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ORIGINAL ARTICLE

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Narrowed abrogation of the Angelman syndrome critical interval on human chromosome 15 does not interfere with epigenotype maintenance in somatic cells

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Abstract Human chromosome 15q11–q13 involves a striking imprinted gene cluster of more than 2 Mb that is concomitant with multiple neurological disorders manifested by Prader–Willi syndrome (PWS) and Angelman syndrome (AS). PWS and AS patients with imprinting mutation have microdeletions, which share a 4.3 kb short region of overlap (SRO) at the 5′ end of the paternal *SNURF-SNRPN* gene in PWS, or on the maternal allele, which shares a 880 bp SRO located at the 35 kb upstream of the *SNURF-SNRPN* promoter in AS. Recent studies have revealed an essential role of PWS-SRO in the postzygotic maintenance of the appropriate epigenotype on the paternal chromosome. For AS-SRO, however, there is insufficient experimental evidence exists to determine the direct functions. Here we show that the complete deletion of AS-SRO does not cause any anomalies of imprinted gene expression or DNA methylation on the mutated human chromosome 15, further supporting the idea that AS-SRO is dispensable for post implantation imprint maintenance. This implies that AS-SRO is not essential for the robust epigenotype preservation in somatic cells.

Keywords Genomic imprinting · DT40 cell shuttle system · AS-SRO · Imprinting center · DNA methylation

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Introduction

Prader–Willi syndrome (PWS) is characterized by infantile hypotonia, gonadal hypoplasia, obsessive/compulsive behavior, and neonatal feeding difficulties followed by hyperphagia leading to profound obesity. Angelman syndrome (AS) is characterized by severe mental retardation, absent speech, ataxic gait, and a happy demeanor. These distinct neurogenetic disorders are caused by the loss of function of oppositely imprinted genes in human chromosome 15q11–q13 (Jiang et al 1998; Nicholls et al. 1998; Horsthemke et al. 1999; Mann and Bartolomei 1999). PWS is due to a lack of paternal genetic information on human chromosome 15q11–q 13, caused most frequently by a ~4 Mb de novo paternal deletion or maternal uniparental disomy (UPD). By

contrast, AS usually results from a lack of maternal 15q11–q13 genetic information, ~4 Mb de novo maternal deletion, or paternal UPD. In addition, mutations in a single gene, *UBE3A*, which is maternally expressed in specific regions of the brain, occur in some AS patients (Kishino et al. 1997; Matsuura et al. 1997; Rougeulle et al. 1997; Vu and Hoffman 1997). In rare cases of either syndrome, neither parental chromosome displays a gross abnormality, but the maternal chromosome carries a paternal imprint or the paternal chromosome carries a maternal imprint (Buiting et al. 1995; Dittrich et al. 1996; Saitoh et al. 1996). Such patients typically have microdeletions; these imprinting mutations have helped to define an imprinting center (IC) thought to act in *cis* to regulate the chromatin structure, DNA methylation, and gene expression at 15q11–q 13 (Reis et al. 1994; Sutcliffe et al. 1994; Yang et al. 1998). In PWS imprinting mutations with fully characterized deletion breakpoints, the shortest region of deletion overlap (PWS-SRO) is less than 4.3 kb, spanning the first exon and promoter of *SNURF-SNRPN* (Buiting et al. 1995; Dittrich et al. 1996; Saitoh et al. 1996). Similarly, the AS-SRO has been narrowed to 880 bp at a site 35 kb upstream of *SNRPN*, which includes exon U5 of the alternative 5′ *SNURF-SNRPN* transcripts (Buiting et al. 1995, 1999; Dittrich et al. 1996; Saitoh et al. 1996; Buiting et al. 1999; Farber et al. 1999; Ohta et al. 1999). These observations have led researchers to suggest that the IC is comprised of a bipartite structure that regulates the erasure and reestablishment of parental imprints in the germline. The PWS-SRO and AS-SRO would thus be required for a maternal-to-paternal imprint switch during spermatogenesis or a paternal-to-maternal imprint switch during oogenesis.

Recent data indicate that the maternal imprints on paternal chromosome 15q11–q13 with the PWS-SRO deletion can also be acquired during or after fertilization (Bielinska et al. 2000; Bressler et al. 2001; El-Maarri et al. 2001). As with the PWS-SRO, the role of the AS-SRO must also be considered further. For example, the alternative 5′ *SNURF-SNRPN* transcripts included in the AS-SRO itself may act in *cis* to regulate imprinting switching (Dittrich et al. 1996; Farber et al. 1999). Alternatively, *trans*-acting factors specific to the female germline may interact with the 5′ *SNURF-SNRPN* transcripts to cause heterochromatin formation (Dittrich et al. 1996; Schweizer et al. 1999; Shemer et al. 2000). Whether or not the AS-SRO element transcripts are conserved in the mouse is as yet unknown; indeed, some observations suggest that human and mouse imprinting mechanisms have diverged (Blaydes et al. 1999). Genetic studies on AS patients with non-IC deletion implicate that some imprinting defects on maternal chromosome 15 are caused by a failure to maintain the imprint postzygotically since, in AS patients, the paternally imprinted maternal chromosome region is inherited from either the maternal grandfather or the maternal grandmother. Moreover, the maternal allele of the *SNRPN* CpG island displays a paternal methylation pattern in unfertilized human oocytes and biparental complete hydatidiform moles (El-Maarri et al. 2001, 2003). Thus, abnormal methylation on the maternal chromosome is not due to an error in erasing the imprinting marks, but rather abnormal methylation reflects an error in the reprogramming process and/or in the reestablishment or postzygotic maintenance of the new maternal marks. The results suggest that, like the PWS-SRO, the AS-SRO may also influence the postzygotic maintenance of the maternal imprint. To date, there is insufficient evidence to determine if the AS-SRO (including all or a part of AS-SRO) is directly required for postzygotic maintenance of the maternal imprint in human chromosome 15.

