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Restoration of Human β-Globin Gene Expression in Murine and Human IVS2–654 Thalassemic Erythroid Cells by Free Uptake of Antisense Oligonucleotides

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ABSTRACT

Correct human β-globin mRNA has been restored in erythroid cells from transgenic mice carrying the human gene with β-globin IVS2–654 splice mutation and from thalassemia patients with the IVS2–654/β^E genotype. This was accomplished in a dose- and time-dependent manner by free uptake of morpholino oligonucleotide antisense to the aberrant splice site at position 652 of intron 2 in β-globin pre-mRNA. Under optimal conditions of oligonucleotide uptake, the maximal levels of correct human β-globin mRNA and hemoglobin A in patients' erythroid cells were 77 and 54%, respectively. These levels of correction were equal to, if not higher than, those obtained by syringe loading of the oligonucleotide into the cells. Comparison of splicing correction results with the cellular uptake of fluorescein-labeled oligonucleotide indicated that the levels of mRNA and hemoglobin A correlate well with the nuclear localization of the oligonucleotide and the degree of erythroid differentiation of cultured cells. Similar but not as pronounced results were obtained after the oligonucleotide treatment of bone marrow cells from IVS2–654 mouse. The effectiveness of the free antisense morpholino oligonucleotide in restoration of correct splicing of IVS2–654 pre-mRNA in cultured erythropoietic cells from transgenic mice and thalassemic patients suggests the applicability of this or similar compounds in in vivo experiments and possibly in treatment of thalassemia.

β-Thalassemia, a genetic blood disorder that affects hundreds of thousands of people worldwide, is caused by more than 200 mutations in the β-globin gene that lead to deficiency of β-globin and adult hemoglobin A. Severe cases of β-thalassemia result in pronounced anemia, bone deformities, hepatosplenomegaly and, if left untreated, death (National Institutes of Health, 1995; Schwartz et al., 2000).

Bone marrow transplantation offers the only cure for thalassemia but its application is limited (Schwartz et al., 2000). Current treatment requires life-long blood transfusions combined with iron chelation. Although effective, the treatment is cumbersome, especially for pediatric patients; in underdeveloped countries, where the incidence of the disease is high, its cost is prohibitive (Schwartz et al., 2000). Experimental treatments, such as stimulation of Hb F synthesis with hydroxyurea (Olivieri and Weatherall, 1998; Olivieri, 1999; Fucharoen and Winichagoon, 2000) and gene therapy (Rivella and Sadelain, 1998; Russell and Lieberhaber, 1998; Li et al., 1999; May et al., 2000), although very promising, are still not a clinical reality. Thus, development of alternative treatments is clearly needed.

Among the mutations responsible for β-thalassemia, some of the most common are splicing mutations found in intron 1 (IVS1–5, –6 and –110) (Busslinger et al., 1981; Spritz et al., 1981; Fukumaki et al., 1982; Treisman et al., 1983; Cheng et al., 1984; Kazakian and Boehm, 1988) and intron 2 (IVS2–654 and –745) (Treisman et al., 1983; Cheng et al., 1984; Dobkin and Bank, 1985; Kazakian and Boehm, 1988; Huang et al., 1994) of the β-globin gene. These mutations generate

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ABBREVIATIONS: Hb, hemoglobin; BM, bone marrow; IMDM, Iscove's modified Dulbecco's medium; FBS, fetal bovine serum; epo, erythropoietin; SCF, stem cell factor; SL, syringe-loaded; 2′-O-MOE, 2′-*O*-(2-methoxy)ethyl; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; RT-PCR, reverse transcription-polymerase chain reaction; BFU, burst-forming unit.

aberrant splice sites and splicing pathways with the correct splice sites remaining potentially functional (Sierakowska et al., 1997, 1999) (see Fig. 1). A common mutation (HbE), which leads to production of hemoglobin E, also leads to aberrant splicing of β-globin pre-mRNA (Weatherall and Higgs, 1993; Miller and Baehner, 1995).

We have previously shown that blocking of the mutation-activated aberrant splice sites in intron 2 of the β-globin gene (IVS2–654, –705, and –745) with modified antisense oligonucleotides induced the splicing machinery to revert to the correct splice sites and produce correct β-globin mRNA and β-globin polypeptide (Sierakowska et al., 1996, 1999; Kole, 1998). Similar results were also obtained for IVS1–5, –6, and –110, and HbE mutants (Dominski and Kole, 1993; Shirohzu et al., 2000; T. Suwanmanee and R. Kole, unpublished observations). The correction of splicing of thalassemic β-globin pre-mRNA was accomplished in cell free extracts (Dominski and Kole, 1993) in stable cell lines transfected with the mutated β-globin gene (Sierakowska et al., 1996, 1997, 1999; Schmajuk et al., 1999) and in erythroid mononuclear cells isolated from peripheral blood of β-thalassemic patients (Lacerra et al., 2000).

High level of correction of splicing of β-globin mRNA in erythroid cells from human patients has been achieved with the application of morpholino oligonucleotides (Lacerra et al., 2000). This type of oligonucleotide is resistant to nucleases and does not promote cleavage of target RNA by RNase H. Because its backbone lacks negative charges present on internucleotide linkages of DNA- or RNA-based oligonucleotides, its uptake and pharmacokinetic properties are expected to be different from those of standard oligonucleotides (Summerton and Weller, 1997). Although this compound cannot interact with cationic lipid transfection reagents, we have found that it can be effectively delivered to cultured cell lines and human primary erythroid cells by methods such as scrape and syringe loading (Summerton and Weller, 1997; Schmajuk et al., 1999; Lacerra et al., 2000). It has been previously shown that with these methods of delivery, which temporarily distort the cell membrane, ensuring its permeability to oligonucleotides, the morpholino oligonucleotides were more effective in repair of β-globin pre-mRNA splicing than oligonucleotides with other backbones (Schmajuk et al., 1999; Lacerra et al., 2000).

In this study, to lay the groundwork for in vivo experiments, we have sought to restore proper function of the IVS2–654 thalassemic β-globin gene by free, unaided uptake of the oligonucleotides into the cells. We found that this approach resulted in effective repair of aberrant splicing in IVS2–654 murine and human erythroid precursors. In cells from thalassemia patients, the formation of hemoglobin A was also detected. These results suggest that the free uptake of morpholino oligonucleotides should be applicable to in vivo studies on the thalassemic mouse model and, possibly, to future clinical treatment of thalassemia patients.

