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The necdin gene is deleted in Prader–Willi syndrome and is imprinted in human and mouse

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Human chromosome 15q11–q13 contains genes that are imprinted and expressed from only one parental allele. Prader–Willi syndrome (PWS) is due to the loss of expression of one or more paternally expressed genes on proximal human chromosome 15q, most often by deletion or maternal uniparental disomy. Several candidate genes and a putative imprinting centre have been identified in the deletion region. We report that the human necdin-encoding gene (*NDN*) is within the centromeric portion of the PWS deletion region, between the two imprinted genes *ZNF127* and *SNRPN*. Murine necdin is a nuclear protein expressed exclusively in differentiated neurons in the brain. Necdin is postulated to govern the permanent arrest of cell growth of post-mitotic neurons during murine nervous system development. We have localized the mouse locus *Ndn* encoding necdin to chromosome 7 in a region of conserved synteny with human chromosome 15q11–q13, by genetic mapping in an interspecific backcross panel. Furthermore, we demonstrate that expression of *Ndn* is limited to the paternal allele in RNA from newborn mouse brain. Expression of *NDN* is detected in many human tissues, with highest levels of expression in brain and placenta. *NDN* is expressed exclusively from the paternally inherited allele in human fibroblasts. Loss of necdin gene expression may contribute to the disorder of brain development in individuals with PWS.

INTRODUCTION

Prader–Willi syndrome (PWS) is a neurobehavioural disorder characterized by severe hypotonia and failure to thrive in infancy, followed by hyperphagia and developmental delay (1). Approximately 70% of affected individuals have a microscopic cytogenetic deletion of their paternal 15q11–q13 region, while the remainder have uniparental maternal disomy, submicroscopic deletions or other rearrangements. The parent of origin dependence of the phenotype is thought to reflect the uniparental pattern of expression of genes in the 15q11–q13 region, a phenomenon known as genomic or gametic imprinting (2). In the PWS region, this imprinting is proposed to be controlled by an imprinting centre (3). Three paternally expressed genes, *SNRPN* (4), *IPW* (5) and *ZNF127* (6), as well as two paternally expressed fragments, PAR-1 and PAR-5 (7), have been identified in the deletion interval.

The deletions of proximal human chromosome 15 in individuals affected with PWS occur exclusively on the paternally inherited chromosome. This has led to the hypothesis that the genes responsible for the PWS phenotype lie within the deletion region and are expressed only from the paternal allele. Although three paternally expressed genes have been identified within the deletion region, no correlation between the loss of expression of a specific PWS region gene and the PWS phenotype has been shown. Furthermore, no affected individuals have been identified in whom a mutation affects the expression of only one PWS region gene, suggesting that PWS is a contiguous gene syndrome in which the loss of expression of several genes is required for full manifestation of the disorder.

We have now localized the human gene encoding necdin (gene locus *NDN)* to the PWS deletion region and have shown that it is imprinted and expressed from only the paternal allele in normal human fibroblasts. We have localized its murine orthologue *Ndn* to central mouse chromosome 7, just telomeric to *Snrpn*, and demonstrated that it too is paternally expressed. Since mouse necdin protein is implicated in terminal differentiation of neurons, we hypothesize that necdin deficiency in individuals with PWS may cause part or all of their neurological deficit by interference with normal brain development.

RESULTS

Localization of the 'human NECDIN-related protein mRNA'

We reasoned that additional imprinted genes may lie within the PWS deletion interval, and undertook a search for transcribed sequences in the region between the two imprinted genes *ZNF127* and *SNRPN*. A contig of yeast artificial chromosome (YAC) clones derived from the proximal end of contig WC15.0 (8) and from smaller non-chimeric YACs previously mapped to the deletion interval (9) was established by analysis of sequence-tagged site (STS) marker content (Fig. 1). Expressed sequence tag (EST) SGC30582 was selected from among expressed sequences in the Human Gene Map (10) that had been localized between 15pter and *D15S156* by radiation hybrid mapping but had not been placed on the YAC map. By PCR analysis of the YAC contig, we found that EST SGC30582 was present in overlapping

