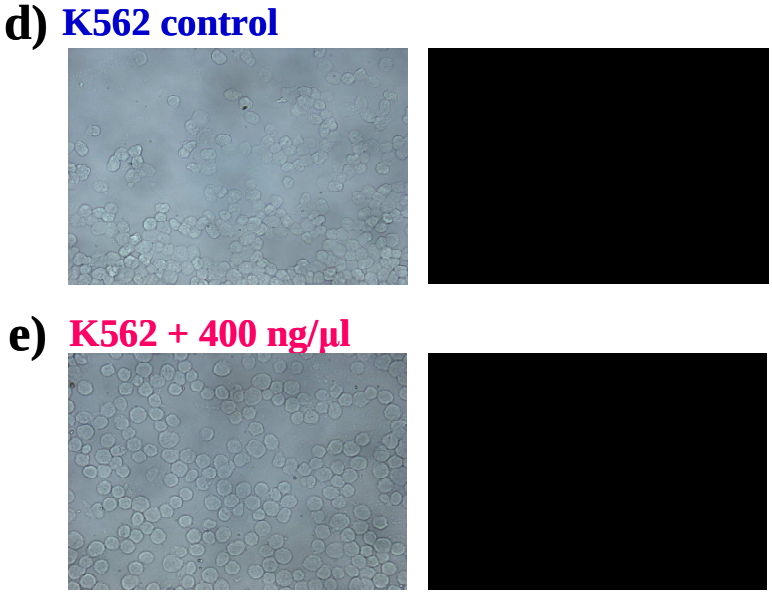


Figura 36 – Effects of *geneticin* (*G418*) on the β-globin production in original K562 cells. In figure, are presented diagrams obtained through FACS analysis of original K562 cells, labelled with *βglobin-PE* MoAb: (**a**) untreated; (**b**) incubated for 3 days with 400 ng/µl G418. For each samples are reported: cells distribution diagram based on morphological parameters *FSC* and *SSC* (**I**) and histogram relating the emitted red fluorescence to the number of beaming cells (**II**). Data on the percentage of cells emitting red fluorescence and the intensity of that fluorescence are shown in table (**c**). Moreover, there are reported the images obtained at fluorescence microscope of the cells labelled with *βglobin-PE* and fixed on a slide: control K562 not treated (**d**), K562 incubated for 72 hours with 400 ng/µl G418 (**e**).

treated with 30 µl of *poly-L-lysine* diluted 1:10, to facilitate cell adhesion to glass. Cells were then fixed with 4% *formalin*. It was therefore possible to analyse the cells using a fluorescence microscope, which permits, through the emission of fluorescent light and with the appropriate filter, to verify the presence of red fluorescence. As shown in **figure 36d**, the cells treated with G418 did not emit any fluorescence, as well as the untreated cells.

The obtained data appear to be discordant with each other, because the FACS was unable to find a slight increase in the proportion of fluorescent cells, which, however, was not brought out by the immunohistochemistry. This can be explained considering that the FACS sensitivity is greater than the human eye perceptivity, and taking into account that the increase in the proportion of red cells and fluorescence intensity is really minimal.

Evaluation of the effects of *geneticin* on β-globin production in wt3 βwt-globin and m5 $\beta^0$39-globin K562 cell clones

The wt3 βwt-globin and m5 $\beta^0$39-globin K562 cell clones cells were plated, as previously explained, in 4 wells: an untreated control and three samples treated with scalar concentrations of *geneticin* (100 ng/µl, 200 ng/µl and 400 ng/µl). To have a positive control for the red fluorescence, we decided to label with *βglobin-PE* MoAb erythroid precursor cells, isolated from healthy donors and treated with erythropoietin, which express the β-globin gene at good levels. After the usual 3 days of incubation, cells were subjected to the permeabilization treatment and incubated with *βglobin-PE* antibody and then divided in order to analyse their ability to emit red fluorescence, which depends on the amount of β-globin chains present, using both the FACS and fluorescence microscope.

FACS analysis

First of all, we decided to treated the wt3 K562 clone to be used as a control of the *geneticin* activity, because it did not express $\beta^0$39-globin mRNA but only βwt transcripts at high level which should not be affected by the aminoglycoside

action. We considered this experiment as a further control, besides that performed using original K562, just because the wt3 K562 cells present a high number of βwt-globin genes and so a potential enhancing effect of the *G418* on gene expression can be point out more easily.

The FACS analysis of these cells led to further steps in the calibration of the instrument, because they express two different fluorescences (GFP and PE), which have different wavelengths of emission and, for this reason, are recognized by two different photomultipliers. It may, however, happen that a negligible radiation of the wrong colour arrives to a sensor, because of the optical filters and amplitude of the fluorochrome emission band. This problem can be rectified through the system of signal electronic correction called compensation: process that aims to subtract from a specific fluorescence channel a fixed signal relative to the emission of another fluorochrome [Brando, 1990].

After configuring parameters as previously described using original K562, which can not emit any fluorescence, we compensated the green (*FL1*) and red fluorescence (*FL2*) channels, according to the fluorescence emitted by the unlabelled wt3 K562 cells (GFP) and erythroid progenitors labelled with *βglobin-PE* MoAb, respectively.

Figure 37 presents all the diagrams obtained in the FACS analysis of our samples: wt3 K562 clone cells untreated (**a**) and treated with 100 ng/μl (**b**), 200 ng/μl (**c**) and 400 ng/μl *G418* (**d**). Comparing *FSC* - *SSC* cytograms (**I**) we had the confirmation of the aminoglycoside action on the cell morphology, as in the sample treated with the greatest concentration (**d**) the resulting cloud of cells is moved up and enlarged. Analysing, however, the *βglobin-PE* - GFP cytograms (**II**) it is possible to observe that the cloud is almost in the same position, unless a slight shift up in the samples treated with the two greatest concentrations of *G418* (**c and d**). This trend is confirmed by histograms of red fluorescence (**IV**), where it is possible to note a minimal movement of the peak of wt3 K562 clone cells treated with 200 ng/μl and 400 ng/μl G418 compared to untreated cells. To facilitate the visualization of the peaks shift, we set up an unique histogram for the red fluorescence with the peaks of all samples (**figure 37e**). In addition, through the analysis carried out by the instrument, we could obtain precise data on both the

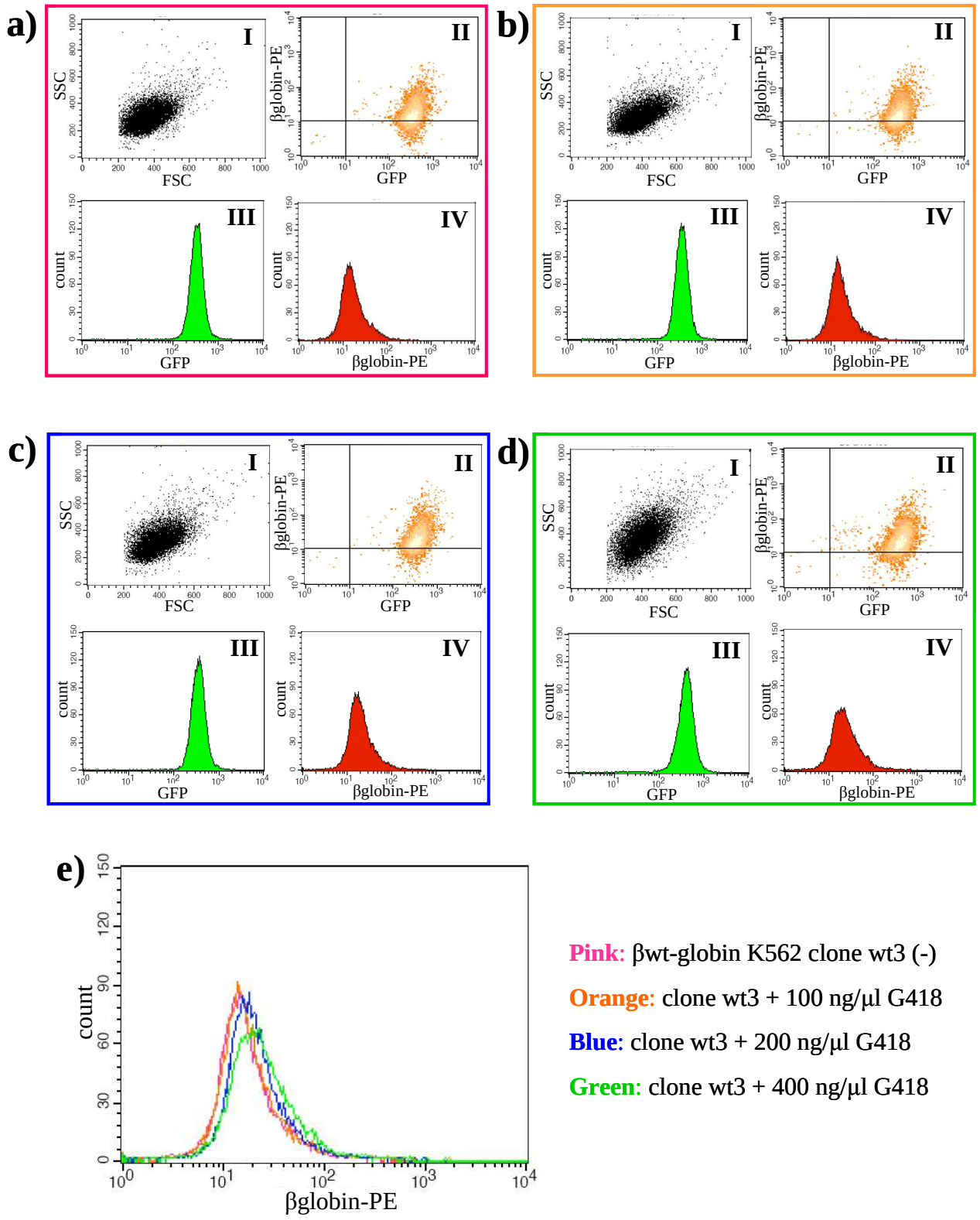


Figura 37 – FACS analysis of wt3 βwt-globin K562 cell clone, treated with scalar concentrations of *geneticin* (*G418*) and labelled with the *βglobin-PE* MoAb. In figure, are reported diagrams obtained through FACS analysis of wt3 βwt-globin K562 cell clone untreated (**a**) or incubated for 3 days with 100 ng/µl (**b**), 200 ng/ µl (**c**) and 400 ng/µl *G418* (**d**). For each sample are reported: cells distribution diagram based on the morphological parameters *FSC* and *SSC* (**I**); cells distribution diagram based on the emitted fluorescences, with the green (GFP) and red (PE) fluorescences on the x-axis and y-axis, respectively (**II**); histograms relating the emitted fluorescence (**III**, GFP; **IV**, PE) to the number of emitting cells. Peaks obtained in the four histograms with the red fluorescence in the x-axis were, then, overlayed in a unique histogram (**e**).

proportion of red fluorescent cells and fluorescence intensity. These data are summarised in the histogram in **figure 38**: as expected, the untreated wt3 K562 clone cells presented a high proportion of red fluorescent cells, corresponding to the proportion of β-globin containing cells, and a quite high median of the fluorescence. Such parameters did not vary significantly in the treated samples, confirming the data previously obtained with original K562 cells.

At this point, we were ready to verify the translational read-through effects of *G418* on m5 $\beta^0$39-globin K562 cell clone. **Figure 39** presents all the diagrams obtained by FACS analysis of our samples: m5 K562 clone cells untreated (**a**) and treated with 100 ng/μl (**b**), 200 ng/μl (**c**) and 400 ng/μl *G418* (**d**). Comparing *FSC - SSC* cytograms (**I**) we had a further confirmation of the aminoglycoside action on the cell morphology, as in the sample treated with the greatest concentration (**d**) the cloud of cells is, also in this case, moved up and enlarged and moreover it is possible to note the presence of a greater amount of dead cells. Instead, the *βglobin-PE* - GFP cytograms (**II**) show a marked shift in the cloud of cells beyond the threshold set for the red fluorescence, proportionally to the concentration of *G418* added to the cells. This trend is confirmed by the histograms of red fluorescence (**IV**), where it is possible to note a gradual movement of the peak of cells beyond the fluorescence threshold of 10^1, whereas peaks in the GFP fluorescence histograms remain invariable. To facilitate the visualization of such shift and its dependence on the concentration of *G418*, we set up an unique histogram for the red fluorescence with the overlay of the samples peaks (**figure 39e**). The data on both the proportion of red fluorescent cells and intensity of fluorescence shown in **figure 40** represent the averages of three independent determinations: as expected, the untreated m5 $\beta^0$39-globin K562 cell clone presented very small proportion of red fluorescent cells and fluorescence median. However, such parameters increased in m5 clone cells treated with G418, proportionally to the increase of the concentration, reaching a maximum (about 50%) in the case of cells treated with 400 ng/μl *G418*.

Taking into account that the red fluorescence corresponds to the presence of β-globin chains inside the cells, we could affirm that the aminoglycoside *geneticin*

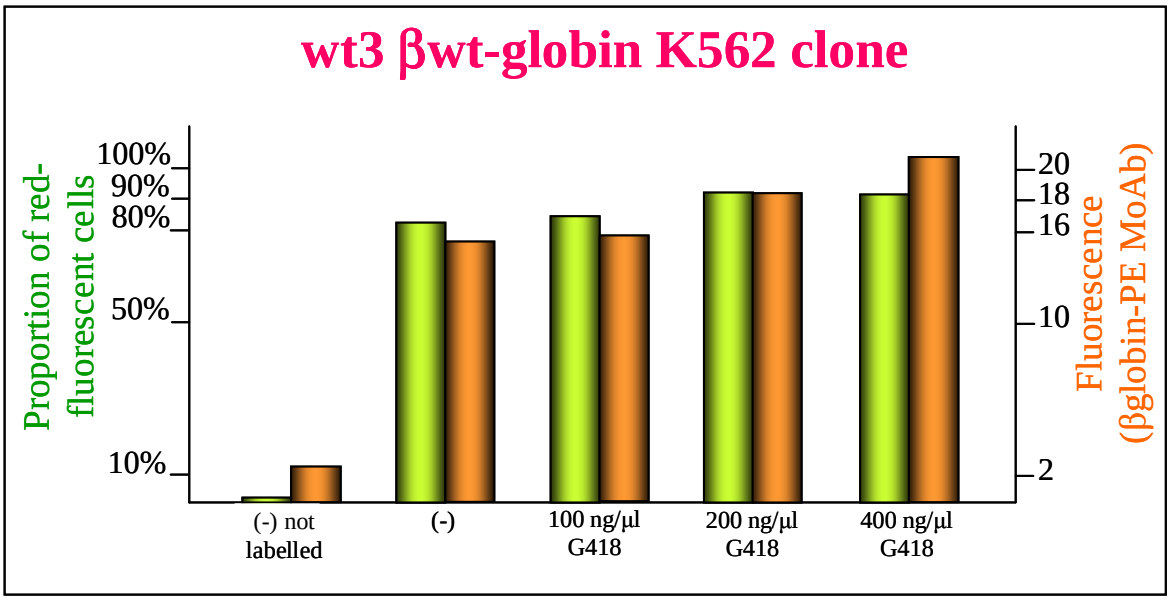


Figure 38 – Effects of increasing concentrations of *geneticin* (*G418*) on the β-globin production in wt3 βwt-globin K562 cell clone. On the histogram are presented both the proportion of red-fluorescent cells (green boxes), corrisponding to the proportion of β-globin containing cells, and the median of the emitted red fluorescence (orange boxes) of the wt3 βwt-globin K562 cell clone, untreated or incubated for 3 days with increasing concentrations (100 ng/µl, 200 ng/µl and 400 ng/µl) of *G418*. Cells were labelled with the *βglobin-PE* MoAb and analyzed by FACS. On the right part of the histogram are shown the data refering to the clone wt3 untreated and unlabelled. The data represent the averages ± SD of three indipendent determinations.

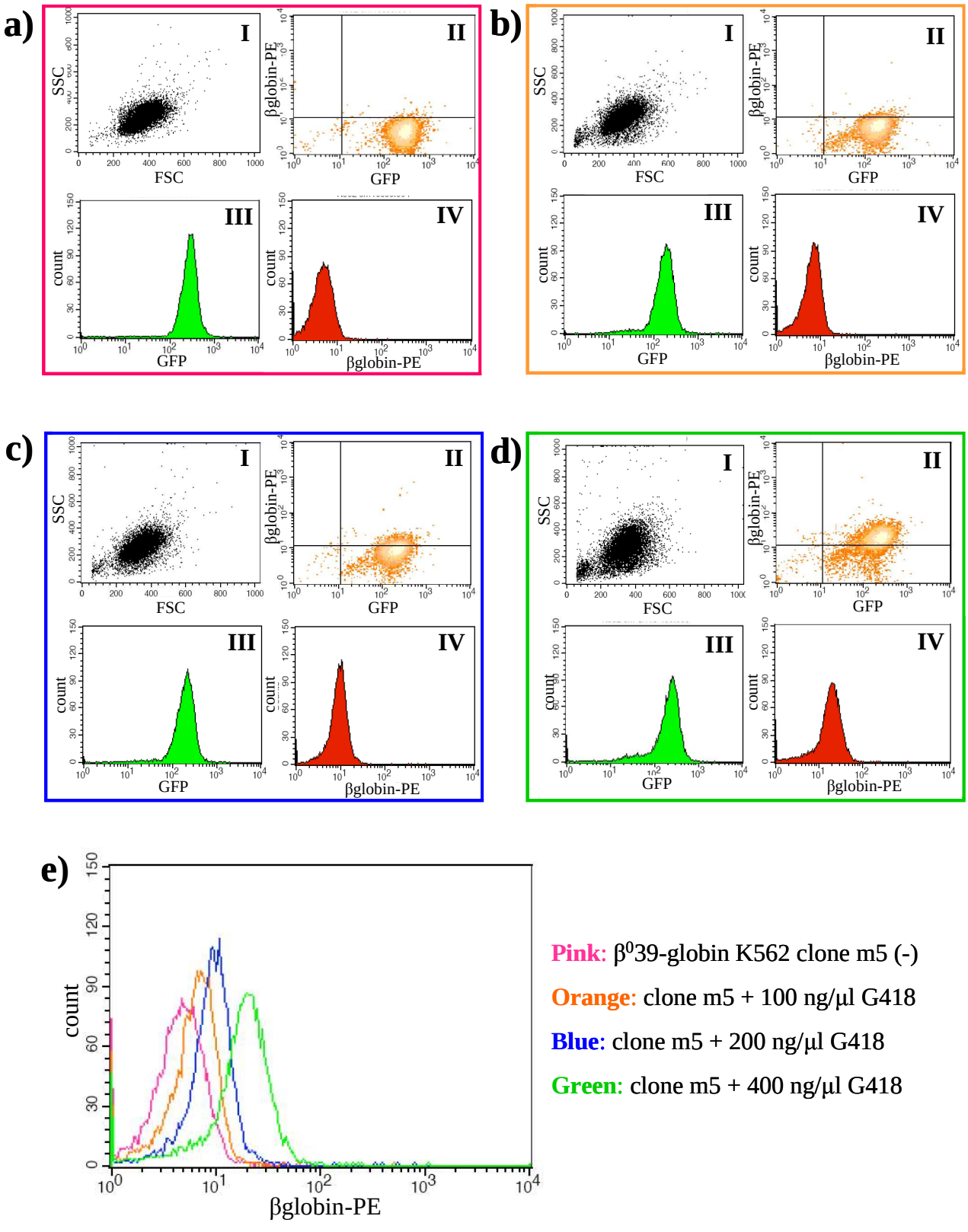


Figura 39 – Representative FACS analysis of m5 β⁰39-globin K562 cell clone, treated with scalar concentrations of *geneticin* (*G418*) and labelled with the *βglobin-PE* MoAb. In figure, are reported diagrams obtained through FACS analysis of m5 β⁰39-globin K562 cell clone untreated (**a**) or incubated for 3 days with 100 ng/µl (**b**), 200 ng/µl (**c**) and 400 ng/µl *G418* (**d**). For each sample are reported: cells distribution diagram based on the morphological parameters *FSC* and *SSC* (**I**); cells distribution diagram based on the emitted fluorescences, with the green (GFP) and red (PE) fluorescences on the x-axis and y-axis, respectively (**II**); histograms relating the emitted fluorescence (**III**, GFP; **IV**, PE) to the number of emitting cells. Peaks obtained in the four histograms with the red fluorescence in the x-axis were, then, overlayed in a unique histogram (**e**).

