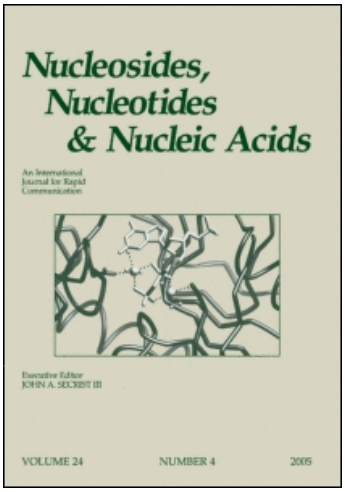

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Restoration of ß-Globin Gene Expression in Mammalian Cells by Antisense Oligonucleotides That Modify the Aberrant Splicing Patierns of Thalassemic Pre-mRNAs

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RESTORATION OF ß-GLOBIN GENE EXPRESSION IN MAMMALIAN CELLS BY ANTISENSE OLIGONUCLEOTIDES THAT MODIFY THE ABERRANT SPLICING PATTERNS OF THALASSEMIC PRE-mRNAs.

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ABSTRACT: Antisense 2'-methoxy-oligoribonucleotides targeted to aberrant splice sites in two thalassemic human ß-globin pre-mRNAs, IVS2-654 and IVS2-705, expressed in HeLa cells efficiently restore correct splicing with the use of Lipofectamine, Cellfectin and DMRIE-C, in effect reactivating a defective ß-globin gene.

Numerous reports indicate that antisense oligonucleotides offer great promise as modulators of gene expression. This is shown by the effects of antisense oligonucleotides in cell culture[1] and in recently reported animal studies[2-4] and by the fact that antisense research has progressed to the stage of clinical trials [5,6]. In most studies these compounds were hybridized in a sequence specific manner to mRNA, leading in consequence either to its subsequent degradation by cellular RNase H or to a block in its translation. Oligonucleotides were also targeted against specific regions in DNA (antigene oligonucleotides) where they formed triplex structures and inhibited transcription by RNA polymerase II[1]. Both antisense and antigene oligonucleotides led to downregulation of targeted genes and reduction of the intracellular level of undesirable gene products.

A significant stumbling block in the usefulness of antisense oligonucleotides is their poor ability to penetrate the cellular membrane. This problem has been addressed by chemical modifications of the oligonucleotides[1] and more successfully by design of a number of transmembrane carriers. These included cationic liposomes such as Lipofectin[7] and other formulations[8-12] but also poly-lysine[13], poly (isohexylcyanoacrylate) nanoparticles[14] and polyamidoamino dendrimers [15,16].

Antisense oligonucleotides can be used not only for downregulation of the gene but also for restoration of its activity. In a modification of the antigene approach, double stranded chimeric RNA.DNA oligonucleotides have been recently used to remove a point mutation from an alkaline phosphatase gene located on extrachromosomal plasmid DNA[17]. We have developed a novel approach in which antisense oligonucleotides were used to suppress aberrant splicing and restore correct splicing of pre-mRNAs transcribed from the

human ß-globin gene damaged by mutations that cause ß-thalassemia, a genetic blood disorder. The oligonucleotides were effective in restoring correct splicing of pre-mRNA in several thalassemic mutations in cell free splicing extracts[18,19] and in mammalian cells expressing a thalassemic ß-globin mutant, IVS2-654[10]. Here we describe the optimization of restoration of correct splicing in the IVS2-654 mutant with several liposomal carriers and application of this approach to treatment of mammalian cells expressing an analogous thalassemic pre-mRNA variant, IVS2-705.

MATERIALS AND METHODS

HeLa cell lines expressing IVS2-654 or IVS2-705 human ß-globin genes were cultured in MEM supplemented with 5% fetal calf and 5% horse sera. For all experiments cells were plated in 24 well plates at 10^5 cells per well 24 hrs before treatment.

