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Restoration of Correct Splicing of Thalassemic β-Globin Pre-mRNA by Modified U1 snRNAs*

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Linda Gorman, Danielle R. Mercatante, and Ryszard Kole‡

From the Lineberger Comprehensive Cancer Center and Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599

The T→G mutation at nucleotide 705 in the second intron of the β-globin gene creates an aberrant 5′ splice site and activates a 3′ cryptic splice site upstream from the mutation. As a result, the IVS2-705 pre-mRNA is spliced via the aberrant splice sites leading to a deficiency of β-globin mRNA and protein and to the genetic blood disorder thalassemia. We have shown previously that in cell culture models of thalassemia, aberrant splicing of β-thalassemic IVS2-705 pre-mRNA was permanently corrected by a modified murine U7 snRNA that incorporated sequences antisense to the splice sites activated by the mutation. To explore the possibility of using other snRNAs as vectors for antisense sequences, U1 snRNA was modified in a similar manner. Replacement of the U1 9-nucleotide 5′ splice site recognition sequence with nucleotides complementary to the aberrant 5′ splice site failed to correct splicing of IVS2-705 pre-mRNA. In contrast, U1 snRNA targeted to the cryptic 3′ splice site was effective. A hybrid with a modified U7 snRNA gene under the control of the U1 promoter and terminator sequences resulted in the highest levels of correction (up to 70%) in transiently and stably transfected target cells.

The use of antisense oligonucleotides for down-regulation of gene expression is well documented. The oligonucleotides are most frequently targeted to mRNA where they block translation or lead to degradation of the message by RNaseH (1). Alternatively, to inhibit transcription, oligonucleotides are designed to form triplex structures with the sequences in promoter regions of DNA. Both strategies result in down-regulation of the targeted genes and may be useful in the treatment of cancer (2–4), viral infections (5–7), and other diseases (8–11).

A variety of antisense RNAs were also used for down-regulation of gene expression. They were especially effective when the antisense function of the targeting RNA was combined with ribozyme activity (12–14). In another approach, antisense RNAs were designed as an external guide sequence that activated endogenous RNaseP, resulting in degradation of the targeted message (15–17). To promote stability, the antisense constructs were usually embedded within larger stable RNA molecules such as mRNAs, tRNAs, and small nuclear RNAs (snRNAs)[1] (18–22). This combination not only stabilized the RNAs but also directed them either to the cytoplasm (mRNA and tRNA) or to the nuclei (snRNAs).

Work in this laboratory showed that in addition to down-regulation of target genes, antisense oligonucleotides could restore the activity of genes inactivated by mutations that affect splicing of pre-mRNA and result in genetic disorders. Restoration of gene activity was accomplished by targeting the splice sites created or activated by several mutations in thalassemia (23–26), cystic fibrosis (27), and in a mouse *mdx* model of Duchenne muscular dystrophy (28). Thus, modification of splicing by treatment with antisense oligonucleotides provides a potential alternative to gene therapy protocols involving the replacement of defective genes.

Treatment of patients with genetic diseases with antisense oligonucleotides, which do not remove the mutation but rather repair the defective pre-mRNA, would require lifelong periodic administrations. This drawback would be alleviated if the patients were subjected to treatment with vectors stably expressing the appropriate antisense RNAs. Under these conditions the effects of RNAs should result in long term, if not permanent, restoration of gene expression.

A group of snRNAs, U snRNAs, which in cells form ribonucleoprotein particles (snRNPS) and are involved in numerous RNA processing reactions (29–31), appear particularly attractive as carriers for antisense sequences effecting correction of splicing. They are localized in the nucleus (the site of splicing), are stable, are expressed at relatively high levels, and most importantly, interact with their natural RNA targets by base pairing via complementary, *i.e.* antisense nucleotides. Indeed, we have shown previously that stable expression of U7 snRNAs, which are normally involved in the processing of the 3′-ends of histone pre-mRNAs (32–35), but which were modified to target splice sites in the human β-globin gene, led to permanent restoration of correct splicing of thalassemic β-globin pre-mRNA (19). In this report we have investigated whether U1 snRNA may be used as an effective modifier of splicing.

The design of U1 snRNA as an antisense agent for modification of splicing was based on several considerations. There are approximately 1×10^6 copies of U1 per cell as compared with 5×10^3 copies of U7 snRNA molecules (29). Because there are 30 functional U1 genes (36) and only one U7 gene (37, 38), the expression of U1 snRNA per gene copy is still approximately 6-fold higher than that of U7, making U1 potentially a more attractive antisense carrier than the modified U7 snRNA. Ex-

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‡ To whom correspondence should be addressed: University of North Carolina,Lineberger Comprehensive Cancer Center, CB no. 7295, Chapel Hill, NC 27599-7295. Tel.: 919-966-1143, Fax: 919-966-3015; E-mail: kole@med.unc.edu.

