Evidence for translational regulation of the imprinted Snurf–Snrpn locus in mice

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In studies of genomic imprinting in the Prader–Willi/Angelman domain, an agouti coat color cassette was inserted into the downstream open reading frame (ORF) of the imprinted bicistronic Snurf–Snrpn locus in the mouse. The fusion gene was maternally silenced, as is Snurf–Snrpn, and produced a tan abdomen only when inherited paternally in otherwise-black mice. A screen for dominant epigenetic or genetic events was performed with ENU mutagenesis, using a strategy whereby variation in abdominal color was scored at weaning. One mouse with maternal origin of the fusion gene had a tan abdomen and had an imprinting defect resulting in loss of both maternal methylation and silencing of the fusion gene. One mouse with paternal origin of the fusion gene was completely yellow and was found to have an ATG-to-AAG mutation in the initiation codon of the upstream ORF encoding SNURF. Northern blotting, immunoblotting, and transfection studies indicated that the ATG-to-AAG mutation causes a 15-fold or more increase in translation of the downstream ORF in two fusion constructs, and it is likely that similar translational control affects the normal Snurf–Snrpn transcript as well.

INTRODUCTION

We are investigating the molecular genetics of genomic imprinting with particular focus on the homologous regions of mouse chromosome 7C and human chromosome 15q11–q13; these regions include the genes responsible for Prader–Willi syndrome (PWS) and Angelman syndrome (AS) in the human. PWS and AS are distinct neurogenetic disorders caused by deficiency of paternal or maternal gene expression, respectively, from human chromosome 15q11–q13 (1,2). AS is caused by maternal deficiency for UBE3A, which encodes E6-AP ubiquitin-protein ligase (3,4), but the gene or genes whose deficiency cause(s) PWS are not known. The SNURF–SNRPN locus in human and the Snurf–Snrpn locus in mouse are bicistronic, with the upstream open reading frame (ORF) encoding a highly conserved protein SNURF (5), and the downstream ORF encoding small nuclear ribonucleoprotein-associated polypeptide N (SmN), a protein involved in mRNA splicing (6–8). In both species, the upstream region of the bicistronic gene is associated with a CpG island that encompasses the promoter and is heavily methylated exclusively on the maternal allele, which is tightly silenced. Mice heterozygous or homozygous for a null mutation for the SmN ORF do not show lethality or any other obvious phenotype (9).

We have undertaken a gene-fusion strategy combined with mutagenesis in the mouse with the goal of identifying mutations or epigenetic events affecting genomic imprinting. We placed an agouti coat color cassette under the control of the imprinted Snurf–Snrpn promoter so that events affecting imprinting could be detected through coat color variation. The common alleles at the agouti locus in laboratory mice are agouti (A), which is dominant and yields a brown coat color, and the recessive nonagouti (a) allele, which yields a black color in homozygotes. The agouti locus is attractive for study because extremely low levels of expression yield a phenotypically significant change in coat color compared with homozygotes for the extreme nonagouti allele (aε), a null mutation (10). Several dominant mutations that cause more widespread expression or ectopic production controlled by a transgene with a heterologous promoter are associated with yellow coat color, obesity, diabetes and susceptibility to tumors, all through overexpression or ectopic expression of agouti protein (11–13).

In this report, we demonstrate that the agouti reporter cassette was placed under the control of the Snurf–Snrpn promoter, and that the switching of imprinted gene expression was preserved.

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of the upstream ORF revealed a 15-fold increase in translation of the downstream ORF.