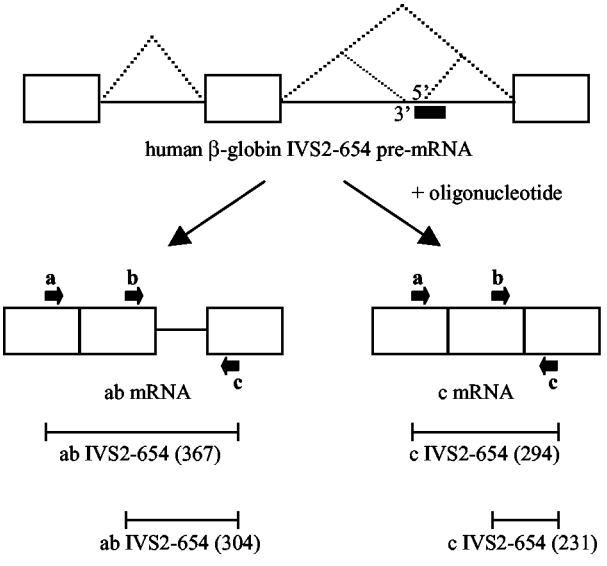


Fig. 1. Correction of splicing of thalassemic human β-globin IVS2–654 pre-mRNA by oligonucleotide targeted to the aberrant 5′ splice site. Boxes, exons; solid lines, introns; dashed lines, correct and aberrant splicing pathways. The aberrant 5′ splice site created by the IVS2–654 mutation and the cryptic 3′ splice site activated upstream are indicated. Thick bar, oligonucleotide antisense to the aberrant 5′ splice site; arrows, primers used in the RT-PCR reaction. The length (in nucleotides) of the appropriate RT-PCR products generated on aberrantly (ab) and correctly (c) spliced mRNAs are shown below each diagram. Forward primers **a** and **b** were used for patient and murine RNA, respectively.

Materials and Methods

Mouse Erythroid Cells. Bone marrow (BM) cells of 3-month-old "knock-in" transgenic mice, a model for human IVS2–654 β-thalassemia (Lewis et al., 1998) were collected by flushing the femurs and tibiae with Iscove's modified Dulbecco's medium (IMDM) containing 2% fetal bovine serum (FBS) (StemCell Technologies, Vancouver, BC, Canada). The cells were sedimented at 1500 rpm for 8 min, treated with ammonium chloride solution (StemCell Technologies), to lyse the red blood cells and washed twice with above medium. The purified BM cells were suspended at 2.7×10^6 cells/ml in IMDM containing 15% plasma-derived serum (Animal Technologies, Tyler, TX), 1% bovine serum albumin (StemCell Technologies), 5% protein free hybridoma medium (Invitrogen, Carlsbad, CA), 100 μM 2-mercaptoethanol, 2 mM L-glutamine, 50 μg/ml ascorbic acid (Sigma), 300 μg/ml human iron-saturated transferrin (StemCell Technologies), 100 units/ml penicillin-streptomycin, 3 units/ml recombinant human erythropoietin (epo) (Amgen, Thousand Oaks, CA), and 25 ng/ml recombinant mouse stem cell factor (SCF) (R & D Systems, Minneapolis, MN), and cultured in 5% CO_2 at 37°C at 0.8×10^6 cells per 0.3-ml well in 96-well plates; 30% of the medium was replaced daily with fresh medium containing epo and SCF.

Human Erythroid Cells. Blood samples were obtained from three Thai patients with thalassemia intermedia, IVS2–654/β^E, with informed consent according to Thai and United States regulations. The total mononuclear cells were isolated by Ficoll gradient (lymphocyte separation medium, ICN/Cappel, Aurora, OH) according to manufacturer's instructions and purified from the remaining red blood cells with the ammonium chloride solution. After two washes with IMDM containing 2% FBS, the cells were suspended at 3×10^6 cells/ml of above medium containing 30% FBS (StemCell) and other components as used with mouse BM cells with the exception of hybridoma medium, human transferrin, and ascorbic acid. The cells were plated at 3×10^6 cells/ml per well in 24-well plates.

Oligonucleotide Treatment. Two techniques, syringe loading (SL) (Lacerra et al., 2000) and free uptake, were used to introduce into the cultured cells the 18-mer morpholino and 2′-*O*-(2-methoxy) ethyl (2′-O-MOE) oligonucleotides, ON-654 (5′-GCTATTACCTTA-ACCCAG) antisense to the aberrant 5′ splice site in the IVS2–654 pre-mRNA. A morpholino oligonucleotide, ON-705 (5′-CCTCUUAC-CUCAGUUACA) targeted to the aberrant 5′ splice site in the IVS2–705 β-globin pre-mRNA served as a negative control. Free uptake of oligonucleotides was obtained by adding the oligonucleotide at the proper concentration to the culture medium. Oligonucleotides were

prepared and purified by Gene Tools (Philomath, OR) and ISIS Pharmaceuticals (Carlsbad, CA).

For intracellular oligonucleotide localization, the mononuclear cells isolated from a healthy subject and cultured as above were treated by both syringe loading and free uptake with fluorescein isothiocyanate (FITC) and Texas Red labeled 18-mer morpholino (5′-CCUCUUACCUCAGUUACA) and 2′-O-MOE (5′-TTATTCTTTAGAATGGTG) oligonucleotides, respectively. Treated cells were washed twice with PBS, loaded at 10^5 cells per well coated with polylysine (BD Bioscience, Bedford, MA) of printed microscope slide (Carlson Scientific, Peotone, IL) and kept in a moist chamber for 1 h. Subsequently the cells were fixed by flooding the slides with 2% *p*-formaldehyde for 15 min, followed by washing with PBS and mounting with Vectashield or Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA).

Isolation and Analysis of Human β-Globin mRNA. The total cellular RNA was isolated with 200 ng TRI-Reagent (Molecular Research Center, Cincinnati, OH) and analyzed by reverse transcription (RT)-PCR using rTth DNA polymerase (PerkinElmer Life Sciences, Norwalk, CT) and 0.2 μCi of [α-^{32}P]dATP per sample at 18 to 25 cycles. Assay of human β-globin mRNA in treated mouse BM cells was performed with forward and reverse primers spanning positions 21 to 43 of exon 2 (Fig. 1, primer **b**) and positions 6–28 of exon 3 (primer **c**) in human β-globin gene, respectively. The aberrant and correct splicing of human IVS2–654 β-globin pre-mRNA in the compound heterozygote IVS2–654/β^E was detected by using a forward allele-specific β^A primer **a** (GCAAGGTGAACGTGGATGAAGTTGGTGTTG, positions 50–79 of β-globin exon 1) and the reverse primer **c**. The RT-PCR products were separated on 7.5% nondenaturing polyacrylamide gel and detected by autoradiography. No product was detectable without the reverse transcription step. Linearity of the RT-PCR response was demonstrated as in Lacerra et al. (2000).

