BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 11–16 (1998)
ARTICLE NO. RC989406

Suppression of the 5′ Splice Site Mutation in the Nagase Analbuminemic Rat with Mutated U1snRNA

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Received August 5, 1998

Nagase analbuminemic rats (NAR) are deficient in serum albumin due to skipping of the albumin exon H caused by a mutation in the intron HI. This mutation deletes nucleotides from +5 to +11 in the 5′ splice site region, where it interacts with U1snRNA. To determine whether the mutation can be suppressed by the compensatory base substitution in U1snRNA, we constructed mutated U1snRNA genes with various degrees of complementarity to the mutated 5′ splice site. Several mutated U1snRNA genes activated the mutated 5′ splice site of the intron HI, when cotransfected with the albumin minigene derived from NAR. *In vivo* activity of these mutated U1snRNAs correlated well with the predicted thermodynamic stability. Since mutation in the 5′ splice site is one of common causes of genetic defects in human (5), these data indicate that NAR is a good model system to examine the possibility of gene therapy using a mutated U1snRNA.

The splicing of nuclear mRNA precursor (pre-mRNA) mediated by the accurate recognition of conserved sequences, including a 5′ splice site (5′ss), a branch site, and a 3′ splice site, by a large number of factors (1–4). Mutations that affect these sequences disturb the splicing and may cause exon skipping or activation of a cryptic splice site, which accounts for more than 5% of all genetic defects (5).

Nagase analbuminemic rats (NAR), which were established from a stock of Sprague–Dawley rats (SD) (6), are deficient in the production of serum albumin due to aberrant splicing of the albumin pre-mRNA, causing exon H skipping (7–9). Genetic analysis revealed a 7 bp deletion in the 5′ss region of intron HI at position +5 to +11 (7). Analysis of the splicing pattern of the cloned NAR albumin minigene spanning from exons F to J confirmed the exon H skipping upon transfection in culture cells (10).

The deletion mutation found in NAR changes nucleotides within the 5′ss consensus at positions +5 and +6. One possible mechanism of exon H skipping was that the two-base alterations in the 5′ss reduce the base-pairing with U1snRNA, which binds to the pre-mRNA in the early steps of splicing reaction (11–13). Since a compensatory base-substitution in U1snRNA was found to suppress inactivation of 5′ss due to certain point mutations (14–17), we were interested in whether a base-substituted U1snRNA could suppress the deletion mutation found in NAR.

To address this question, we constructed expression vectors of mutated U1snRNAs and investigated their effects on splicing of the NAR minigene mRNA in culture cells. We found that several mutated U1snRNA suppressed the deletion mutation and it was well-correlated with the thermodynamic stability between the mutated U1snRNA and the 5′ss.

MATERIALS AND METHODS

Plasmid. The albumin minigenes derived from NAR and SD were described previously (10). A human U1snRNA gene was cloned by genomic PCR as a transcription cassette containing its own promoter and termination signal (20, 21). A series of base-substitutions was introduced in the U1snRNA gene by the Kunkel's method (22). The mutated U1snRNA gene was subcloned in an *Eco*RI site of pEUK-C1. The mutants were designated by the introduced base-substitutions and their positions from the 5′ end of U1snRNA.

Cell culture and transfection. COS-1 cells were cultured with Dulbecco's modified minimum essential medium supplemented with 10% fetal calf serum. Transfection was carried out with 700 ng of plasmid and 1000 μg/ml of DEAE-dextran (Pharmacia) (22). pSVβ was transfected in parallel and transfection efficiency was monitored by *in situ* staining of β-galactosidase with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

RNA analysis. Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (23) and the integrity of RNA preparation was verified electrophoretically. Single stranded cDNA was synthesized with 3 μg of total RNA, oligo-dT primer and 200 units of Superscript II (BRL) at 42°C for 60 min. cDNA of the minigene-derived transcripts was amplified semi-quantitatively by

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0006-291X/98 $25.00

PCR under the following conditions: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for 23 cycles. Primers used for the analysis were as follows: for the albumin minigene, HS 5′-CGA TCC TCC TGC CTG CTA CG-3′, FB 5′-GCG GAT CCA TTC CCC AAT GCT GAG TTC G-3′ and JB 5′-GCG GAT CCT GCC TCC ACG AGA GTT-3′; for the b-actin, hAS 5′-ATC CGC AAA GAC CTG TAC GC-3′ and hAR 5′-TGT GTG GAC TTG GGA GAG GA-3′; for the U1snRNA plasmid, ES3 5′-ATA CTG TTG ACG GGA AAC GC-3′, EA 5′-CAT TCT AGT TGT GGT TTG TCC-3′. The products were resolved on a 3% agarose gel (NuSieve:SeaKem = 2:1, FMC) or an 8% polyacrylamide gel. PCR products were sequenced to determine exon/exon junctions. Transfection of human U1snRNA expression vectors was monitored by RT-PCR with the ES3/EA primers, which can amplify the vector specific transcript derived from the backbone plasmid.

