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ARTICLE

A novel imprinted gene, encoding a RING zinc-finger protein, and overlapping antisense transcript in the Prader–Willi syndrome critical region

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We describe a complex imprinted locus in chromosome 15q11–q13 that encodes two genes, *ZNF127* and *ZNF127AS*. The *ZNF127* gene encodes a protein with a RING (C_3HC_4) zinc-finger and multiple C_3H zinc-finger motifs, the former being closely related to a protein from variola major virus, the smallpox etiological agent. These motifs allow prediction of ZNF127 function as a ribonucleoprotein. The intronless *ZNF127* gene is expressed ubiquitously, but the entire coding sequence and 5′ CpG island overlaps a second gene, *ZNF127AS*, that is transcribed from the antisense strand with a different transcript size and pattern of expression. Allele-specific analysis shows that *ZNF127* is expressed only from the paternal allele. Consistent with this expression pattern, in the brain the *ZNF127* 5′ CpG island is completely unmethylated on the paternal allele but methylated on the maternal allele. Analyses of adult testis, sperm and fetal oocytes demonstrates a gametic methylation imprint with unmethylated paternal germ cells. Recent findings indicate that *ZNF127* is part of the coordinately regulated imprinted domain affected in Prader–Willi syndrome patients with imprinting mutations. Therefore, *ZNF127* and *ZNF127AS* are novel imprinted genes that may be associated with some of the clinical features of the polygenic Prader–Willi syndrome.

INTRODUCTION

A specific subset of mammalian genes are marked during gametogenesis in a way that leads to the differential expression of these genes from each of the parental alleles during somatic development (1). This phenomenon, termed genomic imprinting, is an exception to the Mendelian tenet that identical genes inherited from each parent have an equal effect on the development of offspring. Over 20 endogenous genes in the mouse and in the human have been found to be imprinted and, as a rule, the imprint is evolutionarily conserved when analyzed in both species (1–11). The nature of the differential modification is likely to be complex, involving both gene-specific and chromosomal domain events such as DNA methylation, chromosome condensation and DNA replication (1–3). In some instances, the somatic regulation of imprinted genes has been shown to be controlled by local events involving enhancer competition (12) or antisense gene regulation (13), but for other imprinted genes, parental regulation involves a novel imprint switch mechanism during gametogenesis that acts over a large domain of multiple imprinted genes (3).

Imprinted genes play an etiological role in several human diseases, such as Beckwith–Wiedemann syndrome (6), Prader–Willi syndrome (PWS) and Angleman syndrome (AS) (3), among others (14). Human chromosome 15q11–q13 is among the best-studied imprinted regions and is associated with several very different neurobehavioral syndromes. PWS is characterized by neonatal hypotonia, subsequent hyperphagia with severe obesity, hypogonadism, short stature, mild to moderate mental retardation with learning disabilities and abnormal behavior, including an obsessive–compulsive disorder, and mild facial dysmorphism (15). AS patients typically display ataxia, severe mental retardation with a severe speech impairment, seizures, hyperactivity and a happy disposition with frequent outbursts of inappropriate

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laughter (16). PWS and AS arise from a diverse set of genetic mechanisms of opposite parental origin, including 15q11–q13 deletions, uniparental disomy (UPD), mutations in the imprinting process and, in the case of AS, single gene mutations (3).

The precise molecular pathogenesis of PWS is unknown, but each of the mechanisms resulting in PWS leads to loss of paternal gene expression (3). In contrast, loss of maternal gene expression leads to AS, and single gene mutations in *UBE3A* (E6-AP) have been found in familial and sporadic AS patients (17,18). This gene is expressed from the maternal allele only in the mammalian brain (19–21) and the clinical features of AS thus arise from loss of the imprinted E6-AP E3 ubiquitin ligase. The *UBE3A* mutations were found within a subgroup of ~5% of AS patients in whom the inheritance patterns had been predicted to indicate a single, maternally expressed gene. However, an equivalent class of PWS patients has not been found, suggesting that mutations in single paternally expressed genes would only give some PWS features and such patients are not presently recognizable. Therefore, it is thought that multiple, contiguous imprinted genes may each have additive roles in the classical PWS phenotype (3).

The *SNRPN* gene, encoding the small nuclear ribonucleoprotein SmN subunit putatively involved in splicing in the postnatal brain, was the first gene shown to be functionally imprinted within 15q11–q13, with expression from the paternal allele only (22–25). Only one other paternal-only expressed gene within 15q11–q13 has been identified that encodes a putative protein product, *NECDIN* (*NDN*) (26,27), a member of the *MAGE* gene family. Other non-coding transcripts within 15q11–q13 are imprinted and paternally expressed and include the *IPW* gene (28), several alternative 5′ *SNRPN* transcripts that may play a role in the imprinting mechanism (29) and several ESTs (*PAR5* and *PAR1*) (24) that likely represent non-functional transcriptional read-through of the *SNRPN* and *IPW* genes, respectively (30; unpublished data).

Previously, we reported identification of a cDNA, DN34, that showed differential DNA methylation between the maternal and paternal chromosomes of PWS and AS patients (31). We report here that this complex locus encodes two overlapping antisense transcripts that are differentially expressed in different tissues. One of these, *ZNF127*, is a functionally conserved, retroposed gene encoding a novel zinc-finger protein of the C_3HC_4 and C_3H (C, Cys; H, His) families. We also demonstrate that *ZNF127* is functionally imprinted in human tissues where it is expressed exclusively from the paternally inherited chromosome.

RESULTS

Identification of the human *ZNF127* and *ZNF127AS* genes

The human DN34 cDNA detects a DNA methylation imprint at the *D15S9* locus within the PWS/AS region (31). Restriction enzyme mapping using human genomic DNA showed that the 3 kb cDNA spans a genomic interval of 5 kb, with a single 2 kb putative intron and each of the cDNA and genomic intervals flanked by *Eco*RI restriction enzyme sites (Fig. 1a–e). Therefore, the cDNA cloning method has resulted in deletion of the 5′- and 3′-ends of the transcript from which the DN34 cDNA clone derived. A genomic clone spanning the putative intron was isolated by PCR and sequenced to confirm the presence of sequences conforming to consensus splice donor and splice acceptor sites at either end of a 2 kb intron (Fig. 1e). We also confirmed by isolation of the orthologous mouse locus (32) that the splice acceptor site was highly conserved (Fig. 1e), suggesting that the gene structure is conserved. Nucleotide sequence of the sense strand of the 3.0 kb cDNA revealed no substantial open reading frame (ORF) nor significant similarity to known sequences in the database. Since the 3′-end of the cDNA is rich in A-T nucleotides, we assume that this represents the 3′-end of this transcript.

Surprisingly, a long ORF potentially encoding a 507 amino acid polypeptide was identified on the antisense strand of the DN34 cDNA (Figs 1b and 2a). We demonstrated that the sense strand encoding the ORF was expressed as an mRNA by multiple techniques, including northern analysis, RT–PCR, 5′- and 3′-rapid amplification of cDNA ends (RACE) and database searching (see below). This gene was designated *ZNF127*, as the encoded polypeptide includes a series of zinc-finger motifs (see below). The gene encoding the DN34 cDNA was designated *ZNF127AS* to signify its antisense orientation to the *ZNF127* gene.

The transcription initiation site for the *ZNF127* gene was mapped by 5′-RACE to a single A nucleotide, at a consensus CAP site, with a 109 bp 5′-untranslated region (5′-UTR) (Figs 1b and d and 2a). This is at the beginning of a typical mammalian CpG island, which spans a 0.7–1.0 kb region at the 5′-end of the *ZNF127* gene, based on a G+C content >60% and an observed:expected ratio of CpG frequency of >0.6 (33; Figs 1a and 2a). Therefore, and as confirmed below, the methylation imprint previously detected by the DN34 cDNA (32) corresponds to the 5′-end of the *ZNF127* gene. The 5′-region of *ZNF127* is notable for potential transcription factor motifs for PEA3 at –60 and –90, an SRE site at –390 and, at positions –500 and –750, two copies of the SRF motif (AACAAAG), which is the recognition site for the SRY and related HMG transcription factors (34); however, most putative binding sites are within the CpG island just inside the transcription unit, such as a C/EBP site at +260, a testis-R site at +300 and two AP2 sites at +330 and +350. The *ZNF127* gene is intronless and the 3′-UTR extends 1.1 kb into the *ZNF127AS* intron (Fig. 1b and d). While there are three potential polyadenylation signals (Fig. 2a), at present we only have evidence for utilization of the second one by 3′-RACE experiments (Fig. 1d). Consistent with these observations, we identified two *ZNF127* expressed sequence tags (GenBank accession nos T05078 and AA310871) (35) by database searches, the former of which maps in the 3′-UTR of the *ZNF127* gene (Fig. 2a).

