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Françoise Watrin[a]
Nathalie Roëckel[a]
Laurence Lacroix[a]
Cécile Mignon[a]
Marie-Geneviève Mattei[a]
Christine Disteche[b]
Françoise Muscatelli[a]

[a] Unité INSERM 406, Faculté de Médecine, Marseille, France;
[b] Department of Pathology, University of Washington, Seattle, Wash., USA

The Mouse *Necdin* Gene Is Expressed from the Paternal Allele Only and Lies in the 7C Region of the Mouse Chromosome 7, a Region of Conserved Synteny to the Human Prader-Willi Syndrome Region

Key Words
Necdin gene, mouse
Imprinting
DNA methylation
Asynchronism of replication
Prader-Willi syndrome

Abstract
Prader-Willi syndrome (PWS) is a neurogenetic disorder resulting from the loss of paternal expression of gene(s) localized in the 15q11-q12 region. A new human gene encoding a putative protein with high homology to the mouse NECDIN protein has recently been characterized and mapped to chromosome 15q11-q12. It is expressed from the paternal allele only, suggesting its potential involvement in PWS. We now report the localization of the mouse *Necdin* gene in a region of conserved synteny to the human PWS region. We demonstrate the paternal specific expression of *Necdin* in the mouse central nervous system, and show that parental alleles display a differential methylation profile in the coding region. Finally, fluorescence in situ hybridization analysis reveals an asynchronous pattern of replication at the *Necdin* locus. These results clearly demonstrate imprinting of the mouse *Necdin* gene. Mouse models will be powerful tools in the study of human PWS phenotype and imprinting mechanisms.

Introduction

Isolation of cDNA clones from neurally differentiated embryonal carcinoma P19 cells led to the identification of the murine *Necdin* gene [1, 2]. Although sequence analysis does not make it possible to predict a function for the 325-amino acid residue NECDIN protein, immunohistochemical studies have shown that NECDIN is a nuclear protein, expressed in virtually all postmitotic neurons of the central nervous system, from early stages of neurogenesis until adulthood [3, 4]. *Necdin* mRNA is expressed in neurally differentiated embryonal carcinoma cells, but not in proliferative neuron-like cells originating from tumors [3]. Moreover, induced ectopic expression of NECDIN in NIH3T3 cells leads to an arrest of cell growth without reduction of cell viability [5]. It has been proposed that NECDIN might therefore be involved in the intranuclear events by which neurons become permanently quiescent [5].

A human *NECDIN* cDNA clone was recently isolated, leading to the characterization of the human gene [6], which has been mapped to the human 15q11-q12 region

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Dr. Françoise Watrin
Unité INSERM 406, Faculté de Médecine
27, Bd Jean Moulin
F-13385 Marseille Cedex 5 (France)
Tel. +33 4 91 78 68 94, fax +33 4 91 80 43 19, e-mail watrin@voltaire.timone.univ-mrs.fr

involved in the Prader-Willi syndrome (PWS). PWS is a neurogenetic disorder, which results from the absence of paternal expression of gene(s) localized in 15q11-q12 [7]. It occurs with paternal deletion of the 15q11-q12 region [8, 9] or with maternal disomy for chromosome 15 (Chr 15) [7]. Rarer cases of PWS displaying imprinting abnormalities have also recently been characterized, and attributed to small deletions in a putative imprinting control center [10–13]. PWS is characterized by severe transient hypotonia and feeding problems in newborns, hyperphagia and obesity developing in early childhood, hypogonadism, short stature, craniofacial dysmorphism and mental retardation. Up to recently, five imprinted sequences displaying exclusive paternal expression were characterized in the 15q11-q12 region, including *ZNF127* [14], *SNRPN* [15–17], *IPW* [16], *PAR1* and *PAR5* [10] but none of these have been demonstrated to be involved in PWS [18]. Human *NECDIN* is localized in the PWS region, and displays all the characteristics of an imprinted gene [6]. *NECDIN* has therefore been proposed to be a new candidate gene involved in the etiology of PWS, this hypothesis being strengthened by its complete lack of expression in brain RNA from PWS patients.