The phosphorothioate 2'-O-methoxy-oligoribonucleotides (prepared and purified at Hybridon, Inc.,Worcester, MA) were used. The oligonucleotide 5'ss, GCUAUUACCUUAACCCAG, was targeted to the aberrant 5' splice site of IVS2-654 mutant. The 3'ss oligonucleotide, CAUUAUUGCCCUGAAAG, was targeted to the 3' cryptic splice site of IVS2-705 mutant. An oligonucleotide with random sequence was used as control. The cells were treated with oligonucleotides complexed with Lipofectamine[20], Cellfectin or DMRIE-C[11] (Life Technologies) for 6 or 10 hours, as indicated. 36 hours after termination of treatment the cells were harvested for RNA or protein analyses.

The RNA and protein analysis were carried out exactly as described previously[10].

RESULTS

In ß-thalassemia, a genetic blood disorder that affects a large number of people in the Mediterranean basin, Middle East, South East Asia and Africa, splicing mutations are responsible for the majority of cases worldwide[21]. As a model system of clinical importance, we have studied a C to T mutation at nucleotide 654 (IVS2-654) and a T to G mutation at nucleotide 705 (IVS2-705) of intron 2 of the ß-globin gene. Both these mutations create aberrant 5' splice sites and activate upstream the same cryptic 3' splice site in ß-globin pre-mRNAs. As shown in FIG. 1 the splicing pathway is modified, even though the correct splice sites remain potentially functional, and the resulting mRNA retains a fragment of the intron.

We have found that blocking of the aberrant splice sites with antisense 2'-methoxy-oligoribonucleotides or their phosphorothioate derivatives forced the splicing machinery to reselect the correct splice sites and induce the formation of correct ß-globin mRNA and polypeptide, hence restoring the gene function[10,18,19]. The 2'-O-methyl derivatives were chosen since they hybridize well to their target sequences and are very stable in cellular

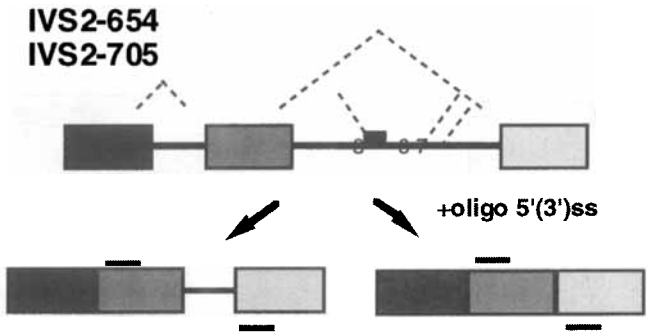

FIGURE 1. Splicing of human ß-globin IVS2-654 and IVS2-705 pre-mRNAs in the presence of antisense oligonucleotides. Boxes - exons; heavy lines - introns; dashed lines indicate splicing pathways; numbers 6 and 7 - the aberrant 5' splice sites created by IVS2-654 and-705 mutations, respectively; 3' indicates the cryptic 3' splice site activated upstream in both IVS2-654 and -705; heavy bar -oligonucleotide antisense to the aberrant 3' splice site; light bars above and below exon sequences indicate primers used in the RT-PCR reaction. The retained fragment of the intron is 73 and 124 nucleotides in length in IVS2-654 and-705, respectively.

environment. Moreover, in contrast to commonly used oligodeoxynucleotides or phosphorothioate oligodeoxynucleotides, they do not promote cleavage of hybridized RNA by cellular RNase H[22]. The latter property is crucial to the success of the experiments since treatment with an unmethylated oligonucleotide would have led to degradation of the ß-globin pre-mRNA and removal of the splicing substrate[1,23].