RESULTS

Placing the agouti cDNA under the control of the imprinted Snurf–Snrpn promoter

The major transcript for the mouse Snurf–Snrpn is derived from 10 exons within 22 kb of genomic DNA (14) and encodes two ORFs, both expressed exclusively from the paternal chromosome. The smaller upstream ORF encodes the 71-amino-acid SNURF protein and extends from exon 1 to within exon 3 (15). A larger downstream ORF starts in exon 4 and encodes the 240-amino-acid SmN protein. Using homologous recombination in mouse embryonic stem (ES) cells, the mouse agouti cDNA was placed in the position of the downstream ORF. The mutation left intact a 10 kb genomic region of potential regulatory significance from exon 1 to exon 3 (Fig. 1A). The expected SNURF/agouti transcript would continue to encode the SNURF protein in the upstream ORF and the agouti protein in the downstream ORF. Although there should be a fusion transcript, no fusion protein is expected. Inclusion of the rabbit β-globin polyadenylation signal with the agouti cassette should terminate the SNURF/agouti fusion RNA. Although the ATG initiation codon for the SmN ORF in exon 4 remains downstream of the agouti and Neo cassettes, leaving the downstream ORF potentially intact, the modified and greatly enlarged exon 3 is likely to become the last exon for the fusion transcript. The intent was to produce a SNURF/agouti fusion RNA that would result in expression of the agouti protein under
the control of the Snurf–Snrpn promoter. The targeting vector was electroporated into AB2.2 ES cells, and clones were identified by Southern blot analysis using 3′ and 5′ flanking probes (Fig. 1A and not shown, respectively). The targeted ES cells were injected into C57BL/6J blastocysts. Male chimeras were bred with C57BL/6J females, and germline transmission of the Snurf/agouti fusion was obtained (Fig. 1B). The heterozygous progeny with a 129/SvEv and C57BL/6J mixed background (Fig. 2A, generation I) were further crossed with C57BL/6J mice to transfer the agouti fusion gene onto the homozygous nonagouti (a/a) background (Fig. 2A, generation II). Only mice of a/a genotype were selected for further study.

Switching of the methylation pattern is preserved

There is a differentially methylated 6 kb region at the 5′ end of the mouse Snurf–Snrpn gene extending upstream and downstream from exon 1 and including the CpG island (14,16). The maternal allele of the gene is methylated at the CpG island and silenced, while the paternal allele is unmethylated and expressed. Figure 2 shows an analysis of the methylation status of differentially methylated sites (SacII and BssHII within intron 1 of the gene; see Fig. 1A) in three generations of mice carrying the agouti fusion gene. Genomic DNA from mice I-1, II-2 and III-2 (Fig. 2A) as well as from the 129/SvEv control was digested with HindIII alone or in combination with the methylation-sensitive enzymes SacII or BssHII. As shown in Figure 2B, the fusion allele was methylated when inherited on the maternal chromosome and unmethylated when on the paternal chromosome. Thus, the differential methylation of the CpG island switched from the paternal unmethylated to the maternal methylated state and from the maternal methylated to the paternal unmethylated state, as would be expected for the normal Snurf–Snrpn promoter.

Imprinting of expression is retained for the fusion allele

RT–PCR analysis was used to examine the RNA expression of the wild-type and fusion alleles. Figure 2C and 2D show that only the paternally inherited allele – whether the wild-type or the fusion – was expressed in each mouse. The fusion RNA was expressed in the skin of mice I-1 and III-1 (see pedigree in Fig. 2A) carrying the paternally inherited fusion gene. When the fusion gene was maternally inherited, it was silenced, and the normal RNA encoding SNURF and Smn was expressed from the paternal wild-type allele (Fig. 2D, mouse II-2). Thus, the differential methylation and expression for the fusion gene are preserved and mimic the natural Snurf–Snrpn locus.
The agouti fusion allele yields an imprinted coat color effect

The effect of the SNRPN/agouti fusion gene on coat color is demonstrated in Figure 2E and 2F. When the fusion gene was inherited from a heterozygous male, the offspring carrying the fusion gene demonstrated a distinctly lighter, tan abdominal coloration that was clearly different from the completely black, nonagouti (a/a) littermates without the fusion gene (Fig. 2E). In contrast, when the fusion gene was inherited from a heterozygous female, the offspring with and without the fusion gene were indistinguishable and entirely black (Fig. 2F). Mice homozygous for the fusion allele were fertile and indistinguishable from those heterozygous for the fusion gene on the paternal chromosome.