In conclusion, *D15S9* encodes a complex genomic locus with two antisense genes: the *ZNF127AS* gene represented by the DN34 cDNA and, on the other strand, the *ZNF127* gene. Remarkably, the entire *ZNF127* coding sequence and CpG island promoter is part of a *ZNF127AS* antisense exon, with only the 3′-end of *ZNF127* within an intron of *ZNF127AS*.

Orientation of the *ZNF127*/*ZNF127AS* genes

To determine the orientation of the *ZNF127*/*ZNF127AS* genes with respect to other PWS/AS genes, we mapped various probes within the 360 kb YAC 254B5 by pulsed-field gel electrophoresis (PFGE). This YAC spans the *ZNF127* gene and the proximal PWS/AS breakpoint region, with the right YAC end at a centromeric location (36; W. Gottlieb and R.D. Nicholls, unpublished data). Therefore, the left YAC end represented by

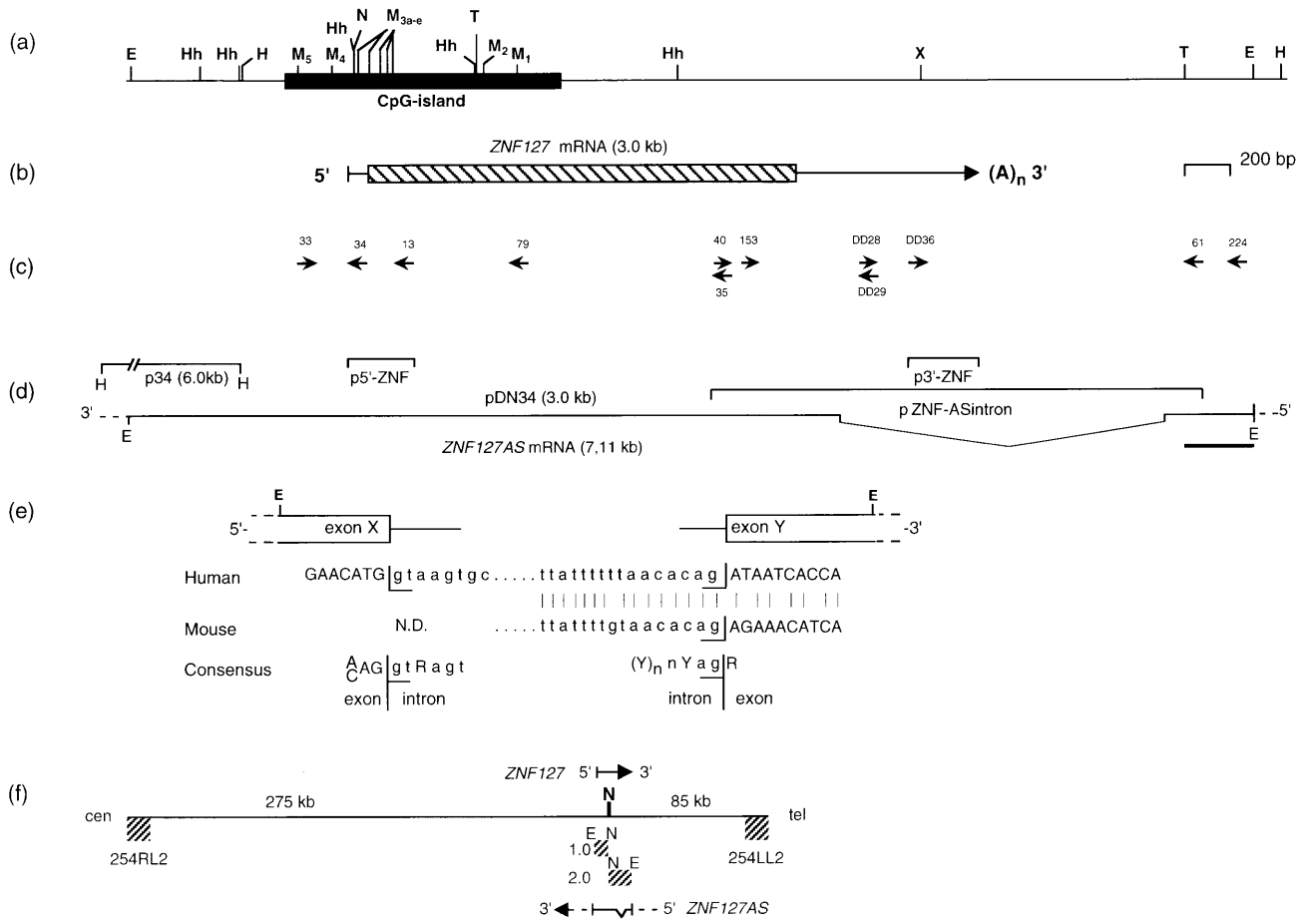


Figure 1. Antisense gene structure of the human *ZNF127* and *ZNF127AS* genes. Illustrated schematically are: (**a**) a physical map of the *D15S9* locus and location of the *ZNF127* CpG island; (**b**) the positioning of the 3 kb intronless *ZNF127* transcript; (**c**) the PCR primers utilized in this study; (**d**) the position of genomic, cDNA and PCR clones, as well as the positioning of two exons for the antisense *ZNF127AS* transcript; (**e**) the exon–intron boundary sequences for two exons X and Y of the antisense *ZNF127AS* gene; (**f**) the centromere (cen)–telomere (tel) orientation of the two genes. *Msp*I/*Hpa*II (M) restriction enzyme numbers in subscript refer to the site numbers in previous DNA methylation analyses (31). The direction of transcription of the *ZNF127* and *ZNF127AS* genes are indicated (b and d–f) and the open reading frame of *ZNF127* is shown by a hatched box in (b). p34 is a genomic clone representing the original *D15S9* locus (68), pDN34 is a 3 kb cDNA for the *ZNF127AS* transcript, p5′-ZNF and p3′-ZNF are RACE clones obtained using reverse transcription primers specific for the *ZNF127* gene and pZNF-ASintron is a genomic PCR clone. The *ZNF127AS*-specific exon probe (exon X) is shown by a black bar in (d) and probes by hatched boxes in (f). E, *Eco*RI; H, *Hin*dIII; Hh, *Hha*I; M, *Msp*I/*Hpa*II; N, *Not*I; N.D., not determined; R, purine; T, *Taq*I; X, *Xba*I; Y, pyrimidine.

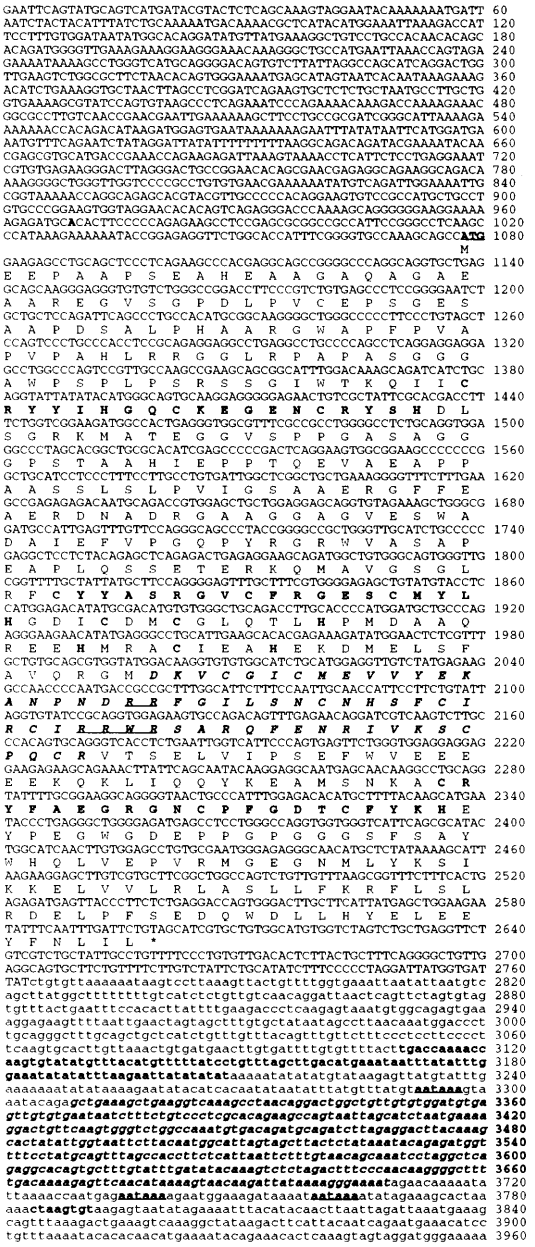
probe 254LL2 maps to a telomeric location. This probe (254LL2) hybridizes to the same 85 kb *Not*I fragment in YAC 254B5 as does a 2 kb probe from the 3′-end of the *ZNF127* gene (Fig. 1f). In contrast, a 1 kb probe from the 5′-side of the *Not*I site in *ZNF127* detects a 275 kb fragment in YAC 254B5 (Fig. 1f). Combined, these data indicate that the 5′→3′ orientation of *ZNF127* is centromere–telomere and, hence, that the *ZNF127AS* gene is transcribed in the opposite direction (Fig. 1f).

