TRANSFECTION OF HOMOPYRIMIDINE THIRD STRAND BINDING PROBES INTO HUMAN CELLS AND THEIR SPECIFIC BINDING TO CHROMOSOMAL TARGETS

by

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

To utilize homopyrimidine strands as highly specific third strand binding agents for delivery of reactive reagents to modify mutant target residues at unique sites in the human genome, we have explored methods for transfecting such short deoxyoligomers into HeLa and K562 cells, and then demonstrated that those strands do, in fact, bind to the correct target sequences. This work is part of a long term effort to develop a general method for correcting point mutations responsible for human diseases that is presently focused on the β-globin gene mutation that causes Sickle Cell Anemia. While transfection of linear duplex and plasmid DNA into human cells is a well-established procedure, this is not the case for single deoxyoligonucleotide strands 20-40 residues long (and without added carrier plasmid or viral vector), as we require for gene mutation correction. After exploring a variety of transfection reagents, one was found that is effective at non-toxic levels. To monitor their entry into cells, the strands were labeled with the fluors FITC or TMR. Probe strands with 5' psoralen and 3' FITC or TMR

termini were transfected into cells, and were observed to enter nuclei rapidly. After 23 hours, colchicine was added to halt cell progression in metaphase; an hour later the cells received a pulse of UVA light to covalently link the probes wherever they might be bound. After bursting the cells in hypotonic solution, their chromosomes were spread on glass slides and examined by confocal microscopy. Using probes specific to centromeric sequences of chromosomes 11 and 17, and probes to the β-globin gene in chr #11 and the Her2/neu and Topo2 genes, both in chr #17, highly specific probe binding to the correct chromosome centromeric and arm regions was observed. In addition, transfection kinetics and efficiency were determined using fluorescence activated cell sorting (FACS).

Keywords: Triple Forming Oligonucleotides, transfection, sickle cell anemia, chromosome, modified oligonucleotides

İNSAN HÜCRESINDEKI SPESIFIK KROMOZOMLARA HOMOPÜRIMIDIN ÜÇÜNCÜ BAĞ PROBUNUN TRANSFER EDILMESI VE BAĞLANMASI.

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ÖZ

İnsan genomundaki belirli bölgelerinde meydana gelen nokta mutasyonların tamirini hedef alan reaktif ajanların spesifik mutasyon bölgelerine bağlanmasında homopürimidin dizisinin kullanımını ve bu dizilerin insan HeLa ve K562 hücrelerine transfer edilmesine yönelik metod geliştirilmesi. Orak hücreli anemiye neden olan β globin genindeki nokta mutasyonunun düzeltilmesini amaçlayan metodun geliştirlmesine yönelik uzun süreçli çalışmanın bir parçasıdır. 20-40 nükleotid uzunluğunda ve tek dizin olan deoxyoligonükleotidin plazmid olmadan ve viral vektörsüz olarak hücre içerisine transfer edilmesindeki temel amaç gen mutasyonlarının düzeltilmesidir. Bu deoxtoligonükleotidi hücre içerisine transfer etmek için toksik olmayan transfer ajanları kullanılmıştır. Bu küçük gene parçalarını hücre içersinde görüntülemek için dizinler FITC ve TMR ile işaretlenmiştir.5' uzunda psoralen, 3' ucunda FITC veya TMR taşıyan probe dizini hücre içerisine transfer edildi ve çekirdek içerisine hızlı girişi gözlemlendi. 23 saat sonra hücre bölünmesini metafaz aşamasında durdurmak için colchicine eklendi. Hücre içerisine transfer edilen probların kovalent bağ oluşturabilmesi için UVA ışığa maruz bırakıldı. Hücrelerin hipotonik solisyon ile patlasınından sonra kromozomları lam üzene yayıldı ve Konfokal mikroskop ile incelendi. 11. ve 17. krozomların sentromerik bölgelerine, β globilin, Her2/nue ve topo II gen bölgelerine spesifik probların kullanılmıştır. Yüksek spesifik özelliğe sahip bu probların doğru gen bölgelerine bağlandıkları gözlemlenmiştir. Buna ek olarak transfer kinetiği ve transfer oranı FACS ile belirlenöiştir.

Anahtar Kelimeler: Triple Forming Oligonucleotides, transfection, sickle cell anemia, chromosome, modified oligonucleotides.

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LIST OF SYSMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

dsDNA	double strand DNA
TFO	Triple Forming Oligonucleotides
T	Tymidine
G	Guanine
A	Adenine
C	Cytosine
N3	N3 position
N7	N7 position
Ps	Psoralen
β	Beta
FITC	Fluorescin 5- isothiocyanate
TMR	Tetramethylrodamine 5- and 6 isothiocyanate
O.D	Optical Density
PI	Propidium iodide
min	Minute
PAGE	Polyacrylamide Gel Electrophoresis
µl	Microliter
ml	Milliliter
ng	Nanogram
ºC	Celcius
rpm	Rotar per minute
RBC	Red Blood Cell

CHAPTER 1

INTRODUCTION

The long term objective of the research in which I was involved is to develop a general approach to repairing in stem cells ex vivo point mutations responsible for particular human diseases. For this purpose the transversion mutation in the β-globin gene responsible for Sickle Cell Anemia was selected as the demonstration case. The approach to the repair of this gene was to design a deoxyoligonucletotide strand that would bind primarily by nucleic acid triplex formation with singular specificity to the target duplex sequence and nowhere else in the human genome. That strand would be used to deliver the photoreactive reagent, psoralen, attached to the delivery oligomer by a linker of such length that upon irradiation the psoralen could only attack the mutant T residue in the coding strand of the β-globin gene. This psoralen-delivery strand was successfully tested in a linear model DNA target system, and then in a plasmid model system. All this was accomplished prior to my thesis research.

My goal was to develop conditions and find reagents that will afford efficient transfection of the psoralen-delivery strand into human cells. Towards this end, I investigated its transfection and intranuclear binding to chromosomal target sites in HeLa and K562 cells.

CHAPTER 2

REVIEW OF LITERATURE

2.1 GENE THERAPY

The term "gene therapy" has been in use for about 20 years, and over this time it has evolved from a mere notion that the genetic determinant of an inherited disease might be correctible to a number of strategies for doing so [1,2} and, currently, to many clinical trials that are in progress. From the very beginning, Sickle Cell Anemia was an object of such study [3,4]. The most developed approach involves introduction of a correct copy of the defective gene into the recipient cells using viral delivery vectors [5,6], retroviral or adeno-associated. This approach requires that a number of obstacles be overcome: an immune response to the viral delivery vectors, multiexxon genes and complex regulation of gene expression, all of which can result in insufficient levels of the expression of the inserted gene or, as recent human clinical trials have shown, even to unanticipated dire consequences due to misinsertion of the correct gene copy into the genome.

Repair of a defective gene (rather than insertion of a correct one by viral transfer) makes it possible to circumvent problems related to viral vector misinsertion of the intended gene. With repair, the defect is corrected right on the chromosome and expression of the repaired gene is regulated by the intrinsic cellular regulatory mechanisms. Triplex-mediated site-specific gene repair is particularly suitable as a strategy for correcting point mutations in situ [1,7,8]. It is based upon specific chemical modification at the mutation site coupled to damage recognition and its (error-prone) correction by the cellular repair machinery, and can thereby lead to the replacement of a mutant base pair by a wild-type one. Psoralen, a photoreactive moiety that upon irradiation can covalently modify T (or C to lesser extent) residues, can be

used for correcting point mutations involving that residue, as is the case for Sickle Cell Anemia. Briefly, a third strand containing a tethered psoralen moiety binds to a specific target sequence via triplex formation. After irradiation, the psoralen photoadducts (monoadducts, when psoralen forms a covalent bond with only one strand, or crosslinks where psoralen binds covalently to both duplex strands) are either misread during bypass replication (which is believed to be the main reason for monoadduct-induced mutagenesis [9,52] or repaired via error-prone nucleotide excision repair[52,44], leading to a highly specific mutation spectrum, often to the transversion T·A→A·T at the targeted site [9,52,45].

With increasing information about cellular processing of triplex-delivered psoralen and the resulting light-induced photoproducts, it has become apparent that there are variations in this process in different cell lines, and that the size of the delivery strand and the structure of the triplex formed may also play an important role in the resulting mutation spectrum[10,12]. Some cell lines, for example, are severely deficient in the removal of psoralen crosslinks[11]. In addition, the presence of triplex by itself, even without covalent psoralen photoadducts, sometimes promotes site-specific mutagenesis[13,14]; third-strand-delivered psoralen-induced mutation spectra may therefore differ depending on whether the third strand remains intact or is instead excised.

A necessary step in developing the triplex-based therapeutic approach is successful single-stranded oligonucleotide delivery into cell nuclei and their specific binding to a target site on a particular chromosome. A number of strategies for such oligonucleotide delivery are currently available, including permeabilization of the plasma membrane with digitonin [75,76], coupling of oligomers to polycations such as polylysine[17], and use of cationic lipids or cholesterol complexed to the oligonucleotide[18,19] . Recently, some new transfection agents, e.g., [20] cytofectin have been developed for oligonucleotide delivery into mammalian cells with good efficiency and low toxicity. Some of these strategies have been successfully used for targeting of psoralen-linked third strand to chromosomal DNA, suggesting that chromatin structure is not a significant barrier to the desired binding [21,23]. CD34+ cells have also been successfully transfected [24,25].