To develop an in vitro resource for the investigation of imprinted loci in humans, we have generated a series of monochromosomal cell hybrids that retain single human chromosomes of defined parental origin (Kugoh et al. 1999). This in vitro assay system has led to the identification of multiple transcripts that are differentially expressed from the maternal and paternal chromosomes (Mitsuya et al. 1999; Meguro et al. 2001a, 2001b; Nakabayashi et al. 2002; Yamada et al, 2004). In addition, targeting experiments by using a shuttle system with DT40 cells demonstrates that the human *LIT1* locus is required for the somatic maintenance of the paternal epigenotype at the imprinted gene cluster 11p15.5 in somatic cells (Horike et al. 2000). Here we constructed a complete somatic deletion of AS-SRO on human chromosome 15. The expression of imprinted genes and methylation pattern at 15q11–q13 remained completely unchanged in somatic cells lacking AS-SRO, implying that none of the AS-SRO interval is required for imprint maintenance in the soma. Given these observations and the evidence from previous studies implying that AS-SRO may play an essential role in the postzygotic maintenance of the proper epigenotype on the maternal chromosome, potential insights into the etiology of AS are discussed.

Materials and methods

Cell cultures

DT40 cells were cultured at 40°C in RPMI1640 medium with 10% fetal calf bovine serum (FCS, Hyclone, Logan, UT, USA), 1% chicken serum, and 50 μM 2-mercaptoethanol (2-ME, Sigma, St. Louis, MO, USA). RPMI medium and chicken serum were obtained from Invitrogen (Carlsbad, CA, USA). Mouse A9 cells containing a paternal or maternal human chromosome 15 tagged with pSV2 *bsr* or pSV2 *neo*, respectively, which were used as fusion donors for chromosome transfer, were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% calf serum (CS, Hyclone) in the presence of 3 μg/ml blasticidin S hydrochloride (BS, Funakoshi, Tokyo, Japan) or 800 μg/ml geneticin (G418, Invitrogen). Chinese ham-

ster CHO cells, which were used as fusion recipients for chromosome transfers, were cultured at 37°C in F-12 nutrient mixture (Invitrogen) with 10% CS.

Microcell-mediated chromosome transfer

Transfer of human chromosome 15 from mouse A9 hybrids to DT40 cells was performed via microcell-mediated chromosome transfer (MMCT), as described previously (Horike et al. 2000) with slight modifications. A9 hybrids (1×10^8 total) in twelve 25 cm^2 flasks were treated with 0.05 μg/ml of colcemid to micronucleate donor cells, which were then purified by digestion with 10 mg/ml of cytochalasin B (Sigma) and centrifugation. The microcell pellets were resuspended in 24 ml of serum-free DMEM, filtered sequentially through 8-, 5-, and 3-μm polycarbonate filters (Whatman, Brentford, Middlesex, UK), and precipitated by centrifugation at 1,800 rpm for 5 min. The purified microcells were then resuspended in 1 ml of serum-free DMEM containing 50 μg/ml of phytohemagglutinin (PHA, BD Biosciences, San Jose, CA, USA). The microcells were attached to 1×10^7 DT40 cells propagated as an adherent monolayer on the poly-L-lysine-coated wells by centrifugation at 1,500 rpm for 5 min, then the cells were fused within a 50% (w/v) polyethylene glycol 1,500 solution (Roche Diagnostics, Indianapolis, IN, USA) for 1 min. The fusions were diluted in 48 ml medium and divided into a 24-well plate for 24 h. DT40 microcell hybrids were selected in 15 μg/ml BS or 1,500 μg/ml G418 and used as fusion donor cells for subsequent chromosome transfer. CHO microcell hybrids containing a modified human chromosome 15 were obtained by polyethylene glycol-mediated fusion of 5×10^6 CHO cells with microcells purified by centrifugation from 1×10^9 DT40 hybrid cells maintained on poly-L-lysine-coated 25 cm^2 flasks. The fusions were then selected in 6 μg/ml BS or 800 μg/ml G418, and expanded.

