The snoRNA HBII-52 absent in Prader-Willi Syndrome patients regulates the alternative splicing of the serotonin receptor 5-HT_{2C}R

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To see a World in a Grain of Sand

And a Heaven in a Wild Flower

Hold Infinity in the palm of your hand

And Eternity in an hour.

William Blake
'Auguries of Innocence',1794

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This work, like most others reflects in its utmost simplicity the positives coming out of a long arduous effort. What it fails to reveal, are the feelings attached to the periods of continued mental conflicts, indecisiveness and despair. Also, it conceals within, the spurts of hopes and excitements at every small accomplishement, the comfort of being together in times of desperation and the bliss of unraveling new horizons. There are many people to whom I owe the fulfillment of this dissertation.

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PUBLICATIONS

Parts of this work are included in the following publications:

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Tang Y., Novoyatleva T., Benderska N., **Kishore S.**, Thanaraj A. and Stamm S. (2005) Analysis of Alternative Splicing *In Vivo* using Minigenes. *Handbook of RNA Biochemistry,* WILEY-VCH Verlag GmbH & Co. KHaA, Weinheim.

CONTENTS

ABBREVIATIONS

5-HT_{2C}R	serotonin receptor 2C
ADAR	adenosine deaminase that act on RNA (1, 2 or 3)
ADATs	adenosine deaminase that act on tRNAs
AMP	adenosine mono phosphate
AS	Angelman Syndrome
ASF	alternative splicing factor
ATP	adenosine 5'-triphosphate
bp	base pairs
BP	break point
BSA	bovine serum albumin
CBs	Cajal bodies
cDNA	complementary DNA
CMV	cytomegalovirus
dH_2O	distilled water
DMEM	dulbeco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotidtriphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ESE	exonic splicing enhancer
EST	expressed sequence tag
FCS	fetal calf serum
fRNA	functional RNA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-Proteins	guanosine triphosphate binding protein
GPCR	G-Protein coupled receptor
HBII-52	human brain specific snoRNA II-52
HEK	human embryonic kidney
HIV	Human immunodeficiency virus
hnRNP	heterogenous nuclear ribonucleoprotein
Ig	immunoglobin
kDa	kilodalton
MBII-52	mouse brain specific snoRNA II-52
mRNA	messenger RNA
miRNA	microRNA
ncRNA	non-coding RNA
NMD	nonsense mediated decay
ORF	open reading frame
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PFC	prefrontal cortex
PMSF	phenylmethanesulfonyl fluoride
PTC	premature termination codon
PWS	Prader-Willi Syndrome
RBII-52	rat brain specific snoRNA II-52
RBM	RNA binding motif
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RRM	ribonucleic acid recognition motif
rRNA	ribosomal RNA
RSV	respiratory synctial virus
RT-PCR	reverse transcription followed by polymerase chain reaction
RUST	regulated unproductive splicing and translation
SDS	sodium dodecyl sulfate
SF	splicing factor (1 or 2)
siRNA	small interfering RNA
SmB	small nuclear ribonucleoprotein associated protein B
SMN	survival motor neuron gene (1 or 2)
SmN	small nuclear ribonucleoprotein polypeptide N
snmRNA	small non-messenger RNA
snoRNA	small nucleolar ribonucleic acid
snoRNP	small nucleolar ribonucleoprotein
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein paricle
SNRPN	small nuclear ribonucleoprotein polypeptide N
SNURF	SNRPN upstream reading frame
SR-protein	serine-arginine- rich protein
SSRI	selective serotonin reuptake inhibitor
stRNA	small temporal RNA
TBE	tris-borate-EDTA buffer
TE	tris-EDTA
TEMED	N,N,N’,N’-tetramethylethylenediamine
tRNA	transfer RNA
UPD	uniparental disomy
UTR	untranslated region

ZUSAMMENFASSUNG

Das Prader-Willi Syndrom ist eine komplexe Erbkrankheit, die durch den Verlust von Genexpression einer Region auf dem langen Arm von Chromosom 15 hervorgerufen wird. Diese Region ist elterlich geprägt, d.h. nur das Allel des Vaters wird exprimiert. Bisher ist nicht klar, wieso der Verlust dieser Genregion auf dem menschlichen Chromosom 15q11-q13 das Prader-Willi Syndrom (PWS) hervorruft. Nur einige wenige Gene dieser als IC-SNURF-SNRPN bezeichneten Region werden in Proteine übersetzt. Diese Proteine konnten als Ursache für PWS ausgeschlossen werden. Die meisten Gene dieser Region beherbergen Gehirn-spezifische snoRNAs, die nicht in Proteine übersetzt werden. Eine dieser snoRNAs, -HBII-52- kommt in 47 fast identischen Kopien vor. HBII-52 zeigt eine 18 nt lange Basenkomplementarität zum alternativ gespleißten Exon Vb des Serotonin Rezeptors 5-HT_{2C}R. Es ist bekannt, dass Exon Vb an fünf Stellen durch Editierungsenzyme (ADARs) von Adenosin zu Inosin editiert wird. Diese Editierung fördert den Einbau von Exon Vb.

In dieser Arbeit wird gezeigt, dass HBII-52 in vivo an ein Spleiß-silencing Element in Exon Vb bindet und hierdurch den Einbau von ExonVb in Abwesenheit von Editierung fördert. Die HBII-52 snoRNA wird nicht von PWS Patienten exprimiert und deswegen ist bei ihnen die Spleiß-Regulation des Serotonin Rezeptors gestört. Durch alternative prä-mRNA Prozessierung werden mindestens 25 Isoformen aus dem Serotonin Rezeptor Gen gebildet. Diese Isoformen unterscheiden sich in ihrer Antwort auf Serotonin. In der Arbeit wird gezeigt, dass die Isoform, die am stärksten auf Serotonin antwortet, bei PWS Patienten stark vermindert exprimiert ist.

Diese Korrelation zeigt an, dass PWS Patienten einen Defekt in der RNA Prozessierung haben, der durch das Fehlen von snoRNAs aus der IC-SNURF-SNRPN Region hervorgerufen wird. Die Ergebnisse könnten erklären, warum PWS Patienten positiv auf selektive Serotonin Wiederaufnahme Hemmer (SSRI) reagieren. Die Ergebnisse zeigen weiterhin, dass eine snoRNA die Prozessierung einer mRNA, die auf einem anderen Gen lokalisiert ist, regulieren kann. Ein besseres Verständnis andere snoRNAs, die in der IC-SNURF-SNRPN Region liegen, könnte weitere molekulare Defekte, sowie mögliche Therapieansätze aufzeigen.

ABSTRACT

The Prader-Willi syndrome is a complex congenital disease caused by the loss of paternal gene expression from a maternally imprinted region on the long arm of chromosome 15. It is not clear how the lack of paternal expression of imprinted genes located on the human chromosome 15q11-q13 causes Prader-Willi syndrome (PWS). Only a few of the genes in this IC-SNURF-SNRPN region are translated into proteins. They were ruled out as a cause for PWS. Most of the genes in this region encode brain specific snoRNAs that are not translated into proteins. One of these snoRNAs, –HBII-52-, present in 47 nearly identical copies, exhibits sequence complementarity to the alternatively spliced exon Vb of the serotonin receptor 5-HT_{2C}R. Exon Vb inclusion is promoted by the Adenosine to Inosine editing at 5 different sites (A-E) by editing enzymes ADARs.

Here, we show that *in vivo* HBII-52 binds to a splicing silencing element in exon Vb and promotes its inclusion without RNA editing. PWS patients do not express this snoRNA and as a result their splicing regulation of the serotonin receptor is disturbed. Due to alternative pre-mRNA processing, at least 25 different mRNA isoforms can be produced from the serotonin receptor gene. They differ in their response to serotonin treatment. We could show that PWS patients do not express the serotonin 5-HT_{2C} receptor isoform responding strongest to serotonin. This shows that they have a defect in pre-mRNA processing caused by the absence of snoRNAs located in the IC-SNURF-SNRPN region. These results could explain why PWS patients respond positively to selective serotonin reuptake inhibitors.

Our results show that a snoRNA can regulate processing of a mRNA expressed from a gene located on a different chromosome. They indicate that the loss of gene expression of the Prader-Willi Syndrome imprinted region could cause a defect in pre-mRNA processing. Understanding of the mechanism of other snoRNAs lying in this critical region might show how the deregulation of chromosome 15q11-q13 causes molecular defects resulting in PWS and could identify putative drug targets.

1. INTRODUCTION

The basic premise of molecular biology revolves around the "Central Dogma" that describes the flow of genetic information hard-wired in DNA to individual transportable cassettes composed of messenger RNA or mRNA (transcription) and finally from each mRNA to the protein(s) which the RNA encodes (translation). Major insights from recent research have shown that not all transcribed RNA code for proteins. Although a large fraction of human genome is transcribed, only 1.2% of it encodes protein. Around 98% of the transcriptional output in humans and other mammals consists of non-protein-coding RNAs (ncRNAs) from the introns of protein coding genes and the exons and introns of non-protein-coding genes (Mattick and Makunin 2005).

In most mammalian cells, only a part of the DNA is transcribed to produce primary RNA transcript, and only a minor portion of the primary transcipt survives the RNA processing steps. RNA processing cumulatively defines all the modifications the pre-RNA undergoes until its degradation by the cell machinery. Post transcriptional modifications of eukaryotic RNA can be achieved in several stages:

a) 5' capping reaction replaces the triphosphate group at the 5' end of mRNA chain with a modified GTP (7-methylguanosine or m^7G) which protects it from ribonucleases and also helps recognition of mRNA by ribosome during translation.
b) Splicing excises the intervening non-coding sequences (introns) from the pre-mRNA and joins the flanking coding regions (exons). Pre-mRNA can be spliced in different ways (alternative splicing) producing distinct transcripts which can add to the protein diversity. A different form of splicing can as well occur in pre-ribosomal RNA (rRNA) and transfer RNA (tRNA) genes.
c) Editing changes the nucleotide compositions of the mRNA which alters its protein coding information. Editing of tRNAs are also very frequent.
d) Polyadenylation at the 3' end increases the stability of the mRNA transcript.
e) Base or sugar modifications of RNA can fine tune its interaction with other RNAs or with proteins. Most common sugar modifications occur in rRNA, tRNA and several small nuclear RNAs (snRNAs).

f) Mature RNAs are recognized by their sequences and modifications and are either transported to special compartments for storage or exported to cytoplasm for translation.

g) "Non functional" RNAs are immediately degraded whereas the functional RNAs are relatively stable but are eventually degraded by a number of RNAses.

This chapter introduces few post transcriptional modifications (splicing, editing, 2'-O-methylation and pseudouridylation) of primary RNA transcripts and their regulation. Functional relevance of a certain class of non-coding RNAs is addressed and potential role of a novel brain specific C/D box snoRNA MBII-52 as a regulator of pre-mRNA alternative splicing has also been discussed. Post transcriptional modifications are subsequently described specifically in context of the human serotonin receptor 2C and their role in regulation of receptor's function is elaborated. Finally, implications of the post transcriptional modifications of 5-HT_{2C}R pre-mRNA to Prader-Willi Syndrome are addressed in detail, which forms the basis of the work presented later.

1.1. Pre-mRNA Splicing

Almost all protein coding genes in higher eukaryotes are interrupted by non-coding sequences (introns) that must be precisely excised from the pre-mRNA such that the flanking regions (exons) are spliced together to yield mature, functional mRNAs.

Introns are marked by three conserved sequence elements (Table 1) which allow intron recognition and their subsequent removal: 5' splice site, branch point and 3' splice site that is preceded by a polypyrimidine stretch.

Table 1. Sequence elements marking major class (GT-AG) intron

Elements	Consensus Sequence*
5' donor splice site	YRG/GURAGU
3' acceptor splice site preceded by a polypyrimidine stretch	Y_{12}NYAG /
Branch point located 18-200nt upstream of the 3' splice site	YNYURAY

*Symbols used: Y-Pyrimidine, R-Purine, N-any nucleotide, slash (/) denotes the exon-intron border, Invariant nucleotides are underlined.

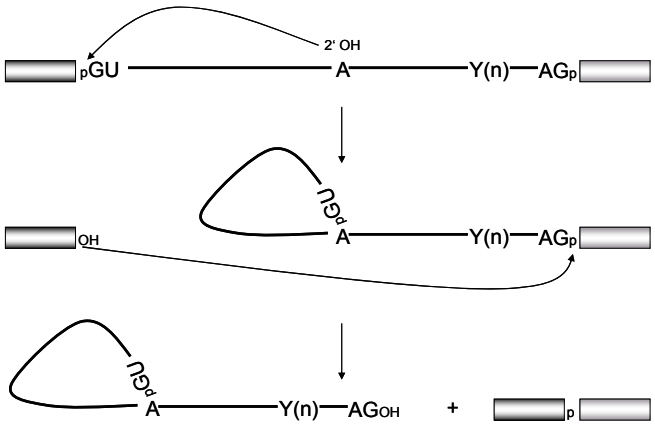


Figure 1. Pre-mRNA splicing reaction.

The major class introns (U2 type) have highly conserved dinucleotides at the 5' and 3' termini (GT and AG respectively). Metazoans can also use another distinct minor intron class (U12 type) that have AT and AC termini. Analysis of splice junction pairs from GenBank annotated mammalian genes showed that 98.71% conformed to canonical GT-AG, 0.56% to non-canonical GC-AG and 0.73% to other non-canonical splice termini (Burset et al., 2001).

Splicing occurs through two sequential trans-esterification reactions involving a dynamic multi component complex called spliceosome, assembled from five subcomplexes called small nuclear ribonucleoprotein particles (snRNPs U1, U2, U4, U5 and U6 in major class U2 type spliceosome; snRNPs U11, U12, U4, U5 and U6 in minor class U12 type spliceosome). Junctions between an intron and its flanking exons, namely the 5' and the 3' splice sites are precisely identified by the spliceosome on the pre-mRNA. Splicing reaction proceeds by an orchestrated formation and disruption of RNA-RNA, RNA-protein and protein-protein interactions, which lead to exon ligation and release of intron lariat. In the first step, nucleophilic attack by the 2' hydroxyl group of a conserved adenosine residue at the Branch point, cleaves the 5' exon-intron junction generating a free 3' hydroxyl group on the upstream exon as well as a branched lariat intermediate. In the second step, the 3' intron-exon junction is attacked by the 3' hydroxyl of the 5' exon, displacing a lariat intron and ligating the exons (Figure 1).

In major class introns, commitment of pre-mRNA to splicing pathway occurs upon formation of the E complex. Assembly of the E complex involves the recognition of 5' splice site, the polypyrimidine tract and 3' splice site by U1 snRNA, U2 auxiliary factor 65 (U2AF65) and U2 auxiliary factor 35 (U2AF35) respectively. The branch point is recognized by the splicing factor1 (SF1). Several non-snRNP splicing factors such as serine/arginine rich

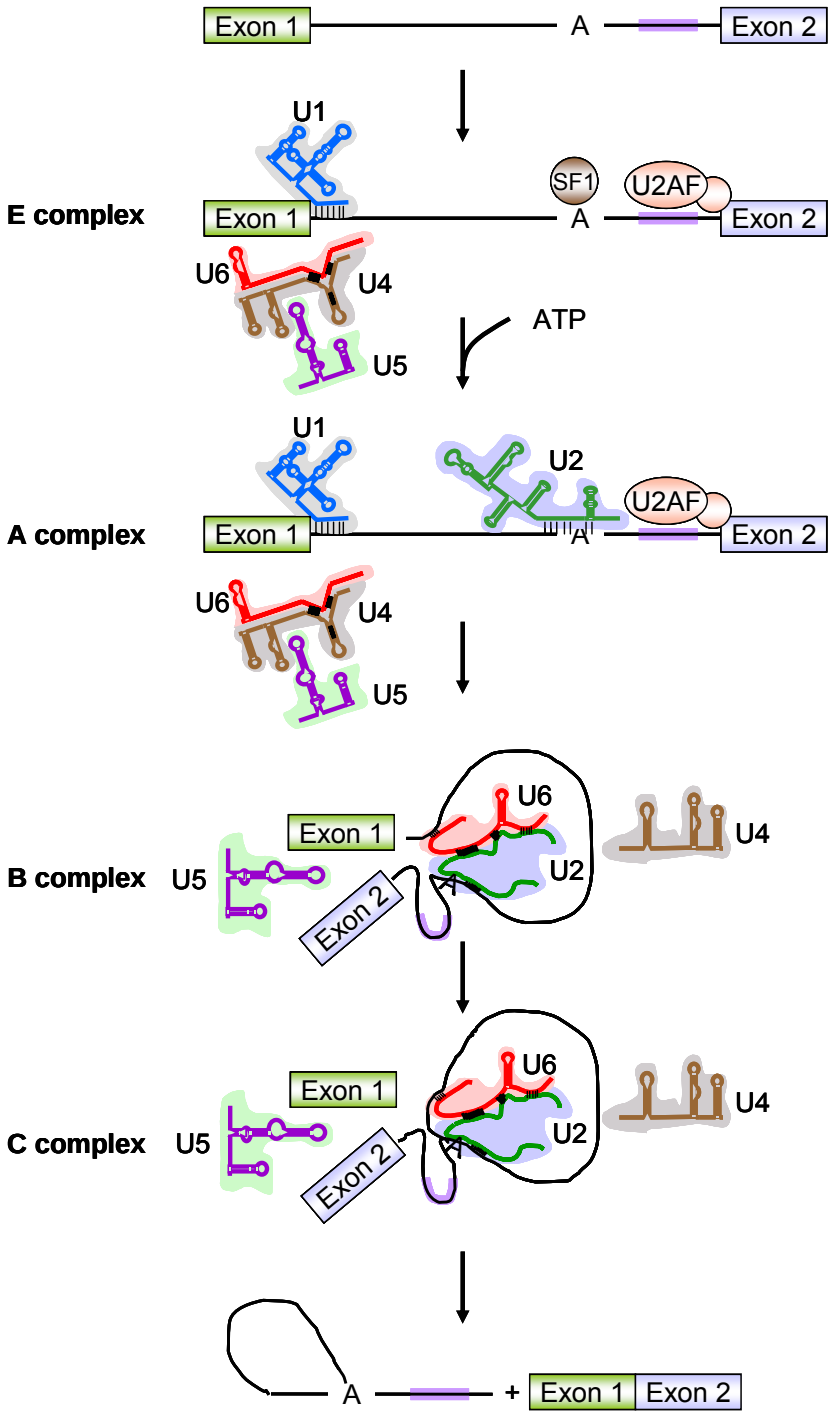


Figure 2. Spliceosome formation and rearrangement during the splicing reactions. (Figure adapted from Patel et al., 2003).

(SR) proteins also associate to the pre-mRNA at this step. In addition, U4/U6∗U5 tri-snRNP can associate with the first exon near the 5' splice site in the E-complex. This association is ATP dependent. Next, ATP dependent base pairing of U2 snRNP with the branch point, leads to the formation of A complex. Formation of next complex B, involves major rearrangements of the snRNP components associated with the pre-mRNA. U6/U4 duplex is disrupted and a new duplex between U6 and the 5' splice site is formed, displacing the U1 snRNP. 5' splice site is brough close to the branch point and the 3' splice site through U6/U2 snRNA base pairing and interaction of U5 snRNP with both exons near splice sites. At this point, U4 snRNP leaves the complex and the first catalytic step of the splicing occurs, creating the intron lariat. Finally, U5 snRNP base pairs with both 5' and 3' exons, thus positioning the ends of the two exons for the second step of splicing. After the second step has been completed, the ligated exons and the lariat intron are released and the spliceosomal components dissociate and are recycled for further rounds of splicing. Figure 2 schematically depicts the spliceosomal assembly, the formation of catalytic spliceosome and the excision of the intron from the pre-mRNA.

1.1.1. Exon recognition and intron bridging:

Introns are marked by short loosely conserved sequences located near their 5' and 3' termini. It is remarkable how the splicing assembly can with such precision locate relatively small exons in a pre-mRNA, excise huge intervening introns and splice exons to generate mature mRNA. The sequences of the splice sites and the branch points are degenerate and are described only by short consensus sequences. These sequences are insufficient for the intron recognition and removal. The specificity can be generated by the presence of additional regulatory elements (silencers or enhancers) within the exon or introns. These elements are again characterized by the loose consensus sequences and this degenerancy prevents from interfering with the coding capacity of the exons. These regulatory elements could bind to several proteins and either promote the splice site usage (enhancers) or suppress it (silencers).

Proteins binding to regulatory sequence elements can be classified into two groups: serine/arginine rich (SR) proteins or heterogeneous nuclear ribonucleoproteins (hnRNPs). RNA recognition by these proteins occurs through one or more RNA recognition motifs (RRMs). The interaction between individual splicing factor and the regulatory sequence is

weak to facilitate easy disloding of the factors, post splicing. As the interaction is weak and not highly specific, different SR and SR like proteins can act through the same regulatory elements and influence the same splice sites. Higher specificity is also achieved by protein–protein interactions that allow simultaneous binding of multiple proteins to RNA. It is well known that SR or SR-like proteins can promote the formation of complexes containing U1 snRNP bound to the 5' splice site and U2 snRNP bound to the pre-mRNA branch site. They can also facilitate the recruitment of U4/U6 and U5 snRNPs. In addition, SR and SR-like proteins can bridge the introns by interacting with themselves and the core spliceosomal components.

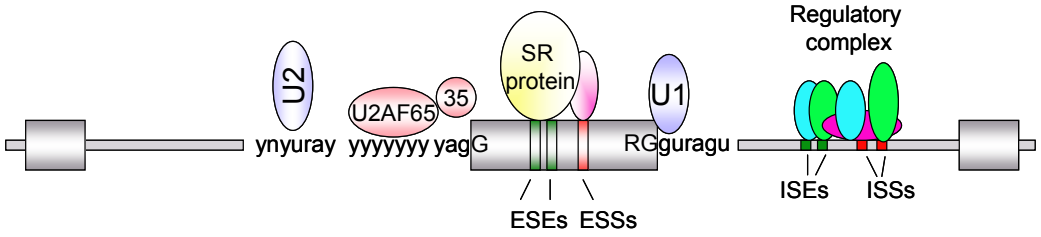


Figure 3. Classical and auxiliary splicing elements and splice factors. 5' Splice site (RGguragu), 3' splice site $(y)_{10}$ncagG and branch point (ynyuray) are indicated (y=c or u, n=a,t,c or g). Auxiliary splicing regulatory elements within exons (Exonic splicing enhancers: ESEs; Exonic splicing silencers: ESSs) and within introns (Intronic splicing enhancers: ISEs; Intronic splicing silencers: ISEs) are depicted as shaded lines colored in exons (thick cylinders) and introns (thin rods) respectively.

In short, serine/arginine rich domains (RS domains) serve as protein-protein interaction modules that recruit other components of the splicing machinery (Bourgeois et al., 2004) or could also be involved in direct RNA contacts to promote spliceosome formation (Shen and Green 2004). The activity of serine/arginine rich domains (RS domains) of the SR proteins is regulated through its phosphorylation and dephosphorylation by several kinases and phosphatases. This phosphorylation is important for the ability of the RS domains to interact with each other and for the activities of the SR proteins in splicing. hnRNPs can influence splice site selection by blocking the access to certain splice sites. hnRNP proteins, in general, antagonize SR and SR-like proteins function to activate splicing. Hence exon recognition and splice site selection is a combinatorial process involving both positive and negative signals provided by SR and SR-like proteins and hnRNP proteins respectively (Figure 3).

1.1.2. Alternative splicing:

An average human gene is 27,000 nucleotides long and consists of 8.8 exons of ~145 nucleotides that are separated by 7.8 introns (Stamm et al., 2005, Lander et al., 2001). More than 90% of the pre-mRNA is removed as introns and only about 10% of the pre-mRNA is retained as the exon in the final spliced transcript. The large number of exons per gene enables the splicing machinery to splice in different sets of exons from a single pre-mRNA, generating different types of mRNA from a single pre-mRNA, a phenomenon referred to as alternative splicing. EST based database analysis indicates that 35-65% of human genes are subjected to alternative splicing which contributes significantly to human proteome complexity and explains the numerical disparity between the low number of human protein coding genes (~26,000) and the number of human proteins (~90,000) (Modrek and Lee 2002). To generate this diverse proteome, several posttranscriptional mechanisms are used. Alternative splicing is one major mechanism to enhance the information contained within the gene and to control its expression. For instance, the Drosophila Down Syndrome Cell Adhesion molecule (DSCAM) gene can potentially generate more than 38,000 different mRNAs by alternatively splicing exons at four clusters (Celotto and Graveley 2001). Alternative splicing is observed in all tissues but tissue specific splicing is most commonly observed in brain cells (Xu et al., 2002, Stamm et al., 2000). Comparative analysis amongst distinct species shows that the mRNA splicing mechanism is well conserved throughout evolution (Ast G. 2004).

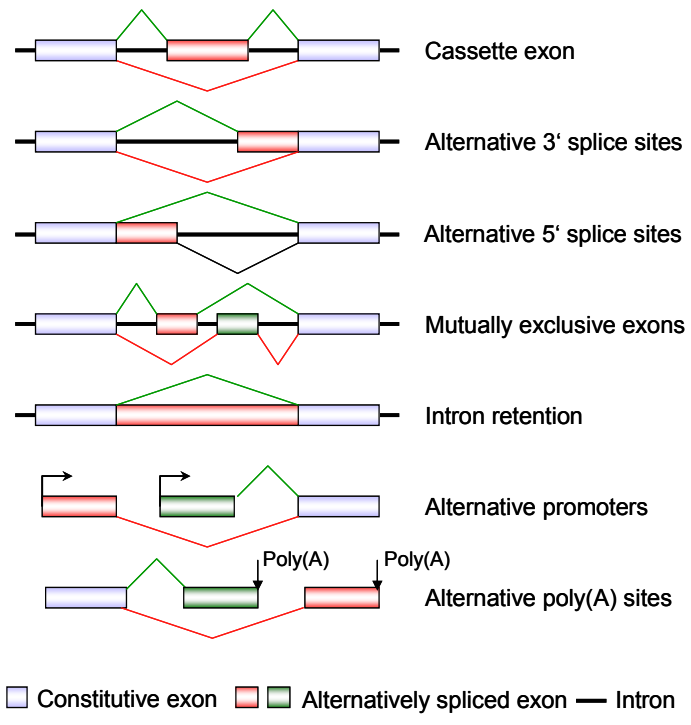


Figure 4. Types of alternative splicing.

There are five major modes of alternative splicing: cassette exons, alternative 5' splice site, alternative 3' splice site, mutually exclusive cassette exons and intron retention. Recently, alternative promoter and alternative poly A sites have been classified separately. The different types of alternative splicing have been depicted in Figure 4. Exon skiping (or cassette exon) accounts for 38% of the alternative splicing events conserved between human and mouse genomes. Alternatively 3'ss and 5'ss account for 18% and 8% of the conserved events, respectively. Intron retention is responsible for less than 3% of the alternative splice events conserved between human and mouse genomes. Other more complex events, like mutually exclusive events, alternative transcription start sites, multiple polyadenylation sites or a multiple combination of the earlier mentioned basic patterns, account for the remaining 33% of the alternatively spliced events (Ast G. 2004). An estimated 75% of all alternative splicing patterns change the coding sequence (Zavolan et al., 2003), indicating that alternative splicing is a major mechanism enhancing protein diversity.

1.1.3. Regulation of alternative splicing:

Tissue and developmental stage-specific alternative splicing is regulated by trans-acting factors. Thus, differences in the activities or amounts of general splicing factors and/or gene-specific splicing regulators during development or in different tissues may cause differential patterns of splicing. Few tissue specific factors like neuron specific RNA binding factor Nova-1 (Jensen et al., 2000) and brain and testis specific SLM1 (Stoss et al.,) have been previously described. Also, several external stimuli like cellular stress, receptor stimulation and changes in neuronal activity, have been identified that can change the alternative splicing patterns.

It has also been shown that expression levels of several ubiquitously expressed general splicing factors like SR-proteins and hnRNP A vary from one tissue to the other. This could have profound effect on the tissue specific splicing. Apart from the expression level of the splicing factors, their post translational modifications also play a significant role in the alternative splicing regulation.

Protein phosphorylation has been shown to modulate the alternative splicing of a number of exons both *in vivo* (Hartmann et al., 2001) and *in vitro* (Prasad et al., 1999). Changes in the phosphorylation state of the splicing fators can influence their ability to

interact with RNA (Chen et al., 2001) and with other proteins (Hartmann et al., 1999). Protein phosphorylation controls the release of the SR-proteins from the storage compartments in the nucleus (Wang et al., 1998) and causes relocalization of hnRNP proteins to the cytoplasm (van der Houven van Oordt et al., 2000). In this way phosphorylation can alter the active concentration of the splice factors. Since the splicing factors can sometimes regulate coherent biological fuctions, it is likely that changes in their activity can influence the expression of different seemingly unrelated genes.

1.1.4. Function of alternative splicing:

Gene regulation through alternative splicing is more versatile than regulation through promoter activity. Variant transcripts generated through alternative splicing, similar to those initiated from distinct promoters, are often tissue and/or development specific, resulting in effects seen only in certain cells or developmental stages. However, changes in promoter activity alter predominantly the expression levels of the mRNA. In contrast, changes in alternative splicing can modulate transcript expression levels by subjecting mRNAs to nonsense-mediated decay and alter the structure of the gene product by inserting or deleting novel protein parts. The structural changes fall into three categories: introduction of stop codons, changes of the protein structure and changes in the 5' or 3' untranslated region. The effects caused by alternative splicing range from a complete loss of function to subtle effects that are difficult to detect. Functional relevance of pre-mRNA alternative splicing with respect to encoded protein have been summarized below.

1.1.4.1. Introduction of stop codons

mRNAs containing premature termination codons (PTCs) present more than 50-55 nt upstream of the last exon-exon junction can be degraded by Nonsense Mediated Decay (NMD). About 25-35% of alternative exons introduce frameshifts or stop codons into the pre-mRNA (Stamm et al., 2000; Lewis et al., 2003). Since approximately 75% of these exons are predicted to be subject to nonsense-mediated decay, an estimated 18-25% of transcripts are switched off by stop codons introduced in alternative splicing and nonsense mediated decay (Lewis et al., 2003). This process, which has been termed RUST for Regulated

Unproductive Splicing and Translation, currently represents the function of alternative splicing with the most obvious biological consequences (Figure 5).

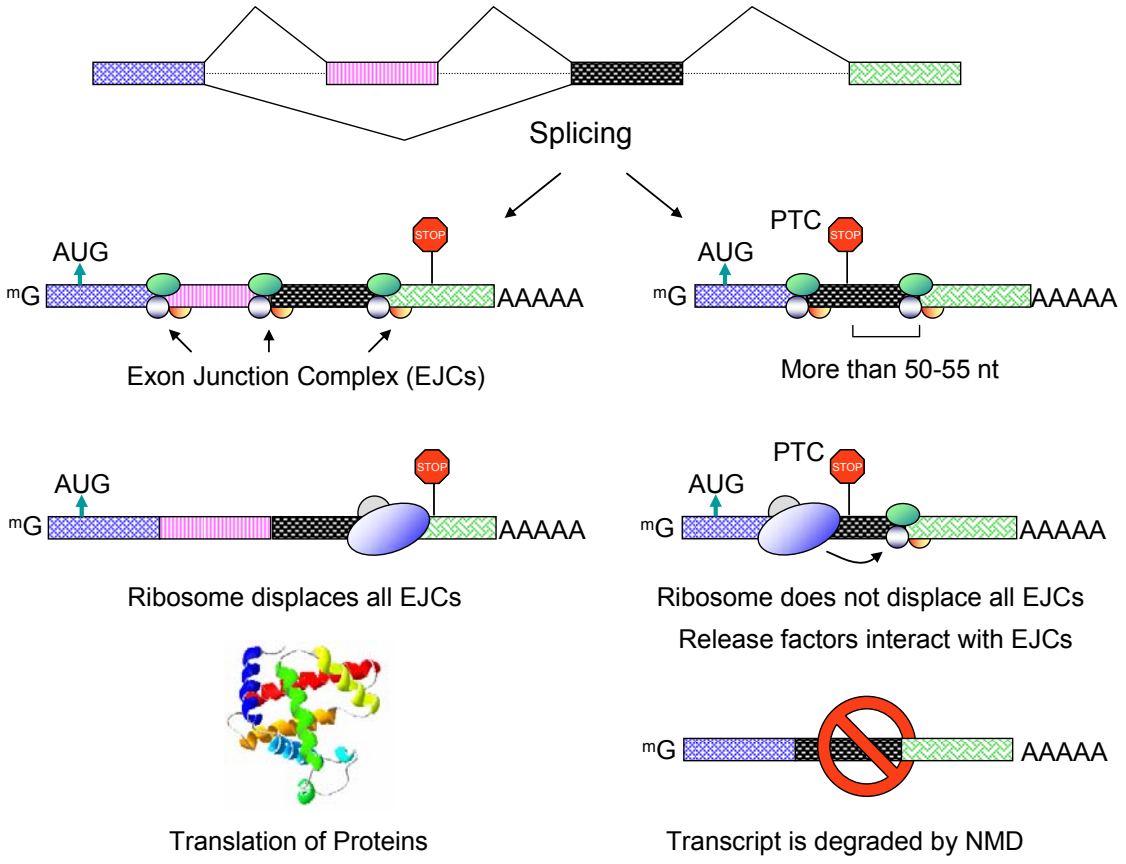


Figure 5. A current model for NMD in mammalian cells. A PTC ≥50 nucleotides (nt) upstream of the last exon-exon junction activates NMD. Translation causes the displacement of the EJCs deposited upstream of each splice junction. However, termination of translation upstream of one or more EJCs triggers NMD by recruitment and activation of factors released by Ribosomes. Termination in the last exon results in displacement of all EJCs and a stable mRNA. (Figure adapted from http://compbio.berkeley.edu/people/ed/rust/)

1.1.4.2. Addition of new protein parts:

Approximately 75% of alternative splicing events occur in the protein coding regions of the mRNAs (Okazaki et al., 2002; Zavolan et al., 2003). Changes in the protein primary structure can alter the binding properties of proteins, influence their intracellular localization and modify their enzymatic activity and/or protein stability by diverse mechanisms.

1.1.4.3. Binding properties

Alternative splicing can altogether delete binding domains or introduce structural changes by inserting protein sequences, that can abolish binding activity of a protein. For instance, alternative splice variants of Thyroid Stimulating Hormone (TSH) receptor are unable to bind TSH (Ando et al., 2001). In some cases, alternative splicing can also create novel binding domains or control the number of multiple binding motifs to modulate protein binding affinities. For example, in tenascin C, alternative splicing can alter the number of fibronectin type III domains which regulates binding to fibronectin (Puente Navazo et al., 2001). Similarly, alternative splicing could as well regulate transcription by abolishing the DNA binding domain of transcription factors.

1.1.4.4. Intracellular localization

Alternative splicing can influence the intracellular localization of numerous proteins, usually by influencing localization signals or regulating the interaction of proteins with membranes. Deletion or interruption of transmembrane domains of membranous proteins can cause them to accumulate in the cytoplasm or to be secreted in the extracellular space. Non-membrane bound soluble isoforms can lose their ability to transduce signals (Tone et al., 2001) and become less stable (Garrison et al., 2001). Alternative splicing can regulate the localization of proteins in various subcellular sites and organelles. Proteins can be sequestered into compartments, where they perform no function. This mechanism is widely used for receptor molecules and alternative splicing can regulate their retention in membrane-enclosed compartments. For example, the inclusion of an endoplasmatic reticulum retention signal in the metabotropic Glutamate Receptor 1B reduces the cell surface expression of this receptor and restricts its trafficking (Chan et al., 2001). Also sub-localization of proteins within organelle can be regulated by alternative splicing. In the nucleus, proteins can be present in different nuclear substructures, such as in the nucleoplasm and speckles, due to alternative splicing (Nishizawa et al., 2001).

1.1.4.5. Protein and mRNA stability

Inclusion of alternate protein domains can regulate the half-life of proteins. Protein stability can be altered due to autophosphorylation that signals the degradation of receptor

molecules. For example, the interleukin-1 receptor-associated kinase, this autophosphorylation-dependent degradation is isoform-specific, leading to a molecule that is not down-regulated by its ligand (Jensen and Whitehead, 2001). The effect of alternative splicing-dependent protein stability has been studied for the *fosB* gene, which generates a shorter isoform that is more stable than the full-length protein. This isoform accumulates in brain in a region-specific manner in response to many types of chronic behavioral changes (Nestler et al., 1999).

Some examples have been described where alternative splicing changes the properties of the mRNA. Alternative splicing events occuring in 5' and 3' UTRs may change the stability of the RNA. For example, alternative exons in the 5' UTR of the HIV-1 virus can either promote or inhibit the nuclear degradation of their mRNA, which regulates HIV-1 gene expression (Krummheuer et al., 2001).

1.1.4.6. Posttranslational modifications

Posttranslational modifications can be dictated by alternative splicing, usually by generating consensus sites for phosphorylation, glycosylation, palmitoylation or sulfatation. For instance, isoform-dependent phosphorylation of the potassium channel Kv4.3 allows the modulation of outward currents by the alpha-adrenergic system via protein kinase C (Po et al., 2001). In addition, binding between a kinase and its substrate can be regulated by alternative splicing, e.g. binding of the SR-protein kinase 1 to scaffold attachment factor B (Nikolakaki et al., 2001).

1.1.4.7. Influence on protein function:

Alternative splicing can modulate affinity, substrate specificity and catalytic activity of enzymes. Inclusion of a stop codon prior to the active center or deletions of protein parts that are necessary for catalysis are frequent mechanisms to regulate enzymatic activity. Also, alternative splicing can either completely abolish or modulate the function of a protein by inserting novel sequences into intracellular domain, as in the case of CD46 (Purcell et al., 1991) or the prostaglandin receptor EP3 that differ in their coupling efficiencies to adenylate cyclase (Harazono et al., 1994). Similarly, interaction of transcription factors with DNA can be modified by alternative splicing, which contributes to transcriptional regulation.

1.2. RNA editing

Unlike RNA splicing, where the sequence of the RNA is changed by removing a large block of contiguous sequence, RNA editing alters only a few nucleotides to change the RNA sequence from that encoded in the DNA. Hence, RNA editing is more like a fine tuning mechanism. Though, the modifications by RNA editing appear to be minimal, the effect that it has can be profound. For example, RNA editing can create, delete, or alter the meaning of the codon, create a splice site or alter RNA structure. In addition, some RNA editing can repair or correct the information coded by the genome and others can act to diversify the mechanism.

In nuclear encoded mRNAs, RNA editing is of two types. While both of them involve deamination of encoded nucleotides, deamination of Cytidine (C) can create Uridine (U) and deamination of Adenosine (A) can create Inosine (I) (Figure 6). As Inosine has base pairing capabilities as Guanosine, it is recognized as Guanosine by the cell machinery. In primates, the RNA editing from A to I is more frequent (Eisenberg et al., 2005).

RNA editing is catalyzed by members of an enzyme family known as adenosine deaminases that act on RNA (ADARs). ADARs that deaminate adenosines act on RNA that is completely or largely double stranded. For this, they have variable numbers of double-stranded RNA (dsRNA) binding motifs (dsRBMs) followed by a highly conserved C-terminal catalytic domain. Until now, three different kinds of mammalian ADARs have been known, namely, ADAR1, ADAR2 and ADAR3. Though functionally ADAR1 and ADAR2 are identical, they may have slightly different specificities. In vertebrates, the ADAR1 is expressed as a long and short form, though the mechanism that leads to the generation of these forms can differ between organisms. In humans, alternative splicing at the 5' end of the ADAR1 mRNA can generate two distinct promoters which in turn, can generate the long and the short form of the protein. The long form is produced from an interferon-inducible promoter, and in contrast to the nuclear localization typical of most ADARs, this protein is a nucleo-cytoplasmic shuttling protein and can also be found in the cytoplasm. Since the genes induced by the interferon are important in establishing the anti-viral state, the long form of ADAR may function in viral defense (Bass BL 2002).

