Am. J. Hum. Genet. 63:1009–1014, 1998

Imprinted Expression of *SNRPN* in Human Preimplantation Embryos

John Huntriss,[1] Robert Daniels,[1] Virginia Bolton,[2] and Marilyn Monk[1]

[1]Molecular Embryology Unit, Institute of Child Health, and [2]King's Assisted Conception Unit, Department of Obstetrics and Gynaecology, King's College, London

Summary

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are two clinically distinct neurogenetic disorders arising from a loss of expression of imprinted genes within the human chromosome region 15q11-q13. Recent evidence suggests that the *SNRPN* gene, which is defective in PWS, plays a central role in the imprinting-center regulation of the PWS/AS region. To increase our understanding of the regulation of expression of this imprinted gene, we have developed single-cell–sensitive procedures for the analysis of expression of the *SNRPN* gene during early human development. Transcripts of *SNRPN* were detected in human oocytes and at all stages of preimplantation development analyzed. Using embryos heterozygous for a polymorphism within the *SNRPN* gene, we showed that monoallelic expression from the paternal allele occurs by the 4-cell stage. Thus, the imprinting epigenetic information inherited in the gametes is recognized already in the preimplantation embryo. The demonstration of monoallelic expression in embryos means that efficient preimplantation diagnosis of PWS may be made by analysis for the presence or absence of *SNRPN* mRNA.

Introduction

The *SNRPN* gene encodes SmN, a 29-kD spliceosomal protein associated with small nuclear ribonucleoprotein particles. It is expressed predominantly in brain and heart (McAllister et al. 1988), and expression is imprinted in human and mouse, with only the paternal allele being transcribed in somatic tissues (Cattanach et al. 1992; Leff et al. 1992; Reed and Leff 1994). The human *SNRPN* gene maps to the smallest deletion region associated with a neurological disorder, Prader-Willi syndrome (PWS), thus implicating the involvement of *SNRPN* (Leff et al. 1992). The disease phenotype is associated with paternal deletions or maternal uniparental disomy in the chromosome region 15q11-q13 (for a review, see Glenn et al. 1997). Further PWS cases are caused by disruption of the imprinting center upstream from the *SNRPN* transcription-start site (Buiting et al. 1995); alternative transcripts of the *SNRPN* gene are believed to be critical in resetting the correct epigenetic information on the imprinted genes within the PWS/AS region during gametogenesis (Dittrich et al. 1996).

During the early stages of mouse development, there is variation in the time of establishment of monoallelic expression of different imprinted genes. Monoallelic expression from the paternal allele of *Snrpn* is observed in the mouse, from the 4-cell stage onward (Szabo and Mann 1995). Similarly, expression of the mouse imprinted *U2afbp-rs* gene is monoallelic from the paternal allele in 2-cell–stage preimplantation embryos (Latham et al. 1995). In contrast, biallelic expression of the imprinted *Igf2r* gene occurs at all stages of mouse preimplantation development (Szabo and Mann 1995), and the switch to monoallelic maternal expression occurs near the time of implantation (Lerchner and Barlow 1997). These data suggest that the differential epigenetic information inherited from the gametes and involved in the process of establishment of monoallelic expression for the imprinted genes exerts its effect at different stages during early development. Therefore, the mechanisms involved in the recognition of imprinting modifications may be diverse. In this work we have determined whether monoallelic *SNRPN* expression occurs during human preimplantation embryonic development.

Material and Methods

Human Samples

Sperm.—Anonymized sperm samples were provided by the Assisted Conception Unit, King's College Hospital, London (ACU). Sperm samples were washed in PBS, and groups of ~50 sperm were isolated in droplets of PBS under oil, by means of finely pulled glass pipettes.

Follicle cells.—Anonymized follicle cells, obtained,

Received December 8, 1997; accepted for publication July 20, 1998; electronically published August 31, 1998.

Address for correspondence and reprints: Dr. John Huntriss, Molecular Embryology Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, United Kingdom. E-mail: J.Huntriss@ich.ucl.ac.uk

© 1998 by The American Society of Human Genetics. All rights reserved.
0002-9297/98/6304-0013$02.00

with consent, from patients attending the ACU were centrifuged briefly at 12,000 *g*, and samples containing ∼50 cells were isolated in droplets of PBS under oil.