Targeted disruption of the human AS-SRO locus

The targeting was designed to delete the AS-SRO region completely. The 4.2-kb 5′ and 3.5-kb 3′ homologous flanking sequences were subcloned from DNA fragments obtained by PCR amplification of human genomic DNA. The 4.2-kb product was amplified with the oligonucleotide primers 5′-CGGGGTACCCCAAGCCTGGCTAACTTTTG-3′ and 5′-TTCGGGGCCCCAAGCCCCCAGGTGATACTA-3′. The 3.5-kb product was amplified with the primers 5′-CGCGGATCCTGCAGGTGCGTTTTTTTATG-3′ and 5′-AAGGAAAAAAGCGGCCGCCATTTCCAAGAGGGCTGAAG-3′. Stepdown PCR was carried out as follows after incubation at 95°C for 3 min, with annealing temperature being reduced by 2°C every three cycles: 95°C for 45 s, 68°C for 30 s, and 68°C for 4 min. Twenty-six subsequent rounds of PCR were carried out as follows: 95°C for 45 s, 62°C for 30 s, and 68°C for 4 min. *Kpn*I-*Apa*I and *BamH*I-*Not*I digested products were cloned into pBluescript II SK^- (Stratagene). Next, the selectable marker pGK $puro^R$ cassette was inserted into the *Kpn*I site of the vector. 1×10^7 DT40 cells containing a paternal or maternal human chromosome 15 were collected in 0.8 ml phosphate-buffered saline and electroporated at 550 V and 25 μF (Gene-Pulser, Bio-Rad, Hercules, CA, USA) with 25 μg targeting vector, which was linearized at the *Apa*I site. Double-drug selection in 0.5 μg/ml puromycin (Sigma) plus 15 μg/ml BS or 1,500 μg/ml G418 was initiated after 24 h. The correctly targeted DT40 hybrid clones screened by PCR analysis were confirmed by Southern blot analysis using a PCR-generated probe. The amplification primers for the 5′ region-specific probe were 5′-CCTGCTGATTGGTCCATTTT-3′ and 5′-GAAGCCACCTGGACTTCGTA-3′. Those for the 3′ region-specific probe were 5′-TACGGAATCCTAAGGCATGG-3′ and 5′-CAAAGAGCAGCAGCATGGAGGAT-3′.

Cytogenetic analysis

Conventional chromosome preparations for cytogenetic analysis followed standard procedures to identify a morphologically intact human chromosome 15 in metaphase spreads after chromosome transfer. Chromosomes were stained with Quinacrine plus Hoechst 33258 (Sigma) and the karyotypes of at least 20 well-banded metaphase cells were analyzed. Fluorescence in situ hybridization (FISH) was performed on fixed metaphase spreads of each cell hybrid type using digoxigenin-labeled (Roche Diagnostics) human COT-1 DNA (Invitrogen) and biotin-labeled pGK $puro^R$ plasmid DNA, essentially as described (Kugoh et al. 1999). After chromosomes were counterstained with DAPI (Sigma), images were captured via the CytoVision Probe System (Applied Imaging, San Jose, CA, USA), and figures were prepared for reproduction using Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA).

Assessment of allelic expression

Total RNA was extracted using the RNeasy minikit (Qiagen, Valencia, CA, USA) and treated with RNase-free DNase I (Nippon Gene, Toyama, Japan) to remove contaminating DNA. First-strand cDNA synthesis was carried out with an $oligo(dT)_{15}$ primer (Roche Diagnostics) and M-MLV reverse transcriptase (Invitrogen). PCR was performed with AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) using a stepdown protocol, as described previously (Meguro et al. 2001a). Reaction products were analyzed by electrophoresis in a 2.0% agarose gel and stained with SYBR Green I (Molecular Probes, Eugene, OR, USA). Primer sequences used to detect parental-allele-specific expression on 15q11–q13 are available upon request.

Methylation analysis

The DNA methylation state of the *SNRPN* CpG island was examined by Southern blot, as described previously (Meguro et al. 1997). Genomic DNA was extracted by standard phenol-chloroform extraction methods, and 5 μg of genomic DNA was digested with the appropriate restriction enzymes. The resulting fragments were separated on a 0.8% agarose gel. The 4.2-kb *Xba*I fragment labeled with [$\alpha-^{32}$ P]dCTP was used as a probe for the *SNRPN* CpG island, and the DNA blots were imaged with a BAS-2500 bioimaging analyzer (Fuji Film, Tokyo, Japan).

Results

Targeted deletion of the AS-SRO on maternal and paternal human chromosome 15

We used gene targeting to delete the AS-SRO region on human chromosome 15 in recombination-proficient DT40 cells. We had previously established monochromosomal hybrids that contain a maternal (hChr15M) or paternal (hChr15P) human chromosome 15 tagged with the pGK *neo* or pSV2 *bsr*, respectively (Kugoh et al. 1999). In this study, the tagged hChr15M and hChr15P independently transferred from these cell hybrids into recombination-proficient DT40 cells using MMCT. Two to 3 weeks after fusion, we isolated 12 G418-resistant clones that contained hChr15M and 27 BS-resistant clones that contained hChr15P. The presence of human chromosome 15 sequences in each of these hybrid clones was confirmed by PCR using the sequence-tagged site markers WI15852 (*P*-locus) and SGC35648 (*ZNF127*) on 15q11–q13. Twelve clones of each type were analyzed for the presence of intact human chromosome 15 by double staining with Quinacrine plus Hoechst 33258. All clones but one were found to contain a single, intact copy of hChr15M or hChr15P and were chosen for further analysis.