[1] The abbreviations used are: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; nt, nucleotide; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HIV, human immunodeficiency virus; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

periments reported here show that U1 snRNA targeted to the 3′- but not the 5′-splice site could be used for antisense repair of aberrantly spliced thalassemic human β-globin pre-mRNA.

EXPERIMENTAL PROCEDURES

Cell Lines—The HeLa cell line carrying the thalassemic IVS2-705 human β-globin gene (39) and the cell lines stably expressing the modified U7 snRNAs (19) were grown in minimum essential medium modified for suspension cells (S-MEM), 5% fetal calf sera, 5% horse sera, 50 μg/ml gentamicin, and 200 μg/ml kanamycin. Cotransfection of the HeLa IVS2-705 cells with a plasmid carrying a hygromycin-resistance gene and a U1.U7.324 snRNA expressing plasmid (see below and Fig. 5*A*) in the presence of LipofectAMINE (8 μg/ml, Life Technologies, Inc., as recommended by the manufacturer), was used to generate the stable cell line. Stable transfectants were isolated after selection in media containing 250 μg/ml hygromycin.

U1 snRNA Constructs—The wild type U1 plasmid (U1.wt) was a kind gift from Dr. W. Marzluff (University of North Carolina). It contains the mouse U1 snRNA gene, U1 promoter, and 3′ sequences. In the U1.309, U1.318, and U1.324 constructs, the natural 9 nucleotide sequence (nt. 3–11) complementary to pre-mRNA 5′ splice sites was replaced with a 9-, 18-, or 24-nucleotide sequence complementary to the cryptic 3′ splice site activated by the IVS2-705 mutation. In the U1.Beta and U1.524 construct, the same 9-nucleotide sequence (nt. 3–11) was replaced with 168 nucleotides from the second intron (IVS2-549-717) or 24 nucleotides complementary to the 5′ splice site, respectively. In the U1.U7.324 construct, nearly the entire U1 gene (nt. 3–161) was replaced with the U7.324 gene, whereas the *U1* promoter and 3′ sequences were retained. Polymerase chain reaction (PCR)-based mutagenesis methods were used in all the above constructions (40, 41). Refer to (19) for U7.324 construction.

Transient Expression of Modified U1 and U7 snRNAs—For all experiments, HeLa IVS2-705 cells were plated 24 h before treatment in 24-well plates at 10^5 cells per 2-cm^2 well. All HeLa cell lines were grown in S-MEM, 5% fetal calf sera, 5% horse sera, 50 μg/ml gentamicin, and 200 μg/ml kanamycin. The cells were treated for 10 h with the modified snRNA plasmids (indicated in the figure legends) complexed with 8 μg/ml LipofectAMINE or 8 μg/ml DMRIE-C (Life Technologies, Inc.). Unless otherwise indicated, the RNA was isolated 24-h post-transfection. Note that the variability of efficiency of transfection between experiments may be responsible for the differences in the effects of U7.324 seen in Figs. 2*B*, 3, 4*A*, and 6*A*. This variability is much less pronounced within a single experiment, and therefore the comparisons of the efficiency of correction within an experiment are more accurate.

RT-PCR Analysis—Total RNA was isolated using TRI-Reagent (MRC, Cincinnati, OH). RNA (200 ng) was analyzed by reverse transcription-PCR (RT-PCR) using rTth DNA polymerase as recommended by the manufacturer (PerkinElmer Life Sciences). To maintain the linear concentration-dependent response, PCR was carried out for 18 cycles (42) with the addition of 0.2 μCi of [α-^{32}P]dATP to the PCR reaction mixture. Correction of human β-globin pre-mRNA was detected with forward and reverse primers spanning positions 21–43 of exon 2 and positions 6–28 of exon 3, respectively, in β-globin mRNA. RT-PCR products were separated on 8% non-denaturing polyacrylamide gels. The gels were dried and autoradiographed with Kodak BioMax film.

RNase Protection Assay—The assay was performed using the RPA II Ribonuclease Protection Assay Kit (Ambion Inc., Austin, TX) with probes antisense to the regions containing the U1.324, U1.524, or U1.U7.324 snRNA genes. The probes included 258 nucleotides complementary to the U1.324 or U1.524 plasmids and 145 nucleotides from the U1.U7.324 plasmid. Probes generated from U1.524 and U1.324 (mouse U1a) hybridize with endogenous human U1 snRNA (U1a) with 1-base pair mismatch resulting in a 152-base pair band after RNase digestion. U1.524 and U1.324 snRNAs are detected as 176-base pair bands. The digestion of the U1.U7.324 (mouse U7) probe generates a 62-nucleotide *U7*-protected band; this probe does not hybridize with endogenous human U7 snRNA.

Following transient transfection, 1 μg of total cellular RNA or 1 μg of tRNA was hybridized at 44 °C overnight with 0.5 μl of radiolabeled probe in the hybridization buffer. Following RNase treatment, samples were separated on 5% non-denaturing polyacrylamide gels. The gels were dried and autoradiographed with Kodak BioMax film.

