Hum Genet (2004) 114 : 553–561
DOI 10.1007/s00439-004-1104-z

ORIGINAL INVESTIGATION

**Maren Runte · Peter M. Kroisel
Gabriele Gillessen-Kaesbach · Raymonda Varon
Denise Horn · Monika Y. Cohen · Joseph Wagstaff
Bernhard Horsthemke · Karin Buiting**

SNURF-SNRPN and *UBE3A* transcript levels in patients with Angelman syndrome

Received: 1 August 2003 / Accepted: 6 February 2004 / Published online: 10 March 2004

Abstract The imprinted domain on human chromosome 15 consists of two oppositely imprinted gene clusters, which are under the control of an imprinting center (IC). The paternally expressed *SNURF-SNRPN* gene hosts several snoRNA genes and overlaps the *UBE3A* gene, which is encoded on the opposite strand, expressed – at least in brain cells – from the maternal chromosome only, and affected in patients with Angelman syndrome (AS). In contrast to *SNURF-SNRPN*, imprinted expression of *UBE3A* is not regulated by a 5′ differentially methylated region. Here we report that splice forms of the *SNURF-SNRPN* transcript overlapping *UBE3A* in an antisense orientation are present in brain but barely detectable in blood. In contrast, splice forms that do not overlap with *UBE3A* are of similar abundance in brain and blood. The tissue distribution of the splice forms parallels that of the snoRNAs encoded in the respective parts of the *SNURF-SNRPN* transcript. Using a quantitative PCR assay, we have found that the ratio of *SNURF-SNRPN*/*UBE3A* transcript levels is increased in blood cells of AS patients with an imprinting defect, but not in AS patients with a *UBE3A* mutation or an unknown defect. Our findings are compatible with the assumption that imprinted *UBE3A* expression is regulated through the *SNURF-SNRPN* sense-*UBE3A* antisense transcript.

Nucleotide sequence data reported are available in the GenBank databases under the following accession numbers: AY362862 (RT-21, exons 23, 24, 25, 25.1, 26), AY362863 (RT-22I, exons 33, 34, 34.1, 35), AY421730 (RT-22II, exons 33, 34, 34.2, 35), AY362864 (RT-23, exons 74, 75, 76a, 76b, 77, 78), AY362865 (RT-24, exons 84, 85, 86a, 86b, 87, 88), AY362866 (RT-25, exons 98, 99, 99.1, 100, 101, 102)

M. Runte · G. Gillessen-Kaesbach · B. Horsthemke
K. Buiting (✉)
Institut für Humangenetik, Universitaetsklinikum Essen,
Hufelandstraße 55, 45122 Essen, Germany
e-mail: Karin.Buiting@uni-essen.de

P. M. Kroisel
Institut für Medizinische Biologie und Humangenetik,
Universitaet Graz, Graz, Austria

R. Varon
Institut für Humangenetik, Charité, Humboldt Universität Berlin,
Berlin, Germany

D. Horn
Institut für Medizinische Genetik, Charité,
Humboldt Universität Berlin, Berlin, Germany

M. Y. Cohen
Medizinische Genetik, Kinderzentrum München,
München, Germany

J. Wagstaff
Departments of Biochemistry and Molecular Genetics
and Pediatrics, University of Virginia,
Charlottesville, Virginia, USA

Introduction

Prader-Willi syndrome (PWS; MIM176270) and Angelman syndrome (AS; MIM105830) are two distinct neurogenetic diseases that are caused by the loss of function of imprinted genes in 15q11-q13. The imprinted domain consists of two oppositely imprinted gene clusters, which are under the control of a bipartite imprinting center (IC). The IC overlaps the paternally expressed *SNURF-SNRPN* gene. This gene and the other paternally expressed genes (*MKRN3*, *MAGEL2*, *NDN* and several C/D box small nucleolar (sno) RNA genes) are candidate genes for PWS. Two genes located telomeric to these genes (*UBE3A* and *ATP10C*) are expressed preferentially from the maternal chromosome. Maternal-only expression of the *UBE3A* gene, which is affected in AS, is restricted to brain cells, although Herzing et al. (2002) provided evidence for preferential maternal expression in fibroblasts, lymphoblasts and neuronal precursor cells by RNA fluorescence in situ hybridization. Whereas maternal silencing of *MKRN3*, *MAGEL2*, *NDN* and *SNURF-SNRPN* is brought about by promoter methylation, *UBE3A* and *ATP10C* lack a 5′ differentially methylated region (DMR) and it has been suggested that paternal silencing of *UBE3A* in brain might involve a brain-specific antisense transcript (Rougeulle et al. 1997; Vu and Hoffman 1997). We have recently shown that the *SNURF-SNRPN* locus, which is transcribed from

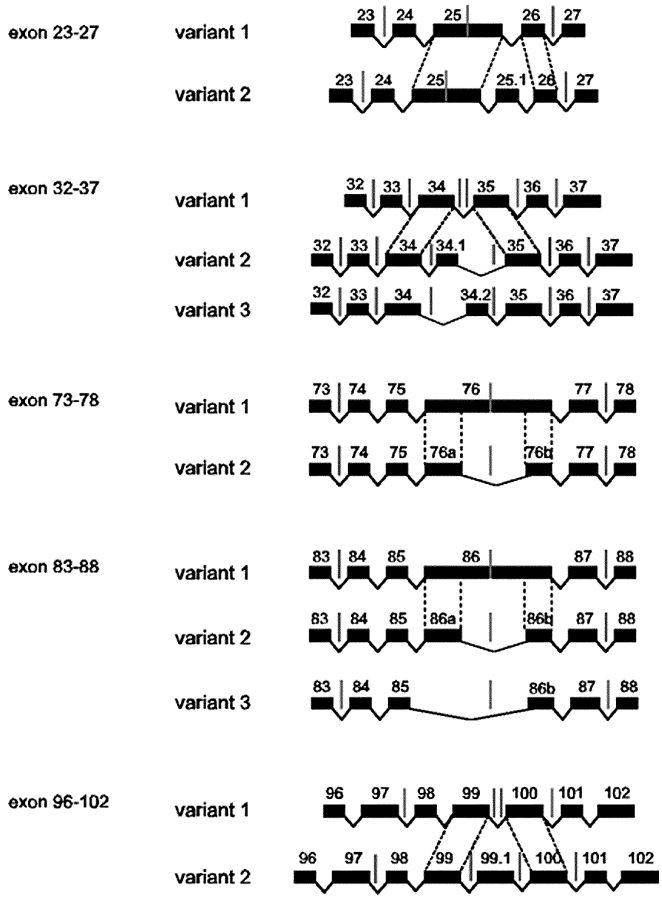

Fig. 1 Schematic view of known (*1*) and novel splice variants (*2* and *3*). The *black boxes* represent *SNURF-SNRPN* exons. The snoRNA genes are indicated as *grey vertical* lines