ENU mutagenesis and identification of variant mice

Ethylnitrosourea (ENU) is proven to be an effective mutagen in male mice (17). We utilized two breeding schemes with ENU mutagenesis of male mice to screen for events affecting imprinting (Fig. 3). In the first scheme, ENU-mutagenized nonagouti C57BL/6J males were bred to females homozygous for the fusion gene. If no mutation occurred, all of the offspring should be black, and all should be heterozygous for the silenced maternal fusion gene. Mice deviating from the expected were considered ‘variant’ on the grounds that genetic and epigenetic events could not be distinguished, and one variant mouse was found in 3186 mice screened. This mouse (V1) showed a tan abdomen indistinguishable from the phenotype when the fusion gene was of paternal origin.

In the second breeding scheme, ENU-mutagenized male mice that were homozygous for the fusion gene were bred to nonagouti (a/a) females. The expectation would be that all of the offspring would be heterozygous for the paternally expressed fusion gene and should have the tan abdominal color. Examination of 1905 mice indicated that all but three animals had a tan abdomen. One variant mouse (V2) was completely yellow — consistent with a high level of agouti expression. The other two mice (V3 and V4) lacked the tan color on the abdomen and were consistent with decreased or absent expression of the fusion gene. The lack of clear heritability has impaired further studies of the V3 and V4 mice, but methylation analysis and sequencing of all exons of the SNURF/agouti transcript did not identify any variations to explain the phenotype, and northern blotting with cultured fibroblasts did not identify any aberration compared with the littermates.

An imprinting defect in the V1 mouse

The difference in coat color compared to littermates was noted for the V1 mouse immediately upon the initial growth of hair. When held by the tail, the V1 mouse demonstrated abnormal limb clasping behavior, which may indicate some degree of neuromuscular abnormality, but no other abnormalities were detected. Beginning at 12 weeks of age, the mouse became obese, reaching a weight of 55 g. Despite numerous attempts to breed this animal with a variety of female mice, no vaginal plugs were found and no pregnancies occurred. The mouse was discovered dead at 23 weeks of age. Southern blot analysis of tail DNA from the V1 mouse was strikingly different from that of the littermates. While the littermates had inherited an unmethylated wild-type copy of the Snurf-Snrpn locus and a methylated and silenced copy of the fusion gene, both alleles were completely unmethylated in the V1 mouse (Fig. 4). This
pattern was consistent with the fact that the color of the mouse was indistinguishable from that of animals with paternal inheritance of the fusion gene. This demonstrated that the V1 mouse did represent an event affecting genomic imprinting, but it was not clear if this represented an epigenetic event (i.e. a change in methylation not accompanied by a change in nucleotide sequence) or a mutation (i.e. a change in nucleotide sequence).

We next tested the methylation status at the necdin (Ndn) locus, which is approximately 1 Mb distant from Snurf–Snrpn and is known to be part of the same imprinted domain. Normally, there is differential methylation at the Ndn locus, with a region of full methylation on the maternal allele and no methylation on the paternal allele. In the case of the V1 mouse, the pattern was abnormal and different from that of the littermates, with decreased, but not absent, methylation of the maternal allele. Thus, the event in the V1 mouse involved a complete defect in genomic imprinting as measured by methylation at the Snrpn CpG island, and a partial, apparently mosaic, defect in genomic imprinting as measured by methylation at the Ndn locus. The pattern of methylation was normal for the H19 and Igf2r loci (not shown).

Figure 4. Methylation analysis of the V1 mouse compared with littermates and parents. DNA from each mouse was digested with a standard restriction enzyme (H. HindIII) alone or in combination with a methylation-sensitive enzyme (Sc, SacII). Southern blotting was performed using probes for Snurf–Snrpn and Ndn. Normally, the maternal allele for each locus is resistant to digestion by the methylation-sensitive enzyme. Designations are as follows: WT, wild-type; Ag-F, agouti fusion; unme, unmethylated. Genotypes for mice are indicated with the maternal allele on the left as follows: +/+ , wild-type; F/F, homozygous fusion; F/+ , maternally inherited fusion gene, F'/+ , mouse V1.