Protein Analysis—Transfected cells were treated with hemin (10 μM, Fluka, Switzerland) in serum free medium for 4 h immediately preceding the isolation of protein. Blots of proteins separated on a 10% Tricine-SDS-polyacrylamide gel (43) were incubated with polyclonal affinity purified chicken anti-human hemoglobin IgG as primary antibody and rabbit anti-chicken horseradish peroxidase-conjugated IgG as secondary antibody (Accurate Chemicals, Westbury, NY). The blots were developed with an ECL detection system (Amersham Pharmacia Biotech).

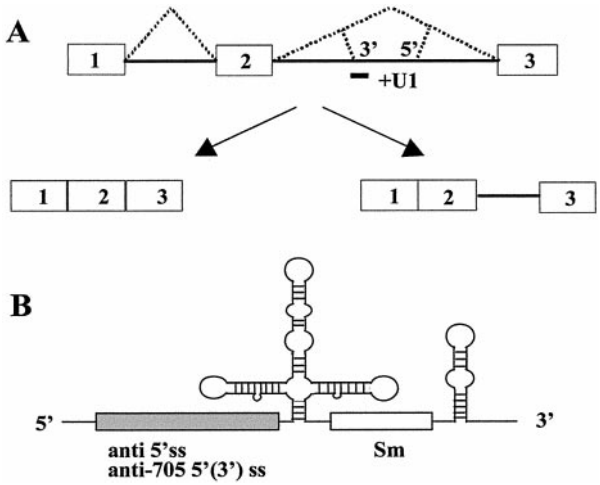

FIG. 1. *A,* correction of aberrant splicing of IVS2-705 pre-mRNA by modified U1snRNA. *Box*, exons; *line,* introns. The *dotted lines* represent correct and aberrant splicing pathways. Modified U1 snRNA (*heavy bar*) is targeted to the cryptic 3′ splice site. *B*, structure of U1 snRNA constructs. Wild-type U1 snRNA includes two stem loops, the Sm sequence (*open box*) and a sequence antisense to the 5′ splice sites of pre-mRNA (*shaded box*). In anti-705 U1 snRNAs, this sequence is replaced with antisense sequences to the aberrant 3′ or 5′ splice sites in the β-globin gene. The U1 promoter and 3′-end forming regions flank the gene.

Image Processing—All autoradiograms were captured by a Dage-MTI CCD72 video camera (Michigan City, IN), and the images were processed using NIH IMAGE 1.61. NIH IMAGE was also used for quantification of the autoradiograms. Correctly spliced β-globin mRNA was quantified by densitometry of the autoradiograms, and the results were expressed as the percentage of correct product relative to the sum of the correct and aberrant products. The results were corrected to account for the 2-fold higher [^{32}P]dAMP content of the PCR product derived from aberrantly spliced mRNA than that from correctly spliced mRNA.

RESULTS

U1.324 snRNA Targeted to the Aberrant 3′ Splice Site in IVS2-705 pre-mRNA—A T→G mutation at nucleotide 705 of intron 2 in human β-globin pre-mRNA (IVS2-705) generates an aberrant donor (5′) splice site and activates a cryptic acceptor (3′) splice site 126 nucleotides upstream from the mutation. As a result, IVS2-705 pre-mRNA is spliced incorrectly, and the β-globin mRNA retains a fragment of the intron (Fig. 1*A*). This fragment prevents proper translation of β-globin leading to reduced levels of hemoglobin and to β-thalassemia (44).

Success in utilizing modified U7 constructs that had the 18 nucleotide anti-histone pre-mRNA sequence replaced with the anti-IVS2-705 sequence, as well as contained a modified Sm sequence (SmOPT), prompted the modification of U1 snRNA as an antisense carrier. To block aberrant splicing and induce correct splicing of the IVS2-705 β-globin pre-mRNA, a series of modified U1 snRNAs, containing sequences antisense to the aberrant 3′ splice site were generated (Fig. 1*B*). The length of the antisense sequences in the constructs ranged from 9 to 168 nucleotides.

Transfection of HeLa cells stably expressing IVS-705 β-globin pre-mRNA with U1.309 resulted in barely detectable correction of β-globin splicing (Fig. 2*A*, *lane 2*). In the U1.309 construct, the 9 nucleotides, which in native U1 snRNA are antisense to the 5′ splice site, were replaced with nucleotides complementary to the cryptic 3′ splice site in the β-globin