Oocytes.—Oocytes that had failed to be fertilized after in vitro fertilization (IVF) treatment were donated by patients attending the ACU and were anonymized at the source. Oocytes were scored as being unfertilized if a male pronucleus did not form 24 h after the IVF treatment. Zonae pellucidae were removed, in the manner described for the embryos (see below).

Preimplantation embryos.—Anonymized embryos derived from IVF and considered to be unsuitable for transfer were isolated, and the zona pellucida of each was removed by treatment with acid Tyrode's solution (pH 2.0–2.4; Sigma) under oil. Care was taken to ensure removal of all maternal cumulus cells and paternal sperm from each embryo.

Fibroblasts and lymphocytes.—Fibroblasts were obtained from J. Rainer (Institute of Child Health) and were isolated in PBS by means of finely pulled glass pipettes. Lymphocytes derived from thumb-prick samples were isolated, by means of a lymphocyte-separation medium (ICN), into aliquots containing ∼50 cells.

Sample preparation for genomic PCR.—Prior genomic PCR of the *SNRPN* gene in parental sperm and follicle cells was performed to distinguish parental alleles by means of a common polymorphism in exon 2 (Giacalone and Francke 1994). Samples (0.5 μl) containing ∼50 cells were added to 1.5 μl lysis buffer (1 μl proteinase K [125 μg/ml], 0.5 μl SDS [4×10^{-4}% {w/v}]), were centrifuged briefly at 12,000 *g*, and were overlaid with 1 drop of mineral oil (Sigma). The cells were lysed during incubation at 37°C for 1 h, followed by inactivation, at 98°C for 15 min, of the proteinase K activity.

Sample preparation for RT-PCR.—Oocytes, embryos, lymphocytes, and fibroblasts were added to 1.5 μl lysis buffer (5 mM DTT [Sigma], 1 U RNAsin [Boehringer]/ml, 0.8% IGEPAL [Sigma]), were centrifuged briefly at 12,000 *g*, were overlaid with 1 drop of mineral oil (Sigma) and were stored at −70°C. Prior to reverse transcription (RT), samples were held at 80°C for 5 min and then immediately were transferred to ice.

Reverse Transcription

RT was performed on samples (previously lysed in 1.5 μl RT lysis buffer) in a total volume of 5 μl comprising the cell lysates and 40 U SuperScript II reverse transcriptase (GibcoBRL), 0.9 mM of each deoxynucleotide (Pharmacia), 2.25 μg random hexamers (BRL), 5 mM DTT (BRL), and 2 U RNAsin (Boerhinger). Negative controls, from which reverse transcriptase or added sample were omitted, were included.

PCR Amplification

For *SNRPN* expression analysis in oocytes and preimplantation embryos, first-round PCRs were performed with primers S3 and S4 (fig. 1), with 2.5 units Ampli*Taq* DNA polymerase (Perkin-Elmer) in 10 mM Tris-HCl pH 8.3 containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin in a total volume of 25 μl (two cycles of 93°C for 5 min, 60°C for 1 min, and 73°C for 2 min, followed by 30 cycles of 93°C 1 min, 60°C for 1 min, and 73°C for 2 min). One microliter of first-round PCR product was then transferred to 24 μl reaction mixture, for the second-round amplification, under the same conditions, except for the substitution of primers S1 and S2 (fig. 1). Products were analyzed on a 2% NuSieve/2% agarose gel in 1 × Tris-borate EDTA buffer (90 min at 120 V). Primer sequences were as follows: S1, 5′-TAC CAG CTG GTG TGC CAA TT-3′; S2, 5′-CTC TTC CCT GTG GAG TCA TT-3′; S3, 5′-TTG GTA GGG CAG CTG GTA GA-3′; and S4, 5′-ACA GCA GCA GCT GCT ACA GT-3′.