Quantitative PCR. Amplification profile of RT-PCR was monitored by adding [α-^{32}P]dCTP into the reaction. The products were separated by 3.5% native polyacrylamide gel electrophoresis. The radioactivity of each band was quantified by the BAS2000 IP reader (Fuji). To mimic the proportional amplification during thermal cycling, subcloned PCR fragments were mixed in various ratios and subjected to competitive amplification with end-labeled primers. The radioactivity of PCR fragments were measured by BAS2000.

Calculation of thermal stability. The binding stability of U1snRNAs with a 5′ss sequence was calculated according to the Turner's stability rule (24, 25). The application of this rule was based on the observation that substitution of pseudouridine with uridine did not change the thermodynamic stability between RNA oligonucleotides having a 5′ free end of U1snRNA and 5′ss consensus sequences (26).

RESULTS

The mutation in the intron HI of the albumin gene was a 7-bp deletion at position + 5 to +11 (Fig. 1). To investigate whether the mutation responsible for exon H skipping could be suppressed by restoration of base-pairing with U1snRNA, we introduced two-base substitutions in the 5′ free end of the U1snRNA gene (Fig. 1), and designated the mutant as C3G4.

Suppression of the 5′ss deletion mutation with the mutated U1snRNA and associated novel cryptic splice site activation. The effect of C3G4 on exon H skipping was studied by cotransfection with the NAR albumin minigene into COS-1 cells. The structure of minigene mRNA was first examined by RT-PCR analysis, using the two sets of primers HS/JB and FB/JB (Fig. 1). With the HS/JB primers, the NAR albumin minigene gave no product due to exon H skipping, whereas the SD albumin minigene produced the expected 197-bp fragment (Fig. 2A, lanes 2 and 5). Following cotransfection with C3G4, three fragments named H1, H2, and H3 were detected (lane 4). Sequence analysis revealed that H1 was a normal splicing product (compare lane 4 and 5). Therefore, C3G4 activated the mutated 5′ss in the NAR minigene.

On the contrary, H2 and H3 contained a 5′ part of the intron HI between exons H and I and their 5′ cleavage sites corresponded to +84 and +117 bases in the intron HI (Fig. 2B). Alignment of these sequences with the rat albumin gene revealed that both sites were similar to the 5′ss consensus sequence (Fig. 2B).

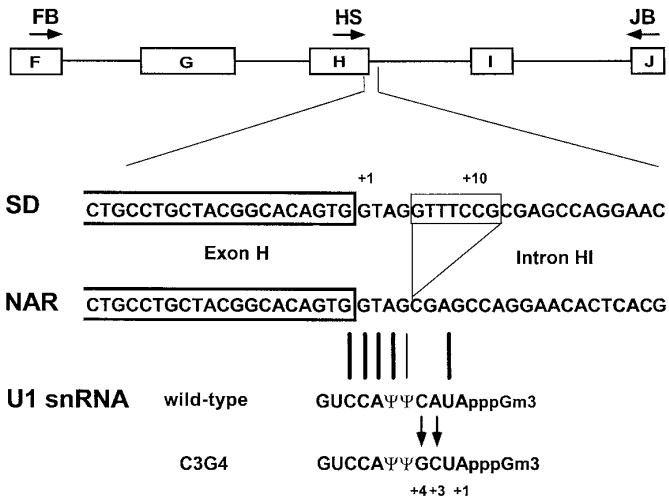

FIG. 1. The deletion mutation in the albumin gene and complementary base-substitution in U1snRNA. The structure of the albumin minigene spanning exons F to J is shown at the top. Exons are depicted as boxes and introns as connecting lines. The arrows indicate the sites of primers used for RT-PCR analysis of minigene mRNA. The sequences of the exon H/intron HI boundary in wild-type (SD) and mutated (NAR) are shown in the middle; the 7 base-deletion is boxed in the intron HI of SD sequence from +5 to +11. The base-pairing between the 5′ss of the NAR intron HI and wild-type U1snRNA is indicated with vertical lines. Thick lines represent standard base-pairings and a thin line G:U pairing. The 5′ end of U1snRNA is shown in the 3′ to 5′ direction to align with the 5′ splice site. The pseudouridine residues of U1snRNA at position 5 and 6 are shown as Y. The base-substitution at +3 and +4 from AC to CG, indicated with vertical arrows, restores base-pairing with the 5′ splice site of the NAR intron HI, and generated a mutated U1snRNA designated as C3G4.