Mouse models of PWS genotype would be valuable tools for the study of this syndrome. Based on the conservation of synteny between human 15q11-q13 and murine chromosome 7 central regions, we would expect mouse *Necdin* to be localized in the described imprinted domain of the central chromosome 7 [19–21]. Maternal duplication for this region leads to an early postnatal lethality, possibly associated with feeding difficulties [22]. This imprinting effect has been suggested to correspond to the human PW phenotype [21, 22]. As a first step in determining whether mouse *Necdin* is involved in the mouse imprinting effect observed in neonatal mice bearing a maternal duplication of the central region of chromosome 7, we determined *Necdin* chromosomal location. *Necdin* is localized in the 7 C region of the mouse genome. Moreover, we show that it is maternally imprinted, and displays a paternal-specific monoallelic expression in the central nervous system of the developing mouse embryo and in the adult brain. Differential methylation as well as replication asynchrony of parental *Necdin* alleles are observed. Mouse models in which *Necdin* is inactivated could allow to assess the potential involvement of the lack of NECDIN in both the mouse imprinting effect observed in neonatal mice bearing a duplication of the central region of chromosome 7 and in the etiology of human PWS.

Material and Methods

Mice

Adult C57BL/6 mice were purchased from IFFA CREDO. Outbred *Mus spretus* males were kindly provided by Jean-Louis Guenet (Pasteur Institute, Paris, France). Outcrosses and backcrosses were performed at the hospital animal facility.

Isolation of DNA and RNA

Genomic DNA used for the methylation studies was isolated using standard methods [23]. Tail DNA used for the genotyping of N2 mice was prepared according to the protocol described by Laird et al. [24]. Total RNAs were prepared by the single-step RNA isolation method developed by Chomczynski and Sacchi [25], using Trizo® reagent (Gibco-BRL). Prior to reverse transcription (RT), purified RNAs (10 μg) were treated with 2 units of RNase-free DNase RQ1 (Promega) for 30 min at 37 °C, in a final volume of 20 μl. The DNase was then inactivated at 80 °C for 10 min.

Genotyping of Backcross Progeny

100 ng of tail DNA were used for PCR amplification. The primers were as follows: Nec-1859 (S) 5′-TCT GGA GCA GGC CAG AGC TC-3′ (nucleotides 1859–1878) and Nec-2420 (AS) 5′-TGC TAA GTG CCT ACA CTG AG-3′ (nucleotides 2420–2401). These primers amplify a sequence of 561 bp which includes a polymorphic *Taq*I site (position 2258). Conditions of amplification are described in RT-PCR analysis. After purification (Quiagen), the PCR products were digested with *Taq*I, fractionated in a 1.5% agarose gel and visualized by ethidium bromide staining.

Isolation of Mouse Necdin Genomic Phages

A PCR product amplified from mouse genomic DNA with primers Nec-1010 (S) 5′-CGA CTG TGA GAT GCA GGA CAG C (nucleotides 1010–1031) and Nec-1879 (AS) 3′-GAG CTC TGG CCT GCT CCA GA (nucleotides 1859–1879) was used to isolate genomic clones from a 129/Sv mouse phage genomic library (Clontech). This PCR fragment covers the almost complete *Necdin* coding sequence. Four phages were isolated and checked by restriction analysis and PCR, among which the phage 15 contained the longest genomic fragment (15 kb).

Southern Analysis

10 μg of digested genomic DNAs were separated by gel electrophoresis through a 1% agarose gel, in 1 × TBE buffer, denatured for 20 min in 0.5 *M* NaOH, 1.5 *M* NaCl, and transferred to a positively charged membrane (PALL Biodyne B) by capillarity in 0.4 *M* NaOH for 24 h. Hybridizations were performed in Church solution with 1.5 × 10^6 cpm/ml of ^{32}P-random-labeled DNA probes (^{T7}QuickPrime kit; Pharmacia) at 65 °C for 18 h. Filters were washed at a final stringency of 0.2 × SSC, 0.1% SDS at 65 °C and exposed to X-ray film at –70 °C for various lengths of time. Probes are described in figure 2a.

RT-PCR Analysis

cDNAs were synthesized from 2 μg of total DNase-treated RNA in a final volume of 20 μl, using 1 μg of oligo (dT) 12–18 (Pharmacia), and SuperScriptTM II Rnase H reverse transcriptase (Gibco-BRL), in presence of 20 units of RNasin (Promega). cDNAs prepared from 100 ng of total RNA were used for PCR amplification. Primers for PCR amplification of the mouse *Necdin* cDNAs are described in Genotyping of Backcross Progeny. Amplifications were performed in

a final volume of 50 µl using 1 × *Taq* DNA polymerase buffer, with 3% DMSO, 0.25 µ*M* of sense and antisense oligonucleotides, 0.1 m*M* dNTPs and 1 unit of *Taq* DNA polymerase (DynaZyme^{II} DNA Polymerase, Finnzymes, Oy). Following denaturation at 94 °C for 5 min, thirty cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C were performed. After purification (Quiagen), the PCR products were digested with *Taq*I, fractionated in a 1.5% agarose gel and visualized by ethidium bromide staining.