For identification of embryos heterozygous for the *Bst*UI restriction-enzyme–site polymorphism in the *SNRPN* gene, 12 sets of parental sperm and follicle samples were initially screened utilizing an SDS/ proteinase K–treated sample in the first-round amplification. PCR was performed as described above, with primers N1 and N4 substituted for the first-round PCR and with primers N5 and N6 substituted for the second-round PCR, for these parental samples (see fig. 3). Genomic PCR products were digested with 10 units *Bst*UI enzyme (+) (New England Biolabs) at 60°C overnight, and the products were electrophoresed on 2% NuSieve agarose gels (90 min at 120 V), to identify parents homozygous for the opposite alleles. Oocytes and embryos from informative couples were then analyzed for monoallelic *SNRPN* expression, by use of primers N2 and N5 (spanning the intron; see fig. 3) for the second-round RT-PCR and by *Bst*UI enzyme treatment of RT-PCR products, as described above. Control reactions for *Bst*UI digestion following RT-PCR were performed on fibroblasts treated in the same fashion. Primer sequences were as follows: N1, 5′-GCT CCA TCT ACT CTT TGA AGC-3′; N2, 5′-CTT GCT ACT CTT GCC AAC AGT-3′; N4, 5′-GGC CAT CTT GCA GGA TAC ATC-3′; N5, 5′-GCC CAG CTT GCA TTG TTA CTA-3′; and N6, 5′-CAC CAA ATC CAA TGT CCA CAG-3′.

Results

Single-cell–sensitive procedures for the detection of *SNRPN* transcripts were developed using human lymphocytes and fibroblasts and then were applied to demonstrate *SNRPN* expression in human preimplantation

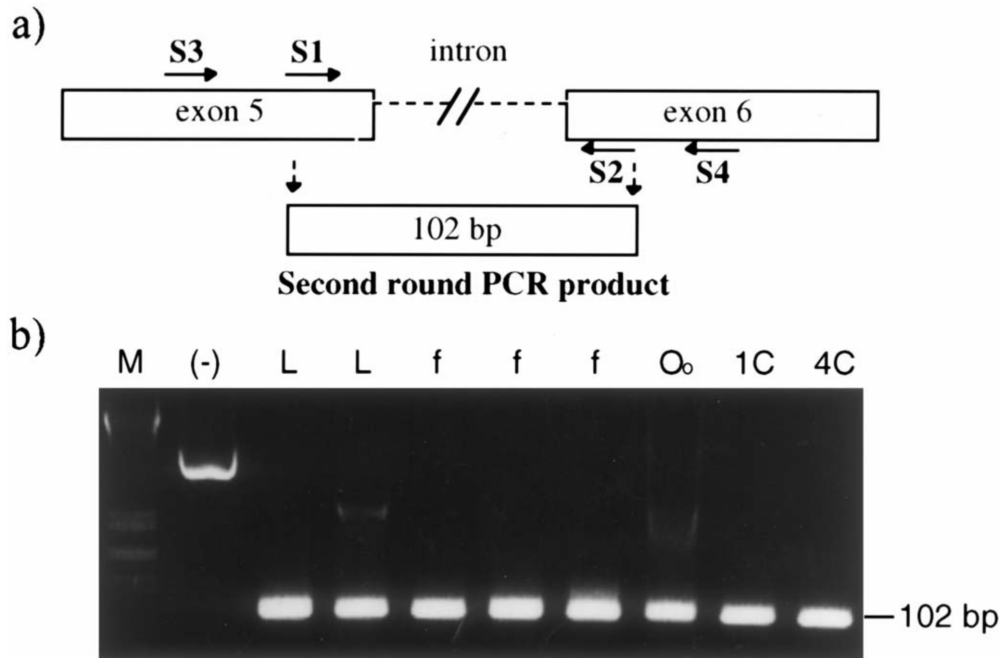

Figure 1 RT-PCR of the exon 5/exon 6 region of the human *SNRPN* gene. *a*, Scheme of *SNRPN* gene and primers used for *SNRPN* expression analysis by RT-PCR. Rectangles represent exons, and arrows represent primers. *b*, Results for two samples of human lymphocytes (lanes L) (∼50 lymphocytes isolated from thumb-prick samples), three single fibroblast cells (lanes f), an oocyte (lane Oo), and 1-cell–stage (lane 1C) and 4-cell–stage (lane 4C) preimplantation embryos. *SNRPN* transcription was observed with a high degree of efficiency in all four oocytes tested and at all stages of preimplantation development (at the 1-cell to 8-cell stage, in 10 of 11 embryos). A control, in which RT has not been performed, is shown in lane (-). PCR products were verified by sequencing.

embryos (fig. 1). *SNRPN* transcripts analyzed by RT-PCR were detected, with a high degree of efficiency, in unfertilized oocytes (in all four samples analyzed), and at all stages of preimplantation development in embryos (at the 1-cell to 8-cell stage, in 10 of 11 analyzed).

