The snoRNA HBII-52 Regulates Alternative Splicing of the Serotonin Receptor 2C

Shivendra Kishore, *et al.*

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The snoRNA HBII-52 Regulates Alternative Splicing of the Serotonin Receptor 2C

Shivendra Kishore and Stefan Stamm*

The Prader-Willi syndrome is a congenital disease that is caused by the loss of paternal gene expression from a maternally imprinted region on chromosome 15. This region contains a small nucleolar RNA (snoRNA), HBII-52, that exhibits sequence complementarity to the alternatively spliced exon Vb of the serotonin receptor 5-HT_{2C}R. We found that HBII-52 regulates alternative splicing of 5-HT_{2C}R by binding to a silencing element in exon Vb. Prader-Willi syndrome patients do not express HBII-52. They have different 5-HT_{2C}R messenger RNA (mRNA) isoforms than healthy individuals. Our results show that a snoRNA regulates the processing of an mRNA expressed from a gene located on a different chromosome, and the results indicate that a defect in pre-mRNA processing contributes to the Prader-Willi syndrome.

Small nucleolar RNAs (snoRNAs) are non–protein-coding RNAs that are 60 to 300 nucleotides (nt) long and that function in guiding 2′-O-methylation and pseudouridylation in ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), and tRNAs (*1*). HBII-52 is a brain-specific C/D box snoRNA. These snoRNAs have the conserved boxes C [(A/G)UGAUGA] and D (CUGA) near their 5′ and 3′ ends, respectively. HBII-52 is located on the IC-SNURF-SNRPN locus on human chromosome 15q11-13. In this region, 47 almost-identical copies of HBII-52 snoRNA genes are localized between unrelated, non–protein-coding exons. The locus is maternally imprinted (*2*). The loss of paternally expressed genes in 15q11-13 results in the Prader-Willi syndrome (PWS), which is characterized by neonatal muscular hypotonia and failure to thrive. In early childhood, patients develop hyperphagia, obesity, and hypogonadism. Further, they develop behavioral problems and are mentally retarded.

HBII-52 lacks any complementarity to an rRNA, snRNA, or tRNA. However, its antisense element exhibits complementarity to the alternatively spliced exon Vb of the serotonin receptor 5-HT_{2C}R, which is a seven-transmembrane receptor located on the X chromosome (Fig. 1, A and B). Exon V of the serotonin receptor contains at least two alternative 5′ splice sites, giving rise to exon Va– and Vb–containing isoforms. Exon Vb encodes the second intracellular loop of the receptor, which is crucial for G protein binding. Skipping of exon Vb causes a frame shift, resulting in a receptor that is truncated after the third transmembrane domain (Fig. 1A) (*3*). The alternative exon Vb is part of a 5-HT_{2C}R pre-mRNA region that is subject to RNA editing (*4*). At least five sites (A to E) in exon Vb can be edited from A to I. Exon Vb editing promotes its inclusion but changes the amino acid sequence of the intracellular loop (*5*). This change decreases serotonin efficacy of receptors generated through RNA editing 10- to 100-fold (*3*). Sequence comparison showed that the antisense element of HBII-52 and its complementary target sequence within the serotonin receptors are almost fully conserved between mammalian species (fig. S1, A to C). In contrast, there are nucleotide exchanges at 19 positions in other regions of the II-52 snoRNAs (fig. S1B), suggesting a functional relevance of the antisense element sequence.

