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Genetics

Identification of a silencing element in the human 15q11-q13 imprinting center by using transgenic *Drosophila*

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ABSTRACT **Prader–Willi syndrome (PWS) and Angelman syndrome are neurogenetic disorders caused by the lack of a paternal or a maternal contribution from human chromosome 15q11-q13, respectively. Deletions in the transcription unit of the imprinted *SNRPN* gene have been found in patients who have PWS or Angelman syndrome because of a parental imprint switch failure in this chromosomal domain. It has been suggested that the *SNRPN* exon 1 region, which is deleted in the PWS patients, contains an imprint switch element from which the maternal and paternal epigenotypes of the 15q11-q13 domain originate. Using the model organism *Drosophila*, we show here that a fragment from this region can function as a silencer in transgenic flies. Repression was detected specifically from this element and could not be observed with control human sequences. Additional experiments allowed the delineation of the silencer to a fragment of 215 bp containing the *SNRPN* promoter region. These results provide an additional link between genomic imprinting and an evolutionary conserved silencing mechanism. We suggest that the identified element participates in the long range regulation of the imprinted 15q11-q13 domain or locally represses *SNRPN* expression from the maternal allele.**

Prader–Willi syndrome (PWS) and Angelman syndrome (AS) involve oppositely imprinted genes on human chromosome 15q11-q13: the paternally expressed PWS gene(s) and the maternally expressed AS gene (1). A candidate gene for AS has been identified recently (2, 3) whereas the gene(s) for PWS remains elusive. One imprinted gene from the PWS critical region is the gene encoding the small nuclear ribonucleoprotein polypeptide N (*SNRPN*), which is expressed exclusively from the paternal allele (4–7).

The *SNRPN* transcription unit has been found to contain small deletions in several PWS and AS families where the syndrome originates from an imprinting defect (8–12). In AS patients of this group, the smallest region of deletion overlap (Fig. 1*A*, ASSRO) is located several hundred kilobase pairs centromeric to the AS gene. The ASSRO is very close to exon BD3 of the paternally transcribed, noncoding BD transcripts of *SNRPN* (Fig. 1*A*; ref. 11). On the other hand, all deletions in PWS-imprinting mutation patients involve a region around exon 1 of *SNRPN* (Fig. 1*A*, PWSSRO; ref. 11). It has been suggested that the BD transcripts are required for a switch from the paternal to the maternal epigenotype that would fail in AS patients whereas the region around *SNRPN* exon 1 might contain a switch initiation site from which both the maternal and paternal epigenotypes originate (11). Alternatively, it has been proposed that the PWSSRO region might be required for erasing maternal and paternal epigenotypes in both germ lines (13).

The fruit fly *Drosophila melanogaster* has proven to be a valuable genetic model for studying epigenetic mechanisms of gene regulation. Epigenetic phenomena, like position effect variegation and homeotic gene silencing, have been subjected to a genetic dissection. Many of the genes involved were found to encode chromatin-regulating factors (14). Because no covalent modification of DNA has been observed in *Drosophila*, these results imply that silencing can be sustained by other epigenetic mechanisms. Indeed, it has been shown recently that an imprinting element from the mouse *H19* flanking region functions as a silencer in *Drosophila* (15), suggesting that the fly is a suitable model to investigate certain mechanistic aspects of genomic imprinting.

We show here that a fragment from the region around the *SNRPN* exon 1 can function as a parent-of-origin-independent silencer in transgenic flies. Repression was specific for this element and could not be observed with nonimprinted human sequences. Additional transgenes resulted in the delineation of the silencer to a 215-bp sequence containing the *SNRPN* promoter region (16). We propose that the observed silencing is based on an evolutionary conserved mechanism that also is used for allele-specific repression of mammalian imprinted genes.