centromere to telomere, overlaps the *UBE3A* gene, which is transcribed from telomere to centromere (Runte et al. 2001). The *SNURF-SNRPN* sense-*UBE3A* antisense transcription unit spans more than 460 kb and has more than 148 exons, including the previously identified *IPW* exons (59–61; Fig. 2b). It also serves as a host gene for the paternally expressed snoRNA genes, which are located within introns of the transcription unit. Some of these snoRNA genes occur as single copies (*HBII-436*, *HBII-13*, *HBII-438A* and *HBII-438B*), whereas two snoRNA genes are present as multicopy gene clusters containing 27 copies (*HBII-85*) and 47 copies (*HBII-52*). The snoRNAs occur predominantly in brain, but are present in other tissues also, except for *HBII-52*, which is brain-specific (Cavaillé et al. 2000; Runte et al. 2001).

To gain further insight into a possible role of the *SNURF-SNRPN* sense-*UBE3A* antisense transcript in the regulation of imprinted *UBE3A* expression in brain, we have now investigated the steady-state level of the different transcripts in brain and blood cells of normal individuals as well as in blood cells of AS patients with an imprinting defect or a *UBE3A* mutation. In the context of these investigations, we have also identified novel exons of the *SNURF-SNRPN* transcription unit.

Patients and methods

Patients

All patients involved in this study were seen by experienced clinicians. PWS-1 and PWS-2 belong to a large Austrian family with a familial IC deletion, which was reported before by Buiting et al. (2000) and El-Maarri et al. (2001). PWS-1 is individual IV.1 and PWS-2 is individual IV.7 in the pedigree shown in El-Maarri et al. (2001). The fathers F1 and F2, who are carriers of the IC-deletion, are the individuals III.2 and III.15, respectively. Patients AS-ID2 and AS-ID71 were diagnosed to have typical AS and an imprinting defect but no IC deletion. In seven patients presenting with typical features of Angelman syndrome all four possible molecular defects (deletion 15q11-q13, upd(15)pat, imprinting defect, *UBE3A* mutation) had been excluded. In addition, a *MECP2* mutation (Rett syndrome) was excluded. In four patients with AS (AS-U1, AS-U2, AS-U3a and 3b), the disease is caused by a frameshift or nonsense mutation in the *UBE3A* gene.

PCR analysis and cloning of novel splice variants

PCR on fetal brain cDNA (Clontech, Palo Alto, Calif.) was performed according to standard conditions with an annealing temperature of 58 °C using the following specific primers for *SNURF-SNRPN* exons: Ex23F (5′-AACATCCTGGAGTTGGTGTTG-3′) and Ex26R (5′-GTTTCTCAGAAGCATCGACCAT-3′), Ex74F (5′-CCTTGGTGGGCCGTGAACTGAAT-3′) and Ex78R (5′-ATACCAGGATGACAGGAAGGACCTG-3′), Ex84F (5′-GAGCTGAAGCTCAGGCCCTTCCTG-3′) and Ex88R (5′-GCTAAGCACCTGGCTGAGGGACATT-3′), Intr34F (5′-GCATGGAAGGAAGGACTGTG-3′) and Intr34R (5′-CCAAGGATCGCACAAGAAGA-3′), Intr39F (5′-TACATTCCTTGGAAAGCTGAACA-3′) and Intr39R (5′-CATATGGAAGTCATCATCGATCC-3′), Ex97F (5′-ACCAGATGGTGAGCCCAGAGGAAGA-3′) and Ex102R (5′-TGGGAAACTGGCTGACAGACACTGG-3′). In the case of multiple PCR products, these products were subcloned in the pGEM-T Easy PCR vector (Promega, Mannheim, Germany).

Southern blot analysis

Southern blot analyses were performed with two PCR derived probes, NG73/74 and NG79/80, which identify DNA sequences 48 kb and 55 kb distal to exon 12. Both probes were generated by PCR using primers NG73 (5′-GCGTGTTATGATACGGTCAA-3′) and NG74 (5′-ACAAAATTCAAACTCAACCTCAA-3′) for probe NG73/74 and with primers NG79 (5′-GTGCTTGTTTGGATCGATGA-3′) and NG80 (5′-TGCCCTCTTCGGAGAGATAG-3′) for probe NG79/80. The annealing temperature for both reactions was 60 °C. Two micrograms of DNA was digested with *Eco*RV and *Sac*I, resolved on 1% agarose gels, and analyzed by Southern blot hybridization. Probes were labeled by random oligonucleotide priming and ([32P] dCTP (NEN DuPont, Boston, Mass.). Autoradiography was performed at –80 °C with intensifying screens and Kodak XAR films. To confirm the junction fragment for the distal deletion breakpoint in *Sac*I-digested DNA, the Southern blots were rehybridized with probe L48.3p1p2, which was previously shown to detect the junction fragment for the proximal deletion breakpoint (Buiting et al. 2000).

Sequence analysis

The cloned PCR products were sequenced with the vector-specific primers SP6 and T7. To detect the nonsense mutation in exon 15 of the *UBE3A* gene, we used the same reverse primer for DNA and RNA (MutEx15R, 5′-TCTGTGTCTGGGCCATTTTT-3′), but a different forward primer for DNA (MutEx15dF, 5′-TGAATGCCAAACTGAAACCA-3′) and RNA (MutEx15rF, 5′-AACTACA-

GAATATGACGGTGGCTA-3′) for PCR (58 °C annealing temperature). Sequencing reactions were performed with fluorescence-tagged dideoxynucleotides (BIGDye Kit) and the Taq cycle sequencing procedure and analysed on an ABI Prism3100 DNA Genetic Analyzer (ABI, Foster City, Calif.).