For exact deletion of AS-SRO, we constructed a targeting construct in which the 880-bp of AS-SRO was replaced by the pGK *puro*^{R} cassette. This vector was introduced into the DT40 hybrid clones with a hChr15M or hChr15P by electroporation. Thirty puro^r/G418^r clones, designated DT40(15M$^{\Delta AS\text{-}SRO}$), and 18 puro^r/BS^r clones, designated as DT40 (15P$^{\Delta AS\text{-}SRO}$), were obtained from DT40 cell donors that retained intact single human chromosomes (Fig. 1a). Next, attempts were made to separate random insertion from homologous recombinants. Drug-resistant DT40 clones were initially screened by PCR for the presence of the AS-SRO or the puromycin-resistance gene. The PCR screening identified seven hChr15M and six hChr15P that were potentially correctly targeted (data not shown). Genomic DNA from four clones of each type was then analyzed by Southern blotting. If there was homologous recombination, the 5′ probe would detect a 6.1-kb *Eco*T22I fragment whereas random insertions would yield a wild-type 7.2-kb fragment and additional fragments of unspecified size. Targeted clones were also checked at the 3′ end, where a >10-kb *Apa*I fragment in wild-type DT40 cells is converted to a 5.7-kb fragment in targeted clones (data not shown). Three hChr15M and two hChr15P homologous recombinants were obtained (Fig. 1b). FISH with a probe derived from the plasmid pGK *puro*^{R} confirmed that the targeting construct integrated at the assigned site of the 15q11–q13 region (Fig. 2).

Expression and methylation-state analysis on 15q11–q13

To assess the function of the targeted alleles, we transferred correctly modified human chromosomes hChr15M$^{\Delta AS\text{-}SRO}$ and hChr15P$^{\Delta AS\text{-}SRO}$ as well as normal intact human chromosomes hChr15M and hChr15P into CHO cells via MMCT. After fusion and selection, we obtained 25 cell hybrids containing hChr15M$^{\Delta AS\text{-}SRO}$, 32 with hChr15P$^{\Delta AS\text{-}SRO}$, 29 with hChr15M, and 27 with hChr15P. Five clones of each were characterized by PCR and FISH (Fig. 2, and data not shown) and used for further studies on expression and DNA methylation studies. To test whether targeted deletion of AS-SRO affects the imprinting status of the imprinted domain, RT-PCR analysis was performed on RNA from cell hybrids that contained either hChr15P$^{\Delta AS\text{-}SRO}$ or hChr15M$^{\Delta AS\text{-}SRO}$ as well as from control cell hybrids that contained hChr15P or hChr15M. We examined the allelic expression profiles of multiple oppositely imprinted genes: *NDN*, *SNRPN*, *UBE3A*, and *ATP10C*. *IPW* was also included to assess the allelic expression of small nucleolar RNAs (snoRNAs), both of which can be transcribed from the same promoter. *NDN*, *SNRPN* and *IPW*, which are paternally expressed genes, were expressed from only clones that contain hChr15P$^{\Delta AS\text{-}SRO}$ or hChr15P (Fig. 3). *UBE3A* and *ATP10C* are imprinted with preferential maternal expression in the brain but are biallelically expressed in other tissues (Rougeulle et al. 1997; Vu and Hoffman 1997; Herzing et al. 2001; Meguro et al. 2001b). Consistent with this, *UBE3A* and *ATP10C* were equally transcribed from both paternal and maternal chromosomes. Biallelic expression was also evident in cells with mutated chromosomes. These results clearly indicate that the AS-SRO deletion has no significant effect on the expression of imprinted genes on human chromosome 15.

To assess the epigenetic modifications on the modified chromosomes, DNA methylation studies of the *SNRPN* CpG island were subsequently performed by Southern blot hybridization. Genomic DNA was digested with *Xba*I and the methylation-sensitive restriction enzyme *Not*I, and probed with a *SNRPN* exon 1 DNA fragment in order to detect the expected 3.0- and 0.9-kb signals (unmethylated DNA) or the 4.2-kb signal (methylated DNA). The unmethylated 3.0- and 0.9-kb and the methylated 4.2-kb signals were