Gene Mapping by in situ Hybridization
In situ hybridization experiments were carried out using lymphocyte metaphase spreads prepared from a WMP male mouse (gift from J.-L. Guenet). Concanavalin A-stimulated lymphocytes were cultured at 37 °C for 72 h with 5-bromo-deoxyuridine added for the final 6 h of culture (60 µg/ml of medium). The mouse lambda phage 15 was biotin-labeled by nick translation using biotin-16-dUTP, annealed with a 150-fold excess amount of Cot-1 DNA (Gibco-BRL), and hybridized to metaphase spreads at a final concentration of 10 µg/ml of hybridization solution, as previously described [26, 27]. The hybridized probe was detected by means of fluorescence isothiocyanate-conjugated avidin (Vectors Laboratories; No. A-2011). Chromosomes were counterstained and R-banded with propidium iodide diluted with antifade solution pH 11 [28]. A total of 50 metaphase cells were analyzed.

FISH-Based Replication Assay
Replication timing studies were carried out by in situ hybridization on concanavalin A-stimulated WMP mouse lymphocytes nuclei, using biotin-16-UTP-labeled phage (mouse *Necdin* phage 15) or cosmids (*H19* cosmid, gift from L. Dandolo; *AF4* cosmid, gift from P. Isnard and M. Djabali) as described previously [29]. After hybridization and washings, slides were incubated sequentially with avidin-FITC (Vector Laboratories; No. A-2011), biotinylated antiavidin antibody (Vectors Laboratories; No. BA-0300) and avidin-FITC to visualize the hybridization dots. Chromosomes were counterstained with propidium iodide diluted in antifade reagent. Fluorescence in situ hybridization (FISH) patterns in over 150 interphase nuclei were scored for each slide and probe. FISH experiments were repeated 3 times.

Mouse Necdin Accession Number
The mouse *Necdin* accession number was D76440 [1].

Results

Chromosomal Localization of the Mouse Necdin *Gene*
Mouse *Necdin* was mapped by hybridization of the biotin-labeled *Necdin* genomic phage 15 on lymphocyte metaphase chromosomes prepared from a WMP male mouse, in which all the autosomes except the 19th are in the form of metacentric robertsonian translocations. A total of 50 metaphase cells were analyzed, among which 85% showed specific fluorescent spots on the C region of murine chromosome 7 (data not shown). Radioactive in situ hybridization of a PCR-amplified fragment corresponding to the *Necdin* coding region (primers described in Isolation of Mouse Necdin Genomic Phages), cloned in a BlueScript vector, was simultaneously performed. A single peak of hybridization was detected in the 7B5-D1 region (data not shown). These results allowed us to map the *Necdin* gene to the murine 7C region, a region of known conserved synteny with the human 15q11-q12 region [30–32].

Paternal Allele-Specific Expression of the Mouse Necdin *Gene*
Primers were designed to amplify a fragment of 561 bp from the 3′ untranslated region of the mouse *Necdin* gene. Sequence analysis of PCR products amplified from *M. musculus domesticus* (C57BL/6 strain) and *M. spretus* genomic DNA identified two single base pair differences between the two sequences, one of which destroys a *Taq*I restriction site in the *M. spretus*-derived copy (fig. 1a, b). To determine whether *Necdin* shows a parental specific monoallelic expression, adult brain RNA was isolated from F1 mice derived from matings between C57BL/6 female and *M. spretus* male, reverse-transcribed and digested with *Taq*I. As shown in figure 1c, the adult brain RNA is exclusively derived from the *M. spretus* paternal allele in F1 mice. Imprinting of *Necdin* was also investigated in the mouse 12.5-day embryo. RNA from the whole head of the embryo was prepared, reverse-transcribed and digested with *Taq*I. In the 12.5-day embryonic brain as in the adult brain, *Necdin* is expressed from the paternal allele only, being the *M. spretus* allele (fig. 1c). In order to determine if the imprint observed at the *Necdin* locus was not due to a selective amplification of the *M. spretus* allele, F1 female were backcrossed to C57BL/6 male, the progeny genotyped, and adult brain RNA analyzed. In adult brain RNA isolated from N2 mice carrying a maternally derived *M. spretus* and a paternally derived C57BL/6 *Necdin* alleles (genotyping not shown), *Necdin* was exclusively expressed from the paternal allele, being in this case the C57BL/6 allele (fig. 2b). These results demonstrate that in the mouse brain, *Necdin* can be expressed from both *M. spretus* and C57BL/6 *Necdin* alleles, only if inherited from the father, and eliminate a potential selective amplification of the *M. spretus* or C57BL/6 cDNAs in mice derived from interspecific crosses. Moreover, they show that the maternal imprint at this locus can be erased and reset from one generation to the other.