Injection of Repaired Thalassemic Mouse BM Cells into Wild-Type Mouse. IVS2–654 mouse cultured BM cells were treated by syringe loading with morpholino oligonucleotide (ON-654) and after harvesting and washing with PBS were suspended at 70×10^6 cells/0.4 ml and injected intraorbitally into a wild-type mouse. Blood aliquots (50 μl) were collected from the tail vein immediately preceding and at 4 h and 1, 2, 3, and 5 days after injection, and the RNA was isolated with TRI-reagent-BD (Molecular Research Center) and analyzed as above.

Isolation and Analysis of Hemoglobins. Assay of human hemoglobin A in oligonucleotide-treated cultured human mononuclear cells of the compound heterozygote IVS2–654/β^E was performed by cellulose acetate electrophoresis and immunodetection. The hemoglobin from 3×10^6 washed cells was extracted with 40 μl of hemolysate reagent and separated on Titan III-H cellulose acetate strips (76 × 60 mm) alongside standard hemoglobins. The electrophoresis protocol and materials were from Helena Laboratories (Beaumont, TX). The cellulose acetate strips were stained with 0.5% Ponceau S, destained with 5% acetic acid, and subsequently processed by immunodetection (Dominski and Kole, 1993) using polyclonal affinity-purified chicken antihuman hemoglobin IgG and rabbit anti-chicken horseradish peroxidase-conjugated IgG (Accurate, Westbury, NY) as primary and secondary antibodies, respectively. The blots were developed with an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). All autoradiograms were scanned using Adobe Photoshop and figures were generated with Adobe Illustrator (Adobe Systems, Mountain View, CA). NIH Image 1.61 software (http://rsb.info.nih.gov/nih-image/) was used for quantitation.

Results

Aberrant Splicing of Human β-Globin pre-mRNA in IVS2–654 β-Thalassemia. A C-to-T mutation at nucleotide 654 of intron 2 of the human β-globin gene generates in the transcribed pre-mRNA a GU dinucleotide, which forms a 5′ splice site at nucleotide 652. The same mutation activates a cryptic 3′ splice site 73 nucleotides upstream. These aberrant 5′ and 3′ splice sites are used in conjunction with the normal, unaltered splice sites at the ends of the intron, leading to retention of a portion of the intron in the spliced β-globin mRNA (Fig. 1, left). This aberrant splicing pathway prevents correct translation of β-globin, resulting in thalassemia. Blocking the aberrant splice sites with antisense oligonucleotides prevents aberrant splicing, causing the spliceosome to form at the normal splice sites. This reverses the effect of mutation, generating correctly spliced mRNA (Fig. 1, right), which is translated into full-length β-globin. In this study, we have used an 18-mer morpholino oligonucleotide targeted to the aberrant 5′ splice site (ON-654) to treat both BM cells from a thalassemic IVS2–654 mouse (Lewis et al., 1998) and the peripheral mononuclear cells from IVS2–654 thalassemic patients.

Correction of IVS2–654 pre-mRNA Splicing by Syringe-Loaded Morpholino Oligonucleotide in Murine Erythroid Progenitor Cells. The mouse expressing the human β-globin IVS2–654 thalassemic transgene models the molecular defect and pathological symptoms of IVS2–654 β-thalassemia. The expansion of early erythroid progenitor target cells was promoted by culturing the BM isolates in the presence of epo and SCF. Syringe loading of the cells with the oligonucleotide ON-654 18 h after cell plating resulted in a dose-dependent correction of IVS2–654 pre-mRNA splicing. RT-PCR of total RNA isolated 24 h after treatment indicates that the level of correctly spliced β-globin mRNA increased linearly in cells treated with 2.5 to 45 μM oligonucleotide; concomitant decrease in the aberrant RNA is also apparent (Fig. 2A, lanes 3–6). At the highest oligonucleotide concentration, the level of correct RNA reached approximately 60%. This value takes into account that the ratio of ^{32}P-labeled adenosine nucleotides in aberrantly/correctly spliced RNA is 1.57. This result shows that the oligonucleotide driven shift in splicing of human β-globin pre-mRNA from aberrant to correct is possible not only in cell lines (Sierakowska et al., 1996, 1997, 1999; Schmajuk et al., 1999) or mononuclear cells from peripheral blood (Lacerra et al., 2000) but also in precursor cells from murine BM.

The 18-h culture followed by 24-h oligonucleotide treatment used in the above experiment was found to be optimal in the time course analysis of splicing correction (Fig. 2B, lane 3). The restoration of correct splicing was much lower when the oligonucleotide was introduced immediately after plating (lane 2) or at any later time of culture. In fact, the percentage of correct mRNA slowly decreased with oligonucleotide treatments on days 2 to 12 of culture (Fig. 2B, lanes 4–9). The sequence specificity of the antisense nucleotide treatment is evident from the control, syringe-loaded at 18 h of culture with 18-mer morpholino oligonucleotide targeted to the region of the intron centered at nucleotide 705 (ON-705). Treatment with this oligonucleotide did not produce any correct β-globin mRNA (Fig. 2B, lane 10). ON-705 efficiently corrected splicing in another thalassemic mutant (IVS2–705) in which an aberrant 5′ splice site is created by a mutation at this site (Sierakowska et al., 1997, 1999; Schmajuk et al., 1999). Treatment with ON-705 provides a stringent test for sequence specificity of the antisense effects because this oligonucleotide hybridizes to the β-globin intron 2, with

a single mismatch, 51 nucleotides downstream from the IVS2–654 mutation, and is complementary to the IVS2–654 splice site with six mismatches, if G-U or G-T base pairing is taken into account.

To test the persistence of expression of the corrected β-globin mRNA, cells were syringe loaded at 18 h of culture and harvested 24 to 72 h later (Fig. 2C, lanes 3–5). As expected, a high yield of corrected RNA was obtained at the first time point (Fig. 2C, lane 3; equivalent to results shown in Fig. 2, A, lane 6, and B, lane 3), but was significantly diminished after 48 and 72 h (Fig. 2C, lanes 4 and 5). The persistence of the correction effect was also tested in vivo by intraorbital sinus injection of the treated IVS2–654 BM cells into a wild-type mouse. RT-PCR of total RNA from blood collected from the tail vein immediately preceding injection did not show any human β-globin mRNA (Fig. 3, lane 4). Both the aberrant and correct mRNAs appeared 4 h after injection and persisted at approximately the same level for additional 20 h (lanes 5 and 6); on the following day (lane 7), however, only a small amount of correct mRNA remained. Interestingly, although both the correctly and aberrantly spliced mRNAs became undetectable on day 3 (lane 8), the aberrantly spliced human β-globin mRNA reappeared 5 days after the injection (lane 9; see *Discussion*).