RNA preparation and RT-PCR

Total RNA from peripheral blood was prepared according to the protocol of the PAXgene Blood RNA System (QIAgen, Hilden, Germany) or QIAamp RNA Blood Mini Kit (QIAgen). RNA was treated with DNase to remove residual traces of genomic DNA. RNA from human fetal brain was purchased from Invitrogen (Carlsbad, Calif.). RT-PCRs were performed with the GeneAmp RNA-PCR kit (ABI, Foster City, Calif.). Total RNA was reverse transcribed using random hexamers. The cDNA products were amplified by 35 cycles of PCR. To determine the expression status at several positions of the *SNURF-SNRPN* transcription unit, we performed RT-PCR with the following specific primers: *SNURF-SNRPN* exons 1–2 (RN134 and RN175; annealing temperature 60 °C; Sun et al. 1996); *SNURF-SNRPN* exons 57–58 (TaqIPWF 5′-CAGGAAAGATCAAAACGATGCA-3′, TaqIPWR 5′-TCCAAAGGAGGCAGTTGGAT-3′; annealing temperature 58 °C), *SNURF-SNRPN* exons 63–65 (Ex63–65F 5′-CTGAAGCTCAGGCCATTCCT-3′; Ex63–65R 5′-GTCTTCCTCCAGGCTCACTG-3′; annealing temperature 60 °C), *SNURF-SNRPN* exons 142–143 (TaqUBE3AasF 5′-CACAGCTGACACACCCAGATATC-3′; TaqUBE3AasR 5′-CCCTGAAGTTTCCTTGAAGTTGTT-3′; annealing temperature 60 °C); *SNURF-SNRPN* exons 147–148 (MRts10–11F 5′-AGAAAAGGCGCAATGAAAGA-3′, Ex148R 5′-GGAGAAGGGCCATAGACTCC-3′, annealing temperature 60 °C). To check the integrity of the RNA, we used primers for the *ß-actin* gene with an annealing temperature of 56 °C (Buiting et al. 2001).

Real-time quantitative reverse transcription (QRT-PCR)

QRT-PCR for exons 57–58 of the *SNURF-SNRPN* transcription unit was performed in a 20-µl reaction volume containing 1 µl of cDNA, 300 nM of each primer, 250 nM of the TaqMan probe and 1×TaqMan Universal PCR Master Mix (ABI, Foster City, Calif.). The primers and probes were designed using the Primer Express software (version 2.0; ABI). To prevent detection of DNA, each probe spanned an exon-exon boundary. *SNURF-SNRPN* exons 57–58: primer TaqIPWF and TaqIPWR (see RNA preparation and RT-PCR), probe 5′-FAM-TGCAAGTGTGATTGGTCCAGATAGCTGC-DQ-3′. The QRT-PCR for *UBE3A* was performed with the ABI assay-on demand (assay-ID Hs00166580_m1 (ABI). The PCR reaction (20 µl) contained 1 µl cDNA, 1 µl TaqMan Universal PCR Master Mix and 1 µl mix of primers and probe. Both probes contained a FAM reporter dye connected to the 5′ end. At the 3′ end, the probe for exons 57–58 of the *SNURF-SNRPN* transcript carried a darkQuencher dye, whereas the probe for *UBE3A* contained a TAMRA quencher dye. PCR was carried out using the ABI Prism 7000 Sequence Detection System (ABI). The reactions were performed in duplicates. Standard curves for both assays were performed with 50 ng, 25 ng, 10 ng, 5 ng, 2.5 ng and 1 ng of the fetal brain cDNA. The relative expression levels were calculated by division of the mean starting concentrations (c0) of *SNURF-SNRPN* and *UBE3A*.

Fragment-length analysis in patients with a *UBE3A* frameshift mutation

To detect the 2-bp deletion in exon 10 of the *UBE3A* gene, we used the same reverse primer for DNA and RNA (MuEx10R; 5′-FAM-CCTCCCTCATCAACTCCTTG-3′), but different forward primers: MutEx10dF (5′-TTTCCATTTACATACAAAAAGTCAA-3′) for DNA and MutEx10rF (5′-CGCATGTACAGTGAACGAAGA-3′) for RNA. To detect the 4-bp deletion in exon 12 of *UBE3A*, we used MutEx12R (5′-FAM-TTCCTGTTTTCATTTGTAATTGG-3′) as a reverse primer for DNA and RNA, with MutEx12dF (5′-TGGTGGCCTCAATTTACCAT-3′) as a forward primer for DNA, or MutEx12rF (5′-AACTTTTCGTGACTTGGGAGA-3′) as a forward primer for RNA. The annealing temperature was 58 °C. PCR products after 20 and 35 cycles were analyzed on an ABI Prism 3100 Genetic Analyzer (ABI) and the Genescan and Genotyper software.

Nomenclature

Gene symbols used in this article follow the recommendations of the HUGO Gene Nomenclature Committee (Povey et al. 2001).

Results

Identification of novel *SNURF-SNRPN* exons

Typically, snoRNA genes reside within an intron of a host gene, and an intron harbors only one snoRNA gene. In Runte et al. (2001), we described that three snoRNA gene copies appeared to be located in an exon (*HBII-85* gene copy 5 in exon 25, *HBII-52* copy 7 in exon 76 and *HBII-52* copy 13 in exon 86), and that four introns appeared to harbor two snoRNA genes (*HBII-85* gene copies 13 and 14 in intron 34, *HBII-85* gene copies17 and 18 in intron 37, *HBII-85* gene copies 19 and 20 in intron 39 and *HBII-52* gene copies 21 and 22 in intron 99).