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1859 tc tggagcaggc cagagctctg cgggaggcta atcttgctgc ccaggccccc

cgcagcagtg tctctgagga ctaaaaaggt ccaggggcac actgatagtt tctgacccat

actagggctg tgtaagggtg gggttgagtc attagagtat cccaaatcca cagtgcagta
                                             t (M. Spretus)

tttcatgtat aatttttaag ttttccatac agtgcttttg taccttgtaa tgctattcat

ttgtgtactc gtgtagtgtt taagattgat gcatgtgtga taagtatttg gtactttcac

ttttgtgctt tcgtgcattt ttgtacaaga gatgtgctgt gctaaacttg tgaaatacat

tgaggtgttc tgtatcttgt tcctttgtat gggactgatg atctgtatcg acaaagaagg
                                                      a (M. spretus)

ccctggagag ttagcaggac ttaacagcaa cgcagacctg agcaagagaa aggtcaaggc

ctttctccat atgacttcaa ctggcacagg aagcatccat gtggaatgga ctgatttgaa

ctggactgtt ctcagtgtag gcacttagca 2420
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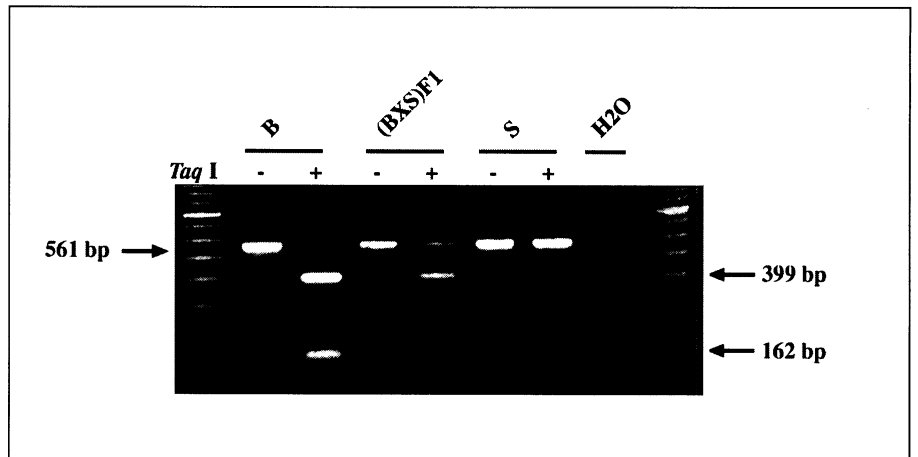
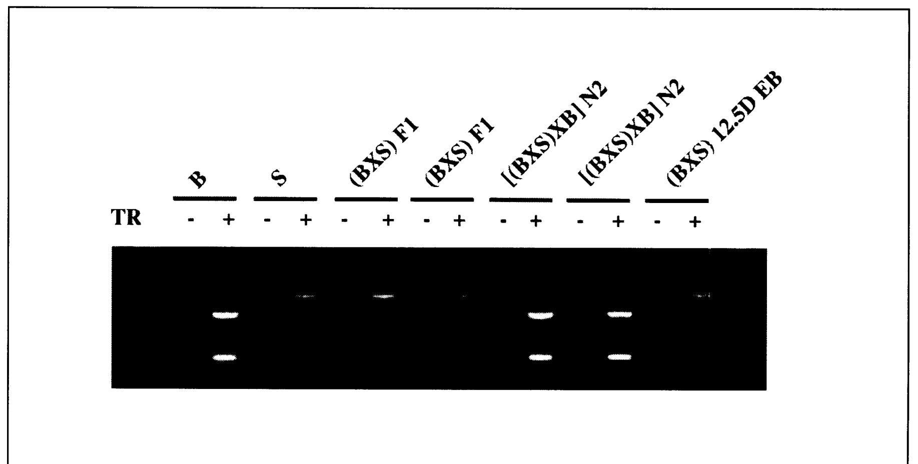
a