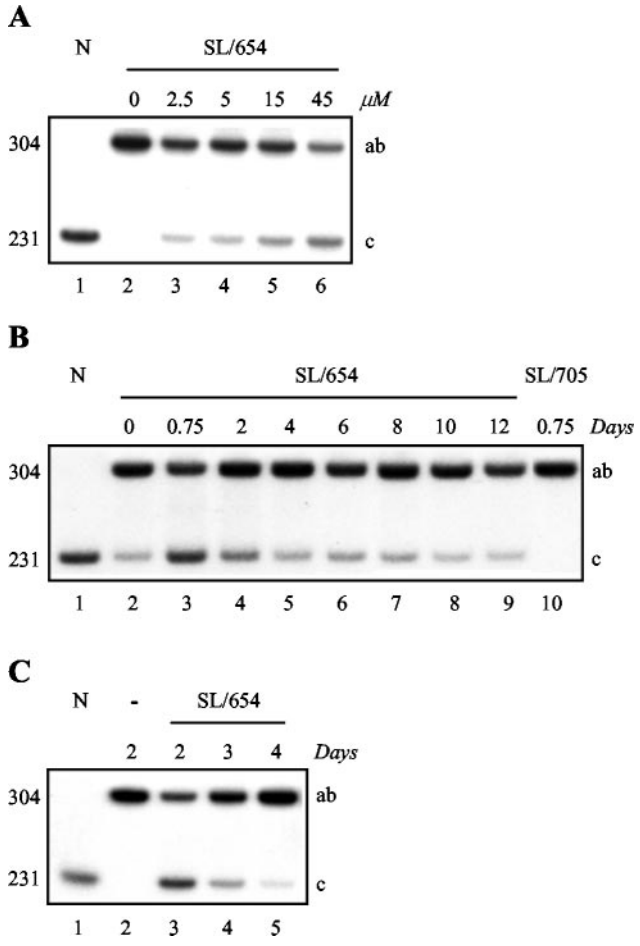


Fig. 2. Correction of splicing of IVS2–654 β-globin pre-mRNA in thalassemic mouse BM cells syringe-loaded with morpholino oligonucleotide (ON-654). RT-PCR analysis of total RNA. A, dose-dependence. The cells were treated at 18 h of culture, harvested 24 h after treatment and the total RNA was analyzed by RT-PCR (lanes 2–6). Lane 1, RNA from normal human blood (N). The numbers on the left indicate the size in nucleotides of the RT-PCR products representing the aberrantly (ab, 304) and correctly (c, 231) spliced human β-globin mRNA. Concentration (micromolar) of ON-654 is indicated above each lane. Unless otherwise indicated, the same designations are applied to subsequent figures. B, time course. Cells were syringe loaded with 45 μM ON-654 for 24 h on 0 to 12 days of culture (lanes 2–9). Lane 10, control cells syringe-loaded at 18 h with 45 μM morpholino oligonucleotide antisense to the IVS2–705 5′-splice site (ON-705). C, time course of decay of correct β-globin mRNA in cells syringe loaded with 45 μM ON-654 at 18 h of culture and harvested on days 2–4 (lanes 3–5). Lane 2, cells syringe treated without oligonucleotide.

Free-Uptake of Morpholino Oligonucleotide into Murine Erythroid Precursors. Syringe-pumping of the cells temporarily disturbs or damages the cellular membrane, allowing the oligonucleotide antisense to the IVS2–654 mutated splice site to enter the cells and translocate to the nucleus, where it affects splicing. Because this treatment is not applicable to in vivo studies, we have tried to deliver the negatively charged 2′-*O*-methyl- or 2′-*O*-methoxy-ethyl-oligoribonucleotides into murine BM cells, using a variety of cationic lipid or dendrimer molecules as carriers. These experiments failed because the carriers were either ineffective or toxic to the erythroid progenitor cells (data not shown). The delivery of free morpholino oligonucleotides under the conditions favoring syringe loading (i.e., 18-h BM culture, followed by 24 h treatment with 45 μM oligonucleotide, was also ineffective in correction of splicing of IVS2–654 pre-mRNA (Fig. 4A, lane 3 and data not shown). This result was disappointing because detectable correction of splicing of an IVS2–654 thalassemic mutant, modeled in HeLa cell line, was achieved by free uptake even at concentrations as low as 1 μM morpholino oligomer (Schmajuk et al., 1999).

Figure 4A shows that mouse BM cells cultured for 2 to 11 days in a medium containing 45 μM 654-18 mer morpholino oligonucleotide displayed significant, time-dependent accumulation of correct β-globin mRNA (lanes 3–8). The effect was sequence specific, because no repair of aberrant splicing was detected in cells cultured in the presence of 45 μM oligonucleotide targeting the IVS2–705 5′-splice site (lane 10). As could be expected, even at the highest level of correction, after 11 days of treatment (lane 8), the effect was lower than that in syringe-loaded cells at 18 h (lane 9), in which the membrane barrier was presumably bypassed. Nevertheless, this is an important result showing for the first time that free morpholino oligonucleotide can affect splicing in primary erythroid precursor cells.

Recent results showed that 2′-MOE oligonucleotides are effective in vivo, especially in hepatocytes (Zhang et al., 2000), even though their poor nuclear uptake renders them ineffective in correction of splicing in HeLa cells (Sazani et

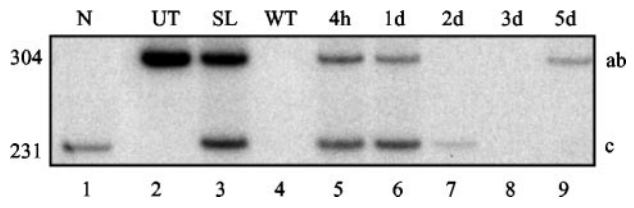


Fig. 3. Expression of human β-globin mRNA in peripheral blood of wild-type mouse injected with IVS2–654 mouse repaired BM cells. Lane 2, untreated cultured BM cells of IVS2–654 mouse (UT); lane 3, BM cells repaired with ON-654; lane 4, blood of a wild-type mouse (WT) before cell injection; lanes 5–9, blood collected 4 h and 1, 2, 3, and 5 days, respectively, after injection of cells repaired as in lane 3. Lane 1, analysis and size designations as in Fig. 2A.