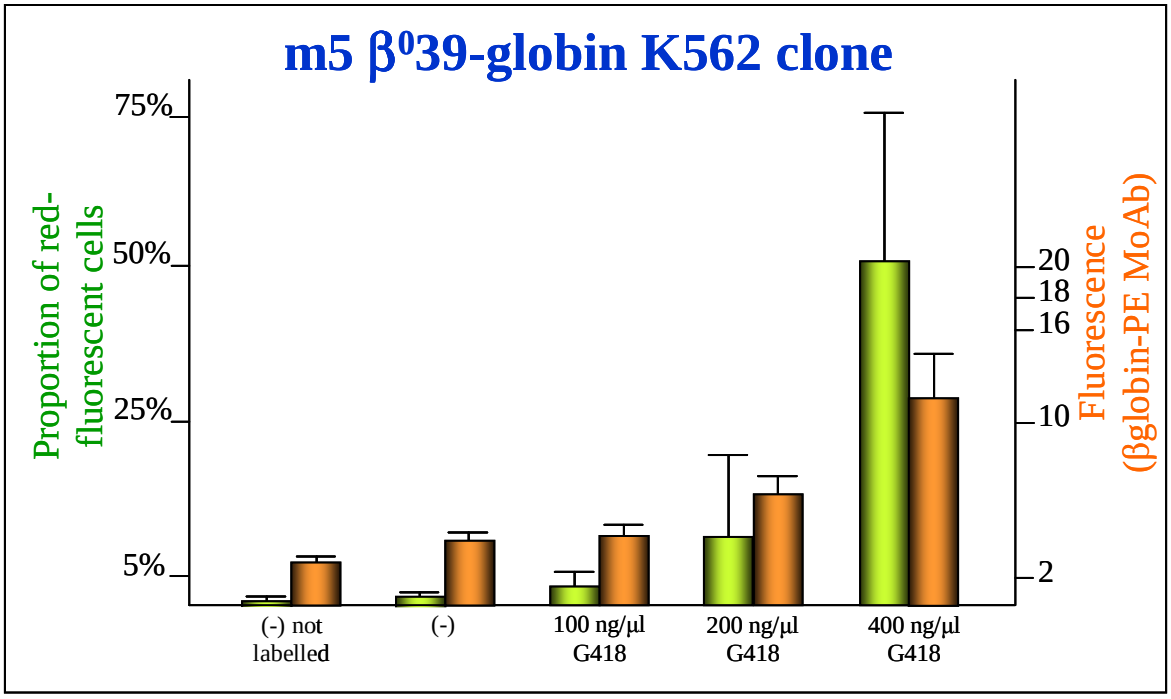


Figure 40 – Effects of increasing concentrations of *geneticin* (*G418*) on the β-globin production in m5 β⁰39-globin K562 cell clone. On the histogram are presented both the proportion of red-fluorescent cells (green boxes), corrisponding to the proportion of β-globin containing cells, and the median of the emitted red fluorescence (orange boxes) of the β⁰39-globin K562 clone wt3, untreated or incubated for 3 days with increasing concentrations (100 ng/µl, 200 ng/µl and 400 ng/µl) of *G418*. Cells were labelled with the *βglobin-PE* MoAb and analyzed by FACS. On the right part of the histogram are shown the data refering to the m5 clone untreated and unlabelled. The data represent the averages ± SD of three indipendent determinations.

was able to induce a translational read-through of the $\beta^0$39 nonsense mutation, determining an increase in the production of β-globin.

Immunohistochemistry analysis

In parallel with the FACS analysis, the same cells were fixed on a slide and then analyzed using a fluorescence microscope to visually assess the emission of red fluorescence. **Figure 41** shows the images of the original K562 (**a,b**) and erythroid precursor (**c,d**) cells, respectively the negative and positive control of the immunohistochemistry assay, the wt3 βwt-globin K562 cell clone untreated (**e,f**) and treated (**g,h**) with 400 ng/μl *G418* and the m5 $\beta^0$39-globin K562 cell clone untreated (**i,l**) and treated (**m,n**) with the same concentration of the aminoglycoside. From these images, it appears immediately obvious that the addition of *geneticin* determined no increase in the red fluorescence emitted by the treated wt3 clone but clear increment in the red fluorescence emitted by the treated m5 clone, indicating an increase in the β-globin containing cells. This result can be consider a validation of what previously observed by FACS analysis.

Although the fluorescence microscope is a method much less sensitive than the flow cytometer, it was, however, possible to confirm the presence of β-globin chains in $\beta^0$39-globin K562 cells treated with *G418*, explaining at these levels only through a corrective action by the aminoglycoside on the translation of the $\beta^0$39 transcript.

Evaluation of the effects of *geneticin* on the content of β-globin mRNA in wt3 βwt-globin and m5 $\beta^0$39-globin K562 cell clones

Once proved the effects of *geneticin* as translational corrector of the $\beta^0$39 nonsense mutation, we wondered if the treatment with *G418* prevents or, at least, limits the decrease in $\beta^0$39-globin mRNA abundance attributable to NMD, as already reported in several papers [Bedwell et al, 1997; Holbrook et al, 2004]. In fact, aminoglicosides, binding the decoding centre of the ribosome and decreasing

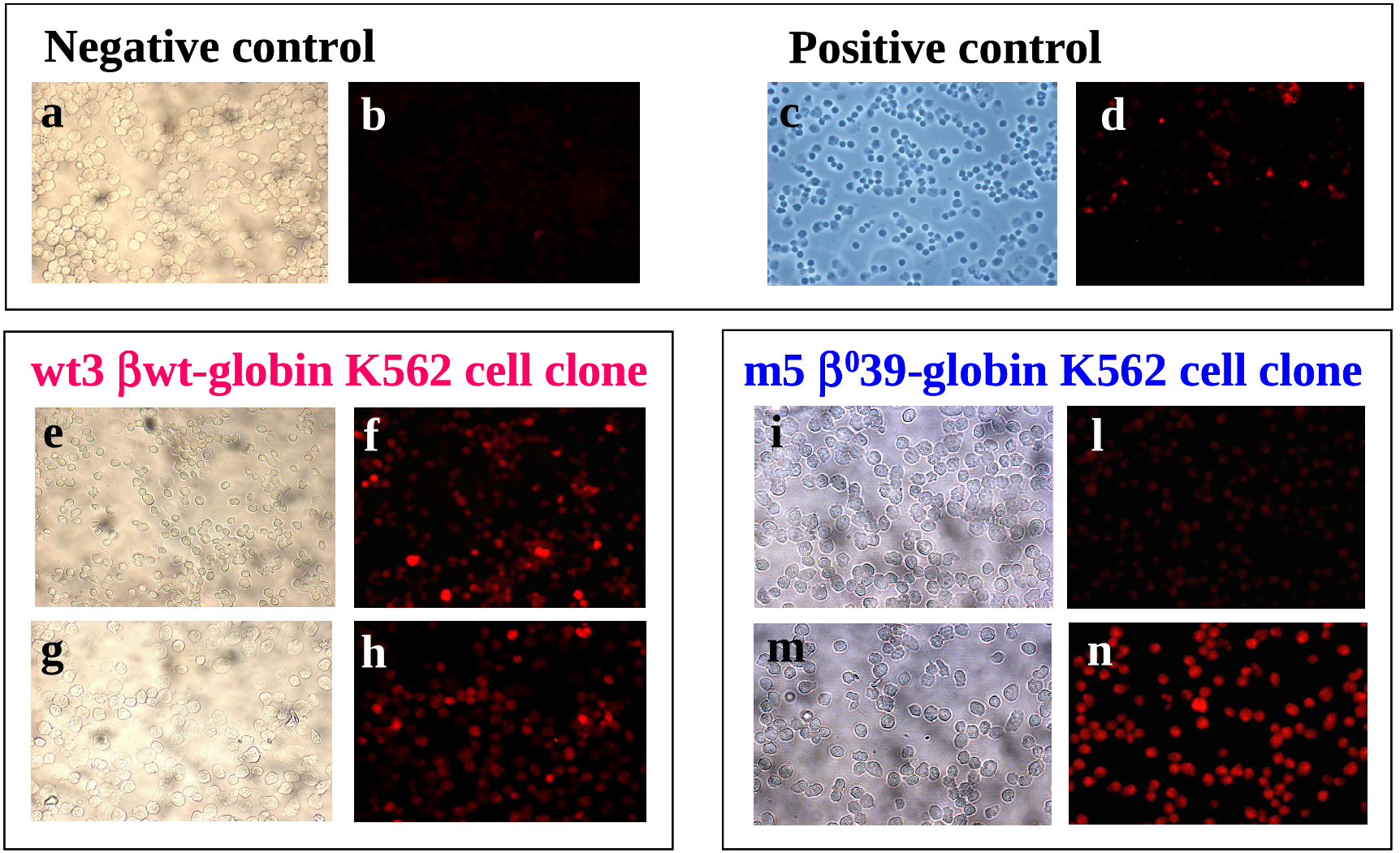


Figure 41 – Immunohistochemistry analysis of wt3 βwt-globin and m5 $\beta^0$39-globin K562 cell clones treated with 400 ng/µl *G418*. In figure are shown the immages obtained at the fluorescence microscope of the cells labelled with *βglobin-PE* and fixed on a slide. **a,b**) negative control: original K562; **c,d**) positive control: erythroid precursor cells of healthy donor; **e-h**) wt3 βwt-globin K562 cell clone untreated (**e,f**) and incubated for 3 days with 400 ng/µl *G418* (**g,h**); **i-n**) m5 $\beta^0$39-globin K562 cell clone untreated (**i,l**) and incubated for 3 days with 400 ng/µl *G418* (**m,n**).

the accuracy requirements for codon-anticodon pairing, suppress stop codons and, instead of chain termination, an amino acid is incorporated into the polypeptide chain. Thus, transcripts containing PTCs are not recognized by the NMD machinery.

In this respect, we performed a quantitative real time RT-PCR assay on the RNA isolated from logarithmically growing wt3 and m5 K562 clones cells, untreated and treated with scalar concentrations of *G418* (100 ng/μl, 200 ng/μl and 400 ng/μl). The reactions were performed as previously explained, using the Genotyping Kit so as to discriminate between the two β-globin transcripts (**table 2**), especially in the case of the m5 $\beta^0$39-globin K562 cell clone, and the GAPDH kit to normalize the data. The quantification of the β-globin gene expression in the treated samples was carried out relatively to the untreated sample, used as standard, and so the β-globin mRNA content in treated cells was determined as a fold respect to control cells (standard).

The obtained data are presented in **figure 42** as averages ± SD of three independent determinations. It is easy to note that the treatment with *G418* determined no significant increase in the β-globin mRNA content in wt3 clone cells, also at the greatest concentration, but a clear increment in the β-globin mRNA content in m5 clone cells, proportionally to the increased concentration of the aminoglycoside. Such result confirms that *geneticin* is able to partially rescue the nonsense $\beta^0$39 transcripts, leading to a production of full length proteins.

Evaluation of the effects of *geneticin* on the protein expression in m5 $\beta^0$39-globin K562 cell clone

Since Manuvakhova et al [2000] demonstrated that the sequence context beyond the termination codon can influence the level of read-through induced by several aminoglycosides and other translation factors present in the complete translation system may also influence the overall efficiency of translation termination, we decided to evaluate if *geneticin* can affect the recognition by the ribosome of premature as well as normal stop codons, resulting in a substantial alteration of the protein expression.

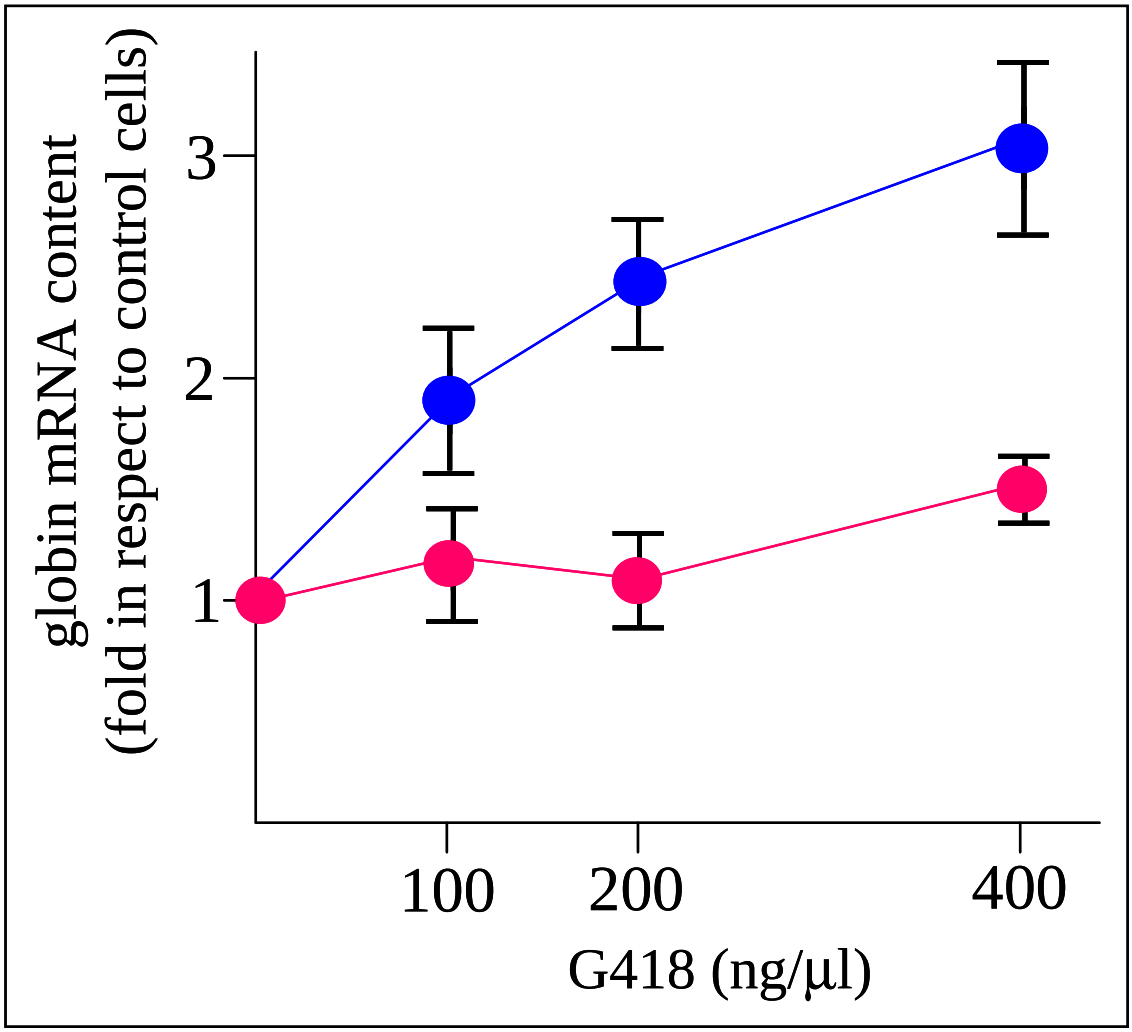


Figure 42 – Effects of increasing concentrations of *geneticin* (*G418*) on the content of β-globin mRNA in wt3 βwt-globin and m5 $\beta^0$39-globin K562 cell clones. The curves show the β-globin content in wt3 βwt-globin (pink circles) and m5 $\beta^0$39-globin (blue circle) K562 cell clones, untreated or incubated for 3 days with increasing concentrations (100 ng/µl, 200 ng/µl and 400 ng/µl) of *G418*. The fold of mRNA content of treated respect to untreated cells was determined by quantitative real time RT-PCR using primers amplifying GAPDH and β-globin mRNA sequences. Two different probe were used for the β-globin mRNA: *Pβwt* and *P$\beta^0$39* contained in the Genotyping Kit and labelled with *FAM* and *VIC*, respectively. The data represent the averages ± SD of three independent determinations.

In this regard, some colleagues performed two-dimensional gel electrophoreses using cytoplasmic extracts from m5 $\beta^0$39-globin K562 clone cells untreated and treated with 400 ng/μl *G418*. Proteins in the extracts were separated first by their isoelectric point using 7 cm long pH 3 – 10 immobilized linear pH gradient strips and then according to the molecular weight on acrylamide gels, which were finally stained with Bio-Safe Coomassie Stain (Bio-Rad, Hercules, CA, USA). For each sample four different gels were carried out in order to confirm that any changes were real and not due to variables in a single electrophoresis. In **figure 43** are shown two representative gels obtained with extracts from m5 K562 clone cells untreated (**a**) and treated with 400 ng/μl *G418* (**b**), where it is possible to note that only 5 out of 350 spots significantly increased or decreased their intensity following treatment with *geneticin*. Two of these spots are reported in **figure 43** (**c and d**). These preliminary results allow us to say that treatment with G418 negligibly affect protein expression in our m5 K562 clone.