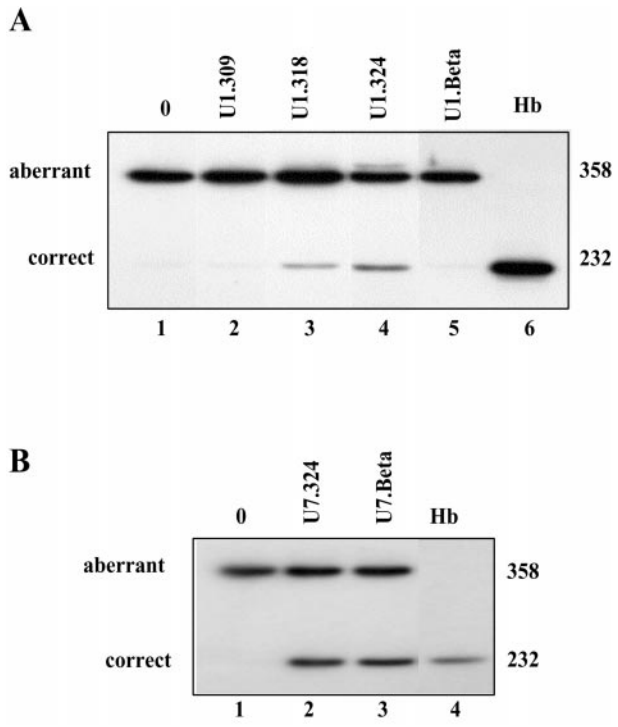

FIG. 2. **Effect of the length of antisense sequences in U1.3 snRNA on correction of IVS2-705 pre-mRNA splicing.** *A*, RT-PCR assay of total cellular RNA from IVS2-705 HeLa cells transfected with U1 constructs. *Lane 1*, mock transfection. *Lanes 2–4*, U1.309, U1.318, and U1.324 snRNAs directed against the 3′ cryptic splice site with respectively, a 9-, 18-, or 24-nucleotide antisense sequence. *Lane 5*, U1.Beta, with a 168-nucleotide antisense sequence spanning two putative branch sites, the 3′ cryptic splice site and the aberrant 5′ splice site. *Lane 6*, RNA from human blood (*Hb*). *B,* effect of antisense length in modified U7 snRNAs on correction of IVS2-705 pre-mRNA splicing. *Lane 1*, mock transfection; *lane 2*, U7.324, with a 24-nucleotide sequence antisense to the 3′ cryptic splice site; *lane 3*, U7.Beta; *lane 4*, RNA from human blood (*Hb*). The sizes (in nucleotides) of PCR bands representing aberrantly and correctly spliced mRNAs are indicated on the *right*. The same designations are used in RT-PCR assays shown in Figs. 3, 4, 5, and 6. Quantitation of these results took into account the approximately 2-fold higher [^{32}P]dAp content of the aberrant PCR product than that of the correct one.

intron. This modification does not change the overall length of U1 snRNA and therefore should not destabilize it. Thus, the negative result suggests that the antisense sequence was not able to compete with the splicing factors that assembled at the 3′ splice site during aberrant splicing of IVS2-705 pre-mRNA. This assumption was further supported by the findings that the levels of splicing correction by U1.318 and U1.324, containing, respectively, 18 and 24 nucleotide antisense sequences (Fig. 2*A*, *lanes 3* and *4*) were increased in a manner commensurate with the length of the inserted sequence. Further elongation of the antisense sequence to 168 nucleotides (U1.Beta, Fig. 2*A*, *lane 5*), abolished rather than enhanced correction of splicing. The β antisense sequence, which extends through the putative branch points and the aberrant 3′and 5′ splice sites in the IVS2-705 β-globin intron, was expected to interfere with aberrant splicing on the basis of its effect when placed in the modified U7 gene (U7.Beta, Fig. 2*B*, *lane 3*). Unlike in U1 snRNA, the U7.Beta and U7.324 corrected splicing to a similar degree (Fig. 2*B*, *lanes 2* and *3*). This suggests that the long antisense sequence affects the structure, stability, and/or interaction of the U1 and U7 particles with the target sequences in a very different manner.

We have compared in more detail the ability of the two similar U7 and U1 snRNAs, *i.e.* those with 24 nucleotide antisense sequences, U7.324 and U1.324, to correct IVS2-705

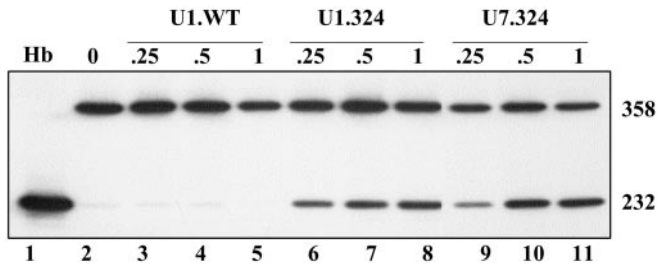

FIG. 3. **Correction of aberrant splicing by U1.324 and U7.324 snRNA.** IVS2-705 cells transiently transfected with 0.25, 0.5, and 1 μg of U1 or U7 snRNA constructs (*top*). *Lane 1*, RNA from human blood (*Hb*); *lane 2*, mock transfection; *lanes 3–5*, cells transfected with wild type U1 snRNA gene (*U1.WT*); l*anes 6–8*, and *9–11*, cells transfected with U1.324 and U7.324 constructs, respectively. All other designations are as described in the legend to Fig. 2.

pre-mRNA splicing. For both snRNAs, transient transfection of IVS2-705 HeLa cells resulted in dose-dependent correction of splicing (Fig. 3, *lanes 6–11*). Quantitation of the results by densitometry (see "Experimental Procedures") showed that at 1 μg of DNA plasmids per 10^5 cells, the level of correction was as high as 56 and 70% for the U1.324 and U7.324 constructs, respectively. The effect of U1.324 was dependent on the antisense sequence because the transfection of the cells with a plasmid expressing wild-type U1 snRNA did not restore correct splicing of β−globin IVS2-705 pre-mRNA (Fig. 3, *lanes 3–5*).