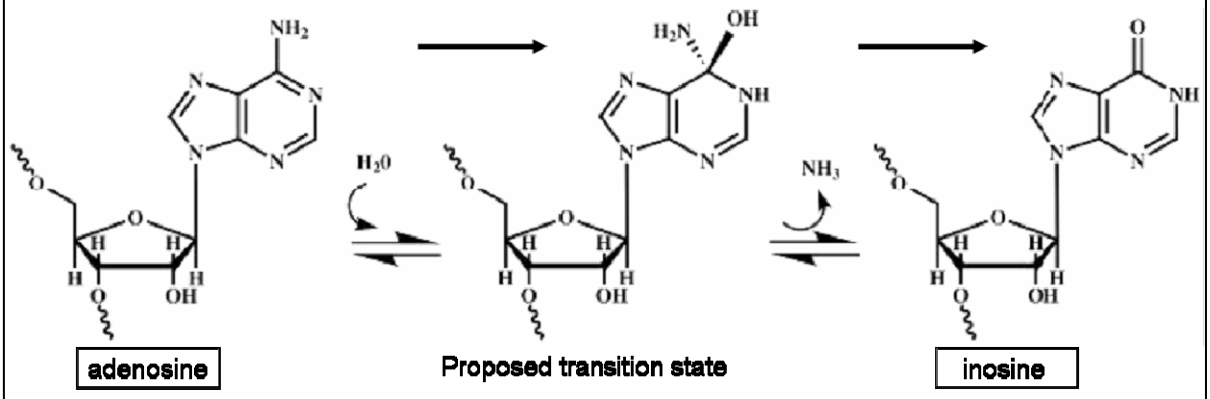


Figure 6. ADARs convert adenosines to inosines by hydrolytic deamination. (Figure modified from Bass BL 2002.)

RNA editing within the coding sequences can alter codon meaning and so more than one protein isoform can be synthesized from a single gene. Amino acid changes generated this way can fine tune the affinity of the encoded protein with its interacting protein or ligand as in the case of G-protein coupled serotonin receptor 2C where the editing can alter the recceptor's ability to bind to serotonin. Surprisingly, most of the RNA editing by ADARs targets the noncoding and the intronic sequence of the mRNA, the role for which is still unclear. The ADAR expression levels, and hence the RNA editing, is highly prevalent in tissues of the nervous system. Though, homozygous knockout mice for ADARs are lethal, heterozygotes may produce subtle defects that are more difficult to discern. Knockouts in *D. melanogaster* and *C. elegans* are viable but show subtle behavioral defects related to locomotion and chemotaxis respectively (Bass BL 2002).

Apart from RNA editing in mRNA, many organisms also express adenosine deaminases that act on tRNAs (ADATs). ADATs are similar to ADARs but without dsRBMs.

Most studied effects of RNA editing in mammals include the Q to R editing within the ion channel in the pore loop of glutamate receptors that can alter the ion permeability of the channel. Combinations of one or more at 5 different editing sites in the serotonin receptor 2C can generate several edited isoforms with a decreased constitutive activity towards the agonist. Similar RNA editings are also very prominent in the neurotransmitter gated Sodium channel and Chloride channels, though, the consequences of editing are not well characterized. Recent studies on the serotonin receptor 2C show that RNA editing can as well

influence the alternative splicing of its pre-mRNA. Most interestingly, mammalian ADAR2 can act on its own pre-mRNA to create a new splice site. The alternative splicing results in a 47 nt insertion that causes a frameshift with the production of a inactive isoform. Hence, ADAR2 can auto-regulate itself by editing its own pre-mRNA (Rueter et al., 1999).

1.3. Regulatory role of non-coding RNAs

Cell from all known organisms contain two different kinds of RNAs. mRNAs that are translated into protein and the non-messenger or non coding RNAs (nmRNAs or ncRNAs) which cannot be translated into proteins. Until recently, the non-coding RNA fraction was considered mainly non-functional with the exception of common infrastructural RNAs involved in protein synthesis, transport and splicing. Introns have long been regarded as evolutionary debris with intronic RNA assumed to be simply degraded after splicing excision. In mammals, the non-protein-coding transcripts were presumed to be 'transcriptional noise'. However, a significant proportion of ncRNAs appears to be stable in eukaryotic cells. Some excised introns have half-lives comparable with mRNA and are even exported from the nucleus to the cytoplasm. Whole chromosome tiling chip arrays have shown that the range of detectable ncRNAs in human cells is much larger than can be accounted for by mRNAs and that there appear to be roughly equal numbers of protein-coding and non-coding transcripts regulated by common transcription factors in the human genome.

Non-coding RNAs (ncRNAs) produce transcripts that function directly as structural, catalytic or regulatory RNAs rather than expressing mRNAs that encode proteins. Table 2 summarizes the abbreviations for various classes of functional non-coding RNAs that are known.

ncRNAs are generally small ranging from 20nt to 300nt and are therefore termed small, non-messenger RNAs (snmRNAs). Based on their size, structure or sequence motifs, interacting proteins or their subcellular localization, snmRNAs can be regrouped into several specific RNA classes, namely, transfer RNAs (or tRNAs), small nuclear RNAs (or snRNAs), small nucleolar RNAs (or snoRNAs), microRNAs (or miRNAs) or small interfering RNAs (or siRNAs). Transfer RNA (tRNA) serves as the adaptor molecule that aligns the correct amino acid on the mRNA template during the elongation of protein chains. snRNAs play

important roles in the biogenesis of cellular RNAs such as in splicing and in 3' end processing. snoRNAs are relatively short guide RNAs that mediate nucleotides modifications in rRNA and facilitate cleavage of rRNA precursors. miRNAs and siRNAs are both ~21-23 nt long, and can regulate gene expression, or in specific, induce translational repression by binding to mRNAs via antisense elements through distinct cellular mechanisms.

Table 2. Various known classes of non-coding RNAs.

Abbreviations	Function
fRNA	Functional RNA; synonymous with non-coding RNA
miRNA	MicroRNA; putative translational regulatory gene family
ncRNA	Non-coding RNA; all RNAs other than mRNA
rRNA	Ribosomal RNA, forms the protein translation machinery
siRNA	Small interfering RNA; active molecules in RNA interference
snRNA	Small nuclear RNA; includes spliceosomal RNA
snmRNA	Small non-messenger RNA; synonymous with small non-coding RNAs
snoRNA	Small nucleolar RNAs; involved in ribosomal RNA modification
stRNA	Small temporal RNA
tRNA	Transfer RNA; invloved in protein translation

1.3.1. Small nucleolar RNAs (snoRNAs)

Pre-rRNA maturation in eukaryotes not only includes a series of endonucleolytic and exonucleolytic cleavages but also covalent modification of a definite subset of rRNA nucleotides that fine tunes their folding and interaction with other ribosomal proteins. In eukaryotes, a substantial proportion of the snmRNAs identified so far belong to the two expanding subclasses of metabolically stable RNAs. On the basis of their conserved sequence elements and associated proteins, they are classified as C/D and H/ACA snoRNAs, and direct site specific 2′-*O*-ribose-methylation and pseudouridylation respectively, of rRNA or spliceosomal snRNAs. These modifications are founds at about 50-100 sites per vertebrate ribosomes and occur in highly conserved and functionally important regions of the rRNA. Additionally, such modifications in spliceosomal snRNAs are confined to sequences critical for splicing, especially those which make contacts with pre-mRNA or with other snRNAs (Massenet et al., 1998). Although, a small number of snoRNAs (eg. U3 snoRNA) can take

part in pre-rRNA cleavages that are essential for viability, the vast majority of these snoRNAs functions in the posttranslational modification of rRNA nucleotides and are dispensable for growth (Cavaille et al., 2000).

The specific sites to be modified by both classes of snoRNAs are identified by formation of a specific duplex at the modification site. Both class of snoRNAs associate with specific common core proteins to form small ribonucleoprotein particles and function through a common protein enzyme, methylase (fibrillarin) or pseudouridine synthase. Since these guide RNAs in eukaryotes accumulate within the nucleolus, they are designated as snoRNAs. Interestingly, some of the C/D box or H/ACA box snoRNAs involved in the modification of the Pol II-transcribed spliceosomal snRNAs, U1, U2, U4 and U5, do not accumulate within the nucleolus but are exclusively found within the Cajal bodies (or CBs), a subnuclear compartment in which they appear to interact with their RNA substrates. This subset of modification guides has been called small CB-specific RNAs or scaRNAs. Novel cellular RNAs targeted by snoRNAs also include tRNAs in Archaea as well some potential mRNAs and few other RNA species. Recently, C/D and H/ACA snoRNAs have also been shown to direct modifications of the Pol III transcribed spliceosomal U6 snRNA which is thought to transit through the nucleolus (Ganot et al., 1999). Computational genomic searches and experimental RNomics findings have now identified more than 200 snoRNAs in vertebrates alone (Bachellerie et al., 2002) most of which have no known RNA targets. Increasing number such “orphan” guide RNAs with no identified cellular RNA target, adds another level of complexity to the biological roles of snoRNA guides, especially when expression of some of these are tissue specific and sometimes also genomic imprinted.

1.3.2. Structure and function of methylation guide C/D box snoRNA

An average C/D box snoRNAs is 60-100 nt long and contains two short sequence motifs, box C (RUGAUGA) and box D (CUGA) located in the vicinity of their 5’ and 3’ termini respectively (Figure 7). The two motifs are brought together by a typical 5’ and 3’ stem loop structure (box C/D motif) involving 4-5 nucleotides at both ends, which is critical for snoRNA processing, stability, methylation activity, nucleolar localiaztion and 5’ cap hypermethylation (Filipowicz and Pogacic 2002). In addition, they also contain a less conserved matching set of internal sequence element of the box C (termed as box C’) and

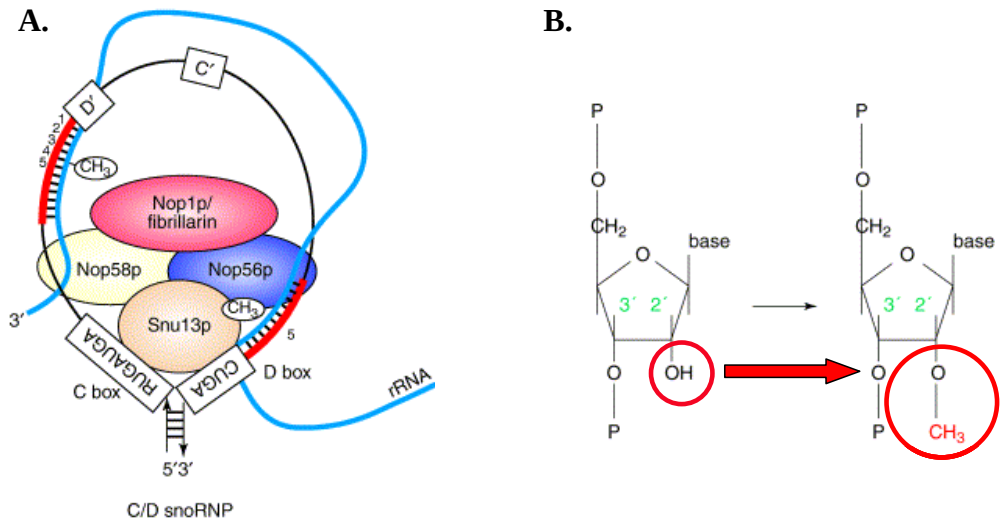


Figure 7. C/D box small nucleolar ribonucleoproteins (snoRNPs). (A) C/D box guide snoRNA and the core associated proteins. **(B)** C/D box snoRNPs guide 2'O-methyl modifications of their target nucleotide. (Figure modified from Brown JWS et al., 2003)

box D (box D') motifs and in their central and 5' region respectively. The C' and D' box are generally found within 3-9 nucleotides of one another or are predicted to be brought together through the formation of an internal stem-loop (Kiss-Laszlo et al., 1998). Immediately upstream from their D or D' box are one or two antisense elements, around 10-21 nt in length, which bears complementarity to the rRNA modification site. In the extended duplex formed with the substrate, box D or D' is located precisely five nucleotides from the residue that base-pairs to the rRNA nucleotide to be modified (Kiss-Laszlo et al., 1996). It has been experimentally demonstrated that the antisense element associated with the box D or D' is the sole determinant of the site of methylation as an artifical C/D box snoRNA carrying an appropriate antisense element is sufficient to target a novel ribose methylation on the predicted pre-rRNA nucleotide and also, to a lesser extent, to RNA-polymerase II transcripts (Cavaille et al., 1996). The ability of C/D box snoRNPs to direct two modifications by making use of two seperate antisense regions, suggests its symmetric nature. Interestingly, the snoRNPs are asymmetric with each box C/D motif interacting with distinct protein subset (Cahill et al., 2002).

1.3.3. Structure and function of pseudouridylation guide H/ACA box snoRNA

H/ACA snoRNAs are generally 120-140nt long and are characterized by two conserved sequence elements H box (ANANNA) and the 3'-terminal ACA sequence (a trinucleotide always found three nucleotides away from the 3' end, Figure 8). They share a common secondary structure consisting of hairpin-hinge-hairpin-tail structure (Ganot et al., 1997), with the conserved sequence elements essential for snoRNA processing, stability, pseudouridylation activity and nucleolar localization (Terns and Terns, 2002). Conserved motifs, box H and ACA are located in the hinge and tail, respectively. Each H/ACA snoRNA contains an appropriate bipartite guide sequence in the internal loop of one or both of the two large hairpin domains. The two stems forming the 9-13 bp bipartite guide duplex precisely flank the substrate uridine which remains accessible for isomerization. The conserved distance between the target uridine and the downstream H or ACA box of the snoRNA is 14-16nt. In contrast to C/D box snoRNPs, electron micrographic studies of H/ACA snoRNPs reveal a symmetric bipartite particle with an estimated mass consistent with two copies of each protein (Watkins et al., 1998a)

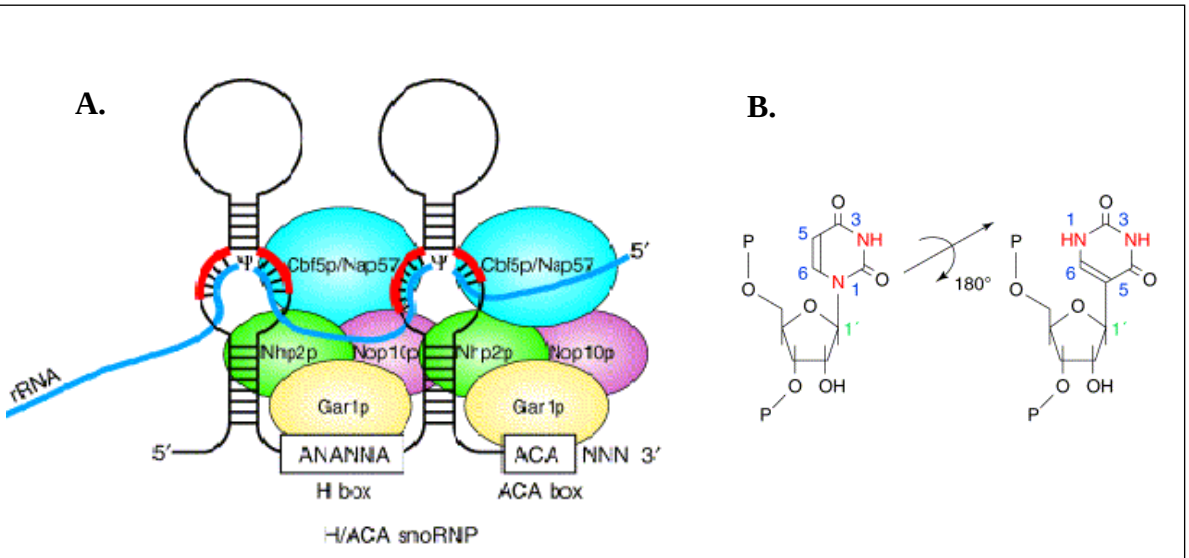


Figure 8. **H/ACA box small nucleolar ribonucleoproteins (snoRNPs). (A)** H/ACA box guide snoRNA and the core associated proteins. **(B)** H/ACA box snoRNPs guide pseudoridylation of their target Uridine (Figure adapted from Brown JWS et al., 2003).

1.3.4. Biogenesis and assembly of snoRNP particles

Both C/D box and H/ACA box snoRNAs share unusual genomic organization and modes of biosynthesis (Figure 9). In vertebrates, they are encoded within the introns and are not independently transcribed but processed from the pre-mRNA introns, in most cases by exonucleolytic digestion of the debranched lariat (Figure 10). In yeasts, only a few of them are intronic and most are synthesited from independent mono-, di- or polycystronic RNA transcripts processed by endo- and exonucleases (Figure 10). In higher plants, clusters of multiple different snoRNA genes transcribed as polycistronic precursors are widespread. Quite a number of genes hosting intronic snoRNA guides for rRNA modifications, code for proteins involved in ribosome biosynthesis or function, which suggests that this particular gene organization might provide a regulatory link between partners in the same biological process. Intriguingly, on the other hand, a number of vertebrate host genes lack any direct relationship with translation, some of them even being apparently devoid of protein-coding

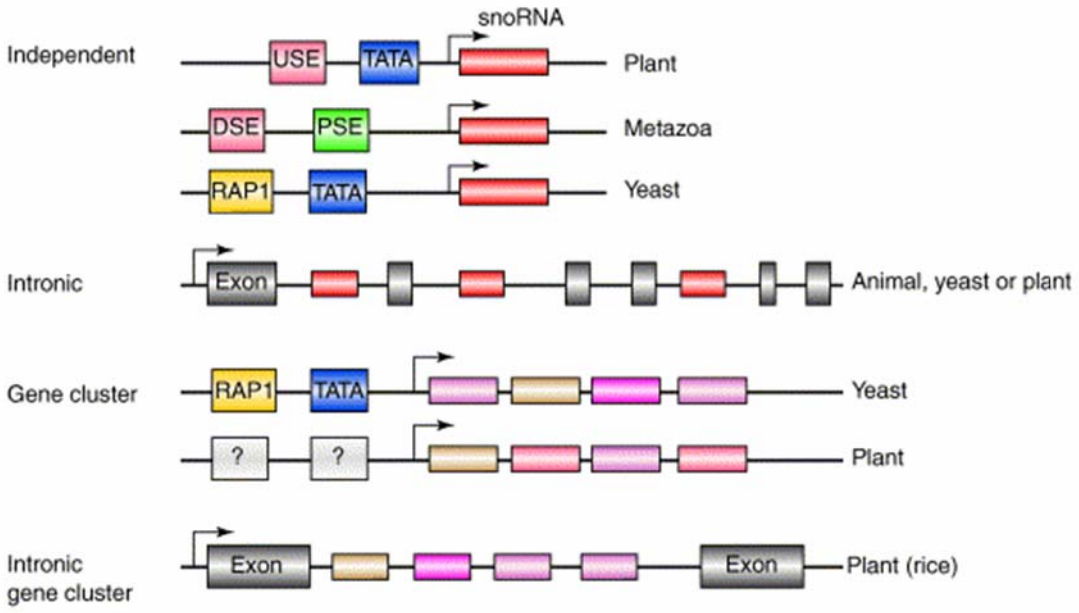


Figure 9. Gene organization and expression of snoRNAs. Genes encoding snoRNAs are transcribed as independent units or found in introns (intronic) or as gene clusters (polycistronic). Exons and potential transcription signals are indicated. Abbreviations: DSE, distal sequence element; PSE, proximal sequence element; USE, upstream sequence element. (Figure reproduced from Brown JWS et al., 2003).

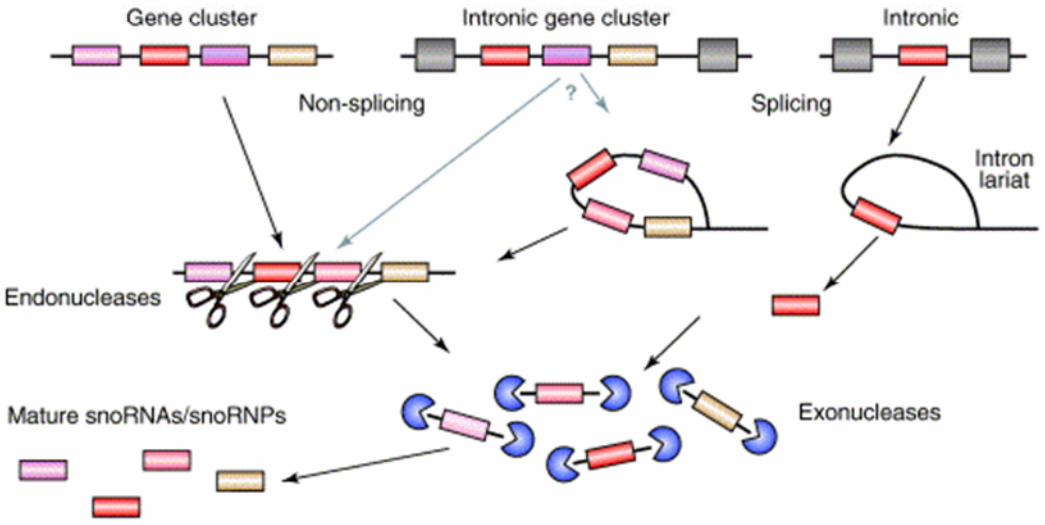


Figure 10. Processing of snoRNAs. Polycistronic and intronic pre-snoRNA transcripts are processed by either a splicing or a non-splicing pathway. Linear polycistrons require endonucleolytic cleavage followed by exonucleolytic trimming. The binding of snoRNAs by core proteins at the earliest stages of processing ultimately blocks the exonucleolytic activity to generate mature snoRNPs. (Figure reproduced from Brown JWS et al., 2003).

potential. However, all vertebrate genes hosting an intronic snoRNA guide for rRNA modification belong to the family of actively transcribed housekeeping genes termed 5′ TOP (terminal oligopyrimidine) genes, which could provide the basis for a coordination of snoRNA biosynthesis at the transcriptional level. In yeast too, promoter regions of mono- or polycistronic snoRNA genes and genes hosting intronic snoRNAs share common control elements, pointing to coordinated transcription.

Irrespective of their mode of transcription, correct processing and nucleolar localization of the mature guide snoRNAs are directed by conserved boxes C/D and H/ACA through bound snoRNP proteins.

1.3.5. Associated proteins

Both types of guide snoRNAs function as small ribonucleoprotein particles (snoRNPs), each one consisting of a site-specific snoRNA associated with a small set of proteins common to each guide family.

The box C/D snoRNPs contain four evolutionary conserved core proteins; fibrillarin (Yeast Nop1p) Nop56p, Nop58p and 15.5kDa protein (Yeast Snu13p). Fibrillarin, which exhibits amino acid sequence motifs characteristic of *S*-adenosyl-Met (SAM)-dependent

methyltransferases, is the likely snoRNA-guided modifying enzyme, as point mutations in the methylase-like domain disrupt all rRNA methylations. 15.5 kDa protein binds specifically to the C/D structural motif, termed as a K-turn. This protein which binds the same structural motif in U4 within the U4/U6.U5 tri-snRNP complex is also an integral component of this spliceosomal complex (Nottrorr et al., 1999), raising the possibility that mRNA splicing and snoRNA synthesis may be linked (Bachellerie et al., 2002).

The core proteins common to H/ACA snoRNPs include proteins dyskerin (Yeast Cbf5p), Gar1p, Nhp2p and Nop10p, all essential for the pseudouridylation reaction. Cbf5p is likely to correspond to the catalytic component of the H/ACA-snoRNA-guided modification, based on the presence of signature motifs for pseudouridine synthase and deleterious effects of point mutations in these motifs (Lafontaine et al., 1998, Zebarjadian et al., 1999). Based on amino acid sequence homologies, Cbf5p appears closely related to TruB, which catalyzes the pseudouridine formation in the T loops of virtually all tRNAs.

Recently, a pair of highly conserved nucleoplasmic proteins related to each other which have both DNA helicase activity and are linked with chromatin remodeling and transcription, p50 (Rvb2) and p55, were observed to bind to a model box C/D snoRNA *in vitro*, suggesting that they function at an early stage of snoRNP biogenesis in the nucleoplasm (Newman DR et al., 2000). Remarkably, depletion of p50 impairs assembly or trafficking of C/D snoRNPs and H/ACA snoRNPs as well.

1.3.6. Cellular RNA targets for eukaryotic snoRNA guides

Around thirty 2'-O-methylations and twenty four pseudouridylations occur collectively in mammalian U1, U2, U4, U5 and U6 spliceosomal snRNA. Interestingly, these modifications are mainly located in the snRNA segments involved in intermolecular RNA-RNA interactions or conformational switches during spliceosomal assembly and function, suggesting their role in pre-mRNA splicing control (Massenet et al., 1998). Several snoRNAs predicted to guide modifications in snRNAs U1, U2, U4 and U5 colocalize in Cajal bodies, a nuclear organelle located close to nucleolus which is involved in biogenesis of snRNAs and snoRNAs. Newly synthesized snRNAs are proposed to transit through the CBs before and after their cytoplasmic stage of snRNP assembly, suggesting that the modifications of these snRNAs take place in the CBs.

A novel brain specific C/D box snoRNA MBII-52, shows striking 18nt phylogenetically conserved antisense element to 5-HT_{2C}R mRNA which is exclusively expressed in brain. Hence, it is very much likely that the snoRNAs can as well target an mRNA (Cavaille et al., 2000).

With an increasing number of 'orphan snoRNAs', the functions of which are still unknown, the possibilities of snoRNAs targeting a still wider range of cellular RNAs with diversified functions cannot be undermined.

1.3.7. Brain specific snoRNAs and genomic imprinting

Increasing number of recently identified snoRNAs (expecially C/D Box family), exhibit a tissue specific expression pattern, being mainly expressed within the brain. Remarkably, genes of all of them with the exception of MBI-56 (the gene encoding MBI-56 is embedded within the second intron of the serotonin receptor 2C, hence its expression correlates with the serotonin receptor 2C's expression), are subjected to genomic imprinting, an epigenetic phenomenon that restricts gene expression to only one chromosome, either the paternal or maternal allele. For example, imprinted snoRNAs like HBII-13, HBII-52 and HBII-85 are located on human chromosome; locus 15q11q13 (Figure 11). HBII-52 and HBII-85 are arranged in to two tandem arrays of 47 and 27 nearly identical snoRNA gene copies respectively, whereas HBII-13 is encoded by a single gene copy. In humans, this locus as well encodes other imprinted snoRNAs like HBII-346, HBII-347 and HBII-348. MBII-346, the mouse homolog of HBII-346 has also been identified, but MBII-347 and MBII-348 have not been detected so far. The genes encoding these snoRNAs are maternally imprinted, so only they are expressed only from the paternal chromosome.

Interestingly, human locus 15q11q13 is also associated with two different neurological disorders: Prader-Willi (PWS) and Angleman Syndrome (AS) that result from the paternal or maternal loss of expression within this region. Human 14q32 and mouse distal 12 locus, contain tandemly repeated arrays of novel, maternally imprinted tissue-specific C/D snoRNAs. Interestingly their antisense elements exhibit a substantial level of inter-copy divergence in each repeat array, supporting the notion that the tandemly repeated gene organization could provide the basis for a functional diversification of some C/D snoRNAs (Cavaille et al., 2002).

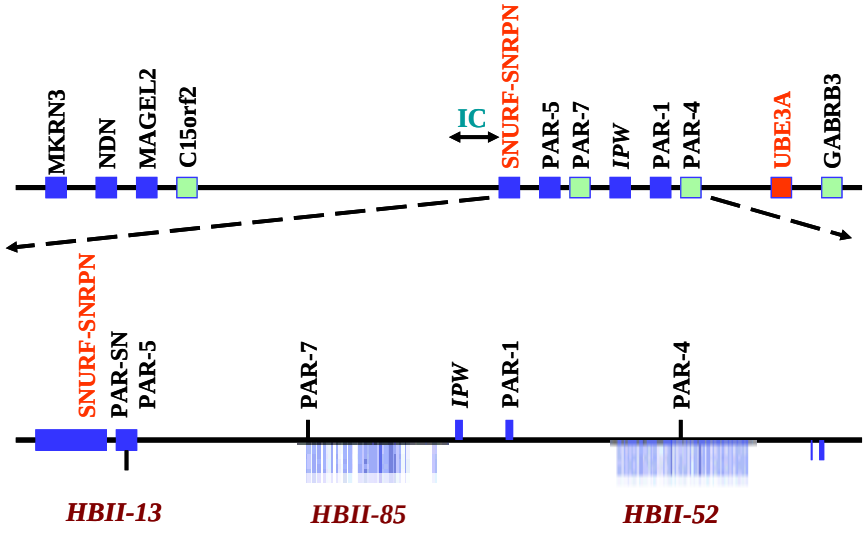


Figure 11. Organisation of brain specific snoRNA in the q11q13 locus on Human chromosome 15.

1.3.7.1. MBII-52: A unique brain specific C/D box snoRNA

The Mouse brain specific snoRNA MBII-52 was isolated from the mouse cDNA library. Identification of several clones differing from each other by a few bases, suggested that they were expressed from a multiple copy gene. The Human orthologue, designated HBII-52 was also detected exclusively in the brain. Within the different regions of the brain, MBII-52 was expressed at roughly similar levels except in choroid plexus where it was not expressed at all. Sequence analysis of PAC clones screened with a MBII-52 probe identified 1.9-kb PstI restriction fragment, present in multiple copies which are only slightly divergent. MBII-52 was later found to be expressed in the intron of the G2/G1 exon of the IPW (Imprinted in Prader-WIlli) gene in mouse chromosomal region 7C. HBII-52, the human orthologue of MBII-52, maps to chromosome 15q11-13. This region is involved in two diverse genetic defects: Prader-Willi (PWS) and Angelman (AS) syndrome. PWS is a neurogenetic disease resulting from a deficiency of a paternal gene expression and associated with significant developmental, behavioural and mental problems. Regularly spaced 47 HBII-52 copies of 1.9 kb units were identified within a 99kb region.

The antisense element of HBII-52 is unique due to its outstanding length, its perfect conservation in humans and mice, and the identity of target RNA involved. HBII-56 exhibits

an 18nt complementarity to a Serotonin receptor 2C mRNA, which is also a host gene from the HBI-36 snoRNA. Moreover, the complementarity lies in the coding region of the gene which is also subjected to both alternative splicing and Adenosine to Inosine RNA editing by ADARs at five vicinal sites. Coincidentally, the base to be modified by the HBII-52 corresponds precisely to one of the sites (C site) that undergo ADAR editing. Adenosine modification at this site can dramatically interfere with its deamination to inosines. Also, an alternative splice donor site is located only 13nt upstream of the potential HBII-52 pairing site in the mRNA sequence. Interestingly, the alternatively spliced mRNA form is mostly enriched in the choroid plexus, the only brain area devoid of HBII-52 snoRNA. Hence, HBII-52 snoRNA might be involved in the regulation of the alternative splicing of the 5-HT_{2C}R heterogenous nuclear RNA.

1.4. Serotonin 5-HT receptors

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter that affects a wide range of psychological and physiological processes in the mammalian central and peripheral nervous systems (Wilkinson and Dourish, 1991) as well as in a number of nonneuronal tissues in the gut, cardiovascular system and blood (Hoyer et al., 2002). 5-HT has been implicated in numerous disease states including depression, anxiety, social phobia, schizophrenia, migraine, hypertension, pulmonary hypertension, eating disorders, vomiting, irritable bowel syndrome (IBS) and obsessive-compulsive and panic disorders (Hoyer et al., 2002). The diverse effects of serotonin are mediated through specific binding to a variety of membrane bound receptors. Pharmacological, electrophysiological, and molecular biological studies indicate the existence of at least 14 subtypes of serotonin receptors in humans, namely 5-HT_{1A} , 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-HT_{1E} , 5-HT_{1F} , 5-HT_{2A} , 5-HT_{2B} , 5-HT_{2C} , 5-HT_3 , 5-HT_4 , 5-HT_{5A} , 5-HT_{5B}, 5- HT_6 , and 5-HT_7 (Hoyer et al., 1994). Apart from this, multiple splice variants (5-HT_4, 5-HT_7) or several RNA edited isoforms (5-HT_{2C}) have been identified (Hoyer et al., 2002). With the exception of 5-HT_3 receptor, which is a ligand-gated ion channel, individual receptor subtypes mediate their effects by coupling to different intracellular second messenger signaling systems via guanosine triphosphate binding (G) proteins.

1.4.1. Human Serotonin receptor 2C (5-HT_{2C}R)

The 5-HT_{2C} (previous named 5-HT_{1C}) receptor (5-HT_{2C}R) belongs to the family of seven transmembrane-containing G-protein-coupled receptors (GPCR) and is mapped to human X chromosome band q24. Its mRNA expression is detected only in choroid plexus, various regions of the brain, and spinal cord and appears to be developmentally regulated. Mice lacking the 5-HT_{2C}R show compulsive like behaviour (Chou-Green et al., 2003), eating disorder and epilepsy. 5-HT_{2C}R are linked to phospholipase C and regulates the production of phospholipase C-generated second messengers (Conn *et al.,* 1986), promoting the hydrolysis of membrane phospholipids and a subsequent increase in the intracellular levels of inositol phosphates and diacylglycerol. Serotonin 2C receptor can modulate cyclic AMP accumulation, can regulate K^+ and Cl^- channels, can both inhibit and stimulate nitric oxide (NO) levels and can also regulate mitogenesis (Raymond et al., 2001). Recently, Serotonin 2C receptor has also been shown to help the secretion of hormones like Vasopressin and Oxytocin (Jorgenson H et al., 2003). The 5-HT_{2C}R expressed from various cloned cDNAs can bind serotonin agonists and antagonists and elicit an increase in intracellular Ca2+ concentrations in response to serotonin. Studies to date implicate the function of this receptor in the actions of a broad range of psychoactive compounds including appetite suppressant, antidepressant, antipsychotic, anxiolytic, psychostimulant and psychedelic drugs (Giorgetti et al., 2004). Hence, this receptor plays a significant role in mediating tonic inhibition of neuronal network excitability.

1.4.2. 5-HT_{2C}R: Genomic structure and its alternative spliced variants

The entire human serotonin 5-HT_{2C} receptor gene consists of 4775 nt. It contains a 728-nt 5'-untranslated region (UTR) upstream from the ATG start codon, followed by a 1374-nt open reading frame (ORF) encoding a protein of 458 amino acids with an estimated molecular mass of 53 kDa. The cDNA has a long 3'-UTR of 2670 nt. This 3'-UTR is AT-rich (65.6%). A total of eight copies of the AUUUA motif, which confers message instability to various short-lived transcripts including those encoding cytokines, lymphokines, and proto-oncogenes are found in the 3'-UTR of the 5-HT_{2C}R mRNA. This suggests that it may be involved in the regulation of human 5-HT_{2C}R gene expression. Several potential transcription factor-binding sites are present in the 5' UTR of this gene. These include

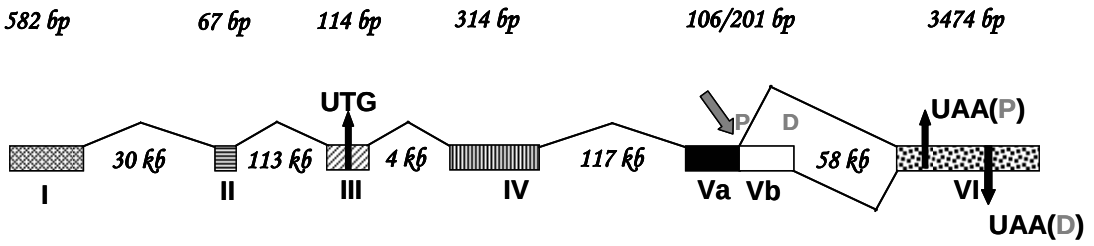


Figure 12. Structure of the human 5-HT2C receptor. The lengths of the exons and the intervening introns have been shown. The location of proximal and distal splices sites are indicated by P and D. Start (AUG) and two stop codons (UAA (P) and UAA(D)) resulting from the usage of the proximal or distal splice site are indicated. The alternative exon Vb is indicated as a white box.

binding sites for the basic region helix–loop–helix (bHLH) and bHLH-zipper proteins (defined as the E-box sequence motif), NF-IL6 binding sites, AP2 binding sites, TCF-1 binding sites, AP1 binding site, GCF binding site, HNF-5 binding site, LFA1 binding site, NF-E1 binding site and NF-$_K$B binding site. In addition, a sequence of the O motif of the immunoglobulin gene enhancer and a γ-interferon-responsive element were found in this region (Xie et al., 1996).

The entire 5-HT$_{2C}$R gene from exon I to exon VI spans at least 326 kb of DNA. It contains six exons and five introns (Figure 12). Recently, 5-HT$_{2C}$R mRNAs from humans (M81778, DR003480) have identified a 91 nucleotides novel exon in the 5' UTR between exon II and III. Surprisingly, a completely unrelated novel exon has been identified between Exon II and III of the mouse 5-HT$_{2C}$R gene (mRNA: BC098327). All introns appear to be large, with the largest one (Intron II) being ~117 kb. The 5' leader region comprises exons I and II and a part of exon III. The coding region of the 5-HT$_{2C}$R cDNA spans from part of exon III to exon VI. Although many GPCRs do not contain introns in their coding regions, the coding sequence of the human 5-HT$_{2C}$R gene is interrupted by three introns. The majority of the exon VI sequence constitutes the long 3`-UTR. In addition, the positions of the intron/exon junctions are conserved between the human and the rodent genes. The homology between the human and the rodent counterparts extends also into their promoter regions, including several potential transcription sites indicating a conserved control of its gene expression by similar cis- and trans-acting elements.

The 5HT$_{2C}$R gene transcribes at least three different alternatively spliced isoforms (Figure 13). Apart from the major, long isoform RNA2 that encompasses the entire coding

region, there is a minor shorter variant RNA1,which contains a 95 nucleotide deletion in the region coding for the second intracellular loop and the fourth transmembrane domain of the receptor. This deletion leads to a frameshift in the reading frame downstream from the deletion and a premature termination codon at nucleotide positions +840 to +842. This short human 5-HT_{2C}R cDNA codes a putative protein of 248 amino acids with 96 specific residues on the carboxyl terminus. Intriguingly, the ratio of the short isoform over the 5-HT_{2C}R RNA was higher in choroid plexus than in normal brain tissue. The third variant RNA3 contains an extra 90 nucleotides sequence of Intron V. Splicing joins the alternative splicing donor site located in the intron V with the exon VI. This partial intron retention allows a lysine residue to be added followed by a stop codon present in intron V. This variant has a truncated short C terminus. Expression of these variant isoforms in different cell types failed to reveal any serotonergic ligand binding. These results raise the possibility of differential regulation of the 5-HT_{2C}R gene expression in different neural tissues.

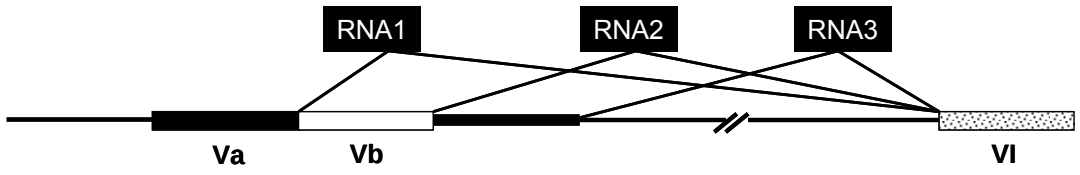


Figure 13. Splicing pattern of the 5-HT2CR. Pre-mRNA of 5-HT2CR could splice in three ways: RNA1 (Exon Vb is excluded), RNA2 (Exon Vb is included) and RNA3 (a partial intron V is retained).