The *ZNF127* gene encodes a putative zinc-finger protein

The human ZNF127 507 amino acid ORF has five putative zinc-finger motifs spaced throughout the molecule (Fig. 2a). Just distal of a central location, ZNF127 has a typical C_3HC_4 or RING zinc-finger motif, a motif more commonly found at the N-termini of proteins (37). The motif spans 58 amino acids in ZNF127 and is related to similar zinc-fingers from multiple proteins of diverse species, with a consensus $E/DX_2CXICXEX_3E/DX_{5–18}CX$-$HXF/YCX_2CIX_2WX_{6–13}CPXCR$ (Fig. 2b). Most strikingly, the ZNF127 RING zinc-finger is 53% identical (66% similar) to the D4R RING zinc-finger protein of variola virus (Fig. 2b) and the orthologous protein from related orthopoxviruses such as mouse ectromelia virus p28 (data not shown).

ZNF127 also has three C_3H zinc-finger motifs at N-terminal, central and C-terminal locations (Fig. 2a). Since the first recognition in a mouse immediate early nuclear protein, Nup475 (38), this motif has been found in dozens more polypeptides from yeast to human with variable copy number from one to seven. The C_3H motif contains highly conserved cysteine, histidine, aromatic and glycine residues at defined positions, forming a consensus $CX_{1–2}Y/FX_{2–5}GX_{1–2}CX_2GX_2CXY/FXH$ (Fig. 2c). A third cysteine- and histidine-rich motif, C_2H_2CH, is also present in the ZNF127 polypeptide between the second C_3H and RING motifs (Fig. 2a). Nevertheless, other closely related motifs do not occur in the databases. A potential basic nuclear

(a) *ZNF127* gene



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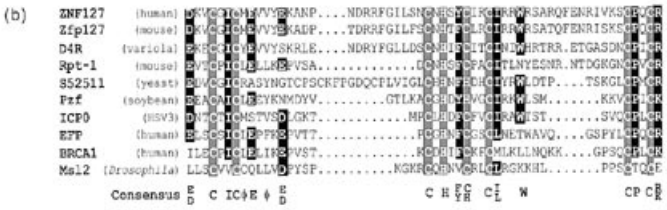

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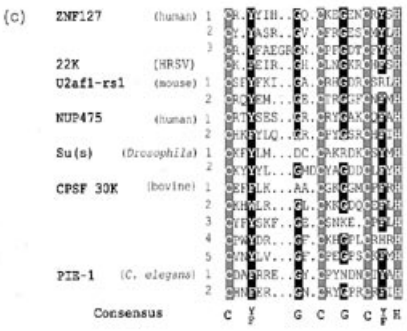

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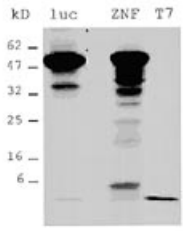

Figure 2. The human *ZNF127* gene and encoded zinc-finger polypeptide. (**a**) Nucleotide sequence of the *ZNF127* gene and flanking regions, with conceptual translation. The nucleotide structural features highlighted include, in the 5′→3′ orientation for the *ZNF127* sense strand, the transcription start site of the *ZNF127* gene (nt 969, bold), the intron sequence of *ZNF127AS* (lower case), the conserved putative unstable mRNA element in the 3′-UTR of *ZNF127* (bold), three potential polyadenylation signals (bold and underlined), EST02966 (bold and italic) and an 8 nt sequence conserved in the mouse gene just 5′ of the poly(A)$_n$ addition (bold). The putative structural features of the ZNF127 polypeptide include, in order, the methionine initiation codon (bold and underlined), three C_3H putative zinc-finger motifs and additional conserved cysteine or histidine residues (bold), the RING or C_3HC_4 zinc-finger motif (bold and italics), putative nuclear localization signal (underlined) and stop codon (asterisk). The GenBank accession no. for human *ZNF127* is U19107. (**b**) The RING zinc-finger motif of ZNF127 and additional representative proteins. The human and mouse ZNF127 RING zinc-finger motifs are most significantly related to that from the D4R protein of variola virus (PIR D36837). Other RING zinc-finger sequences and database accession nos are: mouse Rpt-1, PIR A30891; yeast S52511, PIR S52511; soybean Pzf, PIR S49445; human herpesvirus ICP0, PIR I27215; *Drosophila* Msl2, PIR S55554; human BRCA1, PIR U14680; human EFP, GenBank D21205. (**c**) The C_3H zinc-finger motif of ZNF127 and other proteins. The proteins shown contain from one to five copies of the C_3H motif. The GenBank database accession nos are: HSRV 22K, M11486; U2af1-rs1, D17407; NUP475, M92843; Su(s), X59364; CPSF, U96448; PIE-1, U62896. (**d**) *In vitro* transcription and translation of *ZNF127* cDNA. T7 promoted constructs containing either firefly luciferase (luc), human ZNF127 (ZNF) or an empty vector (T7) were transcribed and translated *in vitro*. The translation products were visualized by [^{3}H]leucine incorporation and autoradiography. Molecular weight marker migration distances are indicated.

localization signal occurs in the RING zinc-finger, and other domains previously implicated in transcriptional activation are found in ZNF127, such as an acidic region of five glutamic acid residues between the RING and the third C_3H motifs and the N-terminal region rich in Pro, Ala, Gly and Ser residues (Fig. 2a).

The AUG start codon of the *ZNF127* mRNA (GCaGCCaugG; Fig. 2a) is present in an optimal context for translation initiation (GCCRCCaugG) (39). To demonstrate that the intronless *ZNF127* transcript can be efficiently translated and thus is likely to encode a functional polypeptide, *in vitro* translation analysis was performed. Major translation products of the expected sizes were found for ZNF127 and the luciferase control, but not in the negative control (Fig. 2d). Minor bands most likely represent truncated or degraded products. These data clearly show that *ZNF127* RNA is efficiently translated in rabbit reticulocyte lysates *in vitro* and is therefore likely to be robustly translated *in vivo* as well.

Tissue-specific expression patterns of *ZNF127* and *ZNF127AS*

The *ZNF127* gene is expressed ubiquitously as an ~3 kb transcript in all tested human adult tissues, with the highest level in testis (Fig. 3a). In human fetal tissues, a DN34 cDNA probe specific for exon X of the overlapping antisense *ZNF127AS* transcript (Fig. 1e and f) detects unique transcripts of 7 and 11 kb predominantly in the brain and lung (Fig. 3b). When a probe common to both *ZNF127* and *ZNF127AS* is used on the same northern blot of human fetal tissues (Fig. 3c), the 3 kb *ZNF127* transcript is detected at significantly greater levels than the two larger transcripts observed with a *ZNF127AS* probe alone. Although *ZNF127AS* was not detectable in most adult tissues by northern blot analysis, long exposures of autoradiograms from northern blots of brain tissues showed expression of the 11 kb transcript only in cerebellum, the 7 kb transcript in frontal lobe and temporal lobe and both transcripts in cerebral cortex, medulla, putamen, occipital pole and spinal cord (data not shown).

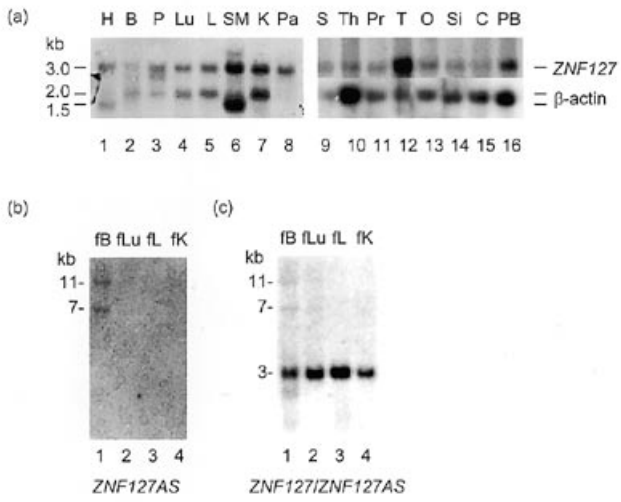

Figure 3. Expression analysis of the antisense *ZNF127* and *ZNF127AS* genes. mRNA expression for (**a**) *ZNF127* in adult human tissues, (**b**) *ZNF127AS* in fetal human tissues and (**c**) both transcripts in human fetal tissues. Multi-tissue northern blots containing poly(A)$^+$ RNAs were hybridized in (a) and (c) with a probe common to both genes (RN33-RN35; Fig. 1c) and in (b) with a probe unique to *ZNF127AS* (Fig. 1d). For (a), β-actin was used as a control probe. Tissue sources: B, brain; C, colon; f, fetal; H, heart; K, kidney; L, liver; Lu, lung; O, ovary; P, placenta; Pa, pancreas; PB, peripheral blood leukocytes; Pr, prostate; S, spleen; Si, small intestine; SM, skeletal muscle; T, testis; Th, thymus.