In agreement with earlier studies (*3*), we found that the rat ortholog of HBII-52, RBII-52, and 5-HT_{2C}R exon Vb are expressed in all areas of the brain, except the choroid plexus (fig. S2A). Thus, there is a positive correlation between RBII-52 expression and exon Vb usage. To test this correlation functionally, we performed cotransfection experiments with an exon Vb splicing reporter and an MBII-52 expression clone, derived from the mouse ortholog (*6*). We generated a minigene of the 5-HT_{2C}R (pRSV-5HT) comprising exons IV, Va, Vb, and VI. The snoRNA was expressed from the construct pCMV-MBII-52 (cmv, cytomegalovirus) that contained MBII-52 and two flanking exons (Fig. 2A) (*2*). We transfected an increasing amount of pCMV-MBII-52 in Neuro2A cells and detected the accumulation of an increasing amount of MBII-52 snoRNA of the expected size by Northern blot analysis (Fig. 2C). We then cotransfected the pRSV-5HT reporter minigene together with an increasing concentration of pCMV-MBII-52. By using primers 5aF and 6R located in the exons Va and VI for polymerase chain reaction (PCR) analysis, we amplified only the mRNA isoform lacking exon Vb. We therefore used the isoform-specific primer 5bF, located in exon Vb, which allowed the detection of the alternative exon in this assay. The usage of exon Vb was significantly increased when the concentration of MBII-52 was increased ($P < 0.0002$) (Fig. 2B). Often, neuron-specific exons are not properly used when reporter constructs are expressed in immortalized cell lines (*7*, *8*). We therefore activated the distal splice site by mutating it into the mammalian consensus (pRSV-5HTcons) (fig. S2B). This allowed us to use the primers 5aF/6R located in the flanking exons Va and VI to detect the increase of exon Vb usage (Fig. 2B). Because these primers amplify both isoforms, the products can be directly compared. An increase of MBII-52 snoRNA resulted in a significant increase of exon Vb inclusion from 5 to 20% ($P < 0.0004$). These experiments are consistent with MBII-52 promoting exon Vb inclusion of the 5-HT_{2C} receptor pre-mRNA.

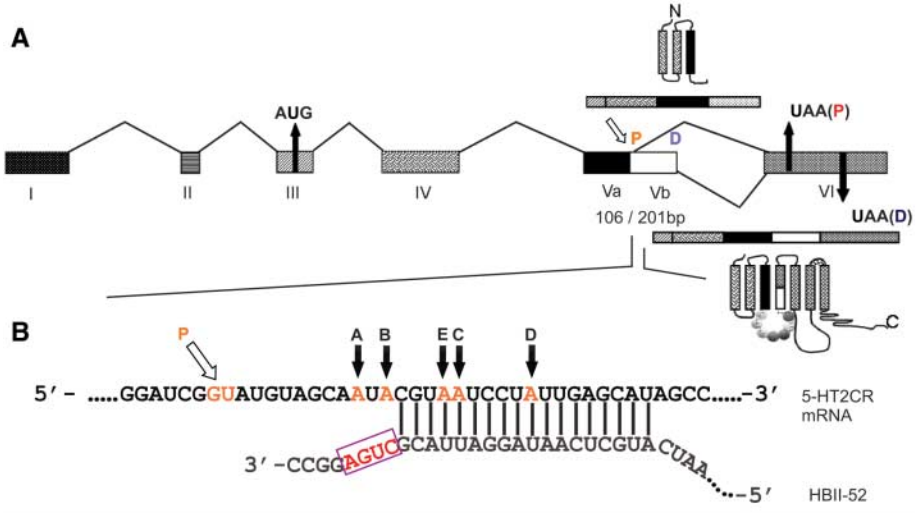

Fig. 1. Complementarity between HBII-52 snoRNA and 5-HT_{2C}R receptor. (**A**) Structure of the human 5-HT_{2C}R receptor. P and D indicate the location of proximal and distal splice sites, respectively. UAA(P) and UAA(D) are the stop codons resulting from their usage. Exons are indicated as boxes with roman numbers. The mRNA isoforms and the resulting proteins are schematically shown (the white box indicates alternative exon Vb). The amino acids encoded by the alternative exon Vb are indicated in the second intracellular loop. (**B**) Base complementarity between the antisense element of the human HBII-52 snoRNA and the human 5-HT_{2C}R receptor. Solid arrows indicate the A → I editing sites (A to E). Open arrow, proximal splice site; box, D box.

Institut für Biochemie, Emil-Fischer-Zentrum, Friedrich-Alexander Universität Erlangen-Nürnberg, Fahrstraße 17, 91054 Erlangen, Germany.