The presence of *SNRPN* transcripts in unfertilized mature oocytes probably is due to the earlier activity of the *SNRPN* alleles during oogenesis, since we expect the maternal allele (inherited as a silent allele) to be inactive in mature oocytes. To determine whether this was the case, we analyzed *SNRPN* transcripts in unfertilized oocytes heterozygous for a *Bst*UI polymorphism in exon 2 of the *SNRPN* gene (Giacalone and Francke 1994). The scheme for the PCR reactions for RT-PCR and genomic PCR, and the *Bst*UI digestion to show the two alleles, is given in figure 3*a*.Figure 2 shows the results of RT-PCR analysis of *SNRPN* expression from oocytes from two patients heterozygous (fig. 2*a* and *b*) for the polymorphism. In each case, the *SNRPN* transcripts are derived from both parental alleles, strongly suggesting that the transcription had occurred at an earlier time during oogenesis, when the imprint on the maternal allele had been erased. The presence of transcripts in the unfertilized mature oocytes therefore appears to be due to earlier transcription during oogenesis.

To determine both the onset of zygotic gene transcription and whether preferential paternal allele expression occurs in preimplantation embryos, we used the same *Bst*UI restriction-site polymorphism to distinguish the transcripts from maternal and paternal alleles in embryos. Prior to the embryo analysis, parental samples (follicle cells and sperm) from the ACU were screened by nested PCR and subsequent digestion with the *Bst*UI enzyme, to determine polymorphic alleles in the parents and thus to predict informative heterozygous embryos. *SNRPN* expression then was assessed in single heterozygous 4-cell–stage preimplantation embryos, by RT-PCR analysis followed by digestion of PCR products by *Bst*UI. Figure 3*b* shows that *SNRPN* expression is monoallelic and expressed only from the paternal allele in these embryos. The absence of maternally inherited transcripts in the embryos also shows that the *SNRPN* transcripts present in the unfertilized oocyte are degraded by the time that the embryo reaches the 4-cell stage. It remains possible that monoallelic expression of *SNRPN* is initiated earlier, at the 2-cell stage, although, because