GENTAMICIN

Since *geneticin* is poorly used in clinical trials because of its high toxicity, we decided to verify if *gentamicin*, which shows a clear read-though activity [Barton-Davis et al, 1999; Keeling et al, 2001; Du et al, 2002; Wilshanski et al, 2003; Howard et al, 2004] and lower toxicity, allowed a significant translational read-through of the $\beta^0$39-globin mRNA in our cellular model.

Evaluation of the toxicity of *gentamicin*

Of course, as with *geneticin*, we first checked the cellular toxicity and growth inhibition effects of *gentamicin* on our model, so as to identify a range of concentrations to be used without seriously damaging the cells. Since we previously determined that there were not significant differences in treatment

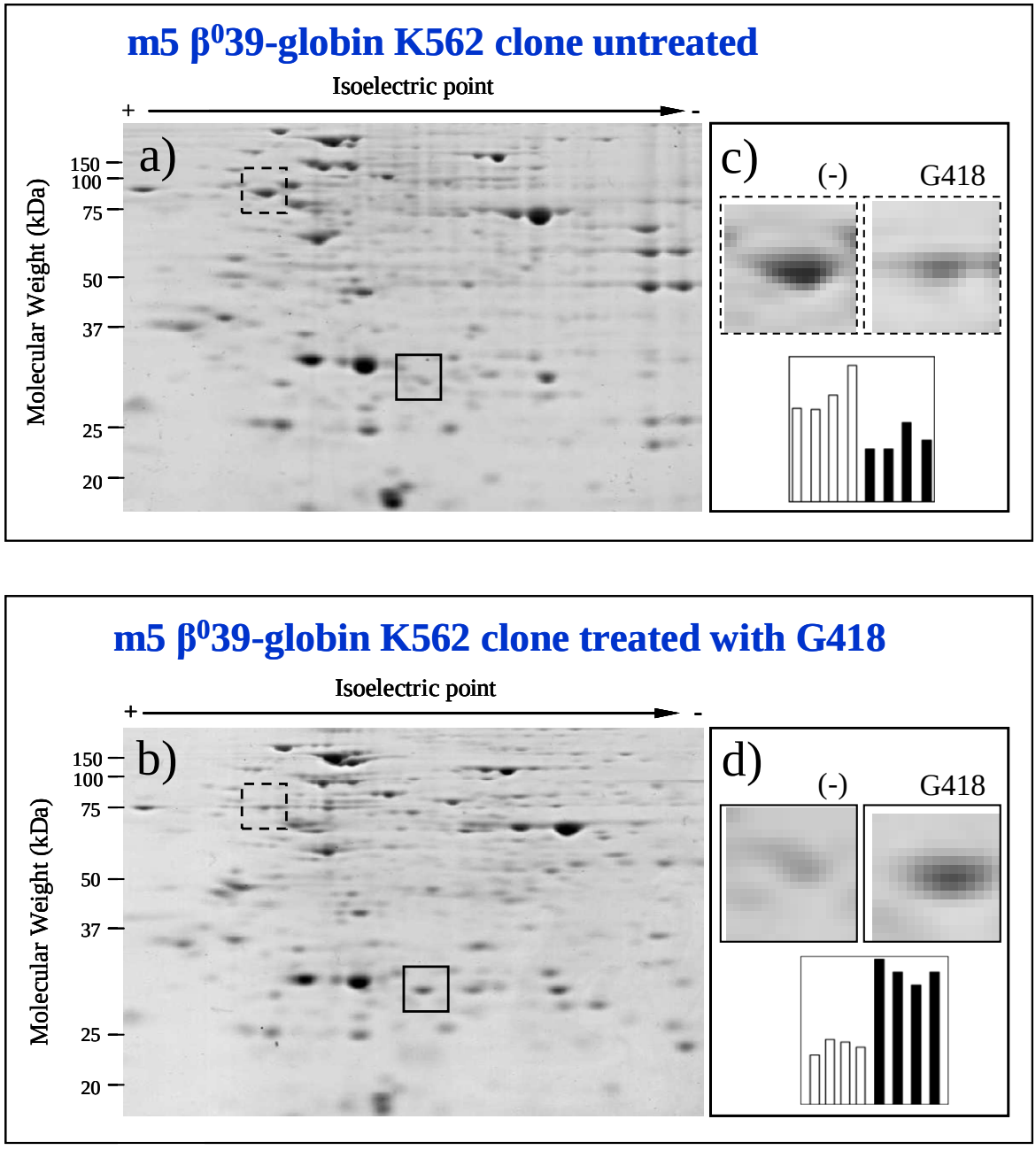


Figure 43 – Proteomic analysis of m5 β⁰39-globin K562 cell clone treated with *genetic* (*G418*). Two-dimensional electrophoresis of the cytoplasmatic extracts of m5 β⁰39-globin K562 cell clone untreated (**a**) and treated with 400 ng/µl *G418* (**b**) stained with Bio-Safe Coomassie Stain. Examples of a decreased (**c**) and increased spot (**d**) after the treatment, identified through the analysis of four different gels. For the assay a 7 cm long pH 3 – 10 immobilized linear pH gradient strips were used.

response between wt3 and m5 K562 clones, we decided to perform a curve of growth inhibition using only m5 $\beta^0$39-globin K562 clone cells, which were plated and treated as previously described, but using scalar concentrations of *gentamicin* from 0 to 2 μg/μl.

The obtained data are summarized in the graph on **figure 44**, where it easy to note that the resulting curve is linear with a minimum slope and so we could not identify a *gentamicin* concentration determining 50% cell growth inhibition (IC50). At this point, since *gentamicin* showed very low toxicity effects on cell growth, we decided to carry out the treatment using concentrations already used by other investigators [Pinotti et al, 2006]: 600 ng/μl, 800 ng/μl, 1 μg/μl, 1.2 μg/μl and 1.4 μg/μl.

Evaluation of the effects of *gentamicin* on β-globin production in original K562, wt3 βwt-globin and m5 $\beta^0$39-globin K562 cell clones

Having regards to the greater sensitivity shown by FACS compared to immunohistochemistry, we decided to only use the flow cytometer to determine the effects on β-globin chain production after treatment with the aminoglycoside.

As done with *geneticin*, also in this case, we first treated the control cells with *gentamicin*, so as to determine if this compound had enhancing effects on gene expression. Original K562 cells were treated with the two greatest concentrations (1.2 μg/μl and 1.4 μg/μl) of *gentamicin* while the wt3 βwt-globin K562 clone cells were treated with all the decided concentrations of the aminoglycoside (600 ng/μl, 800 ng/μl, 1 μg/μl, 1.2 μg/μl and 1.4 μg/μl). These cells were plated, treated for 72 hours, labelled with *βglobin-PE* MoAb and analysed by FACS as previously described. In **figure 45** are summarized the data regarding the proportion of red-fluorescent cells and fluorescence medians obtained analysing the original K562 (**a**) and wt3 clone (**b**) cells untreated and treated with *gentamicin*. No significant differences are demonstrated between untreated and treated original K562 cells, meaning that *gentamicin* is not able to induce gene expression, in these cells. Whereas in the case of the wt3 K562 cell clone, it is possible to note that the proportion of red-fluorescent cells varies independently from the *gentamicin*

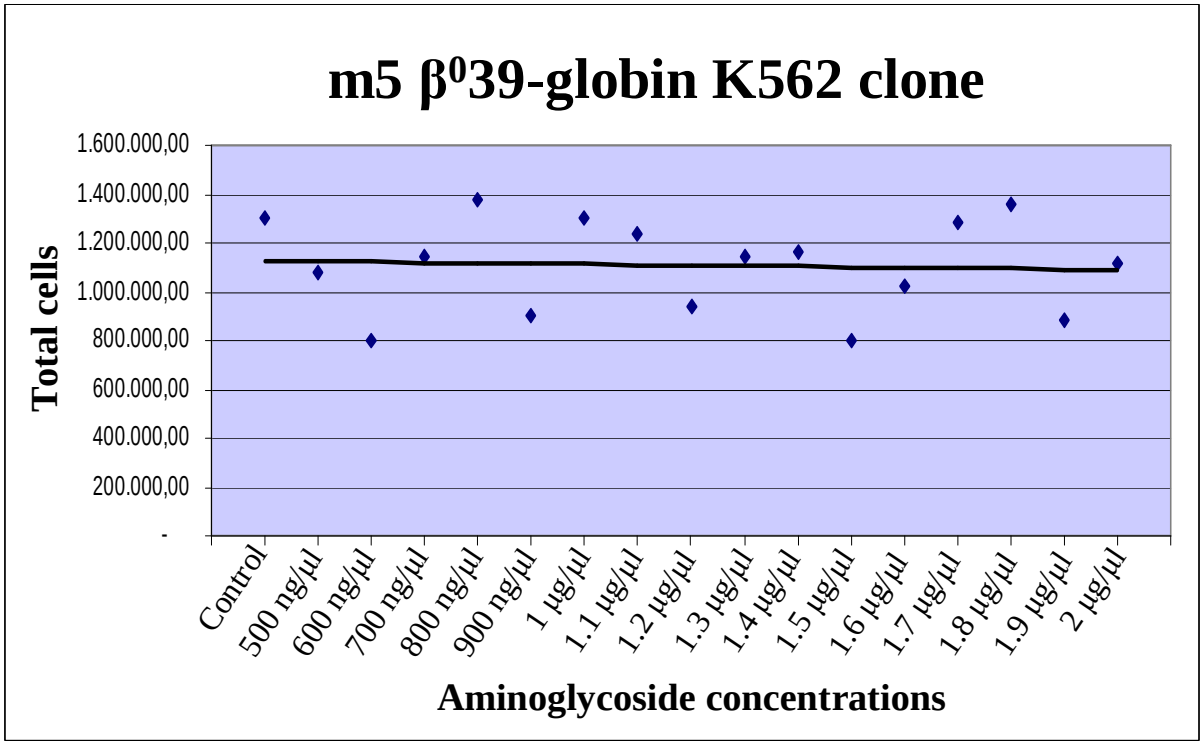


Figure 44 – Inhibition effects of *gentamicin* on m5 β⁰39-globin K562 cell clone. The cells were incubated for 3 days at increasing concentrations of *gentamicin* and, then, counted using the Bürker chamber. On the x-axis are reported the concentrations of *gentamicin* while on the y-axis the total cells counted.

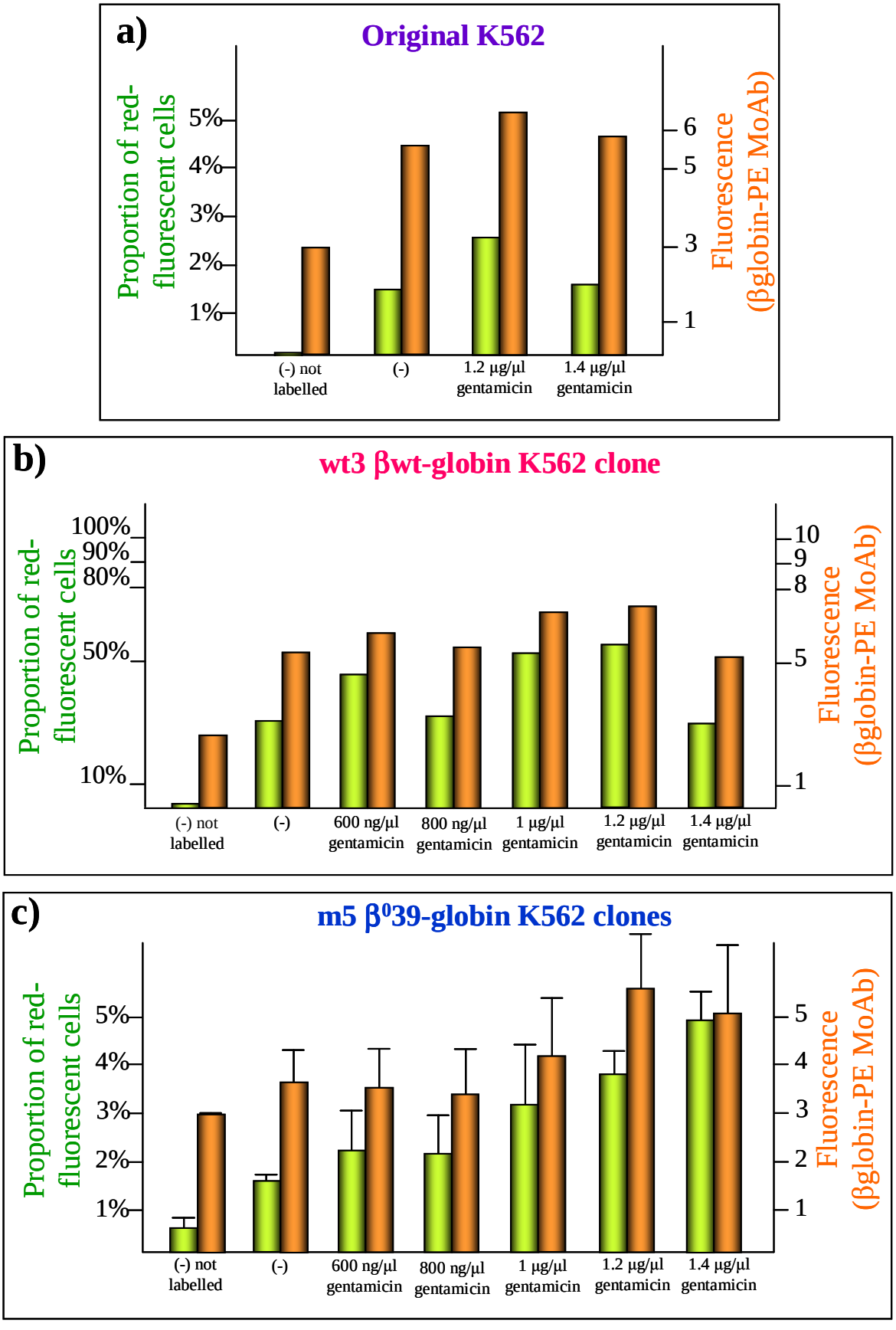


Figure 45 – Effects of increasing concentrations of *gentamicin* on the β-globin production in original K562, wt3 βwt-globin and m5 $\beta^0$39-globin K562 cell clones. On the histograms are presented both the proportion of red-fluorescent cells (green boxes), corresponding to the proportion of β-globin containing cells, and the median of the emitted red fluorescence (orange boxes) of the original K562 (**a**) untreated or treated with high concentrations of gentamicin (1.2 μg/μl and 1.4 μg/μl), wt3 βwt-globin (**b**) and m5 $\beta^0$39-globin (**c**) K562 cell clones, untreated or incubated for 3 days with increasing concentrations (600 ng/μl, 800 ng/μl, 1 μg/μl, 1.2 μg/μl and 1.4 μg/μl) of *gentamicin*. Cells were labelled with the *βglobin-PE* MoAb and analyzed by FACS. On the right part of the histograms are shown the data refering to the K562 (**a**), clone wt3 (**b**) and m5 (**c**) untreated and unlabelled. The data represent the averages ± SD of three indipendent determinations.

concentration, which lead us to believe that the increased presence of β-globin containing cells in same treated samples should not depend on a possible induction effect on gene expression of the compound. This conclusion is confirmed by the fact that the median of the fluorescence emitted by the clone cells do not vary significantly after treatment with *gentamicin*.

At this point, we treated the m5 $\beta^0$39-globin K562 clone cells with scalar concentrations of *gentamicin* (600 ng/μl, 800 ng/μl, 1 μg/μl, 1.2 μg/μl and 1.4 μg/μl) with the aim to verify a possible translational correction effect of this aminoglycoside on the nonsense $\beta^0$39 mutation. The cells were plated, treated, labelled with *βglobin-PE* MoAb and analysed by FACS as formerly explained. The data shown in **figure 45c** are the averages ± SD of three independent determinations and indicate a clear increase in the proportion of red-fluorescent cells and fluorescence intensity after treatment with *gentamicin*, proportionally to the concentration increase. As expected, this increment shown to be much smaller than that obtained after treatment with *G418*. So, we can confirm that geneticin is a stronger translational read-through inducer respect to *gentamicin*.

OTHER AMINOGLYCOSIDES

Given the existence of other types of aminoglycosides, different from those previously analyzed, we decided to test the translational corrective action of some of them on our $\beta^0$39-thalassemia cellular model. We analysed the effects on the β-globin chains production of seven aminoglycosides (*neomycin*, *paromomycin*, *kanamycin*, *amikacin*, *streptomycin*, *tobramycin*, *higromycin*), which showed almost no corrective activity. Only three of them (*amikacin*, *streptomycin* and *tobramycin*) are reported as examples.

Evaluation of the toxicity of *amikacin*, *streptomycin* and *tobramycin*

As done with *gentamicin*, we carried out an inhibition curve for each aminoglycoside to check their toxicity and inhibition effects on cellular growth and find their IC50, using the m5 $\beta^0$39-globin K562 cell clone. The cells were

plated as previously described, incubated with scalar concentrations of aminoglycoside, from 0 to 1.2 μg/μl, and counted after 3 days. The data obtained by the treatment with *amikacin*, *streptomicyn* and *tobramycin* are summarized in the graphs in **figure 46**, where it easy to note that the treatment with the three aminoglycosides (*amikacin* (**a**), *streptomycin* (**b**) and *tobramycin* (**c**)), did not induce any inhibition in cell growth. For this reason, we decided to use these compounds at the following concentrations (similar to *gentamicin*): 800 ng/μl, 1 μg/μl and 1.2 μg/μl.