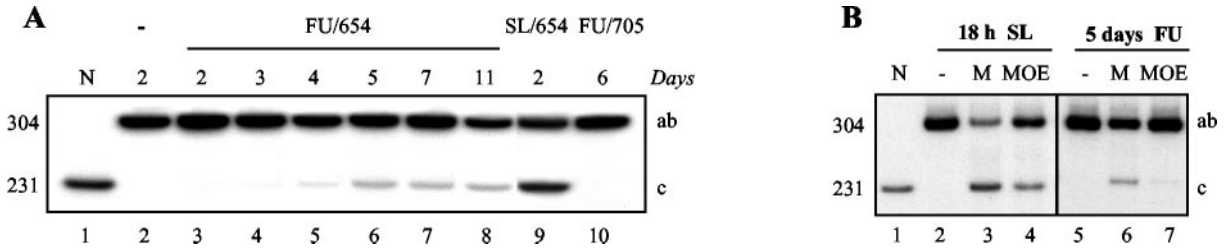


Fig. 4. A, correction of splicing of IVS2–654 β-globin pre-mRNA in thalassemic mouse BM cells by free uptake (FU) of morpholino ON-654. Lanes 3–8, cells treated with 45 μM ON-654 for 2 to 11 days, respectively; lane 9, cells collected on day 2 after syringe loading (SL) with 45 μM ON-654 at 18 h of culture. Lanes 2 and 10, control cells cultured for 2 days in the absence of oligonucleotide and for 6 days in the presence of ON-705, respectively. B, treatment of thalassemic mouse BM cells with morpholino (M) and 2′-O-MOE (MOE) oligonucleotides. Lanes 3 and 4, cells syringe-loaded with 45 μM and MOE at 18 h of culture. Lanes 6 and 7, cells cultured for 5 days in the presence of 45 μM M and MOE, respectively. Lanes 2 and 5, untreated cells.

al., 2001) Thus, we decided to test the uptake and antisense effects of these compounds in erythroid cells.

Syringe loading of 45 μM 18-mer 2′-O-MOE ON-654 led to significant correction of IVS2–654 splicing (Fig. 4B, lane 4), which was less efficient than that achieved with morpholino oligonucleotide in a side-by side experiment (Fig. 4B, lane 3). 2′-O-MOE derivative was even less effective in free uptake; after 5 days of treatment, only trace amounts of corrected β-globin mRNA accumulated (Fig. 4B, lane 7), much less than in morpholino-treated cells (Fig. 4B, lane 6). See also Fig. 7B.

Restoration of Correct Splicing and Hemoglobin A Expression in Human Thalassemic Erythroid Precursors. Encouraging results with mouse BM cells prompted us to test the effects of free uptake of morpholino oligonucleotide on erythroid precursor cells from thalassemic patients (i.e., the cells that constitute the ultimate target of antisense therapy of IVS2–654 thalassemia). Previous work from this laboratory has shown that mononuclear cells from peripheral blood have to be cultured for at least 8 (or preferably 12) days before significant repair of IVS2–654 splicing can be effected by the syringe-loaded morpholino oligonucleotide (Lacerra et al., 2000). Therefore, the mononuclear cells from peripheral blood of a patient with IVS2–654/β^E thalassemia were treated with the oligonucleotide containing media on day 12 of culture and maintained with the oligonucleotide until day 15. Under these conditions, dose-dependent correction of IVS2–654 splicing was easily detected (Fig. 5A, lanes 3 and 4). In fact, quantitation of the data showed that after 3 days of treatment, the level of correction at 45 μM oligonucleotide (lane 4) was approximately 40%, twice as high as that obtained by a prolonged treatment of murine BM cells (Fig. 4A, lane 8).

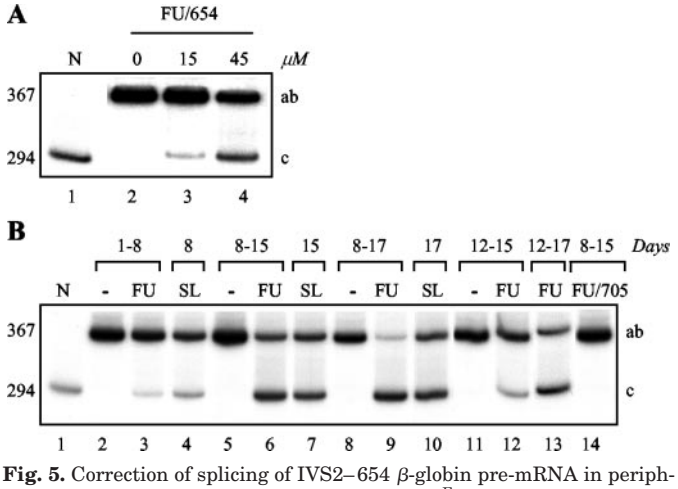


Fig. 5. Correction of splicing of IVS2–654 β-globin pre-mRNA in peripheral blood mononuclear cells from IVS2–654/β^E thalassemic patient by free uptake of ON-654. A, dose-dependence. Lane 1, RNA from normal human blood; lane 2, untreated cells at 15 days of culture; lanes 3 and 4, cells treated during 12 to 15 days of culture with 15 and 45 μM ON-654, respectively. RT-PCR was carried out with primers **a** and **c** (see Fig. 1). The size, in nucleotides, of RT-PCR products is indicated on the left. B, time course of free uptake of 45 μM ON-654. The duration of culture/treatment is indicated at the top of the figure. Lanes 2, 5, 8, and 11, control cells cultured in the absence of ON-654; lanes 3, 6, 9, 12, and 13, free uptake of ON-654. Lanes 4, 7, 10, syringe loaded (SL) cells. Lane 14, free uptake of control, ON-705 oligonucleotide.

To compare the efficacy of free uptake and syringe loading of morpholino oligonucleotides in repair of β-globin pre-mRNA as a function of erythropoietic differentiation, the treatments were performed at different time points during culture. The experiments were carried out on blood samples from three IVS2–654 patients and the extent of correction was quantitated by densitometry of the RT-PCR autoradiograms (Table 1).

The results from a single patient illustrated in Fig. 5B, lane 4, and the quantitative data from three patients (Table 1) show that syringe loading of oligonucleotides within the first 8 days of culture resulted in inefficient correction of splicing, as observed previously (Lacerra et al., 2000). Moreover, continued exposure of the cells to free 45 μM oligonucleotide during 1 to 8 days of culture resulted in even lower levels of correction than that effected by a single syringe loading (Fig. 5B, compare lanes 3 and 4). This is due to the initially low level of erythroid precursors (see below and Lacerra et al., 2000), resulting in few target cells able to freely accumulate the added oligonucleotide. In contrast, in the cells from this patient, the free uptake of oligonucleotide applied during days 8 to 15 of culture led to very efficient correction of splicing (Fig. 5B, lane 6), which seemed to be higher than in cells syringe-loaded at day 15 of culture (Fig. 5B, lane 7). Quantitation of the data from three IVS2–654 patients confirmed this observation (Table 1).