Examples of successful delivery of a third strand to the cell nucleus and its binding to chromosomal targets have so far been limited to all-purine third strands that form stable triplexes at physiological pH [16]. However, "molecular crowding" in cell nuclei, that

appears to stabilize some protein complexes and non-canonical DNA structures, may conceivably affect the thermodynamics of triplex formation [27,28], and may prove to be an important factor for stabilizing all-pyrimidine third strand triplexes, in which C residues must be protonated for third strand binding at physiological pH, far removed from the intrinsic pKa of ~4.5 for that base. All these considerations are encouraging for the development of a triplex-based approach to gene therapy for Sickle Cell Anemia; and as will be seen in this thesis, our relevant findings on transfection of the psoralen delivery strands into two human cell lines in tissue culture are consistent with that expectation.

2.2 SICKLE CELL ANEMIA

Sickle cell disease is a hereditary blood disorder that affects the red blood cells. People with this disease have red blood cells that mostly contain an abnormal type of hemoglobin. Hemoglobin is the main substance of the red blood cells, helping them carry oxygen from the air in the lungs to different parts of the body. Normal red blood cells are smooth and round, and are shaped like doughnuts, so they can pass easily through blood vessels; they have a lifetime of about 120 days. Sickle Cell diease is especially prevalent in Africa and in the Middle East. In the United States more than 70,000 people have sickle cell anemia (Homozygous); and about 2 million Americans, one in 12 African Americans - have sickle cell trait (are heterozygous for the trait).

Thus, sickle cell anemia is a blood disorder resulting from homozygous point mutation in the β-globin gene, causing a single glutamic acid residue (encoded by the wild-type sequence (GAG) to be replaced by valine (GTG) [29,30]. This mutation causes deoxygenated hemoglobin to polymerize, forming long fibrils which distort the flexible disc-like red blood cells into rigid crescent-or sickle like shapes [31]. Red blood cells carry oxygen throughout the body to nourish tissues and sustain life. They are the most abundant cells in the human body. When they are healthy, they look like tiny flexible inner-tubes. This unique shape and small size serve many functions. The tubular shape provides a large surface area to absorb oxygen. It also offers the flexibility necessary to squeeze through the capillaries, which are the tiny blood vessels that connect arteries to veins [88].

Oxygen is carried within the red blood cells by hemoglobin, a complex molecule composed of 2α and 2β protein subunits and heme, which contains iron. Thus, sickle-cell

disease is caused by a genetic defect in this important molecule. A normal red blood cell contains *hemoglobin A*; in a sickle red blood cell, a variant of this molecule exists called *hemoglobin S* (S for sickle) [88].

As noted, hemoglobin S results from a single amino acid substitution, valine in place of glutamic acid, in the β-chain of hemoglobin (Hb S). As as a result, polymerizes on deoxygenation, producing less deformable sickle red blood cells (SS RBCs) that can obstruct blood vessels [32]. More than 20 years ago, it was demonstrated that SS RBCs display increased adherence to endothelial cells [33,34]. Subsequent studies recognized the importance of several adhesion pathways in these interactions [35,36], and revealed that young (low density) SS RBCs are more adherent to the endothelium than dense (often irreversibly sickled) SS RBCs [37,38].

The sickle-cell disease process is triggered when red blood cells become deprived of oxygen. In everyone -- both those with and without the disease -- hemoglobin loses its oxygen in a number of ways. To sustain life, oxygen regularly passes from red blood cells to the tissues, where it is needed to perform vital functions. So, hemoglobin becomes deoxygenated as a result [39,40].

Blockage of blood vessels (vaso-occlusion) can cause the spleen to swell and destroy its ability to fight infection; vaso-occlusion also leads to stroke frequently in children with sickle cell anemia. A vascular necrosis of active bone marrow causes bone pain crises, which may also be caused by vaso-occlusion. The most dangerous consequence of sickle cells is acute chest syndrome, which leads to death in many cases; though this condition is not well understood, it seems to be most frequently brought on by infection [43,44]. Though some patient die early from acute chest syndrome, others can remain largely unaffected by severe symptoms till mid-life; genetic background clearly plays a role in sickle-cell prognosis, but the mechanism of epistatic interactions is unclear [30].

The most common treatments of sickle cell anemia are psychological: avoiding stress factors that precipitate acute chest syndrome, practicing distracting pastimes and learning to manage pain can all have markedly beneficial effects for patients [31]. Both adults and children can be treated with hydroxyurea, a drug that increases the concentration of fetal hemoglobin (which is not coded by the mutant β-globins gene); hydroyurea is quite effective

in reducing pain, frequency of acute chest syndrome and stroke, but it may have a long-term carcinogenic effect and cannot be safely used during conception or pregnancy, an especially problematic time for sickle cell patients [30,31]. The only curative treatment for sickle cell anemia is bone marrow transplantation, which has been performed successfully in many cases; however, as with any organ transplant, finding a compatible donor is a difficult and rate-limiting step.

Performing gene therapy on embryonic stem cells to restore the wild type-β-globins gene would circumvent the need to find a marrow donor match, since the patient's own cells could be therapeutic. This thesis will deal with the advancement of a promising method for repair of the sickle cell mutation through a novel approach to gene therapy using triplex-forming oligonucleotides. In particular, my experimental focus involved the development of a method to transfect into human cells in tissue culture the deoxyoligonucleotide strand required to induce the genetic change.

2.3. TRIPLEX STRUCTURE

Triplex-forming deoxyoligonucleotides [TFOs] bind in the major groove of a classic Watson- Crick duplex at polypurine·polypyrimidine stretches in a sequence-specific manner. The binding specificity of TFOs makes them potential candidates for use in directed genome modification [41]. Binding of a third strand to a nucleic acid duplex was shown to be a highly specific process [90]. In general, a triple helix [triplex] is formed when an oligonucleotide composed of either all-purine or all-pyrimidine residues binds to a homopurine·homopyrimidine stretch of duplex, though this binding can occur according to different triplex binding motifs [59].

Specific base recognition and binding between native double-stranded DNA [dsDNA] and complementary [89] single-stranded DNA (ssDNA) of mixed base sequence is presented. Third-strand binding, facilitated and stabilized by photoreactive chemical triplex forming oligodeoxyribonucleotides containing thymine and 5-methylcytosine [60] can form pyr.pur.pyr-type triplexes with double-stranded DNA. Unlike triplexes whose third strands contain thymosine and deoxycytidine, the stability on these triplexes is dependent on pH because the 5-methyl cytosine [61] must be protonated to bind to a G·C base pair.

2.3.1. Third Strand Binding

Strong and sequence-specific triple-helix formation of oligonucleotides with genomic DNA can selectively interfere with gene expression at the level of transcription and is therefore of interest in medicinal chemistry and biotechnology [42,43].

When the triplex-forming oligonucleotide (TFO) is made up of pyrimidines, cytosine* guanine·cytosine (C*G·C) and thymine*adenine·thymine (T*A·T) base triplets are formed, where a dot represents the duplex's Watson-Crick base pairing and an asterisk *represents third strand binding by the formation of Hoogsteen hydrogen bonds [45].
The molecular recognition of a DNA duplex via a triplex forming oligonucleotide [TFO] in either the parallel [46,47] or the antiparallel [48,49] binding motif is limited to homopurine–homopyrimidine DNA sequence tracts. Despite considerable efforts over the past decade to overcome this sequence restriction by TFO design, a general solution to the problem still remains elusive [50].

As noted, in the parallel motif of triplex formation, a third strand comprised of thymine and protonated cytosine residues binds in the major groove of a Watson-Crick duplex via Hoogteen hydrogen bonds. Third strand probes include 5-methylcytosine [^{5m}C] instead of cytosine residues, which enables pyrimidine parallel motif triplexes to form closer to physiological pH, as opposed to pH 5.5 if the cytosine is not methylated [51]. The additional methyl substituent in 5-methyl-cytosine favors triplex formation by improving hydrophobic base stacking interactions and donating electron density to the ring of the base, which facilitates protonation of N3, which is crucial for hydrogen bonding to N7 of the guanine residue in C*G·C triplets (Figure 1).

However, this type of triplex has been thought in the past to be stable only at acidic pH since N_3 of the cytosine residues on the third strand must be protonated in order to form the required pair of Hoogsteen hydrogen bonds [Figure 1]. However, it was recently shown in the Fresco laboratory that such binding does take place under the macromolecular crowded conditions in the cell nucleus, which fosters protonation of C residues at physiological pH (unpublished information) [57].