The imprinting defect was essentially equivalent to those seen in some Angelman patients. In about half of the human cases of imprinting defects, small deletions of the upstream portion of the IC (AS-IC) are found, but we found no such deletion in the V1 mouse on searching the region of exon 1 and 40 kb upstream (data not shown). Since the event affecting the V1 mouse altered the maternal chromosome, it is quite likely, but not certain, that it represented a spontaneous (i.e. not ENU-induced) loss of silencing of the maternal allele. Direct sequencing of genomic DNA for exons 1–3 failed to identify any mutations.

An ATG-to-AAG mutation in the V2 mouse

The founder V2 mouse was completely yellow, and all of its offspring inheriting the fusion gene were also yellow. Further breeding on a non-agouti (a/a) background demonstrated that the yellow fusion allele (F'y) resulted in a yellow mouse when paternally inherited and in a black mouse when maternally inherited (Fig. 5A,B). The offspring of male mice homozygous for the F'y allele mated to wild-type females were all yellow, having inherited F'y from the male parent. The offspring of
Figure 5. Imprinted inheritance and characterization of the yellow fusion allele. Genotypes are shown with the maternal allele on the left. (A) Paternal inheritance of the yellow (Fy) allele produces a yellow mouse. (B) Maternal inheritance of the yellow (Fy) allele produces a black mouse. (C) Direct DNA sequencing of reverse orientation from a subclone with the ATG-to-AAG mutation. (D) Northern blotting with a probe for exons 1–3 of Snurf for analysis of brain RNA from two mice each as follows: yellow with genotype +/Fy for paternal origin of the AAG yellow fusion allele, tan abdomen with genotype +/-F for paternal origin of the ATG fusion allele, and black with genotype F/+ for maternal origin of the ATG fusion allele (silenced). Assuming polyadenylation of the fusion transcript at the poly(A) site following the agouti cassette, the transcripts are of the expected sizes; the wild-type RNA is slightly larger (1.6 kb in the right two lanes) than the agouti fusion RNA (1.3 kb in the left four lanes). There was no significant difference between the Fy (left two lanes) and F (middle two lanes) alleles in abundance of the fusion transcript. (E) Western blotting with an antibody to agouti protein for analysis of brain extracts from mice with paternal inheritance of the yellow (Fy) or original (F) fusion alleles. The upper band is not related to the fusion gene and is present in mice lacking the fusion allele. (F) Transfection analysis of bicistronic constructs. The SNURF ORF starting with ATG or AAG was inserted between the SV40 promoter and the luciferase cassette. N and N* are NcoI sites regenerated or destroyed respectively. pGL3 pr is the control plasmid with no upstream ORF before the luciferase cassette.
female mice homozygous for the $F^g$ allele mated to wild-type males were all black, having inherited $F^g$ from the female parent. Since homozygous males and females were fertile, the mutagenesis scheme could be performed using yellow mice homozygous for the $F^g$ allele. As is seen with ectopic or excessive expression of agouti, the yellow mice were obese with a mean weight of about 50 g for mice older than 3 months of age compared with 33 g for controls.

Amplification and direct sequencing of genomic DNA from the V2 mouse demonstrated an ATG-to-AAG mutation (Fig. 5C) in the initiation codon for the upstream ORF that encodes SNURF. This alteration is consistent with the known mutagenic profile for ENU. This mutation suggested the possibility that the expression of agouti in the downstream ORF might be affected at a translational level. Northern blotting using a cDNA probe covering exons 1–3 of Snurf distinguishes the 1.6 kb Surfn transcript from the 1.3 kb fusion transcript, and shows no increase in fusion transcript on comparing mice inheriting the yellow AAG fusion allele on the paternal chromosome with mice carrying the original ATG fusion allele on the paternal chromosome (Fig. 5D). Immunoblotting with an antibody to agouti protein was used to compare yellow mice with the AAG fusion allele on the paternal chromosome with tan mice with the ATG fusion allele on the paternal chromosome. Using extracts from brain, a strong signal of the expected size—the start and stop codons for the agouti ORF are from agouti—was obtained with the AAG fusion allele mice, while no band was visible with the ATG fusion allele mice (Fig. 5E). A faint signal for the ATG fusion allele was seen with higher levels of extract.