1.4.3. 5-HT_{2C}R editing by ADARs and its functional implication

The 5-HT_{2C} receptor is a G-protein-coupled receptor whose pre-mRNA is a substrate for base modification via hydrolytic deamination of Adenosines to yield Inosines (Burns et al., 1997). Five Adenosines (named A, B, E, C, and D editing sites), located within a stable stem loop exonic sequence encoding the putative second intracellular domain of the 5-HT_{2C} receptor, can be converted to Inosines (Figure. 14). ADAR enzymes responsible for Adenosine to Inosine editing showed that A and B sites were selectively edited by ADAR1 and the D site by ADAR2a and ADAR2b, whereas the C site was edited although much less

efficiently by both ADAR1 and ADAR2a or ADAR2b (Wang et al., 2000, Liu et al., 1999a). This editing can alter the coding potential of three triplet codons and permits generation of 24

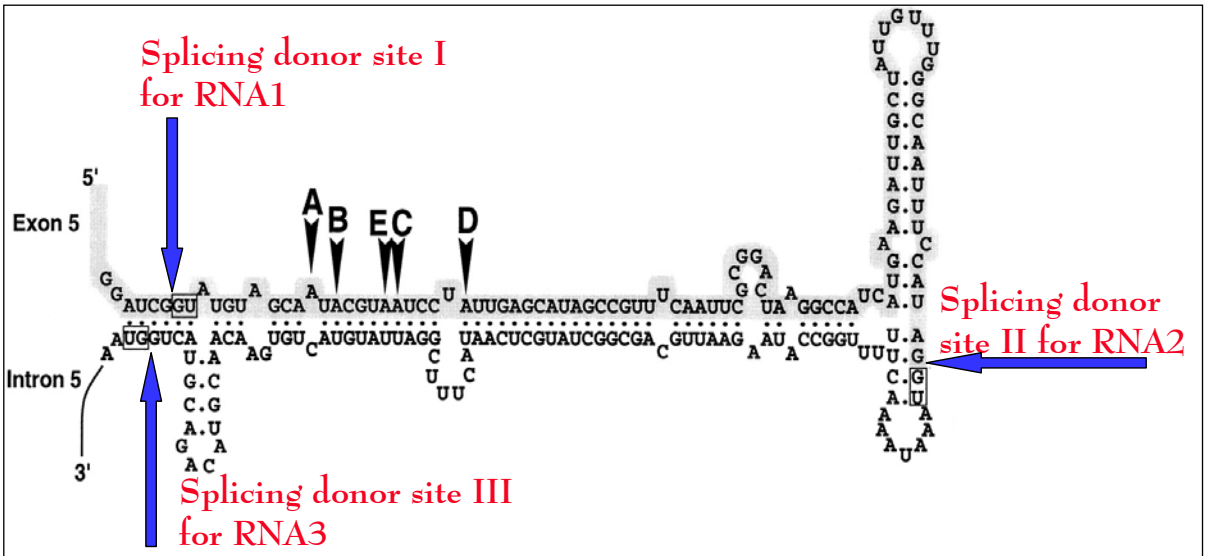


Figure 14. The dsRNA structure formed around exon 5/intron 5 of the human 5-HT2CR gene transcript. Exon 5 sequences are lightly shaded. Three alternative splicing donor sites (I–III), which lead to synthesis of RNA1, RNA2, or RNA3 respectively, are indicated by arrows, and the dinucleotides GU are boxed (Figure adapted from Wang Q. et al., 2003).

different receptor isoforms ranging from the unedited Ile156 Asn158 Ile160 (INI) to the fully edited Val156 Gly158 Val160 (VGV). Fully edited transcripts and partially edited transcripts that include at least editing of the E (previously C') site or the E and C sites differ from nonedited receptors in their lowered affinity for serotonin, hence, reduced ability to activate G-protein (Burns et al., 1997, Niswender et al., 1999, Wang et al., 2000). These differences in the G protein coupling efficiency by RNA editing can be accounted by the differences in the conformational properties of the second intracellular loop (IL2) in the 5-HT_{2C} receptors (Visiers et al., 2001). As a result, >60% of all mRNAs encode serotonin 2C receptors with reduced constitutive and agonist-stimulated activity. Moreover, the stem loop region containing the five editing sites, not only includes the normal 5' splice site of the intron V and but is as well flanked by two alternative splice sites. Recently, Flomen et al. 2004 and Vitali et al. 2005 have identified novel F and G editing sites in the intronic part in the stem loop. Since the intron is excised after the splicing, the role of editing in the intronic region of

the receptor is not very clear though it is assumed that there might be a definite link between the RNA editing and the post transcriptional regulation.

Since pre-mRNA editing precedes splicing, it has been previously shown that editing in the serotonin 2C receptor pre-mRNA not only can alter the coding sequence, but can also profoundly affect relative splicing at the proximal and the distal alternative splice site.

Using minigenes carrying A to G substitution mutations to mimic RNA editing, it has been shown that splicing increasingly favoured RNA2 over RNA1 as the number of editing mutations increased. Clonal analyses from human brains have revealed that each of the RNA3 cDNA contained complete editing at all five sites (Wang et al., 2000). Thus, when editing is inefficient, increased splicing at proximal GU1 splice site to give inactive RNA1, may act as a control mechanism to decrease the biosynthesis of the INI isoform and thereby limit constitutive activity. Marion et al., 2004, provide evidence that the fully edited VGV isoform showing lowest level of constitutive activity was fully expressed at the cell surface under basal conditions and was rapidly internalized in the presence of agonist. In contrast, the non edited INI isoform was constitutively internalized proportionally to their level of constitutive activity. This shows that RNA editing can also dictate the cellular distribution of the receptor and directly influence the interaction of the receptor with the components of the desensitization process.

Moreover, in mouse forebrain neocortex, sustained activation of 5-HT_{2C} receptors leads to significantly increased E-site editing and results in increased expression of mRNA isoforms encoding receptors with reduced basal constitutive activity and decreased agonist affinity and activity. Conversely, depletion of serotonin decreases E- and C-site pre-mRNA editing and increases the pool of mRNA encoding receptor deamination isoforms with the highest constitutive activity and the highest affinity for serotonin. Hence, 5-HT_{2C}R pre-mRNA editing is regulated by the cognate neurotransmitter domain itself and thus serves as a mechanism to maintain normal response properties of 5-HT_{2C} receptors during periods of sustained changes in synaptic input. Substantial changes in the editing preference has been found in the prefrontal cortex (PFC) of depressed suicide victims that result in the predominant expression of the edited isoforms that couples least efficiently to G proteins. However, they are not a consequence of antidepressant treatment. Depression is thought to be associated with lower serotonin-mediated neurotransmission in the PFC, suggesting that the

regulation of 5-HT_{2C}R pre-mRNA editing is altered in depression (Iwamoto et al., 2005, Iwamoto and Kato 2003). Hence, increased stress reactivity, a major psychopathological feature of depression, has been found to be a powerful determinant of 5-HT_{2C}R pre-mRNA editing and its regulation by serotonin.

1.5. Prader-Willi syndrome (PWS)

Prader-Willi syndrome (PWS) is a clinical disorder linked to abnormalities in inheritance of chromosome 15q11-q13. At the molecular level, genes within a 2MB domain spanning half of the 15q11-q13 are imprinted, with expression of these genes dependent on the sex of the parent-of-origin. PWS results from the absence of expression of the paternally derived alleles of maternally imprinted genes in a critical region of SNRPN locus. Loss of maternal UBE3A brain specific expression in the same locus results in Angelman syndrome (AS). The PWS/AS locus is conserved in organization and function with the homologous mouse chromosome 7C region. Two distinct stages can be observed for the patients afflicted with PWS. The first type shows restricted fetal movement, fetal growth retardation, respiratory distress and severe hypotonia at birth. The infants fail to thrive in the postnatal period and tube feeding is necessary. The second phenotype appears at around the age of two. Individuals show hyperphagia in early childhood with abnormal satiety response leading to obesity. Short stature, small hands and feet, a narrow biparietal diameter of the head and impaired sexual development make up the physical phenotype. The mechanisms underpinning the physical phenotype include growth hormone deficiency and central hypogonadism. Ritualistic behaviors, mild-moderate mental retardation, temper tantrums, obsessive-compulsive mannerism, and skin picking are frequently noted, together with an abnormal sleep pattern, poor temperature regulation, and a high pain threshold. For some, especially those whose syndrome is caused by Uniparental Disomy (UPD) of chromosome 15, a third phenotype might emerge in early adult life characterized by the development of severe affective psychotic illness.

1.5.1. Inheritance in PWS

The majority (65%-75%) of the PWS cases are due to de novo deletions spanning 4-4.5 MB of 15q11-q13, although few patients have smaller or larger deletions. Recombination

between duplicated sequences (termed duplicons) can lead to deletions spanning from either breakpoint 1 (BP1) to breakpoint 3 (BP3) or from breakpoint 2 (BP2) to breakpoint 3 (BP3) (Figure 15). Around 20%-30% of the PWS genetic abnormality results from maternal uniparental disomy (matUPD). Microscopic deletions of a genetic element termed the imprinting center (IC) or abnormal imprint accounts up for 5% of the PWS cases. Though rare, balanced translocations occur in ~0.1% of the cases. (Figure 15) (Nicholls et al., 2001, Schule et al., 2005)

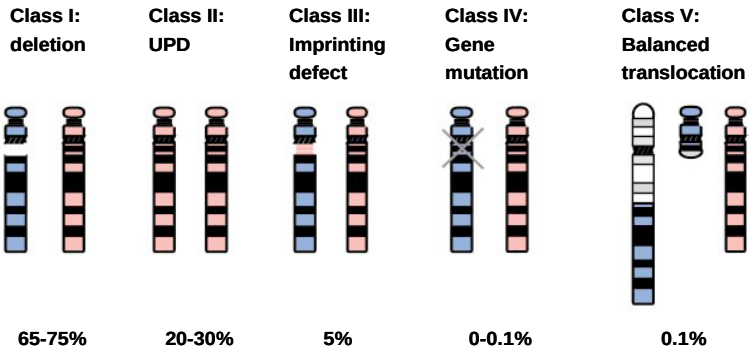


Figure 15. Complex genetic inheritance in PWS. Shown are the five genetic classes of PWS, with the parental origin (blue, paternal; pink, maternal) and frequencies observed for each. UPD denotes maternal uni-parental disomy (Picture reproduced from Nicholls and Knepper 2001).

1.5.2. Imprinted PWS domain

The multiple paternally expressed imprinted genes associated with PWS (Figure 16) maintain the imprint in most or all tissues. Genetic evidence based in micro deletions in ID in the human and balanced translocation patients has implicated the central SNURF-SNRPN locus as a strong candidate for a major role in PWS. This locus encodes at least four functions, including elements of the cis-acting regulatory region termed the imprinting center (IC), two independent proteins SmN and SNURF, and a small nucleolar RNA HBII-13.

The encoded SmN and SNURF polypeptides and HBII-13 snoRNA are expressed *in vivo* in human and mouse but are absent in PWS and PWS mouse models, demonstrating that this is an imprinted polycistronic locus. SmN is encoded by exons 4-10 SNRPN (small nuclear ribonucleoprotein) and is a core spliceosomal protein involved in mRNA splicing in the brain where it replaces the otherwise constitutively expressed SmB'/B proteins. In the PWS mice, the absence of SmN protein is compensated by the upregulation of SmB'/B. SNRPN is the best described gene likely to cause some of the manifestations of PWS. As it is

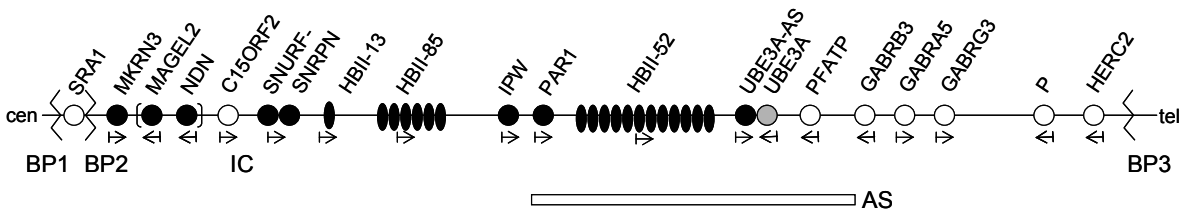


Figure 16. Human Chromosome 15q11-q13 genomic structure. The parental origin (black, paternal; grey, maternal; open circles, biparental) and transcriptional orientation of genes are indicated. The human 4Mb 15q11-q13 region flanked by PWS/AS common deletion breakpoints (BP, zigzag lines). The open bar represents a 1 Mb deletion causing AS only on maternal inheritance. Abbreviations: centromere (cen), telomere (tel) and imprinting center (IC).

a ribosome associated protein that functions in controlling gene splicing, it may be pivotally involved in the control of synthesis of some proteins, particularly those related to hypothalamic function.

SNURF (SNRPN Upstream Reading Frame) is a putative imprinting control element (IC) for the region (Gray et al., 1999). It encodes a 71 amino acids nuclear localized protein that is enriched in arginine residues that could bind to RNA. Potential functions for SNURF include regulation of SmN or of the imprinting process. Disruption of this gene, e.g., through chromosomal translocation, results in failure to imprint the SNRPN gene. HBII-13 snoRNA is encoded within the intron 12 of the SNURF-SNRPN transcript.

Several additional paternally expressed imprinted genes that encode proteins have been identified as a clister of intronless genes in 15q11-13 and mouce 7C, including two MAGE protein family members, NDN and MAGEL2, and MKRN3. The gene NDN encodes MAGE family NECDIN protein that has been shown *in vitro* to suppress cell proliferation.

NECDIN can bind to transcriptional factors p53, E1A, E2F1 nd SV40 large T antigen. The function of MAGEL2 is still not known. MKRN3 encodes a polypeptide with a RING zinc finger and multiple C3H zinc finger motifs, suggesting an unknown function as a ribonucleoprotein. IPW is an imprinted gene that does not encode a protein. Par1, Par5 and PW71 are other imprinted anonymous transcripts with unknown function.

Amongst the non-imprinted genes in this locus include GABRB3, GABRA5 and GABRG3, which are all the receptor subunit genes for the neurotransmitter GABA (γ-amino butyric acid). A loss of another non-imprinted OCA2 or P gene, which codes for a tyrosine

transporter, is responsible for the skin and ocular hypopigmentation in 50-70% of the PWS affected individuals (Gardner et al., 1992).

1.5.3. snoRNAs and PWS

Multiple brain specific C/D box snoRNA genes have been identified in the commonly deleted region between SNRPN and UBE3A. The snoRNA genes are contained in the introns between noncoding exons of a long pre-mRNA transcript that initiates at the SNRPN promoter. The snoRNA molecules are produced only when the pre-mRNA is processed, and thus their expression is imprinted and paternal chromosome–specific. Two of them, PWCR1/HBII-85 and HBII-52 snoRNA, have 27 and 47 copies, respectively. Three single copy snoRNAs, HBII-13, HBII-436 and HBII-437, and HBII-438 with two copies were also found in this region (Cavaille et al., 2000). 27 copies of HBII-85 are spread over 99kb and 47 copies of HBII-52 are spread over 55kb of this 15q11-q13 or mouse 7C chromosomal locus.

The HBII-52 snoRNA coding sequences show only 3% sequence divergence between copies, but 1.9kb repeat units diverge an average of 22% from the consensus and share no homology with the MBII-52 repeats, other than the highly conserved snoRNA coding sequence. In contrast, the HBII-85 coding sequences each differ on average by 20% and the repeat units are less evenly spaced than for HBII-52 genes. The mouse MBII-85 genes have more homogenous and highly conserved structure, with only 2%-4% divergence between copies. All six families of snoRNAs show lack of expression in the PWS tissues from human or from a PWS mouse model (Cavaille et al. 2000).

Most C/D box snoRNas are ubiquitous and function as guide RNAs to direct 2'-O-ribose methylation in rRNA. In contrast, the snoRNAs on the 15q11-q13 locus are brain specific and do not have a region complementary to rRNA. For HBII-52/MBII-52, a guide region exists with an 18nt complementarity to the serotonin receptor 2C mRNA in a region involved in Adenosine-to-Inosine editing and close to a site for alternative splicing. This suggests that snoRNA MBII-52, that is absent in the patients suffering from PWS, can influence the regulation of Serotonin receptor 2C pre-mRNA. This claim is strengthened by the fact that PWS patients respond to SSRI (selective serotonin reuptake inhibitors) treatment. It is likely that defect in serotonergic system is a contributing cause of PWS.

The potential RNA targets of the other PWS snoRNAs are unknown. It is still not clear how lack of some of these imprinted snoRNAs especially MBII-85 can cause neonatal lethality in the Prader-Willi syndrome mouse models (Ding et al., 2005). All this suggests the indispensable role of small nucleolar RNAs in regulating higher brain functions.

2. RESEARCH OVERVIEW

Tiling arrays and other experiments based on characterizing new cDNAs have provided consistent evidence that a larger percentages of eukaryotic genomes are being transcribed than can be accounted by the current state of genome annotations. Around 98% of the transcriptional output in humans is small non-coding RNA (ncRNA). These small RNAs seem to be involved in roles that require highly specific nucleic acid recognition without complex catalysis, such as in directing post-transcriptional regulation of gene expression or in guiding RNA modifications. HBII-52 is a brain specific small nucleolar RNA that bears 18nt complementarity to serotonin receptor 2C mRNA. This complementarity region is 13nt downstream of the alternatively spliced site and overlaps with several sites edited by ADARs.

The first part of the following work shows that the HBII-52 can regulate the alternative splicing of serotonin receptor 2C pre-mRNA. Based on experimental evidences in the second part, a possible mechanism of HBII-52 mediated alternative splicing is proposed. We also show that Prader-Willi Syndrome patients, that express no HBII-52, have a deregulated serotonin receptor 2C, which possibly contributes to the syndrome. Towards the end, using bioinformatics approaches, we searched for putative targets for other snoRNAs missing in the Prader-Willi syndrome. Overall, the results presented here show the importance of HBII-52 on the regulation of serotonin receptor 2C, hence emphasizing the vital role small non-coding RNAs play in the proper functioning of eukaryotic cells.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Product	Supplier	Product	Supplier
30% Acrylamide/Bis solution	Sigma	Nonidet P-40 / Igepal CA-630	Sigma
Agar	GibcoBRL	dNTPs	Invitrogen
UltraPure agarose	Invitrogen	Paraformaldehyde	Merck
Ampicilin	Sigma	Phenol: Chloroform: Isoamyl Alcohol	Sigma
Aprotinin	Sigma	PMSF	Sigma
[γ^{-32} P]-ATP	Amersham	Potassium chloride	Merck
Benzonase	Sigma	2-Propanol	Roth
Boric acid	Roth	Protease Inhibitor Cocktail	Sigma
Brilliant Blue R 250	Sigma	Protein A Sepharose	Amersham
Bromophenol blue	Merck	RNase Inhibitor	Roche
Chloramphenicol	Sigma	SDS	Sigma
Chloroform: Isoamyl alcohol	Sigma	Sepharose CL-4B	Pharmacia
di-Sodiumhydrogen phosphate	Merck	Sodium acetate	Merck
DMSO	Sigma	Sodium chloride	Roth
DTT	Merck	Sodium dihydrogen phosphate	Merck
EDTA	Merck	Sodium fluoride	Sigma
Ethanol	Roth	Sodium hydroxide	Merck
Ethidium bromide	Sigma	Sodium orthovanadate	Sigma
Ficoll 400	Fluka	TEMED	Sigma
Glycerol	Sigma	Tris base	Aldrich
Kanamycin	Sigma	TRIzol	Sigma
Magnesium chloride	Merck	Triton X-100	Sigma
Magnesium sulfate	Sigma	Tween 20	Sigma
β-Mercaptoethanol	Merck	Xylene cyanole FF	Sigma

3.1.2. Enzymes

Product	Supplier	Product	Supplier
T4 PNK	NEB	FastLink T4 DNA Ligase	Biozym
T7 DNA Polymerase	NEB	Platinum Pfx polymerase	Invitrogen
AmpliTaq DNA polymerase	Roche	Restriction Enzymes	NEB
DpnI	NEB	RNAse H	USB
DNAseI	Boehringer	SuperScript II	Invitrogen

3.1.3. Cell lines and media

Cell Line	Description	ATCC number
HEK293	Human embryonic kidney transformed with adenovirus 5 DNA	CRL-1573
Neuro-2a	Neuroblastoma from mouse brain	CCL-131
Rat glial cells	Primary neurons	Established In-house
SH-SY5Y	Homo sapiens brain Neuroblastoma	CRL-2266
C6	Rat brain glail cells	CCL-107
ECPC-4	Mouse Choroid Plexus	Riken Cell Bank
F11	hybrid of mouse neuroblastoma N18 cells and dorsal root ganglia	-
Ntera2	Human teratoma derived cell line	CRL-1973
B104 P4	Human Tumor cell lines of neuronal orgin	-
H4 wt	Human neuroglioma	-
P19	Murine embryonal carcinoma cell line	CRL-1825
C3H	Astrocytes glial cell line	-
BL6	Astrocytes glial cell line	-

All cell lines except ECPC-4 and Rat glial cells were maintained in DMEM supplemented with 10% fetal calf serum (both from GibcoBRL). ECPC-4 was maintained on RPMI medium supplemented with 10% fetal calf serum (both from GibcoBRL). Primary Rat glial cells were maintained on Neurobasal medium with 2% B27 supplement. For sub-culturing immortalized cell lines, 1 X Trypsin/EDTA (GibcoBRL) was used.

3.1.4. Bacterial strains and media

Strain	Genotype	Reference
XL1-Blue MRF'	Δ*(mcr*A)183 Δ*(mcr*CB-*hsd*SMR-*mr*ɪ) 173 *end*A1 *sup*E*44 thi-1 rec*A1 *gyr*A*96 rel*A1 *lac* [F′ *pro*AB *lac*I^q ZΔ M*15* T*n10* (Tet^r)]	(Bullock et al., 1987)
TOP10 cells	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *Φ80lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (*Str*^{R}) *end*A1 *nup*G	Invitrogen
DH5α-T1^R	F$^-$ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi-1 gyr*A96 *rel*A1 *ton*A	Invitrogen

LB MEDIUM (1L):

10 g NaCl
10 g tryptone
5 g yeast extract

LB AGAR (1L):

10 g NaCl
10 g tryptone
5 g yeast extract
20 g agar

3.1.5. Antibiotics

Antibiotic	Stock solution		Working concentration	
	concentration	Storage	liquid culture	Plates
Ampicilin	50 mg/ml in H2O	4°C	100 µg/ml	100 µg/ml
Chloramphenicol	30 mg/ml in ethanol	–20°C	15 µg/ml	30 µg/ml
Kanamycin	20 mg/ml in H2O	4°C	20 µg/ml	20 µg/ml

3.1.6. Antibodies

Antibody	Organism	Source
anti GFP	Mouse	Roche
anti-mouse IgG	Sheep	Amersham

3.1.7. Plasmids

3.1.7.1. Clones from the Stamm lab collection or outside sources

Name	Backbone	Description	Reference
15.5-pCDNA3-YFP	pCDNA3	CMV promoter, YFP-tagged, 15.5 kDa snoRNP protein, Amp^r	E. Bertrand
$5HT_{2C}$	unknown	T7 promoter, 5-HT_{2C}R minigene	A. Hüttenhofer
$5HT_{2C}$ ABCD-Minx	pCDNA3.1	CMV promoter, 5-HT_{2C}R minigene with ABCD editing sites mutated from A → G, Amp^r	A. Hüttenhofer
$5HT_{2C}$ PAC clone no. NO8149	PAC	Contig containing Exon 5a/b and a part of Exon 6 of mouse 5-HT_{2C}R, Kan^r	A. Hüttenhofer
ADAR2	pEGFP	CMV promoter, Kan^r	H. Poulsen
iADAR1	pEGFP-C1	CMV promoter, full length ADAR1, Kan^r	(Poulsen et al., 2001)
iADAR1 C-Terminus	pEGFP-C1	CMV promoter, Kan^r	(Poulsen et al., 2001)
L30-fibrillarin-YFP	unknown	CMV promoter, YFP-tagged, snoRNP protein, Amp^r	E. Bertrand
Nop56-pCDNA3-YFP	pCDNA3	CMV promoter, YFP-tagged, snoRNP protein, Amp^r	E. Bertrand
Nop58-pCDNA3-YFP	pCDNA3	CMV promoter, YFP-tagged, snoRNP protein, Amp^r	E. Bertrand
MBII-52	unknown	CMV promoter, Amp^r	(Cavaille et al., 2000)
pCR-5HT2C	pCR3.1	CMV promoter, 5-HT_{2C}R minigene, Amp^r	M. Gencheva
pCMV-MBI-36	pCDNA3	CMV Promoter, snoRNA MBI-136, Amp^r	A. Hüttenhofer
pDL700-ADAR1	unknown	CMV promoter, editing enzyme, Amp^r	S K Wong

Name	Backbone	Description	Reference
pDL707-ADAR1-L	unknown	CMV promoter, full length cytoplasmic ADAR1, Amp^r	(Wong et al., 2002)
pDL701-ADAR1-S	unknown	CMV promoter, editing enzyme, Amp^r	S K Wong
pEGFP-C2	pEGFP-C2	CMV promoter, Kan^r/Neo^r, f1 ori	Clontech
pKs T7 MBII-85	pBluescript	T7 Promoter, snoRNA MBII-85, Amp^r	A. Hüttenhofer
pMS40-ADAR2	unknown	CMV promoter, editing enzyme, Amp^r	S K Wong
pRCENMBII-52#28	Miscellaneous	CMV promoter, mutant snoRNA MBII-52 with the antisense box swapped with that of MBII-28, Amp^r	A. Hüttenhofer
pRcU3HBII-52 sv40-318U1a	pRC/CMV	Mutant HBII-52 with exchanged antisense element under a U3 promoter	K. Collins
pRcU3HBII-52sv40340U1	pRC/CMV	Mutant HBII-52 with exchanged antisense element under a U3 promoter	K. Collins
pRcU3HBII-52U1	pRC/CMV	Wt snoRNA HBII-52 under a U3 promoter	K. Collins

3.1.7.2. Newly made clones

Name	Backbone	Description
5HT2CEx5LngExonTrap	Exon trap	Sertonin receptor longest minigene w/o flanking constitutive exons.
5HT2CEx5Lng-MG	Exon trap	Sertonin receptor longest minigene with flanking constitutive exons 4 and 6.
5HT2CR-RSSM-MG	Exon trap	Serotonin receptor long minigene with activated distal 5′ splice site.
5HT2CR-SCM-MG	Exon trap	Serotonin receptor long minigene with mutated complementarity site.
5HT2CR-slncr-SXN MG	-	SXN minigene with the wt MBII-52 targeted region as the insert.
C-BOX-snoMut-PCR3.1	pCR3.1	Mutant MBII-52 with the C- Box mutated from TGATGA to GTTAAG.
CTRL-MutMBII52-10bSftSeRe-pcr3.1	pCR3.1	Mutant MBII-52 with anti-sense element identical to the 10 base down stream of the actual MBII52 binding site of 5-HT_{2C}R
CTRL-MutMBII52-13bE1A-pcr3.1	pCR3.1	Mutant MBII-52 with anti-sense element identical to the 13 base down stream of 12S splice site in the E1A minigene
D-Box-snoMut-pCR3.1	pCR3.1	Mutant MBII-52 with the D-Box mutated from CTGA to ACGT.
E1A-TOPO	pCR4 TOPO	E1A minigene for in vitro transcription and subsequent splicing assays.
E1AU1-snoMut-pcR3.1	pCR3.1	Mutant MBII-52 with anti-sense element directed against U1 binding site of alternatively spliced exon

		of the E1A minigene in the 12S/13S junction region.
EX5SHT-5HT2C-pET01	Exon trap	Sertonin receptor shortest minigene
HBII-52-2Copies-TOPO	pCR4 TOPO	two tandem copies of HBII-52.
Intron6 sht-long TOPO	pCR4 TOPO	intronic region of 5-HT_{2C}R downstream of the alternatively spliced exon for subcloning into the short minigene to make it identical to the long minigene.
Kozak-5HT2CR-pcDNA3.1-CT-GFP-TOPO	pcDNA3.1-CT-GFP-TOPO	Sertonin receptor long minigene with Kozak and GFP tag at the C terminus.
MS2-pGEXT4T1	pGEX4T1	Ms2 coat protein (bacteriophage)
Ms2bs2SnoMut-pCDNA3.1	pcDNA3.1	2 copies of MS2 binding sites in between C´and D´box of MBII-52.
Ms2bs4SnoMut-pCDNA3.1	pcDNA3.1	4 copies of MS2 binding sites in between C´and D´box of MBII-52.
Ms2bs6SnoMut-pCDNA3.1	pcDNA3.1	6 copies of MS2 binding sites in between C´and D´box of MBII-52.
Mu5HT2CR-RSSM-TOPO	pCR4 TOPO	Short 5-HT_{2C}R minigene with activated distal 5´ splice site for *in vitro* transcription.
Mut-MBII-52-E1A13BS-pcr3.1	pCR3.1	Mutant MBII-52 with anti-sense element directed against 18 nt shifted 13 bases downstream of the 5' splice site of 12S in the 13S region.
Mut-MBII-52-E1A23BS-pcr3.1	pCR3.1	Mutant MBII-52 with anti-sense element directed against 18 nt shifted 23 bases downstream of the 5' splice site of 12S in the 13S region.
Mut-MBII-52-E1A8BS-pcr3.1	pCR3.1	Mutant MBII-52 with anti-sense element directed against 18 nt shifted 8 bases downstream of the 5' splice site of 12S in the 13S region.
Mut-MBII-52-SR10BS-pcr3.1	pCR3.1	Mutant MBII-52 with the antisense box directed against 18 nt shifted 10 bases downstream to the actual binding site of the MBII-52 on the 5-HT_{2C}R.
Mut-MBII-52-SR-SCM-pcr3.1	pCR3.1	Mutant MBII-52 with the antisense box directed against mutated binding site of the SCM minigene of 5-HT_{2C}R.
Mut-MBII-52-SR5BS-pcr3.1	pCR3.1	Mutant MBII-52 with the antisense box directed against 18 nt shifted 5 bases downstream to the actual binding site of the MBII-52 on the 5-HT_{2C}R.
pET5HT2CRMGsht	Exon trap	Sertonin receptor short minigene
pET5HT2CRMGlng	Exon trap	Sertonin receptor long minigene
RSSCM sht MG	Exon trap	Short 5-HT_{2C}R minigene with combined mutations: distal 5´ splice site activation and the mutations in the MBII-52 complementary region.
RSSM sht MG	Exon trap	Short 5-HT_{2C}R minigene with activated distal 5´ splice site.
SCM-slncr-SXN MG		SXN minigene with the mut-MBII-52-SR-SCM-pcr3.1 targeted region as the insert.
SeReU1-snoMut-pcR3.1	pCR3.1	Mutant MBII-52 targeted against U1 binding site of the alternatively spliced Exon 5 of 5-HT_{2C}R minigene.
SeReU1-8BS-snoMut-pcR3.1	pCR3.1	Mutant MBII-52 targeted 8 bases down-stream to U1 binding site of the alternatively spliced Exon 5 of 5-HT_{2C}R minigene.

SMN2-i7-13BSsnoMut-pCR3.1	pCR3.1	mutant MBII-52 directed 13 bases down stream to the splice site of Ex 7 of the SMN2 minigene.
Sno-PmlI-Topo	pCR4 TOPO	MBII-52 for subcloning into the intron of the SMN2 minigene.
snoRNA-pIIIMS2-2	pIIIMS2-2	snoRNA clone for Yeast-3-hybrid
snoRNA-pCDNA3.1+	pCDNA3.1(+)	MBII-52 expressing clone w/o introns
w/o-K-5HT2CR-pcDNA3.1-CT-GFP-TOPO	pcDNA3.1-CT-GFP-TOPO	Sertonin receptor long minigene without Kozak
MBII-52cC-AS-TOPO	pCR4-TOPO	C/D-Box Mutant MBII-52 for subcloning.
MBII-52cD-AS-TOPO	pCR4-TOPO	
MBII-52cC-AS	pCDNA3.1+	C/D-Box Mutant MBII-52 for subcloning. expressed directly under CMV promoter as an antisence transcript
MBII-52cD-AS	pCDNA3.1+	

3.1.8. Primers

3.1.8.1. Primers used for cloning 5-HT_{2C}R gene

Name	Sequence	Target
EX5_for_NPCR	TGCAGTTTCCTTTTGGAG	Human 5-HT_{2C}R exon 5
EX5_rev_NPCR	TGGTATCAGAACCACAGAGA	
EX6_for_NPCR	TGGAATTTTGTTCTGTCACC	Human 5-HT_{2C}R exon 6
EX6_rev_NPCR	AAAGTACAAGGTTGCAAAGC	
5HT2Cex5_for	AGTCTCGAGTGATCACCATCATCATCCCTA	Human 5-HT_{2C}R exon 5
5HT2Cex5_sht_rev	GGGGGATCCTCTGGGATACATGTAACTTTTCAA	
5HT2Cex5_lng_rev	AGTGGATCCATGCCATTGGTGGAAAACAT	
5HT2Cex6_lng_for	AGTGCGGCCGCCTCTGCCTCCTGGGTTCA	Human 5-HT_{2C}R exon 6
5HT2Cex6_rev	GGGGAGCTCGACCAACAGAAAAATTAAAGAAGAAAA	
SeReEx5f	ATGCTCGAGTTGGATGACGTGTTCTTGTCTTCTC	Human 5-HT_{2C}R exon 5
SeReEx5r	GGGGGATCCTGATCTCAGCTCACTGCAGACTTG	
SeReEx6f	AGTGCGGCCGCATCAAGATCCCAAGAAGATCCACA	Human 5-HT_{2C}R exon 6
SeReEx6r	GGGGAGCTCAAAGAAGGGAGGGAGAGCAAAAAT	
5HT2Cex4-for	GCAGGGCCCTGGCCTGAAATTCCTACTGG	Human 5-HT_{2C}R exon 4
5HT2Cex4-rev	GAGCTCGAGTCAAACTGGGCTCTCCAACT	
5HT2Cex6ShtFor	AGTGCGGCCGCTGACACAATCCACCCTTTGA	Human 5-HT_{2C}R exon 6
5HT2Cex6ShtRev	AGTGAGCTCACTTAGTCCAGGCGGTTCCT	
Ex5SeReXhoIF1	ATGCTCGAGGATTTCCGGGACATGAGAGA	Human 5-HT_{2C}R exon 5
Ex5SeReBamHIR1	GAGGGATCCTGGGGCATGGAAAGTAAAAG	

Ex5SeReXhoIF2	ATGCTCGAGGCAGAATGCCCAACTTCATT	Human 5-HT_{2C}R exon 5
Ex5SeReBamHIR2	GAGGGATCCAGGTTGGGGAATACATGCAA	
Ex5SeReXhoIF3	ATGCTCGAGGGTATGGTGCCACATGTTTG	Human 5-HT_{2C}R exon 5
Ex5SeReBamHIR3	GAGGGATCCACACTGCCTGGCCCTTAGTT	
SeReIN5BamHIF	GGATCCTTGCATGTATTCCCCAACCT	Human 5-HT_{2C}R Intron 5
SeReIN5BamHIF1	GAGGGATCCAGCCTGGGCAACAGAGTG	
SeReIN5BamHIR	GGATCCTTTTTGCCTAGGCCATTGTC	
SeReIn5mBamHIF	GCTGGATCCCAGAATATTAACCTTAGTA	
SREx4GFPTopoF	GCCGCCACCATGGGCCTATTGGTTTGGCAAT	Exon 4 &6 of Human 5-HT_{2C}R
SREx6GFPTopoR	GACTTAGTCCAGGCGGTTCC	

3.1.8.2 Primers used for 5-HT_{2C}R Mutagenesis

Name	Sequence	Generated mutants
Ex5RegSplMutF	TGGGCAATTTCTACAGGTAAGTAAAACT	pRSV-5HTcons
Ex5RegSplMutRev	AGTTTTACTTACCTGTAGAAATTGCCCA	
SeReSnoComMutF	GCAATACGAAAGCCTTTAGACCATAGCCGT	pRSV-5HTsm
SeReSnoComMutR	ACGGCTATGGTCTAAAGGCTTTCGTATTGC	
AltSplSitemutPriF	GGATCGGTTTGTTGCAATACG	-
AltSplSitemutPriR	CGTATTGCAACAAACCGATCC	
MutSeRe*28MG-F	AAACGATCTCAACCTAATAGCCGTTTCAATTCGCGG	-
MutSeRe*28MG-R	TAGGTTGAGATCGTTTTATTGCTACATACCGATCCAGCG	
MuX5RSSMF	CAATATCAACAGGTAAGTATACCTGG	-
MuX5RSSMR	CCAGGTATACTTACCTGTTGATATTG	
SCM MG F	GCAATACGAAAGCCTTTAGACCATAGCCGTTTC	pRSV-5HTsm/cons
SCM MG Rev	GAAACGGCTATGGTCTAAAGGCTTTCGTATTGC	
RSSM MG For	ATTTCTACAGGTAAGTAAAACTTTTTGGCC	
RSSM MG Rev	GGCCAAAAAGTTTTACTTACCTGTAGAAAT	
5HT2Cex4SmaMut	CAGCAATGGCCCGGGACATTAAGAAG	-
SeReMGEx4SmaIF	TCCCCCGGGTGGCCTATTGGTTTGGCAAT	-
5HT2CIN5MutRevSalI	TCGACGTCTGTACGTT	-

3.1.8.3. Primers used for cloning snoRNA gene

Name	Sequence	Target
snoRNA_for	TCCCGGGCTGGGTCAATGATGACAACC	MBII-52 snoRNA
snoRNA_rev	TCCCGGGCCTCAGCGTAATCCTAT	

SnoRNABamHI	CGCGGATCCACTGGGTCAATGATGACAACC	MBII-52 snoRNA
SnoRNAXbaI	TGCTCTAGAGGGCCTCAGCGTAATCCTAT	
G2MBII52For	AGATCTCTGCTTAGGTAAGACATTCTT	G1-G2 exons MBII-52 snoRNA
G1MBII52Rev	CTCGAGTGACCACTTACCTGATTAT	
pacHBII52A1F	ATTGATATCAAGTCGGTCCCAAGAAGGAT	2 tandem copies of HBII-52
pacHBII52A1R	TTTGCGGCCGCGGCCGAATCACACATACCTC	
pacHBII-52B2F	TTTGCGGCCGCGGAGGACACCTTTGCTGAAG	2 tandem copies of HBII-52
pacHBII-52B2R	GAGTCTAGAGCAAATTCCCGGTACAACAC	
pacHBII-52C3F	TATGCGGCCGCTATTTTGGACCTGGGACCTG	2 tandem copies of HBII-52
pacHBII-52C3R	TAATCTAGAGTGGAGCATGCCTTGAATCT	
HBII-52A1SHT-NotI-R	TTTGCGGCCGCCTTCACACCTCGCAAACACA	2 tandem copies of HBII-52
HBII-52B2SHT-XbaI-R	GAGTCTAGACCAGGTCTACCCTGAGACCA	
snoRNA-PmlF	CGCCACGTGACTGGGTCAATGATGACAACC	-
snoRNA-PmlIR	TGCCACGTGGGGCCTCAGCGTAATCCTAT	

3.1.8.4. Primers used for MBII-52 Mutagenesis

Name	Sequence	Generated mutants
snoMutSmaI/BamHI	CGGGATCCGGGTCAATGATGACAACCCAATGTCA	-
	TGAAGACCCGGGAAGGTGATGACATAAAATTC	
5BsftSeResnoMutF-ctrl	TCCTATTGAGCATAGCCGCTGAGGCCCAACCAGGAC	Ctrl-Mut-MBII-52-5BS
5BsftSeResnoMutR-ctrl	CGGCTATGCTCAATAGGAGAATTTTATGTCATCACC TTTCTTCAT	
10BsftSeResnoMutF-ctrl	TTGAGCATAGCCGTTTCACTGAGGCCCAACCAGGAC	Ctrl-Mut-MBII-52-10BS
10BsftSeResnoMutR-ctrl	TGAAACGGCTATGCTCAAGAATTTTATGTCATCACC TTTCTTCAT	
13BsftE1AsnoMutF-ctrl	GTTAGATTATGTGGAGCACTGAGGCCCAACCAGGAC	Ctrl-Mut-MBII-52-E1A-13BS
13BsftE1AsnoMutR-ctrl	TGCTCCACATAATCTAACGAATTTTATGTCATCACCT TTCTTCAT	
5BsftSeResnoMutF	CGGCTATGCTCAATAGGACTGAGGCCCAACCAGGAC	Mut-MBII-52-5BS
5BsftSeResnoMutR	TCCTATTGAGCATAGCCGGAATTTTATGTCATCACCT TTCTTCAT	
10BsftSeResnoMutF	TGAAACGGCTATGCTCAACTGAGGCCCAACCAGGAC	Mut-MBII-52-10BS
10BsftSeResnoMutR	TTGAGCATAGCCGTTTCAGAATTTTATGTCATCACCT TTCTTCAT	
8BSftE1AsnoMutF	CACATAATCTAACACAAACTGAGGCCCAACCAGGAC	Mut-MBII-52-E1A-5BS
8BSftE1AsnoMutR	TTTGTGTTAGATTATGTGGAATTTTATGTCATCACCTT TCTTCAT	