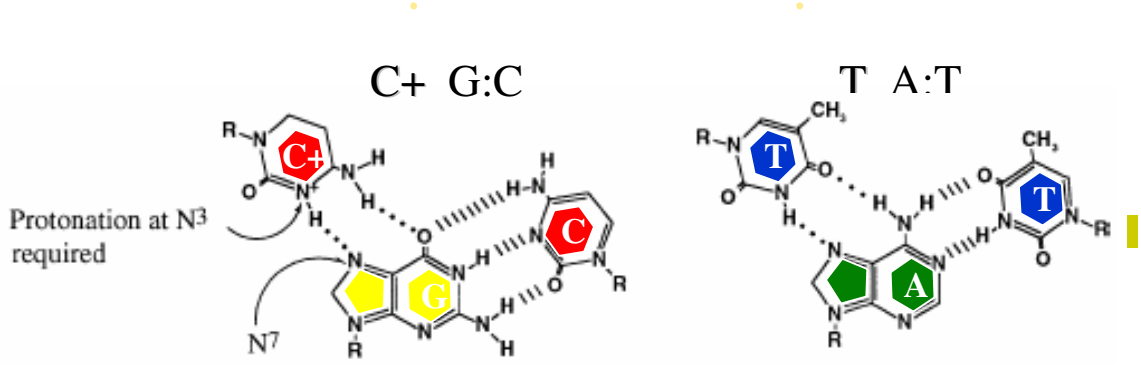

Figure 2.1. Diagram of the pyrimidine parallel motif for triple helix formation. [**a**] Orientation of the third strand in this motif is parallel to the of 5' to 3' orientation of with respect to the purine-rich strand of the duplex target. The structure of the base triplets and the Hoogsteen hydrogen bonds are shown.

A purine TFO can bind antiparallel to the purine strand of the duplex, forming reverse Hoogsteen hydrogen bonds with the duplex's purines, making tripletes A*A·T and G*G·G (Figure 2) [45]. In addition, a thymine/guanine oligonucleotide can bind antiparallel to a duplex purine strand, forming T*A·T and G*G·C triplets through reverse Hoogsteen hydrogen bonding; a T/G third strand actually binds more stably than an all-purine antiparallel third strand (Figure 2). Homopurine third strands can also bind in the parallel orientation (not shown), in which case the third strand a hydrogen bonds to both the A and T of one target base pair and G hydrogen bonds to both the G and C of the other target base pair.

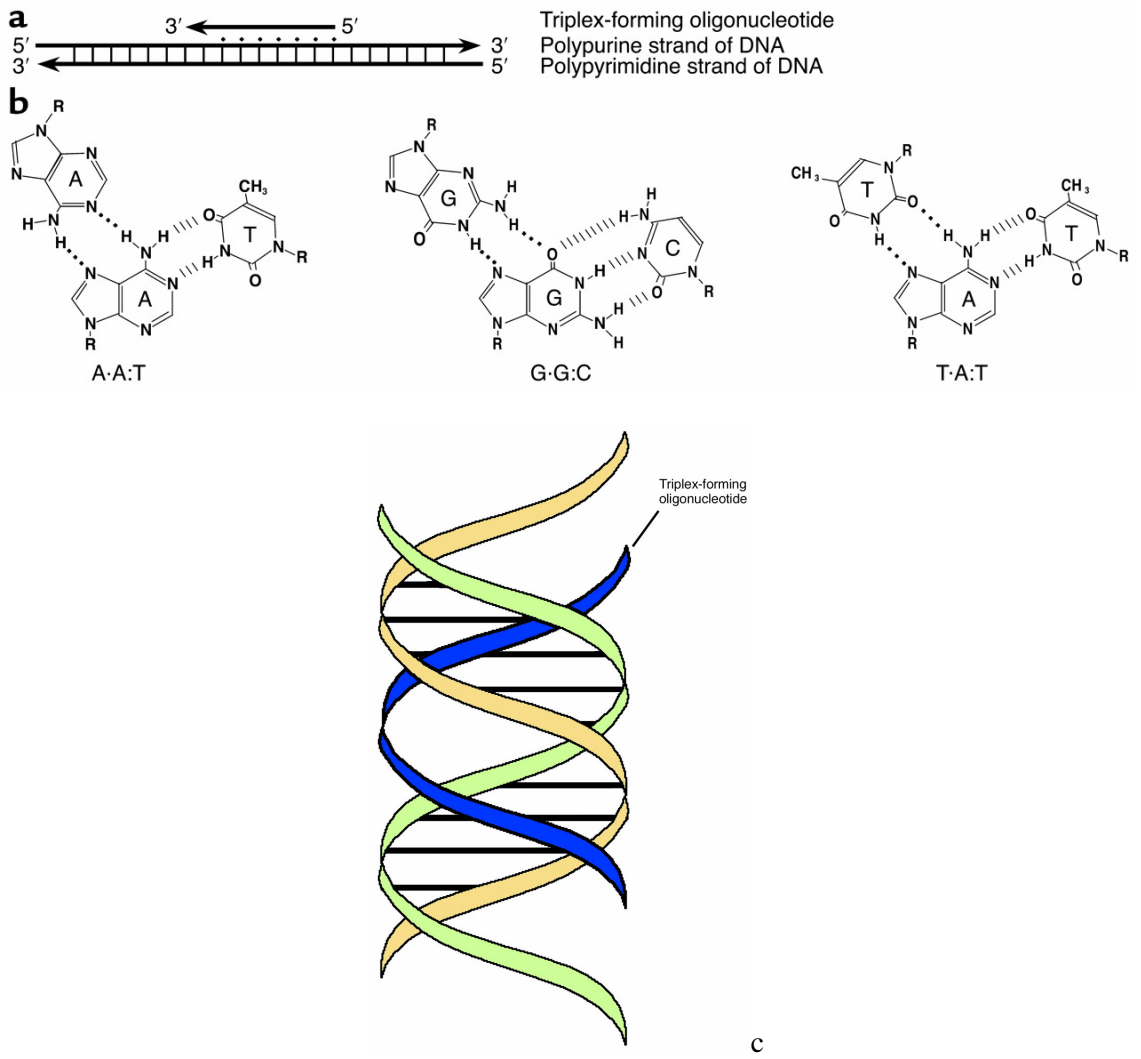

Figure 2.2. Diagram of the purine-antiparallel motif for triple-helix formation. [**a**] Orientation of the third strand in the purine triple-helix motif. Note that the third strand is oriented antiparallel in terms of the 5' to 3' direction relative to the purine-rich strand of the duplex target. [**b**] Base triplets formed in the purine motif and illustration of the reverse Hoogsteen hydrogen bonds that stabilize triple-helix formation. As pictured in the T*A·T triplet [where a dot represents Watson-Crick base pairing and * represents third binding], thymine, a pyrimidine residue, can be incorporated into an otherwise purine TFO in this motif. [**c**] Diagrammatic depiction of a DNA triple helix, with the third strand binding in the major groove.

2.3.2. *Ex vivo* Correction of the Sickle Cell Anemia Mutation

The wealth of sequence information from human and other genome projects highlights the need for facile and efficient strategies for genome sequence manipulation. This requires the selective recognition of duplex DNA, and has stimulated the development of various synthetic candidates for this purpose. Successful reagents could be used for gene knockout, for target validation, to facilitate gene knock in, for strain and transgenic animal construction, and perhaps, for gene therapy. One approach that has been of interest for many years is based on the DNA triple helix [52].

Despite the promising implication of the specificity of third strand binding, however, many obstacles remain to be overcome before this technology can be applied successfully in living systems. Triplexes are not easily stabilized because of the electostatic repulsion of their three negatively-charged phosphate backbones; this problem has been neutralized *in vitro* by augmenting Mg^{2+} concentration above physiological levels [53].

As noted, pyrimidine TFO's bind with difficulty at physiological pH since the cytosine residues must be protonated to form Hoogsten hydrogen bonds. This problem is worsened in sequences containing adjacent cytosines [55] To alleviate it, these residues can be replaced by analogues [54,34]. 5-methylcytosine has had some success in biological assays; the added methyl group acts by helping in base stacking or excluding water from the major groove [56]. To test the latter possibility by examining the intranuclear binding of homopyrimidine third strands that in dilute solution bind effectively at pH 5, less so at pH 6, and insignificantly at pH 7, because their C residues, with an intrinsic pKa of 4.5, must be protonated to bind to G•C target base pairs, highly specific short deoxyoligomer single-stranded sequences with a photoreactive 3′ psoralen moiety and a 5′ fluor were transfected into K562 or HeLa cells [57]. This was on of my major acheivements in this thesis research.

Additional innovations have been applied by others to overcome other obstacles to intracellular triplex stability, but these have not been very successful. Substituting base analogous for guanine and changing the secondary structure of the TFO itself to a circle or a hairpin decrease interference by TFO secondary self-structure formation [53,54,58,62], have been tried. RNA and other novel backbone analogues that conform easily to the shape of the duplex's groove-without distorting its backbone conformation like a DNA-TFO have also had

some success in increasing triplex binding affinity [56,60], but not necessarily in a useful way.

The main strategy of gene therapy has traditionally been focused on gene reproduction. This approach typically involves the introduction of an expression system designed to express a specific protein in the transfected cell [61].

In this connection, we have developed a specific third strand that can bind to the target and invade the adjacent duplex sequence. This strand is specifically photo-coupled to the mutant base in the coding strand, which is then repaired during bypass replication of DNA [62].