C3G4 would be expected to activate these intronic sequences as 5′ splice site in addition to the upstream m5′ss.

Entire structure of the minigene mRNAs were analyzed with the FB/JB primers (Fig. 1). As shown in Fig. 2C, PCR produced comparable amounts of 498 and 631 bp fragments from NAR and SD minigene transfectants, respectively (lanes 2 and 5). In addition to the exon H skipped products present in the NAR minigene alone (lane 2), two fragments, H1′ and H2′, were generated upon cotransfection with C3G4 (lane 4). Sequence of these fragments proved that H1′ and H2′ corresponded to H1 and H2. The exon/exon boundaries in H1′ were identical to those of normal albumin mRNA.

Since these fragments were amplified competitively in a single reaction, the profile of amplification was monitored. It showed proportional amplification of all fragments between cycles 18 to 26 of thermal cycling (Fig 2D). This point was further confirmed by the competitive amplification (see Materials and Methods).

Construction of the base-substituted U1snRNA genes. The activation of the +84 and +117 sites was unexpected, and prompted us to examine whether it was

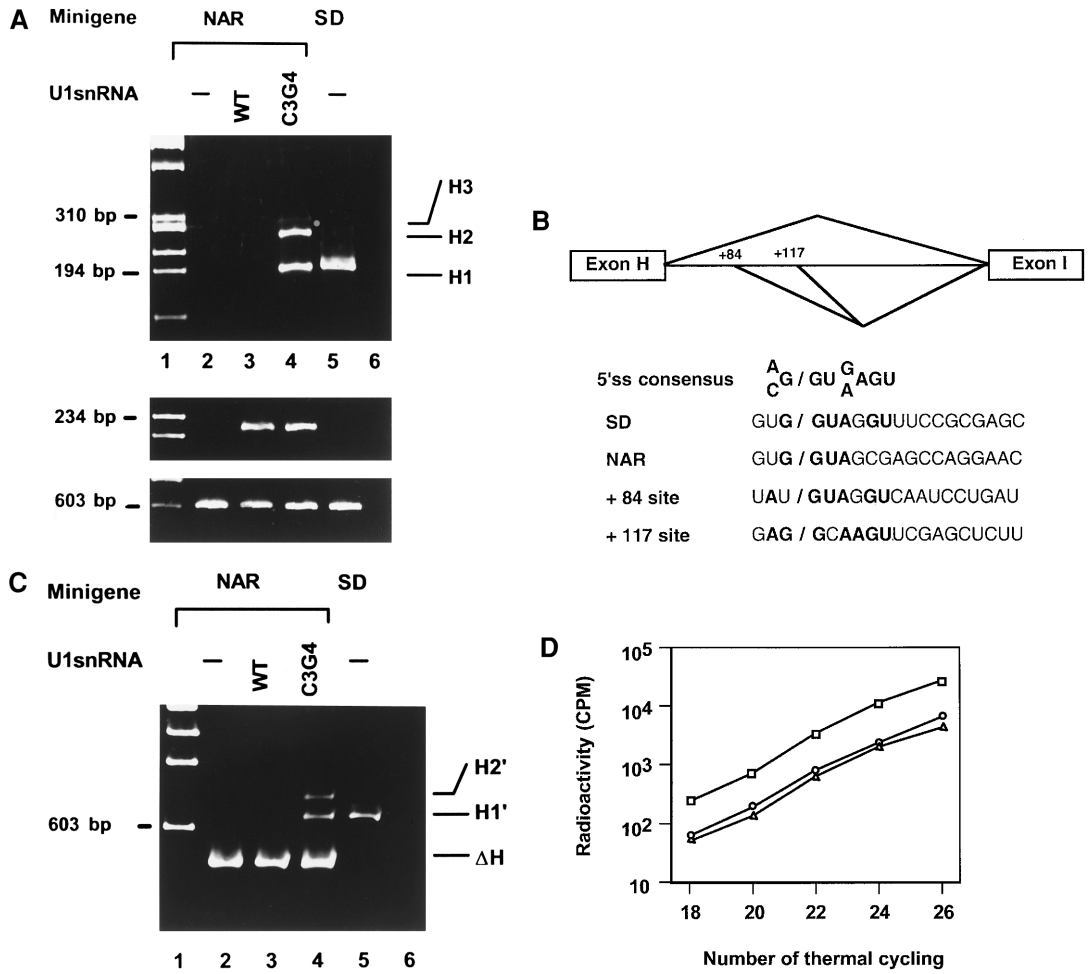

FIG. 2. Suppression of the deletion mutation in the intron HI with the mutated U1snRNA. (A) (Top) RT-PCR analysis of the minigene mRNA using HS and JB primers. The albumin minigenes and the U1snRNA expression vectors was transfected into COS-1 cells in the combinations indicated on the top of the panels. Thermal cycling was performed for 23 cycles. Three fragments present in lane 4 are indicated on the right. Lane 6 is the negative control for PCR without template in the reaction. (Middle) Transfection levels of the U1snRNA expression vectors. The single stranded cDNA was subjected to RT-PCR with the primer set for transcripts from the U1snRNA expression vector. (Bottom) cDNA of b-actin mRNA was amplified for 20 cycles as a control. (B) The position of 5′ cleavage sites in the intron HI detected upon cotransfection with C3G4 is shown schematically. These sequences are aligned with the consensus sequence of the 5′ splice site and/indicates a 5′ cleavage site. Matched bases with the consensus sequence are represented by bold characters. (C) RT-PCR analysis