13BSftE1AsnoMutF	TGCTCCACATAATCTAACCTGAGGCCCAACCAGGAC	Mut-MBII-52-E1A-13BS
13BSftE1AsnoMutR	GTTAGATTATGTGGAGCAGAATTTTATGTCATCACCT TTCTTCAT	
23BSftE1AanoMutF	GTGCCCGGGGTGCTCCACCTGAGGCCCAACCAGGAC	Mut-MBII-52-E1A-23BS
23BSftE1AanoMutR	GTGGAGCACCCCGGGCACGAATTTTATGTCATCACC TTTCTTCAT	
U1SeResnoMutF	GATTACGTATTGCTACATCTGAGGCCCAACCAGGAC	Mut-MBII-52-3BS
U1SeResnoMutR	ATGTAGCAATACGTAATCGAATTTTATGTCATCACCT TTCTTCAT	
U1E1AsnoMutF	ATCTAACACAAACTCCTCCTGAGGCCCAACCAGGAC	Mut-MBII-52-E1A-3BS
U1E1AsnoMutR	GAGGAGTTTGTGTTAGATGAATTTTATGTCATCACCT TTCTTCAT	
SMN2sno13BSF	ACCTTCCTTCTTTTTGATCTGAGGCCCAACCAGGAC	Mut-MBII-52-SMN13BS
SMN2sno13BSR	ATCAAAAAGAAGGAAGGTGAATTTTATGTCATCACCT TTCTTCAT	
SMN2snoU1F	TTTTTGATTTTGTCTAAACTGAGGCCCAACCAGGAC	Mut-MBII-52-SMN-3BS
SMN2snoU1R	TTTAGACAAAATCAAAAAGAATTTTATGTCATCACCT TTCTTCAT	
SeReU1-8BssnoF	CAATAGGATTACGTATTGCTGAGGCCCAACCAGGAC	Mut-MBII-52-8BS
SeReU1-8BssnoR	CAATACGTAATCCTATTGGAATTTTATGTCATCACCT TTCTTCAT	
C-Box mutF	CTGGGTCAAGTTAAGCAACCCAAT	pCMV-MBII52cC
C-Box mutantR	ATTGGGTTGCTTAACTTGACCCAG	
D-box mutantF	TAGGATTACGACGTGGCCCAACCA	pCMV-MBII52cD
D-box mutantR	TGGTTGGGCCACGTCGTAATCCTA	
MBII-52-cC-BamHIF	CGCGGATCCTGGGTCAAGTTAAGCAACCCAATGTCATG	MBII-52cC-AS
MBII-52-cD-XbaIR	TGCTCTAGATGGGCCACGTCGTAATCCTATTGA	MBII-52cD-AS
SMN2-U1-i7F	TCATAATGCTGGCAGACTCTGAGGCCCAACCAGGAC	MutMBII-52-SMN-i7-3BS
SMN2-U1-i7 R	AGTCTGCCAGCATTATGAGAATTTTATGTCATCACCT TTCTTCAT	
SMN2-13Bs-i7F	AGATTCACTTTCATAATGCTGAGGCCCAACCAGGAC	MutMBII-52-SMN-i7-13BS
SMN2-13Bs-i7R	CATTATGAAAGTGAATCTGAATTTTATGTCATCACCT TTCTTCAT	
SCMSRsnoMutF	ATGGTCTAAAGGCTTTCGCTGAGGCCCAACCAGGAC	pCMV-MBII52sm
SCMSRsnoMutR	CGAAAGCCTTTAGACCATGAATTTTATGTCATCACCT TTCTTCAT	

3.1.8.5. Primers used for miscellaneous cloning

Name	Sequence	Target
MS2Pro_for	GGGATCCATGGCTTCTAACTTTACTCAGTTCG	Bacteriophage MS2 protein
MS2Pro_rev	GCTCGAGTTAGTAGATGCCGGAGTTTGC	
MS2bs/SmaI/for	ATTCCCGGGGCGATCCAACATGAGGATC	MS2 binding

Ms2bs/SmaI/rev	TTTCCCGGGCATGGGTGATCCTCATGTTT	site
MS2bsREV	CATGGGTGATCCTCATGTTTT	
MS2bsKpnIF	ATTGGTACCGCGATCCAACATGAGGATC	MS2 binding site
Ms2bsKpnIR	TTTGGTACCACATGGGTGATCCTCATGTTT	
SeReSlncr-SalI-F	TCGACCGTAATCCTATTGAGCATG	5HTR2C-slncr-SXN MG
SeReSlncr-BamHI-R	GATCCATGCTCAATAGGATTACGG	
SeReSCMslncr-SalIF	TCGACCGAAAGCCTTTAGACCATG	SCM-slncr-SXN-MG
SeReSCMslncr-BamHIR	GATCCATGGTCTAAAGGCTTTCGG	

3.1.8.6. Primers used for RT-PCR

Name	Sequence	Target
E1A forward E1A reverse	GTTTTCTCCTCCGAGCCGCTCCGA CTCAGGCTCAGGTTCAGACACAGG	E1A minigene
E1A-Mid-for	TCTTCCACCTCCTAGCCATT	E1A minigene
E1A-x3-For	GTCCGGTTTCTATGCCAAAC	E1A minigene
5HT2CMG_for 5HT2CMG_rev	TATGTCTGGCCTTTACCTAGATATTT TCACCATAATTGTCAACGGGA	Mouse 5-HT_{2C}R minigene
etrap_cDNA_RT 5HT2CMG_for 5HT2Cex6ShtRev	GATCCACGATGCCGCGCT TATGTCTGGCCTTTACCTAGATATTT AGTGAGCTCACTTAGTCCAGGCGGTTCCT	Human 5-HT_{2C}R minigene
etrap_cDNA_RT eTrap-PCR-for eTrap-PCR-rev	GATCCACGATGCCGCGCT GAGGGATCCGCTTCCTGCCCC CTCCCGGGCCACCTCCAGTGCC	Exon-trap vector
SeReAltSplEx5For	GCAATACGTAATCCTATTGAGCA	Human $5HT_{2C}$R Exon 5b
SeReEx5cFor	TTTTGGCCATAAGAATTGCAG	Human $5HT_{2C}$R Exon 5c
SeReEx5c-Mid-F	TCGGATTATGTACTGTGAACAACG	
Rat5HT2Cx6ShtRev	GTTCCTCCTCGGTGTGACCT	Rat $5HT_{2C}$R Exon 6
SeReMGEx6R	GGCTCATTGTCACTGGCTTT	Human $5HT_{2C}$R Exon 6
MuHTR2cX6-148bR	GTGATCACCATAATTGTCAACG	Mouse $5HT_{2C}$R Exon 6
HsMmSeReX4MF	GGTACAAAACTGGCCAGCAC	$5HT_{2C}$R Exon 4
oligo dT	TTTTTTTTTTTTTTTTT	PolyA
BGHRev	TAGAAGGCACAGTCGAGG	BGH polyA
MmSeReX6shtR	CAGTTCCTCCTCGGTGTGAC	Mouse $5HT_{2C}$R Exon 6
HuSMN2-X6-Mr	TGAGCACCTTCCTTCTTTTTG	Human SMN2 Exon 6
SeReEditAr	CTCAATAGGATTACGTAT	$5HT_{2C}$R

SeReEditBr	TGCTCAATAGGATTACGT	isoforms unedited at editing sites A, B, C, D and E respectively in the Exon 5b.
SeReEditCf	GTATGTAGCAATACGTAA	
SeReEditDf	TAGCAATACGTAATCCTA	
SeReEditALngR	GGCTATGCTCAATAGGATTACGTAT	
SeReEditBLngR	GGCTATGCTCAATAGGATTACGT	
SeReEditCLngF	GGATCGGTATGTAGCAATACGTAA	
SeReEditDLngF	CGGTATGTAGCAATACGTAATCCTA	
SeReEditELngF	GGATCGGTATGTAGCAATACGTA	
SCMsno_rev	TCCCGGGCCTCAGCGAAAGCCTTT	MBII-52cm

3.1.8.7. Primers used for Real time PCR analysis of pCMV-5HT ($5HT_{2C}R$) minigene

Name	Sequence	Target
SeReEx5a-Ex6For	CGCTGGATCGGTGTATCAGT	Exon 5a-6 junction
5HT2Cex5a-5bFor	CGCTGGATCGGTATGTAGC	Exon 5a-5b junction
SeReX6b6ShtRev	GGGATAGGAACTGATACACCTATAGAA	Exon 6
SeRex5bReTimeF1	TGTTTGGGCAATTTCTATAGGTG	Exon 5b
SeReEx6ReTimeR1	CGTCAGCGGTATGAAGAAAG	Exon 6
SeReEx6ReTimeR2	CACGAACACCTTTTCTTCGTC	Exon 6

3.1.8.8. Primers used for Sequencing

Name	Sequence	Target
etrap_seq_for etrap_seq_rev	GGATTCTTCTACACACCC TCCACCCAGCTCCAGTTG	Exon trap vector
pIIIMS2-2-seq	TGGCCGGCTGTCTCTATACT	Yeast-3-hybrid vector

3.1.8.9. Primers used for *In vitro* transcription and RNAse protection assay

Name	Sequence	Target
SnoRNAtranscriFor	CCAAGCCTTCTAATACGACTCACTATAGG GAGAGGGTCAATGATGACAACCCA	MBII-52
SnoRNAtranscriRev	GGGCCTCAGCGTAATCCTAT	MBII-52
SCMsnoTranscriR	GGGCCTCAGCGAAAGCCTTT	MBII-52cm
5-HTRTranscriF	CCAAGCCTTCTAATACGACTCACTATAGG GAGATCTGCGCTATATCGCTGGAT	Human $5HT_{2C}R$
5-HTRTranscriR	GAAATTGCCCAAACAATAGCA	Human $5HT_{2C}R$
HuSeReTranscriF	CCAAGCCTTCTAATACGACTCACTATAGGG AGATTGGATGACGTGTTCTTGTC	Human $5HT_{2C}R$
HuSeReTranscriR	AGAACCCGATCAAACGCAAA	Human $5HT_{2C}R$
snoBindRe1R	GCTCAATAGGATTA	MBII-52 complementarity
snoBindRe2R	TCAATAGGATTACG	

snoBindRe3R	ATGCTCAATAGGAT	region on $5HT_{2C}R$
HuSRU1BindReR	CTACATACCGATCC	U1 binding splice site at Exon 5a-5b of $5HT_{2C}R$

3.1.9. Brain Tissues

Human Brain Tissues conforming to 3 Prader-Willi patients and 3 normal controls were obtained from the Maryland Brain and Tissue bank for Developmental Disorders, University of Maryland. Different brain sections were kindly dissected by Prof. Ingmar Blümke, Erlangen.

Different tissues from the rat brain for snoRNA and serotonin receptor expression profile were kindly dissected from the adult rat by Prof. Winfried Neuhuber, Erlangen.

Brain tissues corresponding to depressed patients and normal controls were obtained from Munich.

RNA from mouse tissues introduced to stress and normal controls were obtained from Prof. Giese's Lab, London.

3.2. Methods

3.2.1. Plasmid DNA isolation

Large amounts of ultra pure plasmid DNA was isolated using QIAGEN Plasmid Maxi kit according to the manufacturer's protocol.

Smaller amounts of plasmid DNA was isolated from the alkaline lysis method first described by Birnboim and Doly (Birnboim and Doly, 1979). In brief, bacterial cells carrying the desired plasmid were cultured overnight at 37°C in 5ml LB medium containing the appropriate antibiotics. The cells were harvested by centrifugation for 5 minutes at 12,000 rpm. The pellet was resuspended in 250µl buffer P1. Equal volume of lysis buffer P2 was then added and the solution mixed gently by invertion. The cells were allowed to lyse for 5 minutes, followed by addition of the neutralization buffer P3. The tube was mixed gently by inversion and the solution was maintained on ice for 15 minutes. After centrifugation for 10 minutes at 12,000 rpm, the resulting supernatant was precipitated by adding 1 volume of isopropanol. Plasmid DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes,

washed with 70 % ethanol, air-dried and dissolved in 30µl of TE buffer. All the steps were carried out at room temperature in a conventional tabletop microfuge.

LB MEDIUM:

10g Tryptone
10g NaCl
5g yeast extract

BUFFER P1:

50 mM Tris-HCl, pH 8.0
10 mM EDTA
100 µg/ml RNase A

BUFFER P2:

200 mM NaOH
1% SDS

BUFFER P3:

3M Potassium acetate, pH 5.5

BUFFER TE:

10 mM Tris-HCl, pH 8.0
1 mM EDTA

3.2.2. Electrophoresis of DNA

DNA was resolved on 0.7-2% agarose gels prepared in 1 x TBE buffer. The electrophoresis was run for 80 min at 100 V. The gels were stained for 30 min in 0.5 mg/ml ethidium bromide and visualized under UV light, $\lambda = 260$ nm.

1X TBE:

90 mM Tris-borate
20 mM EDTA

6 X GEL–LOADING BUFFER:

0.25% bromophenol blue
0.25% xylene cyanol FF
15% Ficoll 400 in dH_2O

3.2.3. Elution of DNA from agarose gels

DNA was purified from agarose gels where crystal violet was added to a final concentration of 2 µg per ml to detect DNA under visible light. Individual bands were excised and DNA was extracted using the Qiagen QIAEX II gel extraction kit according to the manufacturer's protocol.

6 X CRYSTAL VIOLET GEL–LOADING BUFFER:

0.25% crystal violet
15% Ficoll 400 in dH_2O

3.2.4. PCR amplification of DNA

A standard PCR reaction to amplify DNA from a plasmid template contained 1-10 ng of plasmid DNA, forward and reverse primers (0.5 µM each), dNTPs (200 µM), 1 x Taq polymerase buffer, 1.5 mM MgCl2 and 1 U Taq polymerase in total volume of 25 µl. When

the amplification was made for cloning purposes, a high-fidelity polymerase, i.e. Platinum Pfx polymerase was used instead of Taq polymerase. The amplification was carried out in a Perkin Elmer GeneAmp PCR System 9700 thermocycler under the following conditions: initial denaturation for 2-4 min at 94°C; 25-35 cycles of 15-30 sec at 94°C, annealing at the Tm of the primers pair, extension of 1 min per 1 kb at 72°C (or 68°C for Pfx polymerase). After the last cycle the reaction was held for 5-10 min at the extension temperature to complete the amplification of all products.

3.2.5. DNA Ligation

When the vector ends were blunt or compatible with each other, the vector was dephosphorylated prior to ligation to prevent self-ligation. To remove 5' phosphates from the vector, 2 U of Calf intestinal phosphatase (CIP, Boehringer) was added to 5 µg of linearised vector in 1 x CIP buffer in 20 µl. The reaction was incubated for 1 hour at 37°C. CIP was subsequently inactivated by heating the reaction to 68°C for 20 min. A typical ligation reaction contained vector and insert at a ratio of about 1:3 (500-1000 ng total DNA), 1 x ligase buffer, 1 mM ATP and 200-400 U T4 DNA Ligase (New England Biolabs) in 15 µl. The incubation was carried out at 16°C for 12-16 h. After that, one third to one half of the ligation mixture was then transformed in *E coli* cells.

3.2.6. Preparation of competent *E. coli* cells

5 ml of LB medium were inoculated with a single bacterial colony and grown overnight at 37°C with vigorous shaking. 4 ml of this culture were transferred to 250 ml LB and grown to early logarithmic phase (OD600 = 0.3-0.6). The culture was centrifuged for 10 min at 2500 rpm at 4°C. The bacterial pellet was resuspended in 1/10 volume of cold TSB buffer and incubated on ice for 10 min. Cells were aliquoted into cold Eppendorf tubes and frozen in liquid nitrogen. Competent bacterial cells could then be stored at -80°C for several months.

TSB BUFFER:

10% PEG 3500
5% DMSO
10 mM $MgCl_2$
10 mM $MgSO_4$
in LB medium, pH 6.1

3.2.7. Transformation of *E. coli* cells

1-10 ng of plasmid DNA or a ligation reaction were added to 20 µl of 5 x KCM buffer and then the volume was brought with water up to 100 µl. Equal volume of competent cells was added. The reaction mixture was incubated on ice for 20 min followed by incubation at RT for 10 min. Then 1 ml of LB medium was added and the bacteria were incubated for 1 h at 37°C with vigorous shaking. Finally cells were plated on LB Agar plates containing appropriate antibiotic. Plates were incubated at 37°C until colonies were visible.

5 X KCM BUFFER:

500 mM KCl
150 mM $CaCl_2$
250 mM $MgCl_2$

3.2.8. Construction of minigenes

Minigenes were constructed as described previously in Tang et al., 2004. In brief, most minigenes were amplified from genomic DNA and contained the alternatively spliced exon and its flanking constitutive exons. In majority of the cases, these parts could be amplified by long-range PCR. If the introns were too long, several kilo-bases flanking the exons were amplified and ligated together to the multiple cloning sites of exon trap vectors (Mobitec, Goettingen, Germany) between two constitutive rat insulin exons. This chimeric gene was then analyzed similar to a genomic construct.

3.2.9. Site directed mutagenesis by overlap extension

Four primers were designed to introduce mutations by this method which was first described by Higuchi et al., 1989. One set of forward F and reverse R primer was complementary to the extreme ends of the DNA template (see Figure 17). The other set of forward MF and reverse MR primer carrying the desired mutation, were complementary to each other and target the site where the mutation was desired. Mutant primers had 8-10 bases on either side of the mutation cassette to allow precise annealing. The first PCR was carried out with Proofreading polymerase to avoid any A-overhang. Individual PCRs were carried out to amplify fragments with primer sets F/ MR and with MF/R respectively.

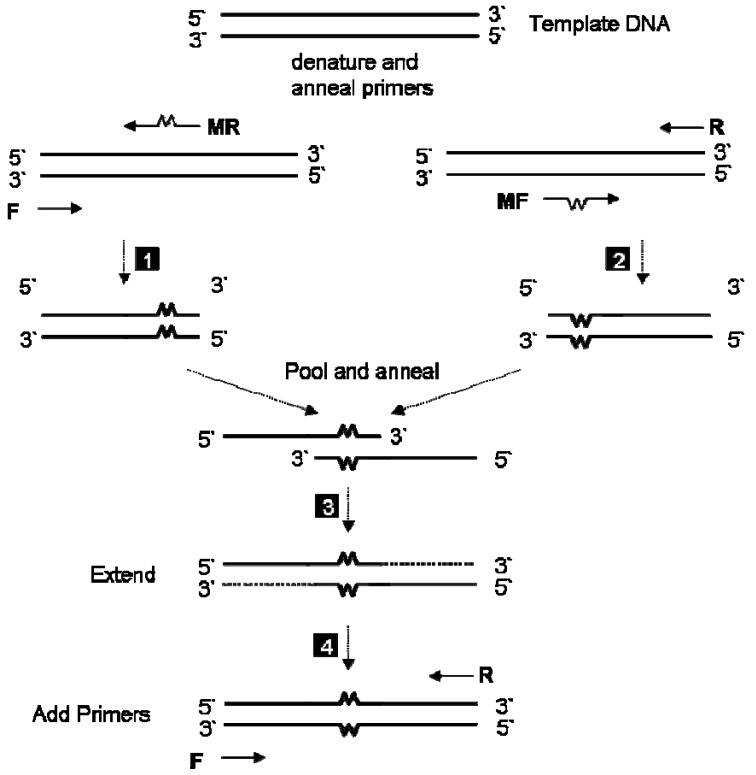


Figure 17. Site-directed Mutagenesis by Overlap Extension. In separate PCR amplification reactions 1 (Primers F and MR) and 2 (Primers MF and R), two partially overlapping fragments of the target gene containing the mutation are amplified. In PCR 3, the denatured products from PCR 1 and PCR 2 anneal at the region of overlap and extend to form full length double –stranded mutant DNA. In PCR 4, the full length mutant DNA is amplified using primers F and R. (adapted from *Molecular Cloning: A Laboratory Manual*, Sambrook and Russel, third edition, 2001).

The amplified fragments were gel eluted to free them from any contaminating DNA template. 200 ng of the individual purified fragments were pooled together and allowed to anneal and extend without any addition of primer with dNTPs (200 µM), 1 x Taq polymerase buffer, 1.5 mM MgCl2 and 1 U Taq polymerase in total volume of 25 µl. The amplification was carried out in a Perkin Elmer GeneAmp PCR System 9700 thermocycler under the following conditions: initial denaturation for 5 min at 94°C; 10 cycles of 30 sec at 94°C, annealing at 50°C, extension of 1 min per 1 kb at 72°C. After the last cycle the reaction was held for 5 min at the extension temperature to complete the amplification of all products. External primers (F and R) were then added and the reaction was again supplemented with 1 U of Taq polymerase. Final PCR was performed with the following conditions: initial

denaturation for 5 min at 94°C; 30 cycles of 30 sec at 94°C, annealing at 60°C and extension of 1 min per 1 kb at 72°C. The last cycle was followed by another 5 min of extension at 72°C. A part of the amplified fragment was run on the Agarose gel and the other subcloned into pCR4 TOPO for sequencing.

3.2.10. Radioactive labeling of 5' DNA ends

About 500 ng of purified DNA fragment (or 5-10 pmol oligonucleotide) were mixed with 1 x T4 polynucleotide kinase buffer, 50 μCi of γ-P 32 -ATP (3000 Ci/mmol) and 10 U T4 polynucleotide kinase (New England Biolabs) in 10 μl. The reaction was incubated for 1 hour at 37°C after which the kinase was inactivated at 68°C for 20 min.

3.2.11. Southern Blotting and hybridisation of DNA

DNA was separated on an agarose gel. After electophoresis the gel was placed in denaturation buffer (1.5 M NaCl/0.5 N NaOH) for 30 min. The DNA was transferred on a nylon membrane in a denaturation buffer for 16 h. The DNA was crosslinked to the membrane on a UV transilluminator. Prehybridisation was carried out for 30 minutes at 68°C in a hybridisation buffer. Hybridisation was done in 10-20 ml of hybridisation buffer for 16 hours at 68°C. The membrane was washed two times in 2 x SSC/0.1% SDS for 10 minutes at 68°C. It was than washed (two times) in 0.5 x SSC/0.1% SDS for 10 minutes at 68°C. Finally, the membrane was washed in 0.1 x SSC/0.1% SDS for 10 minutes at 68°C. The membrane was then exposed to X-ray film or the signal was revealed on a phosphorimager.

Hybridisation buffer:	20 x SSC:
0.5M phosphate buffer, pH 7.2 7% SDS	3 M NaCl 0.3 M Na citrate

3.2.12. Freezing, thawing and subculturing of eukaryotic cells

To freeze, cells were grown to mid logarithmic phase (about 75% of confluence) in 10 cm Petri dishes. They were collected by trypsinization with 1 x Trypsin/EDTA, resuspended in 1 ml of the freezing medium (90% of the growth medium and 10% of DMSO). Vials were placed in Nalge Nunc Cooler giving a cooling rate of ~1°C/min while at -80°C. Cells were stored later in liquid nitrogen.

To thaw, cells were incubated at 37°C. The entire content of the tube was transferred to a 10 cm Petri dish and 10 ml of the growth medium were added. The dish was placed in the incubator at 37°C and 5% CO_2. When cells were attached to the plastic surface, the medium was removed and replaced with fresh one. The cells were maintained in the incubator until ready for the subculturing.

Cells were subcultured after reaching confluence. The monolayer was detached by adding 1 X Trypsin /EDTA and incubating at 37°C until single cell suspension was formed. 1/5 – 1/10 of this suspension was transferred to a new dish and mixed with the growth medium. Cells were maintained in the incubator at 37°C and 5% CO_2.

3.2.13. Transfection of eukaryotic cells

The procedure used for HEK293 cells was based on the one published by Chen and Okayama (Chen and Okayama, 1987). Exponentially growing cells were replated at a density of about 3 x 10^5 cells / 8 cm^2. Growth medium was added and the cells were incubated at 37°C, 5% CO_2 for about 24 h, to reach 60-70% of confluence. For most applications cells were grown in 6-well plates, with 2 ml of growth medium per well. The transfection reaction for one well was made the following way. 1 to 5 µg of expression construct were mixed with 25 µl of 1 M CaCl2 in final volume of 100 µl. Equal volume of 2 x HBS buffer was added drop by drop, with constant mixing. In order to form a precipitate, the solution was allowed to stay at RT for 20 min. After that, it was added to the growth medium. To express the transfected plasmid, cells were grown for additional 24 h at 37°C, 3 % CO_2.

2 X HBS:

280 mM NaCl
10 mM KCl
1.5 mM $Na_2HPO_4 \cdot 2H_2O$
12 mM Dextrose
50 mM Hepes
pH 6.95

3.2.14. *In vivo* splicing assay

To determine the influence of a protein on the splicing of selected minigenes, *in vivo* splicing was performed as described (Stoss et al., 1999; Tang et al., 2005). 1 to 2 µg of the minigene plasmid were transfected in eukaryotic cells together with an expression construct

for the protein. Usually a concentration-dependent effect was assessed. The protein was transfected in increasing amounts, in the range of 0 to 3 μg. To avoid 'squelching' effects, the 'empty' parental expression plasmid containing the promoter was added in decreasing amounts, to ensure a constant amount of transfected DNA. Cells were plated in 6-well plates and transfection (section 3.2.14.) was done 24 hours after plating. After incubation for 14-17 hours at 3% CO_2 total RNA was isolated from the cells (section 3.2.23.).

400 ng of RNA were used in a reverse transcription reaction (section 3.2.24.). The reverse primer used for RT was specific for the vector in which the minigene was cloned, to suppress reverse transcription of the endogenous RNA. To avoid the problem of the amplification of minigene DNA, DpnI restriction enzyme was added into the reverse transcription reaction. DpnI cuts GATC sequence in double-stranded DNA when the adenosine is methylated but does not cut non-methylated single-stranded DNA or cDNA. A control reaction with dH_2O instead of RNA was included.

1/8 of the reverse transcription reactions were used for PCR with minigene-specific primers (section 3.2.24.). The primers were selected to amplify alternatively spliced minigene products. A control reaction with no template (RNA instead of cDNA) was included in the PCR. The PCR programs were optimized for each minigene in trial experiments.

PCR reactions were resolved on a 0.3-0.4 cm thick 2 % agarose TBE gel and the image was analyzed using ImageJ analysis software (http://rsb.info.nih.gov/ij/).

3.2.15. Isolation of total RNA

Total RNA was isolated from eukaryotic cells grown in 6-well plates. Cells were washed with 1 x PBS and the RNeasy Mini kit (QIAGEN) was used according to the manufacturer's protocol. RNA was eluted from the column in 30 μl of RNase-free dH_2O. However this procedure was applied only when the RNA of interest was greater than 200 bases.

Alternatively, for RNA smaller than 200 bases or when in lower concentrations (as in case of RNA immunoprecipitation), RNA was isolated using TRIzol reagent according to the manufacturer's protocol. 15-20 μg of Glycogen per 1 ml was added to the reaction prior to

ethanol precipitation for better recovery. After ethanol precipitation, the RNA pellet was dissolved in 20 µl of RNase-free dH_2O.

3.2.16. RT–PCR

400 ng of total RNA (200 ng/µl), 5 pmol of reverse primer, 40 U of SuperScript II reverse transcriptase, and optionally 4 U of DpnI restriction endonuclease were mixed in 5 µl of RT buffer. To reverse transcribe the RNA, the reaction was incubated at 42°C for 45 min.

1/8 of a typical reverse transcription reaction was used to amplify cDNA. The reaction was held in 25 µl and contained 10 pmol of specific forward and reverse primers, 200 mM dNTPs, 1 x Taq polymerase buffer and 1 U of Taq DNA polymerase. The conditions of the PCR cycles were dependent on the template to be amplified (see section 3.2.22. for conditions of amplifying minigene products from *in vivo* splicing assays).

RT BUFFER:
300 µl 5 X First strand synthesis buffer (Invitrogen)
150 µl 0.1 M DTT (Invitrogen)
75 µl 10 mM dNTPs
475 µl dH_2O

3.2.17. Isolation of nuclear extract and RNA immunoprecipitation

To isolate the nuclear extract, cells were trypsinized 24-36 hours after the transfection (section 3.2.14.) and washed in 30 volumes of PBS. The pellet was then resuspended in one packed cell volume of buffer A and allowed to swell on ice for 15 minutes. Cells were lysed with a 23G hypodermic needle and nuclei were recovered by centrifugation for 20 sec at 12000*g* at RT. The crude nuclear pellet was resuspended in two-thirds of one packed cell volume of buffer C and incubated for 30 min at 4°C with stirring. The nuclear debris was pelleted by 5 min centrifugation at 12000*g*. Collected nuclei were then resuspended in 0.6 ml of NET-Triton, sonicated gently several times on ice and centrifuged. The supernatant contained the nuclear extract.

For immunoprecipitaion, on the day before anti-GFP was allowed to bind to Protein A sepharose in NET-Triton overnight at 4°C. Next day, BSA was added to a final concentration of 1 mg/ml and allowed to incubate at 4°C for another 2-3 hours to block the sepharose beads. Parelly, anti-IgG in NET-Triton was added to the sepharose beads and

allowed to mix at 4°C for a few hours. The nuclear extract was precleared for 1 hour at 4°C with the anti-IgG bound Protein A sepharose. Immunoprecipitation of the desired GFP-Tagged protein from the precleared nuclear extract was done with Protein A Sepharose bound anti-GFP antibody overnight at 4°C, followed by five washes with cold RIPA buffer. RNA was isolated using the TRIzol reagent. After ethanol precipitation, the RNA pellet was dissolved in RNase-free water and DNAase treated as per manufacturer's protocol.

BUFFER A:	BUFFER C:	NET-TRITON:
10 mM HEPES, pH 8.0	20 mM HEPES, pH 8.0	150 mM NaCl
1.5 mM MgC_{12}	25% (v/v) Glycerol	50 mM Tris-HCl, pH 7.4
10 mM KCl	420 mM NaCl	0.1% Triton X-100
1 mM DTT	0.2 mM EDTA, pH 8.0	Protease inhibitors
	1 mM DTT	RNAse inhibitors
	0.5 mM PMSF	

All the three buffers were supplemented with Protease and RNAse inhibitors in appropriate concentrations. The buffers were always prepared fresh and kept on ice for subsequent use.

3.2.18. HBII-52 pull-down assay

This assay has been described in details later in section 4.9.1. using the specific primers used to develop the assay both as a text and as a diagram. In brief, the assay has been summarized in the following paragraph.

Transfection and Crosslinking:

HEK293 cells were transfected with calcium phosphate method. 16 hours after transfection, the cells were washed with serum free DMEM and trypsinised. Trypsinised cells were washed separately with serum free DMEM and isotonic PBS. Finally, the cells were resuspended to a concentration of $8*10^9$ cells/ml in appropriate volume of PBS and transferred to a dish such that the depth was 2-3 mm. AMT psoralen was added to a final concentration of 0.2 mg/ml and incubated on ice for 10 minutes. Cross linking was done in dark room on ice in a cold room under 365 nM Ultra-Violet (UV) radiation for 30 minutes at 4.5 mW/cm^2. A glass plate, ~3-4 mm thick, was placed before the UV source to filter the damaging radiation.

Isolation of Nuclear extract:

Cross linked cells were resuspended in one packed volume of buffer A (10 mM HEPES, pH 8.0, 1.5 mM MgCl2, 10 mM KCL, 1 mM DTT) and the nuclear extract isolated as described in section 3.2.17.

Pull down of crosslinked RNA using Dynal beads:

M-280 streptavidin coated dynal beads was prepared and the biotinylated 2'-O methyl modified RNA oligo was immobilized on the beads as per manufacturer's protocol. The precleared nuclear extract was then incubated with the dynal beads immobilized with the RNA oligo at 4°C for 30 minutes. The beads were washed with NET-triton buffer twice and captured RNA was isolated using Trizol as per manufacturer's protocol. RT-PCR was done with specific primers to detect the crosslinked snoRNA.

3.2.19. RNAse H protection assay

14mer oligomer targeted against the RNA of interest was purchased from MWG Biotech AG (Germany). A standard reaction was carried out in a volume of 30 µl containing 18 µl of HeLa nuclear extract, 3 µl of Buffer D (20% (v/v) Glycerol, 20 mM HEPES-KOH (pH 7.9), 100 mM KCl, 0.2 mM EDTA and 0.5 mM DTT), 3 mM $MgCl_2$, 0.4 mM rATP, 20 mM creatine phosophate, 2.5 Units RNaseH (USB), alpha UTP labelled capped RNA transcript. Since MBII-52 was not endogenously expressed in HeLa cells, the reaction was supplemented with *in vitro* transcribed radiolabeled MBII-52 five times in molar excess than the targeted RNA. It was assumed that the other proteins that interact with the targeted RNA are present in the HeLa nuclear extract in physiological concentration. The mixture was incubated at 30°C for 20 minutes to allow the splicing complex to form. Specific DNA oligos were then added at 40 µM final concentrations and the mixture further incubated at 30°C for 30 minutes. Proteinase K was subsequently added to stop the reaction. The digested RNA was extracted with phenol:chloroform and ethanol precipitated. The pellet was washed with 70% ethanol, air dried and subsequently loaded on denatured 15% polyacrylamide gel containing 8M Urea and the results analysed on X-ray film and phosphorImager.

3.3. Databases and computational tools

Database/software	URL	Description	Reference
ASD	http://www.ebi.ac.uk/asd	The alternative splicing database	(Thanaraj et al., 2004)
Alternative splicing workbench	http://www.ebi.ac.uk/asd-srv/wb.cgi?	Combines Intron Analysis, Regulatory sequences, Exon Finder, Splicing Rainbow, and Blast analysis	(Thanaraj et al., 2004)
ClustalW	http://www.ebi.ac.uk/clustalw/index.html	Multiple Sequence alignment program for DNA or proteins	(Thompson et al., 1994)
ESE Finder	http://rulai.cshl.edu/tools/ESE/	Finds putative binding regions for several splice factors	(Cartegni et al., 2003)
Human BLAT search	http://www.genome.ucsc.edu/cgi-bin/hgBlat	Sequence alignment tool similar to BLAST	(Kent, 2002; Kent et al., 2002)
NCBI BLAST	http://www3.ncbi.nlm.nih.gov/BLAST/	Finds regions of sequence similarity	(Altschul et al., 1990; Altschul et al., 1997)

4. Results

The Serotonin receptor 2C pre-mRNA undergoes several complex post transcriptional modifications to govern precisely the activity of its encoded protein. Alternative splicing at Exon V can regulate the amount of functional protein that carries the second transmembrane loop necessary to elicit response against its agonist. The mRNA coding for this loop region is subjected to multiple editing by ADARs, which can alter the coding signal of one or more amino acids. Editing can further fine tune the receptors' affinity towards serotonin. Edited isoforms show less affinity towards serotonin compared to the non edited isoform. Surprisingly, the serotonin receptor 2C bears 18nt complementarity to a novel brain specific snoRNA HBII-52 in the region 13 nucleotide downstream to the alternative 5' splice site,

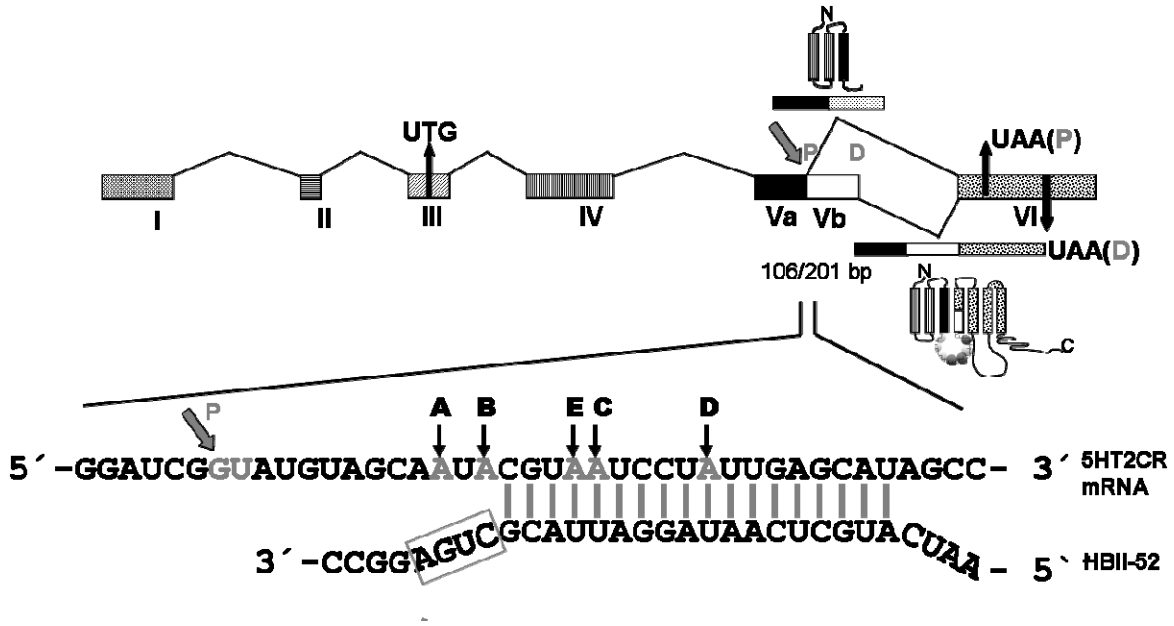


Figure 18. snoRNA HBII-52 bears 18nt complementarity close to the alternatvely spliced site. The structure of the human 5-HT2CR has been depicted (top). Alternate usage of Vb gives rise to two mRNA isoforms that are schematically indicated. Exon Vb skipping results in a truncated receptor shown schematically on the top of the mRNA. Its inclusion generates a full length receptor indicated below the diagram o the mRNA. The upper sequence shows the human 5-HT2CR mRNA. Arrows indicate the editing site (A-E), where A->I editing can occur. An open arrow indicates the proximal spice site. Lines indicate the complementarity between HBII-52 snoRNA and the 5-HT2CR. The D-Box of the HBII-52 is boxed.

which also overlaps with the sites edited by ADARs (Figure 18). Coincidently, the nucleotide predicted to be a target of 2' O methylation by HBII-52 is also a site (site C) for ADAR activity. Substrates analogs have shown that methylation of ribose at 2' can dramatically interfere with the ADAR editing efficiency. Also, both 5-HT_{2C}R and HBII-52 show brain specific expression except in choroid plexus, where HBII-52 is not expressed. In all other regions of brain, the functional exon Vb inclusion isoform of 5-HT_{2C}R predominates, whereas in choroid plexus, the ratio is reversed. Hence, there are several indications that the HBII-52 can have a potential role in the alternative splicing of serotonin receptor 2C. Verification of HBII-52 mediated alternative splicing of serotonin receptor 2C, can open potential areas of gene regulation mediated by snoRNAs. The following study was undertaken to test, if HBII-52 can target serotonin receptor 2C pre-mRNA and hence play a role in the gene's regulation. Also, efforts have been made to confirm if HBII-52 missing in the Prader-Willipatients can have a contributing cause to the development of the disease.