b



c

Fig. 1. Paternal allele-specific expression of the mouse *Necdin* gene. **a** Sequence of *Necdin* genomic DNA amplified with primers Nec-1859 (S) and Nec-2420 (AS). Primer sequences are in italics and underlined. Single nucleotide differences between *M. musculus* (C57BL/6) and *M. spretus* genomic DNA identified by PCR product sequencing are indicated in bold letters. One of these single base pair differences (A in *M. spretus* instead of G in C57BL/6; position 2260) destroys in *M. spretus* a *Taq*I restriction site (underlined) present in C57BL/6. It should be noted that since *Necdin* is an intronless gene, the amplified genomic DNA and cDNAs have the same length. **b** Genomic DNA *Taq*I RFLP analysis. Genomic DNAs from C57BL/6 (B), *M. spretus* (S) and (C57BL/6 × *M. spretus*) (B × S) F1 mice were amplified using primers Nec-1859 (S) and Nec-2420 (AS). *Taq*I-digested (+) and *Taq*I-undigested (–) PCR products were subjected to electrophoresis and visualized by ethidium bromide staining. 100-bp ladder fragments were used as molecular weight markers. The C57BL/6 (B) product containing a *Taq*I site is cleaved in two fragments (399 and 162 bp), whereas the *M. spretus* (S) product is uncleaved (561 bp). **c** Paternal allele-specific expression of *Necdin* in the 12.5-day embryo and adult mouse brains. RT-PCR products from C57BL/6 (B), *M. spretus* (S), (C57BL/6 × *M. spretus*) F1, (B × S)F1, and [(C57BL/6 × *M. spretus*) × C57BL/6]N2, [(B × S) × B]N2, adult mouse brain, and (C57BL/6 × *M. spretus*) F1, (B × S)F1, 12.5-day embryo head, with (+) or without (–) addition of RT, were digested with *Taq*I, subjected to electrophoresis and visualized by ethidium bromide staining.

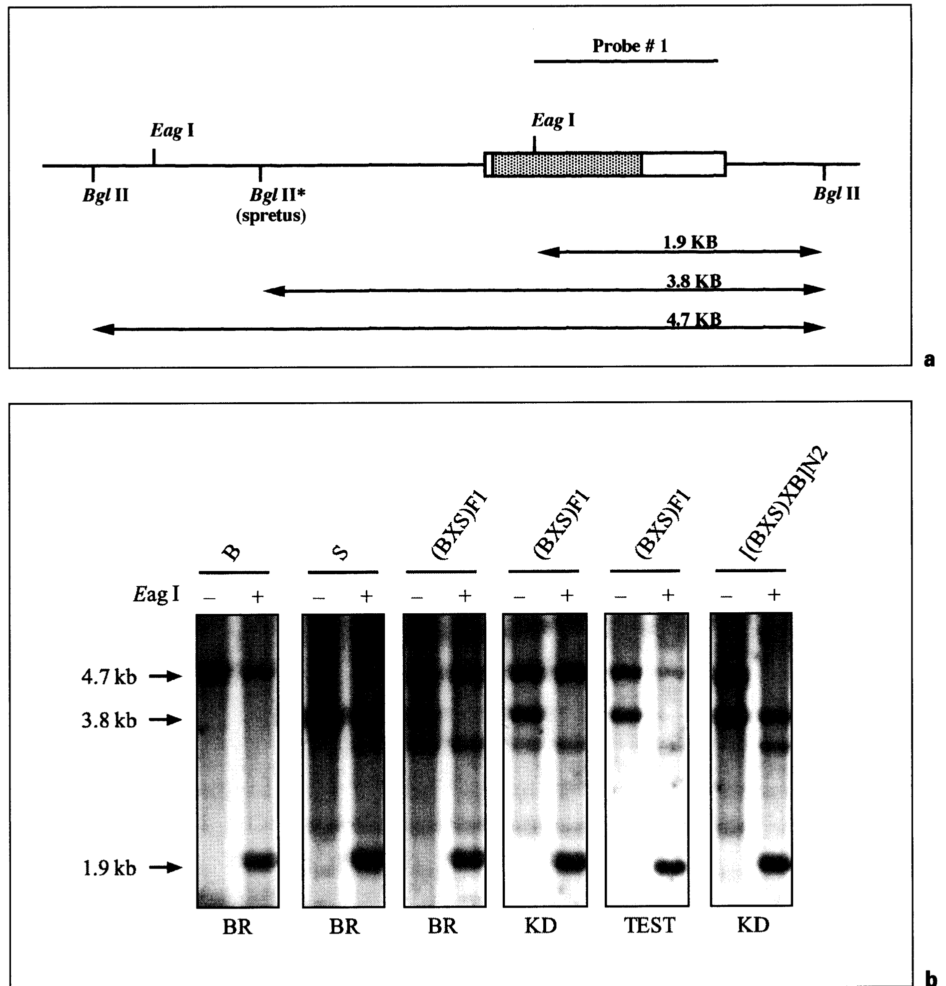
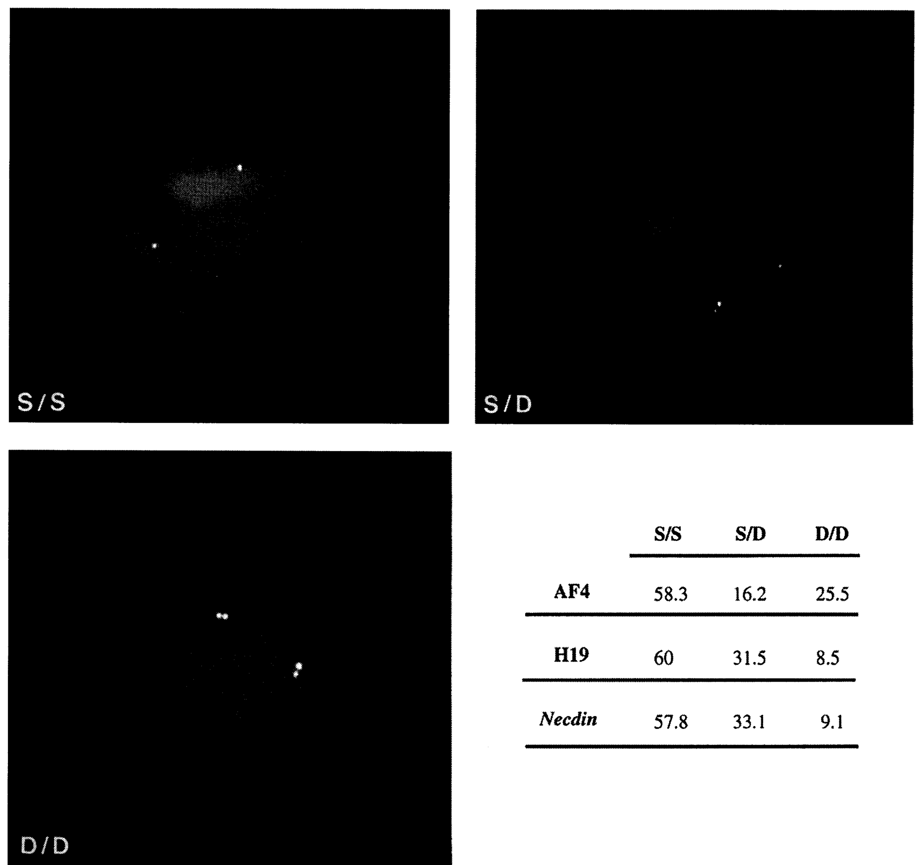