That the intranuclear accumulation of the morpholino oligonucleotide is slow is shown by the fact that additional two days of incubation, days 8–17, led to an increase in correction of IVS2–654 pre-mRNA splicing (Fig. 5B, lane 9, Table 1) while a shorter treatment within the 8–17 period was significantly less effective (days 12–15, lane 12) and again increased with additional time (days 12–17, lane 13). The effect of free ON-654 is sequence specific as no correct splicing of β-globin pre-mRNA is seen with 8–15 days of free uptake of ON-705 morpholino oligonucleotide (lane 14).

TABLE 1

Correctly spliced β-globin mRNA and hemoglobin A (as percentage of total) in cultured mononuclear cells of thalassemic patients

The numbers represent correction after free uptake and syringe-loading with 45 μM morpholino oligonucleotide antisense to the 5′ splice site of IVS2-654 β-globin pre-mRNA.

	Correctly Spliced β-Globin mRNA							Hemoglobin A	
	Free Uptake					Syringe Loading		FU	SL
	Days 1–8	Days 8–15	Days 8–17	Days 12–15	Days 12–17	Day 15	Day 17	Days 8–15	Day 15
				%				%	
Patient A	7.7	64.1	88.7	26.1	68.6	55.5	63.2	24.7	20.3
Patient B	N.D.	80.3	64.0	ND	24.0	35.6	70.6	25.9	24.9
Patient C	N.D.	66.3	77.5	23.4	47.0	69.0	76.3	31.6	31.5
Average		70.2	76.7	24.8	46.5	53.4	70.0	27.4	25.6

N.D., not done.

The mRNA repair (Fig. 5B and Table 1) experiment was followed up by analysis of hemoglobin A formed with the β-globin translated from the newly repaired β-globin mRNA in samples of three IVS2–654/β^E thalassemic patients (Fig. 6). Consistent with RT-PCR results, treatment of cells with free morpholino oligonucleotide for 8 to 15 (lanes 2, 5, and 8) and 12 to 17 (lane 10) days of culture generated high levels of hemoglobin A. Similar levels of hemoglobin A were produced by syringe-loading of the cells (lanes 3, 6, and 11 and Table 1). Note that in Table 1, the amount of hemoglobin A is calculated as percentage of total hemoglobins (Hb E, Hb F, and Hb A). Because the patients are compound heterozygotes for Hb E, only 50% of the total hemoglobin can be restored by repair of IVS2–654 pre-mRNA. Thus the 27% of hemoglobin A shown in Table 1 represents 54% of restorable protein.

Intracellular Localization of Morpholino and 2′-O-MOE Oligonucleotides. To gain insight into the cellular distribution of the morpholino oligonucleotide and to confirm that its intranuclear uptake is responsible for restoration of expression of correct β-globin mRNA and hemoglobin A, the cultured mononuclear cells from a healthy subject were treated with FITC-labeled morpholino oligonucleotide. The delivery was carried out by syringe loading on days 1, 8, and 17 of culture and free uptake on days 13 to 17.

On day 1, in syringe-loaded cells, the FITC label was located in the cytoplasm of relatively large macrophages (Fig. 7, 2). The identity of the cells was confirmed by Wright-Giemsa staining; data not shown). The remaining mononuclear cells, predominantly lymphocytes, were negative. On day 8 of culture, the oligonucleotide now appeared in the majority of the cells, which now differentiated to precursors of the myeloid and erythroid cell lines whereas the number of macrophages decreased to a great extent (Fig. 7A, 4). After 17 days of culture, the precursor cells, especially the erythroid ones, underwent further development and exhibited distinct fluorescence in their nuclei [appreciably stronger than on day 8 (Fig. 7A, 6)], which constituted almost the entire area of the cell (compare DAPI staining, Fig. 7A, 7). The culture also became populated with large megakaryocytes, which avidly accumulated the oligonucleotide. Importantly, free uptake of FITC-labeled oligonucleotide on days 13 to 17 of culture (Fig. 7A, 9) resulted in the same localization as that in the cells syringe loaded on day 17 (Fig. 7A, 6).

The foregoing FITC-labeled oligonucleotide localization supports the quantitative results of the repair experiments (Table 1 and Fig. 5B). The cytoplasmic localization of the oligonucleotide and evident lack of erythroid precursors on day 1 is reflected in the lack of repair of splicing. Likewise, increasing penetration of oligonucleotides into the nuclei on days 8 and 17, in particular the numerous erythroid precursors on day 17, corresponds to the increasing efficiency of the repair of splicing on these days.

To better understand the uptake of morpholino oligonucleotides into human erythroid progenitor cells, we compared it to that of 2′-O-MOE derivatives. FITC-labeled morpholino and Texas Red-labeled 2′-O-MOE oligonucleotides were delivered by syringe loading on day 15 of the culture or by free uptake for days 12 to 15. The relatively short time of this experiment was dictated by the limited availability of labeled 2′-O-MOE oligonucleotide.

Syringe-loaded cells exhibited significant fluorescence after treatment with both oligonucleotides (Fig. 7B, 5–8). Labeled morpholino and 2′-O-MOE oligonucleotides were evident in the cytoplasm and, more importantly, at higher concentration in the nuclei of the erythroid cells (Fig. 7B, 2 and 4, respectively). Notably, for either oligonucleotide, the intracellular distribution was somewhat diffused in both nuclear and cytoplasmic compartments.

In contrast, distribution of the two compounds was different in cells that took up free oligonucleotide during the 12- to 15-day culture. Although the overall concentration of morpholino oligonucleotide seems lower, its intracellular distribution remained similar to that in syringe loaded cells (Fig. 7B, 2). In 2′-O-MOE treated cells the nuclei are clearly devoid of the oligonucleotide, which remains concentrated in cyto-

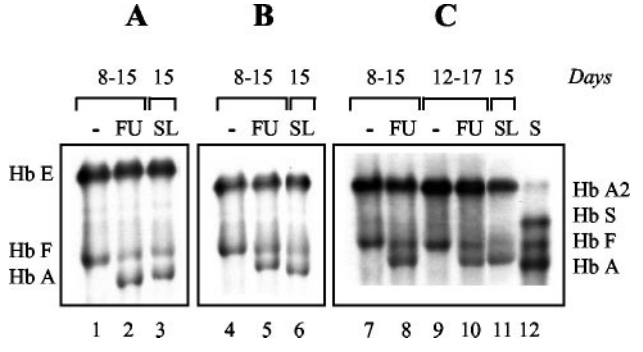


Fig. 6. Restoration of hemoglobin A synthesis in oligonucleotide repaired IVS2–654/β^E patient cells. Cellulose acetate electrophoresis of hemolysates of ON-654 (45 μM) treated cells from three thalassemic patients (A, B, and C). The electrophoresed strips were probed with antihuman hemoglobin antibody. The time of culture/free uptake is shown above the. Lanes 1, 4, 7, and 9, untreated cells; lanes 2, 5, 8, and 10, free uptake of ON-654; lanes 3, 6, and 11, syringe-loaded (SL) cells. Lane 12, hemoglobin standards S, with species of human hemoglobin shown on the right. Electrophoretic mobility of hemoglobins E, F, and A is also indicated on the left.

plasmic speckles (Fig. 7B, 3). These results are concordant with the observation that scrape-loaded morpholino and 2′-O-MOE oligonucleotides corrected IVS2–654 pre-mRNA splicing, whereas the free 2′-O-MOE ON-654 was ineffective (Fig. 4B).