ZNF127 is functionally imprinted in human

Functional imprinting of *ZNF127* was tested in RNA samples prepared from skin fibroblasts of PWS and AS patients with a deletion or UPD, representing gene expression from only the maternal or paternal chromosome 15q11–q13, respectively. Due to the presence of overlapping transcripts, primers for reverse transcription (RT) were designed to obtain specific amplification of either *ZNF127* or *ZNF127AS* in the RT–PCR analysis. No amplification specific for *ZNF127AS* was detectable by this analysis (data not shown). Since the *ZNF127* gene is intronless, PCR amplification resulting from genomic DNA contamination in the RNA samples was ruled out by control reactions without RT (RT–) (Fig. 4). In a multiplex RT–PCR reaction which allows quantitation of allelic expression, *ZNF127*-specific products were detected in fibroblasts from normal and two AS individuals with a deletion or UPD, but not in fibroblasts from four PWS patients with deletion or UPD, whereas the non-imprinted control transferrin receptor (TR) gene was expressed in all samples tested (Fig. 4). These data indicate that *ZNF127* is imprinted and expressed only from the paternal chromosome in fibroblasts derived from neonates to adults.

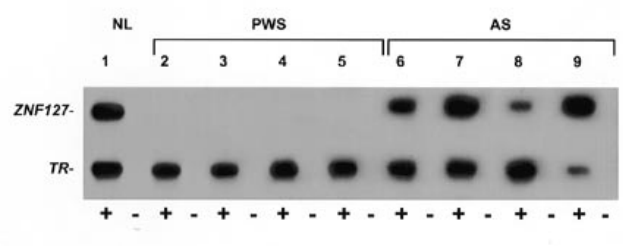

Figure 4. Functional imprinting of the human *ZNF127* gene. Expression analysis of the *ZNF127* and control transferrin receptor (TR) genes were examined by multiplex RT–PCR (+, with RT; –, without RT). NL, normal individual (lane 1); PWS, PWS patients with a paternal 15q11–q13 deletion (PW102, lane 2; PW139, lane 3) or maternal UPD (PW134, lane 4; PW135, lane 5); AS, AS patients with a maternal 15q11–q13 deletion (AS136, lane 6; AS162P.1, lane 7) or with biparental inheritance of 15q11–q13 (AS021, lane 8; AS141, lane 9). AS021 has a karyotype 46, XX, –15, + der(15)t(15;18)(p13::q12.2)mat.

ZNF127 DNA methylation imprints in somatic and germline tissues

Our previous studies of *ZNF127* DNA methylation had only examined peripheral blood leukocytes (31) and uniparental conceptuses such as ovarian teratomas and hydatidiform moles (40). We have further examined DNA methylation at the *ZNF127* CpG island (Fig. 1a) in brain and a range of fetal tissues and germ cells, using the methyl-sensitive enzyme *Hpa*II combined with the methyl-insensitive enzyme *Eco*RI (Fig. 5a). Complete lack of

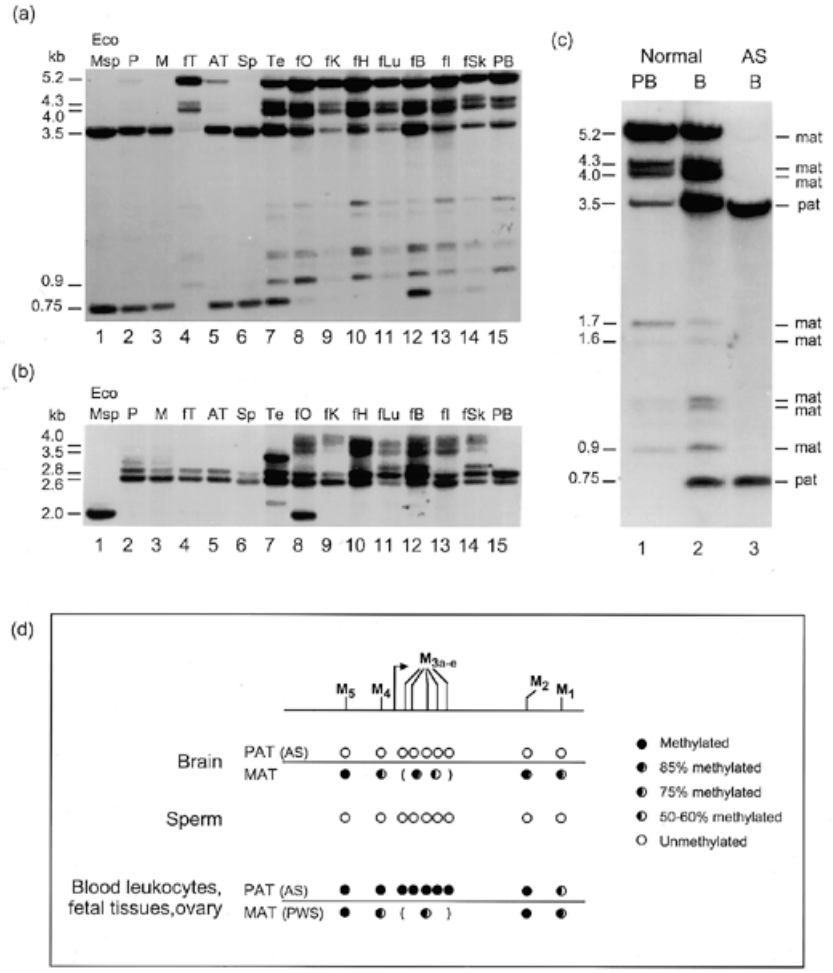

Figure 5. Tissue-specific DNA methylation imprints within the *ZNF127* gene. DNA methylation of (**a**) the *ZNF127* CpG island and flanking sites in adult (A) and fetal (f) tissues, detected by *Hpa*II (double digested with *Eco*RI) and the DN34 cDNA probe, (**b**) the control *P3* gene, detected by a pGD3 probe (40) and (**c**) the *ZNF127* gene in normal and AS brain. (**d**) Summary of allelic DNA methylation patterns in brain, sperm and fetal tissues. Abbreviations for restriction enzymes and tissue sources are as for Figures 1 and 3, respectively. Arrow, transcription start site; filled circles, methylated sites; 50–60, 75 or 85% filled circles, partial methylation; open circles, unmethylated sites; MAT, maternal allele; PAT, paternal allele; fI, fetal intestine; fSk, fetal skin; M, hydatidiform mole; Sp, sperm; Te, teratoma.

methylation is shown by the 0.75 and 3.5 kb fragments on *Eco*RI/*Msp*I double-digestion, since *Msp*I is a methyl-insensitive isoschizomer of *Hpa*II (Fig. 5a, lane 1). The same unmethylated pattern is seen with *Hpa*II in placenta (lane 2), hydatidiform mole (lane 3), adult testis (lane 5) and sperm (lane 6). The latter results indicate that the paternal allele is unmethylated in the mature gamete, as it is after fertilization with a paternal genome only (hydatidiform mole), confirming previous results (40). The fetal ovary sample is hypermethylated at the *ZNF127* locus, as indicated by the lack of a prominent unmethylated fraction (lack of a 0.75 kb fragment in Fig. 5a, lane 8). In contrast, all other genes and loci that have been examined in previous studies from other chromosomes, such as the *P3* gene from the X chromosome (Fig. 5b), have demonstrated a significant unmethylated fraction in the fetal ovary which is representative of the 20–30% of the cells in the ovary that are germ cells (41). Therefore, the *ZNF127* gene appears to have the unique feature of being methylated in female germ cells, although direct analysis of oocytes will be necessary to confirm this suggestion.

Most fetal tissues (Fig. 5a, lanes 8–14) show a pattern of complete (5.2 kb band) and partial (0.9–4.3 kb bands) methylation, similar to that seen in peripheral blood lymphocytes (lane 15). The complete lack of the unmethylated 0.75 kb band indicates that both the paternal and maternal alleles are hypermethylated in non-brain tissues at site M5 (Fig. 5d; 31). In contrast, brain (Fig. 5a, lane 12; also teratoma, lane 7), represents the only fetal tissue with a prominent unmethylated fraction (0.75 kb band) at *ZNF127*. This unmethylated DNA represents the paternal allele, as identified by DNA methylation analysis of an AS brain which demonstrates only the 0.75 and 3.5 kb bands characteristic of completely unmethylated DNA (Fig. 5c, lane 3). In contrast, the maternal allele in brain must be represented by the completely methylated 5.2 kb and the partially methylated 4.0 and 4.3 kb bands (Fig. 5c, lane 2). A summary of DNA methylation patterns at *ZNF127* in brain, sperm and fetal tissues is shown in Figure 5d.