MATERIALS AND METHODS

Recombinant Plasmids. The P element vectors were generated by subcloning fragments of interest in the *Not*I/*Spe*I linker of pUZ (15). pUZC contains a 2.8-kb *Xba*I fragment from the human *EXT1* locus (chromosome 8; ref. 17) including exon 7 plus flanking sequences. The PWSPX fragment (bp −2651 to +538 relative to bp 1 of *SNRPN* exon 1) was PCR-amplified by using *Spe*I cloning primers and was subcloned in pUZ to yield pUZP1 and pUZP2, respectively. pUZP3 contains the PWSDS fragment, a 4.2-kb *Xba*I fragment (bp −203 to +4094), from the human *SNRPN* locus. pUZP1Δ contains a 2.4-kb *Spe*I–*Xba*I subfragment (bp −2651 to −203) of the PWSPX fragment, and pUZP2Δ contains a 0.7-kb *Spe*I–*Xba*I subfragment (bp −203 to +538) of the PWSPX fragment. Base pairs −203 to +12 of a pUZP2Δ clone carrying the human fragment in 5′–3′ orientation were removed by *Not*I digestion to generate pUZPPΔ. The constructs carrying sequences from the human *APP* locus (18) and the *Drosophila Pc* locus (19) each contain a 3-kb cDNA fragment subcloned in the vector pUAST (20).

Transgenic Flies. Fly stocks were maintained at 25°C on standard medium. Transgenic *D. melanogaster* were generated (21) using *white*^{1118} as host and pUChsΔ2–3 as helper plasmid. Transformed flies were identified by rescue of the *white* eye phenotype in the F1 generation after backcrossing to the host

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Abbreviations: PWS, Prader–Willi syndrome; AS, Angelman syndrome; SNRPN, small nuclear ribonucleoprotein polypeptide N; β-gal, β-galactosidase.
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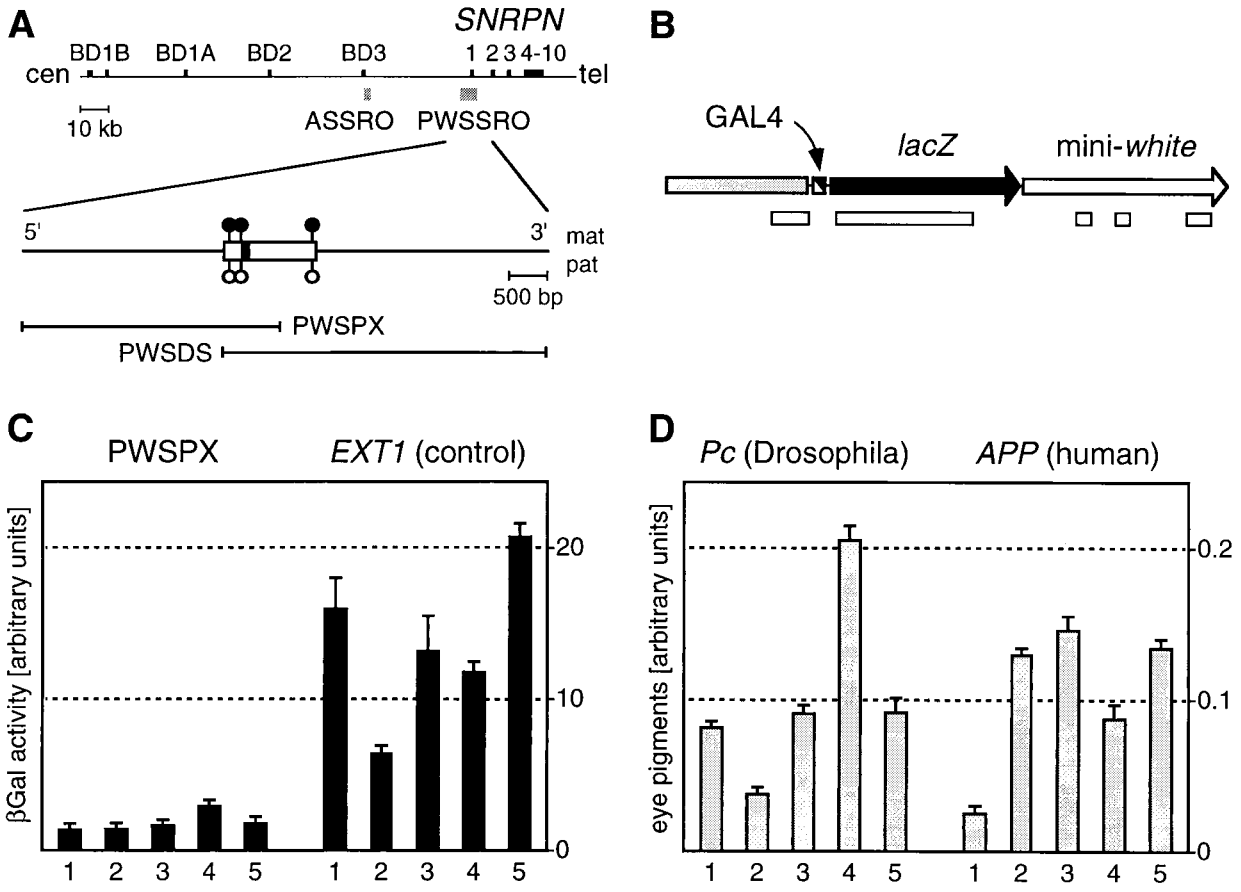