Treatment of HeLa cells for 6 hours with a complex of Lipofectamine and 2'-O-methyl phosphorothioate antisense oligonucleotide targeted to the aberrant 5' splice site efficiently restored correct splicing of ß-globin pre-mRNA in a dose dependent fashion (FIG. 2A). Quantitation of the results showed that at 0.1 and 0.2 µM oligonucleotide the correctly spliced ß-globin mRNA reached, respectively, 21 and 24% of the total (FIG. 2A, lanes 2 and 3). There was a small decrease in the correctly spliced product at 0.3 µM oligonucleotide (19%, FIG. 2A, lane 4) while at 0.4 µM and 0.5 µM oligonucleotide this product was undetectable (FIG. 2A, lanes 5 and 6, respectively). The latter result is possibly due to the fact that the ratio of Lipofectamine to nucleic acid deviated from a narrow range necessary for efficient cellular uptake of the complex[20]. The effect of the antisense oligonucleotide was sequence dependent since an oligonucleotide with random sequence did not restore correct splicing (See FIG. 5).

To test whether the newly generated correctly spliced ß-globin mRNA was translated into full length ß-globin polypeptide, the total protein from oligonucleotide

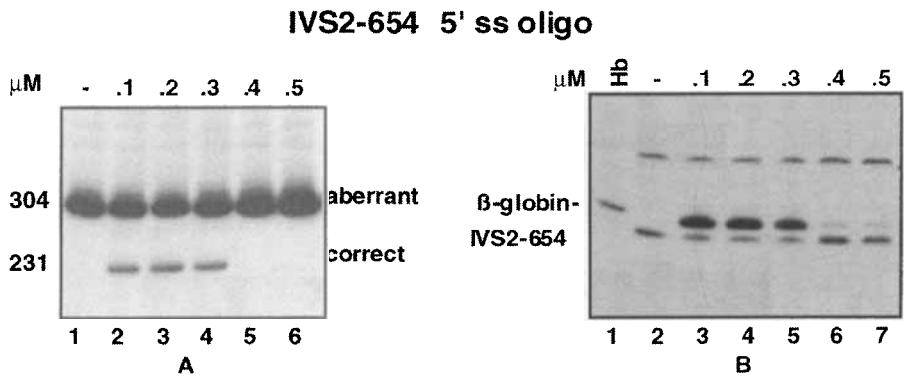

FIGURE 2. A. Dose dependent correction of splicing of IVS2-654 pre-mRNA in HeLa cells by antisense oligonucleotide targeted to the aberrant 5' splice site (5'ss) and complexed with Lipofectamine. Analysis of total RNA by RT-PCR. Lanes 1-6, IVS2-654 HeLa cells treated with increasing concentrations of the oligonucleotide (indicated in micromoles at the top). The numbers on the left indicate the size, in nucleotides, of the RT-PCR products representing the aberrantly (304) and correctly (231) spliced mRNAs. B. Restoration of ß-globin expression by 5'ss oligonucleotide in IVS2-654 HeLa cells treated with hemin prior to isolation of proteins. Immunoblot of total protein with anti-human hemoglobin antibody. Concentration of the oligonucleotide in micromoles is indicated at the top (lanes 2-7); in lane 1 human globin (Sigma) was used as a marker. The positions of human ß-globin and the prematurely terminated ß-globin IVS2-654 polypepeptide are indicated.

treated cells was analyzed by immunoblotting with polyclonal antibody to human hemoglobin. Prior to isolation of protein the cells were treated with hemin which intensifies the ß-globin protein band. This may be due to specific posttranslational stabilization of the full length ß-globin by hemin[24] and/or to an increase in the sensitivity of the immunoassay since the polyclonal anti-hemoglobin antibody may have greater affinity for the ß-globin-heme complex than for ß-globin alone. In agreement with RT-PCR results shown in FIG. 2A, only samples treated with 0.1-0.3 μM oligonucleotide contained significant amounts of full length ß-globin (FIG. 2B, lanes 3-5). There was no ß-globin in untreated, control cells (FIG.2B, lanes 2) and virtually none in those treated with 0.4 μM and 0.5 μM oligonucleotide (FIG.2B, lanes 6 and 7, respectively). Thus, the significant increase in full length ß-globin, roughly parallel to that of the correct ß-globin mRNA, is clearly due to the effect of antisense oligonucleotides on splicing. The specificity of hemin treatment is confirmed by the lack of its effect on the truncated IVS2-654 polypeptide or background protein bands.