*To whom correspondence should be addressed. E-mail: stefan@stamms-lab.net

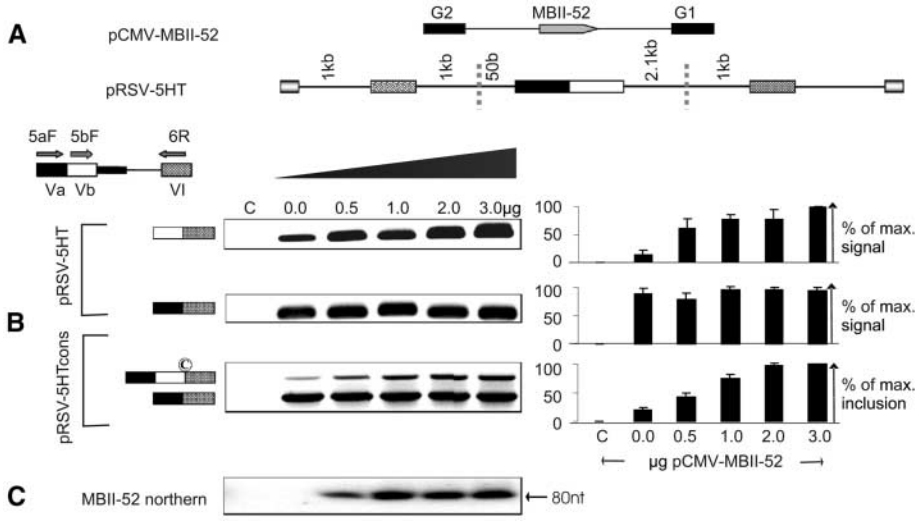

Fig. 2. HBII-52 snoRNA promotes inclusion of exon Vb of the 5-HT_{2C}R. (**A**) Schematic structure of the pCMV-MBII-52 and pRSV-5HT minigenes (fig. S2B). (**B**) Cotransfection of increasing amounts of pCMV-MBII-52 with reporter minigenes in Neuro2A cells. The numbers indicate micrograms of transfected plasmid. The lane labeled C indicates PCR control without reverse transcription. For the wild-type minigene, pRSV-5HT exon-specific primer pairs 5aF/6R and 5bF/6R were used; for the activated minigene, pRSV-5HTcons primers 5aF and 6R that amplify both forms were used. The right panel shows quantification of at least four experiments. At least four independent experiments were quantified by setting the maximum signal (pRSV-5HT) or maximum exon inclusion (pRSV-5HTcons) to 100%. The circled C indicates the consensus 5′ splice site (CAG/GTAAGT). Error bars indicate SD. (**C**) Northern blot showing the increase in MBII-52 expression.