Evaluation of the effects of *amikacin, streptomycin* and *tobramycin* on β-globin production in original K562, wt3 βwt-globin and m5 $\beta^0$39-globin K562 cell clones

Original K562, wt3 βwt-globin and m5 $\beta^0$39-globin K562 clones cells were plated, incubated for 3 days with scalar concentrations of *amikacin*, *streptomycin* and *tobramycin* (800 ng/μl, 1 μg/μl and 1.2 μg/μl), labelled with *βglobin-PE* MoAb and analysed by FACS as formerly explained. The maximum effects obtained after the treatment of the m5 clone cells with these aminoglycosides are summarized in **figure 47a**, where are reported the proportion of red-fluorescent cells and fluorescence medians of the m5 clone cells untreated and treated with 1 μg/μl *amikacin*, 800 ng/μl *streptomycin* and 800 ng/μl *tobramycin*, the most effective concentrations. These results show that *amikacin* and *tobramicin* could not induce any significant increase in the proportion of β-globin containing cells, whereas *streptomycin* determined a slight augment of red-fluorescent cells, demonstrating a weak translational read-through activity. In the table of **figure 47b** are compared the proportions of β-globin containing cells, determined by FACS analysis, of the original K562, wt3 βwt-globin and m5 $\beta^0$39-globin K562 clones cells untreated and treated with *geneticin*, *gentamicin*, *amikacin*, *streptomycin* and *tobramycin*, at the most effective concentration. It is possible to note that the treatment with each aminoglycoside had no effect on the proportion of β-globin containing cells in control cells (original K562 and wt3 clone) whereas some of them, *geneticin* in particular, induced a clear increase in the production of

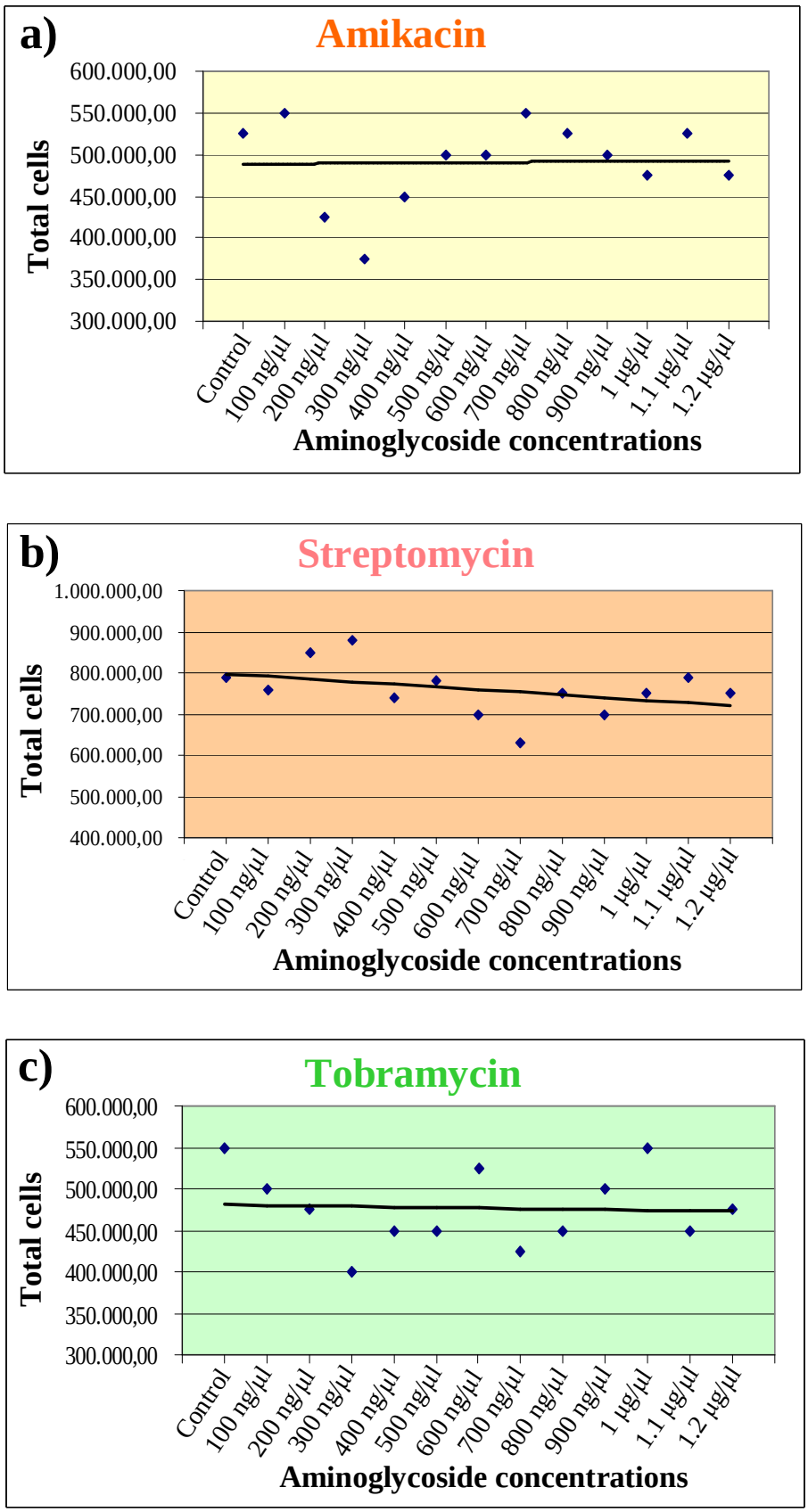
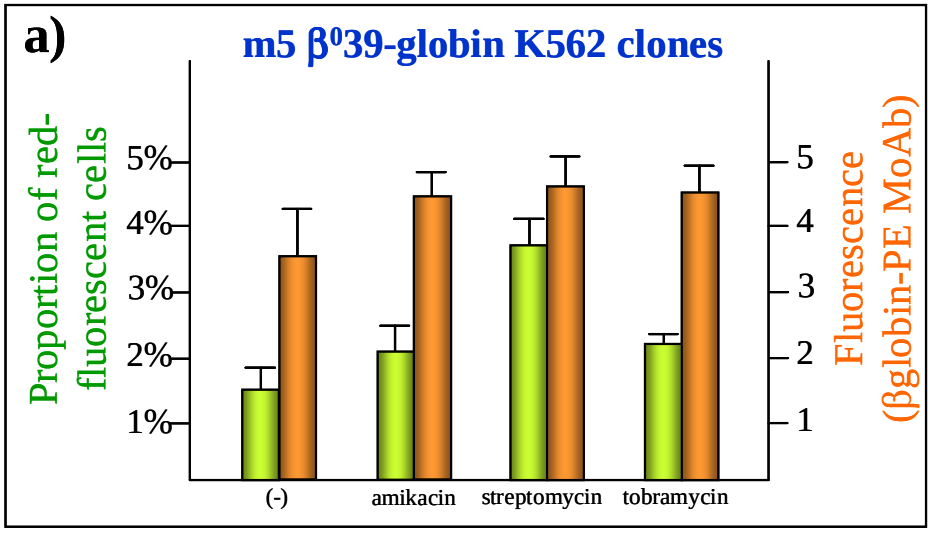


Figure 46 – Inhibition effects of *amikacin*, *streptomycin* and *tobramycin* on m5 $\beta^0$39-globin K562 cell clone. The cells were incubated for 72 hours at increasing concentrations of amikacin (**a**), streptomycin (**b**) and tobramycin (**c**) and, then, counted using the Bürker chamber. On the x-axis are reported the aminoglycoside concentrations while on the y-axis the total cells counted.

a)



b)

Aminoglycoside	Original K562	wt3 βwt-globin K562 clone	m5 β⁰39-globin K562 clone
(-)	1.6 ± 0.3	73.7 ± 18.3	1.5 ± 0.5
geneticin	1.5 ± 0.4	85.3 ± 4.5	51.5 ± 22.3
gentamicin	1.8 ± 0.1	73.4 ± 7.5	5.65 ± 0.7
amikacin	1.5 ± 0.4	67.3 ± 8.5	2.1 ± 0.5
streptomycin	1.4 ± 0.2	64.5 ± 11.4	3.74 ± 0.5
tobramycin	1.1 ± 0.2	70.1 ± 6.8	2.2 ± 0.2

Figure 47 – Effects of aminoglycosides on the β-globin production in original K562, wt3 βwt-globin and m5 β⁰39-globin K562 cell clones. a) In the histogram are presented both the proportion of red-fluorescent cells (green boxes), corresponding to the proportion of β-globin containing cells, and the median of the emitted red fluorescence (orange boxes) of the m5 β⁰39-globin K562 cell clone, untreated or incubated for 3 days with 1 μg/μl *amikacin*, 800 ng/μl *streptomycin* and 800 ng/μl *tobramycin*. Cells were labelled with the *βglobin-PE* MoAb and analyzed by FACS. **b)** In the table are summarized the most significant FACS results obtained with the treatment of original K562, wt3 βwt-globin and m5 β⁰39-globin K562 cell clones with *geneticin* (*G418*), *gentamicin*, *amikacin*, *streptomycin* and *tobramycin*. Results are presented as proportion of β-globin containing cells, detected by FACS analysis. The data represent the averages ± SD of three indipendent determinations.

β-globin chains in our $\beta^0$39-thalassemia cellular model. From these data, we could deduce that aminoglycosides are able to interact with the recognition of premature $\beta^0$39 termination codon by the ribosome, leading to a production of full length proteins.

ISOLATION OF ERYTHROID PRECURSOR CELLS FROM $\beta^0$39-THALASSEMIA PATIENTS

Once determined the ability of some aminoglycosides to rescue $\beta^0$39-globin transcrips from NMD and induce a transcriptional read-through of the premature termination codon with the subsequent production of full length β-globin chains in our $\beta^0$39-thalassemia model, we decided to test the read-though activity of the most active aminoglycoside studied (*geneticin*) on erythroid progenitors isolated from $\beta^0$39-thalassemia patients. For this reason, we had to identify some thalassemia patients carrying the $\beta^0$39 mutation in homozygous, sequencing their β-globin gene alleles.

Genotype characterization of β-thalassemia patients

Following the sign of informed consent, a few ml of blood were collected from β^0-thalassemia patients, so as to extract genomic DNA to be used for sequencing β-globin gene. The DNA extraction was performed using the *QIAGEN Blood & Cell Culture DNA Mini Kit* (QIAGEN), starting from 1 ml of whole blood. The genomic DNAs were used as templates for PCR reactions, performed using two primers designed by us upstream (*BGF*) and downstream (*BGR*) the β-globin gene (**table 1**) and purified with *Microcon® YM-100* (Millipore Corporation). The PCR products were then utilised as templates for sequence reactions using primers amplifying the β-globin gene. The fragments obtained in such reactions were then purified by *Multiscreen*™ (Millipore Corporation), separated by electrophoresis on polyacrylamide gel and analysed using the sequencer *ABI PRISM™ 377 DNA Sequencer* (Perkin-Elmer Applied Biosystems). **Table 1** lists all the primers used, which made it possible to sequence entirely the β-globin gene. In **figure 48a** are

a)

……agacc tcaccctgtg gagccacacc ctagggttgg ccaatctact cccaggagca
gggagggcag gagccagggc tgggcataaa agtcagggca gagccatcta ttgcttacat
ttgcttctga cacaactgtg ttcactagca acctcaaaca gacaccatgg tgcacctgac
tcctgaggag aagtctgccg ttactgccct gtggggcaag gtgaacgtgg atgaagttgg
tggtgaggcc ctgggcaggt tggtatcaag gttacaagac aggtttaagg agaccaatag
aaactgggca tgtggagaca gagaagactc ttgggtttct gataggcact gactctctct
gcctattggt ctattttccc acccttaggc tgctggtggt ctacccttgg accTagaggt
tctttgagtc ctttggggat ctgtccactc ctgatgctgt tatgggcaac cctaaggtga
aggctcatgg caagaaagtg ctcggtgcct ttagtgatgg cctggctcac ctggacaacc
tcaagggcac ctttgccaca ctgagtgagc tgcactgtga caagctgcac gtggatcctg
agaact……

Blue: exons Reverse primer *BGR1*

T: $\beta^0$39 mutation

b)

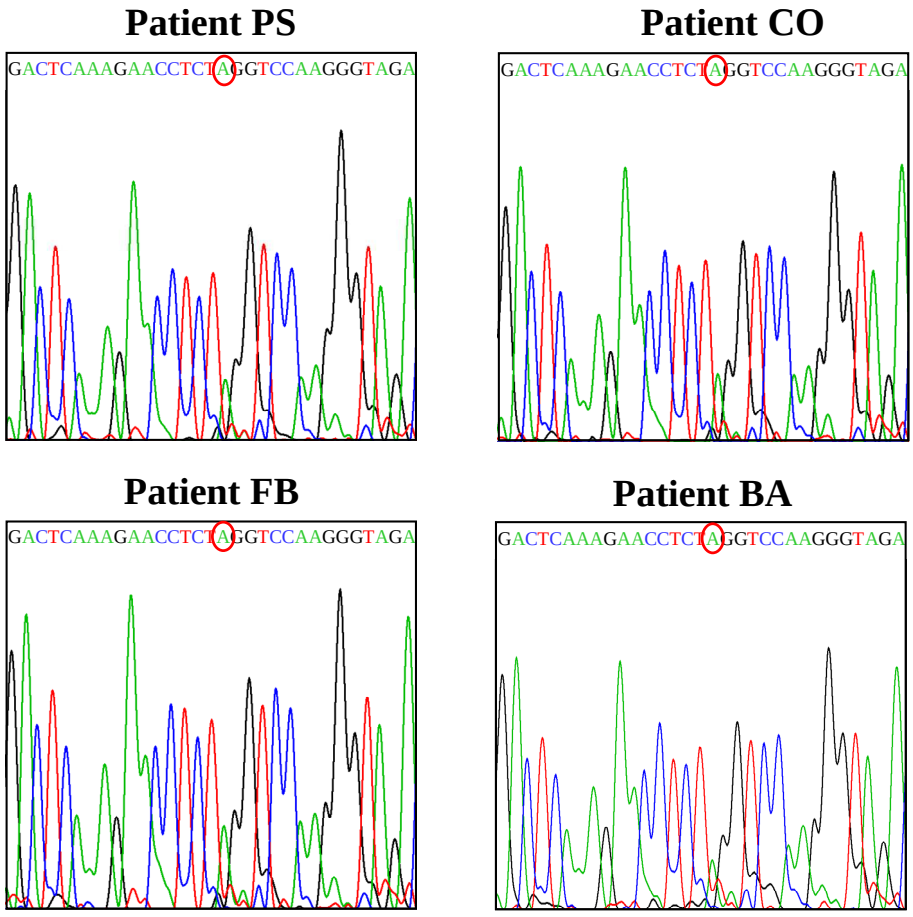


Figure 48 – Sequencing of the second exon of the β-globin gene of $\beta^0$39 thalassemia patients. a) Sequence of the region of $\beta^0$39-globin gene corresponding to 1st and 2nd exons. Box highlights the sequence of the reverse primer used for the sequencing. **b)** Elettropherograms, obtained by the sequencing performed using the reverse primer *BGR1*, of the region containing the $\beta^0$39 (C→T) mutation, highlighted by the circle, of four $\beta^0$39-thalassemia patients.

shown the nucleotide sequence of the first and second exons of the β-globin gene and the primer used to detect the presence of the $\beta^0$39 mutation, which was found as unique point mutation in 4 patients. The obtained chromatograms of the region containing the mutation are reported in **figure 48b**. It is possible to note a single peak in correspondence to the first base of codon 39, indicating either homozygosity for the $\beta^0$39 mutation or heterozygosity for that mutation and a β-globin gene deletion. The homozygosity for the nonsense mutation was confirmed by Dr. Nicoletta Bianchi which performed a quantitative real time PCR to evaluate the number of β-globin genes present into the cells.

Isolation and culture of erythroid precursor cells of $\beta^0$39-thalassemia patients

To isolate erythroid progenitors from $\beta^0$39-thalassemia patients, 20 ml of blood were collected just before the blood transfusion, so as to limit the presence of healthy donor cells. The precursor cells were isolated by centrifugation for gradient density on Lympholyte-H and, after several washing, plated in phase I medium, consisting of α-MEM medium supplemented with 10% FCS, 10% conditioned medium (CM), obtained from cell cultures of bladder cancer (cells 5637), rich in haematopoietic growth factors except the erythropoietin (EPO), and 1 μg/ml cyclosporine A, which has the function to suppress lymphocyte activation and proliferation. In this phase the vast majority of cells are small lymphocytes and only a minute fraction of the cell population are various hemopoietic progenitors. After 7 days of phase I, medium was changed with the phase II medium, consisting of α-MEM supplemented with 30% FCS, 10% deionized BSA, 0.01 mM β-mercaptoethanol, 0.001 mM dexamethasone, 10 ng/ml Stem Cell Factor and 1 U/ml human erythropoietin (EPO), which stimulates the erythroid differentiation. Early in the phase II lymphocytes continue to present the most abundant cell type. Proerythroblasts begin to be discernible after a few days as large, round, smooth cells. As these cells multiply they form clusters. During the intermediate phase, lymphocytes gradually give way to an increasing

proportion of proerythroblasts and basophilic normoblasts, which when indisturbed form aggregates consisting of hundreds of cells. In late phase II, erythroid cells continue to proliferate and mature into polychromatic and orthochromatic Hb-containing normoblasts. Within the intermediate and late phases, the absolute number and proportion of small lymphocytes diminished significantly [Pope et al, 2000].