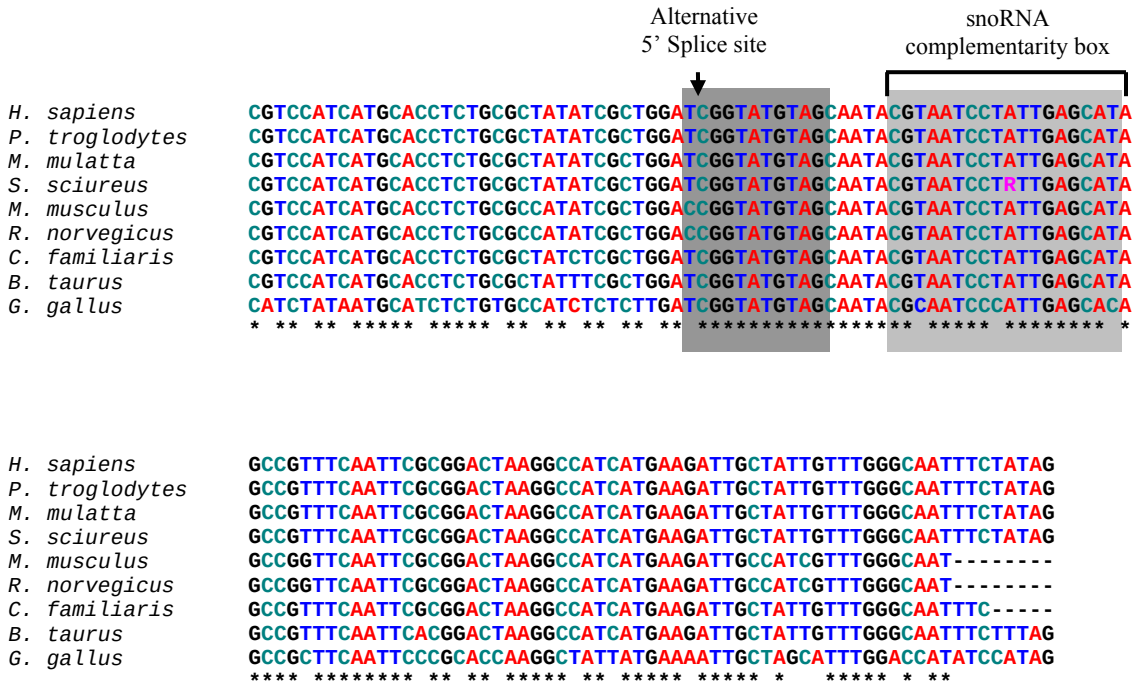


Figure 19. ClustalW sequence alignment of serotonin 2C receptors across various species.

4.1. Complementary regions of both HBII-52 and 5-HT$_{2C}$R are conserved across various species

To test the importance of snoRNA mediated alternative splicing of 5-HT$_{2C}$R gene, sequences from various species were compared using ClustalW sequence alignment software (Figure 19). Sequence comparison shows that HBII-52 and its complementary target sequence within the serotonin receptor 2C are fully conserved across the mammalian species of which the sequence is available.

The only deviation in the serotonin receptor 2C sequence conservation is from non-mammalian chicken which has three mismatches in its complementary element. In addition, the entire alternatively spliced Exon V is conserved across various mammalian species including the proximal 5' splice site, reflecting the importance of this exon in the gene's evolution. This raises the possibility that control of 5-HT$_{2C}$R gene regulation by snoRNAs is only a mammalian feature and hence it is strongly conserved in mammals but only weakly in non-mammalian species.

Similar alignment for all available BII-52 snoRNAs (Figure 20) reveals its high conservation across all the mammalian species, especially with respect to C- and D-Box and the antisense element. Only in *Cannis familiaris*, there is one single C->U alteration at the 2nd

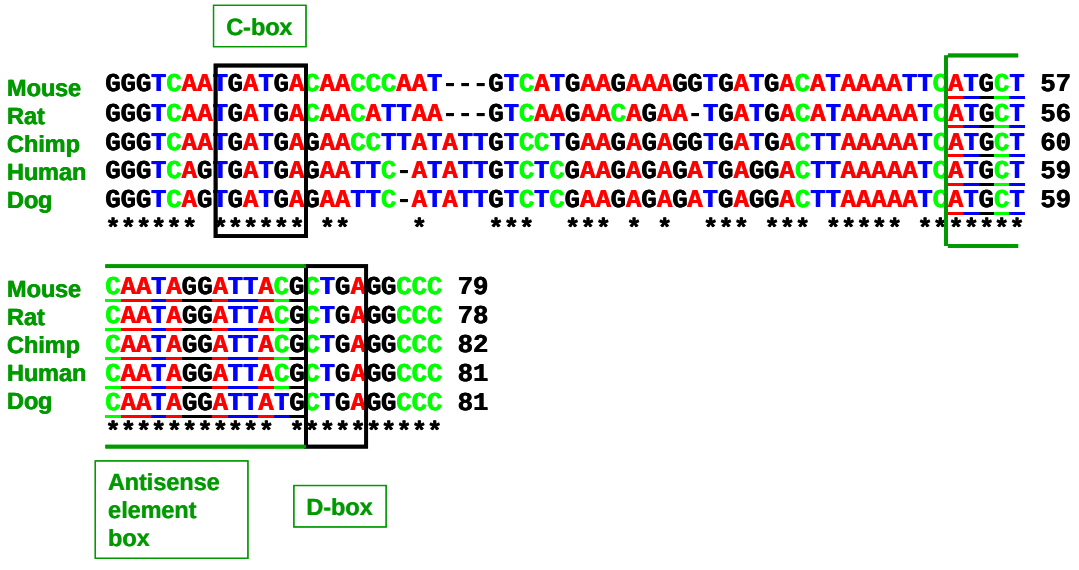


Figure 20. ClustalW sequence alignment of snoRNA HBII-52 across various mammalian species.

position of the 18 nt long antisense element. This exchange still enables G-U wobble base pairing (Varani et al., 2000, Nagaswamy et al., 2000) in the heteroduplex formed by the snoRNA and its mRNA target. In all other mammalian species, there is a perfect 18 nt complementarity. In contrast to the antisense box, other positions of the BII-52 snoRNAs are less conserved. Between the five species, there are nucleotide exchanges at 19 positions, suggesting that the conservation of the antisense-box sequence is functionally relevant. In all

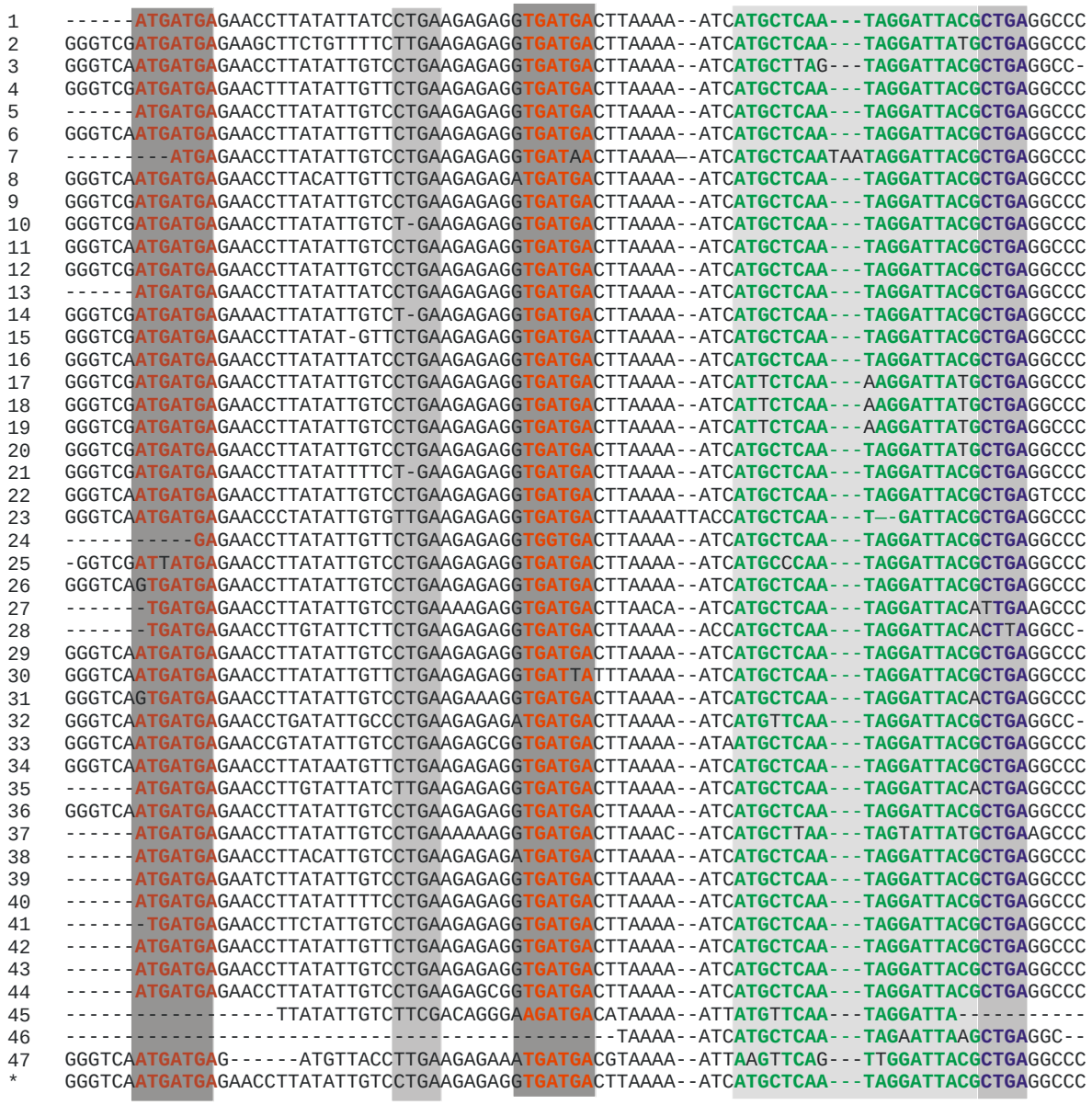


Figure 21. ClustalW sequence alignment showing divergence within 47 copies of HBII-52.

species the complementarity between II-52 snoRNA and exon Vb starts 14 nucleotides downstream of the proximal splice site, whose sequence is fully conserved.

BII-52 snoRNAs are located within the introns and then processed by exonucleases into mature snoRNAs. Interestingly, the neighboring exons from which the snoRNA is excised and processed is not conserved nor is the intronic sequences in which it is embedded.

HBII-52 is arranged in tandem arrays of 47 gene clusters, copies of which are highly similar (~94%) to each other (Figure 21). Even within humans, surrounding sequences in the repeat unit diverge substantially from each other. The organization of snoRNA MBII-52 gene in mouse is similar, indicating its biological importance.

4.2. Alternative splicing of Serotonin receptor is dependent on the HBII-52 expression in brain

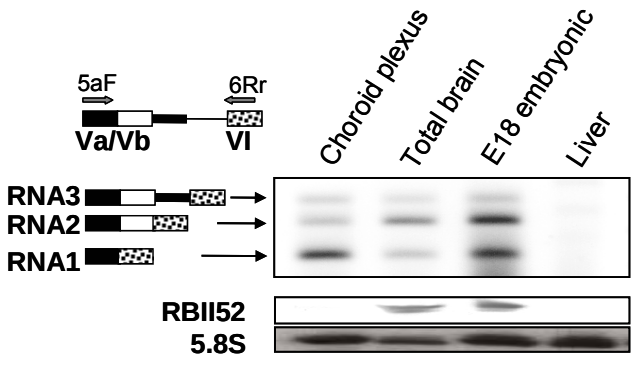


Figure 22. Expression of 5-HT2CR variants and RBII-52 in rat brain. RNA was isolated from the brain parts indicated and analyzed by RT- PCR and northern blot, respectively. The RNA isoforms were detected with RT-PCR using primers 5aF and 6Rr that are schematically indicated on the left. RBII-52 expression was determined by northern blot using 5.8S RNA as a loading control.

To test whether the alternative splicing pattern of the 5-HT_{2C} receptor correlates with the expression of HBII-52 *in vivo*, total RNA was isolated from different areas of the rat brain. RT-PCR of the mRNA was done to analyze the alternative splicing pattern of 5-HT_{2C}R and Northern analysis was done with snoRNA specific primers to detect RBII-52, the MBII-52 ortholog from rat (Figure 22). All the three isoforms; the intron retained variant (RNA3), exon inclusion variant (RNA2) and the exon exclusion variant (RNA1) were detected. Liver as a control showed no expression of 5-HT_{2C}R and RBII-52, both of which are brain specific. 5.8S RNA served as an internal loading control. In agreement with the previously published results, RBII-52 was expressed in all areas of brain except in choroid

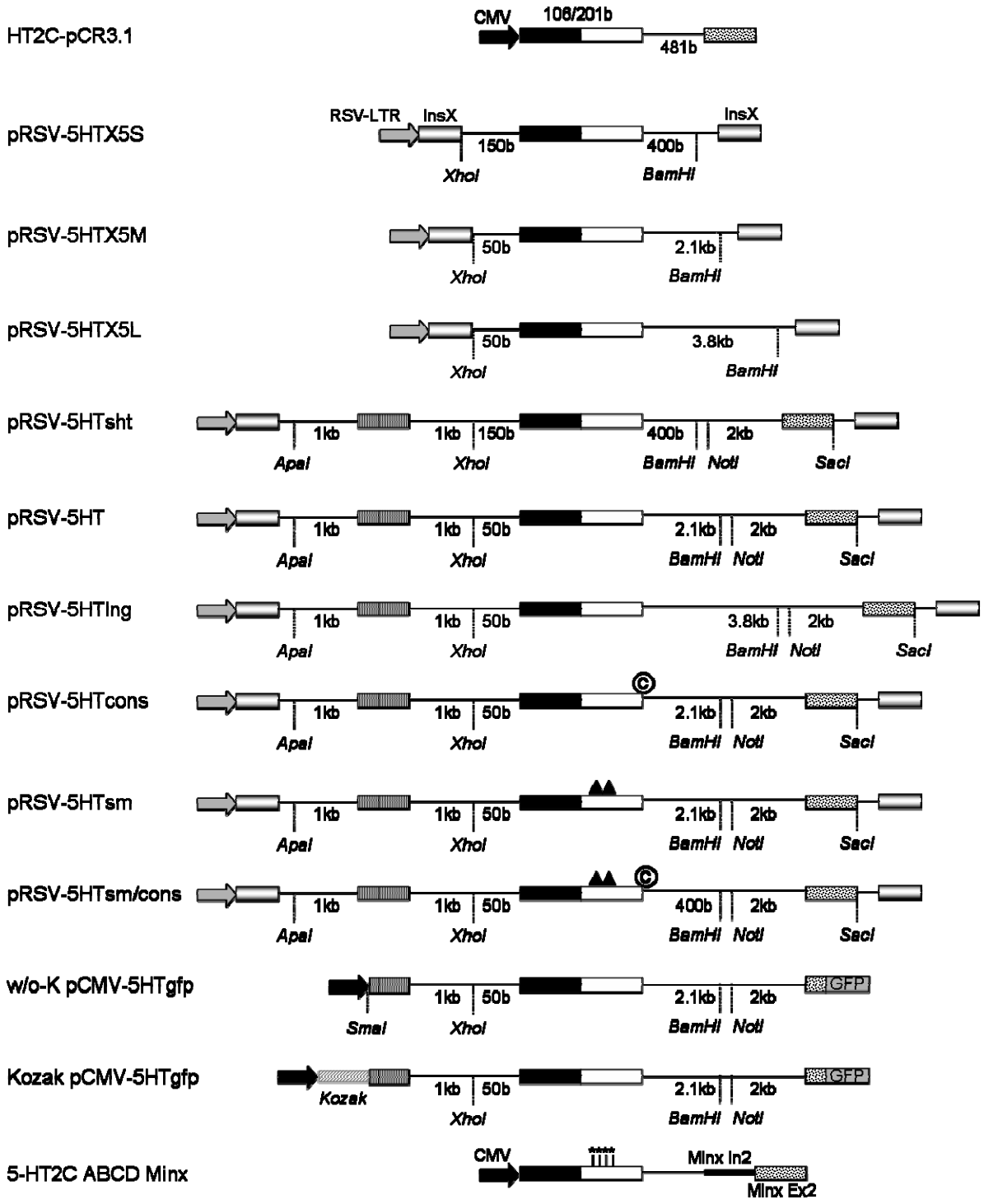


Figure 23. Schematic representation of minigenes used. Exons are shown as boxes, introns as lines. Dashed lines indicate the deletion of large intronic parts. The © indicates the consensus-site mutation of the proximal splice site. Triangles indicate mutations in the antisense and snoRNA complementary box. The features of the minigenes have been detailed in Table 3.

plexus. Surprisingly, Exon Vb is predominantly excluded in choroid plexus where snoRNA RBII-52 is absent. In choroid plexus, the non-functional RNA1 variant is predominant, whereas, in all other brain regions where RBII-52 is expressed, the ratio of functional exon Vb inclusion variant RNA2 over the inactive RNA1 is reversed.

4.3. Minigene Constructs and in vivo splicing assays

To study the role of HBII-52 on the alternative splicing of serotonin receptor 2C pre-mRNA *in vivo*, a minigene approach was employed. Minigenes have proved to be extremely robust and reproducible method to test unknown factors for their involvement in alternative splicing, as well as to study regulatory elements, signal transduction pathways and basic splicing patterns of genes of interest (Tang et al., 2005). This approach involves transfection

Table 3. 5-HT_{2C}R minigene constructs used in the study with their characteristic features.

	5-HT_{2C}R constructs	Features
1	HT2C-pCR3.1	Various 5-HT_{2C}R MG differing in the lengths of their intervening introns.
2	pRSV-5HTsht	
3	pRSV-5HT	
4	pRSV-5HTX5S	
5	pRSV-5HTX5M	
6	pRSV-5HTX5L	
7	pRSV-5HTlng	
8	pRSV-5HTcons	5-HT_{2C}R MG where the distal splice site of the alternatively spliced exon is activated by mutating it to a mammalian consensus
9	pRSV-5HTsm	5-HT_{2C}R MG where HBII-52 complementarity site has been mutated
10	pRSV-5HTsm/cons	5-HT_{2C}R MG where activation of distal splice site (7) and mutations in HBII-52 complementary site (8) are combined.
11	5-HT_{2C} ABCD Minx	5-HT_{2C}R MG with Adenosines at the editing sites A-D in the exon Vb mutated to Guanosine. It is a chimeric construct with the exon Vb followed by a partial intron V and then fused to intron 2 and exon 2 of the Minx gene.
12	w/o-K-pCMV-5-HTgfp	5-HT_{2C}R MG without Kozak sequence in a GFP tagged vector. It was designed to attach MS2 binding sites to detect the localization of receptor RNA.
13	Kozak-pCMV-5-HTgfp	5-HT_{2C}R MG with Kozak sequence in a GFP tagged vector. Protein translated from the Vb exclusion variant is out of frame to C-terminus GFP whereas protein translated from the inclusion variant fluoresces.

of a reporter minigene in eukaryotic cell lines, with one or more splicing regulators. The levels of regulatory factors can be elevated by cotransfecting an increasing amount of cDNA expressing the factor. Equal amounts of total DNA is transfected in all the samples to avoid any "promoter squelching". The effect of the regulatory factors on the minigene is analyzed by performing RT-PCR on the isolated RNA.

The alternatively spliced exon of the human 5-HT_{2C}R gene is flanked by large introns; around 117kb and 58kb on the 5' and 3' end respectively. These large introns are not conserved across species and host several *Alu* repeats. Though the role of these large introns is not clear but it is very likely that they host some splicing regulatory elements. Several minigenes with differences in the size of intervening introns were designed to study the alternative splicing of the serotonin receptor 2C. Various other serotonin receptor constructs that were designed for the study are shown in Figure 23. The features of all the constructs have been detailed in Table 3.

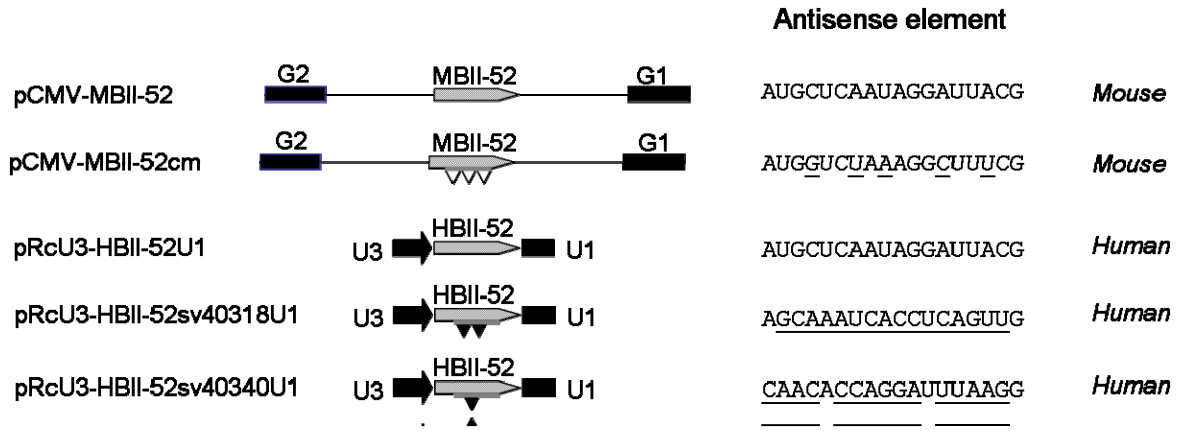


Figure 24. Schematic representation of HBII-52 expression constructs. Exons are shown as boxes, introns as lines. Dark arrows represent U3 promoter. Triangles indicate mutations in the antisense element. MBII-52 constructs harbor the mouse orthologue of HBII-52, which is identical in sequence. All other constructs contain human sequences. The sequences of antisense element for each construct have been shown and the mismatches within this element are underlined.

Figure 24 lists all snoRNA BII-52 constructs used in following experiments. pCMV-MBII-52 contained the natural G2/G1 exons as in mouse genomic DNA with MBII-52 being imbedded in the intron (Figure 25). This allows processing of the intron and release and packaging of the snoRNA MBII-52 as *in vivo*. pCMV-MBII-52cm contained a compensatory mutation in the antisense box such that the artificial snoRNA targets the complementary site mutant pRSV-5HTsm minigene. pRcU3-HBII-52U1 contains the HBII-52 snoRNA directly

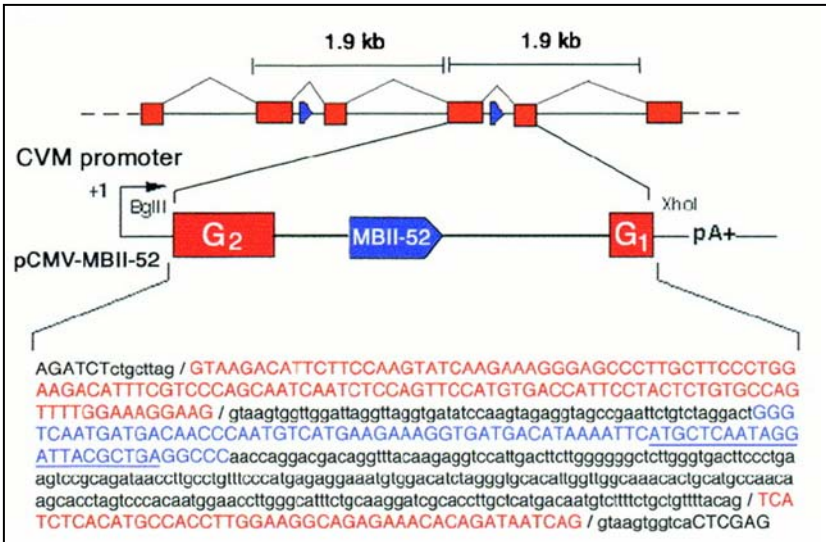


Figure 25. Genomic organization of the tandemly repeated MBII-52 sno RNA genes in mouse. *BglII-XhoI* DNA fragment of the mouse repeat unit was inserted in a eukaryotic expression vector pRCEN downstream of a CMV promter. MBII-52 was processed as a part of the intron. (Picture reproduced from Cavaille et al., 2000.)

transcribed under the control of U3 promoter. Other snoRNA control constructs, pRcU3-HBII-52sc40318U1 and pRcU3-HBII-52sv40340U1 were identical to the pRcU3-HBII-52U1 except that their antisense elements were exchanged with different neutral regions of SV40.

A typical *in vivo* splicing assay was carried out with equal amounts of minigene transfected in the Neuro2A cell line with increasing amounts of the snoRNA construct. Total RNA was isolated after 14-16 hours of transfection. RT-PCR was performed and the splicing pattern was analyzed. Figure 26 enumerates the splicing pattern of individual minigenes in the absence of any snoRNA construct. All the wild type minigenes used the proximal 5' splice site to give the short inactive variant indicating the minimal role of the intron downstream of the alternatively spliced exon. When the distal 5' splice site of the alternatively spliced exon was mutated to the U1 consensus splice site (pRSV-5HTcons), the

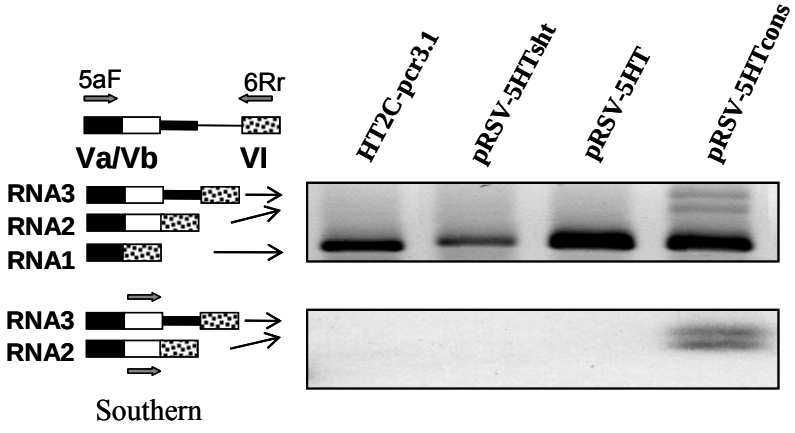


Figure 26. *In vivo* splicing of the reporter minigene constructs in Neuro2A cells. RT-PCR with primers 5aF and 6Rr detects Exon Vb inclusion only with the construct where distal splice site is activated. Inclusion variant was confirmed with Southern blot using oligo specific to exon Vb.

minigene spliced into both the variants though the exon exclusion variant was still predominant. Surprisingly, a third band, equivalent to the intron retained variant could also be consistently amplified. This fragment however could not be cloned for sequencing and also was not detected by southern blot using intron specific primer. Possibly, it is a PCR artifact or a heterodimer.

4.4. snoRNA HBII-52 can influence the alternative splicing of the serotonin receptor 2C in a concentration dependent manner

To analyse the influence of HBII-52 on the alternative splicing of the 5-HT_{2C}R pre-mRNA, cotransfection experiments with splicing reporters were performed. Preliminary cotransfection experiments were performed with pRSV-5HT minigene which contained exons IV, Va, Vb and VI. The intron between exons Vb and VI spanned 3100 nt in the minigene. It was composed of 2.1 and 1 kb from each flanking side. Since the snoRNA MBII-52 derived from mouse and human derived HBII-52 were identical with respect to the major elements, the mouse orthologue MBII-52 was used as a source of snoRNA. The snoRNA was expressed from the construct pCMV-MBII-52 generated from a genomic fragment of the mouse IC-SNURF-SNRPN locus. It contained MBII-52 and two flanking exons. Cotransfection experiments of wild type pRSV-5HT with pCMV-MBII-52 alone or in various combinations of pCMV-MBII-52 and human ADAR1 and ADAR2 constructs showed no inclusion of the alternatively spliced exon (Figure 27).

Increasing amount of the pCMV-MBII-52 construct was then transfected in Neuro2A cells and MBII-52 expression was detected by Northern blot. Northern blot analysis showed that transfection of an increasing amount of pCMV-MBII-52 resulted in an increase of MBII-

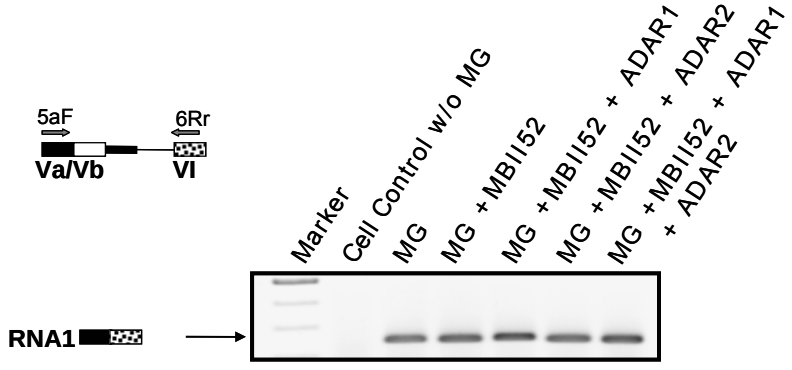


Figure 27. In-vivo splicing assays with the pRSV-5HT minigene cotransfected with various combinations of snoRNA MBII-52 and the editing enzymes ADAR1 and ADAR2. The wild type minigene splices exclusively using the proximal splice site generating only the Exon Vb skipped varaint.

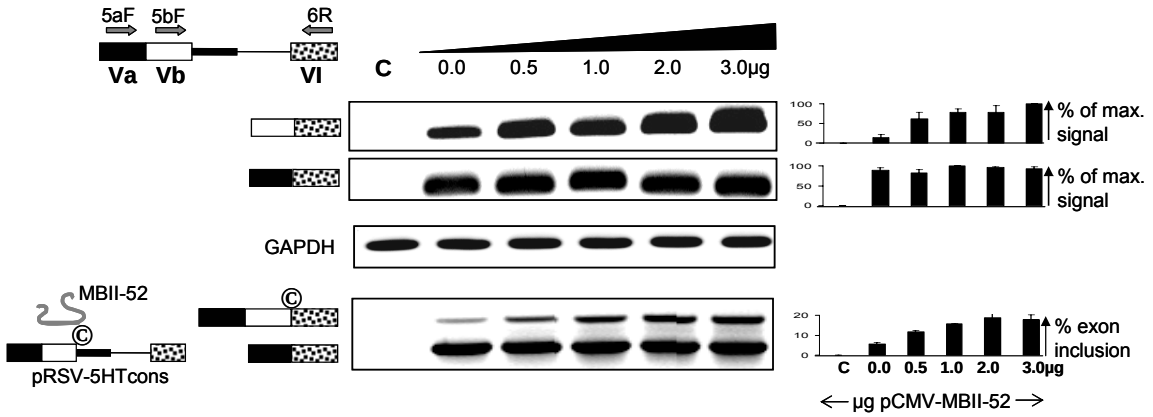


Figure 28. Cotransfection of increasing amounts of pCMV-MBII-52 with the wild type pRSV-5HT and activated pRSV-5HTcons reporter minigenes in Neuro2A cells. Numbers indicate the µg of transfected plasmid. For the wild type minigene pRSV-5HT exon-specific primer pairs 5aF/6R and 5bF/6R were used, for the activated minigene pRSV-5HTcons primers 5aF and 6R that amplify both forms were used. The ethidium bromide stained gels show representative experiments. The quantification of at least four experiments is shown on the right side. The experiments performed with the wild-type minigene pRSV-5HT were quantified by setting the maximum signal to 100%. The ratio between exon inclusion and exon skipping was calculated to quantify the results from the minigene pRSV-5HTcons with the activated proximal splice site. The increase in MBII-52 expression is shown on the bottom by northern blot. C indicated the PCR control without reverse transcription. The structure of the minigene and the resulting PCR products is schematically shown on the left.

52 snoRNA of the expected size. pRSV-5HT reporter minigene was then cotransfected together with an increasing concentrations of pCMV-MBII-52. The mRNA was analysed by RT-PCR.

Using primers 5aF and 6R located in the exons V and VI, only the mRNA isoform lacking exonVb (RNA1) was amplified. However when isoform specific primer 5b, located in Exon Vb was used, inclusion variant (RNA2) could be detected in assay (Figure 26). MBII-52 clearly showed a statistically significant increase ($p<0.0002$, $t=22.4$) in the RNA2 isoform in a concentration dependent manner although the concentration of the RNA2 spliced variant was totally overshadowed when detected along with the major RNA1 isoform. Similar results were obtained with the HBII-52 constructs expressed directly under the U3 promoter. The mutant HBII-52 constructs with the antisense elements exchanged showed no increase in the inclusion variant with the increase in the mutant HBII-52 (Figure 29). Henceforth, for all further experiments MBII-52 was used, as MBII-52 and HBII-52 showed identical response to the human pRSV-5HT minigene.

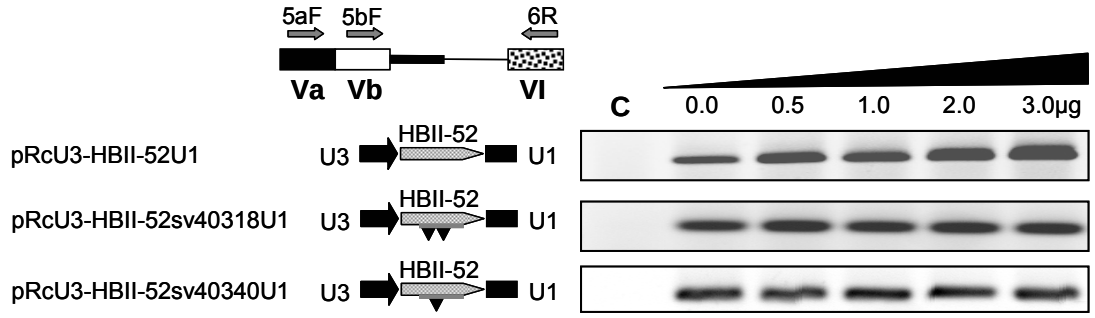


Figure 29. Cotransfection of increasing amounts of HBII-52 constructs with the wild type pRSV-5HT in Neuro2A cells. Numbers indicate the µg of transfected plasmid. RT-PCR using exon-specific primer pairs 5bF/6R were used to detect the ExonVb inclusion. C indicated the PCR control without reverse transcription. The structure of the HBII-52 expressign constructs are schematically shown on the left. pRcU3-HBII-52U1 expresses HBII-52 directly under U3 promoter. Other HBII-52 control constructs pRcU3-HBII-52sv40318U1 and pRcU3-HBII-52sv40340U1 are identical to pRcU3-HBII-52U1 except in their antisense element, which is replaced by sequence from viral SV40 DNA.

It has been earlier shown that neuron specific exons fail to splice efficiently when reporter constructs have been used in the immortalized cell lines, most likely due to a unique combination of splice factors present in the differentiated neurons. Hence, the distal splice site of the minigene was activated by mutating it (TAG/GTAAAT) to a mammalian U1 consensus (CAG/GTAAGT). Cotransfection experiments with the activated consensus mutant (pRSV-5HTcons) still showed that exon Vb was predominantly excluded. However, cotransfection of the snoRNA expressing construct pCMV-MBII-52 caused now an increase of exon Vb usage that could be detected by using primers 5aF/6R located in the flanking exons Va and VI. Since these primers amplified both isoforms that either include or exclude exon Vb, this experiment allowed the direct comparison of the products. An increase in MBII-52 showed a four fold increase (5% to 20%) in the Exon Vb included variant (RNA2) over Exon Vb skipped variant (RNA1), which was statistically significant ($p<0.0001$, $t=11.45$).

It was also confirmed that there was no change in the endogenous alternative splicing pattern of unrelated Tra2beta and YT521-B, suggesting that binding to a target sequence is necessary for HBII-52 function. In addition, the effect due to MBII-52 was not because of general triggering of the cellular splicing machinery (Figure 30).

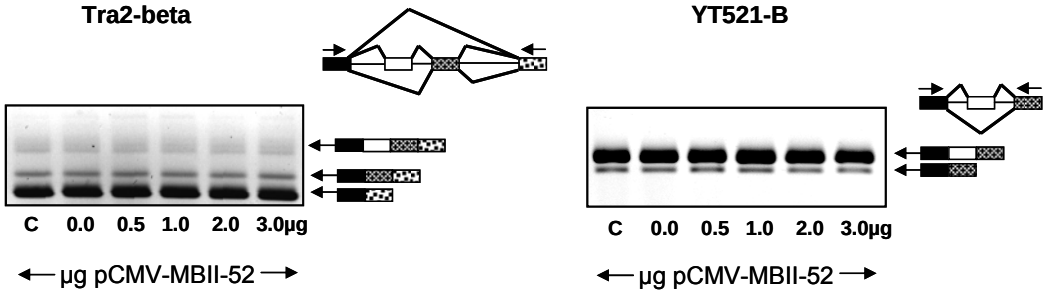


Figure 30. MBII-52 does not change alternative splicing patterns of genes showing no sequence complementarity. An increasing amount of MBII-52 expression construct was transfected into HEK293 cells and the alternative splicing patterns of endogenous genes (Tra2Beta and YT-521B) analysed by RT-PCR. No changes in the splicing pattern were observed. C indicated the PCR control without reverse transcription.

4.5. HBII-52 influences 5-HT$_{2C}$R alternative splicing through complementarity in its antisense box

To investigate whether the complementarity between the snoRNA and 5-HT$_{2C}$R was the sole determinant for the snoRNA mediated alternative splicing of 5-HT$_{2C}$R Exon Vb, five out of eighteen nucleotides of antisense element of MBII-52 were mutated by overlap extension. The antisense box of the resulting clone pCMV-MBII-52cm contained ATG GTC TAA AGG CTT TCG in place of ATG CTC AAT AGG ATT ACG. Similarly, using the same mutation strategy, a compensatory mutation was introduced in the complementary box of the 5-HT$_{2C}$R. The new compensatory mutant pRSV-5HTsm contained CGA AAG CCT TTA GAC CAT changed to CGT AAT CCT ATT GAG CAT in its complementary element.

In vivo cotransfection studies showed that wild type minigene pRSV-5HT had no change in alternative splicing with the mutant pCMV-MBII-52cm (Figure 31A). Also, the mutant pRSV-5HTsm had no effect with the wild type pCMV-MBII-52 (Figure 31B). Surprisingly, when both compensatory mutant constructs, pRSV-5HTsm and pCMV-MBII-52cm were cotransfected, exon Vb inclusion was promoted with the increase in the mutant snoRNA (Figure 31C). This demonstrates that the alternative splicing of the serotonin receptor 2C was clearly a result of the base pairing capabilities of the snoRNA with the receptor.

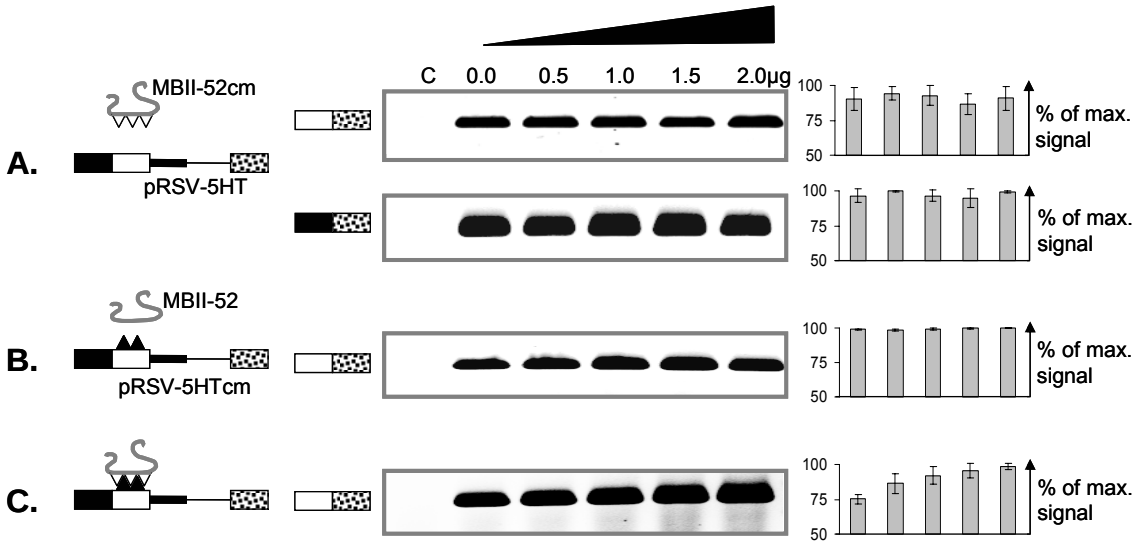


Figure 31. Complementarity drives the HBII-52 mediated Exon Vb inclusion of 5-HT2CR minigene. The constructs schematically shown on the left were cotransfected in Neuro2A cells. Representative ethidium bromide stained gels are shown on in the middle and their statistical evaluation on the right. For the evaluation, the signal with the strongest intensity was taken as 100% and and all other signals were expressed as its percentage. Error bars indicate the standard deviation from at least 5 experiments. **(A).** Upper panel: Cotransfection of the pRSV-5HT and a MBII-52 expression construct (MBII-52cm) whose antisense box is mutated from ATG CTC AAT AGG ATT ACG to ATG GTC TAA AGG CTT TCG. The signal corresponds to the splicing of exon Vb with VI. The signal in the lower panel corresponds to the splicing of exon Va with VI. **(B).** Cotransfection between the compensatory mutation in pRSV-5HT (pRSV-5HTsm), where the antisense box binding region was mutated from CGT AAT CCT ATT GAG CAT to CGA AAG CCT TTA GAC CAT and a wild type MBII-52 expression construct. **(C).** Cotransfection of both compensatory mutants.