To search for additional splice variants of the *SNURF-SNRPN* sense-*UBE3A* antisense transcript, we performed PCR on fetal brain cDNA and cloned and sequenced the multiple products. By this we identified seven novel splice

Table 1 Exon-intron boundaries of the novel *SNURF-SNRPN* exons

SNURF-SNRPN	Size (bp)	Intron	Exon	Intron
Exon 25.1	108	...ggttttccgccag	GTGCACCTCACCTAA.....GGTGGTGGCTGTTAG	gtaaga...
Exon 34.1	254	...tgtccaaaatcag	GTAAAGCCAGAGTTG.....GGATCTCTCCTGAAT	gtaagc...
Exon 34.2	134	...ctgcacttcgcag	GTAGTGTTGGTGTAA.....GGGATCCTCCTGGAT	gtaagt...
Exon 76a	132	...cctgtttccccag	ATGTTGAGCCCAGAG.....CTGCATTGAGCTGTG	gtgagc...
Exon 76b	43	...ttgtgtctttcag	TGAGCTCTTCCACAT.....CATTGACTGGCATAG	gtgagt...
Exon 86a	277	...cctgtttccccag	GTATTGAGCCTGGAG.....TGAGGCCCAGTCTAG	gtgaga...
Exon 86b	45	...ctgtgtctttcag	TGAGCTCTTCCCCAC.....CATTGACTAGCATCG	gtgagt...
Exon 99.1	129	...cctgtcccccag	ATGGTGAGCCTGGAG.....TCCTTCATGGAGCTG	gtgagc...
Consensus		Py(10)cag		gtaagt

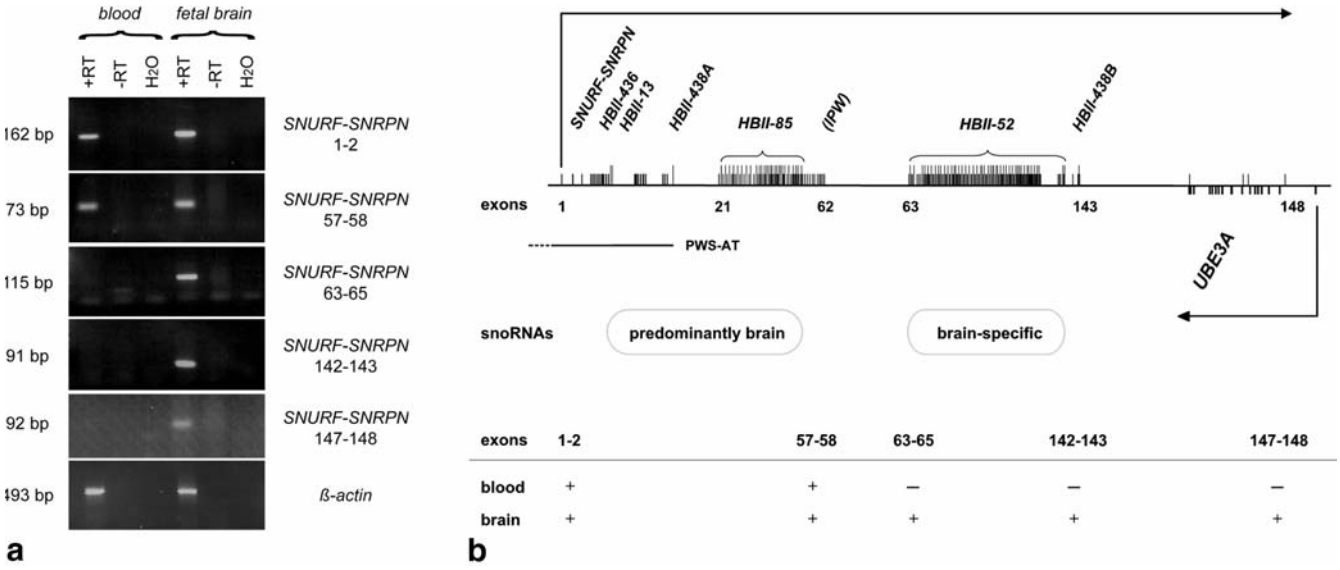

Fig. 2a, b Expression analysis of the *SNURF-SNRPN* transcription unit. **a** RT-PCR analysis of blood and fetal brain RNA. The integrity of the RNA samples was shown by amplification of a 493-bp RT-PCR product from the *ß-actin* locus. *+RT* RT-PCR with reverse transcriptase; *–RT* RT-PCR without reverse transcriptase; H_2O RT-PCR without RNA. **b** Comparison of the expression profile of the snoRNA genes and the *SNURF-SNRPN* transcription unit. Exons of the *SNURF-SNRPN* transcript are shown as *short vertical lines* and snoRNA genes are represented by *long vertical lines*. The exons of the *UBE3A* gene are shown as *vertical lines below the horizontal line*. Orientation of transcription is indicated by *arrows*. The extent of the IC deletion in family PWS-AT is indicated by a *horizontal line*. + RT-PCR positive; – RT-PCR negative

variants (Fig. 1). As we did not want to change the numbering of the exons given in Runte et al. (2001), novel exonic sequences identified within an intron were designated x.1, and exons split into two by a novel intron were designated x.a and x.b. The exon-intron boundaries of the novel *SNURF-SNRPN* exons are shown in Table 1.

Steady-state levels of alternative *SNURF-SNRPN* transcripts in blood and brain cells

Using reverse transcription (RT)-PCR and gel electrophoresis, we have detected transcribed exons 1–2, 57–58, 63–65, 142–143 and 147–148 in RNA from fetal brain. As shown in Fig. 2a, similar amounts of PCR products were obtained in these experiments. In RNA from whole blood we detected exons 1–2 and 57–58 only. Reamplification of exons 63–65, 142–143 and 147–148 gave a PCR product in some experiments, but no product in other experiments (data not shown). These results show that in blood the level of transcripts containing exons 63–148 are below the threshold of detection by these methods.

Ratio of *SNURF-SNRPN*/*UBE3A* transcript levels in blood from PWS patients with an IC deletion

To find out how an IC deletion affects *SNURF-SNRPN* and *UBE3A* transcription, we have studied blood RNA samples from two related PWS patients (PWS-1 and PWS-2), who have an IC deletion on the paternal chromosome, and their fathers (F1 and F2), who have the deletion on the maternal chromosome. From previous studies we knew that the proximal deletion breakpoint is approximately 28 kb upstream of *SNURF-SNRPN* exon 1 and that the distal deletion breakpoint maps within an 80-kb interval between exon 12 and the *IPW* exons (exons 59–61; Buiting et al. 2000; El-Maarri et al. 2001). To map the distal breakpoint more precisely, we performed Southern blot analysis with two different PCR probes, NG73/74 and NG79/80, which map 48 and 55 kb distal to exon 12, respectively. NG79/80 detects an abnormal *Sac*I fragment of 5 kb, indicating that the distal breakpoint is between exons 20 and 21. To verify this result, the Southern blot was rehybridized with probe L48.3p1p2, which was previously used to detect the proximal deletion breakpoint in *Bgl*II digested DNA. And in fact, the same 5-kb *Sac*I junction fragment was identified (data not shown).