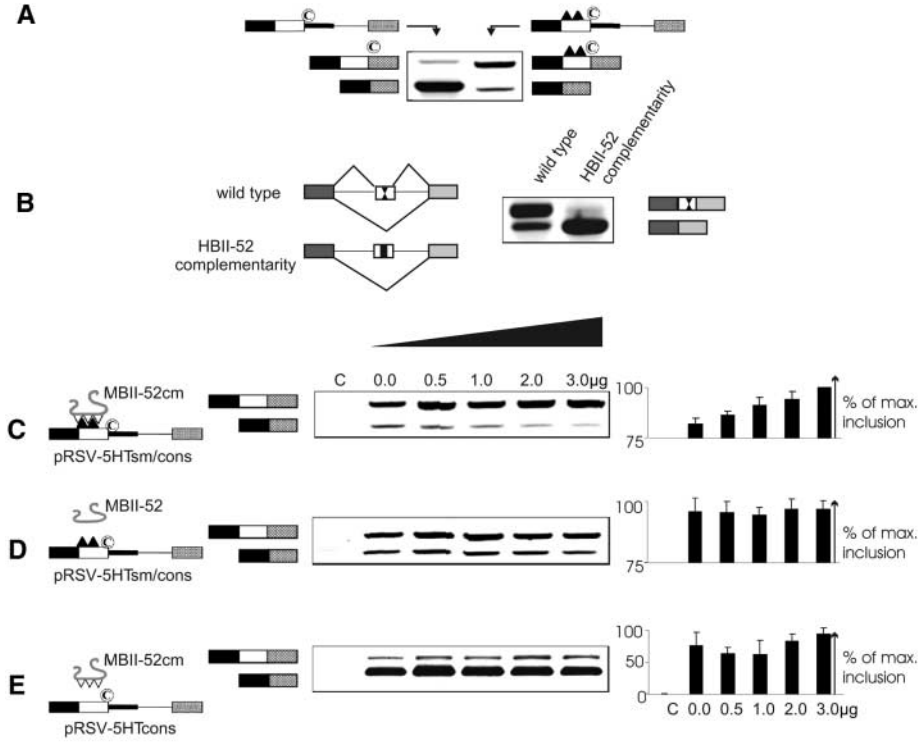

Fig. 3. The snoRNA complementarity region of 5-HT_{2C}R harbors silencing activity that is influenced by snoRNA binding. (**A**) Constructs with the activated distal splice site harboring the wild-type or mutated snoRNA-CR were transfected in Neuro2A cells. The mutation is schematically indicated by triangles on top of exon Vb. The structures of the PCR products are schematically indicated, depending on which minigene was used. The circled C indicates the consensus 5′ splice site. (**B**) The 18-nt snoRNA-CR was introduced into the alternative exon of SXN, a beta-globin based reporter construct, transfected into Neuro2A cells, and analyzed by RT-PCR. (**C** to **E**) Influence of the complementarity between snoRNA and exon Vb on exon Vb usage. The constructs schematically shown on the left were cotransfected in Neuro2A cells. Representative ethidium bromide stained gels are shown (center), and at least four independent experiments are quantified (right). The complementary mutations are indicated by triangles. Error bars indicate SD.

The snoRNA complementarity region (snoRNA-CR) in exon Vb harbors several recently identified putative silencing elements (fig. S3) (*9*). We therefore changed it from CGTAATCCTATTGAGCAT to CGAAAGCCTTTAGACCAT (pRSV-5HTsm/cons, fig. S2B), where the underlined nucleotides indicate those that were mutated. When combined with the consensus 5′ splice site, this mutation now strongly activates exon Vb inclusion (Fig. 3A). The silencing activity was confirmed in a heterologous SXN globin-based reporter system (*10*), where the presence of the snoRNA-CR almost completely silences exon inclusion (Fig. 3B).

We next investigated whether the complementarity between the snoRNA and exon Vb is necessary to influence alternative splicing. We mutated the antisense element of MBII-52 from ATGCTCAATAGGATTACG to ATGGTCTAAAGGCTTTCG (MBII-52cm). This compensatory mutation exhibits sequence complementarity to the mutated snoRNA-CR in pRSV-5HTsm/cons. MBII-52cm further promotes inclusion of exon Vb when cotransfected with the pRSV-5HTsm/cons reporter ($P = 0.004$) (Fig. 3C). In contrast, expressing wild-type MBII-52 snoRNA had no effect (Fig. 3D) on this reporter. Finally, the pRSV-5HTcons reporter containing the original snoRNA-CR was not significantly influenced by the mutated MBII-52cm snoRNA (Fig. 3E), indicating that binding between the snoRNA and exon Vb is necessary for the regulation of alternative splicing.

To test whether HBII-52 exhibits an antisense effect, we mutated the C and D boxes of HBII-52, which prevents formation of a small nucleolar ribonucleoprotein (snoRNP) (*11*), and we used a polymerase II–derived HBII-52 RNA that is not processed into a mature snoRNA. None of these constructs had a significant effect, suggesting that HBII-52 works as an RNP (fig. S4, A to C). We obtained similar results using the minigene constructs with the authentic distal splice site of exon Vb and exon-specific primers (fig. S5), and we did not observe an effect of MBII-52 on the alternative splicing pattern of the unrelated TRA2-beta1 or YT521-B pre-mRNAs (fig. S6).