In order to increase the efficiency of correction of splicing we have increased the time of treatment with liposomal carrier-oligonucleotide complexes from 6 to 10 hrs and, in addition to Lipofectamine, have tested other liposomal reagents, namely Lipofectin, Cellfectin and DMRIE-C. These positively charged lipid compounds had been shown to be efficient in transfection of a number of cell types[11]. Furthermore, DMRIE-C was reported to be an efficient nucleic acids carrier in experimental mice[25].

Extending the time of incubation of HeLa IVS2-654 cells with Lipofectamine-oligonucleotide complex from 6 to 10 hrs increased correct splicing, reaching in a dose dependent fashion the maximum of 30% correction at 0.2 μM oligonucleotide (FIG.3, panel LA, lanes 1-5). As shown in FIG. 2A, at higher concentrations of the oligonucleotide the level of correction decreased. In this and previous experiments the Lipofectamine concentration was 8 μg/ml. Since with Lipofectin used in a similar manner, at concentrations of up to 20 μg/ml, the correction of splicing never exceeded that in the presence of Lipofectamine, the experiments with Lipofectin were discontinued.

Quantitative analysis of the data presented in FIG, 3, panel CLF, showed that treatment of IVS2-654 HeLa cells with the 5' ss oligonucleotide in complex with Cellfectin reached maximally 34% correction at 0.2 μM oligonucleotide (lane 5). Even at only 0.02 μM oligonucleotide correction of splicing attained 15% (lane 2). The highest level of correct splicing of IVS2-654 pre-mRNA, 41%, was achieved with DMRIE-C as a carrier at 0.1 μM oligonucleotide (FIG. 3, panel DM, lane 4). The efficacy of this reagent is even more apparent at lower concentrations of the oligonucleotide with the levels of correctly spliced ß-globin mRNA reaching 23% and 31% at 0.02 and 0.05 μM oligonucleotide, respectively (FIG. 3, panel DM, lanes 2 and 3). With DMRIE-C the effects of the oligonucleotide at 0.05 μM are approximately 2.5 fold higher than those with Lipofectamine.

We have shown previously that correct splicing of IVS2-654 may be restored by targeting the antisense oligonucleotide not only to the 5' splice site created by the mutation but also to the 3' cryptic splice site activated by it[10]. Since, as pointed out above, the same cryptic splice site is activated in another thalassemic mutation, IVS2-705[26], it appeared likely that a single oligonucleotide targeted to the cryptic splice site (3'ss) will be effective in correction of splicing in both mutants. Results presented in FIG. 4 confirm this hypothesis.

The highest levels of correctly spliced product (over 50%) were seen when IVS2-705 HeLa cells had been treated with the 3'ss oligonucleotide in complex with DMRIE-C at concentrations of the oligonucleotide ranging from 0.02 to 0.1 μM (FIG. 4, panel DM, lanes 2-4). Comparable levels of correction were also achieved with the remaining two liposome reagents albeit at higher oligonucleotide concentrations. Correct splicing at 51%