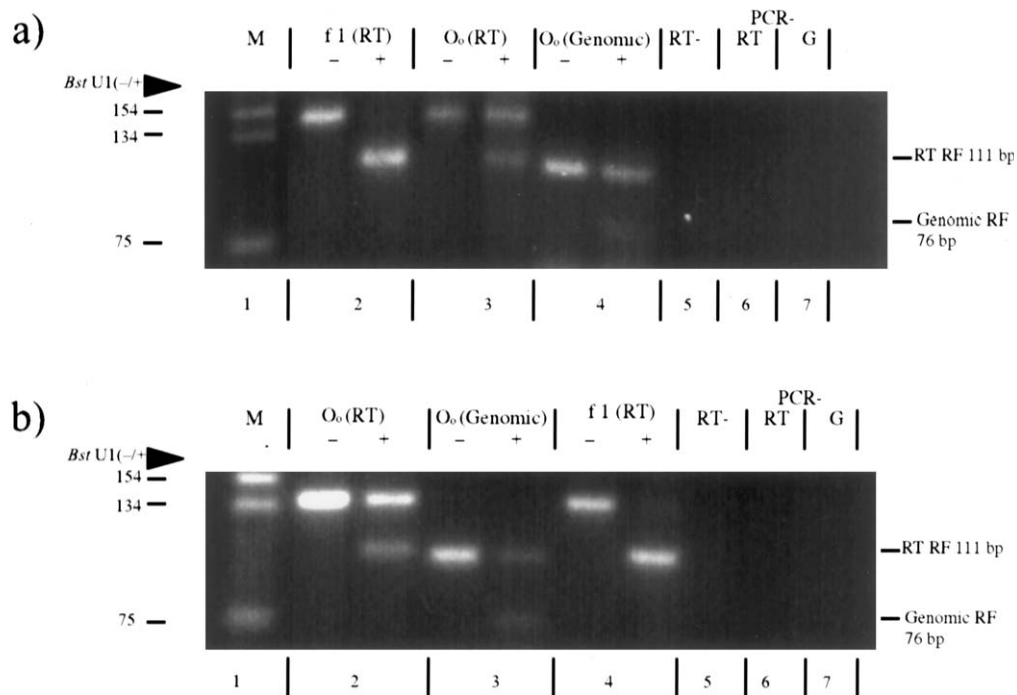

Figure 2 *SNRPN* transcripts in human unfertilized oocytes from two women heterozygous for the *Bst*UI polymorphism; the scheme for the PCR reactions for RT-PCR and genomic PCR, and *Bst*UI digestion, is shown in fig. 3*a*. *a,* Results for patient 1. The RT-PCR product and *Bst*UI digestion products from six pooled oocytes from patient 1 are shown in the two lanes Oo(RT), together with the genomic PCR product and *Bst*UI digestion products that were derived from a separate batch of six pooled oocytes from the same patient, which are shown in the two lanes Oo(Genomic). The products are compared with *Bst*UI-treated RT-PCR products from 10 human fibroblasts derived from an individual homozygous for the allele with the *Bst*UI site, which are shown in the two lanes f1 (RT) and are included to demonstrate completion of digestion. Controls include an RTase-negative sample (lane RT-) and blanks without added sample (the two lanes PCR-) for both RT-PCR (lane RT) and genomic PCR (lane G). The sizes of the major digestion products from the genomic PCR and RT-PCR are indicated to the right of the figure. *b,* Results for patient 2. RT-PCR products and *Bst*UI digestion products from six pooled oocytes from patient 2 are shown in the two lanes Oo(RT), and genomic PCR products and digestion products are shown in the two lanes Oo(Genomic). Results for a control are shown in lane f1 (RT), and results for blanks lacking RT or lacking sample are shown in lane RT- and the two lanes PCR-, respectively.

of the unavailability of earlier-stage embryos, we have not been able to demonstrate this.

Discussion

We have developed techniques for the analysis of allele-specific transcription of the *SNRPN* gene in human preimplantation embryos. Transcripts of *SNRPN* were detected in individual oocytes and at all stages of preimplantation development that were analyzed. Using a polymorphism in the *SNRPN* gene, we have shown that transcripts from both parental alleles are present in unfertilized oocytes, probably because of transcription at an earlier stage of oogenesis, when the inherited maternal imprint had been erased. Similar results have been demonstrated, elsewhere, for the mouse *Snrpn* gene (Szabo and Mann 1995). We have shown that maternal transcripts in the human egg cytoplasm have been degraded by the time that the embryo reaches the 4-cell stage, whenever expression is monoallelic from the paternally inherited allele.

Therefore, as is true for the mouse *Snrpn* gene, the epigenetic information necessary for establishing the monoallelic expression of human *SNRPN* is recognized—and exerts its effect—in preimplantation embryos; that is, the differential gametic epigenetic modifications present on the *SNRPN* alleles in sperm and egg are themselves likely to be part of the mechanism that regulates transcription. In agreement with this, a differential methylation pattern of the 5′ region of the mouse *Snrpn* gene (methylated maternal allele) is established prior to fertilization (Shemer et al. 1997). Furthermore, both the demonstration that methylation of the 5′ region of the human *SNRPN* gene abolishes promoter activity (Huq et al. 1997) and the observation of elevated *Snrpn* mRNA levels in DNA methyltransferase-deficient mice (Shemer et al. 1997) suggest a mechanism whereby such a gametic methylation imprint may lead to silencing of the maternal allele in the preimplantation embryo. In-