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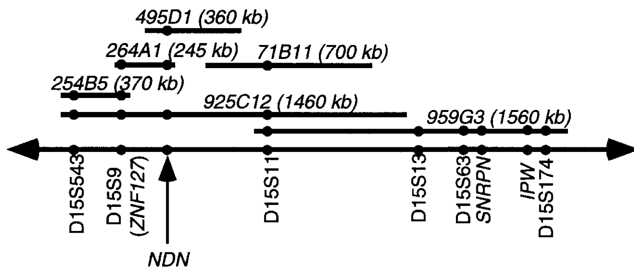


Figure 1. Physical mapping of the *NDN* gene. Mega-YAC clones from the CEPH Mega-YAC genomic library (23) and smaller YACs from the CEPH collection are represented by horizontal bars, with closed circles indicating STSs and genes (italicized) placed by landmark mapping. The centromeric direction is to the left. EST SGC30582, corresponding to the 3′ end of *NDN*, is located between markers *D15S9* and *D15S11*.

YAC clones 925C12, 264A1 and 495D1 from the proximal PWS deletion region (Fig. 1). Additional YACs spanning the rest of the PWS deletion interval were negative for the marker SGC30582 (data not shown).

The EST SGC30582 has 99% sequence identity to the 3′ end of the GenBank locus U35139, defined as a 'human NECDIN-related protein mRNA' (11). Other cDNAs from the IMAGE consortium libraries containing the EST SGC30582 were derived from fetal cochlea, heart and lung, infant brain and senescent fibroblasts. U35139 encodes a putative protein of 321 amino acids that has 82% amino acid identity with the 325 amino acid mouse necdin protein (Fig. 2). We obtained the IMAGE cDNA clone 39127, and found that the 1621 bp insert contained the entire open reading frame of U35139 and most of the 5′ and 3′ untranslated regions (UTRs), including the EST SGC30582. PCR analysis of YAC DNAs with oligonucleotide primers derived from the sequence of clone 39127 verified that the entire cDNA is indeed present within these YACs and therefore is within the PWS deletion region. PCR fragments derived from YAC DNA were identical in size to those derived from the cDNA clones, and sequencing and diagnostic restriction site analysis confirmed that the amplified fragments corresponded to the cDNA 39127 (data not shown). We therefore deduced that U35139 is contained within a single exon. Furthermore, Southern blot hybridization of restriction-digested YAC 925C12 and human DNA with a 1.2 kb *Hin*dIII fragment derived from IMAGE cDNA 39127 revealed strongly hybridizing fragments corresponding to a single locus in YAC 925C12 and in the human genome (data not shown). We conclude that the necdin-encoding gene is a single locus in proximal chromosome 15q, as demonstrated by radiation hybrid mapping of EST SGC30582, localization of the appropriate PCR-amplified fragments to overlapping YACs (Fig. 1) and absence in other YACs from the PWS deletion region.

Chromosomal localization and imprinting of mouse *Ndn*

Mouse necdin (gene locus *Ndn*) was originally identified as a protein encoded by a neural differentiation-specific mRNA, derived from embryonal carcinoma cells (12). The necdin protein is localized to the nuclei of post-mitotic neurons, and is expressed in almost all post-mitotic neurons in the central nervous system from the beginning of neural differentiation and into adult life (13). The *Ndn* locus is present as a single exon in the mouse genome, but had not yet been localized to a specific chromosome. To assess U35139 as a candidate human orthologue of mouse *Ndn*, and to localize *Ndn* within the mouse genome, we genotyped the BSS Jackson Laboratory Backcross DNA Panel Mapping (14). We first identified a DNA polymorphism within the 3′ UTR of *Ndn* by direct sequencing of PCR products amplified from *Mus musculus* strain C57BL/6J and *M.spretus* (SPRET/Ei) DNA. The G→A transition at nucleotide 2260 of the necdin gene sequence (GenBank accession No. D76440) abolishes a *Taq*I restriction site in *M.spretus* that is present in C57BL/6J. By genotyping the BSS cross for this polymorphism, we demonstrated linkage of *Ndn* to central chromosome 7 between the markers *Snrpn* (proximal) and *Tjp1* (distal) (Fig. 3a and b). This is a region of conserved synteny