For a quantitative assessment of the *SNURF-SNRPN* and *UBE3A* transcript levels we performed real-time quantitative reverse transcription (QRT) PCR. We probed exons 57–58 of the *SNURF-SNRPN* gene, which map approximately 100 kb telomeric to the distal IC deletion breakpoint, and the *UBE3A* gene. We did not detect exons 57–58 in the patients' RNA (Fig. 3). In the fathers, the *SNURF-SNRPN*/*UBE3A* transcript ratio was comparable with that of the controls.

Ratio of *SNURF-SNRPN*/*UBE3A* transcript levels in blood from AS patients who have an imprinting defect but no IC deletion

If *UBE3A* is biallelically expressed in blood, the presence of an incorrect paternal imprint on the maternal chromo-

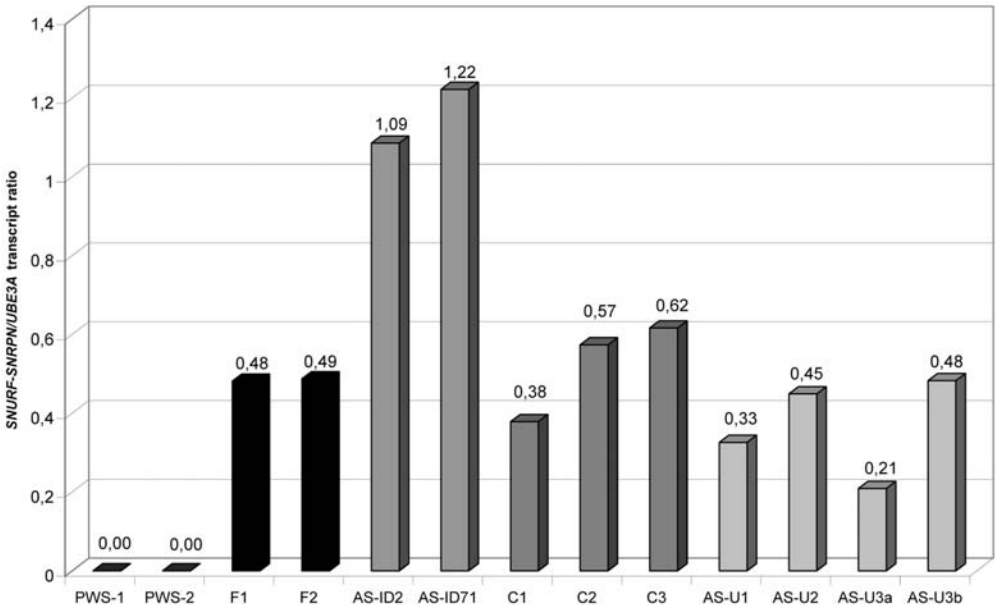

Fig. 3 *SNURF-SNPRN/UBE3A* transcript ratios. Transcript levels in blood RNA were determined by real time quantitative RT-PCR. *PWS-1* and *PWS-2*, PWS patients with an IC deletion; *F1* and *F2*, the fathers of the PWS patients; *AS-ID2* and *AS-ID71*, AS patients with an imprinting defect; *C1–C3*, normal controls; *AS-U1*, *AS-U2*, *AS-U3a* and *AS-U3b*, AS patients with a *UBE3A* mutation

some (AS imprinting defect) should have no effect on the level of *UBE3A* expression, but lead to a twofold amount of the *SNURF-SNRPN* transcript. Indeed, when using the QRT-PCR assay described above, we found a twofold increase in the *SNURF-SNRPN/UBE3A* transcript ratio in two unrelated patients (AS-ID2 and AS-ID71; Fig. 3).

Expression levels of wild-type and mutant *UBE3A* alleles in AS patients

If in AS patients with a *UBE3A* mutation the mutant transcript was subject to nonsense-mediated mRNA decay, we would expect an almost twofold increase in the *SNURF-SNRPN/UBE3A* transcript ratio and the QRT-PCR assay might be used as a screening method for selecting AS patients in whom a deletion, uniparental disomy and an imprinting defect had been excluded for *UBE3A* mutation screening. We studied four AS patients with a *UBE3A* mutation on their maternal chromosome. Two patients had a frameshift mutation: a 2-bp deletion in exon 10 (1559delTC; patient AS-U1) and a 4-bp deletion in exon 12 (1985delCAGA; patient AS-U2). The other two patients were sibs (AS-U3a and AS-U3b) and had a heterozygous G→A substitution in exon 15 (G2304A) leading to a premature termination codon. As shown in Fig. 3, in each of the patients the *SNURF-SNRPN/UBE3A* transcript ratio was not increased, but somewhat decreased.

Although we have no explanation for the decrease in the transcript ratio, the result suggested that the mutant transcripts are not subject to efficient nonsense-mediated mRNA decay. To address this question more directly, we performed fragment-length analysis of blood DNA and RNA in patients AS-U1, AS-U2, and the mother of AS-U2 (Fig. 4a), as well as sequence analysis of DNA and RNA in the patients AS-U3a and AS-U3b (Fig. 4b). For the exon 10 and exon 15 deletions, a different forward primer for DNA and RNA had to be used, because the mutations are close to the exon-intron boundary. Likewise, different primers had to be used for the exon 12 deletion, because the sequence of exon 12 is highly similar to the sequence in two different non-expressed pseudogenes. In patient AS-U1, the peak area of the mutant transcript was 47% of that of the wild-type transcript. This result was independent of the number of PCR cycles (not shown). In patient AS-U2 and his mother, we performed two independent experiments. In the patient, who has the mutation on the maternal chromosome, the peak areas of the mutant transcript were 58% and 43%, respectively, of that of the wild-type transcript. In his mother, who has the mutation on the paternal chromosome, the peak areas of the mutant transcript were 33% and 36%, respectively, of that of the wild-type transcript. Patients AS-U3a and AS-U3b were analyzed by sequencing, because there was no difference in fragment size. As shown in Fig. 4b, the wild-type and mutant transcripts appeared to be of similar abundance.

Ratio of *SNURF-SNRPN/UBE3A* transcript levels in blood from AS patients with an unknown genetic defect

Approximately 20% of patients suspected of having AS have a genetic defect of unknown nature. If *UBE3A* expression were silenced in *cis* by the *SNURF-SNRPN* sense-*UBE3A* antisense transcript, some of these patients might have inappropriate expression of the latter transcript on the maternal chromosome. To test this hypothesis we investigated seven AS patients in whom a deletion, uniparental disomy, an imprinting defect and a *UBE3A* mutation had been excluded. Using the QRT-PCR assay, we found a normal *SNURF-SNRPN/UBE3A* transcript ratio (data not shown) Likewise, we did not detect in blood RNA an upregulation of transcripts containing exons 142–143 (data not shown).