of the entire structure of the minigene mRNA. RT-PCR was performed with the FB and JB primers for 23 cycles. The exon H skipped product is indicated as ΔH and two additional fragments in lane 4 as H1′ and H2′. The SD minigene mRNA is shown in lane 5. (D) Amplification profile of RT-PCR with the FB and JB primers. PCR was performed with cDNA from cotransfectants of the NAR minigene and C3G4 including [α-^{32}P]dCTP (see Materials and Methods). The radioactivity of each fragment was quantitated and plotted as ΔH (square), H1′ (circle), and H2′ (triangle).

unique to C3G4. We were also interested in whether C3G4 was the most efficient mutant for the suppression of the deletion mutation and how the extent of binding stability could activate the m5′ss. To address these questions, a series of U1snRNA mutants were constructed (Fig. 3). These mutants had various degrees of thermal stability to m5′ss, ranging from −7.0 to −15.0 kcal/mol (Fig. 3).

Effect of the mutated U1snRNAs correlated with the predicted binding stability on the mutated 5′ss in vivo. The effect of the mutated U1snRNAs were examined by cotransfecting them with the NAR albumin minigene under the conditions set previously. In addition to C3G4, those that had more than two base-substitutions suppressed the deletion mutation and generated fragments corresponding to H1′ (Fig. 4A). However, the production of additional fragments corresponding to H2′ and H3′ was also observed with these mutated U1snRNAs (Fig. 4A). The relative amounts of PCR fragments was calculated as shown in Fig. 4B. The amount of normal splicing product, as well as total incorporation of exon H, was increased as the predicted stability of the mutated U1snRNA binding to m5′ss increased (Fig. 4C).

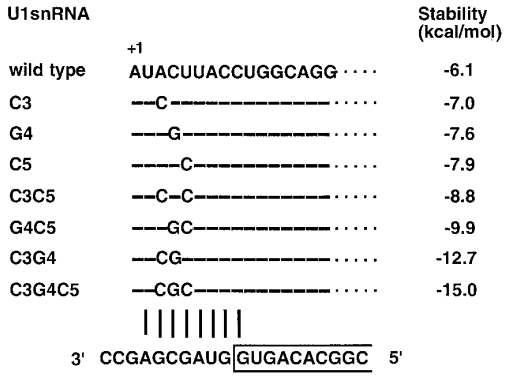

FIG. 3. Base-substituted U1snRNAs. Mutations introduced in the 5′ end of U1snRNA are shown with wild-type counterparts and unchanged bases are represented by horizontal bars. The mutated U1snRNA was named by the introduced base-substitution and their positions from the 5′ end of U1snRNA. Complete base-pairing of C3G4C5 with the 5′ss of NAR intron HI is indicated below. The predicted thermodynamic stability with the 5′ss of the NAR intron HI is shown on the right.