Discussion

The main finding of this work is that free uptake of the morpholino oligonucleotide ON-654 into the human erythroid cells resulted in nearly 80% of correction, a yield higher than that in syringe-loaded cells. Thus, this oligonucleotide was able to penetrate the erythroid precursor cell membrane barrier and translocate to the nucleus, suggesting that similar result should be possible in vivo. In contrast, our attempts of nuclear delivery of free negatively charged oligonucleotides were unsuccessful.

In cultured patient cells, the time course of repair by free uptake of ON-654 oligonucleotide seems to be very slow. In days 1 to 8, the repair is minimal, increasing in days 8 to 15 and even more so in days 15 to 17 (Fig. 5B). The simplest

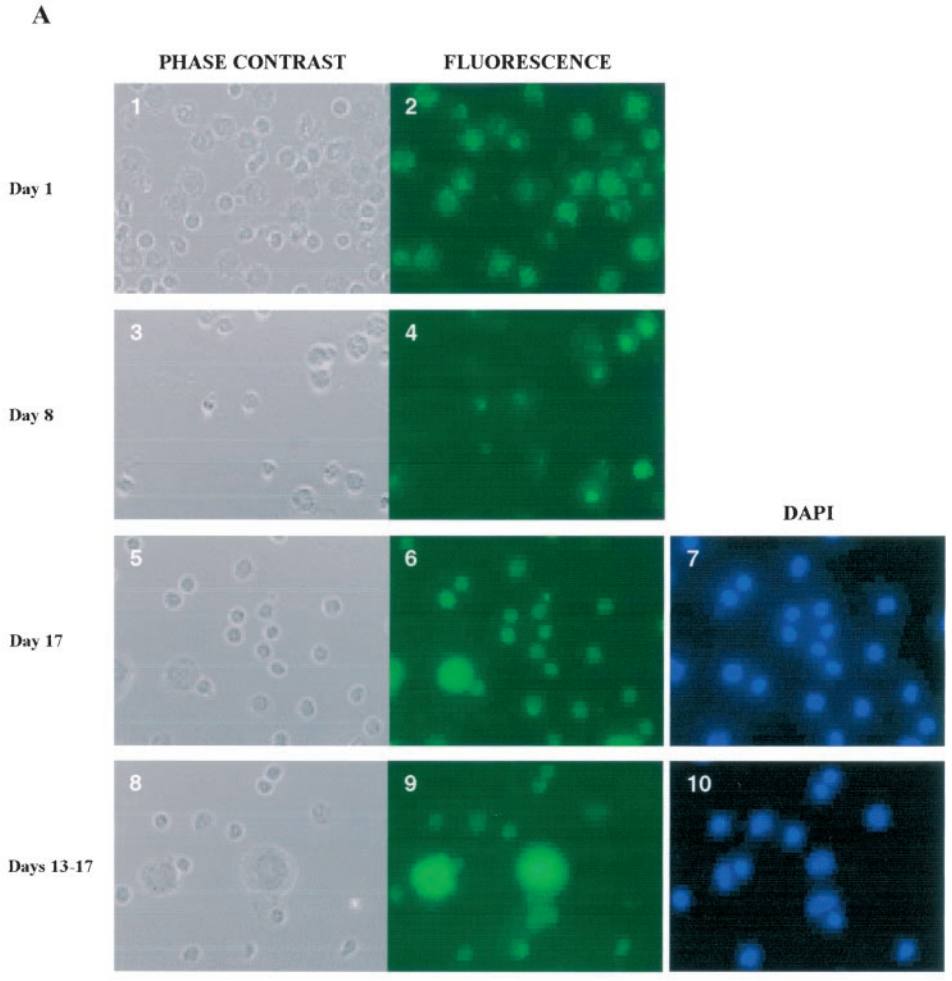
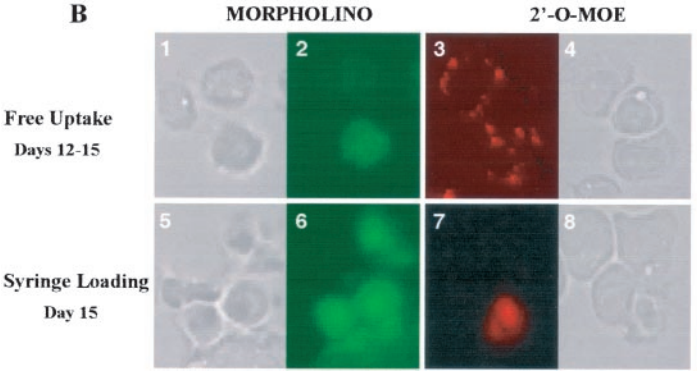


Fig. 7. A, delivery of FITC-labeled morpholino oligonucleotide into cultured human peripheral blood mononuclear cells by syringe loading 24 h before the day of collection (indicated on the left; 1–7) and by free uptake on days 13 to 17 (8–10). 1, 3, 5, and 8, phase contrast; 2, 4, 6, and 9, fluorescence; 7 and 10, DAPI stain. Magnification, 40×. B, delivery of FITC-labeled morpholino (left) and Texas-Red labeled 2′-O-MOE (right) oligonucleotides. Syringe loading on day 15 (5–8) and free uptake on days 12 to 15 (1–4) of culture. 1, 4, 5, and 8, phase contrast; 2, 4, 6, and 7, fluorescence. Magnification, 40×. The cells were deposited on the microscope slide by cytospin to increase their surface observable in the microscope and visualize the cytoplasmic and nuclear compartments.



interpretation of these results is slow uptake of the morpholino oligonucleotide. However, the comparison of free uptake and syringe loading and, in particular, the analysis of intracellular localization of fluorescein-labeled oligonucleotide indicate that selective uptake of this compound and progress in erythroid cell differentiation in the epo- and SCF-driven culture also contribute to these results.