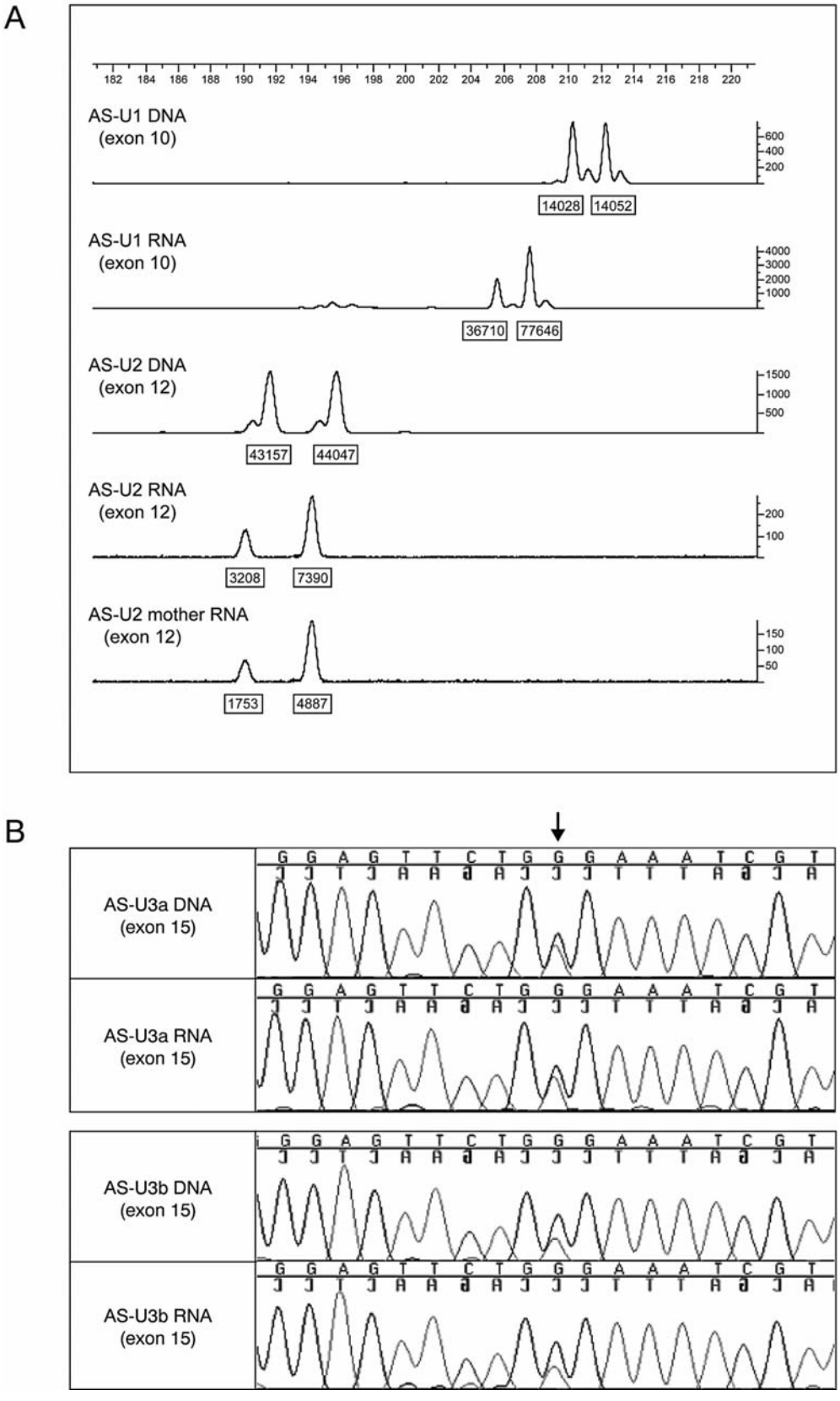

Fig. 4a, b Expression levels of wild-type and mutant *UBE3A* alleles in AS patients. **a** Fragment-length analysis of PCR products from DNA and blood RNA. Patient AS-U1 has a 2-bp deletion in exon 10. Patient AS-U2 and his mother have a 4-bp deletion in exon 12. In the experiment shown, 20 PCR cycles were used. Peak integrals are given *below each peak*. The *first peak* represents the mutant allele. **b** Sequence analysis of DNA and RT-PCR products from sibs AS-U3a and AS-U3b, who have a nonsense mutation in exon 15. For each PCR, 35 cycles were used. The *arrow* indicates the position of the heterozygous G>A substitution

Discussion

Based on cDNA cloning and exon-connection PCR, we have recently identified an extended *SNURF-SNRPN* transcript that serves as a host for multiple small nucleolar RNA species and as an antisense RNA for *UBE3A* (Runte et al. 2001). By studying the expression of this transcript in carriers of a paternally inherited IC deletion that includes the *SNURF-SNRPN* promoter/exon 1 region, we provide further evidence that this transcript is initiated at the IC. This finding confirms the results of Chamberlain and Brannan (2001), who have shown that a deletion of the mouse IC results in the loss of the paternally expressed *Ube3a* antisense transcript. It should be noted, however, that we cannot formally exclude the possibility that the IC deletion has an indirect effect on a hitherto undetected downstream promoter. Also, as the level of transcripts containing exons 59–148 are below the threshold of detection in blood, we cannot exclude the presence of an alternative promoter downstream of exon 59. However, from extensive cloning and sequencing studies there is no indication for the existence of such a promoter. Thus, the low abundance in blood cells of transcripts containing exons 59–148, compared with brain cells, is probably due to tissue-specific splicing or RNA stability and it is impossible, in human patients, to deduce the relative abundance of brain cell transcripts containing exons 59–148 from the relative abundance of blood cell transcripts containing more centromeric exons.

Typically, snoRNA genes reside within an intron of a host gene and an intron harbors only one snoRNA gene. In Runte et al. (2001), we described that three snoRNA genes appeared to be in an exon of the *SNURF-SNRPN* transcription unit and that four introns of this transcription unit appeared to contain two snoRNA genes. As described here, we have detected four additional exons and two additional introns and only three atypical situations are left. We expect that by additional experiments the remaining exons and introns will be found and that the locus completely conforms to the general rule regarding the organisation of snoRNA genes.