Cryptic splice site activation did not correlated with the binding stability of the mutated U1snRNAs. To investigate the role of the mutated U1snRNAs on the activation of the +84 and +117 sites, we compared the binding stability of the mutated U1snRNAs to these sites (Table 1). Except for C5 and C3C5, the mutated U1snRNAs had lower stability than the wild-type U1snRNA. This suggested that the activation of cryptic splice sites did not depend on the direct interaction of the mutated U1snRNAs with the intronic sequences. On the other hand, C5, which activated only the cryptic splice sites, had a markedly higher stability to the +84 site than the wild-type U1snRNA (Table 1). However, when these mutated U1snRNA genes were cotransfected with the SD minigene, no activation of a cryptic splice site was observed (data not shown).

DISCUSSION

Suppression of the 5′ss mutation has been studied with point mutations affecting the 5′ss sequence (15–18). In some cases, the compensatory base-substitution in the 5′ free end of U1snRNA could not restore correct splicing (17). In the NAR minigene, the deletion mutation responsible for the exon H skipping was suppressed by the mutated U1snRNA, although it was accompanied with the novel activation of intronic sequences like a cryptic splice site.

Using the Turner's thermodynamic stability rule (24, 25) to predict the relative strength of the interaction between the 5′ splice site and the mutated U1snRNA, the activation of m5′ss appeared to require a certain levels of binding stability between U1snRNA and m5′ss *in vivo*. The activation was first observed with C3C5, which has a binding stability of -8.8 kcal/mol, and the efficiency was elevated as the predicted stability was increased. Consequently, C3G4C5 was found most effective derivatives that could activated m5′ss.

The induction of novel cryptic splice sites by the mutated U1snRNAs is intriguing. Generally, cryptic splice sites are restricted to the immediate vicinity of the wild-type site and fits to the 5′ss consensus sequence. However, in contrast to common cryptic splice site activation, these sites remained silent in NAR and were located relatively distant from the m5′ss. Although the activation of these cryptic splice sites was observed only when the mutated U1snRNA gene was cotransfected, the mutated U1snRNAs, except for C5, had no particular binding stability to these sites. Therefore, we speculated that the stable binding of the mutated U1snRNA to m5′ss could recruit the splicing machinery to the 5′ss region and promote the splicing reaction using one of three candidate sequences.

When we searched for putative 5′ss sequences around the exon H and intron HI, the +84 site had one of the highest scores, but the +117 site was not listed partly due to the uncanonical GC dinucleotide. Comparing sequences around all GT and GC dinucleotides within the intron HI, the common feature that distinguishes the +84 and +117 sites from others is the presence of a GT at positions +5 and +6 (Fig. 4). These nucleotides were proven to interact with the conserved domain of U6snRNA (18, 19, 27). Several studies demonstrated that the recruitment of the U6snRNP complex to the 5′ss was the rate limiting step of *in vitro* splicing reaction depleted with U1snRNP (28) and the 5′ss activation by U1snRNA bound at a site distant from the 5′ cleavage site (27, 29). Therefore, the presence of GT at +5 and +6 may contribute to the specific selection of these sites as 5′ cleavage sites.

Although it remains to elucidate that the mutated U1snRNA could stimulate the serum albumin production in NAR, we proved that the mutated U1snRNA could suppress the deletion mutation present in the

TABLE 1

Binding Stability between the 5′ End of U1snRNAs and 5′ Splice Sites[a]

	5′ splice site			
U1snRNA	SD	NAR	+84 site	+117 site
Wild type	−9.2	−6.1	−6.3	−7.1
C3	−7.4	−7.0	−4.5	−5.3
G4	−6.1	−7.6	−3.2	−3.8
C5	−10.8	−7.9	−9.7	−3.4
C3C5	−10.8	−8.8	−7.9	−2.0
G4C5	−7.9	−9.9	−5.0	−2.0
C3G4	−6.1	−12.7	−3.2	−3.0
C3G4C5	−7.9	−15.0	−5.0	−2.0

[a] The binding stability is calculated according to Turner's stability rule and the unit is kcal/mol.