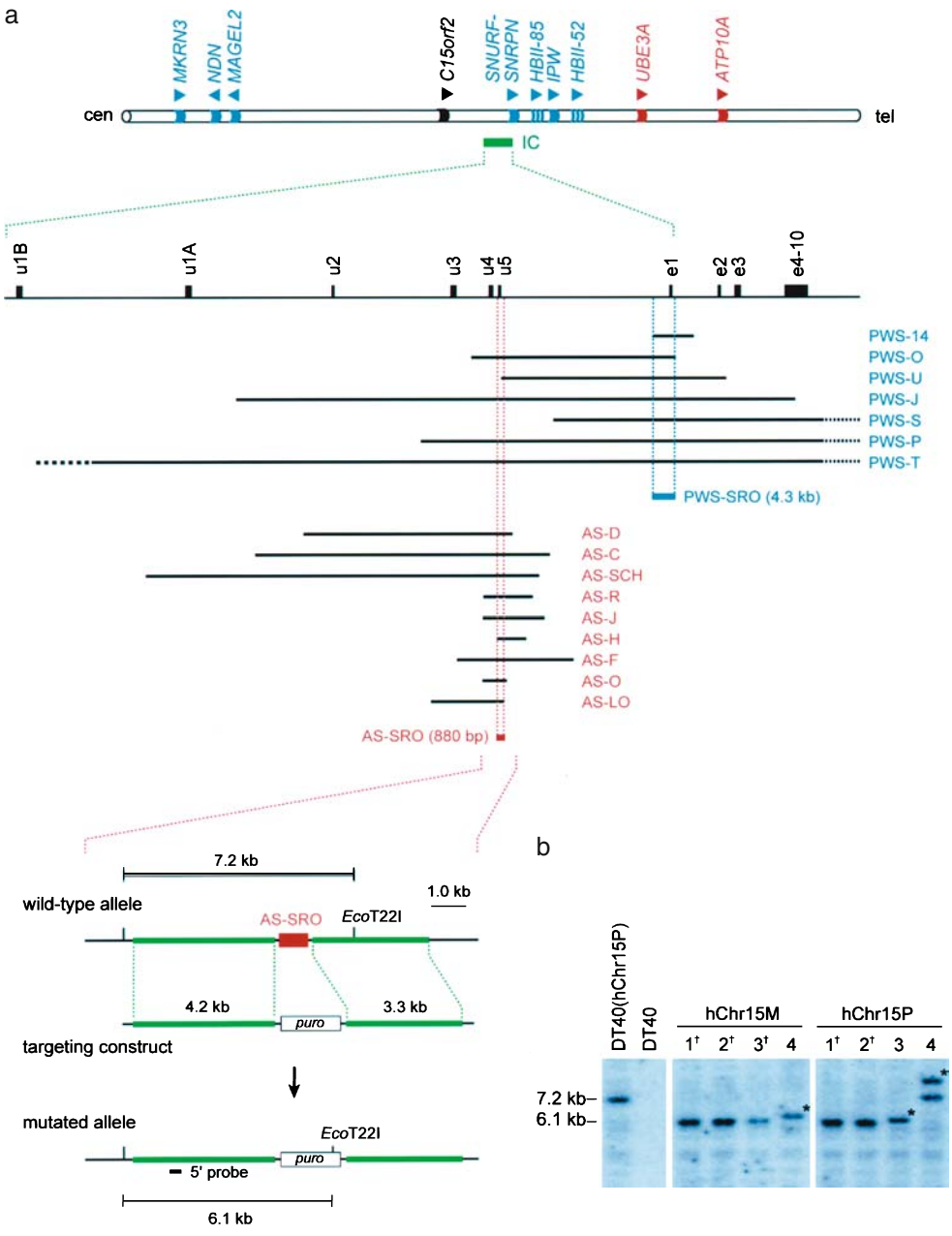

Fig. 1 Strategy for creating the Angelman syndrome short region of overlap (AS-SRO) deletion. **a** Paternally (*blue*) and maternally (*red*) expressed genes or transcripts at the 15q11–q13 imprinted gene cluster and the regions of Prader–Willi syndrome (PWS) and AS deletions are shown (*top*). The *arrowheads* indicate the transcriptional orientation. *Cen* centromere, *tel* telomere. The imprinting center (IC) spans the 150-kb region that includes exons of the alternative 5′ *SNURF-SNRPN* upstream transcripts through the *SNURF-SNRPN* CpG island at exon 1 (*middle*). The shortest region of overlap in PWS and AS deletions (PWS-SRO and AS-SRO) are shown in *blue* and *red*, respectively. The endogenous locus of AS-SRO (880 bp), the targeting vector, and the predicted structure of the locus after targeted integration of the replacement vector are shown (*bottom*). The targeting construct includes pBluescript II SK(-), the 1.7 kb puromycin resistance cassette (*open box*), and 4.2 kb of 5′ and 3.3 kb of 3′ homologous flanking sequences (*green*). The correctly targeted integrants were identified using probe A (*black box*) after *Eco*T22I digestion of genomic DNA, which detects a 7.2-kb band in the untargeted locus (top) and 6.1-kb band in the targeted locus (*bottom*). **b** Southern blot analysis of the AS-SRO deletion. DNA from drug-resistant DT40 cell hybrids, DT40(hChr15P), which have the normal paternal human chromosome 15, and from parental DT40 cells was digested with *Eco*T22I and hybridized with probe A. Clones with *daggers* show only a 6.1-kb targeted signal. The *asterisks* indicate the signals resulting from nonhomologous recombination

observed in cultured normal human fibroblasts consistent with the expected maternal methylation of the *SNRPN* CpG island. In cell hybrids containing the single hChr15M, only the methylated 4.2-kb band was detected. These results demonstrate that the maternal methylation at the *SNRPN* CpG island was retained appropriately in the somatic cell cultures. Similarly, the *SNRPN* CpG island was unmethylated on the