The snoRNAs within the *SNURF-SNRPN* transcription unit show a distinct tissue distribution (Cavaillé et al. 2000; Runte et a. 2001). *HBII-436* and *HBII-13* are expressed in brain, lung and kidney and, to a lower extent, in muscle, heart and liver (Cavaillé et al. 2000; Runte et al. 2001). The expression pattern of *HBII-438A* and *HBII-438B* cannot be tested independently, since both sequences are identical. Their expression pattern resembles that of *HBII-85*; e.g., strongest expression in brain and kidney, weaker expression in muscle and lung and very low expression in liver and heart (Cavaillé et al. 2000; Runte et al. 2001). As shown by Wirth et al. (2001), *HBII-85* is also expressed in blood. In contrast, *HBII-52* was found to be present exclusively in brain (Cavaillé et al. 2000). Thus, there appears to be an increase in brain-specificity of the snoRNAs from centromere to telomere. By determining the steady-state level of transcribed exons of the host gene in blood and brain we have obtained suggestive evidence that the tissue distribution of the splice forms parallels that of the snoRNAs encoded in the respective parts of the *SNURF-SNRPN* transcript. Northern blot experiments performed several years ago (Dittrich et al. 1996; Buiting et al. 1996; Gray et al. 1999) had revealed that exons 1–10 (*SNURF-SNRPN*) are ubiquitously expressed. Here we show by RT-PCR that exons 1–2 and 57–58, which flank the snoRNA genes *HBII-436*, *HBII-13, HBII-438A* and the *HBII-85* gene cluster (Fig. 2a and 2b), are present at a similar level in blood and brain. In contrast, exons 63–65 and 142–143, which flank the *HBII-52* gene cluster, as well as exons 147–148, which represent the most 3′ end of the transcription unit, are barely detectable in blood. These results are in agreement with previous studies showing that *UBE3A* antisense RNA is only detectable in the tissue in which *UBE3A* is imprinted (Vu and Hoffman 1997; Rouguelle et al. 1997; Yamasaki et al. 2003) and support the notion that the antisense RNA silences *UBE3A* in *cis*. There are at least two imprinted genes, *Kcnq1* and *Igf2r*, the imprinted expression of which appears to be regulated through an antisense transcript (Horike et al. 2000; Fitzpatrick et al. 2002; Wutz et al. 1997; Sleutels et al. 2002).

We reasoned that some of the patients who are suspected of having AS, but who do not have any of the known genetic defects, might have inappropriate expression of the antisense RNA from the maternal allele in the absence of methylation changes at *SNURF-SNRPN*. In seven patients tested we have not obtained any evidence for such a mechanism. It should be noted, however, that we could test blood only and that we cannot exclude an imprinting defect of *SNURF-SNRPN* and inappropriate expression of the antisense RNA in brain cells. Mosaic imprinting defects appear to be rather common in Angelman syndrome (Buiting et al. 2003). Therefore, it is possible that some patients have a normal methylation pattern in blood, but abnormal methylation and expression of the *SNURF-SNRPN* sense-*UBE3A* antisense RNA in brain cells.

Herzing et al. (2002) have recently suggested that *UBE3A*, although biallelically expressed in lymphocytes, is more active on the maternal allele. If this finding was true, it would question a role of the antisense transcript in the regulation of imprinted *UBE3A*, because, as shown here, the *UBE3A* antisense part of the *SNURF-SNRPN* transcript is barely detectable in blood. Using two independent methods, we have not been able to substantiate the finding of Herzing et al. (2002). First, we used QRT-PCR to compare the *SNURF-SNRPN*/*UBE3A* transcript ratio in RNA from patients with AS and an imprinting defect to that of normal controls. If the two parental alleles of *UBE3A* are equally active in blood cells, the presence of an incorrect paternal imprint on the maternal chromosome should have no effect on the level of *UBE3A* expression, but result in a twofold amount of the *SNURF-SNRPN* transcript and hence a twofold increase in *SNURF-SNRPN*/*UBE3A* transcript ratio. If, however, as suggested by Herzing et al. (2002), *UBE3A* were partially imprinted and more active on the maternal allele, we would expect a decrease in *UBE3A* transcript levels in AS-ID patients and hence a significant increase in the *SNURF-SNRPN*/*UBE3A* transcript ratio. However, the latter was not the case. Second, we studied the relative transcript levels of the wild-type

paternal and the mutant maternal *UBE3A* allele in AS patients with a *UBE3A* mutation. Two related patients with a nonsense mutation in exon 15 appeared to have equal levels of the mutant and wild-type transcript, although we are well aware that sequence analysis is only semiquantitative. Two patients with a frameshift mutation were studied by quantitative fragment-length analysis and found to have reduced levels (40–60%) of the mutant maternal allele compared with the wild-type paternal allele. In the mother of patient AS-U2, who has the frameshift mutation on the paternal chromosome, the mutant allele was present at a level only slightly lower than that in the patient (~35%). This finding might reflect a small imprinting effect, but it might also be due to experimental error, to inter-individual differences in the expression levels of the wild-type alleles (note that these are different in the mother and the patient), or to inter-individual differences in NMD. In conclusion, if *UBE3A* is imprinted in blood cells, the effect is probably very small.

The absent or moderate reduction of nonsense *UBE3A* transcripts in the patients studied by us indicates that these transcripts are not efficiently degraded by NMD. A general prerequisite for NMD appears to be that the premature stop codon is at least 50 nucleotides upstream the last intron (Byers 2002). This is the case for all three mutations studied here. The premature termination codons are in exons 10, 12 and 15, respectively, of the 16-exon gene, and the exon 15 premature termination codon is located 135 nucleotides 5′ to the last intron. It is unclear at present why some genes escape NMD, whereas in many cases NMD efficiently removes most of the aberrant transcripts. We had hoped that *UBE3A* transcripts might be subject to mRNA quality surveillance, because the QRT-PCR assay might then be an efficient method for the prescreening of "methylation-normal" AS patients. Such a prescreen would be welcome because the rate of detection of a *UBE3A* mutation in patients suspected of having AS is very low (see for example Malzac et al. 1998; Baumer et al. 1999; Fang et al. 1999).