DISCUSSION

The novel genes identified here, *ZNF127* and *ZNF127AS*, map 1–1.25 Mb centromeric of the *SNRPN* gene within the PWS region in chromosome 15q11–q13. The *ZNF127* locus defines the proximal extent of the 1.5 Mb imprinted domain spanning

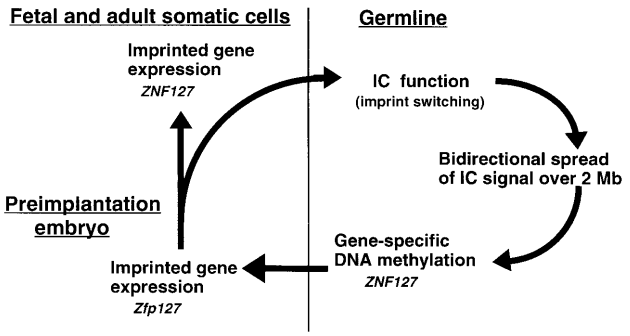

Figure 6. Model for imprinted gene regulation. The regulation of imprinted genes within human chromosome 15q11–q13 begins in the germline, with initiation of imprint switching at the IC (3). The IC signal then spreads bidirectionally across several Mb and is recognized by each target imprinted gene, each of which then switches its own DNA methylation imprint, within germ cells. This gametic mark then regulates allele-specific expression in somatic tissues, beginning in preimplantation development for genes such as the mouse ortholog of *ZNF127*.

cen–5′-*ZNF127*-3′–*NDN*–*IC*–*SNRPN*–*IPW*–tel, which to date contains genes with expression from the paternal allele only. It is likely that additional imprinted genes will be found within uncharacterized intervals from *ZNF127* to the *UBE3A* gene (the latter located ~250 kb telomeric of *IPW*).

Each imprinted gene within chromosome 15q11–q13 is coordinately regulated *in cis* during gametogenesis (3). Analysis of microdeletions at the 5′-end of the *SNRPN* gene in PWS and AS patients with a mutation in the imprinting process has led to identification of an imprinting center (IC) that functions in the initiation of imprint switching and generation of a molecular signal that spreads in a bidirectional manner over the entire 2 Mb imprinted domain (Fig. 6). In the absence of IC function, none of the imprinted genes switches its methylation imprint (3). As a consequence, *ZNF127* and each of the other known paternally expressed genes are silenced in PWS imprinting mutation patients, whereas they are biparentally expressed in AS patients (42). Similarly, the maternally expressed *UBE3A* gene must be silenced in AS imprinting mutation patients in the relevant brain tissue. The precise molecular basis of imprint switching over a large domain is currently unknown (3,29). However, the outcome of the IC-initiated germline process is the setting of gametic DNA methylation imprints at each imprinted gene, including *ZNF127*, which provides the parental epigenetic mark dictating post-zygotic regulation of these imprinted genes during development (Fig. 6). Our finding that the 5′-end of the human *ZNF127* gene is unmethylated in sperm but is likely to be methylated, at least partially, in oocytes suggests that this may represent the gametic imprint for this gene. This finding is consistent with the imprinted expression of mouse *Zfp127* during preimplantation development (Fig. 6; J. Jones, M.T.C. Jong, R.D. Nicholls and B.M. Cattanach, unpublished data).

The *ZNF127* gene is located within an exon and intron of the overlapping antisense gene, *ZNF127AS*, and this arrangement has been conserved since prior to the human–rodent divergence (32). Transcription of both strands of the same DNA locus has been described in a number of eukaryotic genes, in some cases with one gene embedded in an intron of the other gene, but many others having overlapping exons within the transcriptional units. Although the functional role of this type of arrangement has not been determined in most cases, proposed functions for temporal or spatial developmental control of antisense genes include roles in: transcriptional inhibition, with steric hindrance precluding transcription from both strands; interference of splicing, processing of mRNA or nuclear mRNA export; translational inhibition by formation of RNA duplexes in the cytoplasm; or an effect on mRNA stability (43–49).

Intriguingly, overlapping antisense gene arrangements have been found for imprinted genes, as predicted (44), and may thus play regulatory roles in the imprinting process. Nevertheless, the mechanism by which antisense genes exert an effect appears to differ between loci. The mouse *Zfp127* and *Zfp127as* antisense genes are both expressed only from the paternal allele (32,50). In this instance, we propose that transcription of one strand in the human and mouse precludes that on the other strand, as mutually exclusive expression of these two genes has been found in different cell types during development (50 and this study; J. Jones, M.T.C. Jong, R.D. Nicholls and B.M. Cattanach, unpublished data). Another model proposes that antisense transcripts to the *IGF2* gene may prevent production of IGF2 peptide in Wilms tumors despite increased expression of *IGF2* mRNAs (51). A second class of *IGF2* antisense mRNAs occurs at an upstream regulatory region, although the function is unknown (52). Mouse *U2af1-rs1* is an imprinted intronless gene located within an intron of an antisense, non-imprinted gene and, similar to transgenes, may have become imprinted as a consequence of retrotransposition (53). Perhaps the most intriguing mechanism comes from the recent finding that the paternally repressed allele of the imprinted *Igf2r* gene expresses an oppositely imprinted antisense mRNA, *Igf2rAS*, that may function to regulate developmentally controlled imprinting of *Igf2r* (13). Similarly, a paternally expressed antisense RNA may regulate *UBE3A* expression uniquely in the brain (54). Other naturally occuring antisense genes therefore represent antisense gene pairs that may be imprinted.

The presence of multiple classes of zinc-finger motifs within the ZNF127 protein implies diverse functional roles and it is likely that ZNF127 has specific interactions with multiple other molecules. Over 100 RING zinc-finger proteins from diverse species have now been identified (37), with known or suggested functions ranging from transcriptional activation, modulation of locus-specific chromatin complexes, developmental control, signal transduction, peroxisome biosynthesis and cellular transformation. The RING zinc-finger is likely to function in protein–protein interactions and many RING proteins may mediate formation of macromolecular complexes (37); the widely spaced zinc-fingers of ZNF127 would be ideally suited for the latter function.

Several subfamilies of the RING zinc-finger protein family exist, where members contain additional classes of zinc-finger or other protein motifs (37). ZNF127, and two other proteins identified to date, are included in a separate subfamily with C-terminal RING zinc-finger and C_3H motifs. This includes the *Drosophila* unkempt protein (55) and a putative yeast protein (GenBank accession no. 662126). The C_3H zinc-finger motif is similar to the C_2HC zinc-knuckle of retroviral nucleocapsid and related proteins as well as the C_3H zinc-fingers of NAB2 and RNA polymerases (56), which are thought to interact with RNA as part of ribonucleoprotein complexes. The C_3H proteins $U2AF^{35}$ and *Drosophila* Suppressor of sable [Su(s)] are involved in pre-mRNA splicing (57,58), where Su(s) binds RNA, while the

30 kDa subunit of the cleavage and polyadenylation specificity factor (CPSF) is involved in RNA 3′-end processing (59). Interestingly, the *U2af1-rs1* gene, encoding a C_3H protein homologous to U2AF^{35}, is also an intronless, imprinted gene (53,60). Recently, the C_3H protein tristetraprolin was found to bind to the AU-rich mRNA instability motif of the tumor necrosis factor-α mRNA and it was suggested that the C_3H proteins as a class may be RNA-binding proteins (61). Based on the preceding observations, we suggest that the role of the three C_3H motifs in ZNF127 may be to mediate RNA binding as part of a ribonucleoprotein complex in conjunction with the RING zinc-finger.

Remarkably, the RING zinc-finger motif of the D4R protein of variola major virus (62) and of p28 from the closely related mouse ectromelia and cowpox viruses (63) is closely related to the RING motif of ZNF127. The N1R protein from the distant poxvirus, rabbit fibroma virus, is also related to these proteins (64). Based on the close homology of the RING zinc-finger of ZNF127 and the orthopoxvirus proteins, it is possible that an ancestral virus gained the RING zinc-finger from a mammalian host *ZNF127* gene (or close paralog; see ref. 32). Both the viral p28 and N1R proteins have been localized to virus factories (64,65). Disruption of the *p28* gene abolishes the lethality of ectromelia virus for susceptible mice (63) and demonstrates that p28 is essential for viral DNA replication in macrophages in cell culture (65). Furthermore, the variola ortholog D4R is mutated or missing in attenuated vaccinia virus strains (63,64). Given that variola virus is the etiological agent of smallpox, it will be of significance to determine the evolutionary relationship and the potential interaction of viral and host ZNF127-related proteins in infected animal models.