U1.524 snRNA Targeted to the Aberrant 5′ Splice Site—The fact that transfection with U1.324 and U7.324 plasmids led to efficient correction of splicing of IVS2-705 pre-mRNA indicated that the 24-nucleotide antisense sequence did not destabilize the snRNPs, and that it bound to the 3′ splice site strongly enough to inhibit aberrant splicing. Because modified U7.524 snRNA targeted to the aberrant IVS2-705 5′ splice site was effective in correction of splicing (Fig. 4*A*, *lane 3*), it seemed possible that U1.524 might be similarly effective. However, as shown in Fig. 4*A*, *lane 5*, U1.524 did not correct β-globin splicing; other constructs, U1.324, U7.524, and U7.324, were active as expected (Fig. 4*A*, *lanes 2–4*).

To confirm that the inability of the U1.524 construct to correct splicing was not because of lack of its expression, an RNase protection assay was performed (Fig. 4*B*). Densitometry analysis of the autoradiogram indicated that both U1.324 and U1.524 were expressed in transient transfections at comparable levels (Fig. 4*B*, *lanes 3* and *6*). Comparison of the intensity of the bands representing anti-thalassemic and endogenous U1 snRNAs (176 and 152 nucleotides, respectively) indicated that these RNAs were expressed at, approximately, a 1:5 ratio. Thus, assuming that the wild-type U1 snRNA is present at 10^6 copies per cell (29), the amount of modified U1.324 and U1.524 was estimated to equal 2–3 × 10^5 copies per cell.

U7.324 snRNA Expressed from the U1 Promoter—In an attempt to further improve the effectiveness of snRNA-based vectors, we combined the strong U1 promoter and terminator sequences with the coding sequence of the modified U7.324 gene. In transient transfections with 1 μg of plasmid, this construct (U1.U7.324, Fig. 5*A*) was effective, resulting in approximately 70% correction of β-globin splicing (Fig. 5*B*, *lane 2*), whereas the U7.324 gene controlled by the U7 promoter (Fig. 5*B*, *lane 3*) corrected splicing to approximately 60%. This small increase in correction efficiency was consistent with the higher level of U1.U7.324 expression over that of the U7.324 construct (Fig. 5*C*, *lanes 3* and *4*).

The effectiveness of the U7.324 and U1.U7.324 constructs was compared in a time-course experiment in transiently transfected cells. For both constructs, correctly spliced β−globin mRNA became detectable 12-h post-transfection (Fig. 6*A*,

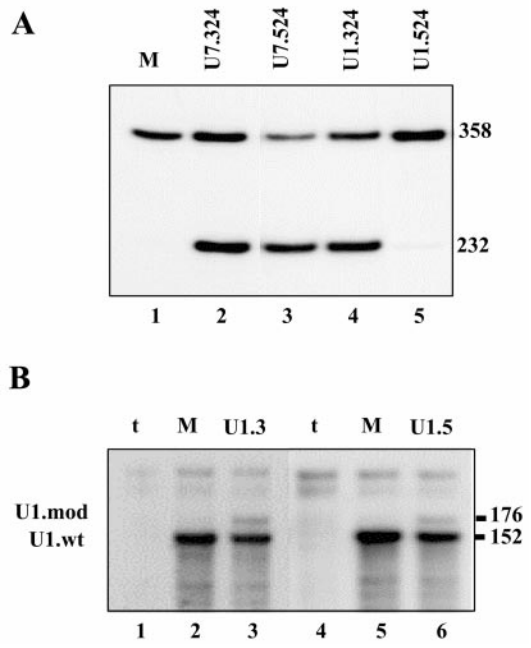

FIG. 4. **RNase protection assay of U1 snRNA expression.** *A*, correction of aberrant splicing. *Lane 1*, mock transfection; *lanes 2* and *3*, cells transfected with 1 μg of U7.324 and U7.524, respectively; *lanes 4* and *5*, cells transfected with 1 μg of U1.324 and U1.524, respectively. *B*, RNase protection assay using RNAs from *A*. *Lanes 1* and *4*, tRNA (*t*) mixed with probe; *lanes 2* and *5*, RNA from mock transfected cells mixed with probe (*M*); *lane 3*, cells transfected with U1.324; *lane 6*, cells transfected with U1.524. *Bands* from modified U1 (*U1.mod*, 176 nucleotides) and wild-type U1 (*U1.wt*, 152 nucleotides) are indicated. In *lanes 1-3* and *4–6*, the probes were transcribed from U1.324 and U1.524 plasmids, respectively, as described under "Experimental Procedures."