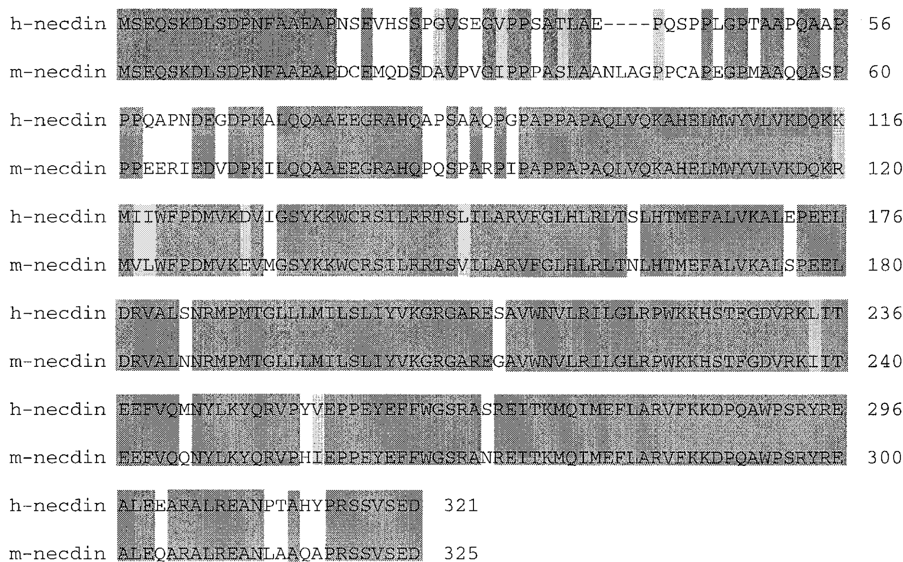
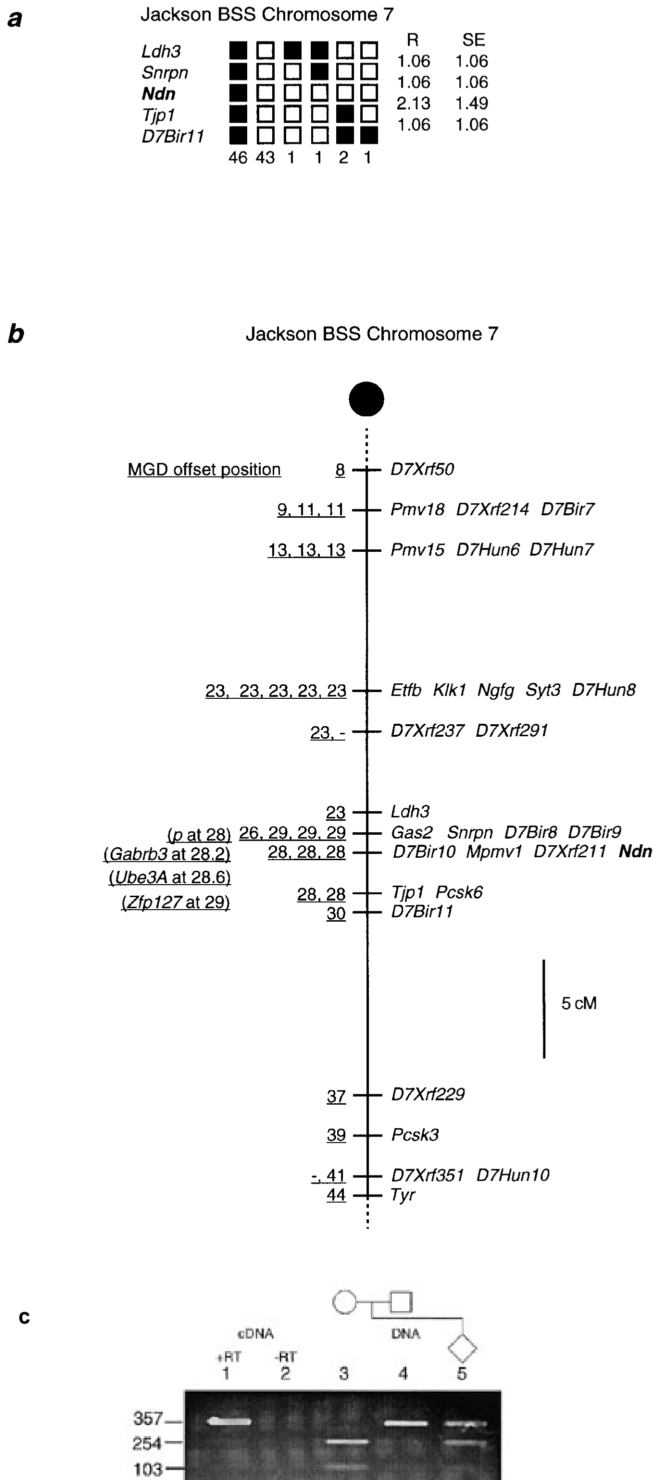