The latter application of TFO's is an approach to gene therapy ideally explored in the sickle cell anemia model since the β- globin gene contains a homopurine stretch appropriate for third strand binding located immediately downstream of the sickle cell mutation [64]. I used a TFO site-directed probe developed in out laboratory and developed conditions to transfect it with a liposomal transfection reagent into HeLa and K562 cells, where it enters the nucleus and binds to its genomic target.[65,66,67]. Psoralen, a photoreactive moiety that upon irradiation can covalently modify T residues[68], was used for correcting the sickle cell point mutation involving that residue. When the psoralen forms a covalent bond with one strand of the target, or crosslinks when psoralen binds covalently to both duplex strands, the sequences is either misread during bypass replication, which is believed to be the main reason for monoadduct-induced mutagenesis [69,70], or repaired via error-prone nucleotide excision repair [37], leading to a highly specific mutation spectrum, often to the transversion T•A→A•T at the targeted site [70,71,72].

The third strand was thus designed in our laboratory to bind to the purines which follow the A→T transversion mutation on the coding strand and then to cross over and bind to the purine stretch which follows on the non-coding strand (Figure 3). The approach extended the length of the binding sequence from 7 to 23 residues [62].

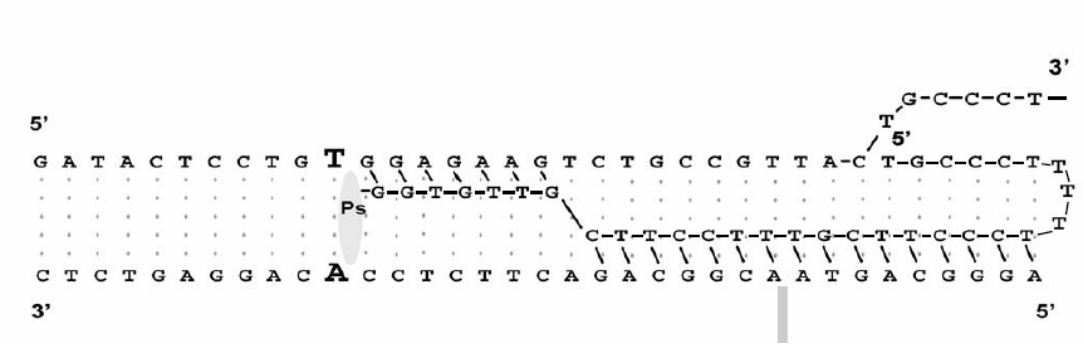

Figure 2. 3. In the upper scheme, the elements of the third strand are shown to bind to the coding strand and then to the non-coding strand. In the lower scheme, the extension on the 5' end of the third strand includes a four residue linker to 5 residues with a capacity to strand invade by complementary base pairing.

However the purine stretch on the non-coding strand also contains one thymine (T) and three cytosine (C) residues, reducing binding affinity. Therefore 5-methyl-cytosine and 5-propynyluracil analogs, which increase binding affinity as discussed above, were incorporated into the TFO. The incorporation of these modified residues has two effects: [i] it decreases TFO self-association, and [ii] it slightly increases triplex stability. The enhanced ability of the modified oligonucleotides containing 7-chloro-7-deaza-2'-deoxyguanosine over the parent oligomer to form triple helices was confirmed by inhibition of restriction enzyme cleavage using a circular plasmid containing the target sequence [74].

As a precursor to moving our sickle cell gene repair project *in vivo*, binding affinity of the third strand was also tested against a plasmid containing the target sequence. Binding was greatly facilitated by the addition of the strand-invading sequence element, since the sticky end with which that element previously formed a duplex is not present in the chromosomal target. As Figure 3 shows, this TFO extension is thought to act by strand invasion [64,74]. This hypothesis is supported by the far greater binding affinity of the third strand to a supercoiled plasmid target than to a relaxed or linearized plasmid (a feature characteristic of strand invasion) [74]. The efficiency with which this psoralen delivery strand binds to a supercoiled plasmid seemed very promising for the prospects of its intracellular application [75].

However, when this strand-switching, strand-invading TFO was tested for mutagenic activity on a plasmid containing the sickle cell –β-globin gene in tissue culture, mutations were not limited to the target site at T_{11} [48]. Some mutations induced by the psoralen moiety were detected hundreds of base pairs away from the intended position. Since successful site-specific mutagenesis has only been reported using TFO's that bind to one strand of the duplex, it is thought that the strand-switching TFO may act like a clamp, preventing separation of the duplex strands by the NER machinery [67,71,77,78].

To explore the feasibility of the triplex-mediated approach for the desired mutation correction, experiments were undertaken under tractable circumstances with a plasmid bearing a fragment of the Sickle Cell β-globin gene [75].

A plasmid photomodified *in vitro* and purified from non-modified plasmid prior to transfection was used for this purpose. Thus, only photomodified plasmid was transfected into cells. Consequently, only plasmid whose psoralen photoadducts were removed by the DNA repair machinery could be replicated. In this way, the maximum repair potential of directed psoralen modification was evaluated. The presence of any non-photomodified plasmid would obscure the true repair potential inherent in the cells. This was followed by experiments showing a significant level of mutation correction that occurs when the treated plasmid was transfected into COS-7 cells [75].

In what follows, I describe my experiments to achieve transfection of the psoralen-delivery strand into human cells in tissue culture, and then its linking to its chromosomal target.

CHAPTER 3

MATERIALS AND METHODS

3.1.MATERIAL

Fluorescin 5- isothiocyanate[FITC, Fig. 3 Sigma-Aldrich, St. Louis, MO]

Tetramethylrodamine 5- and 6 isothiocyanate [TMR/ TRITC, Fig. 3; Molecular Pobes, Eugene OR]

4'6-diamidino-2-phenylindole (DAPI, Sigma)

Propidium iodide (Calbiochem, San Diego, CA)

Transfection reagent [Biotex]

Model B 100AP BLAK-RAY® UV lamp [14W, 320-400nm; UVP, Inc.

TOTO3 (Molecular Probes)

LSM 510 Confocal Microscope

FACS Vantage SE with Turbo Sort II

CO2 incubators (Fisher Scientific)

Model G25 shaker[New Brunswick Scientific Co.,Edison NJ]

Deoxyoligomers were obtained from the Princeton University Synthesis and Sequencing Facility

Lithium per chlorate (Sigma-Aldrich)

Acetone (Sigma-Aldrich)

Butanol (Sigma-Aldrich)

Gel Electrophoresis set

DMEM (Gibco)

RPMI (Gibco)

Fetal Calf Serum (Fisher Scientific)

Sodium Citrate(Sigma-Aldrich)

Colchicine (Sigma-Aldrich)

Pure Water (Aldrich, St. Louis, MO)

Methanol (Aldrich, St. Louis, MO)

Acetic acid (Sigma-Aldrich)

Centrifuge (Ependorf)

K562 Cell Line (ATCC)

HeLa Cell Line (ATCC)

3.2 METHODS

Both the psoralen delivery strand (give the sequence here) and a strand specific for the centromeric target in human chromosome 11 were synthesized by the phosphoramidate method commercially with a psoralen moiety at the 3′ end and a C_7NH_2 residue on its 5′ end so that a fluor could be attached. These oligomers were purified to homogeneity by gel electrophoresis.

Probes:

Ps—C ^{m}C T ^{m}C T T ^{m}C T ^{m}C T ^{m}C C ^{m}C T T T ^{m}C ^{m}C T T C ^{m}C ^{m}C T—FITC

Choromosome 11 centromeric probe labeled with FITC above, and TMR below.

Ps—C ^{m}C T ^{m}C T T ^{m}C T ^{m}C T ^{m}C C ^{m}C T T T ^{m}C ^{m}C T T C ^{m}C ^{m}C T—TMR

Ps—T T T T T ^{m}C T T ^{m}C T T T ^{m}C T T ^{m}C- FITC

Choromosome 17 centromeric probe labeled with FITC above, and TMR below

Ps—T T T T T ^{m}C T T ^{m}C T T T ^{m}C T T ^{m}C- TMR

Ps—^{m}C T T ^{m}C T ^{m}C T ^{m}C C ^{m}C T ^{m}C T T T C—FITC

Her2/nue probes labeled with FITC

Ps—C C A A T C T T T T T T ^{m}C ^{m}C T T T ^{m}C T T ^{m}C T ^{m}C T T T—FITC

Topo II probes labeled with FITC

3.2.1. Fluorescent Labeling

Fluorescin 5- isothiocyanate (FITC, Fig. 4 Sigma-Aldrich, St. Louis, MO) or mixed isomers of tetramethylrhodamine 5- and 6 isothiocyanate (TMR/ TRITC, Fig. 4; Molecular Pobes, Eugene OR) were reacted with the 5′ terminal amino group of the third strands. The fluorescent dye, FITC in acetonitrile or TMR in dimethylformamide was added to the third strand (36 O.D units/ml at 260nm) in sodium carbonate buffer, pH 9. Reactions were allowed to proceed overnight at room temperature with shaking at 180 rpm on a model G25 shaker (New Brunswick Scientific Co.,Edison NJ); they were then neutralized with acetic acid. As the ratio of dye to oligomer was at least 10:1, excess dye was removed by butanol exctraction, and the labeled oligomer was precipitated with 2% lithium perchlorate in acetone. Fluorescently- labeled third strands were purified by PAGE and ethanol precipitation give conditions prior to their use in triplex-forming reactions or for TISH [91].