Each of the multiple paternally expressed genes within chromosome 15q11–q13 is a candidate to play a role in the pleiotropic clinical features of PWS, which is suggested to be a contiguous gene syndrome (3). It will be necessary to identify the complete *ZNF127AS* gene before its role in PWS can be assessed. Since the DNA methylation imprint of *ZNF127* is only complete in brain and germ cells, this suggests that these are critical tissues for *ZNF127* function. Therefore, the *ZNF127* gene may be a candidate for behavioral abnormalities, obesity or hypogonadism and infertility in PWS. While a mouse *Zfp127* knockout is viable, non-obese and fertile (A.H. Carey, M.T.C. Jong, R.D. Nicholls and C.L. Stewart, unpublished data), behavioral studies have not been performed on these mice and it is likely that the presence of related genes (32) suggests some degree of functional redundancy for this gene. Therefore, we cannot at present rule in or out a role for the *ZNF127* gene in some aspect of the PWS phenotype. Determination of which imprinted genes contribute to the complex neurobehavioral phenotypic components of Prader–Willi syndrome will require mouse models and the identification, and phenotypic correlations, of mutations in specific genes within the 15q11–q13 imprinted domain.

MATERIALS AND METHODS

DNA analyses

Isolation of the DN34 cDNA from a human fetal brain library has been described (31). Isolation of the *ZNF127AS* intron (pZNF-ASintron) was by PCR amplification of genomic DNA using primers RN40 (5′-AATTCGCATTAAGAAGGAGCTTGT-CG-3′) and RN61 (5′-CGAAGCTTCAAGTCCATCTTT-GCTCCAC-3′), flanking the splice junctions. PCR parameters were 30 cycles of 1 min at 94°C, 2 min at 65°C and 3 min at 72°C and PCR fragments were cloned using the TA vector (Invitrogen, Carlsbad, CA). DNA preparations used the Minipreps DNA Purification system (Promega, Madison, WI) and DNA sequencing was performed by the dideoxy termination method with the Sequenase Sequencing kit (US Biochemical, Cleveland, OH). Some DNA sequences were confirmed using fluorescent labeled primers and *Taq* DNA polymerase, on an Applied Biosystems model 370A DNA sequencer (Applied Biosystems, Foster City, CA). DNA and amino acid sequences were analyzed using the BLAST (http://www3.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast) and ProSite (http://www/expasy.ch/sprot/scnpsit1.html) programs and one specific for conserved amino acid motifs (66).

YAC DNA isolation in agarose blocks and PFGE analysis were by standard methods, using a hexagonal CHEF-DR III PFGE apparatus (Bio-Rad, Hercules, CA), with switch times of 2.1–27.6 s at 6 V/cm for 16 h. Subsequent analysis and DNA methylation determination was by standard Southern blot and hybridization analyses, as described (25,29,31,42). The isolation of the YAC 254B5 right (probe 254RL2) and left (probe 254LL2) ends by Alu-YAC end PCR will be described elsewhere (J.M. Amos-Landgraf, Y. Ji, W. Gottlieb, T. Depinet, A.E. Wandstrat, S.B. Cassidy, D.J. Driscoll, P.K. Rogan, S. Schwartz and R.D. Nicholls, unpublished data). Probes were labeled by random hexamer priming (Rediprime DNA labelling system; Amersham Life Science, Arlington Heights, IL). Tissue samples were obtained from the following sources: fetal organs except for germline tissues were from a terminated 23-week-old female fetus with a large sacrococcygeal teratoma, but that was otherwise normal; fetal testes and ovaries from normal 17–18-week terminated fetuses; placenta from a term male fetus; testis, sperm and peripheral blood from adult males; brain from a deceased 15-month-old male AS patient with a 15q11–q13 deletion. Full IRB Human Subjects approval and informed consent from the patient or parent was obtained for these and all human studies described below.

Determination of the transcription start site

The transcription start site for the human *ZNF127* gene was mapped using the 5′ AmpliFINDER kit (Clontech, Palo Alto, CA). First strand cDNA was synthesized from poly(A)$^+$ human placental RNA supplied in the kit with a gene-specific primer, RN79 (5′-CTCTCAGTCTCTGAGCTC-3′). Subsequent PCR amplification was performed using the AmpliFINDER anchor primer and either of two gene-specific primers, RN13 (5′-GCGGAGGTGGGCAGGGACTGGAG-3′) or RN34 (5′-GAGGGAGCTGCAGGCTCTTCC-3′), both upstream of RN79. PCR products were cloned into the TA vector and the transformants were screened using radioactive probes. One identical start site was mapped in this way using either RN13 or RN34 for the *ZNF127* gene.

Preparation of RNA samples

Skin fibroblasts were isolated from normal individuals and patients with PWS (1 month to 21 years old) or AS (3–10 years old) by punch biopsies and cultured in RPMI1640 w/HEPES medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 0.25 μg/ml amphotericin B, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Cells were harvested from culture flasks with trypsin and washed with Hank's balanced salt solution (Gibco BRL). Poly(A)$^+$ RNA was prepared from the cell pellets using the PolyATract System 1000 (Promega). To remove genomic DNA contamination in RNA samples, 200 ng of poly(A)$^+$ RNA or 2 µg of total RNA were digested with 1 µl DNase I (1 U/µl; Gibco BRL) for 15 min at room temperature. DNase I was inactivated by the addition of 1 µl of 20 mM EDTA and heating for 10 min at 65°C. The RNA samples were used following phenol–chloroform extraction, isopropanol precipitation and storage at –70°C. For northern analysis, MultiTissue Northern Blots were purchased from Clontech.

RT–PCR and 3′-RACE analysis

DNase I-treated poly(A)$^+$ RNA samples (200 ng) prepared from skin fibroblast cultures were divided into two aliquots and first strand cDNA synthesis for reverse transcription (+) was performed with the Superscript Preamplification System (Gibco BRL) using either oligo(dT), random hexamers or gene-specific primers, as specified. RNA samples in the second aliquot were not reverse transcribed (–) as controls. Subsequent PCR reactions were carried out using *Taq* DNA polymerase in a Perkin Elmer-Cetus DNA Thermal Cycler 480, with PCR parameters 30 cycles of 1 min at 94°C, 2 min at 62°C and 2 min at 72°C. Primers for the *ZNF127* imprinting analysis were RN153 (5′-GTTACCCTTCTCTGAGGAC-3′) and DD29 (5′-CACAGTTTAACAAGTGCAC-3′). The multiplexed control for RT–PCR was the transferrin receptor (TR) gene, located in chromosome 3 (TR-5′, 5′-TTCTCATGGAAGCTATGGGTATCACAT-3′; TR-3′, 5′-CCACCATCTCGGTCATCAGGATTGCCT-3′). RT–PCR for *ZNF127AS* was performed in parallel with TR, due to the low abundance of *ZNF127AS* transcripts, using first strand cDNAs primed with random hexamers and PCR with *ZNF127AS*-specific primers RN224 (5′-CTGAGGTACTTATAGGAGAACCAG-3′) and RN153.

For 3′-RACE, a human fetal brain Marathon-ready cDNA and Advantage cDNA PCR kit (Clontech) was used to amplify the 3′-end of the *ZNF127* gene. DD28 (5′-GTGCACTTGTTAAACTGTG-3′) and AP1 were used for first round amplification with PCR parameters 30 cycles of 30 s at 94°C, 30 s at 52°C and 2 min at 68°C. Then, DD36 (5′-CAAGTGGGTCTGGCCAAATG-3′) and AP2 were used for nested PCR with parameters 30 cycles of 30 s at 94°C, 30 s at 60°C and 2 min at 68°C. PCR products were cloned into the T/A vector and sequenced.

In vitro transcription and translation

The full-length *ZNF127* cDNA was inserted into a vector (pSIT) containing flanking T7 promoter and T7 terminator sequences (67). The T7 polymerase-compatible TNT transcription and translation kit (Promega) was used and supplemented with [^{3}H]leucine for protein analysis (Amersham). Protein electrophoresis was performed by 10% SDS–PAGE in a Mini-Protean II system (Bio-Rad). Autoradiographic exposures were made on RX film (Fuji, Stamford, CT) at –80°C.

ABBREVIATIONS

AS, Angelman syndrome; EST, expressed sequence tag; IC, imprinting center; ORF, open reading frame; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; PWS, Prader–Willi syndrome; RACE, rapid amplification of cDNA ends; RT, reverse transcription; TR, transferrin receptor; UPD, uniparental disomy; UTR, untranslated region.