Figure 2. Alignment of human (h-necdin) and mouse (m-necdin) protein sequences. The necdin proteins share an overall 82% amino acid sequence identity. Identical amino acids are shaded dark grey, conserved amino acids are shaded light grey. The region of identity is uniformly distributed, with the exception of amino acids 19–62, which are less well conserved.

with human chromosome 15q11–q13 and is consistent with our localization of U35139 near human *SNRPN*. The sequence similarity of mouse *Ndn* to U35139 and our localization of the two genes to regions of conserved synteny in human and mouse strengthens our hypothesis that U35139, the human NECDIN-related protein mRNA, is the human orthologue of *Ndn*. The locus represented by cDNA U35139 is therefore designated *NDN*.



The mouse *Ndn* transcript is expressed exclusively in post-mitotic neurons, with no expression detected elsewhere by Northern blot analysis (13). To determine the allelic expression of the *Ndn* gene, we analysed RNA from a mouse heterozygous for the G2260A polymorphism. Tissues were collected from the newborn progeny of a cross between a SPRET/Ei male and a C57BL/6J female. DNA from this F1 interspecies hybrid mouse was analysed by PCR and restriction digestion, demonstrating heterozygosity for the G2260A polymorphism. RNA was extracted from brain of the same animal and amplified by reverse transcription-PCR (RT-PCR). Digestion with *Taq*I showed expression of only the paternally inherited SPRET/Ei allele (Fig. 3c).

Expression studies and imprinting of human *NDN*

To examine the pattern of expression of human *NDN*, we performed a Northern blot analysis with an *NDN* cDNA probe. Hybridization of the 1.2 kb *Hin*dIII fragment from IMAGE clone 39127 to a Northern blot prepared from human RNA from multiple adult tissues revealed a widespread expression of the 1.8 kb transcript (Fig. 4a). When compared with the control probe, expression of *NDN* was highest in brain and placenta although expression was detected in all tissues. A longer minor transcript was also observed in skeletal muscle and a shorter transcript in placenta. Thus the brain specificity of RNA expression in mouse is not conserved in human RNA. Interestingly, *IPW*, another imprinted gene in the PWS deletion region, is also widely expressed in human tissues while the expression of its murine homologue *Ipw* is limited primarily to the brain (15).

We assessed the imprinting status of *NDN* because of its genomic location between the two imprinted genes *ZNF127* and *SNRPN*. Neither *ZNF127* nor *SNRPN* is expressed in cell lines derived from PWS-affected individuals (4,16,17). Our preliminary expression studies indicated that expression of *NDN* was not detectable in RNA from control lymphoblasts or lymphocytes by

Figure 3. (**a**) Haplotype figure from The Jackson Laboratory BSS backcross showing the central part of chromosome 7 with loci linked to *Ndn*. Loci are listed in order, with the most proximal at the top. The black boxes represent the C57BL6/JEi allele and the white boxes the SPRET/Ei allele. The number of animals with each haplotype is given at the bottom of each column of boxes. The percent recombination (R) between adjacent loci is given to the right of the figure, with the standard error (SE) for each R. Missing typings were inferred from surrounding data where assignment was unambiguous. (**b**) Map figure of central chromosome 7, using data from the Jackson BSS cross. The map is depicted with the centromere toward the top. A 5 cM scale bar is shown to the right of the figure. Loci mapping to the same position are listed in arbitrary order. Missing typings were inferred from surrounding data where assignment was unambiguous. Raw data from The Jackson Laboratory were obtained from the World Wide Web address http://www.jax.org/resources/documents/cmdata. Underlined figures to the left of the chromosome bar give corresponding centiMorgan offset positions from the composite map in the Mouse Genome Database (MGD, from the World Wide Web at http://www.informatics/jax.org/mgd.html). The positions of three loci that are not mapped in the Jackson BSS cross are indicated in parentheses. (**c**) Parental origin of *Ndn* gene expression. DNA or cDNA was amplified with primers NEC9F/NEC10R and digested with *Taq*I restriction enzyme. Lane 1, digested RT-PCR product derived from RNA from the brain of a newborn mouse heterozygous for the G2260A polymorphism demonstrating that only the paternal G2260 allele is expressed; lane 2, the same RNA sample as in lane 1 but omitting the reverse transcriptase as a control for DNA contamination; lane 3, digested PCR product from C57BL/6J showing the digested A2260 allele; lane 4, digested PCR product from SPRET/Ei showing the undigested G2260 allele; and lane 5, digested PCR product from DNA from the newborn F1 interspecies hybrid mouse demonstrating heterozygosity for the G2260A polymorphism.