FITC TMR

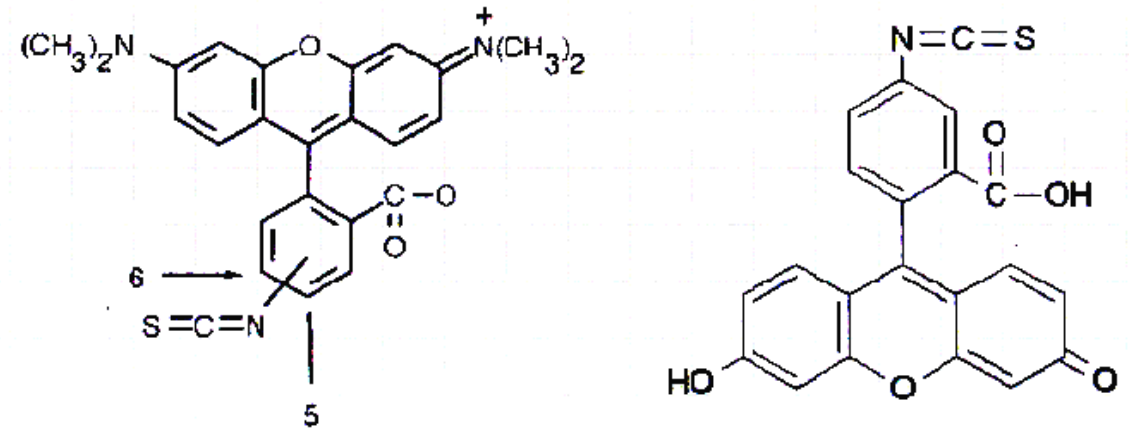

Figure 3.1 Structures of the two fluorescent dyes used to label probes.

3.2.2. Transfection of oligonucleotides into human cells

3.2.2.1. Transfection of Adherent Cells Protocol

In a 12 well tissue culture plate seed 0.4-1.6 x 10^5 (starting point: 0.8 x 10^5) cells per dish in 1ml of freshsuitable complete medium. Incubate the cells at 37°C in a CO_2 incubator until they are at 30-60% real confluency. The required time will vary among cell types, butusually this will take 18-24 hours. The solutions of DNA/RNA and METAFECTENE transfection reagent should have an ambient temperature and should be gently vortexed prior to use. Prepare the following solutions using a cell culture grade 96-well plate or other tubes made of glass,polypropylene or polystyrene:

Solution A: 0.5–1.5µg of DNA/RNA in 50µl medium free of serum and antibiotics;

Solution B: 1.0–6.0µl (lipid : DNA/RNA range of 2–7 : 1) of metafectene transfection reagent in 50µlmedium free of serum and antibiotics. Ratios will need optimizing based on various factors. Mix the solutions gently by carefully pipetting several times. Combine the two solutions, mix gently by carefully pipetting several times (do not vortex or centrifuge !), andincubate at room temperature for 15-20 min. This time is required to form the DNA/RNA-lipid complex. Add the DNA/RNA-lipid complexes to the wells with the cells and mix gently. (If toxicity is a problem because of very sensitive cells, remove the transfection mixture after 3-6 hours and replace it with medium).Incubate at 37°C in a CO_2 incubator. Depending on cell type and promoter activity, assay cell extracts for gene activity 24-72 hours following thestart of transfection.

3.2.2.2. Transfection of Suspension Cells Protocol

In a 12 well tissue culture plate seed 0.2-1.0 x 10^6 cells in 2 ml fresh suitable complete medium. The solutions of DNA/RNA and metafectene transfection reagent should have ambient temperature andshould be gently vortexed prior to use. Prepare the following solutions using a cell culture grade 96-well plate or other tubes made of glass,polypropylene or polystyrene for each transfection:The solutions of DNA/RNA and metafectene transfection reagent should have ambient temperature andshould be gently vortexed prior to use.

Solution A: 0.5–1.5µg of DNA/RNA in 50µl medium free of serum and antibiotics;

Solution B: 1.0–6.0µl (lipid : DNA/RNA range of 2–7 : 1) of metafectene transfection reagent in 50µlm medium free of serum and antibiotics. Ratios will need optimizing based on various factors. Mix the solutions gently by carefully pipetting several times.
Combine the two solutions, mix gently by carefully pipetting several times (do not vortex or centrifuge), and incubate at room temperature for 15-20 min. This time is required to form the DNA/RNA-lipid complex. Add the DNA/RNA-lipid complexes to the cell suspension and mix gently. (If toxicity is a problem by use of very sensitive cells, remove the transfection mixture after 3-6 hours and replace it with medium). Incubate at 37°C in a CO_2 incubator. Depending on cell type and promoter activity, collect cells by centrifugation and assay cell extracts for genen activity 24-72 hours following the start of transfection.

Transfection of HeLa cells was performed in the same manner as for K562, except that the HeLa cells were trypsinized the day before transfection. For both types of cells, 10mg/ml colchicine was added one hour before harvesting.

Following incubation, cells were UV-irradiated in a 4ºC room for 10min using a Model B 100AP BLAK-RAY® UV lamp (14W, 320-400nm; UVP, Inc.) 10cm above the sample.
Cells were pelleted at 2000rpm for 5min, resuspended in 0.5% sodium citrate, and incubated at 37ºC for 25min. Cells were then centrifuged for 7 min at 1000rpm and gently resuspended in 0.5% sodium citrate. Finally cells were fixed with methanol: acetic acid solution (3:1).

3.2.3. Preparation of metaphase chromosome spreads from human cells

Immediately prior to adding the cells, the slides were incubated in steam from a 92ºC water bath for 5min to provide a uniform layer of moisture on the slide. Then the cell suspension was spread evenly on the slide. To maximize spreading, 20µl of acetic acid were added and allowed to cover the surface of the slide; the slide was immediately returned to the steam for 20sec, and then incubated on the surface of a hot plate for 3sec, until the cells appeared grainy. Finally, all slides were counterstained with PI, DAPI or TOTO-3
Two main factors facilitate chromosome spreading: the brief increase in acetic acid concentration when most of the methanol has evaporated, and the rapid exposure of condensed water vapor to the hot surface. The mix of acid and water at this moment, followed by quick drying on a hot surface, provides good chromosome spreading.

CHAPTER 4

RESULTS

Transfection and *in vivo* chromosomal binding

We have tried Initial trials with different transfection agents – provide a list and explain why most were rejected, and why Metafectene was chosen as the most favorable one.

Confirmation of chromosomal specificity of probe binding

Once Metafectene was characterized as the most satisfactory agent and shown not to be cyto-toxic, we set about to establish that the psoralen delivery strand designed to be specific for sickle cell or wild type β globin binds to the correct target on the correct chromosome. (Mustafa, you are summarizing the results and evidence too quickly and in too cursory a fashion: This is not appropriate for a thesis. You have to give the logic at each step. What you have done is too brief and too much a summary, which is not acceptable for a thesis or for a paper.

So far, we have achieved successful transfection and *in vivo* binding of the fluorescently labeled psoralen-delivery strands specific for the β-globin gene site in addition to probes specific for other chromosomes, in two human cell lines. For each probe, the location of the fluorescent signal corresponds to the known approximate location of its target sequence on sister chromatids (Figures. 4.2.-4.5). These observations suggest highly specific probe binding. Additionally, to confirm that the gene-specific probes were binding to the correct chromosomes, each of these probes was hybridized to chromosome spreads simultaneously with probes specific for the appropriate centromere.

Given that we have a probe that is specific for a centromeric target in chromosome 17, and probes for the Her2/neu and the TopoII genes in that chromosome, each probe was labeled with a different fluor, co-transfected into cells and their locations were examined on the resulting chromosome spreads. (Figures 4.1 and 4.2. show the result of these probes bound individually).