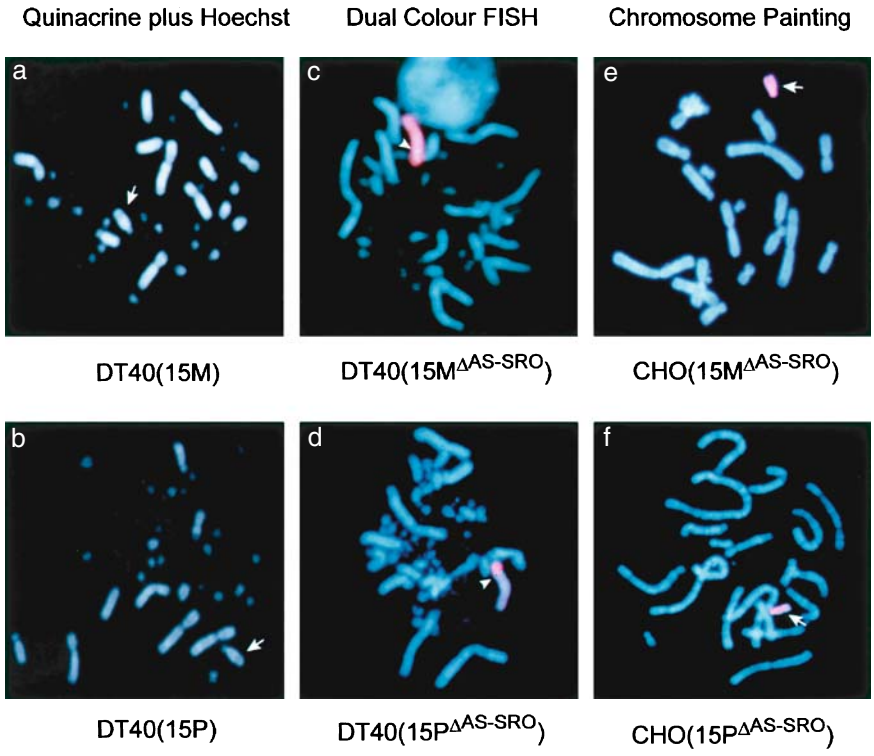

Fig. 2 Cytogenetic analysis of microcell hybrids. **a, b** DT40 (hChr15) containing a paternal or maternal chromosome 15 (*white arrows*). Metaphase spreads were stained with Quinacrine and Hoechst 33258. **c, d** DT40 cell hybrids containing a paternal or maternal modified chromosome 15. These cell hybrids were analyzed by two-color Fluorescence in situ hybridization (FISH) using a digoxigenin-labeled COT-1 probe (*red*) and a biotin-labeled pGK *puro*^{R} probe (*yellow-green*). The integration site of the targeting construct was detected as twin-spot signals on human chromosome 15 (*arrowhead*). **e, f** CHO cell hybrids containing a modified chromosome 15. *White arrows* indicate the transferred modified chromosome 15 as detected with a digoxigenin-labeled human COT-1 DNA probe (*red*)

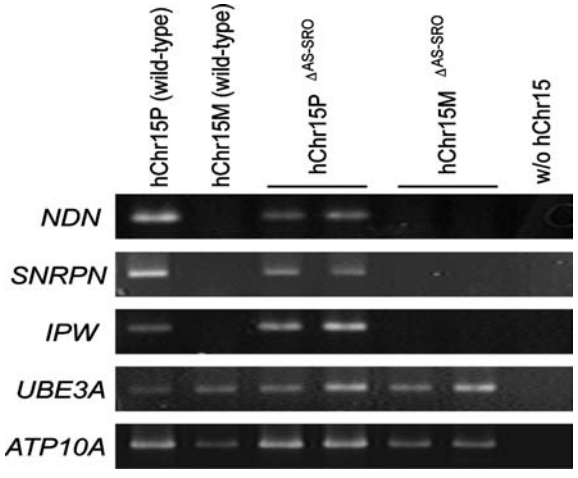

Fig. 3 Allelic expression analysis of imprinted genes on modified human chromosomes lacking Angelman syndrome sort region of overlap (AS-SRO). cDNA from CHO hybrid cells containing a paternal or maternal normal and modified human chromosome 15, as well as paternal CHO cells without hChr15, was amplified using primers for *NDN*, *SNRPN*, *IPW*, *UBE3A*, or *ATP10C*. There was no detectable signal from control reactions that lacked reverse transcriptase (not shown)

mutated paternal chromosome and was methylated in the maternal one. Expression and DNA methylation studies on at least five individual clones gave the same results. Together, these data show that the complete AS-SRO deletion exerted no detectable impairments with respect to imprint preservation in the 15q11–q13 imprinted domain.