It was shown previously that the initial preparation of human mononuclear cells is devoid of erythroid precursors that produce β-globin pre-mRNA (Lacerra et al., 2000). The two types of cells that predominate in the initial culture are monocytes and lymphocytes, with small numbers of myeloid progenitors as well as early erythroid progenitors not yet engaged in β-globin expression. Although it is surprising that the monocytes are stained and lymphocytes are not when syringe-loaded with FITC-labeled morpholino oligonucleotide, neither expresses β-globin pre-mRNA. Thus, it is the absence of appropriate erythroid target cells and not uptake kinetics that is responsible for the lack of detectable β-globin repair on day 1 of culture (H. Sierakowska, unpublished data).

Subjecting the initial culture to epo and SCF increased the population of erythroid cells, as evidenced by the strong expression of IVS2–654 pre-mRNA (Lacerra et al., 2000). Syringe loading on day 8 or free uptake on days 1 to 8 resulted in detectable levels of repaired β-globin mRNA. Accordingly, the syringe loading and free uptake of labeled oligonucleotide exhibited nuclear and cytoplasmic staining in nearly all cells. Nuclear accumulation of morpholino ON-654 oligonucleotide is essential for correction of splicing, a nuclear process. Further culture until day 17 under the same epo-stimulated conditions resulted in very efficient correction of splicing and a change to a more differentiated population of cells with predominately erythroid precursors, which exhibit strong nuclear accumulation of the FITC-morpholino oligonucleotide. Thus, the level of correction observed in a culture of differentiating erythroid progenitors represents a sum of nuclear uptake of the oligonucleotide and the increase in the oligonucleotide targets. Note that in thalassemic patients, in vivo, the population of cells subjected to oligonucleotide treatment will be at a steady state and therefore the level of correction will be determined solely by oligonucleotide uptake and biodistribution.

In human cells, there was a time-dependent increase in the level of β-globin mRNA and hemoglobin A in response to free uptake and syringe loading of oligonucleotides. In contrast, in mouse BM cells, the optimal repair obtained with syringe loading occurred 18 h after plating (Fig. 2B, lane 3) and later diminished. Moreover, in murine cell culture, the correct β-globin mRNA decayed faster than aberrant (see Fig. 2C), while in vivo, when the oligonucleotide-treated cells were injected back into the blood stream, the result was just the opposite (Fig. 3). There are several possible explanations of these results.

It seems unlikely that there are significant differences in the mechanism of action of the oligonucleotide, because in free uptake, the repair of murine and human cells follows roughly the same pattern. Also, similarly to human cells, on day 10 of culture, the BM repair level with the free uptake exceeded that observed with syringe loading (compare lanes 8 in Figs. 4A and 2B). Thus, the oligonucleotide was able to penetrate the cell membrane not only of human but also of murine erythroid cells, albeit not as effectively.

The fact that in murine cell culture optimal β-globin repair effected by syringe loading is seen at 18 h indicates that, in contrast to human culture, the "repairable" erythroid precursors are already present in significant numbers. The relatively rapid decay of correct mRNA might be due to the effective removal of the corrected cells, slightly damaged by syringe loading, by the numerous macrophages present in the culture. The unaffected and newly differentiated cells can be destroyed at a lower rate, resulting in constant amounts of aberrant mRNA. Another more attractive possibility is that the corrected cells differentiate, by day 4 terminating mRNA production. This idea is consistent with the fact that mouse erythropoietic cell culture is known to promote hemoglobinization, albeit very weak, that peaks at 3 to 5 days.

The in vivo results (Fig. 3) suggest that the reinjected corrected and more mature erythroid cells remained in the blood stream within the span of 2 days and subsequently either matured, eventually losing correct β-globin mRNA, or were destroyed by macrophages. Because hemoglobin-producing cells are normally released from the bone marrow into the blood stream, it is unlikely that their disappearance was caused by sequestration in the bone marrow. In contrast, the fact that the aberrant mRNA reappeared on day 5 after injection is best explained by a scenario in which the stem cells and other early unaffected and undifferentiated cells (not yet repairable by ON-654) were first sequestered in the bone marrow, where they were induced to erythropoietic differentiation and released into the blood stream while producing IVS2–654 β-globin mRNA. This is supported by an additional experiment carried out in SCID mice, in which the aberrant RNA remained detectable at a constant level for 47 days, until the mouse was sacrificed (data not shown).

It is notable that the timing of the maximum mRNA repair and hemoglobin A expression (15–17 days of culture) coincides with the time course of in vitro human erythropoiesis (Papayannoupoulou et al., 2000). In media with similar cytokines, the multipotent or very early erythroid progenitor cells [burst-forming unit-erythroids (BFU-E)] produce in about 2 weeks a number of highly multicellular erythroid bursts of hemoglobinized cells, still fairly active in RNA synthesis. At 7 days (i.e., at the peak of colony-forming unit–erythroid formation), the erythroid colony-forming units contain a small number of cells; therefore, there are fewer erythroid precursors in culture. This seems to coincide with lesser repair observed in our population of cultured mononuclear cells from patient peripheral blood. This, in conjunction with the fact that besides stem cells, BFU-E rather than colony-forming unit–erythroids enter the peripheral blood and tend to multiply into thousands of cells, would indicate that the highest RNA repair efficiency and hemoglobin A formation observed at 15 to 17 days is caused mainly by BFU-E–derived erythroid precursors.

Recent findings show that morpholinos, albeit at relatively high concentrations, are freely taken up into the HeLa cell nuclei, where they correct splicing, whereas 2′-O-methyl or 2′-O-MOE oligonucleotides are not (Schmajuk et al., 1999; Sazani et al., 2001). On the other hand, 2′-O-MOE oligonucleotides antisense to Fas mRNA were shown to be effective in vivo in mouse liver, indicating that these compounds were taken-up by the hepatocytes (Zhang et al., 2000). Here, in

primary erythroid cells, which despite in vitro culture, properly differentiated and expressed β- and α-globin RNA and protein, 2′-O-MOE oligonucleotides were ineffective and exhibited typical endocytotic, cytoplasmic localization. In the same cells, morpholino oligonucleotides were taken up not only into the cytoplasm, but also the nuclei, suggesting a different uptake mechanism, presumably effected by the lack of charge on these compounds. Whether or not morpholinos are endocytosed, the nature of the backbone seems to prevent their entrapment in the cytoplasmic, endosomic vesicles, allowing their entry into nucleus. This is important because the nuclear compartment seems to be the main site of antisense activity of many, if not all, oligonucleotides (Kole and Sazani, 2001).

In conclusion, high levels of correction of splicing in erythroid precursor cells in IVS2–654 thalassemia were achieved by free uptake of neutral morpholino oligonucleotides. Thus, for in vivo treatments, additional neutral backbones that seem to have promising uptake characteristics, such as peptide nucleic acids (Sazani et al., 2001) phosphoroamidates (Faria et al., 2001) or locked nucleic acids (Orum and Wengel, 2001), should be tested.

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