Fig. 2. Intragenic *Eag*I differential methylation of the maternal and paternal *Necdin* alleles. **a** Schematic representation of the *Necdin* locus showing the position of the *Bgl*II sites in C57BL/6 and *M. spretus* genomic DNA, the methylation-sensitive restriction *Eag*I sites, and the probe 1 used in the Southern blot analysis. The *Necdin* gene is represented by a rectangle in which the shaded area corresponds to the coding region. The size of the different hybridizing fragments is indicated. **b** Southern blot analysis of the methylation-sensitive intragenic *Eag*I site. *Bgl*II genomic DNA isolated from C57BL/6 (B), *M. spretus* (S), (C57BL/6 × *M. spretus*) outcross progeny (B × S)F1, and [(C57BL/6 × *M. spretus*) × C57BL/6] backcross progeny [B × (S × B)]N2 were further digested with *Eag*I (+) or not (–), fractionated on an agarose gel, and transferred onto a positively charged membrane. The filter was hybridized with probe 1, and exposed to an autoradiographic film for 12 h. Adult brain (BR), kidney (KD) and testis (TEST) DNA were analyzed. Sizes of hybridizing fragments are indicated. In F1 mice, the 4.7- and 3.8-kb *Bgl*II-hybridizing fragments originate from the mother and the father, respectively. In N2 mice, the 4.7- and 3.8-kb *Bgl*II-hybridizing fragments originate from the father and the mother, respectively.