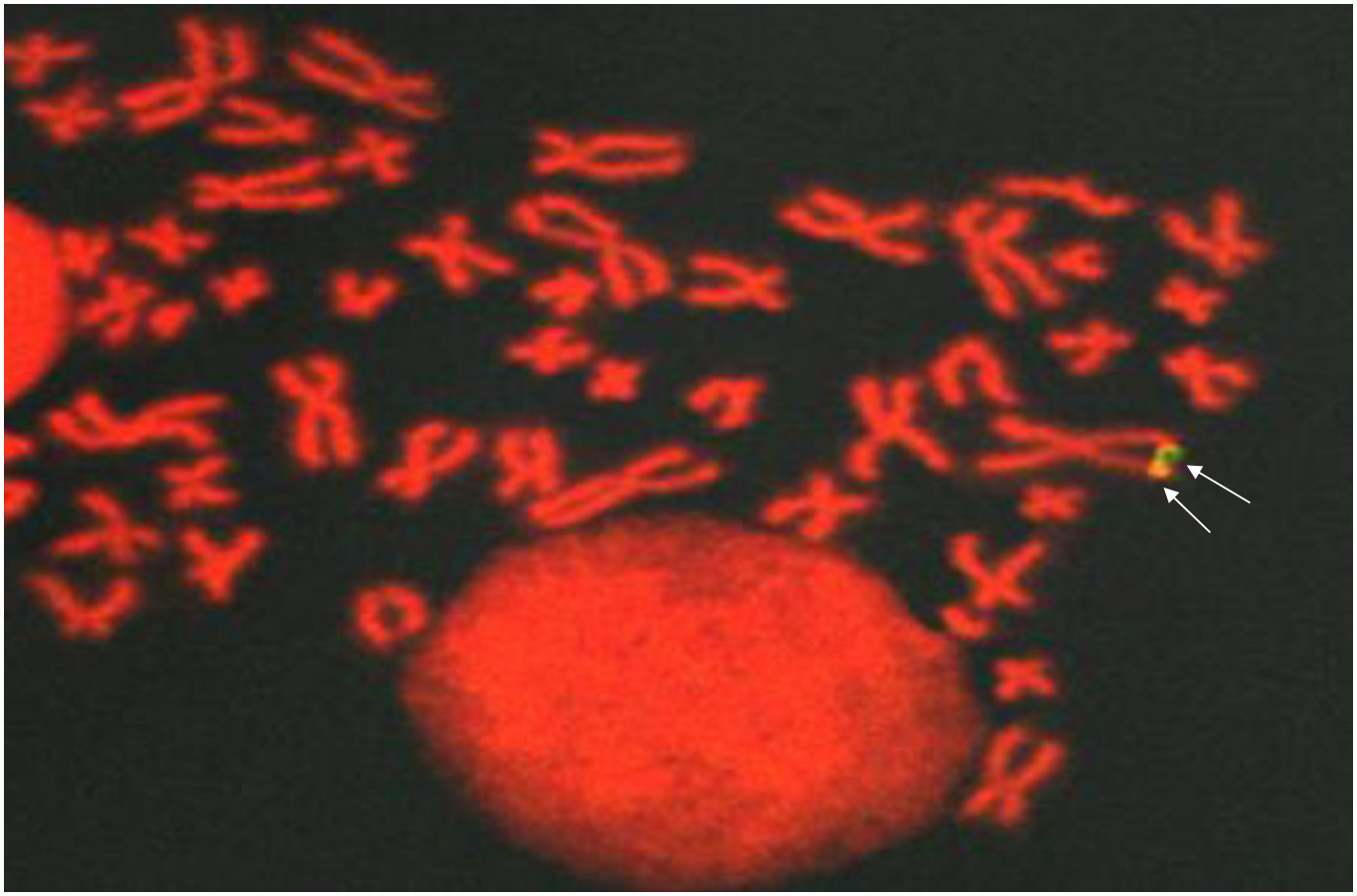

Figure 4.1. *In vivo* binding of psoralen-delivery strands to amplified Her2/neu genes in chromosome 17 of HeLa cells.

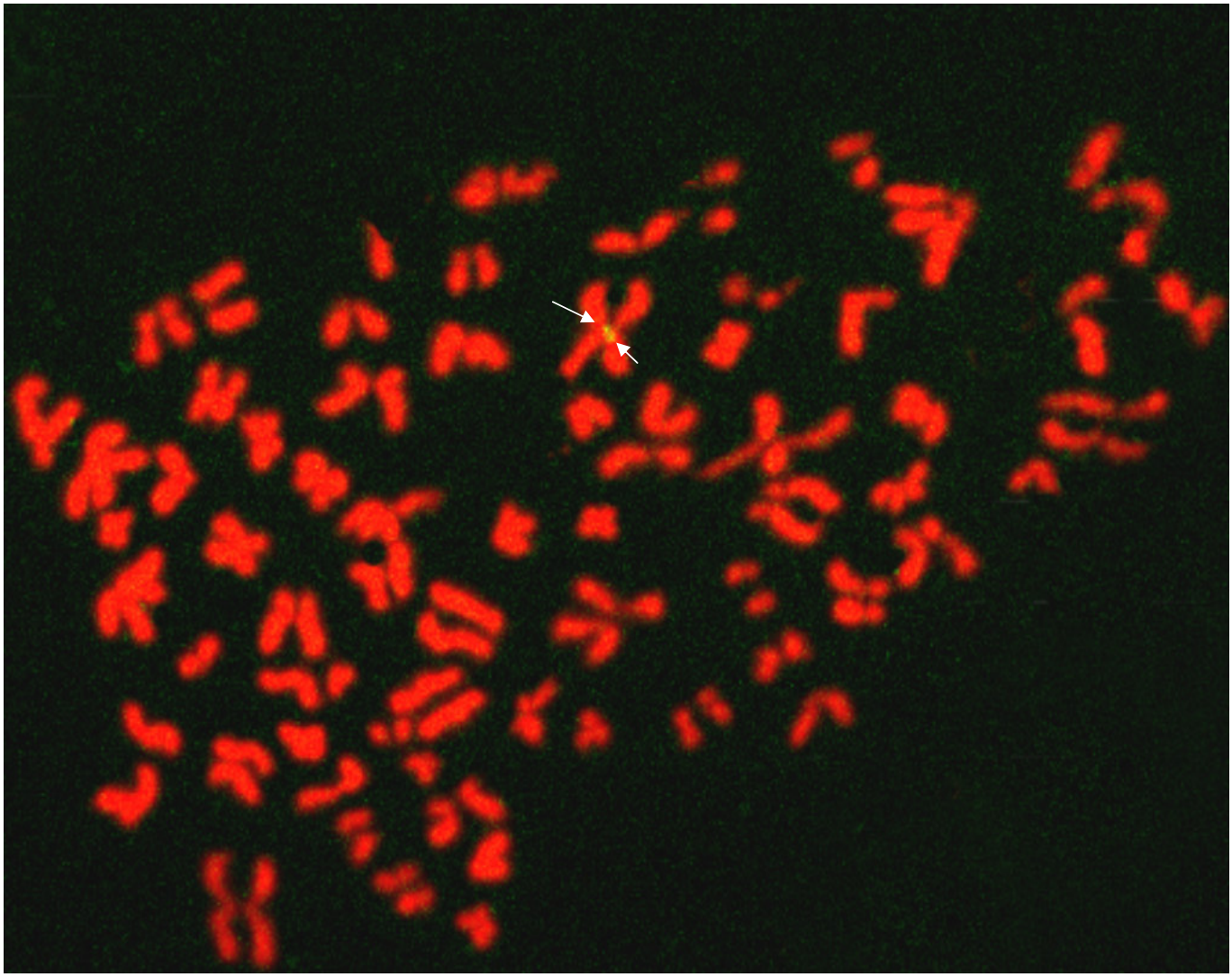

Figure 4.2. *In vivo* hybridization of psoralen-delivery strand to centromeric target on chromosome 17 of K562 cells.