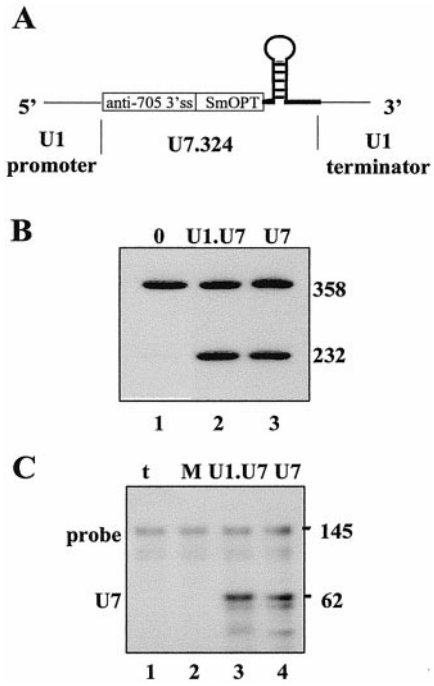

FIG. 5. *A,* structure of U1.U7.324 snRNA construct. U7.324 snRNA includes a stem loop structure, the SmOPT sequence, and a 24-nucleotide sequence antisense to the 3′ cryptic splice and is flanked by the U1 promoter and terminator. *B,* correction of aberrant splicing by U1.U7.324 snRNA. IVS2-705 cells were mock transfected (*lane 1*) or transfected with 1 μg of U1.U7.324 (*lane 2*) and U7.324 (*lane 3*) plasmid DNA. *C,* RNase protection assay. *Lane 1*, tRNA; *lanes 2–4* RNAs from *B* mixed with probe. *Bands* from modified U7 (62 nucleotides) and undigested probe (145 nucleotides) are indicated.

lanes 4 and *5*), and its level increased in a time-dependent fashion at the 24- and 48-h time points (Fig. 6*A*, *lanes 6–9*). There was no further increase 72-h post-transfection (Fig. 6*A*, *lanes 10–11*). At every time point, the level of correction effected by U1.U7.324 snRNA was higher than that by U7.324 snRNA. The average of the results from this figure and Fig. 5 indicates that the U1.U7.324 construct is approximately 30% more effective in splicing correction than its U7-only counterpart.

The RNase protection assay shows that the time course of splicing correction is consistent with the levels of expression of the two constructs (Fig. 6*B*). The U7.324 snRNA is not yet generated 6-h post-transfection but is clearly detectable at 12–72-h time points, and its levels are higher upon transcription from the U1 promoter (Fig. 6*B, lanes 5, 7, 9* and *11 versus lanes 6, 8, 10,* and *12*, respectively). Interestingly, translation of β−globin protein was delayed relative to transcription and splicing because it was not detectable for 24-h post-transfection (Fig. 6*C*, *lanes 2–5*); β−globin was translated 24 h later (Fig. 6*C*, *lanes 6–9*). However, although an increasing time-dependent correction of splicing is confirmed by the Western blot, a quantitative difference between the U7 and U1.U7 constructs is not evident. This may be because of low sensitivity of the Western blot or loading error. Note that this error is eliminated in RT-PCR assays by comparing the ratio of the spliced products.

Because the main advantage of vector-transcribed antisense RNA over synthetic antisense oligonucleotides is the possibility of stable intracellular expression, a U1.U7.324-expressing IVS2-705 HeLa cell line was generated. As expected, correct splicing of IVS2-705 pre-mRNA was restored very efficiently (77%) and persisted at this level throughout the time of culture (Fig. 7). The growth rate of the cell line was comparable with that of the wild-type HeLa cells (data not shown), suggesting that the modified U7 snRNA is not toxic to the cells.

DISCUSSION

Approximately 15% of all point mutations that cause genetic diseases affect splicing of pre-mRNA (45). Moreover, a great majority of genes in higher eukaryotes are transcribed into pre-mRNAs that undergo alternative splicing (46–49). Thus, manipulation of splicing with antisense oligonucleotides and RNAs offers a promising method of modification or repair of spliced mRNAs. On the other hand, splice sites and other sequences involved in pre-mRNA splicing appear to be unlikely targets for the antisense approach because they interact with numerous protein splicing factors and several snRNP particles within the spliceosome (31, 50). Nevertheless, work from this laboratory showed that blocking of aberrant splice sites by antisense agents leads to restoration of correct splicing in pre-mRNAs for β-globin, cystic fibrosis transmembrane conductance receptor (*CFTR*) and dystrophin genes in systems modeling thalassemia, cystic fibrosis, and Duchenne muscular dystrophy, respectively (19, 23, 27, 28, 39). Previous work showed that antisense oligonucleotides targeted to splice site junctions in immediate early genes led to inhibition of replication of herpes simplex virus (52–54). Recently it has been found that modification of alternative splicing of bcl-x pre-mRNA by antisense oligonucleotides sensitized the cells to apoptotic stimuli (55).[2] Clearly, splice sites are accessible to antisense agents, most likely because the spliceosome is formed *de novo* for every splicing event and its interaction with pre-mRNA is very dynamic (31).