Methylation Analysis

To analyze whether the paternal and maternal *Necdin* loci are differentially methylated, several restriction fragment length polymorphisms (RFLP) were identified between C57BL/6 and *M. spretus* species DNA which could allow us to distinguish parental alleles in F1 progeny DNA (fig. 2a). Adult brain DNAs from C57BL/6, *M. spretus* and (C57BL/6 × *M. spretus*) F1 mice were first digested with *Bgl*II, then by *Eag*I which does not cut DNA at its recognition sequence if the internal CpG is methylated. This *Bgl*II digestion generates restriction fragments of 4.7 or 3.8 kb in C57BL/6 or *M. spretus* DNAs, respectively, detected by probe No. 1 on Southern blots (fig. 2b). In F1 DNA digested with both enzymes, the paternally derived 3.8 kb *M. spretus* allele was always totally digested by *Eag*I, whereas the maternally derived 4.7 kb was almost completely resistant to this enzyme. These results indicate a differential methylation status of the *Eag*I site on parental *Necdin* alleles, this site being completely nonmethylated on the expressed paternal allele. DNA from adult kidney in which *Necdin* is not expressed as opposed to DNA from adult brain in which *Necdin* is expressed at high levels displayed the same pattern of differential methylation on parental alleles. These observations suggest that the parental imprint is maintained in all somatic cells, whether they express *Necdin* or not. Moreover, analysis of adult kidney DNA from (C57BL/6 × *M. spretus*) × C57BL/6 backcross progeny showed that nonmethylation of the intragenic *Eag*I restriction site of the *M. spretus* allele is dependent on its paternal inheritance, since this site became methylated when the *M. spretus* allele was maternally inherited in N2 mice. Conversely, this same site became nonmethylated on the C57BL/6 allele of N2 mice, when paternally inherited. Adult testis DNA did not quite display the same pattern of parental allele differential methylation as the one observed in adult brain and



	S/S	S/D	D/D
AF4	58.3	16.2	25.5
H19	60	31.5	8.5
Necdin	57.8	33.1	9.1

Fig. 3. *Necdin* FISH-based replication timing analysis. Nuclei displaying either two isolated hybridization dots (S/S; not yet replicated alleles), both an isolated and a double hybridization dot (S/D; asynchronously replicating alleles) or two double hybridization dots (D/D; two replicated alleles) are shown. Percentages of S/S, S/D and D/D nuclei for the three loci analyzed are indicated.

kidney, the maternal allele being almost completely nonmethylated. The majority of cells in the adult testis being germ cells, this nonmethylation of both maternal and paternal alleles might reflect the resetting of the imprint in the male germ cells.

Asynchronous Replication of Necdin

Asynchronous replication as assayed by 5-bromodeoxyuridine (BrdU) incorporation or FISH has been reported for several classes of monoallelically expressed genes [33–35]. In a first approach to investigate the replication timing of *Necdin*, FISH analysis was performed on mouse lymphocyte interphase nuclei, using the biotin-labeled genomic phage 15. Two additional biotin-labeled mouse cosmid probes were simultaneously hybridized to the same nuclei preparations: an *H19* probe which has been shown by FISH to detect asynchronous replication [34] (positive control) and an *AF4* probe corresponding to the biallelically transcribed *AF4* gene (negative control). For each of these probes, the number of interphase nuclei displaying either two isolated hybridization dots (not yet replicated alleles; S/S), both an isolated and a double hybridization dots (asynchronously replicating alleles; S/D), or two double hybridization dots (two replicated alleles; D/D) was determined (fig. 3). For both the *Necdin* and *H19* probes, 33 and 31%, respectively, of the interphase nuclei exhibited a single-double dot hybridization pattern, whereas this same pattern of hybridization dropped to 16% for the *AF4* probe. Thus, as visualized by FISH analysis, *Necdin* displays a potential asynchronism of replication, a common characteristic of imprinted genes.

Discussion

Previous genetic studies in mice using various robertsonian and reciprocal translocations to generate uniparental disomies and uniparental duplications for whole and selected chromosomal regions, respectively, have defined several imprinting effects, ranging from early embryonic lethality to influences on postnatal growth. Ma-

ternal duplication of chromosome 7 central region has been shown to be associated with postnatal lethality, possibly associated with feeding difficulties, and has been proposed to represent a potential mouse PWS syndrome model [22]. We now report the localization of mouse *Necdin* in the 7 C region of the mouse genome. Since restriction analysis mapping has indicated that human *NECDIN* would be localized distal but close to *ZNF127* and 1–1.5 Mb proximal to *SNRPN* [6], mouse *Necdin* is likely to be localized between *Snrpn* and *Znf127/Dn34*. The mouse *Necdin* locus should therefore lie proximal to the 7 B5-C T9H breakpoint, in the described imprinted domain of central chromosome 7 [19–21]. This hypothesis is further strengthened by our demonstration of mouse maternal *Necdin* imprinting. *Necdin* might therefore be involved in the mouse imprinting effect observed in neonatal mice bearing a maternal duplication of chromosome 7 central region.