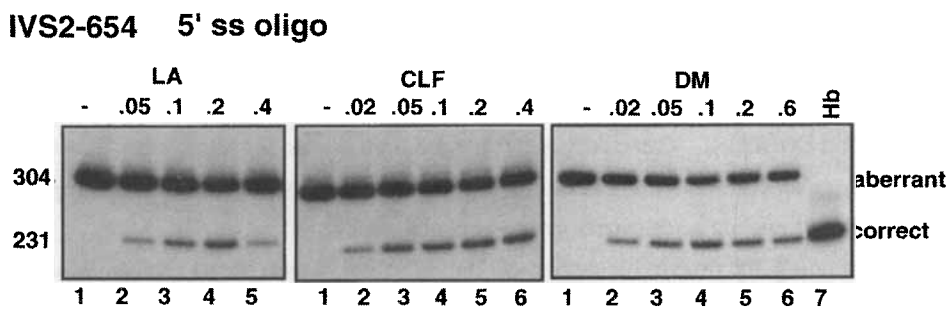

FIGURE 3. Dose dependent correction of splicing in HeLa IVS2-654 cells treated with 5'ss oligonucleotide complexed with 8 μg/ml Lipofectamine (LA), 6 μg/ml Cellfectin (CLF) and 8 μg/ml DMRIE-C (DM) according to Life Technologies protocol. Concentrations of oligonucleotide are indicated in micromoles at the top of each panel. Hb (DM, lane 7), RNA from human blood. All other designations as in FIG. 2.

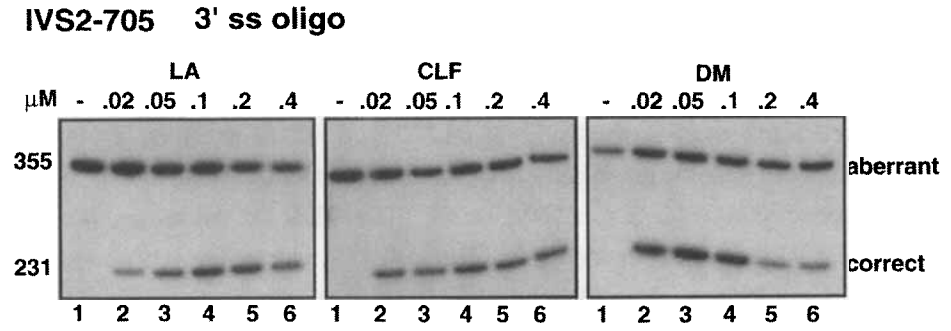

FIGURE 4. Correction of splicing in HeLa IVS2-705 cells treated with an oligonucleotide targeted to the 3' cryptic splice site (3'ss) complexed with Lipofectamine (LA), Cellfectin (CLF) and DMRIE-C (DM), as in FIG. 3. All other designations are as in FIG. 2

level was observed at 0.4 μM oligonucleotide with Cellfectin (FIG. 4, panel CLF, lane 6) and at 48% level at 0.2 μM oligonucleotide with Lipofectamine (FIG. 4, panel LA, lane 5). Nevertheless, it is clear from the data for 0.02 μM oligonucleotide (FIG. 4, lanes 2 in panels LA, CLF and DM) that DMRIE-C is the most efficient of the three in delivering the oligonucleotide to the cells. Since the results with this carrier appear to be at plateau, it is likely that even significantly lower concentrations of the 3'ss oligonucleotide would be quite efficient in restoring correct splicing in IVS2-705 HeLa cells.

To test the specificity of the effects of the oligonucleotide targeted to the 3' splice site (FIG.4), the IVS2-705 HeLa cells were treated with a 2'-methoxy phosphorothioate

oligonucleotide with a random sequence complexed with Lipofectamine, DMRIE-C, and Cellfectin. None of these treatments resulted in detectable correction of splicing (FIG. 5). Furthermore, we have shown previously that an oligonucleotide complementary to the aberrant 5' splice site in IVS2-654 pre-mRNA had no corrective effect on the splicing of a modified control IVS2-654 consensus construct which included two mismatches within the splice site sequence[10]. There was likewise no corrective effect on splicing of IVS2-654 pre-mRNA in cells treated with the oligonucleotide targeted to the 5' splice site of IVS2-705 pre-mRNA. This oligonucleotide is similarly complementary, with two mismatches, to the aberrant IVS2-654 5' splice site and, with a single mismatch, to the intron sequence 44 nucleotides downstream[10]. It should also be noted that none of the tested oligonucleotides affected the growth rate of the treated cells[10] indicating a lack of pleiotropic effects caused, for example, by hybridization to multiple pre-mRNAs or by direct interaction with proteins, as observed in certain instances[27].