Since both the wild type pRSV-5HT and compensatory mutant pRSV-5HTsm minigenes spliced only at the proximal 5' splice site, exact comparison could not be made between the inclusion and the exclusion variant in the same experiment. To facilitate simultaneous detection of both the isoforms, the activated pRSV-5HTcons minigene was used instead of pRSV-5HT. For the compensatory mutant minigene, pRSV-5HTsm was as well activated by mutating its distal 5' splice site to a consensus U1 splice site (pRSV-5HTsm/cons). Both the Exon Vb inclusion and the exclusion variant could now be simultaneously detected and compared in the single assay when the activated minigenes, pRSV-5HTcons and pRSV-5HTsm/cons were used. The new constructs gave similar results as the ones obtained previously. Alternative splicing of pRSV-5HTcons had no effect with

the mutant pCMV-MBII-52cm (Figure 32C). Also, the compensatory mutant receptor minigene pRSV-5HTsm/cons had no effect with the wild type pCMV-MBII-52 construct (Figure 32B). The effect was restored, though to a lesser degreee as against the non activated minigene, when the mutant minigene pRSV-5HTsm/cons was transfected with the mutant pCMV-MBII-52cm containing the compensatory mutation in the snoRNA antisense element (Figure 32A).

Both the results indicate that HBII-52 exerts its effect on the alternative splicing of the serotonin receptor 2C through base pairing. Hence, the guiding antisense box of the snoRNA is crucial for the HBII-52 to function *in vivo*. Interestingly, in pRSV-5HTsm/cons minigene, when both the mutations are combined, the ratio of the spliced isoforms is reversed. pRSV-5HTsm/cons minigene splices predominantly into Exon Vb inclusion variant.

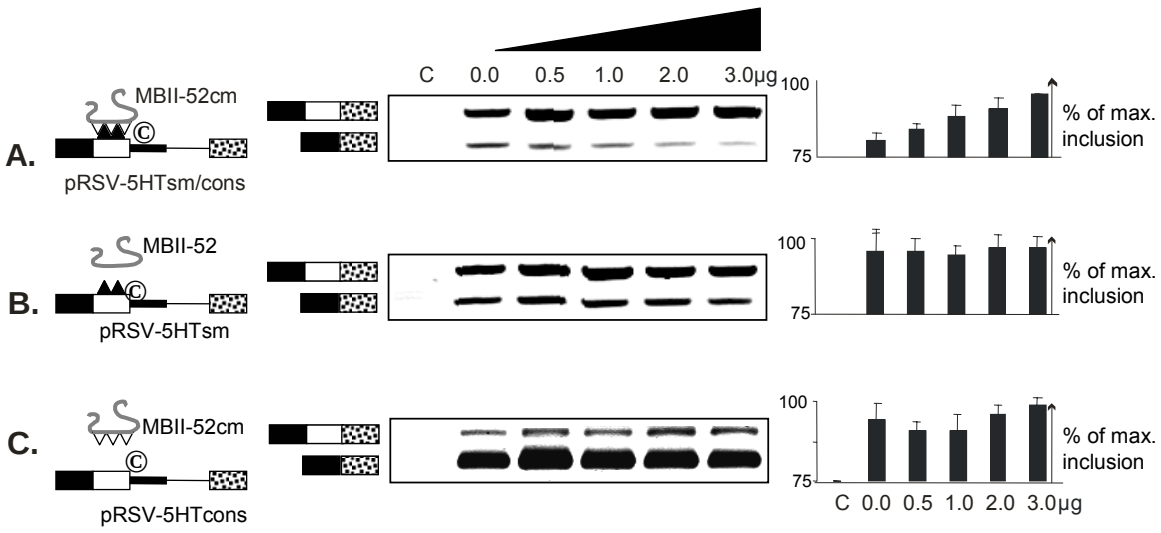


Figure 32. Influence of snoRNA complementarity on 5-HT2CR exon Vb usage. The constructs schematically shown on the left were contransfected in Neuro2A cells. Representative ethidium bromide stained gels are shown on the right. **(A).** Transfection of an increasing amount of the MBII-52cm expression construct with the pRSV-5HTcons minigene. The antisense box of MBII-52cm is mutated from ATG CTC AAT AGG ATT ACG to ATG GTC TAA AGG CTT TCG. **(B).** Transfection of an increasing amount of the wild type MBII-52 expression construct and the pRSV-5HTsm/cons minigene where the antisense box binding region was mutated from CGT AAT CCT ATT GAG CAT to CGA AAG CCT TTA GAC CAT and a wild type MBII-52 expression construct. **(C).** Transfection of an increasing amount of the MBII-52cm expression construct with the pRSV-5HTsm/cons minigene. The mutations are compensatory and both RNAs exhibit sequence complementarity.

4.6. HBII-52 binds to the serotonin receptor on a putative silencer sequence

It is surprising that Exon Vb is predominantly skipped both in case of the wild type pRSV-5HT and the activated pRSV-5HTcons minigene. Whereas, the splicing pattern dramatically reverses (Figure 33) upon combining both the mutations; the mutations in the

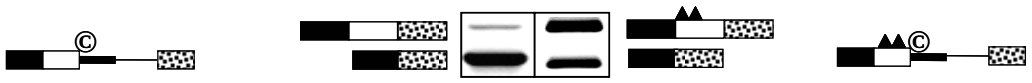


Figure 33. The snoRNA complementarity region of 5-HT2CR harbors silencing activity that is influenced by snoRNA binding. Constructs with the activated distal splice site harboring the wild type or mutated snoRNA complementarity box were transfected in Neuro2A cells. The mutation is schematically indicated by triangles on top of exon Vb. The complementarity box was mutated from CGT AAT CCT ATT GAG CAT to CGA AAG CCT TTA GAC CAT. The structures of the PCR products are schematically indicated on the sides of the ethidium bormide stained gel, depending on which minigene has been used (indicated on sides).

snoRNA binding region and the activation mutation in the distal 5' splice site. Inspection of the snoRNA complementarity region in exon Vb shows the presence of several putative silencing elements that were recently identified in an exon silencer screen (Wang et al., 2004). The sequence overlapped with two potential silencer elements (Figure 34).

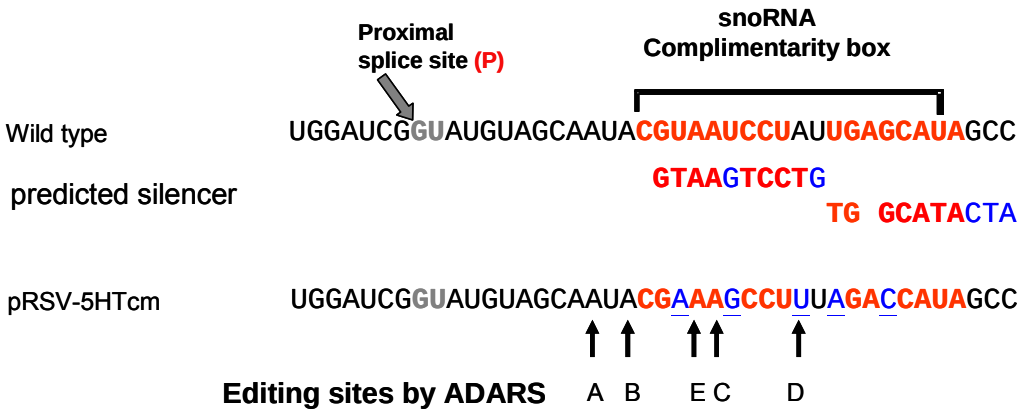


Figure 34. The snoRNA complementarity box contains predicted splicing silencer sequences. The wild type sequence is shown on the top. The proximal splice site and the snoRNA complementarity box are indicated by a box. The GU in bold is the 5' splice site. Arrows indicate A-E editing sites. The next two lines show experimentally verified splicing silencers. The fourth line shows the location of the mutations in the silencer used to generate the silencer mutation construct pRSV-5HTsm. Mutated nucleotides are underlined.

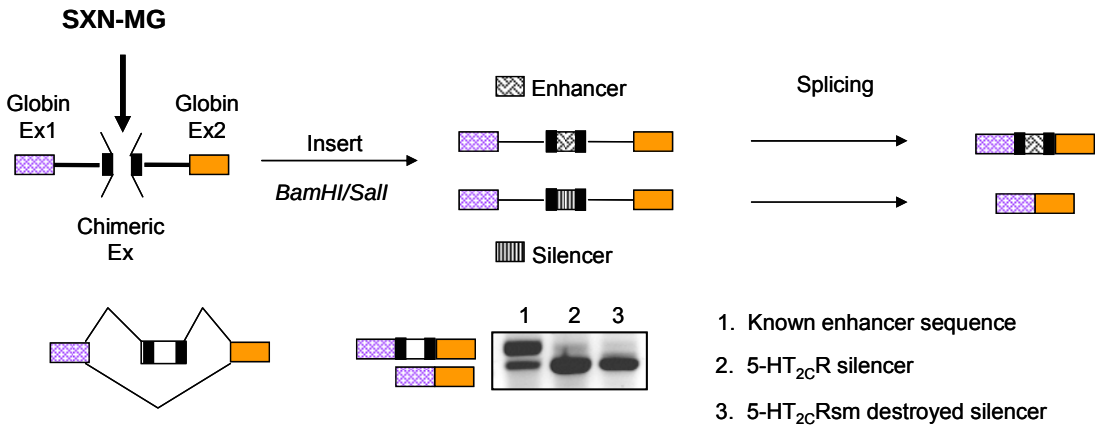


Figure 35. Experimental verification of the silencing element in the complementarity region of 5-HT2CR. The 18 nt snoRNA complementarity region was introduced into the alternative exon of the SXN, a beta-globin based reporter construct (shown on top). Known enhancer element, complementarity box, and mutant in the complementarity box containing constructs were transfected into Neuro2A cells and analyzed by RT-PCR.

The isoforms ratio reversal is probably because of the destruction of a regulatory element, most likely a silencer. Intriguingly, the sites that were mutated did not overlap with the sites edited by ADARs. Editing by ADARs as well promote exon inclusion, with multiple editing favoring more inclusion (Flomen et al., 2004). Conclusively, editing by ADARs as well destroys the silencer. So, it is not the editing at particular sites or specific change from Adenosine to Guanosine that favors exon inclusion, but any change that alters the binding potential of regulatory factors can equally elevate exon Vb usage.

To experimentally confirm that MBII-52 binding region harbors a silencing activity, the sequence was cloned using a *SalI* and *BamHI* restriction site, within a short exon of SXN globin-based reporter system. This SXN minigene is a chimeric construct with a short middle exon, flanked on either side by globin exons (Figure 35). The construct is designed to identify the nature of regulatory element cloned within the middle exon. An enhancer cloned within the middle exon will promote exon inclusion while a silencer would suppress it.

In vivo splicing analysis reveals that the MBII-52 binding sequence suppresses the exon inclusion, suggesting its silencing nature. *In vivo* splicing assays with the SXN minigene containing the predicted 5-HT_{2C}R silencer element and increasing levels of MBII-52 was carried out to determine if MBII-52 could mask the silencer activity in a heterologous

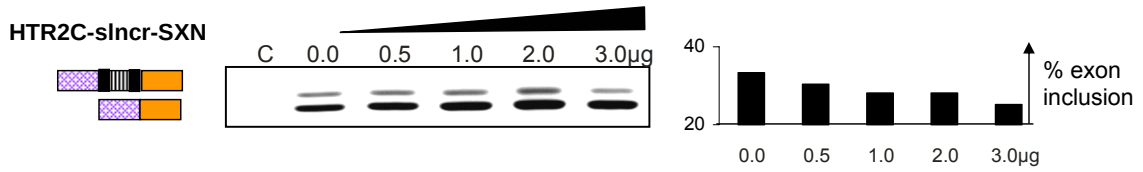


Figure 36. Cotransfection of SXN minigene containing the 5-HT$_{2C}$R complementarity element with increasing amounts of MBII-52.

system. Surprisingly, the exon inclusion, decreased upon MBII-52 addition suggesting a more complicated mode of HBII-52 interaction with its target sequence (Figure 36).

4.7. HBII-52 mutants at C- or D box have no effect on the alternative splicing of 5-HT$_{2C}$R

C/D box play an important role in the structure and function of the C/D box snoRNAs. The mature 5' and 3' termini of many C/D box snoRNAs pair to form a short duplex which together with boxes C and D comprises the terminal core motif. The C/D core motif promotes binding of proteins, such as fibrillarin, Nop56p, Nop58p and the 15.5 kD protein which form the snoRNP. Disruption of the C/D box motif prevents binding of these proteins to snoRNA and prohibits formation of a snoRNP. This motif is also a requirement for both snoRNA processing and nucleolar localization. It has been shown previously that

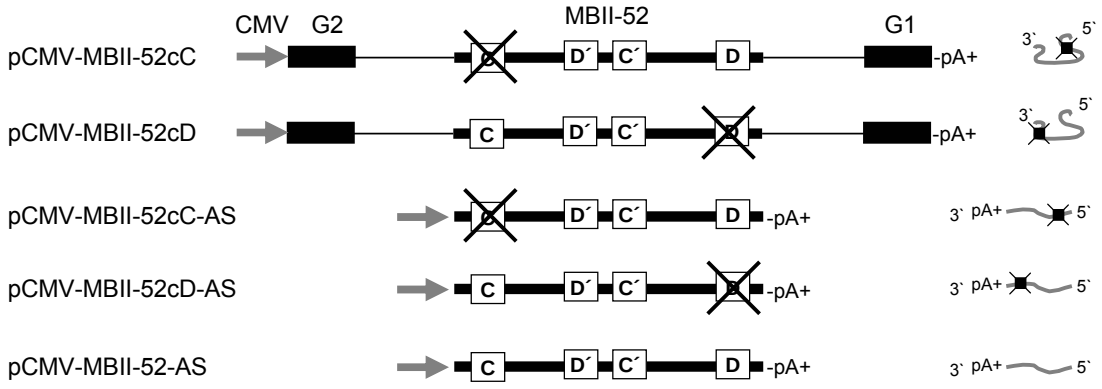


Figure 37. Schematic representation of the C/D box mutant MBII-52 constructs. pCMV-MBII-52cC has mutation ATGATGA to AGTTAAG in its C Box while pCMV-MBII-52cD has the D-Box changed from CTGA to ACGT. pCMV-MBII-52cC-AS, pCMV-MBII-52cD-AS and pCMV-MBII-52-AS are similar to pCMV-MBII-52cC, pCMV-MBII-52cD and pCMV-MBII-52 respectively in their snoRNA sequence but they are not intronically imbedded. They are expressed directly under a CMV promoter with a polyadenylation tail.

any disruption in the C/D motif can lead to their inefficient nucleolar localization and subsequent degradation.

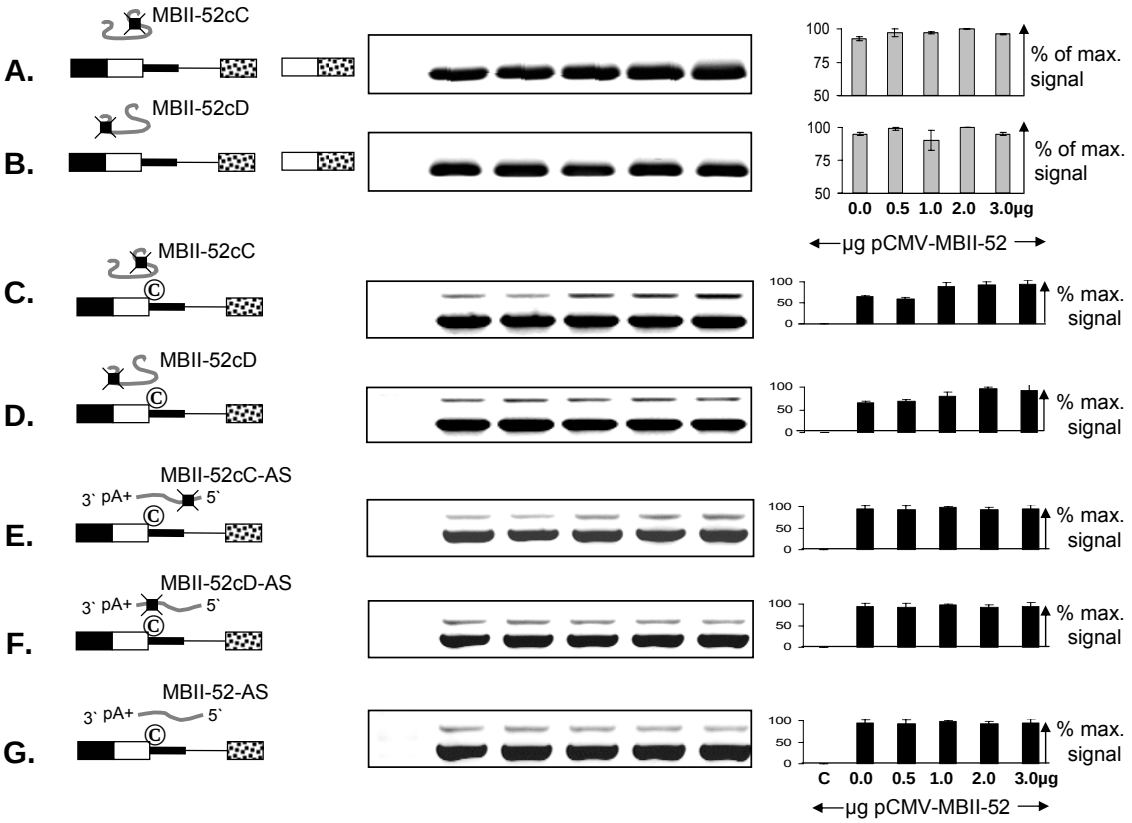


Figure 38. An intact snoRNP that binds to exon Vb is necessary to change 5-HT2CR splice sites. **(A).** Transfection of an increasing amount of MBII-52cC and the pRSV-5HT minigene. **(B).** Transfection of an increasing amount of MBII-52cD and the pRSV-5HT minigene. **(C).** Transfection of an increasing amount of MBII-52cC expression construct and the pRSV-5HTcons minigene. **(D).** Transfection of an increasing amount of MBII-52cD and the pRSV-5HTcons minigene. **(E).** Transfection of an increasing amount of MBII-52cC-AS and the pRSV-5HTcons minigene. **(F).** Transfection of an increasing amount of MBII-52cD-AS and the pRSV-5HTcons minigene. **(G).** Transfection of an increasing amount of MBII-52-AS and the pRSV-5HTcons minigene. "AS" in the snoRNA constructs stands for antisense, where the constructs produce a stable antisense RNA without snoRNP formation.

To rule out non-specific effects from the transfection, we mutated the C and D boxes of HBII-52 (constructs MBII-52cC and MBII-52cD respectively). Separate mutants targeting the C and D box of snoRNA MBII-52 respectively, were constructed using overlap extension method. The C-Box mutant snoRNA pCMV-MBII-52cC had its C-Box exchanged from ATGATGA to AGTTAAG whereas; the D-Box mutant pCMV-MBII-52cD had its D box mutated from CTGA to ACGT (Figure 37). Simiarly, wild type and mutant C/D boxed

snoRNA constructs were made where the snoRNA was transcribed directy under the control of a CMV promoter and with a polyAdenylation signal in the end. Such contructs would not be processed like a snoRNA to form snoRNP but would only serve as stable antisense transcripts. Transfection of such contructs with the minigene could show if the MBII-52 was functional as a snoRNP or mediated its affect through antisense alone.

In vivo splicing assay with the pCMV-5-HT minigene abolishes the effect of snoRNA mediated exon inclusion with either pCMV-MBII-52cC or with pCMV-MBII-52cD. The results are consistent with the activated pCMV-5-HTcons minigene (Figure 38, A-D). This experiment demonstrates that the effect of MBII-52 on the alternative splicing of serotonin receptor 2C is not due to the antisense element alone. The effect is largely due to HBII-52's integrity and its proper nucleolar localization.

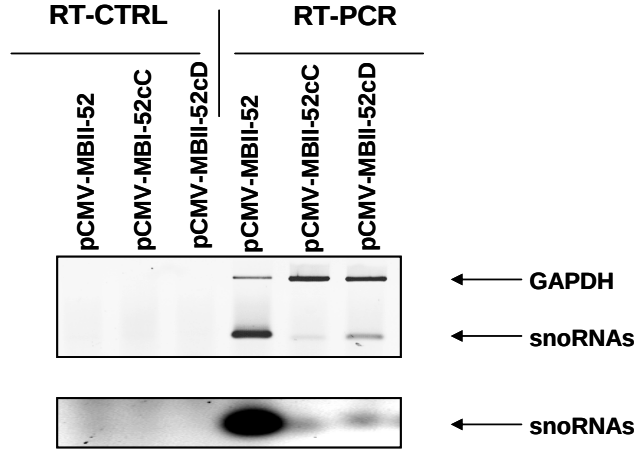


Figure 39. C/D Mutations of MBII-52 express small amounts of snoRNAs. The snoRNAs were detected by RT-PCR after the expression constructs were transfected into HEK293 cells. Primers specific to individual snoRNAs were used. GAPDH was amplified in the same reactions as a loading control. RT-CTRL represents a PCR reaction without reverse transcription. Lower panel shows the RT-PCR of snoRNAs alone.

Since it has been previously reported that any mutations in the conserved C/D motifs of snoRNAs can lead to its instability and immediate degradation, it was confirmed if the C/D box mutant snoRNAs were actually degraded. Equal amounts (4.0 µg) of wild type pCMV-MBII-52, C-Box mutant pCMV-MBII-52cC and D-box mutant pCMV-MBII-52cD constructs were transfected in HEK293 cells and their expression checked with RT-PCR against specific primers. As expected, RT-PCR analysis shows that the level of C/D mutant snoRNAs was drastically reduced when compared to the wild type (Figure 39), indicating the instability of the mutants.

As the C/D box mutants are unstable upon transcription, stable antisense transcripts of MBII-52 and its C/D box mutants were generated directly under a CMV promoter and

with a 3' poly Adenylation tail. Transfection of these stably expressed antisense transcripts with the activated reporter minigene as well showed no effects on the exon inclusion, suggesting that the structural integrity of the MBII-52 snoRNP is essential for the snoRNA mediated exon inclusion (Figure 38 E-G).

4.8. HBII-52 promotes exon inclusion independent of RNA editing mediated by ADARs

It has been previously reported that A->I editing of Serotonin receptor 2C at any one or more editing sites (A to E) can promote exon inclusion. We therefore tested if HBII-52 enhanced exon inclusion by promoting editing cooperatively with ADARs or if it had independent mechanism of governing the exon inclusion. The reporter minigene pRSV-5HT was cotransfected in Neuro2A cells in various combinations with pCMV-MBII-52 and ADARs. The RT-PCR products containing exon Vb sequences were directly sequenced to examine the editing pattern.

As shown in Figure 40, no editing ws observed when minigene was cotransfected with MBII-52 alone. In contrast, editing at D site could be observed when the 5-HT_{2C}R reporter minigene was cotransfected with ADAR2 expressing clones. ADAR1 alone or in combination with MBII-52 did not edit any of the sites. Interestingly, when minigene was cotransfected with ADAR2 and MBII-52, no additional effect was observed. This shows that MBII-52 acts independently of an A->I editing mechanism. Similar results were observed in HEK293 cells. It is noteworthy that as against HEK293 cells, which is kidney derived,

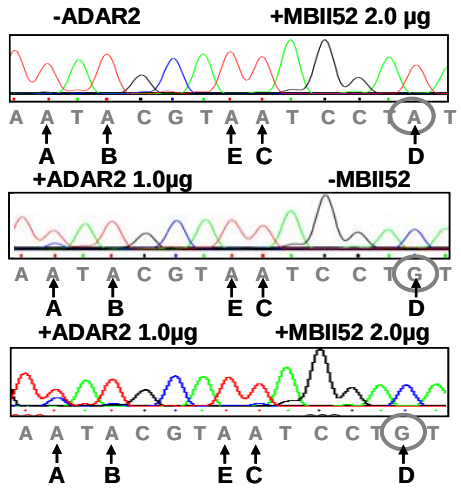


Figure 40. HBII-52 snoRNA promotes inclusion of exon Vb by editing independent mechanism unlike ADARs. HBII-52 snoRNA does not induce A->I editing. pRSV-5HT was cotransfected with MBII-52, or with ADAR2 expression clones, or with both expression clones in Neuro2A cells. Exon Vb sequences were amplified by RT-PCR and directly sequenced. A-D indicates the editing sites. Only in the presence of ADAR2 editing at site D is observed.

Neuro2A cells are derived from brain where the levels of endogenous editing is very high. It has been previously reported that endogenous serotonin receptor undergoes normal editing at all sites in brain derived cell lines. In contrast, the transfected reporter minigenes showed editing only at the D site suggesting a non-native artificial behaviour. However, the data collectively suggests that in addition to the known editing-dependent pathway, exon Vb inclusion can be promoted by HBII-52 snoRNA.

4.9. HBII-52 capture assay

Our experiments imply that HBII-52 binds to exon Vb of the 5-HT_{2C} receptor *in vivo*. Until now, several methods have been described that can allow the identification of the snoRNA methylated nucleotides (Yi-Tao et al., 1997). Due to insensitivity of these assays, many targets have still not been identified. Conventional assays described so far have failed to identify the HBII-52 mediated 2`O-methylation at the C site of 5-HT_{2C}R probably becuase of extremely low efficiency of methylation at the suggested site as against a high background of non methylated receptor RNA.

To show the HBII-52 interaction with the 5-HT_{2C} receptor *in vivo*, an extremely sensitive assay was developed that captures the snoRNA:mRNA complex. The assay has been described in details in the following section (4.9.1).

4.9.1. snoRNA capture assay:

Transfection and Crosslinking: The plasmids expressing the 5-HT_{2C}R and the HBII-52 were transfected in combinations in HEK293 cells that do not express the serotonin receptor 2C and HBII-52 endogenously. HEK293 cells were transfected with calcium phos--phate method as described in the section 3.2.13. Sixteen hours after transfection, the cells were washed with serum free DMEM and trypsinised. Trypsinysed cells were washed separately with serum free DMEM and isotonic PBS. Finally, the cells were resuspended to a concentration of $8*10^9$ cells /ml in appropriate volume of PBS and transferred to a dish such that the depth was 2-3mm. AMT psoralen (Sigma) was added to a final concentration of 0.2 mg/ml and incubated on ice for 10 minutes. The cells were irratiated with UV in the presence of AMT-psoralen to *in vivo* crosslink the interacting RNAs. Psoralen cross linking was done

in dark room on ice under 365nM UV for 30 minutes at 4.5mW/cm^2. Total RNA from the cells was then isolated using TRIzol according to the manufacturer's protocol.

Pull down of crosslinked RNA using Dynal beads: M-280 streptavidin coated dynal beads was prepared and the biotinylated 2'-O methyl modified RNA capture oligonucleotide was immobilized on the beads according to the manufacturer's protocol. The dynal beads were pre-blocked with 1.0 mg/ml final concentration of BSA. Crosslinked 5-HT$_{2C}$R was then affinity purified with exon Va/Vb junction specific biotinylated oligo immobilized on strepdavidin coated dynal beads. For this, total RNA obtained after psoralen cross-linking, was incubated with the dynal beads immobilized with RNA oligonucleotide at 4°C for 30 minutes. The beads were washed with B&W buffer (5 mM Tris-HCL, pH 7.5; 0.5 mM EDTA and 1M NaCl) thrice and captured RNA was isolated using TRIzol according to the manufacturer's protocol and DNAse treated. HBII-52 crosslinked to the 5-HT$_{2C}$R was detected by RT-PCR using a special chimeric oligo which recognized both HBII-52 and the serotonin receptor though the crosslinked junction by binding to the 3' end the HBII-52 snoRNA and the adjacent part of exon Vb (Figure 41).

The chimeric primer amplifies only RNA from a complex of HBII-52 and 5-HT$_{2C}$ exon Vb RNAs, but it does not amplify the individual RNAs (Figure 41, lane 1-2). The complex between HBII-52 and 5-HT$_{2C}$R can only be detected when both 5-HT$_{2C}$ pre-mRNA and MBII-52 snoRNA are present in the same cell and subjected to UV crosslinking (Figure 42, lane 3). Also, no apparent detection of the complex was observed without UV crosslink (Figure 42, lane 4) or when lysates of separately transfected and UV irradiated cells were

Figure 41. Chimeric primer can detect snoRNA in a HBII-52:5HT2CR complex. The sequences of the HBII-52 snoRNA and 5-HT$_{2C}$R RNA surrounding the complementarity region are indicated. The sequence of the C$_R$ primer and the sequence of the biotinylated capture primer are shown and marked by a gray box.

mixed (Figure 42, lane 5). These data provide a direct evidence of an *in vivo* complex between HBII-52 and 5-HT_{2C} exon Vb.

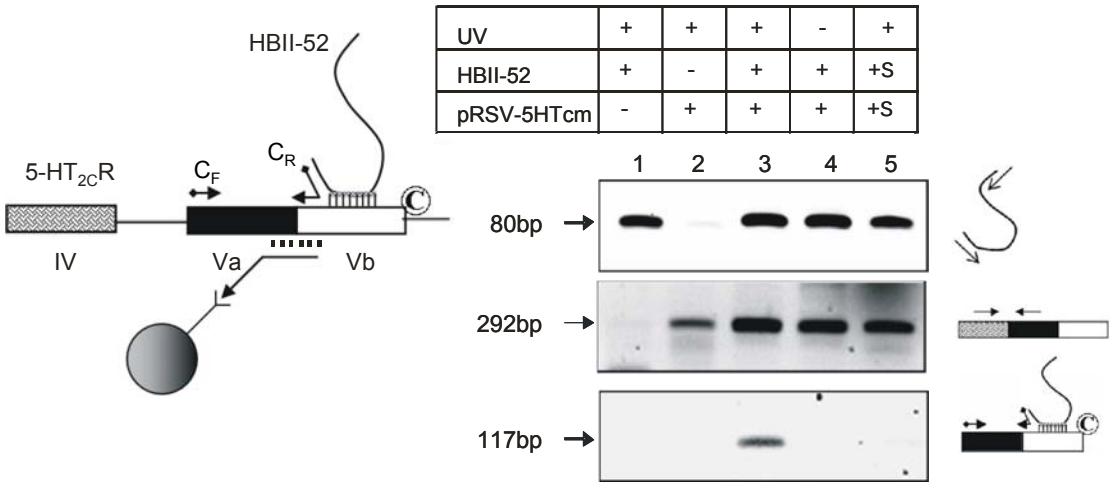


Figure 42. snoRNA capture assay: HBII-52 binds to 5-HT2CR RNA *in vivo*. The experimental strategy is shown on the left: 5-HT2CR nuclear RNA is isolated using a biotinylated oligonucleotide binding to exon Va/b sequences (indicated by dots). The primer pair C_R and C_F specifically amplify complexes between HBII-52 snoRNA and 5-HT2CR RNA, since the primer CR binds only to the complex (see supplemental data S5). The experimental conditions are listed on the right. In lane 5, HBII-52 and 5-HT2CR expression clones were transfected into separate cells, and the resulting lysates were mixed after UV crosslink. The identity of the PCR products is shown on the right (80 nt: HBII-52, 292 nt: 5-HT2CR mRNA, 117 nt: complex-specific amplicon).

4.10. RNAse H assay

To demonstrate the mechanism of HBII-52 mediated alternative splicing, RNAse H protection assay was employed. The enzyme RNAse H, cleaves only the hybrids formed between the RNA and DNA. Figure 43 illustrates the mechanism of RNaseH action.

In the RNAse H protection assay, 5-HT_{2C} receptor RNA was *in vitro* transcribed using α-P^{32}-UTP and capped with tri-methyl-G. Purified labeled and capped 5-HT_{2C}R RNA was added to the HeLa nuclear extract in a typical splicing reaction supplemented with RNAseH. The splicing complex was allowed to assemble on the 5-HT_{2C}R RNA for 10 minutes. Since the HeLa nuclear extract did not contain endogenous levels of MBII-52, the reaction was supplemented with *in vitro* transcribed radio-labeled MBII-52 five times in molar excess than the target RNA. Specific 14mer DNA oligoes were then added to the

reaction to allow binding and subsequent cleavage by RNAse H. The extracted RNA was then run on the gel and analyzed on the phosphoimager (Figure 44).

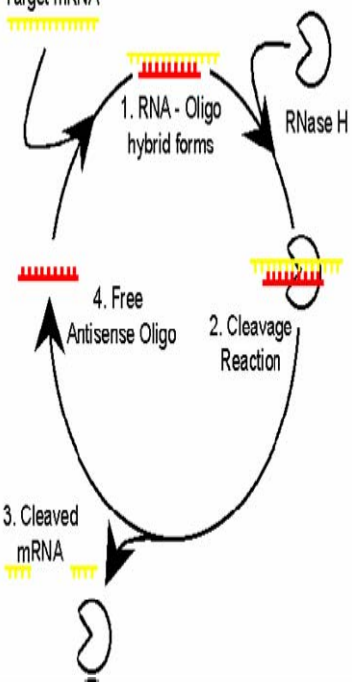


Figure 43. Schematic representation of the basic mode of RNAse H action. RNAse H degrades only a DNA:RNA hybrid.

With increasing amounts of MBII-52, the oligo directed against the snoRNA complementary site on the receptor (sno-cp), showed less and less degradation with RNase H, elucidating that the MBII-52 competes with sno oligo (sno-cp) to bind the complementary site. Since the RNA-RNA hybrid is not cleaved by RNAse H, the targeted receptor mRNA undergoes less degradation. In the absence of MBII-52, the oligo directed against the U1 binding site only partially cleaves the 5-HT_{2C}R mRNA. As the MBII-52 levels are increased, all the mRNA gets cleaved. This indicates that in the absence of MBII-52, U1 occupies the splice site, but as the MBII-52 levels are increased, snoRNA stearically hinders the U1 complex to assemble on the mRNA. Its noteworthy that U1 oligo is much smaller than the U1 complex. So, even when the U1 complex is bumped off, it is still likely that U1 oligo can hybridize to the mRNA.

Complete cleavage was detected with sno oligo, when increasing amounts mutant MBII-52 (MBII-52cm; mutated in the antisense box) was added, indicating that the mutant MBII-52 failed to bind and protect the mRNA from being targeted by sno oligo. This experiment demonstrates that binding of MBII-52 can bump off U1 spliceosomal complex from the proximal 5' splice site and hence facilitate exon inclusion. One major drawback of this experiment is that the experiment could reflect only the antisense effect. C/D box snoRNPs have so far been difficult to be reconstituted *in vitro*. So, it is likely that the experimental effects are largely due to the primary snoRNA structure and not due to the snoRNPs. Nevertheless, this experiment offers one possible mechanism of snoRNA mediated alternative splicing.

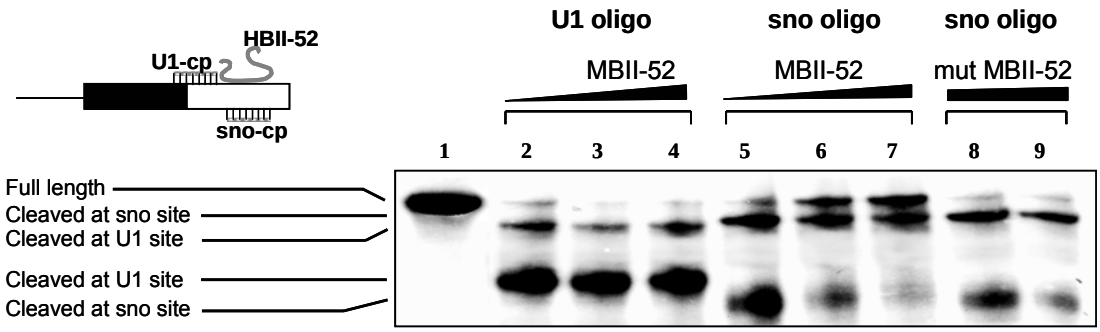


Figure 44. RNAse H assay: HBII52 snoRNA blocks binding of a factor at the proximal site. The RNA construct used is schematically shown on the left. The localisation of the oligonucleotides U1-cp and sno-cp and the HBII-52 binding site is indicated. Lane 1: input RNA, lane 2-4: incubation with U1-cp oligo, addition of snoRNA in lane 3 and 4; lane 5-7: inclubation with sno-cp oligo, addition of snoRNA in lane 6-7; lane 8-9: addition of a snoRNA with a mutated antisense box. The identity of the bands is indicated on the left.

4.11. MBII-52 binding region on 5-HT$_{2C}$R lies at a critical distance for mediating alternative splicing

To determine if HBII-52 influences alternative splicing by stearically hindering the binding of the U1 spliceosomal complex, several artificial snoRNA constructs were made, each directed against a region either upstream or downstream of the actual HBII-52 binding region (Figure 45A). The idea behind this experiment being, artificial snoRNA directed against regions overlapping the U1 binding site will compete with U1 complex in binding to the receptor and hence promote exon inclusion. On the other hand, when the artificially targeted region is moved away from the U1 binding site, the exon would favour exon skipping.

In vivo splicing assays of the artificial HBII-52 constructs with pRSV-5HTcons minigene show that exon inclusion decreased when artificial MBII-52 was targeted on either side of the actual MBII-52 binding site. This indicates that 13nt downstream of the proximal 5' splice site serves as the critical distance for promoting exon inclusion (Figure 45C).

The binding affinity of the artificial snoRNAs to their respective target was measured in terms of their melting point. It was observed that the melting point of the 18nt targeted region of the wild type and all the positional mutants, in general decreased towards the

proximal 5' splice site indicating a possible influence of the target properties on the stability of the MBII-52 interaction (Figure 45B).