Discussion

To explore whether deletion of the AS-SRO affects the maintenance of imprinting in *cis* at the 15q11–q13 imprinted domain, we generated modified human chromosomes that carry a targeted deletion of AS-SRO using recombination-proficient DT40 cells. A substantial number of studies on both PWS-SRO knockout mouse models and PWS-SRO deletion in PWS patients have advanced our understanding of PWS-SRO functions in the soma. Recently, Bielinska et al. (2000) showed that deletion of the *SNURF-SNRPN* exon 1 region on the paternal chromosome of early human and mouse embryos causes somatic switching from a paternal to a maternal epigenotype. The data suggest that PWS-SRO is required for the postzygotic maintenance of the paternal imprint. Furthermore, El-Maarri et al. (2001) demonstrated that sperm DNA from males with a maternally inherited deletion of the PWS-SRO have a normal paternal imprint. Together, these observations indicate that the maternal imprints on paternal chromosome 15q11–q13 with PWS-SRO deletion can also be acquired during or after fertilization. In contrast, the role of AS-SRO has historically been poorly understood. Use of mice as a model for AS-SRO function is closed by the fact that it is still unclear if the AS-SRO element is present in mice. To date, how we think about the AS-SRO is largely based on inferences drawn from the studies from AS patients. This includes the idea that the paternal methylation pattern on maternal chromosome 15 might be due to an error in the reestablishment or the

postzygotic maintenance of the maternal methylation pattern (El-Maarri et al. 2001, 2003). Another interpretation of the findings is that AS-SRO may be correlated with the postzygotic maintenance of the parent's origin imprint, as is the case for PWS-SRO.

To define the role of the AS-SRO, we tested the expression of multiple imprinted genes and analyzed DNA methylation at the *SNRPN* CpG island within 15q11–q13 in cells retaining a human chromosome 15 lacking the AS-SRO. In AS patients with an imprinting mutation, paternally expressed genes (*SNRPN, NDN, IPW*, and others) at 15q11–q13 are transcribed from the mutated maternal allele, which lacks the maternal methylation at the *SNRPN* CpG island. In this study, we found that complete deletion of the AS-SRO does not cause the paternally expressed genes to be expressed from maternal alleles, and furthermore, that the *SNRPN* CpG island within PWS-SRO remains methylated on the modified maternal allele. These findings demonstrate that there are no significant alterations in the imprinting state of the PWS/AS chromosomal domain on the modified maternal or paternal human chromosomes lacking the AS-SRO. This suggests that AS-SRO itself and/or AS-SRO binding protein(s) may be necessary to acquire the maternal imprint at this imprinted domain but not to preserve the parental epigenotype in the soma. This contrasts with our previous observation that targeted deletion of the KvDMR1 imprinting center led to bidirectional activation of the multiple maternally expressed transcripts on the paternal chromosome in somatic cells. Indeed, our findings further support the idea that the AS-SRO might have a role in the establishment of the maternal DNA methylation at PWS-SRO (Kelsey and Reik 1997; Perk et al. 2002; Kantor et al. 2004a, 2004b) using a 1.0-kb minitransgene consisting of AS-SRO and the 160-bp murine *SNRPN* minimal promoter, suggested that AS-SRO is essential for the establishment of the differential imprint state on parental alleles. Therefore, we would propose that in AS patients with an imprinting mutation, the maternal chromosome 15 results from a defect in establishment, not maintenance, of the maternal epigenotype.

What, then, is the role of the AS-SRO in resetting the maternal imprint in the germ line? Upstream exons of *SNURF-SNRPN* were identified, and there is evidence of alternative splicing and promoter usage (Dittrich et al. 1996; Farber et al. 1999). The AS-SRO includes exon U5 of the 5′ transcript. It may be argued that the deletion of U5 is irrelevant (e.g., because several splice variants lack U5). However, we would argue that this transcript could be associated with epigenotype switching since we detected several splice variants that lack U5 (data not shown). Alternatively, the AS-SRO may contain a binding site for a *trans*-acting factor required for the paternal-to-maternal imprint switch (Dittrich et al. 1996; Schweizer et al. 1999; Shemer et al. 2000). This *trans*-acting factor may bind the AS-SRO during oogenesis or during early embryogenesis and interact in *cis* with the PWS-SRO, leading to establishment of the maternal imprint. Further analysis will be required to determine the role of any *trans*-acting factors that participate in the switch mechanism.

The chicken pre-B cell line, DT40, is capable of homologous recombination between exogenous DNA templates and targeted regions with an efficiency of 10–100% (Buerstedde and Takeda 1991). In this study, homologous recombination events that remove the AS-SRO were obtained at a substantial and reproducible frequency of >1 in six puromycin-resistant clones, which is consistent with our previous results (Horike et al. 2000). It has been reported that the genes on human chromosome transferred into DT40 cells can also be targeted by homologous recombination and that the modified human chromosomes can then be transferred back into mammalian cells for functional assays. Transfer of human chromosomes to DT40 cells does not appear to irreversibly alter the expression of genes on those chromosomes (Kuroiwa et al. 2002; Ishida et al. 2002). Thus, changes in expression, DNA methylation state, chromatin structure, replication timing, or other parameters can be attributed to the effects of introduced mutations. In addition, we previously reported use of the DT40 cell shuttle system to show that the human *LIT1* locus can act as a negative regulator in *cis* for coordinating local control of imprinting on chromosome 11p15.5 (Horike et al. 2000). In this context, it is interesting to note that this imprinting center may possess a unidirectional chromatin insulator (Kanduri et al. 2002). Here we performed the expression and DNA methylation analyses at 15q11–q13 in cell hybrids in which the imprinting status on human chromosome 15 was maintained, as evidenced by the proper, cell-type-specific regulation of human genes on the transferred chromosomes (Figs. 3, 4). Furthermore, our results suggest that the AS-SRO might be necessary to acquire the maternal imprint but not to maintain the imprinted state on human chromosome 15q11–q13 in somatic cells.