Immunohistochemical studies have shown that NECDIN is a nuclear protein, expressed in virtually all postmitotic neurons of the central nervous system, from early stages of neurogenesis until adulthood [3, 4], and which might be involved in the intranuclear events by which neurons become permanently quiescent [5]. In developing mouse brain, *Necdin* mRNA has been first detected from day 10.5 in the forebrain area [2], throughout development in most brain areas in which neurons differentiate [3]. Using polymorphisms between *M. musculus* (C57BL/6) and *M. spretus* mice, we have demonstrated that in both the 12.5-day mouse embryo and in the adult, *Necdin* is exclusively expressed from the paternal allele, in the central nervous system. Moreover, we show that the maternal imprint at this locus can be erased and reset from one generation to the other. *Necdin* imprinting does not seem therefore to be gradually relaxed from embryonic stages to adulthood at least in the tissues analyzed, as it is the case for some other imprinted genes which display both developmental- and tissue-specific patterns of imprinting [36–38]. However, a more detailed analysis is underway since we have detected *Necdin* expression by RT-PCR and in situ hybridization analysis in the myotome of developing mouse embryos and in the placenta (data not shown).

A growing body of evidence suggests that differential methylation of the cytosine residue in CpG dinucleotides is involved in the imprinting process [39–41]. Although the exact role of methylation in imprinting remains to be defined, all the imprinted genes that have been examined display a parental specific methylation profile [40–42]. Preliminary analysis of *Necdin* parental alleles methylation status in brain genomic DNA demonstrates that, at least for the intragenic *Eag*I site analyzed, these two alleles are differentially methylated, the expressed paternal allele being completely nonmethylated as opposed to the silent maternal allele being fully methylated. Two additional intragenic *Hpa*II methylation-sensitive sites located 5′ to the *Eag*I site display differential methylation as well, the maternal allele being only partially methylated however (data not shown). The *Eag*I site differential methylation profile is maintained in somatic tissues of the mouse independently of gene expression, as it has been observed for other genes [43]. It is interesting to note that methylation analysis of the same conserved *Eag*I site in human lymphocyte DNA from normal or PWS individuals indicates that this site is also differentially methylated in normal individuals (having both a paternal and a maternal allele) and completely methylated in PWS patients (who present either a deletion in the paternal 15q11-q13 region or an imprinting mutation) [6]. Differential methylation of imprinted genes seen in late embryonic and adult tissues has been shown to be established either de novo after the global wave of demethylation affecting the blastocyst, or earlier during gametogenesis for specific genomic regions which methylation pattern is maintained up to the implantation stage. These particular sequences resistant either to the global wave of demethylation at the blastocyst stage or to de novo methylation later in development could constitute the imprinting signal for distinguishing the parental alleles [40, 41]. In this regard, the almost complete nonmethylation of the *Necdin* intragenic *Eag*I site in adult testis which is composed mostly of spermatogenetic cells suggest that the *Necdin* paternal allele demethylation observed in adult somatic tissues might be established early during spermatogenesis. Further detailed analysis will be required to analyze the methylation profile of this particular site as well as of other sites in *Necdin* promoter region and 5′ and 3′ flanking sequences, during both spermatogenesis and oogenesis, and early embryogenesis.

Asynchronous replication is characteristic of monoallelically expressed genes such as X-linked genes [33, 44], imprinted genes [34, 45–48] and some nonimprinted autosomal genes [35]. Replication timing of *Necdin* was analyzed by FISH on mouse lymphocyte interphase nuclei, as well as those of two other mouse loci: the *H19* and *AF4* genes, respectively, imprinted and biallelically expressed genes. Both *Necdin* and *H19* displayed a similar percentage of a single-double dot hybridization pattern, significantly higher than the one displayed by *AF4*. These results demonstrate that *Necdin* displays a potential

asynchronism of replication, a common characteristic of imprinted regions. Careful comparisons of replication timing data obtained in BrdU incorporation or FISH analyses however suggest that replication asynchrony as visualized by FISH might in some cases reflect parental allelic chromatin structural differences (at least in nonexpressing cells), rather than a real replication asynchrony [33, 47, 49].

One striking difference between the mouse and human genes is their differential pattern of expression. In contrast to mouse *Necdin* which is expressed almost exclusively in the central nervous system [2, 3], *NECDIN* is ubiquitously expressed in human tissues with the exception of peripheral blood leukocytes [6]. However, in situ hybridization analysis of NECDIN in the developing and adult central nervous system of humans suggests that both genes display a similar developmental and cellular expression pattern. In the developing spinal chord for example, both genes are first expressed in similar regions of ventral horns [6; Watrin: unpubl. results]. These observations suggest that, despite their different tissue specificity of expression, human and mouse NECDIN might share a common function at least in the developing and adult central nervous system.

Comparisons of human and mouse promoter sequences and more detailed expression studies are underway. Furthermore, mouse models in which *Necdin* is inactivated should allow to determine NECDIN function and to assess the potential involvement of a lack of NECDIN in both the mouse imprinting effect observed in neonatal mice bearing a duplication of the chromosome 7 central region and in the etiology of human PWS.

Acknowledgments

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