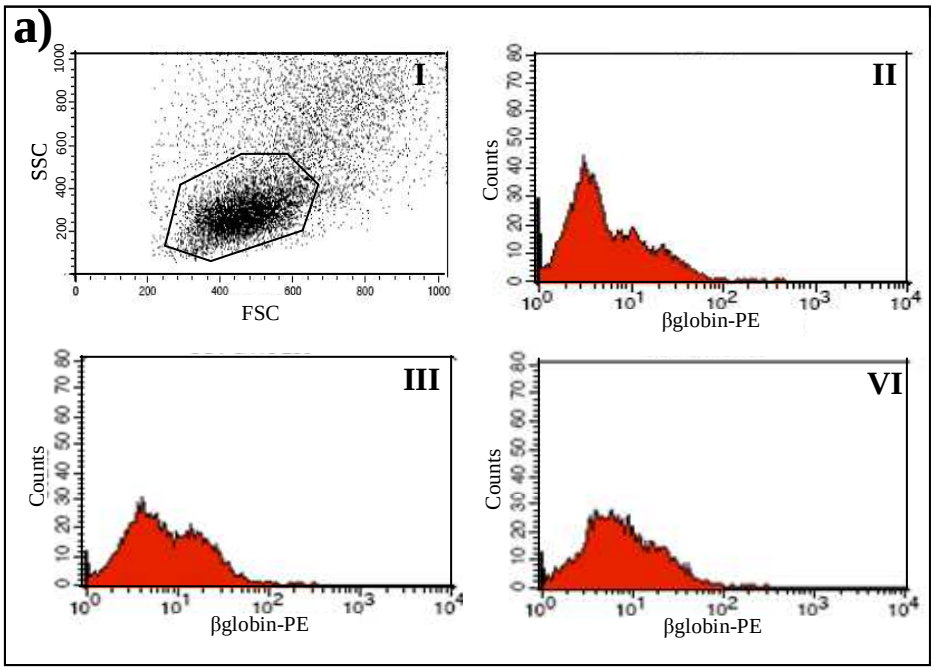
USE OF $\beta^0$39 ERYTHROID PROGENITORS TO CHARACTERIZE THE READ-THROUGH ACTIVITY OF AMINOGLYCOSIDES

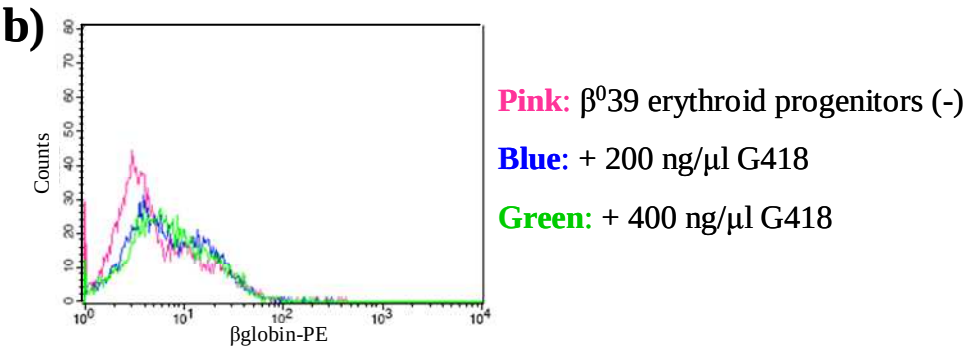
To verify the translational read-through activity of *geneticin* on erythroid progenitors isolated from $\beta^0$39-thalassemia patients, we decided to use the two greatest concentrations of G418 previously used with the cellular model (200 ng/μl and 400 ng/μl). The reason was that we could always have a limited amount of precursor cells so we could not waste them performing an inhibition curve.

Evaluation of the effects of *geneticin* on β-globin production in $\beta^0$39 erythroid precursor cells

Erythroid progenitors, after 7 days of phase II, were collected, plated at 10^6 cells/ml, treated with 200 ng/μl and 400 ng/μl *G418* for 3 days, labelled with *βglobin-PE* MoAb and analysed by FACS as previously described for K562 clones.

In **figure 49a** are reported a representative *FSC* - *SSC* cytogram of untreated $\beta^0$39 erythroid progenitors, which shows a heterogeneous population due to the variability of cells present in this type of cell cultures, as formerly explained. For this reason, we decided to create a gate so as to analyse only the majority of cells, with small size, constituted mostly of erythroid precursor cells. Always in **figure 49a** are shown representative histograms of red fluorescence obtained by FACS analysis of $\beta^0$39 erythroid progenitors untreated (**II**) and treated with 200 ng/μl (**III**) and 400 ng/μl (**IV**) *G418*. In the histogram of untreated cells, it is possible to note that the majority of the cells constitute a peak below the fluorescence





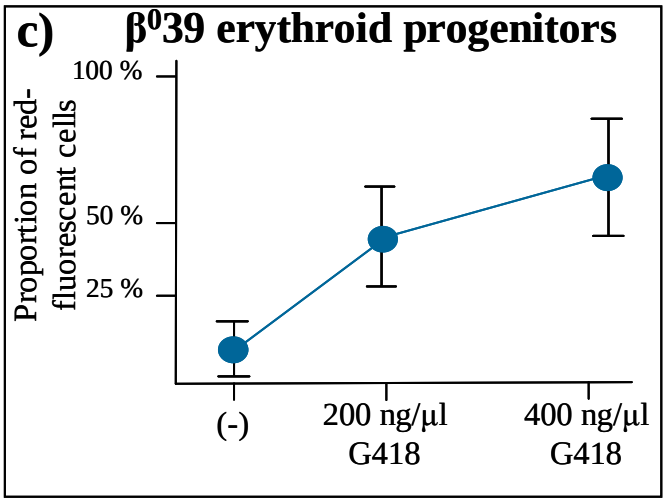


Figure 49 – Effects of increasing concentrations of *geneticin* (*G418*) on the β-globin production in β⁰39 erythroid precursor cells. a) Representative FACS analysis of β⁰39 erythroid progenitors treated with scalar concentrations (200 ng/µl and 400 ng/µl) and labelled with *βglobin-PE* MoAb. In panel (**a**) are reported: diagram of cells distribution based on the morphological parameters *FSC* and *SSC* (**I**); histograms relating the emitted fluorescence to the number of emitting cells of untreated (**II**) and treated (**III**, 200 ng/µl; **IV**, 400 ng/µl) cells. **b**) Overlay of the peaks obtained in the three histograms. **c**) Graph summarizing the proportion of red-fluorescent cells, corresponding to the proportion of β-globin containing cells, of β⁰39 erythroid progenitors untreated and with scalar concentrations of *G418*. The data represent the averages ± SD of three indipendent determinations.

threshold of 10^1, indicating that they are not red-fluorescent cells, whereas a small proportion of cells are slightly shift beyond that threshold, probably due to the presence of contaminating red blood cells in the culture. Comparing all the histograms of red fluorescence demonstrated, after treatment with *geneticin*, a clear shift of the peak of negative cells beyond the threshold of 10^1, indicating an increase in β-globin chains. To facilitate the visualization of this shift, we set up an unique histogram for the red fluorescence with overlay of all the peaks (**figure 49b**). Moreover, in **figure 49c** is reported a graph summarizing the proportion of red-fluorescent cells, corresponding to β-globin containing cells, obtained in three independent treatments of $\beta^0$39 erythroid progenitors with scalar concentrations of *G418*. Such proportion increased significantly and dependently to the *geneticin* concentration and could confirm the ability of such aminoglycoside to induce translational read-through of $\beta^0$39 nonsense mutation, determining a production of β-globin chains.

Evaluation of the effects of *geneticin* on adult haemoglobin (HbA) production in $\beta^0$39 erythroid precursor cells

As aminoglycosides could induce incorporation of a correct as well wrong amino acid at a premature termination codon, producing a full length protein, it could be that such protein is not functional, if the incorporated amino acid produces structural changes.

To verify if β-globin chains produced by $\beta^0$39 erythroid precursor cells treated with *G418* were functional and able to generate HbA, we decided to perform a HPLC analysis, which can give us information about the proportion of HbA respect to the total Hbs content. $\beta^0$39 erythroid progenitors were therefore plated and treated with 200 ng/μl and 400 ng/μl G418, as previously described. After 3 days, they were collected, washed and lysated by incubation for 15 minutes in water at 4°C. The nuclei and membranes were collected in the bottom of a tube by centrifugation and the supernatant, containing Hbs, was loaded on the cation – exchange column *Syncropack CCM 103/25*. In **figure 50a** are shown representative chromatograms obtained by HPLC analysis of $\beta^0$39 erythroid

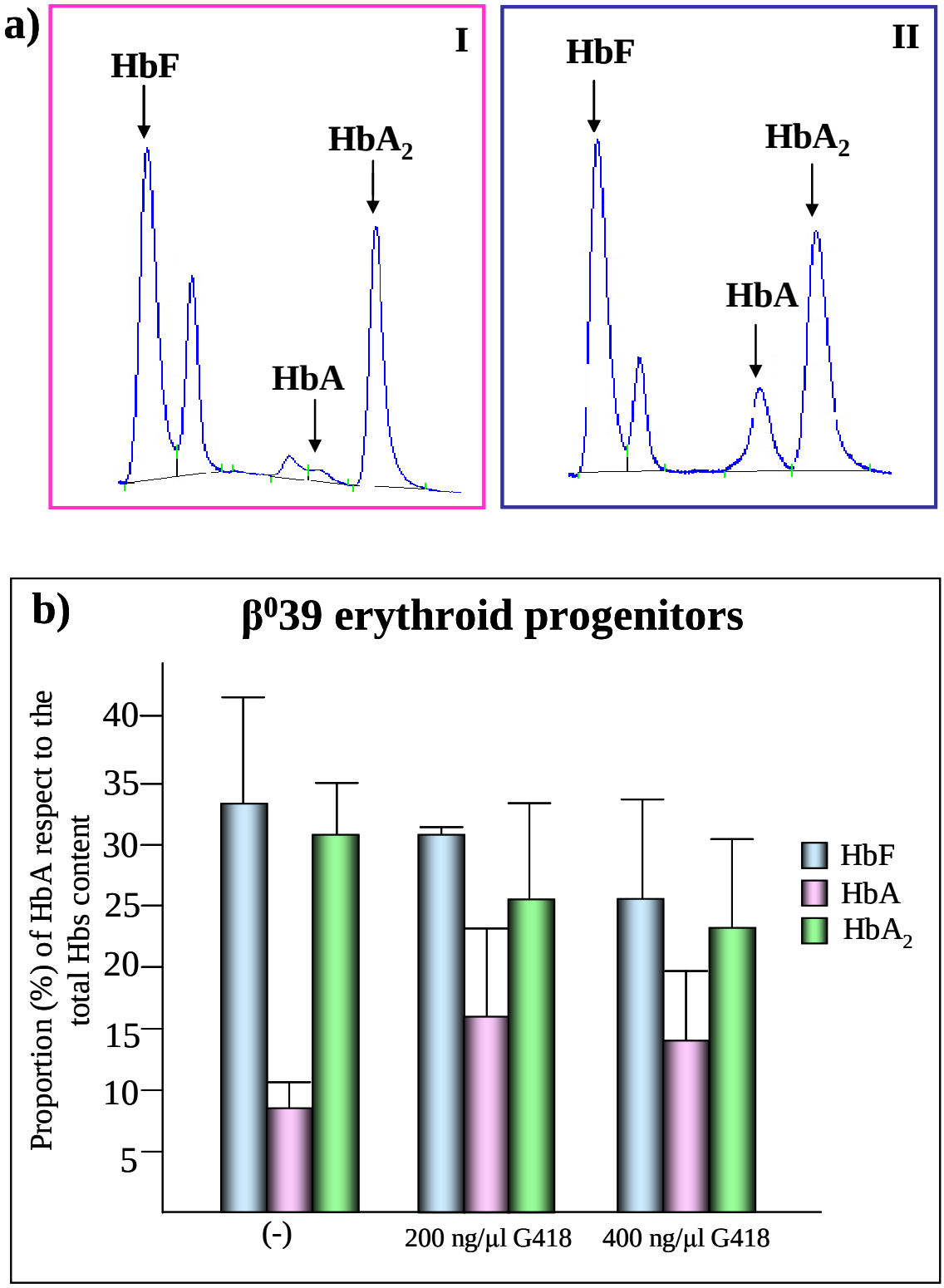


Figure 50 – Effects of increasing concentrations of *geneticin* (*G418*) on the HbA production in $\beta^0$39 erythroid precursor cells. a) Representative chromatograms obtained by HPLC analysis of $\beta^0$39 erythroid progenitors, untreated (**I**) or treated with 400 ng/µl G418 (**II**). **b**) Histogram summarizing the proportion of fetal haemoglobin (HbF), adult haemoglobin (HbA) and type 2 adult haemoglobin (HbA_2) respect to the total Hbs content of the $\beta^0$39 erythroid cells treated with scalar concentrations of *G418* (200 ng/µl and 400 ng/µl). The data represent the averages ± SD of three indipendent determinations.

progenitors untreated (**I**) and treated with 400 ng/μl G418 (**II**). Such histograms show a clear increase of the area percentage of the peak corresponding to HbA after treatment with the aminoglycoside, indicating an increase in the proportion of HbA respect to the total Hbs content and so a production of β-globin chains, able to generate normal HbA. This result was confirmed in three independent experiments, whose data are presented in **figure 50b**, where it is possible to note a clear increase in HbA proportion and a corresponding decrease in the proportion of HbF and HbA_2.

Evaluation of the effects of *geneticin* on the content of β-globin mRNA in $\beta^0$39 erythroid precursor cells

As done with m5 $\beta^0$39-globin K562 cell clone, we demonstrated the capacity of *G418* to rescue the PTC containing transcripts from the degradation by NMD pathway, performing a quantitative real time RT-PCR assay on the RNA isolated from $\beta^0$39 erythroid progenitors, untreated and treated with 200 ng/μl and 400 ng/μl *G418*. The reactions were performed as previously described, using primers amplifying β-globin and GAPDH mRNA sequences (**table 2**). The quantification of the β-globin gene expression in the treated samples was carried out relatively to the untreated sample, used as standard, and so the β-globin mRNA content in treated cells was determined as a fold respect to control cells (standard). In this case, we decided to use the *PFβ* and *PRβ* primers and *Pβ* probe lists in **table 2**, because we did not need to discriminate between βwt and $\beta^0$39 transcripts as $\beta^0$39 erythroid progenitors only produce mutated β-globin mRNA.

The obtained data are presented in **figure 51** as averages ± SD of three independent determinations and show a strong increment in the β-globin mRNA content, proportionally to the increase of the aminoglycoside concentration. Such result confirms that *geneticin* is able to partially rescue the nonsense $\beta^0$39 transcripts, leading to a production of full length proteins.

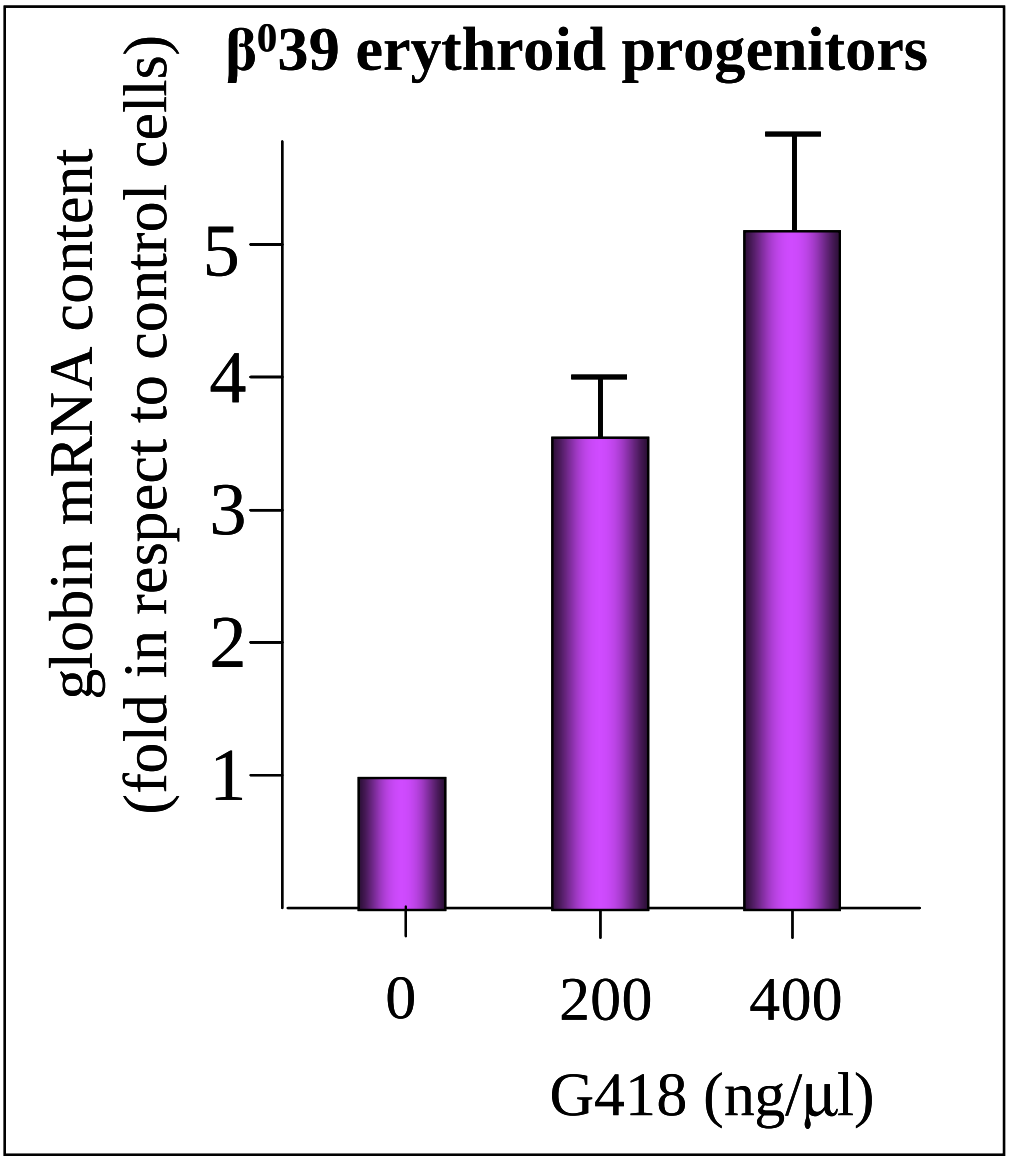


Figure 51 – Effects of increasing concentrations of *geneticin* (*G418*) on the content of β-globin mRNA in β^{0}39 erythroid precursor cells. The histogram shows the β-globin content in β^{0}39 erythroid progenitors, untreated or incubated for 3 days with increasing concentrations (200 ng/µl and 400 ng/µl) of *G418*. The fold of mRNA content of treated respect to untreated cells was determined by quantitative real time RT-PCR using primers amplifying GAPDH and β-globin mRNA sequences.

USE OF pCCL.βwt.PGW LENTIVIRAL VECTOR FOR GENE THERAPY OF β^0-THALASSEMIA

At present, there are two approaches to directly overcome disease caused by nonsense mutations: translational read-through induced by aminoglycosides, already widely characterized in this work, and gene therapy, which, by introducing an exogenous gene into diseased cells, could establish the production of the protein of interest, permanently correcting the disease. On the contrary of read-through approach which requires life-long administrations, gene therapy could be a definitive cure for a recessive disease, as those caused by nonsense mutations. Unfortunately, many problems remained to be solved, as gene silencing and activation of oncogenes.

With the aim to verify the possible use of our lentiviral vector for gene therapy and compare the results with those obtained with aminoglycosides treatment, we decided to transduced erythroid progenitors isolated from homozygous $\beta^0$39-thalassemia patients with *pCCL.βwt.PGW*. Such vector, as already described, presents some important characteristics which make the integration of the transgene into the genome safe and efficient, minimizing the effects of the surrounding chromatin, which could silence its expression, and the enhancing effects of the promoters contained into the vector on oncogenes. The absence of gene silencing was demonstrated in our βwt-globin K562 cell clones, cultured for more than 3 months without a significant reduction in the expression of GFP and β-globin chains.