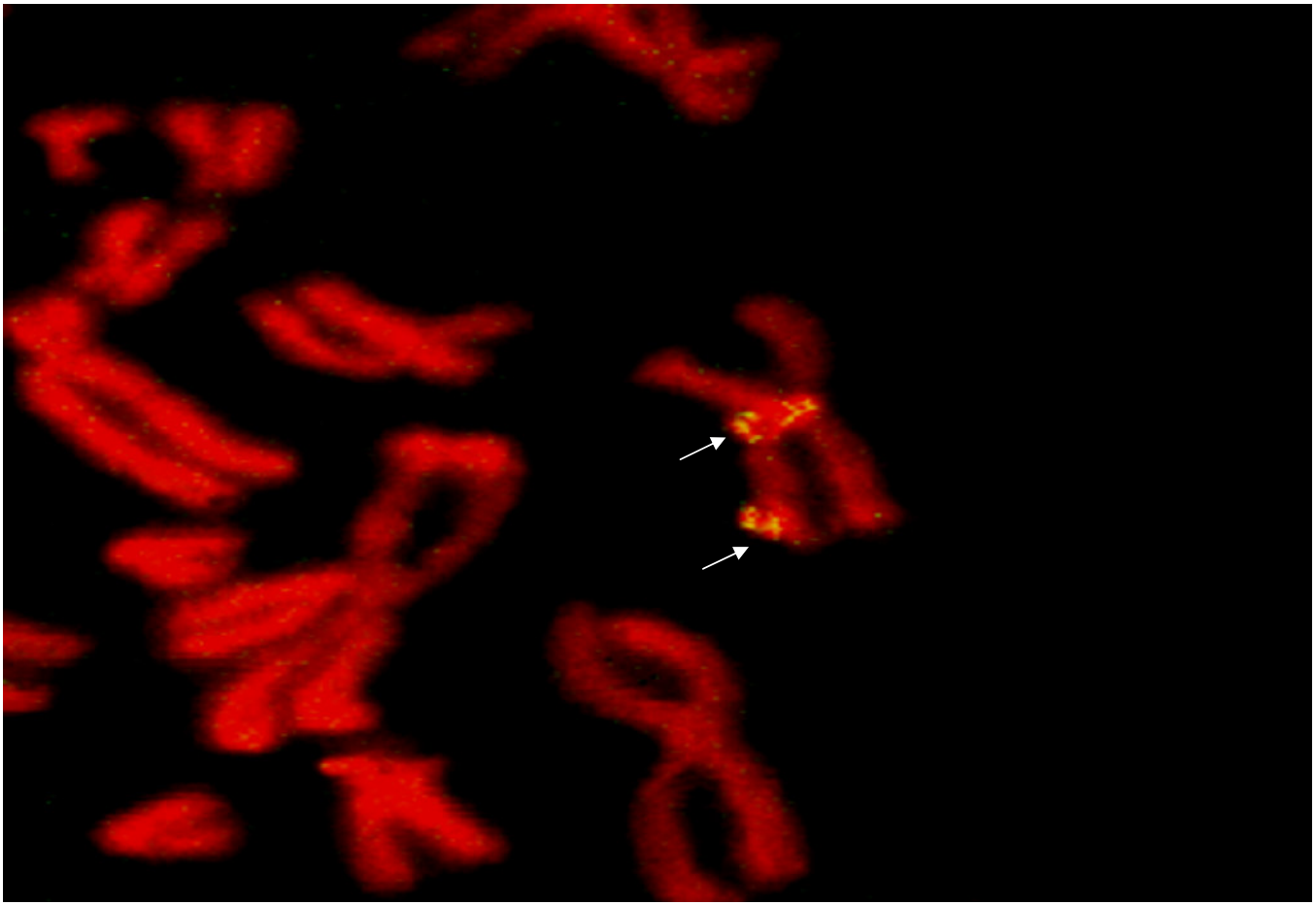

Figure 4.3. Photomicrograph of metaphase chromosomes spread from HeLa cells transfected with a new third strand binding probe specific for an alpha satellite sequence unique to Chromosome 11 and a second probe specific for the beta globin sequence adjacent to the site where the Sickle Cell mutation occurs. The spots to the two alpha satellite binding sites at the centromere of the sister chromatids and to the beta-globin site on one of them.. Both probes are located close to their known chromosomal positions.

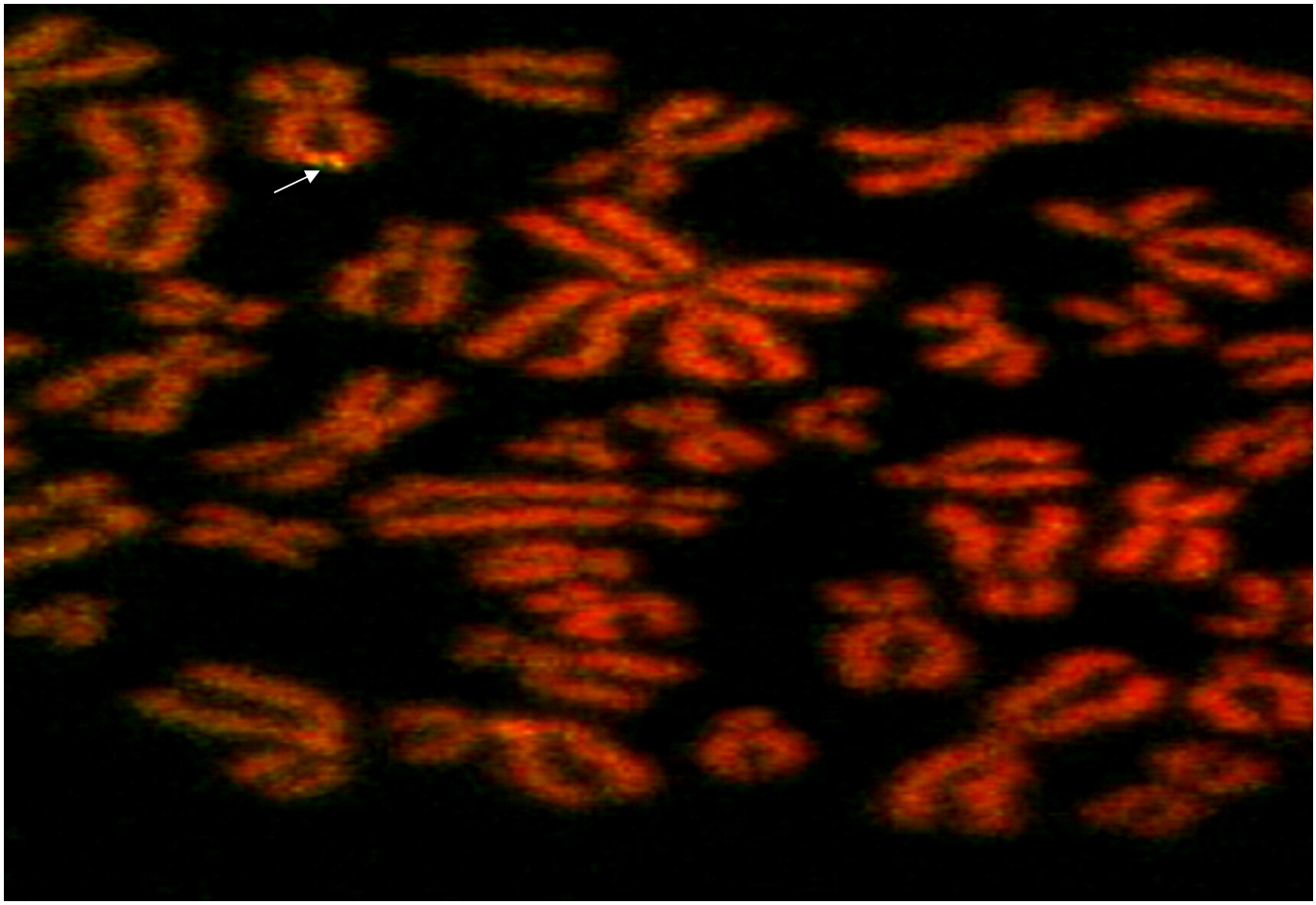

Figure 4.4. *In vivo* binding of psoralen-delivery strands to β-globin gene in chromosome 11 of HeLa cells.

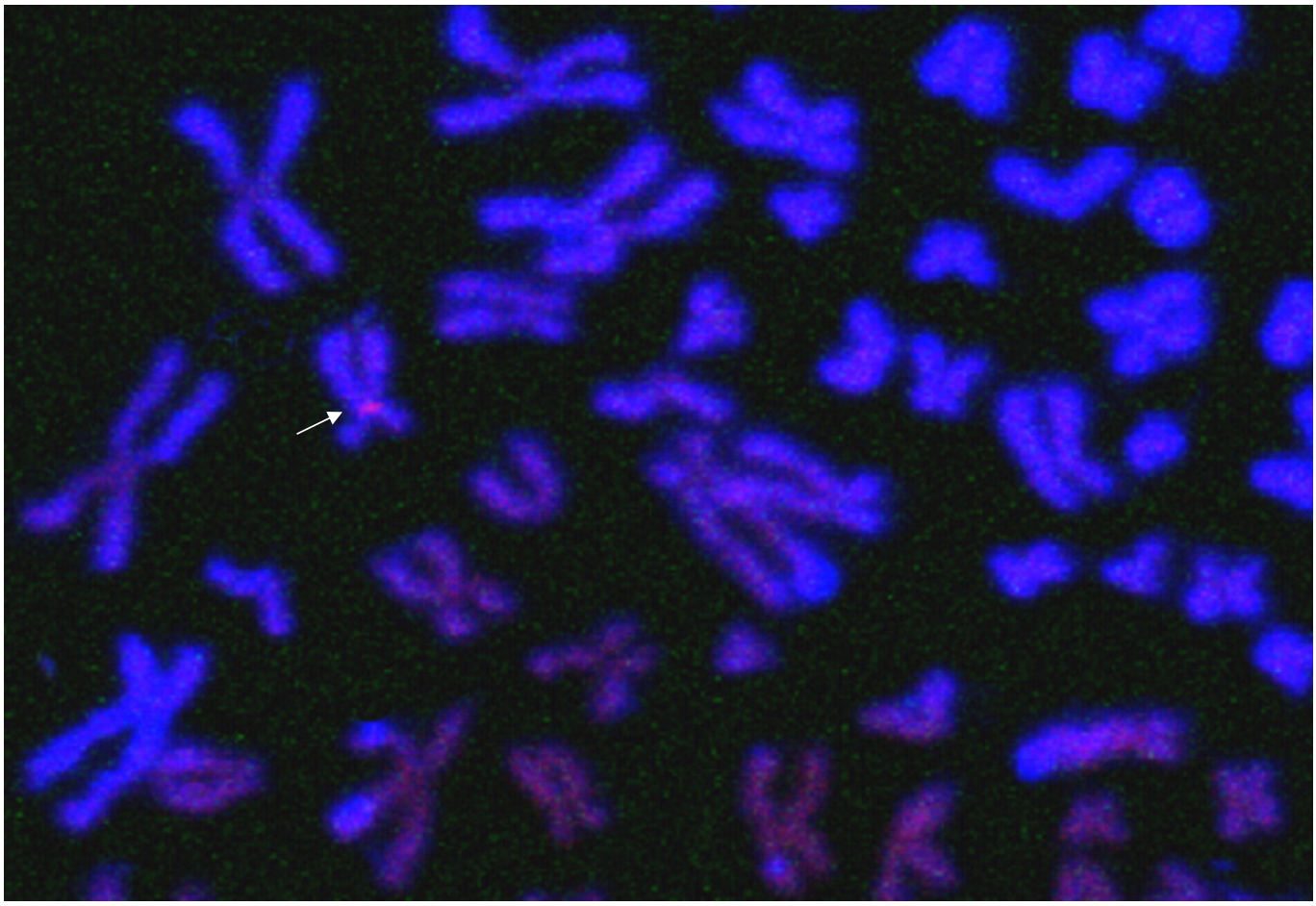

Figure 4.5 Photomicrograph of metaphase chromosomes spread from HeLa cells transfected with the same new third strand binding probe specific for the alpha satellite sequence unique to Chromosome 11. The red spot to the two alpha satellite binding sites at the centromere of the sister chromatids. The probe in this case is labeled with TMR and the background stain is TOTO-3.