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REFERENCES

1. Bartolomei, M.S. and Tilghman, S.M. (1997) Genomic imprinting in mammals. *Annu. Rev. Genet.*, **31**, 493–525.
2. Barlow, D.P. (1995) Gametic imprinting in mammals. *Science*, **270**, 1610–1613.
3. Nicholls, R.D., Saitoh, S. and Horsthemke, B. (1998) Imprinting in Prader–Willi and Angelman syndromes. *Trends Genet.*, **14**, 194–200.
4. Kobayashi, S., Takashi, K., Miyoshi, N., Kuroiwa, Y., Aisaka, K., Tsutsumi, O., Kaneko-Ishino, T. and Ishino, F. (1997) Human *PEG1/MEST*, an imprinted gene on chromosome 7. *Hum. Mol. Genet.*, **6**, 781–786.
5. Miller, N., McCann, A.H., O'Connell, D., Pedersen, I.S., Spiers, V., Gorey, T. and Dervan, P.A. (1997) The *MAS* proto-oncogene is imprinted in human breast tissue. *Genomics*, **46**, 509–512.
6. Reik, W. and Maher, E.R. (1997) Imprinting in clusters: lessons from Beckwith–Weidemann syndrome. *Trends Genet.*, **13**, 330–334.
7. Reisewijk, A., Hu, L., Schulz, U., Tariverdian, G., Hoglund, P., Kere, J., Ropers, H. and Kalscheuer, V. (1997) Monoallelic expression of human *PEG1/MEST* is paralleled by parent-specific methylation in fetuses. *Genomics*, **42**, 236–244.
8. Dao, D., Frank, D., Qian, N., O'Keefe, D., Vosatka, R.J., Walsh, C.P. and Tycko, B. (1998) *IMPT1*, an imprinted gene similar to polyspecific transporter and multi-drug resistance genes. *Hum. Mol. Genet.*, **7**, 597–608.
9. Gould, T.D. and Pfeifer, K. (1998) Imprinting of mouse *Kvlqt1* is developmentally regulated. *Hum. Mol. Genet.*, **7**, 483–487.
10. Kato, M.V., Ikawa, Y., Hayashizaki, Y. and Shibata, H. (1998) Paternal imprinting of mouse serotonin receptor 2A gene *Htr2* in embryonic eye: a conserved imprinting regulation on the *RB*/*Rb* locus. *Genomics*, **47**, 146–148.
11. Qian, N., Frank, D., O'Keefe, D., Dao, D., Zhao, L., Yuan, L., Wang, Q., Keating, M., Walsh, C. and Tycko, B. (1997) The *IPL* gene on chromosome 11p15.5 is imprinted in humans and mice and is similar to *TDAG51*, implicated in *Fas* expression and apoptosis. *Hum. Mol. Genet.*, **6**, 2021–2029.
12. Leighton, P.A., Ingram, R.S., Eggenschwiler, J., Efstratiadis, A. and Tilghman, S.M. (1995) Disruption of imprinting caused by deletion of the *H19* gene region in mice. *Nature*, **375**, 34–39.
13. Wutz, A., Smrzka, O., Schweifer, N., Schellander, K., Wagner, E. and Barlow, D. (1997) Imprinted expression of the *Igf2r* gene depends on an intronic CpG island. *Nature*, **389**, 745–749.
14. Hurst, L. and McVean, G. (1997) Growth effects of uniparental disomies and the conflict theory of genomic imprinting. *Trends Genet.*, **13**, 436–443.
15. Cassidy, S.B. (1997) Prader–Willi syndrome. *J. Med. Genet.*, **34**, 917–923.
16. Williams, C.A., Angelman, H., Clayton-Smith, J., Driscoll, D.J., Hendrickson, J.E., Knoll, J.H.M., Magenis, R.E., Schinzel, A., Wagstaff, J., Whidden, E.M. and Zori, R.T. (1995) Angelman syndrome: a consensus for diagnostic criteria. *Am. J. Med. Genet.*, **56**, 237–238.
17. Kishino, T., Lalande, M. and Wagstaff, J. (1997) *UBE3A*/E6-AP mutations cause Angelman syndrome. *Nature Genet.*, **15**, 70–73.