Moreover, we decided to test the combined effects of gene therapy with the lentiviral vector and fetal haemoglobin induction by treatment with *mithramycin*, a potent HbF inducer [Bianchi et al, 1999; Fibach et al, 2003], to verify if the concomitant increases in HbA and HbF could lead to a haemoglobin content similar to that of a heterozygous subject.

Erythroid precursor cells were isolated from $\beta^0$39-thalassemia patients and cultured in two-phase liquid cultures as previously described. At the 7^{th} day of phase II the cells were transduced with *pCCL.βwt.PGW*. The progenitors were incubated with the volume of lentiviral particles corresponding to a MOI equal to

0.3 and the transduction agent polybrene, for 6 hours at 37°C in humidified atmosphere of 5% CO_2/air, mixing well every 30 minutes, and then plated at 10^6 cells/ml. We decided to use a very low MOI so as to obtain a single infection for each cell, limiting the introduction of exogenous sequences and mimicking the physiological situation. In some experiments the cells were also treated with 30 nM *mithramycin* to induce fetal haemoglobin production. We chose to use this concentration of mithramycin, because it demonstrated to be effective to induce γ-globin expression [Bianchi et al, 1999].

After 5 days the cells were collected and divided in aliquots to be used for: 1) analysis of vector integration by quantitative real time PCR; 2) analysis of the α-, β- and γ-globin expression by quantitative real time RT-PCR; 3) analysis of HbA and HbF by HPLC assay.

Analysis of integration of *pCCL.βwt.PGW* vector in transduced $\beta^0$39 erythroid precursor cells

The evaluation of integration of *pCCL.βwt.PGW* vector in transduced $\beta^0$39 erythroid progenitors permitted us to compare the increases in β-globin mRNA and HbA content with the number of β-globin transgenes integrated into the cellular genome.

In this respect, we extracted and used the genomic DNA as template for real time PCR reactions as formerly explained. Such reactions were performed using *PFβ* and *PRβ* primers and *Pβ* probe, *UBC* and *C21orf104 kits* for allelic quantification (Applied Biosystems) amplifying β-globin, UBC and C21orf104 DNA sequences respectively, the latter two used as reference genes. The choice of the suitable gene for the normalization of PCR analysis in each experiment was based on the slope of the standard curves, namely we chose the gene whose slope was the most similar to the β-globin one. Of course, the DNA extracted from untreated cells was used as standard.

In **figure 52a** are presented the fold of β-globin transgenes integrated into the genomic DNA of erythroid progenitors isolated from three different $\beta^0$39-thalassemia patients and transduced with *pCCL.βwt.PGW* vector (MOI: 0.3) or

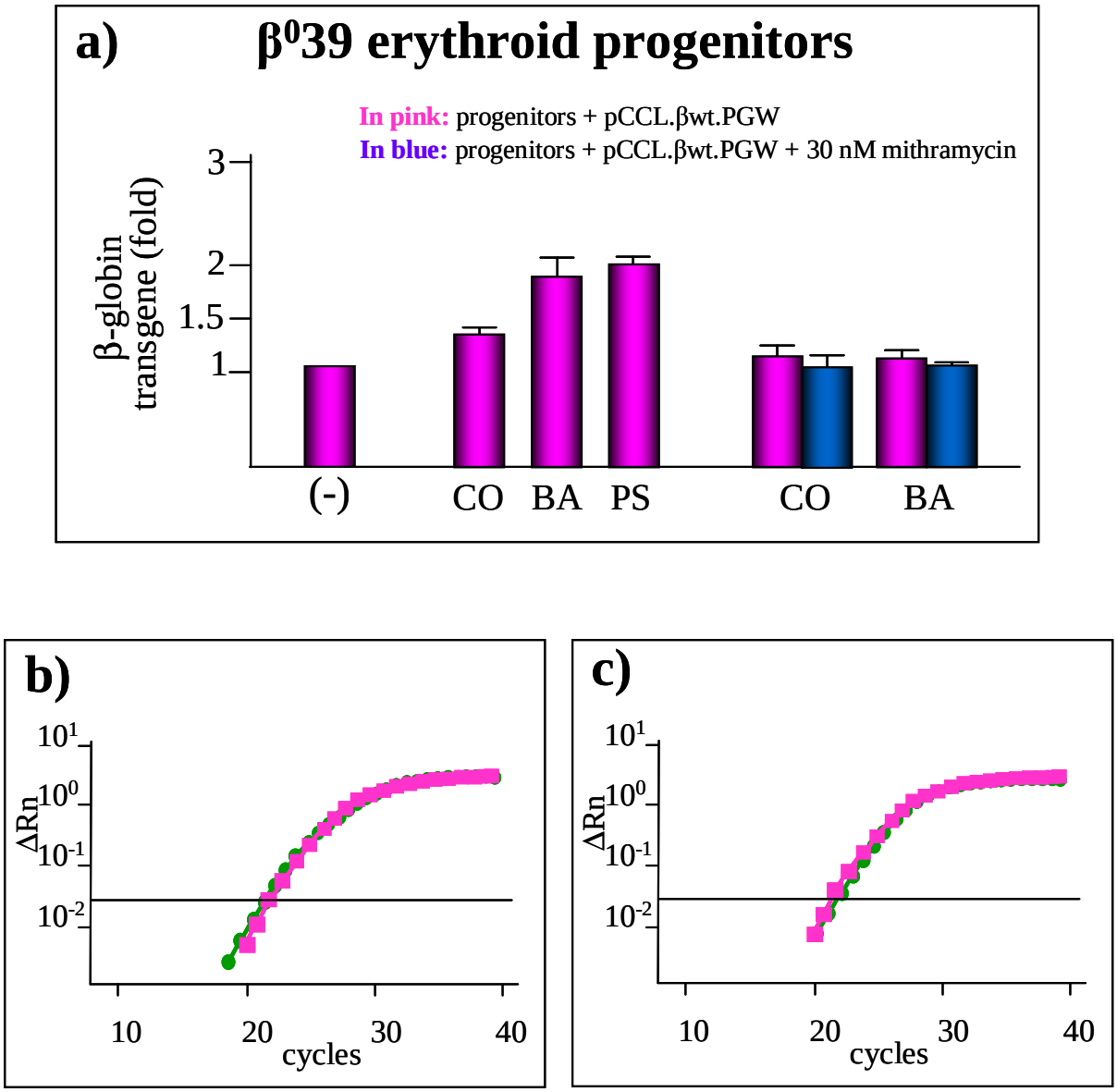


Figure 52 – Analysis of integration of the *pCCL.βwt.PGW* vector in erythroid progenitors from β⁰39-thalassemia patients. a) On the histogram are reported the fold of β-globin transgenes integrated into the genomic DNA of β⁰39 erythroid progenitors transduced with *pCCL.βwt.PGW* (MOI: 0.3) (pink boxes) and β⁰39 erythroid progenitors transduced with the lentiviral vector (MOI: 0.3) and treated with 30 nM *mithramycin* (blue boxes) (average ± SD o three indipendent determinations). **b,c**) Representative profiles of quantitative real time PCR analysis performed on DNA extracted from erythroid precursor cells from patient CO, untreated (green circles) and transduced with pCCL.βwt.PGW (pink squares), using primers amplifying C21orf104 (**b**) and β-globin (**c**) DNA sequences.

transduced with the lentiviral vector and treated with 30 nM *mithramycin*. As it is notable from the histogram, with a MOI equal to 0.3 we obtained at most a 2 time increase in the content of β-globin genes. In **figure 52** are also shown two representative profiles of quantitative real time PCR analysis performed on DNA extracted from erythroid progenitors isolated from patient CO, untreated (**b**) and transduced with pCCL.βwt.PGW vector (**c**), using primers amplifying C21orf104 and β-globin DNA sequences.

Analysis of the α-, β- and γ-globin mRNA content in transduced $\beta^0$39 erythroid precursor cells, treated or not with mythramycin

In β-thalassemia, the synthesis of normal α-globin chains from the unaffected α-globin genes continues as normal, resulting in the accumulation within the erythroid precursors of excess unmatched α-globin. The free α-globin chains are not able to form viable tetramers and instead precipitate in red cell precursors in the bone marrow forming inclusion bodies. They are responsible for the extensive intramedullary destruction of the erythroid precursors and hence the ineffective erythropoiesis that underlies all β-thalassemias [Thein, 2005]. For this reason, it is

very important, for an effective therapy, that the increase in β-like globin genes expression does not coincide with an increase in α-globin genes expression, so as to reduce the precipitation of the α-globin chains, ameliorating the viability of red blood cells.

In this respect, we decided to evaluate the expression of α-, β- and γ-globin genes, the last one mainly because of the treatment with *mithramycin*, a potent HbF inducer, by quantitative real time RT-PCR. The RNA was extracted, reverse transcribed using both *oligodT* and *random* primers and used as template for real time PCR reactions as previously described. **Table 2** lists all the primers and probes used: *PFα*, *PRα* and *Pα*, *PFβ*, *PRβ* and *Pβ*, *PFγ*, *PRγ* and *Pγ* for the amplification and quantification of α-, β- and γ-globin mRNA, respectively. All the probes were labelled with the fluorochrome FAM. As reference genes we used GAPDH or r18S, choosing the suitable one for each experiment depending on the slope of standard curves.

In **figure 53** is presented a histogram showing the α-, β- and γ-globin mRNA content, expressed as fold in respect to control cells (untreated $\beta^0$39 erythroid precursor cells), in $\beta^0$39 erythroid progenitors transduced with *pCCL.βwt.PGW* or trasduced with the vector and treated with 30 nM *mithramycin*. The data represent the averages ± SD of three independent determinations. From these results, it is possible to note that the expression of α-globin gene did not change significantly among the three samples, while the content of β-globin mRNA increased strongly in the progenitors transduced with the lentiviral vector, diminishing after treatment with *mithramycin*, which instead induced a significant increase in the expression of γ-globin genes. In **figure 54** are shown representative profiles of quantitative real time RT-PCR analysis performed on RNA extracted from erythroid precursor cells isolated from $\beta^0$39-thalassemia patient CO, untreated and transduced with *pCCL.βwt.PGW* (**a**) or transduced with the lentiviral vector and treated with 30 nM *mithramycin* (**b**), using primers amplifying r18S, α-, β- and γ-globin mRNA sequences.

Analysis of HbA and HbF content in transduced $\beta^0$39 erythroid precursor cells, treated or not with *mithramycin*

The $\beta^0$39 progenitors were collected, washed and lysated as formerly explained and the supernatant was loaded on the cation – exchange column *Syncropack CCM 103/25*, to perform a HPLC analysis. In **figure 55** are presented chromatograms obtained by HPLC analysis of erythroid precursor cells isolated from CO (**a**) and BA (**b**) $\beta^0$39-thalassemia patients untreated and transduced with *pCCL.$\beta^0$39.PGW* or tranduced with the vector and treated with 30 nM *mithramycin*. In the case of patient CO (**figure 55a**), it is possible to note a strong increase in the area percentage of the HbA peak in the cells transduced with the lentiviral vector, diminishing after treatment with *mithramycin*, which however induced a slight increase in the HbF content. On the contrary, in the case of patient BA (**figure 55b**), there were a significant increase in the area percentage of the HbA peak following transduction with *pCCL.$\beta^0$39.PGW*, which did not decrease

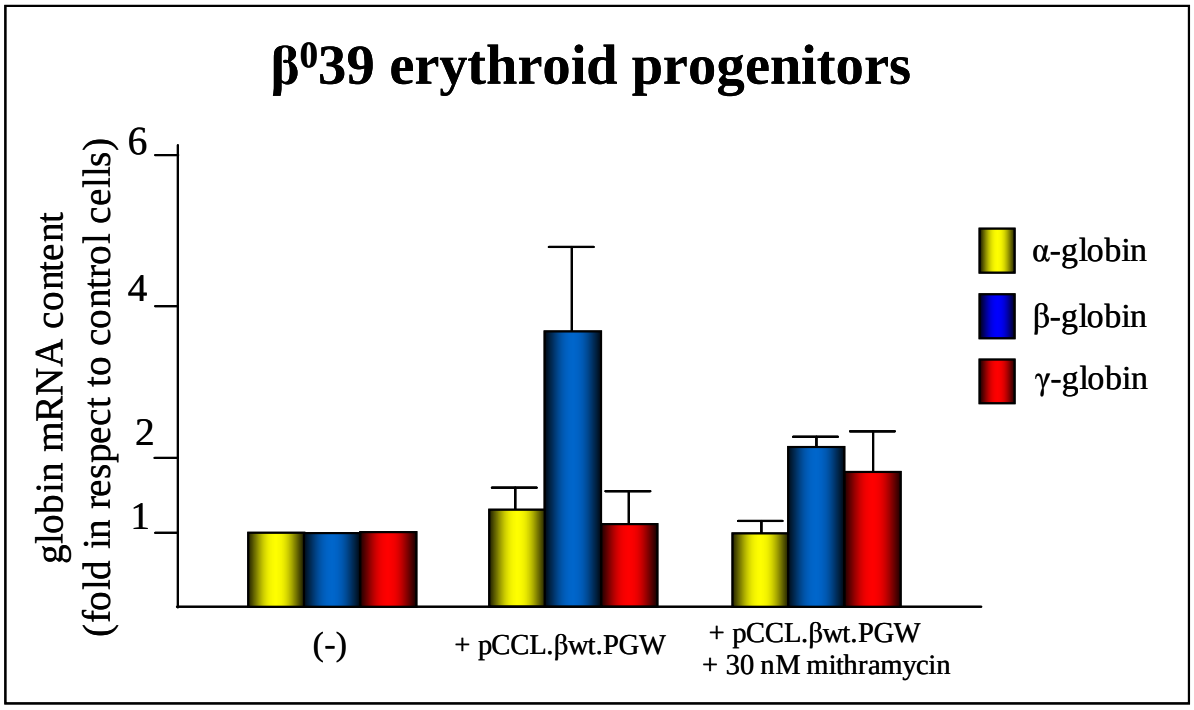


Figure 53 – Analysis of α-, β- and γ-globin expression in erythroid progenitors from β^{0}39-thalassemia patients transduced with *pCCL.βwt.PGW* and treated or not with 30 nM *mithramycin*. On the histogram are reported the α- (yellow boxes), β- (blue boxes) and γ-globin (red boxes) mRNA content (fold in respect to control cells) of β^{0}39 erythroid progenitors transduced with *pCCL.βwt.PGW* (MOI: 0.3) and β^{0}39 erythroid progenitors transduced with the lentiviral vector (MOI: 0.3) and treated with 30 nM *mithramycin* (average ± SD o three indipendent determinations). The fold of mRNA content of treated versus untreated cells was determined by quantitative real time RT-PCR using primers amplifying r18S, α-, β- and γ-globin mRNA sequences.

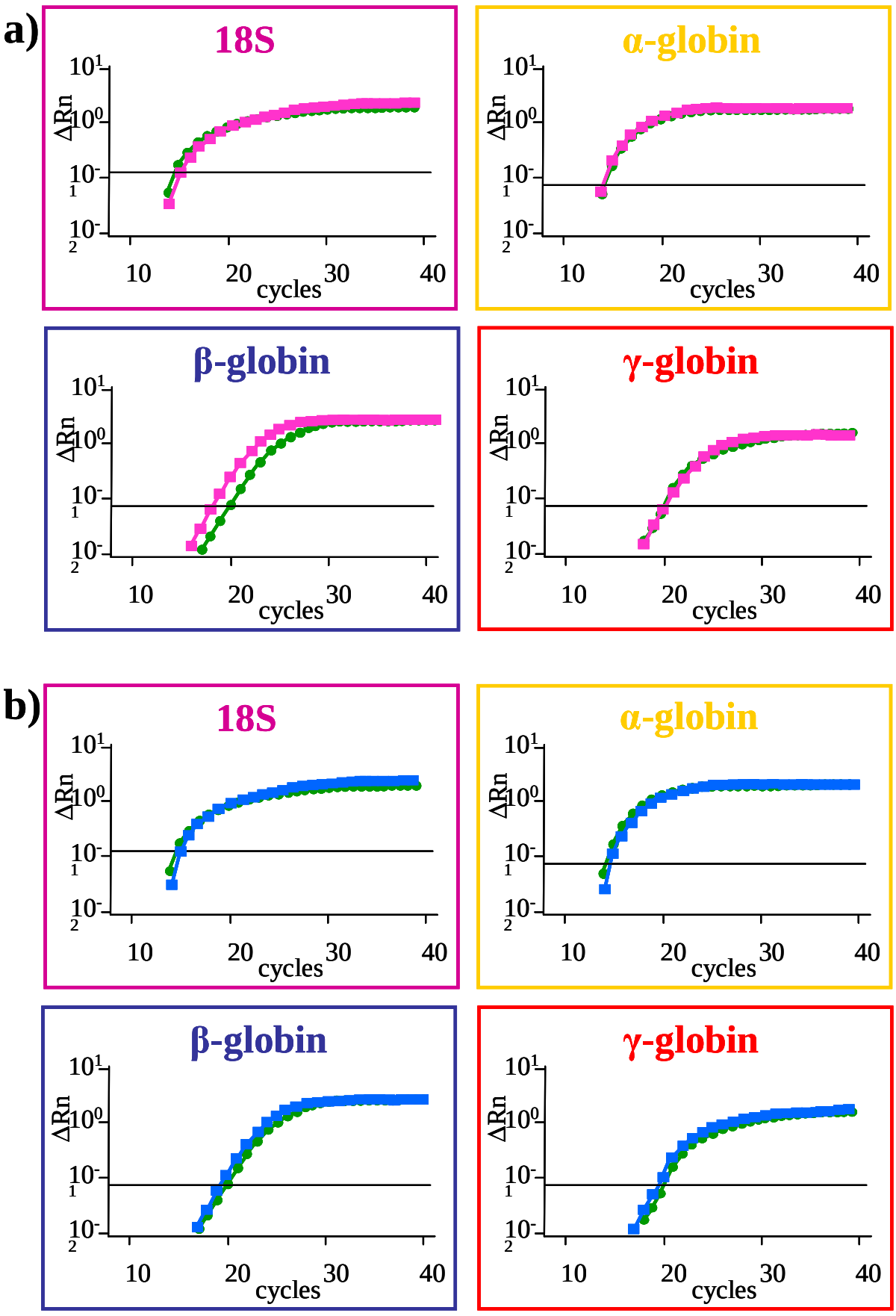


Figure 54 – Representative profiles of quantitative real time RT-PCR analysis of α-, β- and γ-globin mRNA from β^{0}39 erythroid progenitors transduced with *pCCL.βwt.PGW* and treated or not with 30 nM *mithramycin*. Profiles of quantitative real time RT-PCR analysis performed on RNA extracted from erythroid precursor cells from patient CO, untreated (green circles) and transduced with *pCCL.βwt.PGW* (MOI: 0.3) (pink squares) (**a**) or transduced with the lentiviral vector (MOI: 0.3) and treated with 30 nM *mithramycin* (blue squares) (**b**), using primers amplifying r18S, α-, β- and γ-globin mRNA sequences.