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft (grant BU907/1-2). We would like to thank Alexander Hüttenhofer for helpful discussion. We also thank Dagmar Wieczorek for providing us with blood from patients.

References

Baumer A, Balmer D, Schinzel A (1999) Screening for *UBE3A* gene mutations in a group of Angelman syndrome patients selected according to non-stringent clinical criteria. Hum Genet 105:598–602

Buiting K, Dittrich B, Endele S, Horsthemke B (1996) Identification of novel 3′ exons of the human *SNRPN* gene. Genomics 40:132–137

Buiting K, Farber C, Kroisel P, Wagner K, Brueton L, Robertson ME, Lich C, Horsthemke B (2000) Imprinting centre deletions in two PWS families: implications for diagnostic testing and genetic counseling. Clin Genet 58:284–290

Buiting K, Barnicoat A, Lich C, Pembrey M, Malcom S, Horsthemke B (2001) Disruption of the bipartite imprinting center in a family with Angelman syndrome. Am J Hum Genet 68: 1290–1294

Buiting K, Groß S, Lich C, Gillessen-Kaesbach G, El-Maarri O, Horsthemke B (2003) Epimutations in Prader-Willi and Angelman syndrome: a molecular study of 136 patients with an imprinting defect. Am J Hum Genet 72:571–577

Byers PH (2002) Killing the messenger: new insights into nonsense-mediated mRNA decay. J Clin Invest 109:3–6

Cavaillé J, Buiting K, Kiefmann M, Lalande M, Brannan CI, Horsthemke B, Bachellerie JP, Brosius J, Hüttenhofer A (2000) Identification of brain-specific and imprinted small nucleolar RNA genes exhibiting an unusual genomic organization. Proc Natl Acad Sci USA 97:14311–14316

Chamberlain SJ, Brannan CI (2001) The Prader-Willi syndrome imprinting-center activates the paternally expressed murine *Ube3a* antisense transcript, but represses paternal *Ube3a*. Genomics 73:316–322

Dittrich B, Buiting K, Korn B, Rickard S, Buxton J, Saitoh S, Nicholls RD, Poustka A, Winterpacht A, Zabel B, Horsthemke B (1996) Imprint switching on human chromosome 15 may involve alternative transcripts of the *SNRPN* gene. Nat Genet 14:163–170

El-Maarri O, Buiting K, Peery EG, Kroisel PM, Balaban B, Wagner K, Urman B, Heyd J, Lich C, Brannan CI, Walter J, Horsthemke B (2001) Maternal methylation imprints on human chromosome 15 are established during or after fertilization. Nat Genet 27:341–344

Fang P, Lev-Lehman E, Tsai TF, Matsuura T, Benton CS, Sutcliffe JS, Christian SL, Kubota T, Halley DJ, Meijers-Heijboer H, Langlois S, Graham JM Jr, Beuten J, Willems PJ, Ledbetter DH, Beaudet AL (1999) The spectrum of mutations in *UBE3A* causing Angelman syndrome. Hum Mol Genet 8:129–135

Fitzpatrick GV, Soloway PD, Higgins MJ (2002) Regional loss of imprinting and growth deficiency in mice with a targeted deletion of *KvDMR1*. Nat Genet 32:426–431

Gray TA, Saitoh S, Nicholls RD (1999) An imprinted, mammalian bicistronic transcript encodes two indepented proteins. Proc Natl Acad Sci USA 96:5616–5621

Herzing LB, Cook EH Jr, Ledbetter DH (2002) Allele-specific expression analysis by RNA-FISH demonstrates preferential maternal expression of *UBE3A* and imprint maintenance within 15q11-q13 duplications. Hum Mol Genet 11:1707–1718

Horike S, Mitsuya K, Meguro M, Kotobuki N, Kashiwagi A, Notsu T, Schulz TC, Shirayoshi Y, Oshimura M (2000) Targeted disruption of the human *LIT1* locus defines a putative imprinting control element playing an essential role in Beckwith-Wiedemann syndrome. Hum Mol Genet 9:2075–2083

Malzac P, Webber H, Moncla A, Graham JM, Kukolich M, Williams C, Pagon RA, Ramsdell LA, Kishino T, Wagstaff J (1998) Mutation analysis of *UBE3A* in Angelman syndrome patients. Am J Hum Genet 62:1353–1360

Povey S, Lovering R, Bruford E, Wright M, Lush M, Wain H (2001) The HUGO Gene Nomenclature Committee (HGNC). Hum Genet 109:678–680

Rougeulle C, Glatt H, Lalande M (1997) The Angelman syndrome candidate gene, *UBE3A*/E6-AP, is imprinted in brain. Nat Genet 17:14–15

Runte M, Hüttenhofer A, Gross S, Kiefmann M, Horsthemke B, Buiting K (2001) The IC-*SNURF-SNRPN* transcript serves as a host for multiple small nucleolar RNA species and as an antisense RNA for *UBE3A*. Hum Mol Genet 10:2687–2700

Sleutels F, Zwart R, Barlow DP (2002) The non-coding Air RNA is required for silencing autosomal imprinted genes. Nature 415:810–813

Sun Y, Nicholls RD, Butler MG, Saitoh S, Hainline BE, Palmer CG (1996) Breakage in the *SNRPN* locus in a balanced 46,XY,t(15;19) Prader-Willi syndrome patient. Hum Mol Genet 5: 517–524

Vu TH, Hoffman AR (1997) Imprinting of the Angelman syndrome gene, *UBE3A*, is restricted to brain. Nat Genet 17:12–13

Wirth J, Back E, Hüttenhofer A, Nothwang HG, Lich C, Gross S, Menzel C, Schinzel A, Kioschis P, Tommerup N, Ropers HH, Horsthemke B, Buiting K (2001) A translocation breakpoint cluster disrupts the newly defined 3′ end of the *SNURF-SNRPN* transcription unit on chromosome 15. Hum Mol Genet 10: 201–210

Wutz A, Smrzka OW, Schweifer N, Schellander K, Wagner EF, Barlow DP (1997) Imprinted expression of the *Igf2r* gene depends on an intronic CpG island. Nature 389:745–749

Yamasaki K, Joh K, Ohta T, Masuzaki H, Ishimaru T, Mukai T, Niikawa N, Ogawa M, Wagstaff J, Kishino T (2003) Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of *Ube3a*. Hum Mol Genet 12:837–847