In order to document the presumed translational control using transfection studies, the upstream ORF and flanking sequences were introduced upstream of a luciferase cassette. Thus, the 5' portion of the Snurf–Surfn transcript extending from the 5' end to the ATG for the downstream ORF encoding Smn was fused to the luciferase sequence. Identical vectors using an SV40 promoter and varying only by ATG or AAG at the start of the upstream ORF for SNURF were compared (Fig. 5F). The construct with the AAG ORF for SNURF expressed luciferase at approximately 60% of the value for the control plasmid without any insertion of the ORF for SNURF (Fig. 5F). In contrast, the construct with the ATG ORF for SNURF expressed very little luciferase, but the value was detectable above background and was approximately 15-fold less that observed with the AAG fusion construct. All of the data analyzing expression of agouti in mutant mice and measuring the effect on luciferase expression were consistent with the interpretation that the ATG-to-AAG mutation in the initiation codon of the upstream ORF causes an approximately 15-fold increase in translation of the downstream ORF, whether it is agouti or luciferase.

**DISCUSSION**

We have used homologous recombination in ES cells to fuse an agouti coat color reporter cassette to the imprinted Snurf–Surfn promoter in the mouse. After transmission to the germline, mice homozygous for the fusion gene were used to screen for genetic and epigenetic variants affecting expression of the fusion gene. Using this strategy, a variant with loss of DNA methylation and activation of expression for the maternal allele was identified. This variant is analogous to the imprinting defects that cause Angelman syndrome, and most probably represented a spontaneous event affecting the unmethylized maternal allele, although a mutation having a trans-acting effect on the maternal allele cannot be ruled out—particularly since the animal was infertile.

Although the strategy employed in our studies allows rapid screening of mice and represents a start towards the overall goal of detecting mutations affecting imprinting, this approach will fail to detect mutations that are lethal prior to detection of coat color. In addition, detection of recessive mutations would require breeding mice for one additional generation to detect defective maintenance of imprinting and for two additional generations to detect defective resetting of imprints. Although possible, this would require much more extensive effort. As an alternative, we are exploring chemical and retroviral mutagenesis followed by FACS sorting of ES cultured cells expressing enhanced green fluorescent protein (EGFP) under the control of the Snurf–Surfn promoter. While not a complete solution, this approach may facilitate recovery of mutations that would be lethal in vivo, and the retroviral gene-trap mutagenesis may allow recovery of trans-acting mutations.

The yellow mouse was found to have an ATG-to-AAG mutation in the initiation codon of the upstream ORF (uORF) that encodes the 71-amino-acid SNURF protein, and this mutation was designated as the $F^g$ allele. Although we have not directly shown translational control of the Smn ORF in vivo, the results with the SNURF/agouti and SNURF/luciferase fusions suggest strongly that translational control similar to that observed occurs for the native Snurf–Surfn transcript. Bicistronic transcripts with two coding ORFs are relatively rare in mammals, but there are at least 23 reported examples (18). Upstream ORFs may be non-overlapping as in the case of the SNURF and Smn proteins, overlapping and out of frame, or in frame providing N-terminal extensions to the downstream protein, as reviewed elsewhere (18). The uORFs typically reduce translation of the downstream ORF, as demonstrated by the fact that mutations in the ATG initiation codon for the uORF commonly increase translation of the downstream ORF. The increase in translation can be as little as 2-fold as for mouse $\beta_2$-adrenergic receptor (19) or as much as 100-fold in the case of rabbit serum hydroxymethyltransferase (20). The increase in translation for the $F^g$ allele was 15-fold as measured in transfection studies. The evidence for strong translational control of an imprinted gene might seem unexpected, but we believe that the association could be quite compatible with a recently proposed rheostat model of quantitative hypervariability for genomic imprinting (21).