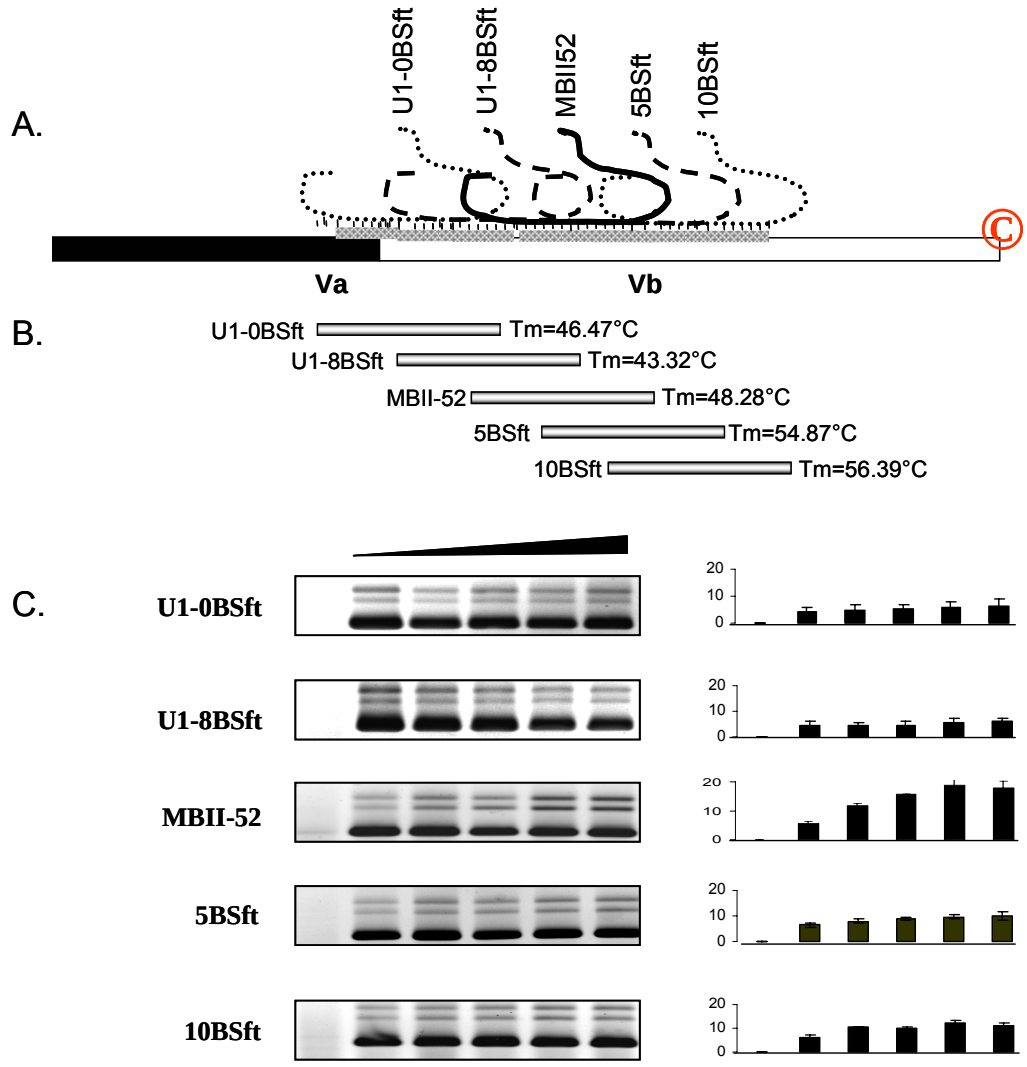


Figure 45. HBII-52 binding region on 5-HT2CR lies at a critical distance for mediating alternative splicing. (A). Several MBII-52 mutant constructs targeting regions either upstream or downstream of the actual MBII-52 binding region are schematically shown. U1-0BSft targets U1 spliceosome binding region (starting 4 nt downstream of Proximal splice site). U1-8BSft target starts 9 nt downstream of Proximal start site. 5BSft and 10BSft target regions start 19nt and 24nt downstream from the Proximal splice site of the 5-HT2CR respectively. (B). Binding affinities of the mutant snoRNA to the various 5-HT2CR targets are calculated as a measure of the melting points of the complementarity regions. (C). Transfection of increasing amounts of mutant and wild type MBII-52 snoRNAs with the activated pRSV-5HTcons construct.

4.12. Artificially targeted snoRNAs can influence alternative splicing in heterologous systems

Artificial snoRNA constructs were designed by exchanging the antisense box of MBII-52 with a guide sequence that targets various regions in the adenoviral E1A minigene close to the alternative 5' splice site. The E1A minigene contains three alternative 5' splice site and two alternative 3' splice sites. The various combinations of all these splicing signals can generate six splice variants, namely, 9S, 10S, 11S, 12S, 13S and the unspliced. Artificial snoRNAs were made by over lap extension in which the antisense element was directed against the fourth exon, 13 nucleotides downstream to the 5' alternative splice site. This exactly mimicked the MBII-52:5-HT_{2C}R system. Another similar construct was made where the mutant snoRNA targeted 8 nucleotides downstream of the alternatively spliced 5' splice site.

In vivo splicing assays of the E1A minigene with the artificial snoRNAs showed decreased usage of 5' alternatively spliced site close to the targeted region, an effect similar

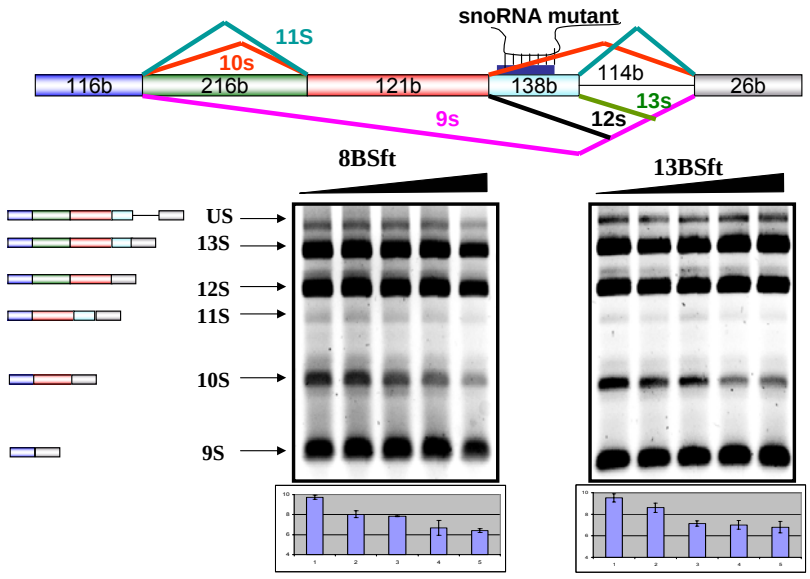


Figure 46. snoRNAs could regulate alternative splicing in a heterologous system. Artificial snoRNAs, E1A-8BSft and E1A-13BSft, target 18nt region starting 9 and 14 nt downstream of an alternative 5' splice site of an adenoviral E1A minigene. Transfection of increasing amounts of E1A-8BSft snoRNA (left) and E1A-13BSft (right) with E1A minigene. The schematic representation of the various spice sites and the splice products have been shown on the top.

to MBII-52 on serotonin receptor 2C. This experiment demonstrates that snoRNAs can function in heterologous systems. In addition, it also confirms the finding that the binding of snoRNAs within a critical region can impair proper functioning of U1 spliceosomal complex.

4.13. In Vivo implications of HBII-52 mediated 5-HT$_{2C}$R splicing: Editing in Prader-Willi patients increases in absence of HBII-52

It is known that the critical imprinting center responsible for Prader-Willisyndrome (PWS) hosts several small nucleolar RNAs including HBII-52. PWS occurs as a loss of paternal gene expression within this region due to deletion of the whole imprinting locus. Several reports have documented the loss of expression of HBII-52 in patients suffering from Prader-Willi Syndrome. Whereas the editing pathway generates 5-HT$_{2C}$R receptor isoforms with amino acid changes in their intracellular binding site to the G-protein, the HBII-52 dependent pathway generates a protein corresponding to the genomic sequence. Hence, in the absence of HBII-52, there would be a reduction in the nonedited 5-HT$_{2C}$R mRNA and increase in the receptor RNA editing. As a consequence, the editing of 5-HT$_{2C}$R mRNA should increase significantly in a PWS patient. To confirm this, we analyzed the mRNA from the patients with PWS and normal controls (Figure 47A). Semi-quantitative RT-PCR was performed with mRNA extracted from hippocampus, where editing is most prevalent. Semiquantitative analysis employed a multiplex primer approach that amplified GAPDH in the same reaction as a loading control. Specific individual primers were designed against each sites with the last 3' nucleotide of the primers complementary to the non-edited nucleotide. As a result, only the non-edited site specific variant is selectively amplified (Figure 47B).

Also, non-edited variants reduced in a PWS patient to 25%, 39%, 74% and 89% at the A, B, C and D sites, when compared with healthy controls. The reduction at the A, B and C sites was statistically significant ($p= 0.018$, 0.025 and 0.019, respectively, Figure 4C)., implying that the editing at individual site increases with loss of HBII-52 expression. RT-PCR confirms that there is no HBII-52 expression in the PWS patients whereas the healthy controls show adequate expression. These findings show a dependency of 5-HT$_{2C}$R alternative splicing 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. Consequently, it signifies the

profound biological role that small nucleolar RNAs could play in the overall development at the cellular level.

Alternatively, direct sequencing was employed to detect the editing in various tissues derived from the PWS patient and normal controls. For this, low cycle (~20 cycles) RT-PCR was performed from the hippocampus RNA. The PCR product was ligated into pCR4-TA vector and later sequenced (Figure 47C).

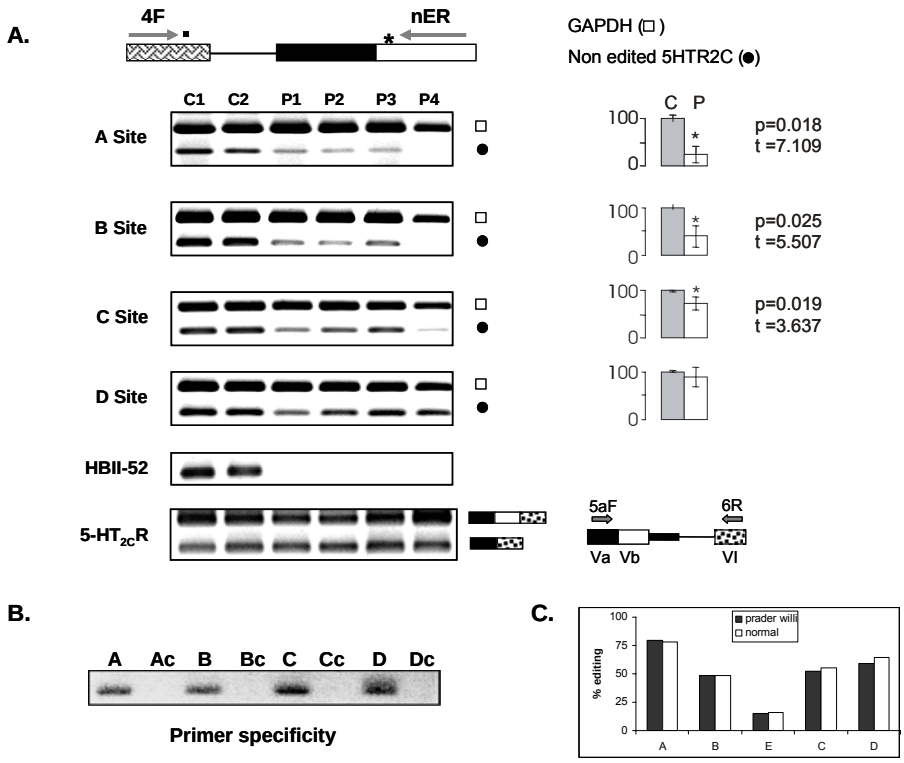


Figure 47. Changes of 5-HT2CR mRNA isoform expression in PWS patients detected by isoform-specific RT-PCR.

(A). RNA from similar hippocampal areas from PWS patients (P) and aged matched controls (C) were amplified by RT-PCR. Primers that are complementary to the non-edited sites, indicated by a star in the scheme on top, were used for amplification. GAPDH was amplified in the same reactions as a loading control. Representative RT-PCR results stained on ethidium bromide 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. The statistical evaluation of the experiment is shown on the right. The amplification of HBII-52 in controls, but not PWS patients confirmed the clinical diagnosis.

(B). Specificity of the edititing-site specific PCR assay: Primers specific for the non-edited A-D sites, respectively were used in all experiments. In lanes A, B, C and D cDNA corresponding to the non-edited version of the receptor was used as amplification template. In lanes Ac, Bc, Cc and Dc we used a cDNA corresponding to the edited version of the receptor.

(C). Clonal analyses of the hippocampus RNA from PWS patient and normal control brain. RT-PCR was done from the hippocampus RNA and the products cloned into pCR4 vector. Several clones from both the patients and the normal controls were sequenced to determine the extent of editing in the diseased and the normal individual.

4.14. Editing in mice subjected to stress

Ventral and dorsal parts of the hippocampus have different significance in the contextual fear conditioning. Rogelj et al. (2003), have earlier shown that there are differential patterns of gene expression in these two regions. Their findings show that there is a statistically significant upregulation of the MBII-52 during consolidation of contextual fear memory, suggesting that MBII-52 may be involved in higher brain functions. Also, studies indicate that 5-HT is involved in some kinds of memory consolidation. Surprisingly, analysis of the expression of 5-HT_{2C}R splice variants show that the short variant was very weakly expressed in the hippocampus and there was no significant difference in the splicing of the receptor during contextual fear conditioning.

Since the study did not take into account the alterations in the editing pattern of 5-HT_{2C}R, it was important to investigate if editing of the receptor and not its splicing, played an important role in the contextual learning.

cDNA isolated from hippocampus of trained (in fear conditioning)and naïve mice were requested from Karl Peter Giese's lab. RT-PCR was carried out using the primers specifically amplifying the non edited variants individually at A, B and C site (Figure 48).

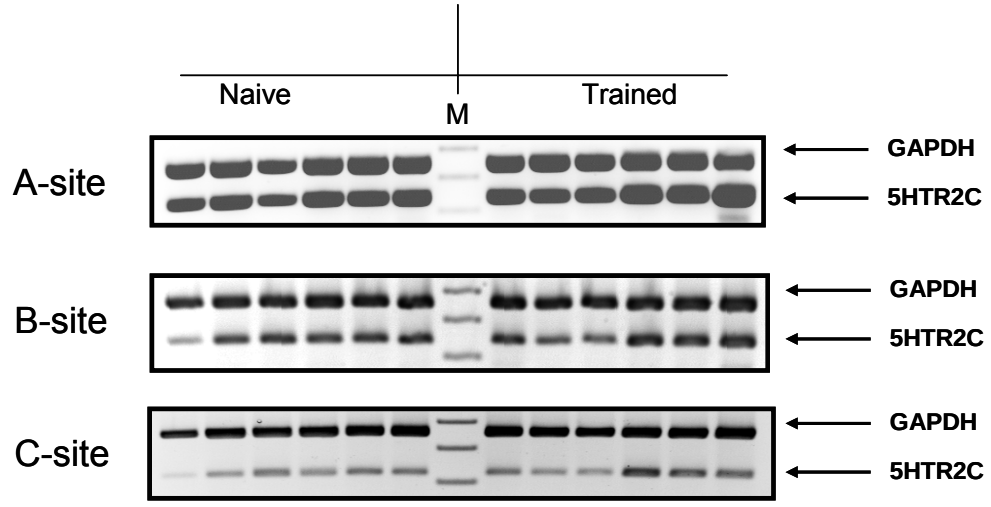


Figure 48. Editing of 5HT2C receptor in naïve and trained mice. Endogenous editing levels at individual sites (A, B and C sites) of the trained and naïve mice were checked by semi-quantitative RT-PCR using primers amplifying only the non-edited sites. GAPDH was used as an internal loading control.

The results show that there is no appreciable difference between the editing pattern of trained and naïve mice with respect to editing sites A, B and C. It is as well likely that the limitations of the experimental methodology, restricts the detection of subtle changes in the 5-HT_{2C}R editing, which might be governing the contextual learning.

4.15. Editing in suicidal and depressed patients

It is generally believed that depression is associated with decreased serotonin levels. In response to lower levels of serotonin, the brain produces more of unedited 5-HT_{2C}R isoforms that have high affinity towards serotonin. Several reports have demonstrated that the 5-HT_{2C}R pre-mRNA editing changes under stress conditions. For example, reduced RNA editing and increased expression of the unedited (INI) 5-HT_{2C}R isoform was detected in Schizophrenia (Sodhi et al., 2001). RT-PCR was performed on RNA from cortex orbitofrontalis of six normal and six suicidal individuals. Primers specifically amplifying non edited sites were used to detect any variations in the editing pattern. Quite contrary to the earlier studies, our results demonstrate no significant difference in the editing pattern of normal and suicidal patients.

4.16. DNA chip analysis

As the mechanism of MBII-52 action is not certain, one plausible mechanism could be that the antisense element sequesters the splice factors away from the splicing reaction. This is consistent with the findings that MBII-52 is brain specific, and forms one of the most highly expressed small RNAs in the brain. Hence, if this were true, MBII-52 could influence the alternative splicing of more than one gene.

To test this hypothesis, total RNA from Neuro2A cells transfected with MBII-52 was sent for DNA chip analysis. pEGFP-C2 transfected Neuro2A cells were taken as a control. Interestingly, DNA chip analysis revealed many important genes involved in splicing could be up or down regulated by the MBII-52. Most prominent amongst them included several hnRNPs, snRNPs and several other splicing factors. The significantly upregulated genes have been shown in red (>0.3); whereas the downregulated genes have been shown in blue (<0.3). Genes with value other than zero in the “Ignore me” column was discared due to experimental inconsistency.

Table 4. Influence of MBII-52 on the expression of Apoptosis related genes

Transcript	median LR	IgnoreMe	Transcript	median LR	IgnoreMe
apaf1_com	-0.217	0	casp6	-0.232	0
apaf-1L	-0.018	0	Casp7	-0.33	0
Ayelet2 (luc7-homolog?)	-0.535	0	casp8	-0.22	0
bak1_altN	0	1	casp9L	-0.006	0
bak1_com	-0.019	0	casp9S	0.197	0
bax_alpha	0.181	0	Ctnnbl1 (NAP)	-0.07	0
bax_kappa	0.224	0	Fas_beta	0	1
bcl2_alpha	0	1	fas_long	0.284	0
bcl2_beta	0.203	0	FLIP-L	0.219	0
Bcl2l13	-0.016	0	FLIP-S	0.038	0
bcl-x_alt2	0.108	0	ICAD_com	0	1
bcl-X_com	0.056	0	ICAD_L	-0.547	0
bcl-x_gamma	0.107	6.905	LARD_altA	0.195	0
bcl-x_long	0.058	0	LARD_altB	0.143	0
BI1	0.091	30.053	madd_alt	0.452	0
Bid	0.256	0	madd_com	0.068	0
casp1	0.148	24.352	Mcl1	0.246	0
casp2_alt	0.769	0	traf2_com	0.069	0
casp2_com	-0.486	0	TRAF2A	-0.1	0

Table 5. Influence of MBII-52 on the expression of helicases

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
Ayelet1 (KIAA-homolog?)	0.066	0	Ddx27_com	-0.114	0
Bat1a	-1.535	0	Ddx41	0.064	0
Ddx15	-0.268	0	Ddx46	0.134	0
Ddx16	-0.158	0	ddx48	0.211	0
Ddx27_alt1	0.198	0	Ddx5 (p68)	-1.638	0
Ddx27_alt2	0.164	0	Ddx9	0.202	0

Table 6. Influence of MBII-52 on the expression of hnRNPs

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
brPTB (ptb2)	-0.191	0	hnRNP K_alt3	-0.798	0
hnRNP A1	-0.665	0	hnRNP K_Pcom	-0.917	0
hnRNP A2/B1-old	-0.219	0	hnRNP L-old	-0.917	0
hnRNP AB_alt1	0	1	Pcbp2_alt	0.072	0
hnRNP AB_alt2	-0.146	0	Pcbp2_com	-1.333	0
hnRNP C	0.115	0	ptbp1_alt1	-0.078	0
hnRNP G	-0.377	0	ptbp1_alt2	0.139	0
hnRNP H1	-1.049	0	Ptbp1_com	-0.754	0
hnRNP K_alt1	-0.905	0	Rbmxrt (hnRNP G)	-0.44	0
hnRNP K_alt2	-0.605	0	snRNP E	0.4	0

Table 7. Influence of MBII-52 on the expression of house keeping genes

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
atubulin1	-0.275	0	Ndufc1	0.592	0
atubulin4	0	1	Pgk1	-0.29	0
bActin	-0.234	0	Rl13a	0.066	0
Gapd	-0.5	0			

Table 8. Influence of MBII-52 on the expression of mRNA processing genes

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
CPSf1	0.063	0	Ncbp2	-0.051	0
Cpsf2	-0.089	0	NLP4	0.375	0
CPSf3	0.016	0	Pabpc1	-1.017	0
Cpsf4	-0.616	0	Pabpn1	0.879	0
Cpsf5	-0.529	0	Papolb	0.612	0
cstf3_alt	-0.22	0	Refbp1	-1.852	0
cstf3_com	-0.957	0	Refbp2	-1.289	0

Table 9. Influence of MBII-52 on the expression of other genes

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
BChE	0	1	PenK1_old	-0.101	0
BCHE-old	-0.083	0	Pon1	0	1
Mapk8	-0.116	0	Pon2	0	1
Mfap1_alt1	0.401	0	Pon3	0	1
Mfap1_alt2	0.283	0	Thyrosine hydorxylase	0	1
Pdyn_old	-0.185	0			

Table 10. Influence of MBII-52 on the expression of other spliceosomal components

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
Ayelet 4	-0.024	0	sam68(Khdrbs1)	-0.344	0
Bcas2	-0.191	0	SLM1	0.185	0
Cd2bp2	0.29	0	SMNRP(sf30)	-0.498	0
Crnk1	0	1	Spop_alt	0.259	0
Dnajc	0.259	0	Spop_com	0.223	0
Fnbp3 (FB11)	0.71	0	Thoc1	0.15	15.66
RBM17	-0.245	0	Wbp11	0.06	0
Rbm8	0.724	0	Wtap	-0.005	0
Rnpc2	-0.162	0			

Table 11. Influence of MBII-52 on the expression of snRNPs

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
Lsm2_com (smx5)	-0.366	0	snRNP D1-old	-0.055	0
Lsm2_Pcom (smx5)	-0.223	0	snRNP d2	0.34	0
Lsm3	0.127	0	snRNP d3	0.472	0
Lsm4	0.745	0	snRNP G	0.267	0

Lsm7	0.108	0	snRNP N_alt1	0.715	0
p14-pending	0.177	0	snRNP N_alt2	0.364	0
Prpf3 (U4/U6-90kD)	0.266	0	snRNP N_alt3	0.053	0
Prpf4 (U4/U6-60kD)	-0.317	0	Tri-snRNP 27kD-pending (RY1?)	0.104	0
Sf3a1	-0.073	0	U1snRNP70_alt	0.313	0
sf3a2_alt1	-0.2	0	U1snRNP70_com	0.003	0
sf3a2_alt2	0	1	U2A'-old	0.104	0
Sf3a2_alt3	0.26	0	U4/U6-20kD-pending_alt1	0.362	0
sf3a2_com	0.139	0	U4/U6-20kD-pending_alt2	0	1
Sf3a3	-0.093	26.385	U4/U6-20kD-pending_alt3	0.599	0
sf3b1 (SAP155)	-0.292	0	U4/U6-61kD	0.055	0
Sf3b4	0.043	0	U5 116 kd-old	-0.136	0
snRNP 1C	0.085	7.446	U5-102kd	0.07	0
snRNP A	0.11	0	U5-15_alt1	0.238	0
snRNP B2	0.083	0	U5-15_alt2	-0.457	0
snRNP B-old	0.354	0	U5-40	-0.195	0

Table 12. Influence of MBII-52 on the expression of genes involved in spliceosomal assembly

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
Cdc5l	0.479	0	Gemin7	0.298	0
gemin2 (sip1)	-0.039	0	Plrg1	-0.419	0
gemin3_alt	0.188	0	prp19	0.513	0
gemin3_com	-0.059	0	Prpf8	-0.278	0
gemin4	0.232	0	Sart1	0.463	0
Gemin5	0.04	0	SKIIP	0.055	0
Gemin6	-0.107	0	Slu7-pending	0.322	0

Table 13. Influence of MBII-52 on the expression of splicing factors phosphorylation

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
Abl1	0.109	0	pp2r2b	0.575	0
Cdc2a	-0.364	0	Ppm1g (pp2c)	-0.051	0
Clk1 (sty)	-0.508	0	Ppp2r5e	-0.376	0
Clk2	-0.136	0	Prpf4b_alt1	-0.323	0
Clk3_alt	-0.036	0	Prpf4b_alt2	-0.033	0
Clk3_com	0.534	0	Prpf4b_com	0	1
Clk4	-0.614	0	SRpK1	-0.049	0
crk7	0	1	SRpk2	-0.296	0
Dusp11	0.177	0	Topor	-0.453	0
NIPP1	-0.862	0			

Table 14. Influence of MBII-52 on the expression of SR and SR-related proteins

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
9G8 (sfrs7)	-0.376	0	SRp55 (sfrs6)-pending	-0.446	0
ASF/SF2 (sfrs1)	-1.259	0	SRp75_alt1	-0.9	0
SC35_5	-1.055	0	SRp75_E3	-0.013	0

SC35_alt	0	1	Srrm1 (srm160)_alt	-0.514	0
SC35_com	-0.508	0	Srrm1 (srm160)_com	0.698	0
SC35-old	0	1	srrm2 (srm300)	0.124	0
SRp20 (sfrs3)	-0.373	0	tra2beta_alt1	-1.294	0
SRp25 (Arl6ip4)	0.458	0	tra2beta_alt2	-1.625	0
SRp30c (Sfrs9)	-0.174	0	tra2beta_alt3	0	1
SRp40 (sfrs5)	-1.158	0	U2af1	-0.107	0
SRp54	-2.043	0	U2AF2	0	1

Table 15. Influence of MBII-52 on the expression of target genes

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
Ache-mE2	0	1	mE1a	1.28	0
Aqp4_alt1	0.074	0	mE1b	0.172	0
Aqp4_alt2	0	1	mE1c	0	1
Aqp4_com	0	1	mE1d	0.081	0
ARS2_alt1	0.349	0	mE1e	-0.061	0
ARS2_com	0.201	0	mE3	0	1
Ars2-pending	0.024	0	mE5	0	1
Ccnl_alt1	0.127	0	mE6	0.366	0
Ccnl_alt2	0	1	mI4	0.052	0
Ccnl_com	-0.621	0	Snca_alt1	0.695	0
Clcn3_alt1	0.109	0	Snca_alt2	0	1
Clcn3_alt2	0.521	0	Snca_com	0	1
Clcn3_com	-0.021	0			

Table 16. Influence of MBII-52 on the expression of genes of unknown functions

Transcript	Median LR	IgnoreMe	Transcript	median LR	IgnoreMe
Ayelet 5	-0.098	0	Ayelet3	0	1
puf60-homolog	-0.09	0			

4.17. Experimental validation of DNA chip results

Considering the error margins within the DNA chip data analysis, it was necessary to support and validate the chip results with the experimental data. One of the few constitutive genes that showed down regulation with MBII-52 over-expression was GAPDH. Semi quantitative PCR of increasing amounts of transfected MBII-52 with endogenous GAPDH as well confirmed that GAPDH is down regulated with increasing concentrations of MBII-52 (Figure 49A).

Similarly, MBII-52 could as well promote Exon 7 exclusion in SMN2 minigene in a concentration dependent manner (Figure 49B).

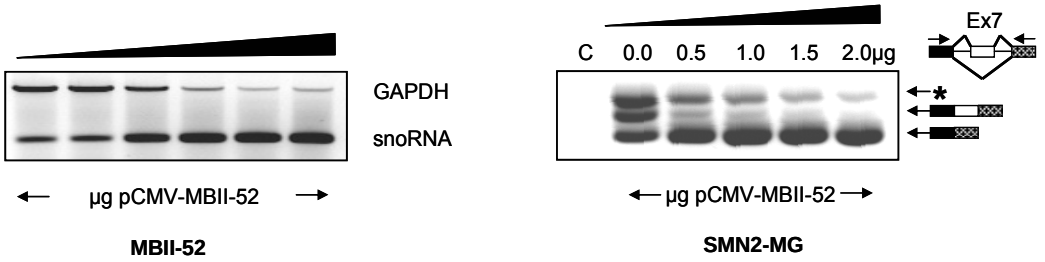


Figure 49. Experimental verification of the CHIP results. (A). Increasing amounts of MBII-52 snoRNA was transfected in the 293 cells and endogenous GAPDH levels were compared by RT-PCR. **(B).** Increasing amounts of MBII-52 was co-transfected with the SMN2 minigene (1.0 µg) and the various splice variants of SMN2 were amplified by RT-PCR.

These results indicate that MBII-52 need not act through its antisense box alone but could function by sequestering the splice factors away from the splice site. These findings are substantiated with the fact that HBII-52 binding region on the receptor hosts a regulatory element. It is likely that the anti-sense element of HBII-52 could as well serve as a potential regulatory sequence which can sequester splice factors away from the reaction site.

5. DISCUSSION

5.1. A conserved mammalian brain specific HBII-52 regulates the alternative splicing of 5-HT$_{2C}$R

All intensively studied gene loci, including those that are imprinted and conventional loci such as IPW locus imprinted in PWS and beta-globin have been shown to contain a majority of non-coding transcripts (Ashe et al., 1997, Charlier et al., 2001, Holmes et al., 2003, Seitz et al., 2003, Nicholls and Knepper 2001). ncRNAs have been implicated in diseases including various cancers and neurological diseases (Mattick JS 2003, Pang et al., 2005) and at least some are processed into smaller functional molecules (van den Berg et al., 2003, Metzler et al., 2004). In most, if not all, cases, their function is based on recognition of RNA or DNA target sequences by specific base pairing. Because of this feature, even short RNAs contain sufficient information to specify individual targets in the genome and the transcriptome, in a much more compact and energy-efficient manner than proteins. Until now, the role of snoRNAs was restricted to rRNA modification in ribosome biogenesis, but it is now evident that they can target other RNAs including tRNAs and snRNAs. This work outlines a new function of snoRNAs. Here, we have shown that a non-canonical brain specific C/D Box snoRNA that is missing in the patients suffering from PWS, can regulate the alternative splice site selection of a G-protein coupled 5-HT$_{2C}$ receptor pre-messenger RNA.

At least 47 identical copies of HBII-52 snoRNA genes are located between unrelated, non-protein coding exons on the paternally imprinted IC-SNURF-SNRPN locus of human chromosome 15q11-13. The antisense box of HBII-52 exhibits 18 nucleotide phylogenetically conserved sequence complementarity to a critical alternative spliced exon Vb of the 5-HT$_{2C}$R pre-mRNA, whose gene itself encodes another snoRNA with unknown target. Homology studies carried across mammalian species prove strong conservation of HBII-52 antisense element and the sequence motifs. The snoRNA complementary region on the receptor is as well conserved between mammalian species. In the non-mammalian species, no orthologs of HBII-52 could be computationally detected, indicating that the HBII-52 mediated regulation is an evolved mammalian feature. It is not surprising as some brain-

specific snoRNAs appear to have evolved recently and to be restricted in their phylogenetic distribution (Cavaille et al.,2001), suggesting their importance in the epigenetic control of behaviour. Interestingly, in some of the non-mammalian species like *Gallus gallus* and *Macaca fascicularis*, the complementary region on the serotonin receptor is still conserved but traces for HBII-52 orthologs in them are yet to be found. It is noteworthy that the constitutive exon Va of serotonin receptor 2C is tightly conserved even in large number of non-mammalian species, where the snoRNA binding region shows no conservation. Also, the number of copies of HBII-52 snoRNA decreases in less evolved mammals showing that HBII-52 underwent multiple gene duplications with evolution. This together suggests that snoRNA mediated regulation of serotonin receptor function is an evolved mammalian feature.

Both the serotonin receptor 2C and HBII-52 show brain specific expression. The alternative splicing pattern of 5-HT_{2C}R correlates with HBII-52 expression *in vivo*. HBII-52 is expressed in all areas of brain except in choroid plexus. In accordance, the exon Vb included functional isoform of 5-HT_{2C}R is strongly expressed than the exon Vb excluded non-function variant, in all areas of brain except in choroid plexus where the ratio is reversed. Minigene analysis reveals that the HBII-52 promotes exon inclusion of 5-HT_{2C}R in a concentration dependent manner. The complementarity between the snoRNA and the exon Vb is necessary to influence alternative splicing of this exon. Mutation in the binding elements of either the snoRNA or the receptor destroys the effect, whereas compensatory mutation in both the constructs restores it. This demonstrates that complementarity drives the alternative splicing. Mutations in the HBII-52 conserved sequence motifs have no effect on the alternative splicing of the receptor's pre-mRNA, indicating that integrity of snoRNA to form snoRNP is necessary to regulate the alternative splicing of 5-HT_{2C}R. Intriguingly, all the serotonin receptor constructs spliced predominantly at proximal splice site which is contrary to the case *in vivo*. Since the upstream and downstream introns flanking the alternative spliced exon are 117kb and 58 kb respectively, a large part of it is truncated in the minigene construct. It is likely that these introns host some splicing elements required for efficient splicing. As the introns flanking the alternatively spliced exon host several Alu repeats and are not conserved even amongst closely related mammalian species, it is still unclear what functions the huge introns can have in the splicing process. Also, it has been

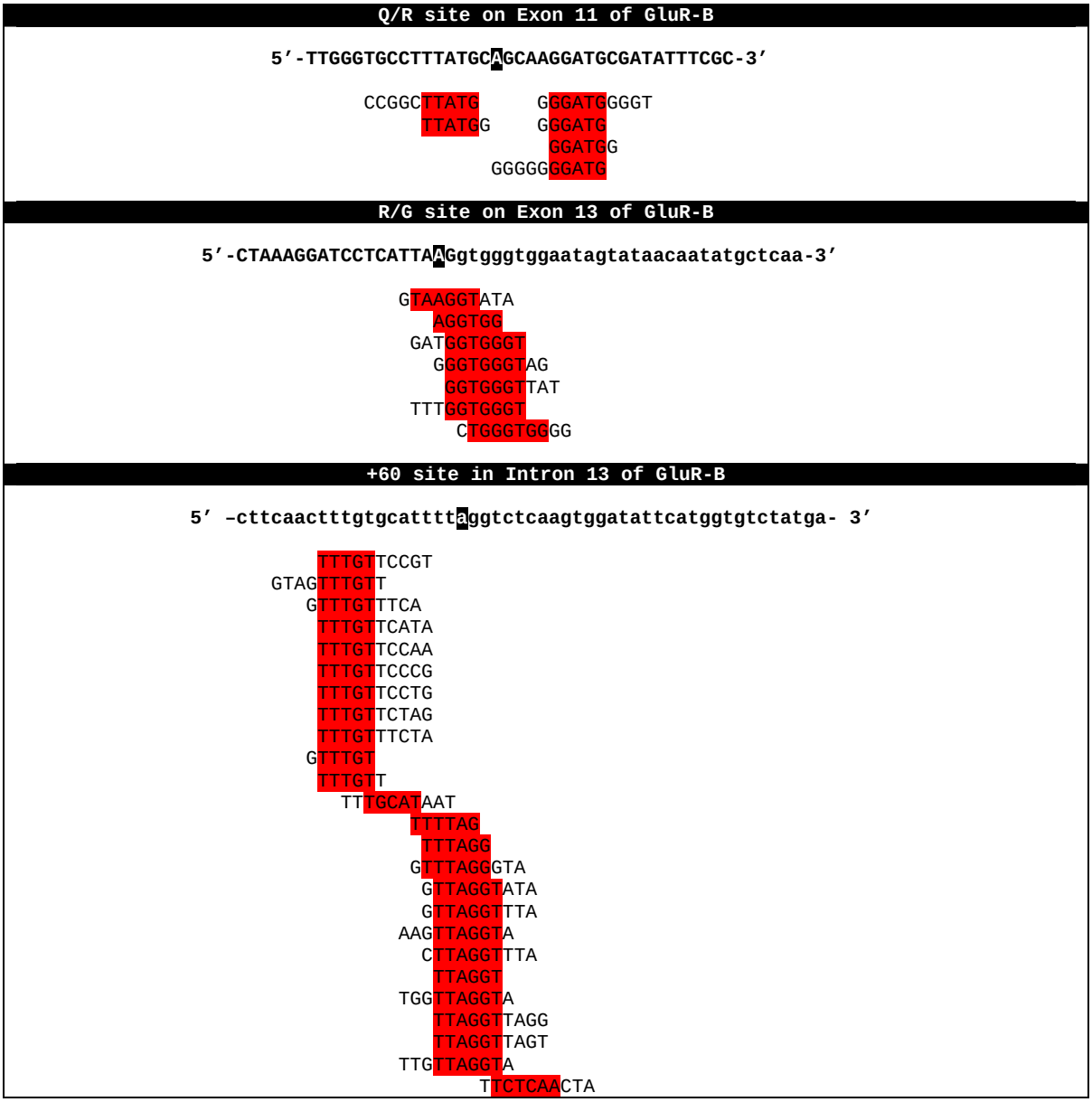
earlier reported that many neuronal genes including serotonin receptor 2C fail to splice correctly in immortalized cell lines, probably because of a unique combination of splice factors that are present in the differentiated neuronal cells. Moreover, in brain, HBII-52 is highly expressed. Generally gene duplication occurs for genes which are constitutively required at high basal levels. There are 47 copies of HBII-52 in the human genome, suggesting its importance. It still has to be checked if the levels of snoRNA in cotransfection experiments are comparable to the levels *in vivo*. It is very likely, that high concentrations of snoRNA are required *in vivo* to influence the proper splicing of the serotonin receptor 2C.

5.2. Splice site selection of the 5-HT$_{2C}$R pre-mRNA is regulated by the silencers located in the snoRNA complementary site

Exon Vb is predominantly skipped both in the wild type and minigene activated at the distal splice site. When the minigene activated at the distal splice site is also mutated at the snoRNA complementary site, the splicing pattern reverses dramatically. Inspection of the snoRNA complementarity region in exon Vb shows the presence of several putative silencing elements that were recently identified in an exon silencer screen. The sequence overlapped with two potential silencer elements. This snoRNA binding sequence was experimentally verified to harbor silencing activity as it suppressed the exon inclusion in a chimeric beta-globin construct. It has been shown earlier that the editing by ADARs promote exon inclusion of the serotonin receptor 2C by an unknown mechanism. Also, simultaneous editing at multiple sites promotes more inclusion than editing at any single site. Interestingly, the mutations that we made in the snoRNA complementary site did not overlap with the editing sites, indicating that exon inclusion is promoted not because of specific editing from A to I by ADARs at defined sites, but any other mutation in this region can as well promote exon inclusion. We have shown that the snoRNA binding region on the receptor harbors regulatory elements. Mutations in this region can destroy this regulatory element and promote exon inclusion. Another well studied system for ADAR editing is Glutamate receptor B (GluR-B), which undergoes A-I editing at 3 sites. The first two editing events take place in Q/R site and +60 hotspot, which lie 25 nt upstream of a constitutive splice site of exon 11 and 60 nt downstream of the same splice site in the intron 11 respectively. The third editing takes place at the R/G site of exon 13, which overlaps with the U1 binding site.

noteworthy that Exon 14 is an alternatively spliced cassette exon, whose exclusion and inclusion produces Flip and Flop variants of the encoded protein respectively.

Table 17. Putative regulatory elements around the edited sites in the Glutamate receptor B.



In addition, it has been previously shown that the mRNA encoding flip variant is subjected to more editing than corresponding flop variant (Liu et al., 1999), suggesting that editing could favor exon 14 skipping in GluR-B pre-mRNA. The argument is strengthened by the fact that in choroid plexus where the editing of both the Flip/Flop variants is minimal, the isoform ratio is close to one. In contrast, in all other regions of the brain, where editing of

GluR-B pre-mRNA is more frequent, the Flip isoform predominates the Flop variant (Liu and Samuel 1999b). Comparison of the ADAR editing site on GluR-B pre-mRNA with known regulatory sequences reveals several regulatory elements around the edited nucleotide (Table 17).

Until now, it was only known that ADARs add to the diversity of the proteome by changing the coding potential of the mRNA from that encoded by the genome. The findings in this work demonstrate a novel role of ADARs in regulating the alternative splicing of the pre-mRNA by weakening the regulatory elements. These findings can as well justify the presence of editing in intronic regions.

Presence of exonic sequence silencers can as well explain how HBII-52 can mediate the alternative splicing of 5-HT_{2C}R pre-mRNA. Binding of snoRNA HBII-52 to the pre-mRNA masks the silencer and promotes exon usage. This inference is also supported by the findings that the compensatory mutations in the mutant HBII-52 and the mutant 5-HT_{2C}R fails to elicit the same degree of splice site usage as the wild type constructs. In the receptor mutated at the snoRNA binding site, the silencers are already weakened. Masking of this site by the compensatory mutant snoRNA has a minimal effect on restoring the snoRNA dependency of the splicing as against the wild type constructs. Moreover, artificial snoRNAs directed on either side of the actual wild type snoRNA binding region show decreased exon Vb usage as against equal amounts of the wild type HBII-52. This demonstrates that HBII-52 binding region on 5-HT_{2C}R lies at a critical distance to influence the alternative splicing. This is evident that the shifting the snoRNA binding region leaves the regulatory element unmasked and the splicing regulation is lost as a result.