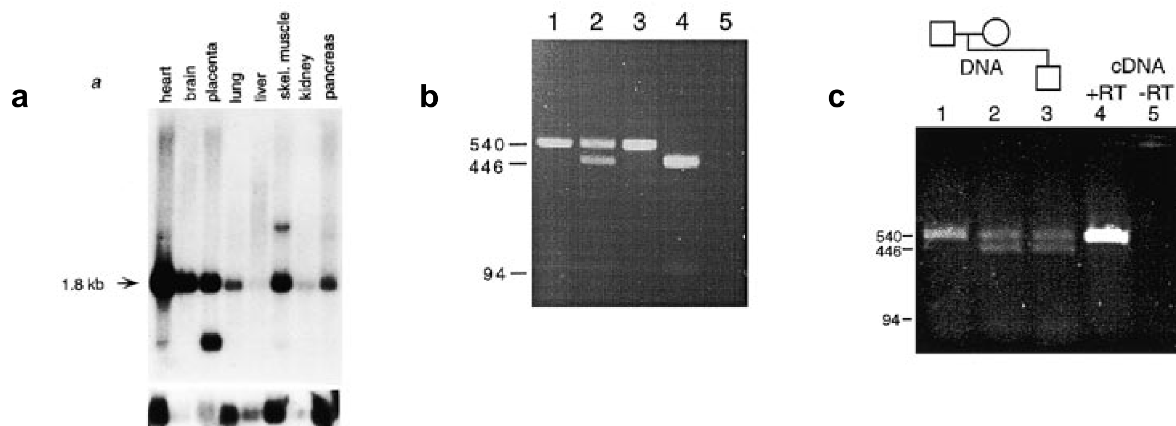


Figure 4. Expression analysis of *NDN* mRNA in adult human tissues. (**a**) Northern blot of adult poly(A)$^+$ RNA was hybridized with a 1.2 kb *Hin*dIII probe representing the first 1236 nucleotides of the IMAGE 39127 cDNA. The probe detects a 1.8 kb mRNA in all tissues. Relative quantities of RNA loaded were estimated by hybridization of the same blot with a ribosomal protein S26 cDNA probe, displayed in the lower panel. (**b**) Imprinting analysis of *NDN* expression in normal human amniocytes. Lane 1, NEC20F/NEC6R 540 bp PCR product amplified from DNA of amniocyte sample 10 (AMN10); lane 2, product from lane 1 digested with *Mbo*I demonstrating heterozygosity for the polymorphism (undigested allele 540 bp, digested allele 446 and 94 bp); lane 3, NEC20F/NEC6R 540 bp RT-PCR product from AMN10 RNA; lane 4, product from lane 3 digested with *Mbo*I demonstrating the presence of only the digested allele of *NDN*; lane 5, NEC20F/NEC6R RT-PCR with no reverse transcriptase as a negative control. (**c**) Paternal origin of *NDN* expression. Lanes 1–3, NEC20F/NEC6R PCR products digested with *Mbo*I demonstrating inheritance of the undigested 540 bp allele from the father; lane 4, NEC20F/NEC6R RT-PCR product from GM01653 RNA digested with *Mbo*I shows expression of only the paternal allele; lane 5, NEC20F/NEC6R RT-PCR with no reverse transcriptase as a negative control.

RT-PCR with primers derived from *NDN* cDNA sequence. We could not, therefore, test whether expression was absent specifically in similar cells derived from PWS-affected individuals.