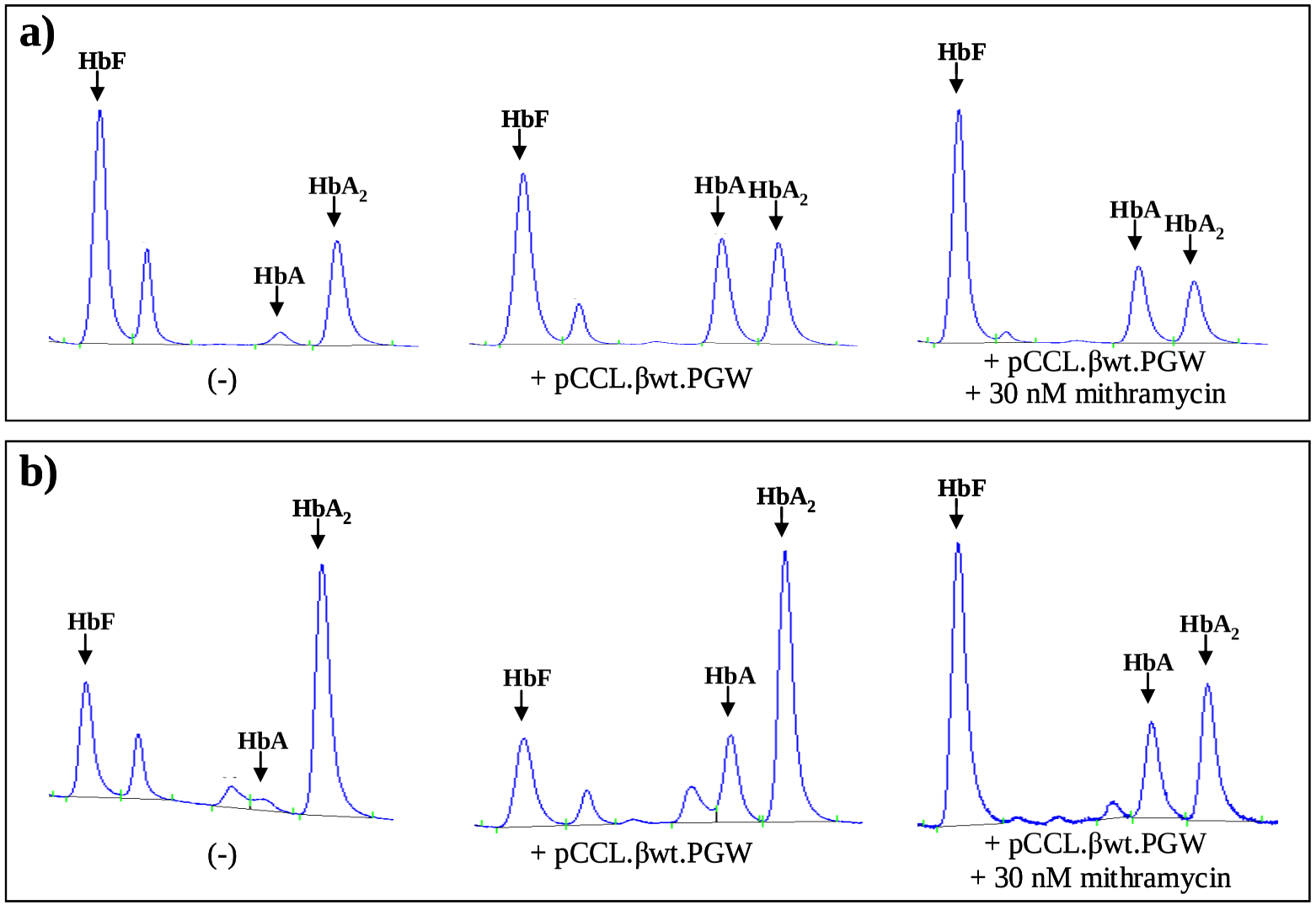


Figure 55 – Chromatograms obtained by HPLC analysis of erythroid progenitors, from two $\beta^0$39 thalassemia patients, transduced with *pCCL.βwt.PGW* and treated or not with 30 nM *mithramycin*. In figure are shown the chromatograms obtained by HPLC analysis of erythroid procursor cells isolated from CO (**a**) and BA (**b**) $\beta^0$39-thalassemia patients and treated with the *pCCL.βwt.PGW* alone or in association with 30 nM *mithramycin*. HbF, fetal haemoglobin; HbA, adult haemoglobin; HbA_2, type 2 adult haemoglobin.

after treatment with *mithramycin*, and a strong increase of the proportion of HbF after treatment with the HbF inducer.

DISCUSSION

The β-thalassemia is an autosomal recessive disease characterized by a reduced or absent synthesis of β-globin chains, caused by the presence of mutations within or near the β-globin gene. Among these is the C → T substitution at the first base of codon 39 in the second exon of the gene. Such mutation, known as $\beta^0$39, belongs to the nonsense mutations, which lead to the formation of stop codons, causing the premature termination of translation. The β-thalassemia caused by the presence in homozygosity of this mutation shows a very serious phenotype, because the synthesis of β-globin chains is totally absent.

Actually, there are two approaches to directly overcome diseases caused by nonsense mutations: gene therapy, meaning introduction of an exogenous gene into diseased cells, and translational read-through induced by aminoglycosides, which decrease the accuracy of translational elongation and reduce the efficacy of the translation termination machinery. With the aim to study and verify the effects and clinical utility of such approaches, we decided to develop lentiviral vectors containing the βwt- or $\beta^0$39-globin gene, to be used the first in gene therapy and both to produce cellular models, suitable for studying aminoglycosides activity *ex vivo*.

In this work, we therefore produced two *ex vivo* cellular models expressing βwt- or $\beta^0$39-globin mRNA and used them to characterized the read-through activity of aminoglycosides. Once verified such activity in our cellular models, we decided to use aminoglycosides to treat erythroid precursor cells isolated from $\beta^0$39-thalassemia patients, which were also used to study the effects of the introduction of exogenous βwt-globin genes, through transduction with the lentiviral vector.

The proposed experimental strategy included the use of a third generation lentiviral vector, which ensures the integration of the β-globin gene in the cellular genome and its expression in a stable way over time. To insert the $\beta^0$39-globin gene into this vector, we carried out a series of cloning, starting from a lentiviral

vector lacking of some important sequences for the stable expression of the transgene (*dTNS9*) [Sadelain, 2002], where we introduced the $\beta^0$39-globin gene, taken from an expression plasmid (*pSL1180DT+$\beta^0$39*), achieved through site-directed mutagenesis. This gene with its promoter was finally transferred into the final construction, obtaining the *pCCL.$\beta^0$39.PGW* vector, which contains a reduced version of the β cluster control region LCR, which allows efficient expression of the gene. Similarly, we introduced the βwt-globin gene, taken from the original *dTNS9* vector, into the third generation lentiviral vector, obtaining the *pCCL.βwt.PGW*.

The insert, present in both the viral vectors, was then sequenced with the purposes to check and determine the presence of the $\beta^0$39 mutation in the *pCCL.$\beta^0$39.PGW* vector, verify that the ends of the insert were intact and ensure the absence of any other type of mutation in both the *pCCL.βwt.PGW* and *pCCL.$\beta^0$39.PGW* vectors. The results of sequencing clearly revealed the absence of anomalies in the nucleotide sequence, except the expected $\beta^0$39 mutation in *pCCL.$\beta^0$39.PGW*.

Once obtained the lentiviral vectors, as being used as infecting agents, they were incapsidated into viral particles using 293T packaging cell line. These cells were transfected with the *pCCL.βwt.PGW* or *pCCL.$\beta^0$39.PGW* lentiviral vector and three other constructs, called helper vectors, which provide structural and regulatory elements, not present in the lentiviral vector and necessary for its expression. The lentiviral vector integrates into the 293T cellular genome thanks to its two *LTR* (*long terminal repeat*) sequences. Then, through the transcriptional machinery of the cell, the viral mRNA is produced, and thanks to the proteins encoded by the three helper vectors, is transported from the nucleus to the cytoplasm and incapsidated. It is obtained, thus, a large amount of viral particles which must be titrated, to be used in the cell infection.

The purpose of the titration is to determine the transducing power of a solution of viral particles, information essential for subsequent infections. For this reason, HeLa cells were transduced with scalar quantities of each virus and then subjected either to FACS analysis to determine the viral titre based on the GFP expression (*GFP titre*), whose gene is also contained in the vector, and to quantitative real

time PCR assay, to determine the titre based on the number of viral vectors integrated into the cellular genome (*DNA titre*).

Once performed the FACS analysis, it was necessary to decide which sample was the most suitable for calculating the *GFP titre*. Since it is essential, for obtaining an accurate titre, that in the majority of the cells took place a single infection, our choice fell on the sample with the percentage of fluorescent cells that mostly approaches the 30%. This decision was based on the assumption that, when only 3 out of 10 cells are fluorescent, in all probability they were infected by a single virus, namely a transducing unit. The obtained viral titres were 3 x 10^5 TU/ml (trasducing units/ml) and 1.8 x 10^5 TU/ml for the *pCCL.βwt.PGW* and *pCCL.$\beta^0$39.PGW* viral particles respectively, low viral titres compared to values found in the literature [Zhang et al, 2004].

In the quantitative real time PCR assay, the genomic DNA extracted from transduced Hela cells were amplified using probes for the β-globin and ^{A}γ-globin genes, the latter used as reference gene. Analyzing the Ct, we were able to calculate, in infected cells, the fold of β-globin compared to ^{A}γ-globin genes, a fundamental information to determine the *DNA titre*, which was 6 x 10^5 TU/ml and 4 x 105 TU/ml for the *pCCL.βwt.PGW* and *pCCL.$\beta^0$39.PGW* viral particles respectively. Such titres were obtained using data on the same samples selected for the *GFP titres*, so that we could make a more accurate comparison with them, previously calculated, because the titre of a lentiviral vector varies according to the conditions of the infective process: the volume of inoculum, the number of target cells, cell type and viability/susceptibility, vector exposure time for uptake and vector half life. Moreover, the proportion of phospholipids present in the cell membranes, whose biosynthesis and degradation are very much dependent on cell type and positions in the cell cycle and/or metabolic activity, can affect the success of an infection, because of their binding to the VSV-G protein. The variation of these parameters can result in a difference in the viral titre in different aliquots of the same viral stock, hence the viral titre is not a value that exactly indicates how many infectious units per ml were added to the cells [Zhang et al, 2004].

By comparing the *GFP titres* with the corresponding *DNA titres*, it is possible to note that the latter were two times the first, because of the limitations of the

flow cytometer, which include the fact that not always a transduced cell emits green fluorescence at levels that exceed the FACS threshold of detection and even assuming that with a percentage of fluorescent cells equal to 30%, in most cases, a single infection happens, this does not exclude the possibility of multiple infections. Such limits may cause an underestimation of the viral titre. Precisely for this reason we decided to use for subsequent infections the *DNA titre*, a choice recommended also in the literature, where the *DNA titre* has been confirmed more precise compared to the *GFP titre* [Sastry et al., 2002].

Once calculated the titre of the viral particles containing the *pCCL.βwt.PGW* or *pCCL.β^{0}39.PGW* vector, we transduced K562 cells, chosen for our models because they express the β-globin gene at very low levels both in their uninduced state as well as after erythroid differentiation stimulated by a variety of chemical inducers, such as cytosine arabinoside [Cortesi et al, 1999], mithramycin [Bianchi et al, 1999; Fibach et al, 2003], rapamycin [Mischiati et al, 2004; Fibach et al, 2006]. To do this infection, we based on the MOI (multiplicity of infection), parameter decided arbitrarily by the researcher, used to predict the viral infectivity in a cell population and, therefore, to assume how many events of gene transfer can occur. Following the infection, we performed cell cloning by limiting dilution of the transduced K562, in order to obtain individual cell clones, so that the resulting cells of the colony possessed all the same genetic characteristics. The obtained clones, seven βwt-globin and six β^{0}39-globin K562 cell clones, identified using a fluorescence microscope, emit fluorescence at different intensities, depending on the number of vectors integrated into their genomes.

We then analysed the integration and GFP fluorescence of the βwt-globin and β^{0}39-globin K562 cell clones by quantitative real time PCR and FACS analysis respectively, obtaining a fold of β-globin transgenes, respect to the reference gene, and a median of the fluorescence intensity for each clone. The choice of γ-globin gene as reference gene was of great importance because the K562 cells, deriving from malignant cells, present most chromosomes in three or more copies [Gribble et al, 2000; Naumann et al, 2001] and thanks to the location of γ-globin gene close to the β-globin gene, the ratio between them in control K562 cells is, without doubt, equal to 1. Moreover, we chose the median as parameter of the fluorescence

intensity, instead of the arithmetic mean, because it is the value that corresponds to the middle item in a ranked list of all measurements and does not necessarily move in response to small numbers of outliers. The results obtained by these analysis show that the two parameters, fold of β-globin transgenes and median of fluorescence intensity, have similar trends, unless a few cases, indicating that the βwt-globin and $\beta^0$39-globin K562 cell clones contained from one to several copies of the vector and that the GFP expression is not dependent on the site of integration, due to the fact the GFP gene is under the control of the constitutive PGK promoter.

At this point, it was very important to determine which kind of β-globin transcript was expressed by the K562 cell clones. For this reason, we performed a non-quantitative and a subsequent quantitative real time RT-PCR analysis using the RNA isolated from each clone and a *Genotyping kit* for the $\beta^0$39 mutation. This kit permits, through two probes specific for βwt-globin and $\beta^0$39-globin sequences, to determine the type of transcript expressed. The obtained data show that the βwt-globin K562 cell clones express only wild type β-globin transcripts whereas the $\beta^0$39-globin K562 cell clones produce two β-globin mRNA, wild type, due to the copies of the endogenous β-globin gene, and mutated, due to the transgenes. In addition, through the quantification of the β-globin gene expression, it was possible to determine that, despite the fact that no silencing effect was noted, no clear relationship was evident between levels of gene expression and number of integration units. This is however expected, since transcription might depend also on the site of integration. Moreover, in $\beta^0$39-globin K562 cell clones the accumulation of β-globin mRNA sequences is affected by the known effect of nonsense mediated mRNA decay (NMD), which can significantly reduce but not totally eliminate the nonsense $\beta^0$39 transcripts. In fact, NMD does not completely ablate expression of mRNAs containing PTCs, and residual expression levels vary considerably between transcripts, cell types and even individuals [Holbrook et al, 2004; Stalder and Mühlemann, 2008]. After comparative analysis of the RT-PCR data, clones wt3 (βwt-globin K562 cells) and m5 ($\beta^0$39-globin K562 cells) were selected for the experiments employing possible corrections with

aminoglycosides, due to the fact that, among the βwt-globin and $\beta^0$39-globin K562 cell clone sets, they express similar, although not identical levels of β-globin mRNA molecules. Interestingly, clone m5 displays higher β-globin gene integration units and GFP production in respect to clone wt3 suggesting, as expected, NMD affecting $\beta^0$39-globin mRNA, although in this case the large number of mutated transgenes present into the cells may induce the saturation of the process, leading to the accumulation of a significant amount of nonsense transcripts.

The positive results obtained in the characterization of the βwt-globin and $\beta^0$39-globin K562 cell clones allow us to validate the βwt-globin expressing and $\beta^0$39-thalassemia *ex vivo* cellular models.

The second part of our work has been focused on the treatment of our cellular models with several aminoglycosides to characterize their read-through activity. As known, the aminoglycosides more effective in the translational read-through, for the treatment of genetic diseases, are *geneticin* or *G418* [Howard et al, 1996; Bedwell et al, 1997, Lai et al, 2004; Pinotti et al, 2006] and *gentamicin* [Barton-Davis et al, 1999; Keeling et al, 2001; Du et al, 2002; Wilshanski et al, 2003; Howard et al, 2004]. Given the ready availability of *geneticin*, we decided to start precisely with that compound.

First of all, we performed control experiments, with the purpose to verify if *geneticin* was able to induce an increase of β-globin chains, due to the induction of β-globin gene expression. So, we treated original K562 cells with 400 ng/μl *G418* and wt3 βwt-globin K562 clone cells with scalar concentrations (100 ng/μl, 200 ng/μl and 400 ng/μl) of the compound, which were decided depending on the inhibition curves performed on wt3 and m5 K562 cell clones. The samples were labelled with the *βglobin-PE* MoAb and the effects of *geneticin* on the β-globin production were determined by both FACS and immunohistochemistry analysis. The FACS data showed a not significant increase in the proportion of β-globin containing cells after treatment with *geneticin* using both the control cell lines.

Increase that was not demonstrated by immunohistochemistry analysis, due to the lower sensitivity of such technique compared to the flow cytometer.

Once determined that *G418* has no effects on the β-globin gene expression, we treated the m5 $\beta^0$39-globin K562 cell clone with increasing concentration (100 ng/μl, 200 ng/μl and 400 ng/μl) of *geneticin*, analysing the effects by FACS and immunohistochemistry. The FACS data showed, in this case, a strong increase in the proportion of β-globin containing cells, proportionally to the *geneticin* concentration, reaching a maximum in the samples treated with 400 ng/μl *G418*, also demonstrated by immunohistochemistry. Since in control experiments only negligible increase of the proportion of β-globin containing cells was detected, we could conclude that the clear increase obtained in the case of m5 $\beta^0$39-globin K562 cell clone was due to the translational correction of the mutated transcript by the aminoglycoside.