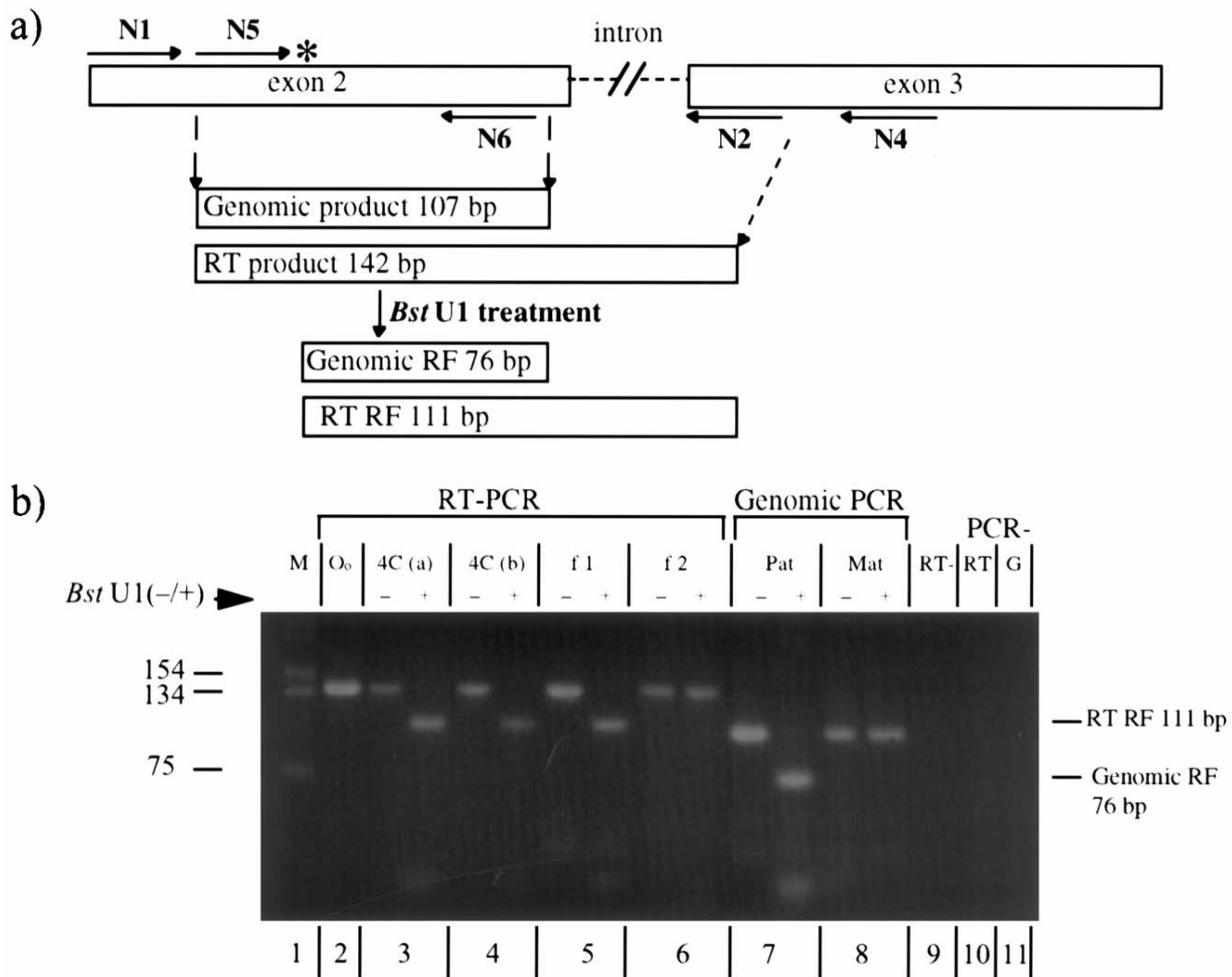

Figure 3 RT-PCR of the exon 2/exon 3 polymorphic region of the human *SNRPN* gene. *a*, Scheme of *SNRPN* gene and primer arrangements used for nested PCR protocols to distinguish maternal and paternal alleles. The asterisk (*) represents the polymorphism disrupting the *Bst*UI site, and the restriction fragments produced from RT-PCR (RT RF) and genomic PCR (Genomic RF) products are indicated. *b*, Results for an informative couple in which each partner homozygous for the opposite polymorphic allele was identified by genomic PCR of sperm and follicle cells are shown (the two lanes Pat [which show results for the father] and the two lanes Mat [which show results for the mother]). The *SNRPN* RT-PCR products and *Bst*UI digestion products (lanes +) from two 4-cell–stage embryos from these parents, which, in both cases, were found to be of paternal origin, are shown in the two lanes 4C (a) and the two lanes 4C (b); despite the clear demonstration of *SNRPN* expression in unfertilized oocytes (lane Oo), there is no evidence for the presence of maternal *SNRPN* transcripts in either 4-cell–stage embryo. Controls include an RTase-negative sample (lane RT-) and blanks (the two lanes PCR-), for both the RT-PCR reaction (lane RT) and the genomic PCR reaction (lane G). The products are compared with *Bst*UI-treated RT-PCR products from single human fibroblasts (the two lanes f1 and the two lanes f2) from individuals homozygous for opposite polymorphic alleles, which are included to demonstrate completion of digestion. PCR products are compared with a 1-kb marker ladder (GibcoBRL) (lane M).

terestingly, the human *IGF2* gene has been shown to be parentally imprinted in human preimplantation embryos. However, in this case, maternal *IGF2* transcripts derived from the oocyte are observed in 4-cell–stage embryos, and monoallelic expression, from the paternal allele, is first observed in the 8-cell–stage embryo (Lighten et al. 1997). Thus, there appear to be differences between human imprinted genes, with respect to both the time of onset of imprinted gene expression and the stability of the maternally derived transcripts of these genes.