18. Matsuura, T., Sutcliffe, J.S., Fang, P., Galjaard, R.-J., Jiang, Y.-H., Benton, C.S., Rommens, J.M. and Beaudet, A.L. (1997) *De novo* truncating mutations in E6-AP ubiquitin-protein ligase gene (*UBE3A*) in Angelman syndrome. *Nature Genet*., **15**, 74–77.
19. Albrecht, U., Sutcliffe, J.S., Cattanach, B.M., Beechey, C.V., Armstrong, D., Eichele, G. and Beaudet, A.L. (1997) Imprinted expression of the murine Angelman syndrome gene, *Ube3a*, in hippocampal and Purkinje neurons. *Nature Genet*., **17**, 75–78.
20. Rougeulle, C., Glatt, H. and Lalande, M. (1997) The Angelman syndrome candidate gene, *UBE3A*/E6-AP, is imprinted in brain. *Nature Genet*., **17**, 14–15.
21. Vu, T.H. and Hoffman, A.R. (1997) Imprinting of the Angelman syndrome gene, *UBE3A*, is restricted to brain. *Nature Genet*., **17**, 12–13.
22. Glenn, C.C., Porter, K.A., Jong, M.T.C., Nicholls, R.D. and Driscoll, D.J. (1993) Functional imprinting and epigenetic modification of the human *SNRPN* gene. *Hum. Mol. Genet*., **12**, 2001–2005.
23. Reed, M.L. and Leff, S.E. (1994) Maternal imprinting of human *SNRPN*, a gene deleted in Prader–Willi syndrome. *Nature Genet*., **6**, 163–167.
24. Sutcliffe, J.S., Nakao, M., Christian, S., Orstavik, K.H., Tommerup, N., Ledbetter, D.H. and Beaudet, A.L. (1994) Deletions of a differentially methylated CpG island at the *SNRPN* gene define a putative imprinting control region. *Nature Genet*., **8**, 52–58.
25. Glenn, C.C., Saitoh, S., Jong, M.T.C., Filbrandt, M.M., Surti, U., Driscoll, D.J. and Nicholls, R.D. (1996) Gene structure, DNA methylation and imprinted expression of the human *SNRPN* gene. *Am. J. Hum. Genet*., **58**, 335–346.
26. MacDonald, H.R. and Wevrick, R. (1997) The necdin gene is deleted in Prader–Willi syndrome and is imprinted in human and mouse. *Hum. Mol. Genet*., **6**, 1873–1878.
27. Jay, P., Rougeulle, C., Massacrier, A., Monda, A., Mattei, M.-G., Malzac, P., Roeckel, N., Taviaux, S., Lefranc, J.-L.B., Cau, P., Berta, P., Lalande, M. and Muscatelli, F. (1997) The human necdin gene, *NDN*, is maternally imprinted and located in the Prader–Willi syndrome chromosomal region. *Nature Genet*., **17**, 357–363.
28. Wevrick, R., Kerns, J.A. and Francke, U. (1994) Identification of a novel paternally expressed gene in the Prader–Willi syndrome region. *Hum. Mol. Genet*., **3**, 1877–1882.
29. Dittrich, B., Buiting, K., Korn, B., Rickard, S., Buxton, J., Saitoh, S., Nicholls, R.D., Poustka, A., Winterpacht, A., Zabel, B. and Horsthemke, B. (1996) Imprint switching on human chromosome 15 may involve alternative transcripts of the *SNRPN* gene. *Nature Genet*., **14**, 163–170.
30. Buiting, K., Dittrich, B., Endele, S. and Horsthemke, B. (1997) Identification of novel exons 3′ to the human *SNRPN* gene. *Genomics*, **40**, 132–137.
31. Driscoll, D.J., Waters, M.F., Williams, C.A., Zori, R.T., Glenn, C.C., Avidano, K.M. and Nicholls, R.D. (1992) A DNA methylation imprint, determined by the sex of the parent, distinguishes the Angelman and Prader–Willi syndromes. *Genomics*, **13**, 917–924.
32. Jong, M.T.C., Carey, A.H., Caldwell, K.A., Lau, M.H., Handel, M.A., Driscoll, D.J., Stewart, C.L., Rinchik, E.M. and Nicholls, R.D. (1999) Imprinting of a RING zinc-finger encoding gene in the mouse chromosome region homologous to the Prader–Willi syndrome genetic region. *Hum. Mol. Genet*., **8**, 795–803.
33. West, R.L. and Maroun, L.E. (1992) CGISLE: a program for CG island detection in DNA sequence data. *Biotechniques*, **13**, 912–913.
34. Nasrin, N., Buggs, C., Kong, X.F., Carnazza, J., Goebl, M. and Alexander-Bridges, M. (1991) DNA-binding properties of the product of the testis-determining gene and a related protein. *Nature*, **354**, 317–320.
35. Adams, M.D., Soares, M.B., Kerlavage, A.R., Fields, C. and Venter, J.C. (1993) Rapid cDNA sequencing (expressed sequence tags) from a directionally cloned infant brain cDNA library. *Nature Genet*., **4**, 373–380.
36. Christian, S.L., Bhatt, N.K., Martin, S.A., Sutcliffe, J.S., Kubota, T., Huang, B., Mutirangura, A., Chinault, A.C., Beaudet, A.L. and Ledbetter, D.H. (1998) Integrated YAC map of the Prader–Willi/Angelman region on chromosome 15q11–q13 with average STS spacing of 35 kb. *Genome Res*., **8**, 146–157.
37. Saurin, A.J., Borden, K.L., Boddy, M.N. and Freemont, P.S. (1996) Does this have a familiar RING? *Trends Biochem. Sci*., **21**, 208–214.
38. DuBois, R.N., McLane, M.W., Ryder, K., Lau, L.F. and Nathans, D. (1990) A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence. *J. Biol. Chem*., **265**, 19185–19191.
39. Kozak, M. (1996) Interpreting cDNA sequences: some insights on studies on translation. *Mamm. Genome*, **7**, 563–574.
40. Mowery-Rushton, P.A., Driscoll, D.J., Nicholls, R.D., Locker, J. and Surti, U. (1996) DNA methylation patterns in human tissues of uniparental origin using a zinc-finger gene (*ZNF127*) from the Angelman/Prader–Willi region. *Am. J. Hum. Genet*., **61**, 140–146.
41. Driscoll, D.J. and Migeon, B.R. (1990) Sex difference in methylation of single copy genes in human meiotic germ cells: implications for X chromosome inactivation, parental imprinting and origin of CpG mutations. *Somat. Cell Mol. Genet*., **16**, 267–282.
42. Saitoh, S., Buiting, K., Rogan, P.K., Buxton, J.L., Driscoll, D.J., Arnemann, J., Konig, R., Malcolm, S., Horsthemke, B. and Nicholls, R.D. (1996) Minimal definition of the imprinting center and fixation of a chromosome 15q11–q13 epigenotype by imprinting mutations. *Proc. Natl Acad. Sci. USA*, **93**, 7811–7815.
43. Spencer, D.A., Gietz, R.D. and Hodgetts, R.B. (1986) Overlapping transcription units in the dopa decarboxylase region of *Drosophila*. *Nature*, **322**, 279–281.
44. Williams, T. and Fried, M. (1986) A mouse locus at which transcription from both DNA strands produces mRNAs complementary at their 3′ ends. *Nature*, **322**, 275–278.
45. Kimelman, D. and Kirschner, M.W. (1989) An antisense mRNA directs the covalent modification of the transcript encoding fibroblast growth factor in *Xenopus* oocytes. *Cell*, **59**, 687–696.
46. Volk, R., Koster, M., Poting, A., Hartmann, L. and Knochel, W. (1989) An antisense transcript from the *Xenopus laevis bFGF* gene coding for an evolutionarily conserved 24kd protein. *EMBO J*., **8**, 2983–2988.
47. Munroe, S.H. and Lazar, M.A. (1991) Inhibition of *c-erbA* mRNA splicing by a naturally occuring antisense RNA. *J. Biol. Chem*., **266**, 22083–22086.
48. Noguchi, M., Miyamoto, S., Silverman, T.A. and Safer, B. (1994) Characterization of an antisense *inr* element in the *eIF-2a* gene. *J. Biol. Chem*., **269**, 29161–29167.
49. Hsieh-Li, H.M., Witte, D.P., Weinstein, M., Branford, W., Li, H., Small, K. and Potter, S.S. (1995) *Hoxa11* structure, extensive antisense transcription and function in male and female fertility. *Development*, **121**, 1373–1385.
50. Cattanach, B.M., Barr, J.A., Beechey, C.V., Martin, J., Noebels, J. and Jones, J. (1997) A candidate model for Angelman syndrome in the mouse. *Mamm. Genome*, **8**, 472–478.
51. Baccarini, P., Fiorentino, M., D'Errico, A., Mancini, A.M. and Grigioni, W.F. (1993) Detection of anti-sense transcripts of the insulin-like growth factor-2 gene in Wilms tumor. *Am. J. Pathol*., **143**, 1535–1542.
52. Moore, T., Constancia, M., Zubair, M., Bailleul, B., Feil, R., Sasaki, H. and Reik, W. (1997) Multiple imprinted sense and antisense transcripts, differential methylation and tandem repeats in a putative imprinting control region upstream of mouse *Igf2*. *Proc. Natl Acad. Sci. USA*, **94**, 12509–12514.
53. Nabetani, A., Hatada, I., Morisaki, H., Oshimura, M. and Mukai, T. (1997) Mouse *U2af1-rs1* is a neomorphic imprinted gene. *Mol. Cell. Biol*., **17**, 789–798.
54. Rougeulle, C., Cardosa, C., Fontés, M., Colleaux, L. and Lalande, M. (1998) An imprinted antisense RNA overlaps *UBE3A* and a second maternally expressed transcript. *Nature Genet*., **19**, 15–16.
55. Mohler, J., Weiss, N., Murli, S., Mohammadi, S., Vani, K., Vasiakis, G., Song, C.H., Epstein, A., Kuang, T., English, J. and Cherdak, D. (1992) The embryonically active gene, *unkempt*, of *Drosophila* encodes a Cys3His finger motif. *Genetics*, **131**, 377–388.
56. Mattaj, I.W. (1993) RNA recognition: a family matter? *Cell*, **73**, 837–840.
57. Murray, M.V., Turnage, M.A., Williamson, K.J., Steinhauer, W.R. and Searles, L.L. (1997) The *Drosophila* suppressor of sable protein binds to RNA and associates with a subset of polytene chromosome bands. *Mol. Cell. Biol*., **17**, 2291–2300.
58. Tronchere, H., Wang, J. and Fu, X.D. (1997) A protein related to splicing factor U2AF35 that interacts with U2AF65 and SR proteins in splicing of pre-mRNA. *Nature*, **388**, 397–400.
59. Barabino, S.M., Hubner, W., Jenny, A., Minvielle-Sebastia, L. and Keller, W. (1997) The 30-kD subunit of mammalian cleavage and polyadenylation specificity factor and its yeast homolog are RNA-binding zinc finger proteins. *Genes Dev*., **11**, 1703–1716.
60. Hayashizaki, Y., Shibata, H., Hirotsune, S., Sugino, H., Okazaki, Y., Sasaki, N., Hirose, K., Imoto, H., Okuizumi, H., Muramatsu, M., Komatsubara, H., Shiroishi, T., Moriwaki, K., Katsuki, M., Hatano, N., Sasaki, H., Ueda, T., Mise, N., Takagi, N., Plass, C. and Chapman, V.M. (1994) Identification of an imprinted U2af binding protein related sequence on mouse chromosome 11 using the RLGS method. *Nature Genet*., **6**, 33–40.
61. Carballo, E., Lai, W.S. and Blackshear, P.J. (1998) Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. *Science*, **281**, 1001–1005.
62. Shchelkunov, S.N., Blinov, V.M., Resenchuk, S.M., Totmenin, A.V., Olenina, L.V., Chirikova, G.B. and Sandakhchiev, L.S. (1994) Analysis of the nucleotide sequence of 53 kbp from the right terminus of the genome of variola major virus strain India-1967. *Virus Res*., **34**, 207–236.

63. Senkevich, T.G., Koonin, E.V. and Buller, R.M. (1994) A poxvirus protein with a RING zinc finger motif of crucial importance for virulence. *Virology*, **198**, 118–128.
64. Upton, C., Schiff, L., Rice, S.A., Dowdeswell, T., Yang, X. and McFadden, G. (1994) A poxvirus protein with a RING finger motif binds zinc and localizes in virus factories. *J. Virol*., **68**, 4186–4195.
65. Senkevich, T.G., Wolffe, E.J. and Buller, R.M. (1995) Ectromelia virus RING finger protein is localized in virus factories and is required for virus replication in macrophages. *J. Virol*., **69**, 4103–4111.
66. Shaw, G. (1993) Identification of novel pleckstrin homology (PH) domains provides a hypothesis for PH domain function. *Biochem. Biophys. Res. Commun*., **195**, 1145–1151.
67. Andreansky, M. and Hunter, E. (1994) Phagemid pSIT permits efficient *in vitro* mutagenesis and tightly controlled expression in *E. coli*. *Biotechniques*, **16**, 626–633.
68. Tantravahi, U., Nicholls, R.D., Stroh, H., Ringer, S., Neve, R., Kaplan, L., Wharton, R., Wurster-Hill, D., Graham, J.M.Jr, Cantu, E.S., Frias, J.L., Kousseff, B.G. and Latt, S.A. (1989) Quantitative calibration and use of DNA probes for investigating chromosome abnormalities in the Prader–Willi syndrome. *Am. J. Med. Genet*., **33**, 78–87.