Several papers reported that treatment with aminoglycosides prevents or, at least, limits the degradation of nonsense transcript through NMD, because these compound, binding the decoding centre of the ribosome and decreasing the accuracy requirements for codon-anticodon pairing, suppress stop codons and, instead of chain termination, an amino acid is incorporated into the polypeptide chain, preventing the recognition of PTC containing mRNA by the NMD machinery [Bedwell et al, 1997; Holbrook et al, 2004]. For this reason, we decided to evaluate the effects of *geneticin* on the content of β-globin mRNA in both wt3 and m5 K562 cell clones, by quantitative real time RT-PCR, performed using the *Genotyping kit*, so as to discriminate between βwt- and $\beta^0$39-globin mRNA sequences. This discrimination was particularly important in the case of m5 $\beta^0$39-globin K562 clone cells, because it permited us to quantify only the amount of the mutated transcripts. The obtained results showed that the treatment with *geneticin* determined no significant increase in the β-globin mRNA content in wt3 clone cells, also at the greatest concentration, but a clear increment in the β-globin mRNA content in m5 clone cells, proportionally to the increased concentration of the aminoglycoside. Such result confirms that *geneticin*, suppressing the $\beta^0$39 premature stop codon, prevents the decrease in $\beta^0$39-globin mRNA abundance attributable to NMD, leading to the production of full length proteins.

Since Manuvakhova et al [2000] demonstrated that the sequence context beyond the termination codon can influence the level of read-through induced by several aminoglycosides and other translation factors present in the complete translation system may also influence the overall efficiency of translation termination, we decided to evaluate if *geneticin* can affect the recognition by the ribosome of premature as well as normal stop codons, resulting in a substantial alteration of the protein expression. In this regard, some colleagues performed two-dimensional electrophoreses using cytoplasmic extracts from m5 $\beta^0$39-globin K562 clone cells untreated and treated with 400 ng/μl *G418*, obtaining that only 5 out of 350 proteins significantly increase or decrease following treatment with *geneticin*, which therefore shows only negligible effects on protein expression in our m5 K562 clone.

As *geneticin*, although shown to be an excellent translational corrector, has many disadvantages, in particular the high toxicity, we decided to check the read-through activity of other aminoglycosides, first of all *gentamicin*. Thus, we treated original K562 cells with 1.2 μg/μl and 1.4 μg/μl *gentamicin* and wt3 βwt-globin and m5 $\beta^0$39-globin K562 clones cells with increasing concentrations of such aminoglycoside (600 ng/μl, 800 ng/μl, 1 μg/μl, 1.2 μg/μl and 1.4 μg/μl), decided depending on the inhibition curves performed on m5 K562 cell clone. Because the FACS analysis showed a greater sensitivity compared to the immunohistochemistry, we decided to limit ourselves to the first in determining the effects on β-globin chain production after treatment with the aminoglycoside. The treatment of control cells with *gentamicin* determined a not significant increase in the proportion of β-globin containing cells, furthermore not dependent to the concentration. On the contrary, treated m5 $\beta^0$39-globin K562 clones cells showed a clear increment in the proportion of β-globin containing cells, proportionally to the concentration of *gentamicin*, but much smaller than that obtained after treatment with *geneticin*, leading us to conclude that also if *gentamicin* presents an obvious read-through activity, *G418* is a stronger translational corrector. Our data on these two aminoglycosides are consistent with those reported by Manuvakhova et al [2000], where *geneticin* and *gentamicin* showed a percentage of read-through equal to 22.1% and 4%, respectively, in the

presence of the tetranucleotide (comprised of the stop codon and the first nucleotide that follows) formed as a result of the $\beta^0$39 mutation.

Unfortunately with the other aminoglycosides tested (*neomycin*, *paromomycin*, *kanamycin*, *amikacin*, *streptomycin*, *tobramycin*, *higromycin*), we did not achieve any results better than those previously obtained with *G418* and *gentamicin*. In any case, our data allow us to conclude that aminoglycosides, to varying degrees depending on the compound, are indeed able to induce translational read-through of the $\beta^0$39-thalassemia mutation, leading to the production of full length β-globin chains.

At this point, we decided to characterized the read-through activity of *geneticin* using a different cellular system, namely erythroid precursor cells isolated from $\beta^0$39-thalassemia patients, whose growth *in vitro* represents more closely than cell lines the *in vivo* situation. In this regards, we sequenced the β-globin gene of several β^0-thalassemia patients and isolated the erythroid progenitors from such individuals resulted homozygous for the $\beta^0$39 mutation. Due to the limited number of erythroid precursor cells obtained from each subject, we decided to treat the cells only with 200 ng/μl and 400 ng/μl *G418*. The FACS analysis of the treated $\beta^0$39 progenitors showed a clear increase in the proportion of β-globin containing cells, dependently to the *geneticin* concentration. Because these cells produce only $\beta^0$39-globin transcripts the yield of β-globin chains can be explained only by the translational read-through activity of the aminoglycoside, which prevents the recognition of the premature termination codon by the ribosome and rescues the nonsense mRNA from the NMD pathway.

As known, the ribosome under the action of aminoglycosides cannot discriminate between the amino acids introduced at the premature stop codon, so amino acids very different from the correct one can be added to the polypeptide chain, leading to the production of not functional proteins. For this reason, we decided to verify if β-globin chains produced by $\beta^0$39 erythroid precursor cells treated with *G418* were able to generate adult haemoglobin, performing a HPLC analysis, which can give us information about the relative concentration of HbA compared to total Hbs. The obtained chromatograms showed a significant increase in the proportion of HbA with a concomitant expected decrease in the proportion

of HbF and HbA_2, respect to the total Hbs content, after treatment with *geneticin*. Such increase, however, was smaller than the increment of the proportion of β-globin containing cells, detected by FACS analysis, due to fact that only a part of the amino acids incorporated by the ribosome at the premature stop codon gave rise to a protein able to interact with α-globin chains to yield a molecule of HbA.

Finally, we decided to evaluate if also in the case of $\beta^0$39 erythroid precursor cells the treatment with *G418* lead to an increase in the β-globin mRNA content respect to control cells, due to the rescue of the PTC containing transcripts from the degradation by NMD pathway. The results obtained by quantitative real time RT-PCR showed, after treatment with *geneticin*, increases in β-globin mRNA content even greater than those achieved in the case of the m5 $\beta^0$39-globin K562 cell clone. Such increases, proportional to the aminoglycoside concentration, confirm the ability of *G418* to reduce the recognition of the nonsense mRNA by the NMD machinery.

In the final part of our work, we decide to verify the possible use of the *pCCL.βwt.PGW* lentiviral vector for gene therapy using $\beta^0$39 erythroid precursor cells. This vector, as already described, presents some important characteristics which make the integration of the transgene into the genome safe and efficient, minimizing the effects of the surrounding chromatin, which could silence its expression, and the enhancing effects of the contained promoters on oncogenes. Moreover, we decided to test the combined effects of gene therapy with the lentiviral vector and fetal haemoglobin induction by treatment with *mithramycin*, a potent HbF inducer [Bianchi et al, 1999; Fibach et al, 2003], to verify if the concomitant increases in HbA and HbF could lead to a haemoglobin content similar to that of a heterozygous subject.

First of all, we quantified the copy number of vectors integrated into the cellular genome of transduced $\beta^0$39 erythroid progenitors, treated or not with 30 nM *mithramycin*, by quantitative real time PCR. We achieved β-globin transgenes folds in transduced versus untransduced cells, varying from about 1.2 to 2. An expected result because of the low MOI used for the infection, with the purpose to

obtain limited introduction of exogenous sequences, mimicking the physiological situation.

Once verified to have reached our goal regarding the number of vectors integrated, we evaluated the α-, β- and γ-globin mRNA content in transduced $\beta^0$39 erythroid precursor cells treated or not with the *mithramycin*. In β-thalassemia, the synthesis of normal α-globin chains from the unaffected α-globin genes continues as normal, resulting in the accumulation within the erythroid precursors of excess unmatched α-globin. The free α-globin chains are not able to form viable tetramers and instead precipitate in red cell precursors in the bone marrow forming inclusion bodies. They are responsible for the extensive intramedullary destruction of the erythroid precursors and hence the ineffective erythropoiesis that underlies all β-thalassemias [Thein, 2005]. For this reason, it is very important, for an effective therapy, that the increase in β-like globin genes expression does not coincide with an increase in α-globin genes expression, so as to reduce the precipitation of the α-globin chains, ameliorating the viability of red blood cells. Our results showed that the α-globin mRNA content remained stable even after infection with *pCCL.βwt.PGW* vector and treatment with *mithramycin*, whereas the β-globin mRNA content clearly increased after transduction with the lentiviral vector, diminishing in the presence of *mithramycin*, which instead caused a significant increment in the γ-globin mRNA content. The reduction of the abundance of β-globin transcripts following the treatment with *mithramycin* can be explained by the fact that this compound is a potent HbF inducer and affects transcription by moving toward the expression of fetal globin genes, reducing the production of β-globin mRNA [Breveglieri et al, manuscript in preparation].

Finally, we performed a HPLC analysis to evaluate the effective production of functional HbA and HbF following transduction with *pCCL.βwt.PGW* vector and treatment with *mithramycin*, using the erythroid progenitors isolated from two $\beta^0$39-thalassemia patients. In the first case, the chromatogram showed a high proportion of HbF and a very low proportion of HbA in control cells. After infection with the lentiviral particles the relative concentration of HbA increased significantly whereas that of HbF remained stable, slightly increasing in presence of *mithramycin*, with a concomitant reduction of the HbA relative concentration.

On the contrary, in the second case, both the proportion of HbF and HbA were low in control cells, but the transduction with *pCCL.βwt.PGW* vector induced a clear increase in the relative concentration of HbA, which remained stable also in presence of *mithramycin*, which instead induced a strong increase in the proportion of HbF.

From the result obtained on the globin transcripts content and the Hbs proportion, we can affirm that our lentiviral vector containing the βwt-globin gene is suitable to perform gene therapy of the $\beta^0$39-thalassemia, particularly when associated with the treatment with *mithramycin*.

Concluding, we can assert that both the therapeutic approaches for the cure of β^0-thalassemias analysed in this work, namely translational read-through and gene therapy, while still presenting many disadvantages, including toxicity of aminoglycosides and the uncertainty of the effects of lentiviral vector on endogenous genes, can be considered techniques with a high curative potential. Of course, in the case of translational read-through is hoped the identification of new compounds with a therapeutic effect similar to but greater than that of aminoglycosides, with less toxicity and oral bioavailability, while in the case of gene therapy, further investigations should be made to eliminate its well known disadvantages, primarily including the possible activation of oncogenes. In this respect, most investigators are studying the development of viral vectors increasingly safe and efficient.

Finally, regarding the production of new translation correctors, in a recent paper by Welch et al. [2007], two high-throughput screens (comprising 800,000 low molecular weight compounds) were performed to identify compounds that promoted UGA nonsense suppression. Chemical scaffolds were identified and optimized through extensive medicinal chemistry efforts. These analyses identified PTC124 (3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid; C15H9FN2O3) as a candidate for further development. The selectivity of PTC124 for premature termination codons, its well characterized activity profile, oral bioavailability and pharmacological properties indicate that this drug may have broad clinical potential for the treatment of a large group of genetic disorders with limited or no therapeutic options.

TABLES

Name	Strand	Nucleotide sequence	Length	Tm (°C)
BGF	forward	5’-GTGCCAGAAGAGCCAAGGACAGG-3’	23	72.1
BGR	reverse	5’-CACTGACCTCCCACATTCCCTTTT-3’	24	69.8
BGF1	forward	5’-AGACCTCACCCTGTGGAGCC-3’	20	67.9
BGR1	reverse	5’-AGTTCTCAGGATCCACGTGCA-3’	21	67.1
BGF2	forward	5’-CTTGATGTTTTCTTTCCCCTTC-3’	22	62.5
BGF3	forward	5’-ACAATCCAGCTACCATTCTGCTTT-3’	24	65.7
BGR3	reverse	5’-AGGCAGAATCCAGATGCTCAAG-3’	22	66.6
BGF4	forward	5’-GCATCTGGATTCTGCCTAATAA-3’	22	61.8
BGF5	forward	5’-GCCTGGCTCACCTGGACA-3’	18	67.9
BGF6	forward	5’-CGCTTTCTTGCTGTCCAATTTC-3’	22	66.7
BGR7	reverse	5’-CTGTCTCCACATGCCCAGTTT-3’	21	66.3
LV1F	forward	5’-GGATCTCGACGGTATCGGTTAACT-3’	24	66.9
LV1R	reverse	5’-GGGTGTGTGCCCAGATGTTCT-3’	21	68.4
LVA	forward	5’-GGTGGTTGATGGTAACACTATGCT-3’	24	64.7
LVB	reverse	5’-TTTTACGGCGAGATGGTTTCTC-3’	22	66.0
LVC	forward	5’-GATTCCGGGTCACTGTGAGTG-3’	21	66.8
LVD	reverse	5’-ATCCCAAAGCTGAATTATGGTAGAC-3’	25	64.0
LVE	forward	5’-TCCTGGGAGTAGATTGGCCA-3’	20	66.7
LVF	forward	5’-ATCCTCCTTTGCAAGTGTATTTACG-3’	25	64.9

Table 1. Synthetic oligonucleotides used as primers in polymerization chain reactions (PCR).

Name	Strand	Nucleotide sequence	Length	Use
PF	forward	5’-CAGGCTGCTGGTGGTCTAC-3’	19	Primer
PR	reverse	5’-AGTGGACAGATCCCCAAAGGA-3’	21	Primer
PFα	forward	5’-CACGCGCACAAGCTTCG-3’	17	Primer
PRα	reverse	5’-AGGGTCACCAGCAGGCAGT-3’	19	Primer
PFβ	forward	5’-CAAGAAAGTGCTCGGTGCCT-3’	20	Primer
PRβ	reverse	5’-GCAAAGGTGCCCTTGAGGT-3’	19	Primer
PFγ	forward	5’-TGGCAAGAAGGTGCTGACTTC-3’	21	Primer
PRγ	reverse	5’-TCACTCAGCTGGGCAAAGG-3’	19	Primer
Pβwt		5’-VIC-AAAGAACCTCTGGGTCCA-TAMRA-3’	18	Probe
$P\beta^{0}39$		5’-FAM-CAAAGAACCTCTAGGTCCA-TAMRA-3’	19	Probe
Pα		5’-FAM-TAGTGATGGCCTGGCTCACCTGGAC-TAMRA-3’	25	Probe
Pβ		5’-FAM-TAGTGATGGCCTGGCTCACCTGGAC-TAMRA-3’	25	Probe
Pγ		5’-FAM-TGGGAGATGCCATAAAGCACCTGG-TAMRA-3’	24	Probe

Table 1. Synthetic oligonucleotides used as primers and probes in real time quantitative PCR.

Nome	Restriction site	Conc.	Buffer	Temp.	Company
ClaI	**AT CGAT TAGC TA**	10 U/µl	*Buffer Y^+/Tango:* 33 mM Tris-acetato pH 7.9, 10 mM Mg-acetato, 66 mM K-acetato, 0.1 mg/ml BSA	37°C	MBI Fermentas
NcoI	**C CATGG GGTAC C**	10 U/µl	*Buffer Y^+/Tango:* 33 mM Tris-acetato pH 7.9, 10 mM Mg-acetato, 66 mM K-acetato, 0.1 mg/ml BSA	37°C	MBI Fermentas
PmeI	**GTTT AAAC CAAA TTTG**	10 U/µl	*Buffer B^+:* 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mg/ml BSA	37°C	MBI Fermentas
SmaI	**CCC GGG GGG CCC**	10 U/µl	*Buffer Y^+/Tango:* 33 mM Tris-acetato pH 7.9, 10 mM Mg-acetato, 66 mM K-acetato, 0.1 mg/ml BSA	30°C	MBI Fermentas
SwaI	**ATTT AAAT TAAA TTTA**	10 U/µl	*Buffer 0^+:* 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA	30°C	MBI Fermentas
XbaI	**T CTAGA AGATC T**	10 U/µl	*Buffer Y^+/Tango:* 33 mM Tris-acetato pH 7.9, 10 mM Mg-acetato, 66 mM K-acetato, 0.1 mg/ml BSA	37°C	MBI Fermentas
SnaBI	**TAC GTA ATG CAT**	10 U/µl	*Buffer Y^+/Tango:* 33 mM Tris-acetato pH 7.9, 10 mM Mg-acetato, 66 mM K-acetato, 0.1 mg/ml BSA	37°C	MBI Fermentas

Table 3. Endonucleases used in cloning experiments.

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ACKNOWLEDGEMENTS

First of all, I would like to acknowledge Prof. Roberto Gambari for the opportunity to work in his group and to study a genetic disease so important for my life. Thanks to Dr. Giulia Breveglieri, Dr. Alessia Finotti, Dr. Vera Cantale, Chiara Chiavelli and Dr. Giordana Feriotto, who have always helped and supported me with a patience that I suppose is endless; to Dr. Nicoletta Bianchi and Dr. Cristina Zuccato, whose assistance in performing real time PCR and erythroid progenitors cultures has been absolutely essential; to Dr. Ilaria Lampronti, Dr. Eleonora Brognara and Dr. Elisa Bono for their help in HPLC analyses, which were, at the begin, totally obscure for me; to Dr. Federica Destro and Dr. Alessandro Canella, for their patience and assistance in carrying out FACS analysis; to Prof. Stefano Rivella and Dr. Laura Breda, for their guidance and enlightening advices; to Dr. Mirko Pinotti, whose recommendations have helped us in the beginning phase of the work. Moreover, I have to acknowledge Dr. Giulia Breveglieri, Dr. Alessia Finotti, Dr. Eleonora Brognara and Dr. Monica Borgatti, for having performed the proteomic analysis in my place.

I have to thank Associazione Veneta per la Lotta alla Talassemia, Rovigo (President Elio Zago), for the continous support to the research on thalassemia, and Dr. Michele Lipucci di Paola for suggestions and encouragement; Fondazione CARIPARO is also acknowledged for sopporting our laboratory.

Naturally, well as colleagues, there are many people that I would like to thank but I have to limit myself to a few of them, including, first of all, my mom and dad, who have made me what I am, the love of my life Riccardo, my grandmas and granddad, my cousins Marcello and Annalisa and my new little cousin Emma. Finally, a big thanks goes to Gilda, a rediscovered childhood friend.

ARTICLES

Inherent in the topic of the thesis:

Salvatori F, Cantale V, Breveglieri G, Zuccato C, Finotti A, Bianchi N, Borgatti M, Feriotto G, Destro F, Canella A, Breda L, Rivella S and Gambari R. Development of K562 cell clones expressing β-globin mRNA carrying the $\beta^0$39 thalassemia mutation for the screening of correctors of stop codon mutations. *Biotechnol. Appl. Biochem.* (in press).

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