To test whether HBII-52 binds to exon Vb of the 5-HT_{2C} receptor in vivo, we used an assay that captures the snoRNA:mRNA complex. Binding of HBII-52 to exon Vb was determined by a PCR assay that uses a chimeric primer binding to the 3′ end of the HBII-52 snoRNA and the adjacent part of exon Vb (Fig. 4A and fig. S7). This primer amplifies only RNA from a complex of HBII-52 and 5-HT_{2C}R exon Vb RNAs, but it does not amplify the individual RNAs (Fig. 4A, lanes 1 and 2). We transfected HEK293 with combinations of

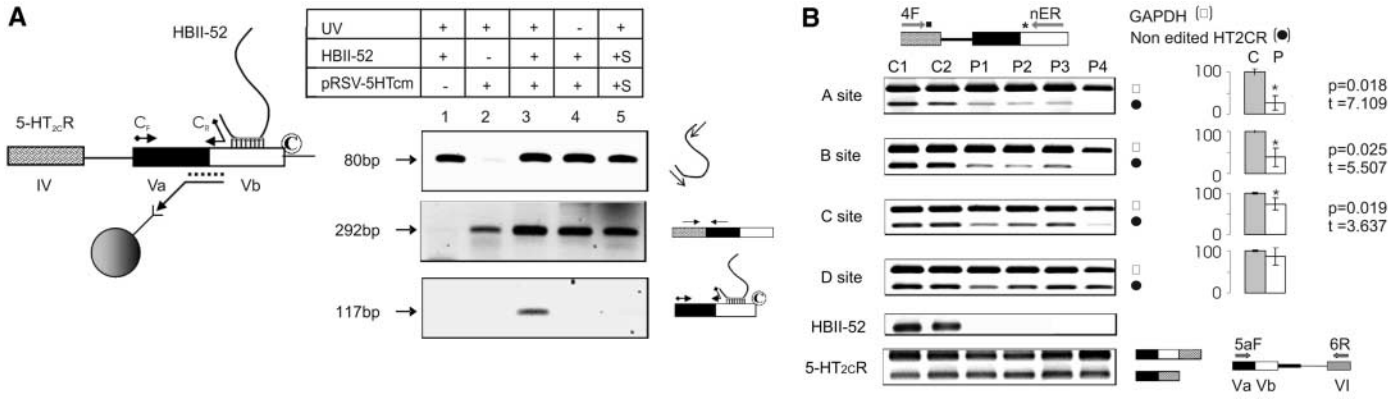

Fig. 4. HBII-52 snoRNA promotes inclusion of nonedited exon Vb by binding to exon Vb RNA. (**A**) HBII-52 binds to 5-HT$_{2C}$R RNA in vivo. The experimental strategy is shown on the left. 5-HT$_{2C}$R nuclear RNA complexes were isolated by using a biotinylated oligonucleotide binding to exon Va/b sequences (indicated by dots). The primer pair C_R (GGGCCUCAGUAUUGCUAC) and C_F (TATGTCTGGCCACTACCTAGATATTT) specifically amplifies complexes between HBII-52 snoRNA and 5-HT$_{2C}$R RNA, because the primer C_R binds only to the complex (fig. S7). The experimental conditions are diagrammed on the right. In lane 5, HBII-52 and 5-HT$_{2C}$R expression clones were transfected into separate cells (+S), and the resulting lysates were mixed after UV irradiation. The identity of the PCR products is shown on the right (80 nt, HBII-52; 292 nt, 5-HT$_{2C}$R mRNA; 117 nt, complex-specific amplicon). (**B**) Changes of 5-HT$_{2C}$R mRNA isoform expression in PWS patients detected by isoform-specific RT-PCR. RNA from similar hippocampal areas from PWS patients (P) and age-matched controls (C) were amplified by RT-PCR. Primers complementary to the nonedited sites, indicated by a star in the scheme on top, were used. GAPDH was amplified in the same reactions. Representative RT-PCR results are shown for each editing site, and their statistical evaluation is shown on the right. The signal was normalized to GAPDH and set to 100% in the control samples (right). The amplification of HBII-52 in controls, but not PWS patients, confirmed the clinical diagnosis (HBII-52). Primers 5aF and 6R amplify similar ratios of isoforms in all samples. Error bars indicate SD.

pCMV-MBII-52 and pRSV-5HTcons constructs and covalently attached base-paired RNA complexes by psoralen crosslinking.