Elsewhere, PWS diagnosis on the basis of the absence of *SNRPN* expression in blood leukocytes has been shown to be reliable, regardless of the underlying mutation (Wevrick and Francke 1996). Our sensitive RT-PCR assay extends this diagnostic approach to small samples (e.g., thumb-prick samples). Moreover, our demonstration of monoallelic *SNRPN* expression at the

4-cell stage, as well as the absence of maternal transcripts derived from the oocyte at this stage, shows that either of the single-cell–sensitive RT-PCR procedures that we have described for *SNRPN* transcripts may be applied for accurate, reliable, preimplantation diagnosis of PWS cases in which *SNRPN* is disrupted.

Acknowledgments

R.D. and M.M. are supported by the Medical Research Council; J.H. is supported by the Birth Defects Foundation.

References

Buiting K, Saitoh S, Gross S, Dittrich B, Schwartz S, Nicholls RD, Horsthemke, B (1995) Inherited microdeletions in the Angelman and Prader Will syndromes define an imprinting centre on human chromosome 15. Nat Genet 9:395–400

Cattanach BM, Barr JA, Evans EP, Burtenshaw M, Beechey CV, Leff SE, Brannan CI, et al (1992) A candidate mouse model for Prader-Willi syndrome which shows an absence of *Snrpn* expression. Nat Genet 2:270–274

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

Giacalone J, Francke U (1994) Single nucleotide dimorphism in the transcribed region of the *SNRPN* gene at 15q12. Hum Mol Genet 3:379

Glenn CC, Driscoll DJ, Yang TP, Nicholls RD (1997) Genomic Imprinting: potential function and mechanisms revealed by the Prader-Willi and Angelman syndromes. Mol Hum Reprod 3:321–332

Huq AH, Sutcliffe JS, Nakao M, Shen Y, Gibbs RA, Beaudet AL (1997) Sequencing and functional analysis of the *SNRPN* promoter: in vitro methylation abolishes promoter activity. Genome Res 7:642–648

Latham KE, Rambhatla L, Hayashizaki Y, Chapman VM (1995) Stage-specific induction and regulation by genomic imprinting of the mouse *U2afbp-rs* gene during preimplantation development. Dev Biol 168:670–676

Leff SE, Brannan CL, Reed ML, Ozcelik T, Francke U, Copeland NG, Jenkins NA (1992) Maternal imprinting of the mouse *Snrpn* gene and conserved linkage homology with the human Prader-Willi syndrome region. Nat Genet 2:259–264

Lerchner W, Barlow DP (1997) Paternal repression of the imprinted mouse *Igf2r* locus occurs during implantation and is stable in all tissues of the post-implantation mouse embryo. Mech Dev 61:141–149

Lighten AD, Hardy K, Winston RML, Moore GE (1997) *IGF2* is parentally imprinted in human preimplantation embryos. Nat Genet 15:122–123

McAllister G, Amara SG, Lerner MR (1988) Tissue-specific expression and cDNA cloning of small nuclear ribonucleoprotein-associated polypeptide N. Proc Natl Acad Sci USA 85:5296–5300

Reed ML, Leff SE (1994) Maternal imprinting of human *SNRPN,* a gene deleted in Prader-Willi syndrome. Nat Genet 6:163–167

Shemer R, Birger Y, Riggs AD, Rhazin A (1997) Structure of the imprinted mouse *Snrpn* gene and establishment of its parental-specific methylation pattern. Proc Natl Acad Sci USA 94: 10267–10272

Szabo PE, Mann JR (1995) Allele-specific expression and total expression levels of imprinted genes during early mouse development: implications for imprinting mechanisms. Genes Dev 9:3097–3108

Wevrick R, Francke U (1996) Diagnostic test for the Prader-Willi syndrome by *SNRPN* expression in blood. Lancet 348: 1068–1069