Our previous work showed that the accessibility of the aberrant 3′ splice site in IVS2-705 pre-mRNA to oligonucleotides

[2] D. R. Mercatante and R. Kole, manuscript in preparation.

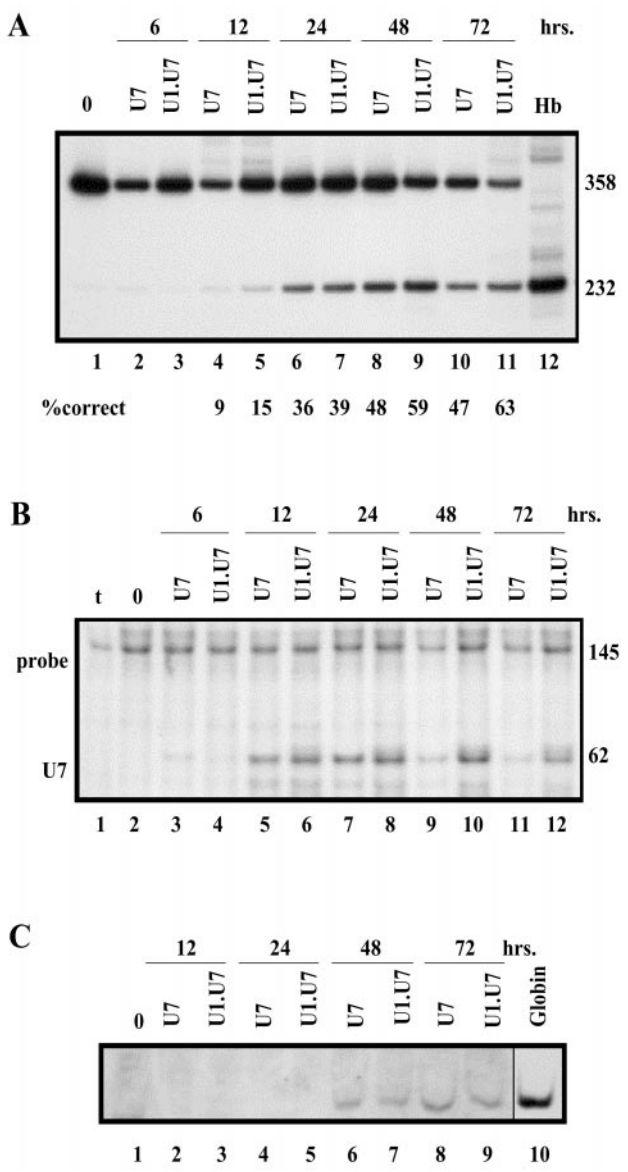

FIG. 6. **Time course of splicing correction by U1.U7.324 snRNA in transiently transfected cells.** *A,* RT-PCR. Total RNA from cells transfected with U7.324 (*lanes 2, 4, 6, 8,* and *10*) and U1.U7.324 (*lanes 3, 5, 7, 9,* and *11*) constructs was isolated at 6-, 12-, 24-, 48-, and 72-h post-transfection (*top*). *Lane 1*, untreated cells; *lane 12*, human blood (*Hb*). Percent correction at every time point is shown *below the panel*. *B,* expression of U7 snRNA. The RNA from transfected cells was analyzed by RNase protection assay. *Lane 1*, tRNA; *lane 2*, RNA from untreated cells; *lanes 3–12*, the same RNA samples as analyzed in *A*, *lanes 2–11*, respectively. *C,* immunoblot. Total protein from transfected cells was isolated 12-, 24-, 48-, and 72-h post-transfection as indicated in *lanes 2–9*. *Lane 10*, human β−globin as size marker.

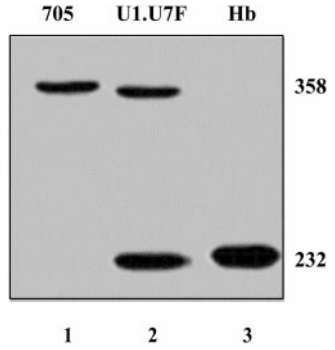

FIG. 7. **Stable correction of IVS2-705 pre-mRNA splicing in cell line 705U1.U7.324.F.** *Lane 1*, RNA from IVS2-705 cell line; *lane 2*, RNA from cell line 705U1.U7.324F; *lane 3*, RNA from human blood (*Hb*).

was ~8 times lower than that of the 5′ splice site (26). The difference in the accessibilities of the 3′ and 5′ aberrant splice sites in IVS2-705 pre-mRNA to modified U7 snRNPs, particles much larger than the oligonucleotides (Ref. 19 and Fig. 4*A*, *lanes 2* and *3*), was not as pronounced. This difference may be because of the fact that the modified U7 snRNP particle, carrying the appropriate Sm proteins, may mimic other snRNPs in the spliceosome assembling on the splice sites. However, because of inappropriate antisense sequences and/or inappropriate structure of modified U7, the spliceosome is dysfunctional, resulting in inhibition of aberrant splicing.