Only when both 5-HT$_{2C}$ pre-mRNA and MBII-52 snoRNA are present in the same cell and subjected to psoralen ultraviolet (UV) crosslinking can a complex between snoRNA and mRNA be detected (Fig. 4A, lane 1 to 3). A complex is not observed without crosslink (lane 4) or when separate cells are transfected, irradiated with UV, and the lysates mixed (lane 5), suggesting that there is an interaction between MBII-52 and 5-HT$_{2C}$ exon Vb in vivo.

This result suggests that in addition to the known editing-dependent pathway of exon Vb inclusion (*5*), HBII-52 snoRNA promotes exon Vb inclusion. HBII-52 snoRNA is absent in patients suffering from PWS, predicting a reduction of the nonedited 5-HT$_{2C}$R mRNA isoform. We analyzed mRNA from PWS patients and performed reverse transcriptase (RT)–PCR using editing site-specific primers and RNA from the hippocampus, where editing is most prevalent. The last 3′ nucleotide of the primers was complementary to the nonedited nucleotide, amplifying only the nonedited 5-HT$_{2C}$R mRNA isoform (fig. S8). We coamplified glyceraldehyde phosphate dehydrogenase (GAPDH) in a multiplex primer reaction as a loading control. We found that the amounts of mRNA-containing nonedited exon Vb sequences at the A, B, C, and D sites were reduced to 25, 39, 74, and 89%, respectively, when compared with healthy controls. RT-PCR showed that HBII-52 was absent in PWS patients but present in control individuals. Amplification of all isoforms with primers in exons Va and VI demonstrates that their overall ratio and amount are not significantly changed (Fig. 4B). These findings show a dependency of 5-HT$_{2C}$R isoform composition on the presence of HBII-52 in a physiological system. They suggest a defect of the 5-HT$_{2C}$R receptor system in patients with PWS.

Our experiments indicate that HBII-52 influences alternative splicing of the 5-HT$_{2C}$R pre-mRNA. HBII-52 snoRNA partially blocks a silencer located in the snoRNA-CR in exon Vb. This silencer can also be weakened by editing exon Vb at positions C, D, and E, explaining why editing promotes exon Vb inclusion (*5*). Psoralen crosslink experiments indicate a transient complex between HBII-52 snoRNA and exon Vb. Similar to other C/D-box snoRNAs (*12*), HBII-52 is localized in the nucleolus (*13*), whereas pre-mRNA splicing occurs in the nucleoplasm where snoRNAs are generated during pre-mRNA splicing. It is therefore possible that they can interact transiently with pre-mRNAs, similar to serine/arginine-rich (SR) proteins that are localized predominantly in nuclear speckles but function outside (*14*). Similar to recent reports, we found no evidence that HBII-52 affects editing of nucleoplasmic 5-HT$_{2C}$R transcripts (fig. S9) (*13*). Our data indicate that HBII-52 promotes the formation of nonedited forms of 5-HT$_{2C}$R mRNA by an editing-independent mechanism, most likely through the masking of a silencer.

Only mRNAs containing exon Vb encode a functional 5-HT$_{2C}$R receptor. Because editing of exon Vb changes the amino acid sequence of the receptor and its coupling to G-proteins, it influences the serotonin response. Isoforms of 5-HT$_{2C}$R mRNA containing nonedited sequences of exon Vb are abnormally low in patients with PWS. Because these 5-HT$_{2C}$R isoforms show the strongest effect to serotonin, this finding explains why PWS patients respond to selective serotonin reuptake inhibitor treatment (*15*, *16*) and points to defects in the serotonergic system as a contributing cause of PWS.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1118265/DC1
Materials and Methods
Figs. S1 to S9
References

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