5.3. HBII-52 promotes exon inclusion independent of RNA editing mediated by ADARs

It has been previously reported that A to I editing of serotonin receptor 2C at any one or more editing site (A to E) can promote exon inclusion. Similar to recent reports (Vitali et al 2005), our cotransfection experiments with reporter minigene show no evidence that HBII-52 affects editing of nucleoplasmic 5-HT_{2C}R transcripts. Our data suggests that in addition to the known editing-dependent pathway, exon Vb inclusion can be promoted by HBII-52 snoRNA (Figure 50). Though, exon Vb editing promotes its inclusion, it changes the amino

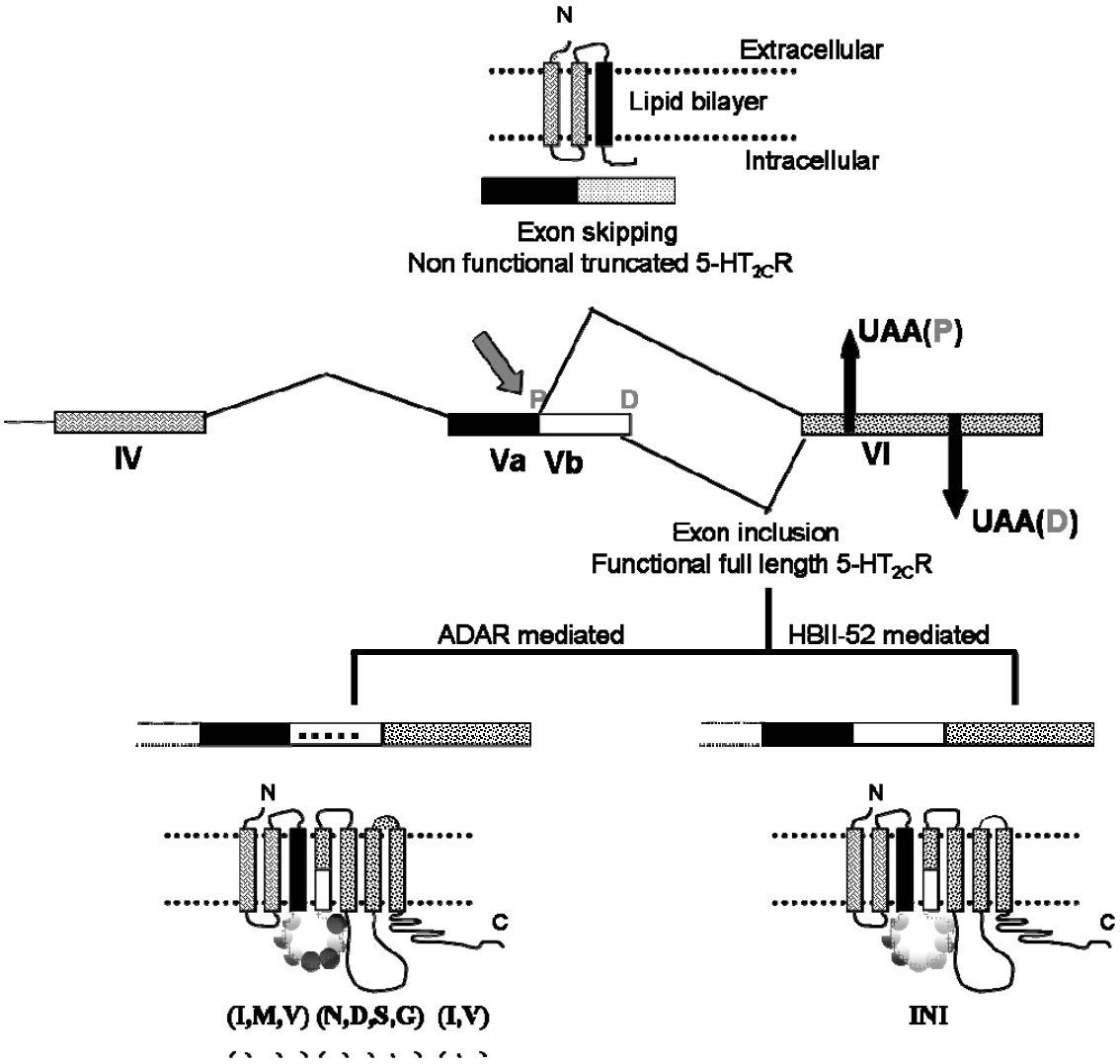


Figure 50. ADARs and HBII-52 can independently promote 5-HT2CR Exon Vb inclusion by regulating the silencing element. ADARs promote A to I editing and destroys the silencer where as HBII-52 binds to the silencer and masks its activity.

acid sequence of the intracellular loop which binds to the G-protein. This decreases serotonin efficacy of the receptors generated through RNA editing 10 to 100 folds. In contrast, HBII-52 plays an indispensable role as HBII-52 dependent pathway generates a protein corresponding to the genomic sequence with maximal serotonin binding affinity. The non edited INI isoform are generally not as abundant as the edited isoforms. The production of INI variants

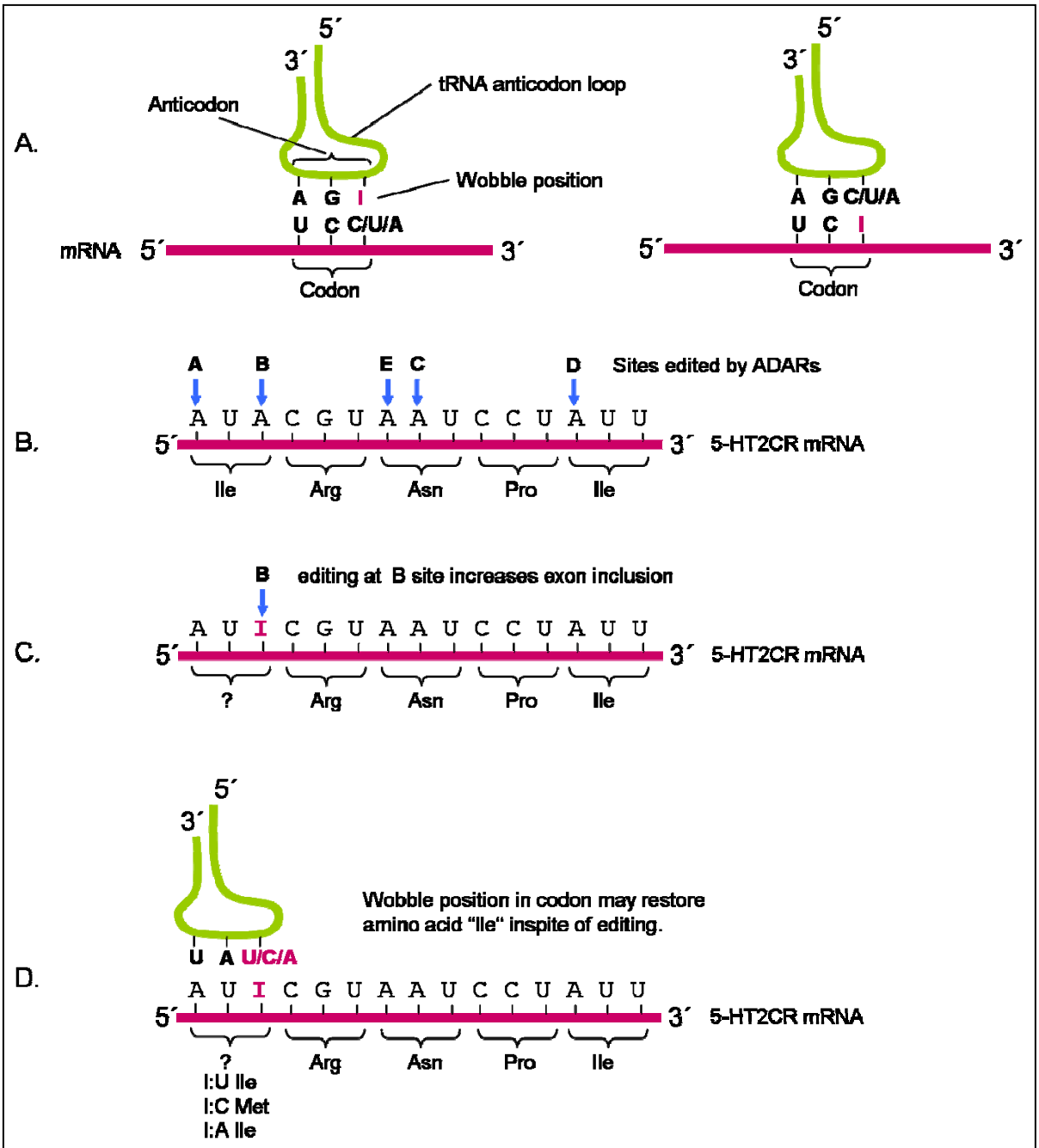


Figure 51. Effect of editing by ADARs could be nullified by the wobble base pairing of Inosine. (A). tRNAs can base pair to different codons due to presence of Inosine at wobble position. **(B).** ADARs could promote exon inclusion of 5-HT_{2C}R while this changes the coding potential of the mRNA. **(C).** Editing at B site can promote exon inclusion of 5-HT_{2C}R. It is special as it lies at the wobble position. **(D).** Presence of Inosine at this B site can facilitate I:U base pairing and bind to Ile carrying tRNA. This can revert the effect of editing while promoting exon inclusion.

increases in the serotonin depleted brains where high sensitivity is required. It is remarkable how HBII-52 can subtly regulate the quantity of non-edited transcripts. One limitation of the

experiment was that the endogenous levels of editing of the receptor could not be reproduced in the reporter constructs. However again, it has to be kept in mind that transfected immortalized cells do not have identical environment as the differentiated neurons.

It is previously shown that Inosines can equally base pair with Uridine, Cytosine and Adenosine with comparable thermodynamic energies. It would be quite interesting if this Inosine comes as the third wobble nucleotide while base pairing with the tRNA, it has the possibility to rectify the editing by binding to Uridine in the wobble position, hence undoing the editing effect. In such case, the edited transcripts especially one at the B site alone, can still through wobble base, code for non-edited protein consistent with that of the genomic sequence (Figure 51). In short, while editing can increase the exon inclusion, the use of Inosine at the wobble position could still code for an unaltered protein from the edited mRNA. Till now, it has only been shown that the tRNAs could have inosine in the anti-condon to allow flexible binding with the mRNA. But this does not rule out the possibility that Inosines in the mRNA codons can not bind to regular anti-codons with flexibility. Though there has been no experimental verification so far, this offers an interesting possibility of a more intricate level of gene regulation.

5.4. Mechanism of HBII-52 mediated alternative splicing

We have developed a novel highly sensitive assay to detect the snoRNA complex with the serotonin receptor pre-mRNA. This complex appears to be transient as it can only be detected after crosslinking with psoralen under UV. This assay shows for the first time the complex formed between a small nucleolar RNA and the messenger RNA indicating that the binding is necessary to mediate the regulation of 5-HT_{2C}R alternative splicing by HBII-52. As the HBII-52 binding element hosts silencer activity, it is likely that the HBII-52 mediated effect effect of exon inclusion is a result of snoRNA masking the silencers (Figure 52). Like other snoRNAs, HBII-52 is also localized in the nucleolus, whereas pre-mRNA splicing occurs in the nucleoplasm. However, snoRNAs originate in nucleoplasm as a byproduct of splicing of their host genes. It is therefore possible that they can interact transiently with pre-mRNAs, similar to SR proteins that are localized predominantly in nuclear speckles but function outside speckles.

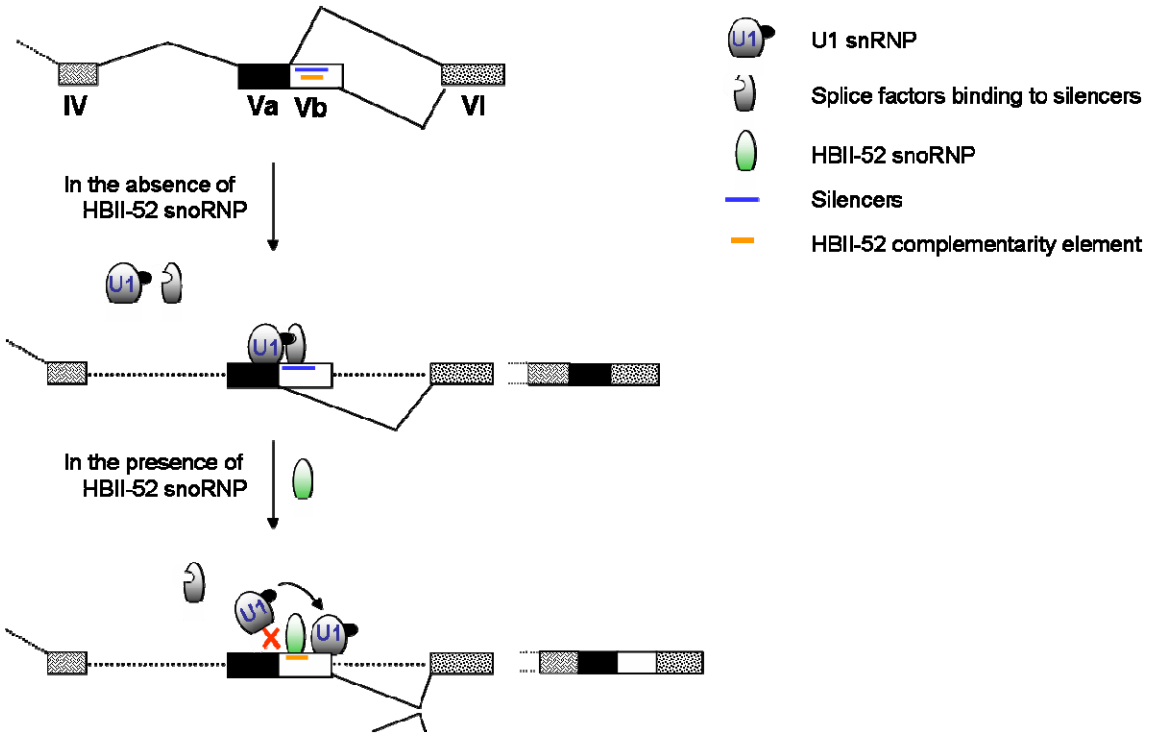


Figure 52. Mechanism of HBII-52 mediated alternative splicing of 5-HT$_{2C}$R. HBII-52 binds to the Exon Vb of the 5-HT$_{2C}$ receptor. This complementarity site hosts several silencers which are masked by the HBII-52 binding.

However it might not be the only way in which the snoRNA can regulate the splicing. DNA chip results clearly indicate that HBII-52 is capable of influencing the expression pattern of a large variety of splicing factors especially hnRNPs, snRNPs and SR and SR related proteins. Interestingly, most of the hnRNPs and the SR and SR proteins are down regulated, whereas the snRNPs and other spliceosomal components are upregulated. This suggests that HBII-52 expression can influence several splicing regulators and in turn regulate the splicing pattern of many other genes. It is possible that the antisense element of HBII-52 can bind to regulatory factors and sequester them away from the splice site, hence influence splicing.

Another relatively less sensitive RNAseH assay showed that binding of HBII-52 on the serotonin receptor 2C stearically hinders the U1 splicesomal from binding to the proximal splice site. Interestingly, both the proximal and distal splice sites of the alternatively 5' spliced Vb exon do not correspond to the U1 consensus. It is not clear how the proximal splice site is totally favored over the distal splice site. Recent studies have shown that U1

associated splice factors bind to sequence downstream of the weak splice site (Puig et al., 1999). It is possible that HBII-52 binding to the 5-HT_{2C}R inhibits these auxiliary factors from binding and hence interfere with proper splicing mediated by U1 spliceosome at the proximal splice site. One limitation of this assay is that so far *in vitro* transcribed RNAs have been difficult to reconstitute into snoRNPs *in vitro*, though some cases have been reported where several snoRNAs like U14 and U15 can be assembled *in vitro* with relative ease (Watkins et al., 1998b, Newman et al., 2000). It was however not confirmed in our experiments whether *in vitro* transcribed HBII-52 could reconstitute into a snoRNP with the Hela Nuclear extract under splicing conditions. It can be speculated that in the absence of snoRNP assembly, it is likely that the results only reflect HBII52 binding to the *in vitro* transcribed 5-HT_{2C}R through antisense effect alone. The situation would be far from portraying the actual *in vivo* conditions. Also, if the HBII-52 functioned by dislodging U1 complex, the positional snoRNA mutants when designed to bind closer to the U1 splice site should decrease the splicing at proximal splice site, which is clearly not the case. The positional snoRNA mutants targeting either side of the actual HBII-52 binding site elicit decreased exon usage.

All the experiments conclusively prove that HBII-52 functions by masking the silencers on the snoRNA complementary site of the 5-HT_{2C}R exon Vb and promotes the exon usage.

5.5. Loss of HBII-52 in PWS patients could be a contributing cause for the syndrome

Our data demonstrates that isoforms of 5-HT_{2C}R mRNA containing non-edited sequences of Exon Vb are abnormally low in PWS patients. This is a natural consequence of the lack of HBII-52 expression in patients suffering from PWS. This snoRNA, like several others is localized in the imprinted region that is deleted in the PWS patients. Since these 5-HT_{2C}R isoforms show the strongest affinity to serotonin, this finding could explain why PWS patients respond to Selective Serotonin Reuptake Inhibitors (SSRI) treatment. Recently, Serotonin receptor 2C has also been shown to help the secretion of hormones like Vasopressin and Oxytocin (Jorgenson H et al., 2003). Also, decreased number and size of cells in anterolateral hypothalamic nucleus of Prader-Willipatients have been reported, whose normal function is secretion of Oxytocin (Swaab et al., 1995). The serotonin

receptor 2C has also been implicated in the control of depression, neuronal excitability, phagia and obesity (McCarthy et al., 2005), which as well characterize Prader-WilliSyndrome. These data collectively points to defects in the serotonergic system as a contributing cause of PWS. For mutated CFTR alleles, it has been shown that their penetrance depends on the genetic background, suggesting that alternative splicing is a genetic modifier of disease. To date, HBII-52 RNA expression could not be detected in Prader-Willi patients. However, two patients were described that lack the paternal HBII-52 snoRNA cluster and suffer from Angelman syndrome, but not PWS. It has not been investigated whether these patients express HBII-52, but it is possible that they either express HBII-52 from the maternal allele by an unknown mechanism or that the alternative splicing defect of the 5-HT_{2C}R gene is modified by a unique composition of splicing regulatory factors in these two patients.

Knockout animal models have shown that separate SNRPN (Yang et al., 1998) and UBE3A (Tsai et al.,1999) knock-out mice show no obvious abnormal phenotype, whereas the lack of Pwcr1/MBII-85 snoRNA is critical for neonatal lethality observed in the PWS model mice (Ding et al., 2005). Ding et al., (2005) have also shown that deletion of snoRNA MBII-52 gene clusters is not lethal in mice. Interestingly, Pwcr1/MBII-85 deletions in humans are not lethal as in mice. The attempted mouse models fail to depict human PWS conditions as they have not survived sufficiently long to learn whether information on pathogenesis can be gleaned from them. Taking together all available data, the contributing cause of HBII-52 in post natal developmental abnormalities in PWS still cannot be undermined.

Recently, using quantitative Real-Time Polymerase Chain Reaction, it has been shown that fear conditioning in mice upregulates MBII-52 expression in the hippocampus, indicating that MBII-52 expression can be regulated by physiological stimuli. This opens the exciting possibility that snoRNAs are involved in regulating higher brain functions by influencing alternative splicing. In contrast, there have been conflicting evidences regarding the involvement of the Serotonin receptor 2C in the memory consolidation (Rogelj et al., 2003).

5.6. snoRNAs other than HBII-52 located in PWS criticial region can potentially regulate alternative splicing of pre-mRNAs

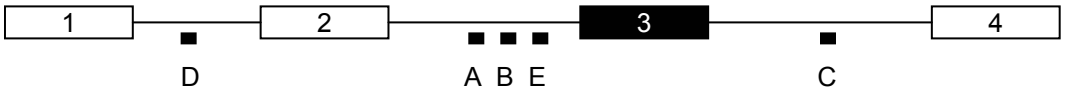
Expression of several genes is affected in the Prader-WilliSyndrome. It has been shown that several hormones like Ghrelin, Orexin, Growth Hormone, Leptin, etc. are deregulated in Prader-Willipatients. Growth hormone has been known to be externally administered to persons suffering from PWS to improve their body composition. Since, most of the genes in the Prader-Willicritical region codes for snoRNAs (HBII-13, -436,-437,-438, -85 and -52) that are not translated into proteins, it is likely that the snoRNAs in this locus can regulate proper RNA processing of genes misregulated in the syndrome. We performed a search for putative snoRNA targets in PWS relevant genes, which revealed extensive complementarities in the intron (Figure 53). The search included a tolerance for a few mismatches and considered Crick-wobble base pairing. It has been earlier shown that a few mismatches can be tolerated in the target recognition for several snRNAs and snoRNAs (eg. U1 binding to the splice site, or HBII-52 binding to 5-HT_{2C}R). The presence of complementarities in clusters in the introns of the relevant genes makes it an unlikely chance phenomenon. A 12 to 14 Watson-Crick complementarites between antisense box and the intron, indicates that such a sequence would occur ever 412 to 414 (~17 to 268 million) base pairs. It is therefore statistically unlikely that several clusters of these binding motifs occur by chance in a single gene. Interestingly, the intronic region flanking the alternatively spliced exon Va of Serotonin receptor 2C showed most significant similarities with all snoRNAs. HBII-52 showed yet another 16 nt complementarity to the intron upstream of alternatively spliced Exon 6 of a brain specific Hypocretin receptor 2 (Orexin receptor 2). Discovery of such complementarities indicate that HBII-52 could be involved in the processing of more than one gene.

Like HBII-52, other snoRNAs related to PWS also showed several regulatory elements in their target regions (Table 18). This shows that many of the orphan snoRNAs could work by binding to silencers or indirectly sequestering the splice factors away from the splice site.

Figure 53. Putative target genes for snoRNAs missing in PWS sufferers. The gene name and size are indicated. Boxes with numbers indicate exons, lines introns. Black boxes indicate alternative exons. Small boxes with letters show sequence complementartities between the antisense boxes of snoRNAs and regions on the pre-mRNAs. The table underneath the gene structure shows these complementarities. The antisense boxes are shown in 3'->5' direction, the RNA sequences in 5'->3' direction. Stars indicate Watson-Crick base pairing. Underlined stars indicate Crick wobble base pairing.

A. GHRL, synonyms: (MTLRP, ghrelin) :

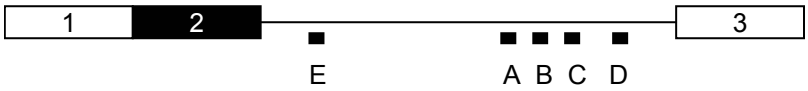
Gene Size: 4.6 Kb



A	HBII-13 TCATTAGTGCAACTCG GHRL AGCAATCCCATTAGAG	B	HBII-52 GCATTAGGATAACTCGTA GHRL CCCACTCCCATTGAACAG
C	MBII-85 AAGGCTACTCTCACCGTCAT GHRL AACTGATTGAGGTGGCAGAA	D	HBII-436 TCACAGTAAAAATTCG GHRL AGCGCCATCTCTGGGC
E	HBII-438 TAACTGTAGACCTTAC GHRL ATTCTCATCTGAAAGA		

B. Growth hormone secretagogue receptor (GHSR) :

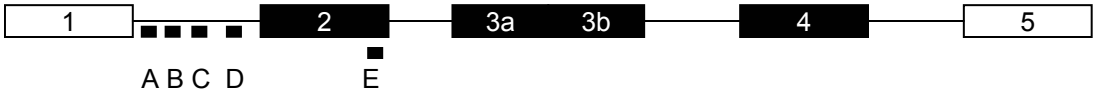
Total Gene : 3.5 Kb



A	HBII-13 TCATTAGTG-CAACTCG GSH-R1a/b AGTAAAAATTGTTGAAC	B	HBII-52 GCATTAGGATAACTCGTA GSH-R1a/b GATAATCCTCTTAGAGAT
C	MBII-85 AAGGCTACTCTCACCGTCAT GSH-R1a/b CACCTACGAGAGTCACATTG	D	HBII-436 TCACAGTAAAAATTCG GSH-R1a/b AATTTTATTTTCAAGC
E	HBII-438 TAACTGTAGACCTTACT GSH-R1a/b CTTGCTTTCTTGAGTGA		

C. Growth hormone1, GH1 :

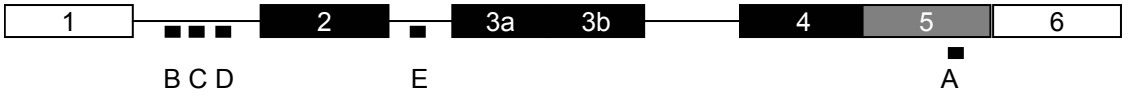
Total gene: 1.8 kb.



A	HBII-13 TCATTAGTGCAACTCG GH1 TAAAATCCCTTTGGGC	B	HBII-52 GCATTAGGATAACTCGTA GH1 TAAAATCCCTTTGGGCAC
C	MBII-85 AAGGCTACTCTCACCGTCAT GH1 TCCTGAGGGGAGAGGCAGCG	D	HBII-436 TCACAGTAAAAA-TTCG GH1 AGTATCGCCATGTAAGC
E	HBII-438 TAACTGTAGACCTTACT GH1 TTTGACACCTACCAGGA		

D. Growth hormone, GH 2:

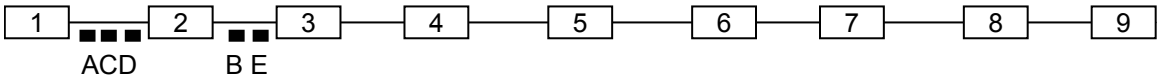
Total gene : 1.8 kb.



A	HBII-13 TCATTAGT--GCAACTCG GH2 AGCAGTCAGGCGCTGACC	B	HBII-52 GCATTAGGATAACTCGTA GH2 TAAAATCCCTTTGGGCAC
C	MBII-85 AAGGCTACTCTCACCGTCAT GH2 TCCTGAGGGGAGAGGCGGCG	D	HBII-436 TCACAGTAA-AAATTCG GH2 AGTATCGCCATCTAAGC
E	HBII-438 TAACTGTAGACCTTACT GH2 GTTGTTTTCTGAAGTGA		

E. GH Receptor:

Total gene: 156 kb.

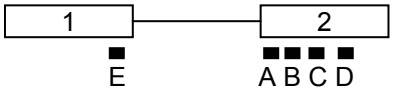


A	HBII-13 TCATTAGTGCAACTCG GHR AGTGACCAGTTTGAGC	B	HBII-52 GCATTAGGATAACTCGTA GHR TACAGTTCTGTTGAGCAT

C	MBII-85 AAGGCTACTCTCACCGTCAT GHR TTCCTATGAAAATGCTAGTT **** **** * ** ****	D	HBII-436 TCACAGTAAAAATTCG GHR AGTGTCATCTTCAAAC ******** ** ** *
E	HBII-438 TAACTGTAGACCTTACT GHR ATTGACACCTAGAATGG ******* ** *******		

F. Leptin Precursor:

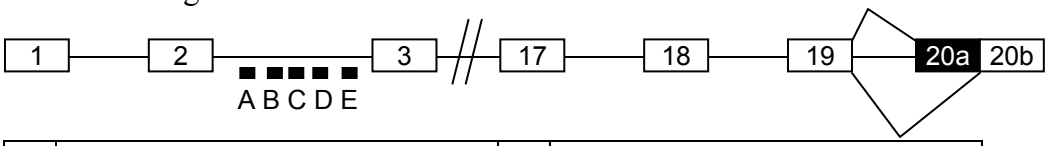
Total gene: 5.8 Kb



A	HBII-13 TCATTAGTGCAACTCG Leptin GGTGGTGGCCCTGAGC ***** * *****	B	HBII-52 GCATTAGGATAACTCGTA Leptin CCTGACCTTATCCAAGAT * *** **** * **
C	MBII-85 AAGGCTACT--CTCACCGTCAT Leptin TTCCTATCATGGAGTGACGGTC **** ** * ***** ****	D	HBII-436 TCACAGTAAAAATTCG Leptin AGTGTCATATGTAGGT ******** * ** **
E	HBII-438 TAACTGTAGACCTTAC Leptin ATTGTCACCAGGATCA **** ** * ***		

G. Leptin receptor :

Gene total length: 216Kb



A	HBII-13 TCATTAGTGCAACTCG Lep-US ACTAATGATGTTGAGC * **** ********	B	HBII-52 GCATTAGGATAACTCGTA Lep-US TAGAATCTTTTTGAGCAT * ***** ********
C	MBII-85 AAGGCTACTCTCACCGTCAT Lep-US TTTCAACTAAGATGGCAATA *** * * * ***** **	D	HBII-436 TCACAGTAAAAATTCG Lep-US AGTATCATTTCTAGGA *** ****** ***
E	HBII-438 TAACTGTAGACCTTAC Lep-US ACTGAAATCTGAAATG * *** ***** ****		

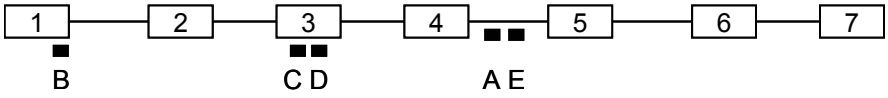
H. Orexin precursor:

Total gene: 1.6 Kb

A	HBII-13 TCATTAGTGCAACTCG Orexin CATCCTCACGCTGGGC * ***** *****	B	HBII-52 GCATTAGGATAACTCGTA Orexin CATCCTCACGCTGGGCAA * * ** * ******
C	MBII-85 AAGGCTACTCTCACCGTCAT Orexin CCCCCAGAAAAAAGGCAATA ** * * * **** **	D	HBII-436 TCACAGTAAAAATTCG Orexin ACTCCTGTTTTCAGGT * * ****** ***
E	HBII-438 TAACTGTAGACCTTAC Orexin AGTGACAGCCAGAAAG * ***** * *** *		

I. Orexin receptor 1, Synonym: (Hypocretin receptor 1) :

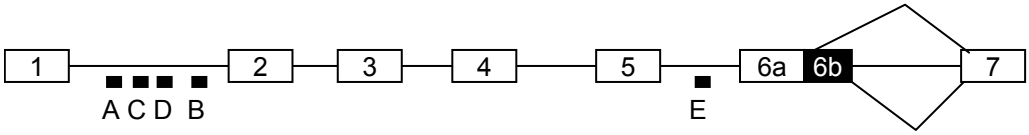
Total Gene: 8.3 Kb



A	HBII-13 TCATTAGTGCAACTCG OXR-1-US ATTACCCACATTAAAT * ** *** ** * *	B	HBII-52 GCATTAGGATAACTCGTA OXR-1-US CGTGGCCCTGGTGGGCAA ***** **** ******
C	MBII-85 AAGGCTACTCTCACCGTCAT OXR-1-US TGTCCGTGTCAGTGGCAGTG * * ** *********	D	HBII-436 TCACAGTAAAAATTCG OXR-1-US AGTGCTAACTCTCAGC **** ** * * ***
E	HBII-438 TAACTGTAGACCTTAC OXR-1-US ATTGGCGTCTGGGCGG ************ *		

J. Orexin receptor 2, synonym: (Hypocretin receptor2) :

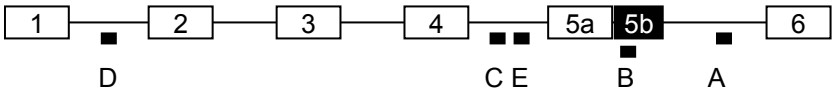
Total Gene: 108 Kb.



A	HBII-13 TCATTAGTGCAACTCG OXR-2-US AAGAATCATGTTAATC	B	HBII-52 GCATTAGGATAACTCGTA OXR-2-US TATAATCTTATTGAGTAG
C	MBII-85 AAGGCTACTCTCACCGTCAT OXR-2-US CTCTGCTGAAAATGACAGGA	D	HBII-436 TCACAGTAAAAATTCG OXR-2-US AGTGCTATTTTTAGAC
E	HBII-438 TAACTGTAGACCTTACT OXR-2-US ATTGCCATTTTGAATGA		

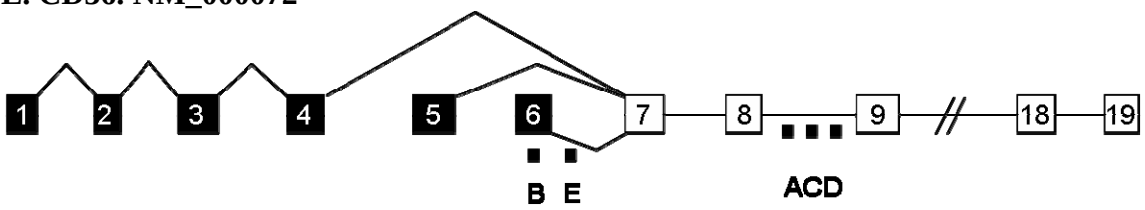
K. Serotonin receptor2C, synonym: (5-HT$_{2C}$R) :

Total gene: 326 kb



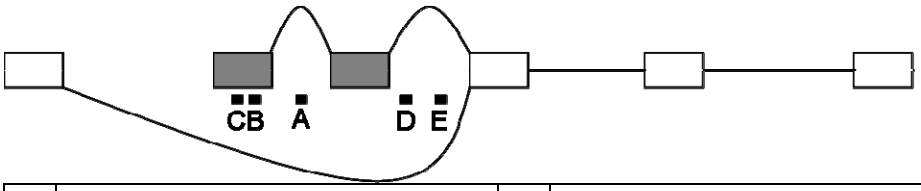
A	HBII-13 TCATTAGTGCAACTCGT 5-HT2CR AGCAATTACGTTGAGCA	B	HBII-52 GCATTAGGATAACTCGTA 5-HT2CR CGTAATCCTATTGAGCAT
C	MBII-85 AAGGCTACTCTCACCGTCAT 5-HT2CR CTCTGATGAAAGTAGTAGTG	D	HBII-436 TCACAGTAAAAATTCG 5-HT2CR AGTGCCATTTTCTGGC
E	HBII-438 TAACTGTAGACCTTAC 5-HT2CR TTTGACATCTCGAATG		

L. CD36. NM_000072



A	HBII-13 TCATTAGTGCAACTCG CD36 AGTAACCATATTAAAG	B	HBII-52 GCATTAGGATAACTCGTA CD36 TTTGATCCTATTAAGAAT
C	MBII-85 AAGGCTACTCTCACCGTCAT CD36 TTTAAATTAAAATAGCAGTA	D	HBII-436 TCACAGTAAAAATTCG CD36 GGTATTGTTTTTGAGC
E	HBII-438 TAACTGTAGACCTTACT CD36 GTTAATATTTAAAGTGA		

M. DUSP4



A	HBII-13 TCATTAGTGCAACTCG DUSP4 TTCAACCACGTTGAAC	B	HBII-52 GCA-TTAGGATAACTCGTA DUSP4 CGTGAATCCCGTTGAGAGT
C	MBII-85 AAGGCTACTCTCACCGTCAT DUSP4 TCCCGGGGAGAGAGGGATTA	D	HBII-436 TCACAGTAAAAATTCG DUSP4 GGAGTTATTCTTAAGT
E	HBII-438 TAACTGTAGACCT-TACT DUSP4 ACTGACACCCAGAGATGA		

M. KCTD12, designated as ESRT in paper, NM_138444

A	HBII-13 TCATTAGTGCAACTCGG EST AGTGAATGACATTGGAC	B	HBII-52 GCA-TTAGGATAACTCGT EST TGAAATCTTCTCAAGCTC
C	MBII-85 AAGGCTACTCTCACCGTCAT EST CTCCGGGGAAGGCGGTTGCA	D	HBII-436 TCACAGTAAAAATTCG EST AATGCCACTTTCATGC
E	HBII-438 TAACTGTAGACCT-TAC EST TTTGAAGCCTGAAATAA		

N. MMP12

A	MBII-13 TCATTAGTGCAACTCG MMP12 AGTAGTCAAGATGGAT	B	HBII-52 GCA-TTAGGATAACTCGTA MMP12 TGTCATCTTATTAATCAT
C	MBII-85 AAGGCTACTC----TCACCGTCAT MMP12 TCCAGATGAGGATCAGTGGTGGTG	D	HBII-436 TCACAGTAAAAATTCG MMP12 AATAACATTTCAAAGC
E	HBII-438 TAACTGTAGACCTTACT MMP12 TTTGTCATTTGGCATGA		

O. PDGFRL

A	HBII-13 TCATTAGTGCAACTCG PDGFRL AGTGACTAGATTGAGC	B	HBII-52 GCATTAGGATAACTCGTA PDGFRL CATATTTTTATTGAATGT
C	MBII-85 AAGGCTACTCTCACCGTCAT PDGFRL CTCTAATGGAGGTGGCAGGG	D	HBII-436 TCACAGTAAAAATTCG PDGFRL TGTATCATTCTTACGC
E	HBII-438 TAACTGTAGACCTTAC PDGFRL ATTTAGATCTGGAGTG		

P. SCG2

A	HBII-13 TCATTAGTGCAACTCG SCG2 AATGATCATTTTGAAT	B	HBII-52 GCATTAGGATAACTCGTA SCG2 TATAATTCTATTGAAAGT
C	MBII-85 AAGGCTACTCTCACCGTCAT SCG2 TTTCAATGTTAGCAATATTA	D	HBII-436 TCACAGTAAAAATTCG SCG2 AATGTTATTTCTGGAC
E	HBII-438 TAACTGTAGACCTTACT SCG2 ATTGATGTCTAAGCTAA		

Q. SNRPN

A	HBII-13 TCATTAGTGCAACTCG SNRPN AGCAATCATGGTAAGC	B	HBII-52 GCATTAGGATAACTCGTA SNRPN TATAATCCCATTGGGCAA
C	MBII-85 AAGGCTACTCTCACCGTC SNRPN TTCCGGTGAGGGAGGGAG	D	HBII-436 TCACAGTAAAAATTCG SNRPN AGTCTCATTTTAAAGC
E	HBII-438 TAACTGTAGACCTTACT SNRPN ATTAATATTTGGAATAA		

Table 18. Putative silencers in the snoRNA binding sites (sense of antisense box)

HBII-13	HBII-52	HBII-436
5′-AGTAATCACGTTGAGC-3′ GTAATGGGGG ACGTTTCCAT AGTTGTTGAC	5′ -CGTAATCCTATTGAGCAT- 3′ GTAATGGGGG CCTATGTTTG GTAGGTATTG	5′ –AGTGTCATTTTTAAGC- 3′ GTTAGTGTAG TGTAGTGTTA TTTAGTGTTA AGTGTA TAGTGT AGTGTT GTTTTT GTAGGTTTTT ATAGGTTTTA CTTTTACCAA TTAGTTTTTA TTTTAG
MBII-85	**HBII-438**	
5′-TTCCGATGAGAGTGGCAGTA-3′ GTTCCG TTCCGT TTTGTTCCGT TCGTTCCGTA TAGTGG GTGGCA GGGGGTGGCA TGGCATACTA	5′ –ATTGACATCTGGAATGA- 3′ AGTTGTTGAC	

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Curriculum Vitae

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Education:

1995	Primary schooling, Don Bosco Academy, India. (91.6%)
1995-1997	Higher Secondary, St. Michael's High school, India. (90%)
1997-2002	Bachelor's and Master's (Dual degree) in Biochemical Engineering and Biotechnology, Indian Institute of Technology, Delhi, Bachelor's CGPA: 7.87 on a scale of 10; Master's CGPA: 8.45 on a scale of 10.)
Jun' 2002-Dec' 2005	Graduate student at Stamm's lab, University of Erlangen-Nürnberg, Germany.

Professional Experience:

May-July' 2000	Industrial Training in Hoechst Roussel Pvt. Vet Ltd., Pune, India.
2001-2002	Research for Master's dissertation in Malaria Group, International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India.

Honors and awards:

2001-2002	Graduate Aptitute Test in Engineering. (94 percentile)
2000	Summer Undergraduate Research Award, sponsored by Industrial Research and Development, IIT Delhi, India
1998-2001	MCM scholarship, IIT Delhi, India.
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1996	Scholarship through National Talent Search Examination, India.

Publications:

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