In view of the observations regarding U7 snRNA it was surprising that the modified U1 snRNA, U1.524, targeted to the aberrant 5′ splice site, was unable to inhibit aberrant splicing. Lack of expression or instability of U1.524 was excluded by the RNase protection assay, which confirmed that the inactive U1.524, and the active U1.324 were produced at similar levels. One possibility is that U1.524 could not compete with wild type U1 for binding to the 5′ splice site because it was expressed at one-fifth of the level of endogenous U1. Because endogenous U1 does not initially interact with the 3′ splice site, it would not compete with U1.324 for binding to its target.

U1.524 ineffectiveness in inhibiting splicing may also be because of the fact that, despite its modification, the construct is still able to support the proper spliceosome assembly at the 5′ splice site. This hypothesis is supported by several reports, which have shown that modified U1 could, albeit inefficiently, restore splicing at defective splice sites or redirect splicing to sequences adjacent to canonical splice sites (56, 57). Of particular interest is the observation that U1 snRNA-targeted 14 nucleotides downstream from the 5′ splice site still promoted correct splicing presumably by forming the so-called commitment complex, which then evolved into a functional spliceosome (56, 58). This interpretation is consistent with the fact the U7 snRNAs targeted to the 3′ and 5′ splice sites were both effective in splicing correction (Fig. 4*A*, *lanes 2* and *3* and Ref. 19) even though their level of expression is likely to be lower than that of U1 snRNA (59).

Several reports showed that modified U1 snRNA could be used for down-regulation of targeted mRNAs (14, 18, 60–62). They were particularly effective in inhibiting HIV replication when a hammerhead ribozyme sequence was incorporated into the U1 snRNA molecule (14, 60, 61). It is notable however, that the most effective constructs were targeted either to the coding sequences or to regions that included the 3′ splice site sequences (14). Recently, an effective anti-HIV U1-ribozyme targeted to the 5′ splice site of REV pre-mRNA was constructed. In this construct, the antisense/ribozyme sequence was located within the body of the U1 molecule whereas the regular 5′ antisense sequence was retained. Thus, the resulting RNA bound simultaneously to the 5′ splice site and to the adjacent sequences, forming a double-target antisense molecule (14). Interestingly, double-target U7 snRNAs were also found to be more effective than the single target ones in correction of splicing of several thalassemic β-globin pre-mRNAs (44). It appears that the arrangement of closely spaced antisense sequences may loop out and deform the targeted pre-mRNA, preventing the formation of the spliceosome and inhibiting aberrant splicing.

Because U6 snRNA has been used as an antisense vector by several groups (20, 63–65) and because it differs from U7 and U1 snRNA in important aspects of metabolism and structure (29), we built a series of U6 constructs carrying either 24 or 168 nucleotide sequences antisense to the aberrant splice sites of

IVS2-705 pre-mRNA. Surprisingly, when transfected into HeLa IVS2-705 cells, all of the constructs failed to correct splicing of IVS2-705 pre-mRNA (data not shown). Notably, in previous work, the effective U6 constructs were not targeted to splice sites. It therefore seems plausible that in our system U6 was not able to compete with the splicing factors for splice site targets. This could be because of the fact that U6 snRNA is incorporated into snRNP particles only in conjunction with U4 snRNA (66–67 and 51), and in its absence U6 snRNA may be unable to access the splice site.

The combination of the promoter and termination sequences from the U1 gene with the modified U7 snRNA sequence increased expression of U7 snRNA and concomitant correction of β−globin pre-mRNA splicing, providing an improvement over the U7.324 vector. Thus, the ability to modify splicing by two types of snRNA molecules, U1 and U7, generated from three different vectors U1, U7 and U1.U7 broadens the possibilities of using the antisense approach as a form of gene therapy and may offer certain advantages in different cell types or tissues.

The obvious next step for this work is to deliver the antisense U1 or U7 constructs in vectors that are stably expressed in stem cells and/or erythroid progenitor cells. If efficient correction of splicing by antisense RNAs were achieved in these cells in a thalassemic patient, a more balanced synthesis of α- and β-globin would have been restored and the clinical symptoms of thalassemia ameliorated. Note that the correction would have occurred in the β-globin pre-mRNA, which was properly transcribed from the gene that remained in its natural chromosomal environment. In consequence, the possibility of overexpression or inappropriate expression of β-globin mRNA, an important consideration in treatment of hemoglobinopathies, would have been precluded. One concludes that repair of defective pre-mRNAs by antisense agents offers an attractive alternative to gene replacement therapy.

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