The same type of co-transfection and probe visualization analysis was performed in order to establish that the transfected psoralen-delivery strand for the Sickle Cell β-globin gene target is in fact bound to chromosome 11. Figures show the individual binding of the β-globin probe and of the chromosomes centromeric probe. You have a photo that shows binding to both the centromere and to β-globin that is much better than the one you show, which is poor.

We have determined that a 24 hour incubation optimizes the efficiency of transfection into K562 and HeLa cells. Transfection efficiency was assessed by fluorescence activated cell sorting (FACS) analysis.

These analyses (Figure 4.6.) suggest that a 24 hour incubation period optimizes the efficiency of transfection of our probes into K562 and HeLa cells. Kinetics of transfection of different probes into HeLa and K562 cells.

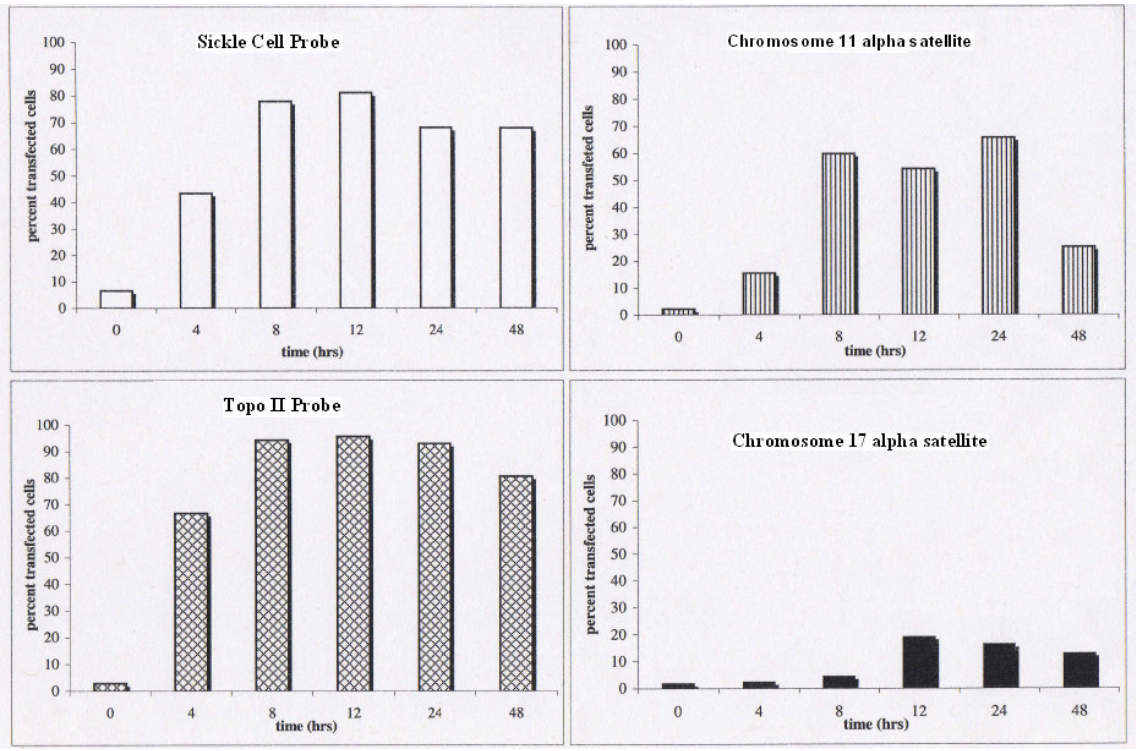

Figure 4.6. Probes transfection efficiency

CHAPTER 5

5.1. DISCUSSION

Our results demonstrate that small homopyrimidine oligodeoxyribonucleotide probe sequences designed for particular targets specifically bound *in vivo* to their targets in the human cell genome as indicated by their fluorescent labels.

Human K562 and HeLa cells were transfected with homopyrimidine deoxyoligonucleotide strands bearing sequences to enable their specific binding to targets in the centromere of chromosome 11 and to the Sickle Cell mutation site in the β-globin gene located near the tip of that choromosome. So they can be visualized in the fluorescent microscope, the strands were each labeled at their 5′-ends with a fluorescent dye. The strands also contained a photoreactive intercalating psoralen moiety, so that upon irradiation they would form a photoadduct with a nearby pyrimidine residue in the target sequence. Conditions were found for efficient transfection of these third strand-binding probes, which were observed to enter the nucleus in a few hours. After extended incubation, followed by treatment with colchicine to block the cells in metaphase, the cells were briefly irradiated to covalently link their psoralen to a pyrimidine residue nearby their binding site. The cells were then burst in hypotonic solution, their chromosomes spread on glass slides, fixed with methanol-acetic acid, and examined in the confocal microscope for the distribution of the probe strands. Probes were in evidence only on the centromeres of the sister chromatids of chromosome 11 and at the tips of these chromatids, where the β-globin gene is known to occur. These observations verify the survival of the probe strands for at least 23 hours in these two human cell types, and the specificity of their binding to the targets designated by their sequences.

While such homopyrimidine probe strands bind well intracellularly, where the pH is known to be very close to neutrality or even higher, they bind poorly or not at all to their

target sequences in dilute solution. Presumably, the intracellular behavior is due to the macromolecular crowding therein.

TFOs have been shown to inhibit both transcription *in vitro* and the expression of target genes in cell culture by binding to a polypurine–polypyrimidine tract in several human gene promoters and in chromatin. Triplex formation in the promoter or coding sequences of several genes have been described previously [78,79].

Traditional gene therapy has utilized both viral and nonviral delivery systems [82,83]. Prolonged transgene expression is dependent on random genomic insertion which is not advisable. Moreover, adenoviral vectors do not integrate, and actively dividing cells are required for retroviral integration, both major restrictions for somatic gene therapy. Also production of viral vectors is time consuming and expensive. Nonviral delivery systems have the potential advantages of being less immunogenic and providing greater ease of production, purity, and standardization of molecules [84].

The limitations associated with gene augmentation have resulted in the pursuit of alternative strategies. Specifically, there is a growing interest in gene repair in which nucleic acids are designed to affect correction of precise genomic mutations [85] rather than replacement of a non-functional gene. Site-specific gene repair has a number of advantages over gene augmentation. First, in situ repair of the targeted mutation allows the gene to remain under the control of its natural regulatory elements. Second, precise repair has the potential to address both recessive and dominant mutations, whereas augmentation is generally restricted to recessive diseases. Third, the repair process usually involves the use of small synthetic molecules, which are typically less immunogenic. In fact, immunostimulatory CpG motifs may contribute significantly to the exaggerated immune states associated with some vectors/constructs [86]. Fourth, the smaller size of repair molecules may increase cell delivery and nuclear translocation, compared with the larger plasmid systems. Finally, effective and permanent gene repair may be achieved with fewer treatments, and possibly even a single treatment.

Performing gene therapy to repair the sickle-cell mutation in embryonic stem cells would be an ideal, curative treatment, and repair by site-directed mutagenesis using psoralen-tethered triplex-forming oligonucleotides [TFO's] represents a unique approach. By

exploiting the specificity with which deoxyoligonucleotides can bind to DNA duplexes to form DNA triplexes, a TFO was previously designed that had a strand- switching binding mechanism: binding a homopurine stretch downstream of the sickle cell mutation on the coding strand followed by a homopurine stretch on the non-coding strand. TFO binding creates a lesion that is recognized and repaired by the cell's nucleotide excision removal pathway [NER], with a certain frequency to the wild-type sequence. Even if some undesirable mutations occur this way, properly repaired stem cells can be selected and propagated and cloned, while the others can be discarded.

Repair of a defective gene [rather than *insertion* of a correct one by viral transfer] makes it possible to circumvent problems related to viral vector misinsertion of the intended gene. With repair, the defect is corrected right on the chromosome and expression of the repaired gene is regulated by the intrinsic cellular regulatory mechanisms. Triplex-mediated site-specific gene repair is particularly suitable as a strategy for correcting point mutations *in situ.* In this respect, our work in this thesis to successfully transfect the psoralen delivery strand to achieve correction of the sickle cell mutation is a useful and important step in creating a mutation correction method for that disease.

CHAPTER 6

CONCLUSIONS

To develop conditions and find reagents that will afford efficient transfection of the psoralen-delivery strand into human primary hematopoietic stem cells. Towards this end, we had investigated its transfection and intranuclear binding into HeLa, K562, also we will investigate transfection of TFO into CD34+ and transgenic mouse primary hematopoietic stem cells containing the human wild type or Sickle Cell β-globin gene.

To develop a protocol for using the psoralen-delivery strand, which binds as well to the wild-type β-globin sequence, for creating human cell lines with the Sickle Cell mutation.

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