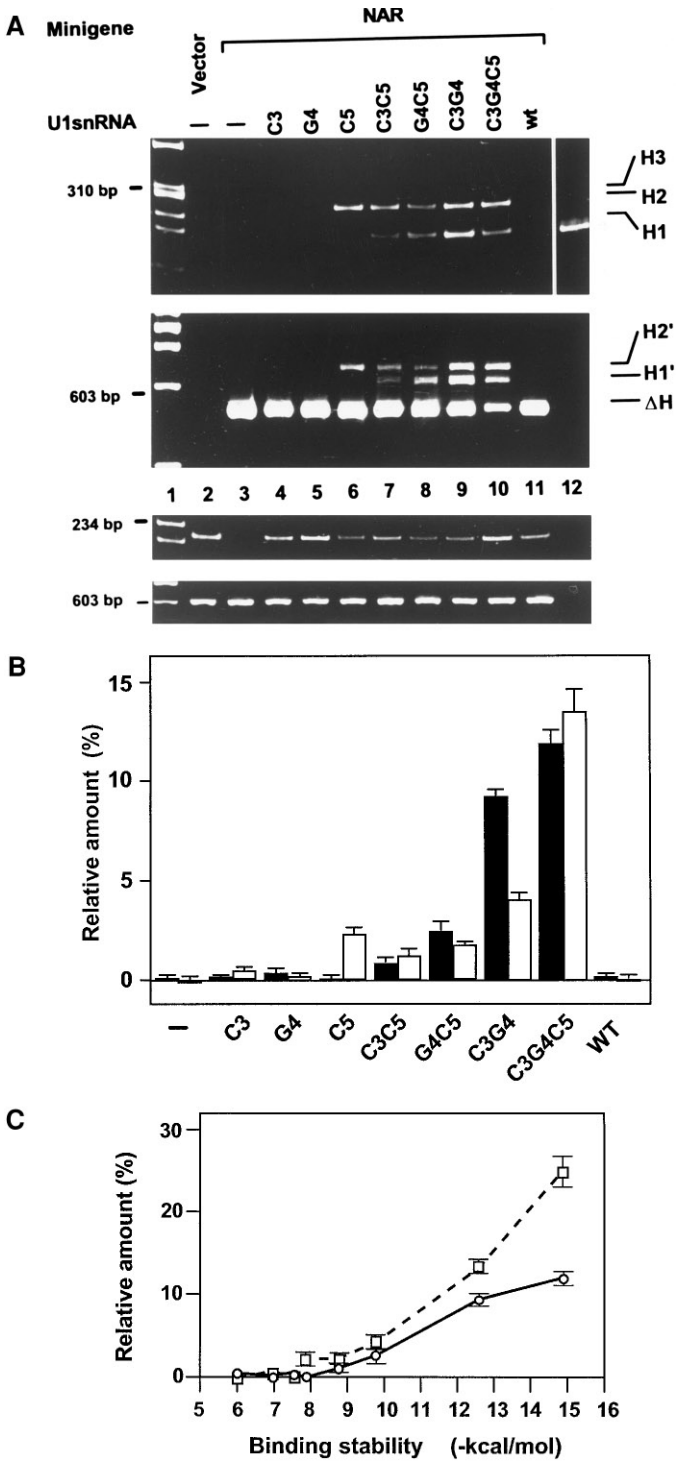

FIG. 4. Effect of the mutated U1snRNAs on splicing of NAR minigene mRNA. (A) RT-PCR analysis of the minigene mRNA using the FB or HS and JB primers. PCR products were indicated on the right. Transfection levels of the U1snRNA expression vectors and the integrity of cDNA are shown as in Fig. 2. (B) The relative amount of H1′ and H2′. RT-PCR was performed with the radiolabeled primer. After electrophoresis, radioactivity of each fragment was quantitated by BAS2000. The relative amounts of H1′ or H2′ against total radioactivity of the three fragments, ΔH, H1′ and H2′, was calculated and represented as a percentile. Closed bars are the relative amounts of H1′ and open bars are that of H2′. (C) Suppression of the deletion mutation and predicted binding stability of the mutated U1snRNA. The relative amounts of H1′ (circles) and the total incorporation of the exon H (H1′+ H2′) (squares) are plotted against the predicted thermodynamic stability of the mutated U1snRNAs. Horizontal scale is the absolute value of the thermodynamic stability.

NAR albumin minigene. Since, in the majority of 5′ splice site mutations, the coding sequence of the affected gene remains intact, the suppression of the 5′ splice site mutation could be an attractive method for gene therapy.

ACKNOWLEDGMENTS

This work was supported in part by a Grant from the Ministry of Health and Welfare of Japan for the second-term Comprehensive 10-year Strategy for Cancer Control and a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan.

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