Expression of *NDN* could, however, be detected in RNA from control fibroblasts and amniocytes. Since we did not have these cell types available from PWS-affected individuals, we sought a DNA polymorphism within the transcribed region to perform the imprinting analysis in unaffected individuals. Monoallelic expression detected in reverse-transcribed RNA despite heterozygosity in DNA would constitute evidence for imprinting. Direct sequencing of the *NDN* PCR product NEC1F/6R amplified from DNA samples from unrelated individuals revealed a C→T transition at nucleotide 916 (numbering according to GenBank accession No. U35139) that introduces an *Mbo*I restriction site and is neutral with respect to the predicted protein. Five individuals (two amniocytes and three fibroblasts) were heterozygous for the polymorphism. We performed RT-PCR on RNA from the heterozygous samples with primers that closely flanked this polymorphism (NEC20F/NEC6R) and digested the PCR-amplified products with *Mbo*I. Since the primers used were within the single exon of the *NDN* gene, the RNA was pre-treated with DNase I to eliminate residual DNA contamination. In RNA from heterozygous amniocytes and fibroblasts, only one of the two parental alleles was expressed (Fig. 4b). A negative control, without reverse transcriptase, showed no sign of amplification derived from contaminating DNA. In one individual for whom parental DNA samples were available and were informative, PCR analysis demonstrated that the C-916 allele was paternally inherited. RT-PCR of RNA from this heterozygous cell line followed by *Mbo*I restriction digestion revealed only the C-916 allele, indicating exclusive expression of the paternally derived *NDN* allele (Fig. 4c). We conclude that the necdin-encoding gene is imprinted in mouse and human and that gene transcription occurs only from the paternal allele in both species.

DISCUSSION

We report the identification of the human orthologue of the mouse necdin-encoding gene within the PWS deletion region. Human *NDN* is transcribed from only one of the two parental alleles in human fibroblasts and amniocytes, indicating that the expression of human *NDN* is imprinted in these tissues. The expression of *SNRPN* and *ZNF127*, located telomeric and centromeric respectively to *NDN*, is also imprinted. Consistent with the observation that imprinted genes have few and small introns (18), human *NDN* is contained within a single exon, like its mouse orthologue. The hypothesis that *NDN* is located near direct repeats and regions of monoallelic methylation like many imprinted genes (19) has not yet been tested.

Imprinting in human chromosome 15q11–q13 is controlled by an imprinting centre, located near the 5′ end of the *SNRPN* gene. Deletions of the imprinting centre cause a failure of genes in this region to switch their genomic imprint during gametogenesis. PWS-affected individuals with deletions <100 kb including the imprinting centre show lack of expression of paternally expressed genes many hundreds of kilobases from the deletion. This implies that the imprinting of multiple genes in the 15q11–q13 interval may be regulated by the imprinting centre, and the loss of expression of one or more paternally expressed genes within the range of the imprinting centre causes PWS. Although we have not yet tested for loss of expression of *NDN* in PWS-affected individuals with microdeletions, we would predict that *NDN* is also regulated by the imprinting centre and would, therefore, not be expressed.

The *NDN* locus in the PWS deletion region is highly likely to be the human orthologue of the mouse *Ndn* based on their map positions within a region of conserved synteny, near *SNRPN/Snrpn* in both genomes. Expression of human *NDN* is widespread, in contrast to mouse *Ndn*. The proteins encoded by the two genes

share a moderate degree of amino acid identity (82%) similar to the average value of 85% calculated in a comparison of 1196 mouse and human cDNAs (20). Both genes are imprinted and expressed exclusively from the paternal allele. This conservation of imprinting and paternal expression is shared by at least two other genes located near *NDN/Ndn* in both species, *SNRPN/Snrpn* (21,22) and *IPW/Ipw* (5,15).

Mouse necdin is a nuclear factor that is expressed in virtually all post-mitotic neurons from the beginning of neuronal differentiation until adulthood. It is not expressed in undifferentiated stem cells and is thus suggested to regulate the permanent arrest of cell growth of post-mitotic neurons during development, or at least serve as a marker for neuronal differentiation from stem cells. Loss of expression of human necdin may result in a phenotype that includes abnormalities in the development of the brain. Since the expression of *NDN* is from the paternal allele, loss of the paternal allele in PWS-affected individuals would leave no functional allele of *NDN*, resulting in a complete loss of necdin gene expression. Our findings suggest that loss of *NDN* gene expression could contribute to developmental delay in individuals affected with PWS.