Upstream ORFs most often occur singly in a transcript, but they can be multiple, as in the case of the mouse retinoic acid receptor $\beta_2$, which has five uORFs that encode peptides of 16–21 amino acids (22). The peptides encoded by mammalian and non-mammalian uORFs are usually very short, as for the hexapeptide of human $S$-adenosylmethionine decarboxylase (23). At least one mammalian uORF is closer to the 71-amino-acid size for the SNURF protein, as shown by the 68-amino-acid peptide of rabbit serum hydroxymethyltransferase (20), although this ORF appears not to be conserved in mouse and...
human (24). The coding sequences of uORFs frequently are conserved between species, and in some cases the coding sequence is documented through mutagenesis studies to be critical for controlling downstream translation (19,22,23). The downregulation often involves interaction of the peptide encoded by the uORF with the translation process in cis, although _trans_ effects can be demonstrated with high concentrations of peptide in _vitro_ (19). Based on the findings for other loci, it is quite possible that the sequence of the SNURF protein is conserved between mouse and human to allow interaction of the protein with the translation process, as in the proposed model for the β2-adrenergic receptor (19). Alternatively, and not mutually exclusively, the SNURF protein might have some other function, since it is reported to be localized in the nucleus (5).

**MATERIALS AND METHODS**

**Construction of the targeting vector**

Overlapping lambda phage clones containing the _Snurf–Snrpn_ gene were isolated from a mouse 129/SvEv genomic library (provided by Dr Allan Bradley, Baylor College of Medicine). To construct the agouti fusion gene vector, a 0.58 kb _CdeI–BamHI_ fragment containing the mouse agouti cDNA was removed from plasmid p284, which was a gift from Dr Gregory S. Barsh, Stanford University Medical Center. This fragment and a 0.46 kb _BglII–HindIII_ fragment containing the rabbit β-globin polyadenylation signal were cloned into the p Bluescript vector (Stratagene, Los Angeles, CA). A neomycin expression cassette with the RNA polymerase II promoter (25) was then inserted downstream of the polyadenylation signal. The 3′ homology region of homology, the targeting vector consisted of a 3.4 kb _EcoRI_ genomic DNA fragment as the 5′ homology region and a 2.7 kb _Sall_ (one end from the polylinker of the phage clone) genomic DNA fragment as the 3′ homology region flanking the agouti and neomycin cassettes. The HSV-tk expression cassette with the rat β-actin promoter (26) was subsequently ligated downstream of the 3′ homology region. The targeting vector was linearized at a unique _SalI_ site outside of the 5′ region of homology, leaving the plasmid backbone attached to the HSV-tk cassette.

**Generation of mice with the SNURF/agouti fusion gene**

The linearized targeting vector (15 μg) was electroporated into 1 × 10⁷ AB2.2 ES cells as described previously (27). Geneticin (G418 sulfate, 200 μg/ml; Life Technologies, Gaithersburg, MD) and FIAU (0.2 μm; Bristol Myers, Atlanta, GA) selections were applied 24 h after plating and maintained for 7–8 days. Colonies resistant to G418 and FIAU were picked, trypsinized, and seeded onto a feeder layer of mitotically inactivated STO cells. Targeted ES cells were injected into C57BL/6 blastocysts and reimplanted into pseudopregnant female mice. Chimeric males were bred with C57BL/6 females. Tail DNA was isolated from brown progeny and analyzed by Southern blot for germline transmission. Southern and northern blotting was performed as described previously (29).