Single human chromosomes retained in CHO hybrids can be introduced via MMCT into any other cells including mouse embryonic stem (ES) cells via MMCT. It was recently suggested, based on a cell culture model of neurogenesis, that *Ube3a* antisense transcripts contain upstream exons and that multiple alternatively spliced *Ube3a* antisense transcripts regulate brain-specific silencing of *Ube3a* (Rougeulle et al. 1998; Chamberlain and Brannan 2001; Yamasaki et al. 2003; Landers et al. 2004). This possibility can be investigated for human chromosomes 15 using the DT40 cell shuttle system and a cell culture model of neurogenesis. Furthermore, it has also recently been reported that assisted reproductive technology (ART), including both in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), could lead to epigenetic defects such as large offspring syndrome (LOS), which is mainly seen in ruminants (Niemitz and Feinberg 2004). There is evidence that ART might cause Beckwith–Wiedemann syndrome, retinoblastoma, and AS (Cox et al. 2002; DeBaun et al. 2003; Maher et al. 2003; Moll et al. 2003; Orstavik et al.

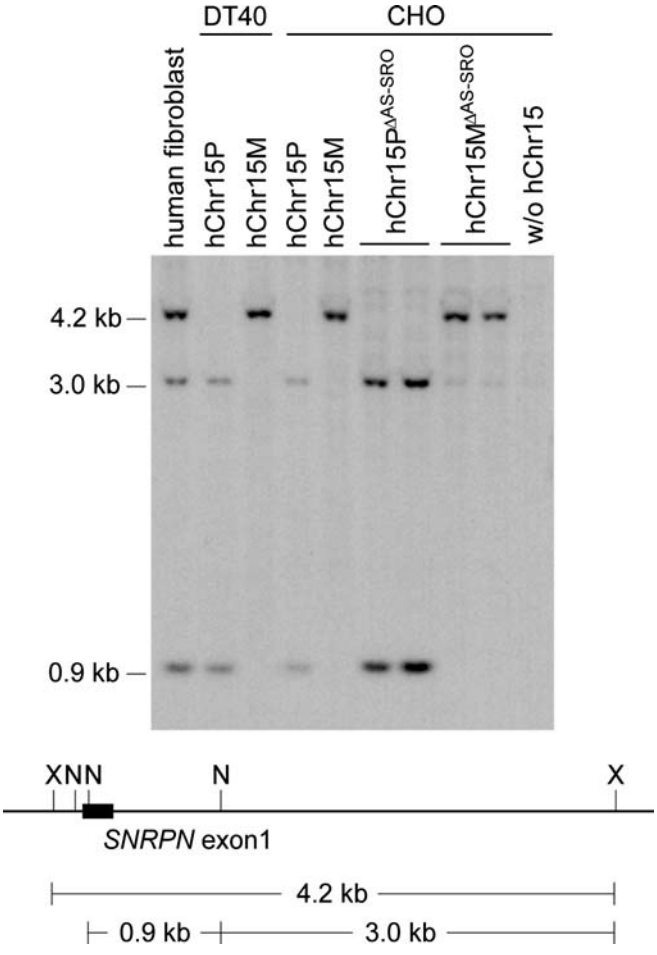

Fig. 4 DNA methylation-state analysis of *SNRPN* CpG island in somatic cell hybrids. Southern blot analysis of the differentially methylated regions of the human *SNRPN* CpG island. Genomic DNA from normal human fibroblasts and cell hybrids was digested with *Xba*I (*X*) and *Not*I (*N*), Southern blotted, and probed with the 4.2-kb *Xba*I DNA fragment including exon 1 of *SNRPN* (*black box*) shown in the restriction maps

2003), perhaps because of embryo culture itself or the media used although there might be other possibilities. In this context, our findings clearly indicate that the robust parental epigenotype at 15q11–q13 can be maintained in somatic cells even if the critical interval is disrupted. In PWS patients with imprinting mutations, it is suggested that the PWS/AS imprinting center may be required to preserve the parental epigenotype on the paternal chromosome during early embryogenesis when the process of global demethylation and de novo methylation takes place (Bielinska et al. 2000; Mann and Bartolomei 2000). In fact, deletion of the imprinting center does not interfere with DNA methylation alterations in ES cell cultures. To address these issues, it would be critical to ascertain the consequences of the defined deletion of AS-SRO using mouse models containing the modified human chromosome used in this study. Thus, our DT40 cell shuttle system should permit a rapid and exclusive dissection of the epigenetic properties of imprinting centers.

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