MATERIALS AND METHODS

EST mapping and analysis

YAC clones are from the CEPH human YAC libraries. YAC clones from contig WC15.0 (http://www-genome.wi.mit.edu), MapPair primers amplifying chromosome 15 ESTs and IMAGE cDNA clones were obtained from Research Genetics. PCR amplification was performed as follows. For each PCR reaction, 50 ng of template DNA was added to a buffer containing 20 mM $(NH_4)_2SO_4$/75 mM Tris–HCl pH 8.8/0.1% Tween-20/0.2 mM dNTPs/1–2.5 mM $MgCl_2$/0.05 U of *Taq* polymerase/0.5 µM each primer. After initial denaturation at 94°C for 5 min, 30 cycles of amplification were performed. Each cycle consisted of denaturation (94°C for 30 s), annealing (50–55°C for 30 s) and extension (72°C for 30s). A final extension was at 72°C for 10 min. Amplification products were electrophoresed on 2% agarose gels and detected by ethidium bromide staining.

Sequencing and polymorphism identification

Oligonucleotide primer pair NEC9F, 5′-GTATCCCAAATCCA-CAGTGC and NEC10R, 5′-CTTCCTGTGCCAGTTGAAGT were designed from the sequence of MUSNCP (encoding mouse necdin) and amplify a 356 bp product in mouse DNA. DNA samples were amplified as described above and the amplification products sequenced directly on both strands using the Thermo-Sequenase® kit from Amersham, incorporating ^{33}P-labelled dideoxynucleotide chain terminators. DNA amplification products were digested with *Taq*I to produce a 356 bp undigested product in C57BL/6J, and 254 and 103 bp digested products in DNA from *M.spretus* (SPRET/Ei). Primer pair NEC20F, 5′-GCCCGAATA-CGAGTTCTTTT and NEC6R, 5′-CACACATCATCAGTCCC-ATA designed from the sequence of U35139 amplify a 540 bp product in human DNA that was sequenced as above. Digestion of the NEC20F–NEC6R PCR product with *Mbo*I produced either a 540 bp undigested product (C-916 allele), 446 and 94 bp digestion products (T-916 allele) or both patterns in a heterozygous individual. Sequence changes between U35139, cDNA clone 39127 and PCR products from human or YAC DNA were as follows. cDNA clone 39127 contains the sequence GGGCG instead of GCGGG at positions 970–974 but this change was not seen in the PCR product from six individuals sequenced and is assumed to be an error in the cDNA clone. A single nucleotide change from U35139 (C→T transition at position 684) was detected in PCR products from all six individuals but did not change the predicted amino acid sequence and is either an error in U35139 or a polymorphism. Finally, the first 19 bp of the U35139 sequence contains 11 changes from the sequence derived from clone 39127 that appear to be sequencing errors in U35139, since they resulted mostly in 2 bp inversions. It was not possible to design a flanking 5′ PCR primer to validate the sequence 5′ to the start codon in human DNA samples. Primers used to amplify YAC DNA and/or human DNA as four overlapping fragments (in the order 5′ to 3′) were as follows: NEC21F, 5′-GCGCAGACAT-GTCAGAACAA and NEC14R, 5′-ATGCTCCTGCACCAC-TTCTT; NEC16F, 5′-ACGAGCTCATGTGGTACGTG and NEC17R, 5′-GAAGGTGGAGTGCTTCTTCC; NEC1F, 5′-AT-GATCCTGAGCCTCATCTA and NEC6R; NEC7F, 5′-TTGTGC-TACCTTTCTTGGATT and NEC8R, 5′-GGTGGGGTTGTATAT-GTGTT.

Expression studies

A randomly primed [^{32}P]dCTP-labelled 1263 bp probe from IMAGE clone 39127 was hybridized to a human multiple tissue Northern blot (Clontech Laboratories). Hybridization was performed in 7% SDS/1% bovine serum albumin (BSA)/0.5 M $NaHPO_4$/1 mM Na_2EDTA (pH 7.2) at 65°C. The final wash of the Northern blot was in 0.1% SDS, 1× SSC at 65°C. Exposure to Hyperfilm (Amersham) was for 16 h at –70°C. Normal human amniocytes were obtained as samples discarded from the cytogenetics laboratory and normal fibroblasts were obtained from the NIGMS. The fibroblast cell line used to demonstrate paternal *NDN* expression was GM01653. DNA was prepared from cells or homogenized tissues by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. RNA from cell lines or homogenized mouse brain was extracted with TRIzol® (Gibco/BRL). Reverse transcription of 1 µg of RNA was performed with Superscript® reverse transcriptase (Gibco/BRL) and random hexamers according to the manufacturer's instructions. PCR amplification of one-tenth of the first strand cDNA was performed as described above, with primers NEC20F and NEC6R for human cDNA and NEC9F and NEC10R for mouse cDNA.

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