DISCUSSION

We showed that splicing pathways can be modified in cultured cells in a sequence specific manner by antisense oligonucleotides using one of several cationic liposomes as a carrier. Two thalassemic human ß-globin pre-mRNAs were used as targets. Although the feasibility of treatment of thalassemic patients with antisense oligonucleotides has yet to be explored, several observations suggest that this approach may be clinically promising.

Correct splicing was restored up to 50% at oligonucleotide concentrations that were easily attained in bone marrow of experimental animals[28]. Note also that 50% levels of hemoglobin are seen in heterozygote thalassemic individuals who are frequently asymptomatic while the status of patients undergoing transfusion therapy, with even lower hemoglobin levels, is markedly improved. Thus, the treatment reported here would be clinically beneficial if it resulted in a similar level of correction of aberrant splicing in thalassemic patients.

ß-globin mRNA and protein are very stable and so are mature erythrocytes, with a life span of about 120 days[1]. Hence, in principle, treatment with antisense oligonucleotides may have an extended effect on the in vivo levels of ß-globin mRNA and blood hemoglobin, thus reducing the need for frequent oligonucleotide administration. In this context it is encouraging that the correctly spliced ß-globin mRNA and protein generated by a single delivery of the antisense oligonucleotide persisted in IVS2-654 HeLa cells for up to 48 hrs[10]. Moreover, we had delivered the oligonucleotides to the nuclei of not only HeLa but also NIH 3T3 based cell lines. This suggests that it should be feasible to find appropriate conditions and/or carriers for delivery of the oligonucleotides into cells of patients, including the targeted nucleated erythroblasts. Since, as shown above, the effects of

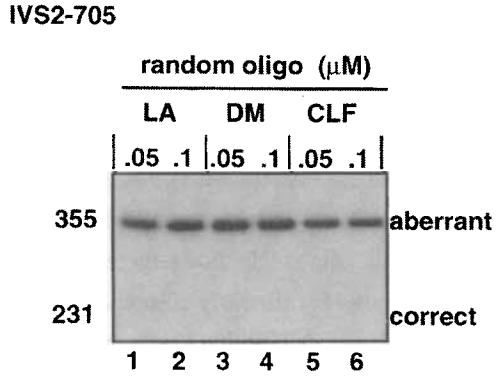

FIGURE 5. HeLa IVS2-705 cells treated with 0.05 and 0.1 μM random oligonucleotide complexed with Lipofectamine (LA, lanes 1 and 2), DMRIE-C (DM, lanes 3 and 4) and Cellfectin (CLF, lanes 5 and 6). Other designations as in FIG. 2.

antisense oligonucleotides are highly specific in cell culture, this specificity will probably be retained in the clinical treatment especially since the nucleated erythroblasts are the only cells containing the target sequence.

The restoration of correct splicing by targeting the cryptic 3' splice site of IVS2-705 pre-mRNA is of particular interest because this splice site is also activated in IVS2-654 and IVS2-745 mutants[1]. Thus, a single oligonucleotide should be effective in correcting aberrant splicing in several types of ß-thalassemia which in clinical setting would translate into a large number of patients. It is also likely that aberrant splice sites activated by other thalassemic mutations[1] will be amenable to this approach. Since splicing mutations are responsible for a large proportion of ß-thalassemia patients[29], it appears that restoration of correct splicing of ß-globin pre-mRNA may offer a useful alternative to current treatments[1] and to potential therapies based on stimulation of fetal hemoglobin production [30,31] or ß-globin gene transfer.

The fact that DMRIE-C significantly enhanced correction of splicing in comparison to the previously tested Lipofectamine[10] is encouraging and suggests that other carriers[9,12,16] may produce even better results. Our earlier observations in cell free splicing extracts, where correct splicing was fully restored for both IVS2-654 and IVS2-705 mutants[23], suggest that neither the interactions of the oligonucleotides with their target sequences nor their competition with splicing factors bound to the same sequence would prevent achieving such a result in animals and perhaps in patients.

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