**RNA analysis**

Total RNA was isolated using the guanidinium thiocyanate/CSCl gradient method (30). For RT–PCR analysis, DNasel-treated total RNA was reverse-transcribed using primer p3 (5′-CTGTTCCACAATAGCCGTTTC-3′) for the wild-type transcripts, p5 (5′-GCGAGGTCTAGAAGGATTTACTCC-3′) for the SNURF/agouti fusion transcript, primer Neo-3 (5′-TGTGCTCTTGCACGATCATC-3′) for the neomycin transgene, and primer HPRT-3 (5′-CTTTCCAGTTAAGGTGAGATC-3′) for the HPRT gene. All PCR was carried out in 50 μl reactions containing 10 μM Tris pH 8.3, 1.5 μM MgCl₂, 50 mM KCl, 200 μM dNTPs, 1 μM primers and 2.5 μl of RT reaction, with cycling conditions of 94°C for 1 min, 59°C for 1 min and 72°C for 1 min for 30 cycles. PCR products were analyzed on a 1.2% agarose gel. The PCR primer sequences are as follows: for the _Snrpn_ wild-type allele, p1 (forward) 5′-TGGTTCTGAGGATGGATTTGC-3′ and p2 (reverse) 5′-CTTTGAAATCCACACCTTGC-3′; for the SNURF/agouti fusion allele, p1 (forward, listed above) and p4 (reverse) 5′-GTGGACGGAATGGAAGGACCCAAGAG-3′; for the _Snurf_ agouti transgene, Neo-1 (forward) 5′-CTTTTTGTCAAGAGCCGACTTCCG-3′ and Neo-2 (reverse) 5′-CTCGATGGATGTTCGTCGTTG-3′; for the HPRT internal control, HPRT-1 (forward) 5′-ATGCC-TAGATTGTGTTTGTATACC-3′ and HPRT-2 (reverse) 5′-GTAGCTCTTCAGCTGTGAAAATCTAC-3′. The _Snrpn_ cDNA probe for exons 1–3 was prepared as described previously (14,16).

**Methylation analysis**

Genomic DNA was isolated by the proteinase K/SDS digestion and phenol/chloroform extraction method (30). For methylation study of _Snurf–Snrpn_, genomic DNA (10 μg) was digested overnight with restriction enzymes as specified in the figure legends. Digested DNA was transferred from 0.8% of agarose gels to a Hybond N+ membrane (Amersham, Newark, NJ) as described previously (30) for Southern blot analysis. The probe for _Snurf–Snrpn_ was a 1.3 kb _BssHII–EcoRI_ fragment located at the 3′ end of the _Ndn_ coding region. Radiolabelled DNA probe was synthesized using the random hexamer method (31), and was hybridized in a buffer containing 0.25 M NaPO₄ pH 7, 0.25 M NaCl, 1 mM EDTA, 10% PEG-8000, 7% SDS and 1% bovine serum albumin. Probes containing repetitive sequences were preassociated using an excess of mouse DNA to quench repeat hybridization (32). Filters were washed to a final stringency of 0.5 × SSC/0.1% SDS at 65°C. Autoradiography was performed at −80°C with intensifying screen and Kodak XA-R film.

**Immunoblotting**

Proteins from brain extracts were separated on 15% polyacrylamide SDS gels and then transferred to nitrocellulose
membranes and incubated with primary rabbit polyclonal antibody to agouti protein (a gift from G. Barsh) at a 1:1000 dilution. The procedure was essentially as described elsewhere (33); visualization of antibody binding was performed using a donkey anti-rabbit secondary antibody with Enhanced Chemiluminescence (Amersham Corp., Arlington Heights, IL) according to the manufacturer’s instructions.

**Transient transfections**

Transient transfection experiments were performed in NIH3T3 cells, using Lipofectamine-Plus (Invitrogen Life Technologies, Baltimore, MD), according to the manufacturer's instructions. Aliquots of 1 μg of pSV-β-gal (Promega, Milwaukee, WI) plasmid were cotransfected with a luciferase reporter plasmid containing a Snurf ORF/ATG or Snurf ORF/AAG. Luciferase and β-galactosidase activity were assayed 48 h after transfection. Luciferase assays were performed with a luciferase assay system (Promega) using a luminometer. β-Galactosidase values were obtained by using a chemiluminescent reporter gene assay system (Gallolight kit, Tropix, Bedford, MA).

**ENU mutagenesis**

Mice were mutagenized with ENU as described previously (34). C57BL/6J male mice and male mice homozygous for the fusion gene received one injection per week of ENU at a dose of 100 mg/kg body weight for three successive weeks.

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