FIG. 1. (*A*) Outline of the *SNRPN* transcription unit indicating regions deleted in AS and PWS patients (ASSRO and PWSSRO, shaded boxes; refs. 10–12). The PWSSRO has been narrowed down recently to ≈3 kb (T. Ohta and R. D. Nicholls, personal communication). Exons are shown as black boxes. Filled and open lollipops in the region of the *SNRPN* CpG island (open box) represent maternally methylated *Not*I sites (32). Fragments investigated in this study (PWSPX and PWSDS) are indicated. (*B*) Schematic outline of the PWSPX transgene. A GAL4 binding cassette (black and white box) immediately 5′ to the promoter mediates binding of the activating protein. The direction of transcription is indicated by arrowheads. Open boxes below the construct denote CpG islands as determined by standard procedures (33) by using GRAIL (34). (*C*) Quantitative determination of *lacZ* expression in GAL4-induced transgenic larvae carrying the PWSPX fragment or a control fragment from the nonimprinted *EXT1* locus. (*D*) Quantitative determination of mini-*white* expression in adult transgenic flies carrying a sequence from the *Drosophila Pc* locus or a sequence from the human *APP* locus immediately 5′ to mini-*white*. Error bars indicate the SD; numbers indicate distinct independent strains.

strain. Only independent founders were used for the generation of stocks. All transgenic strains are homozygous viable and carry the transposon on an autosome. When necessary, transgenes were mobilized by using *w*; ry^{506} Sb^{1} P[ry^{+} Δ2–3]99B/TM6B as a stable source of transposase (22). Integrity, single copy status, and independence of transgenes were confirmed by genomic Southern blots by using standard procedures (23).

***lacZ* and mini-*white* Expression.** Quantitative determination of β-galactosidase (β-gal) activity and red eye pigments was essentially as described (15). All measurements were repeated independently two times. Values from all constructs were compared by using the *t* test. The difference between lines classified as "silenced" and as "not silenced" was always significant ($P < 0.01$) as determined by the *t* test.

Reverse Transcription–PCR Assays. Transgenic strains were crossed with the 1032.hx *GAL4*-enhancer trap line (24) or $white^{1118}$. Total RNA was isolated from wandering third instar larvae by using the S.N.A.P. Total RNA Isolation Kit (Invitrogen) including DNase digestion. cDNA synthesis was performed from 1 μg of total RNA by using the cDNA Cycle Kit (Invitrogen). Control experiments were run in parallel, omitting reverse transcriptase. Ten percent of the cDNA products was amplified by 33 cycles of PCR by using primers specific for *SNRPN* exon 1/intron 1 (GCGGTCAGTGACGCGATGGAGCG and CCGGATCTGGTTCTCCAGAACAAAGGAC) and *lacZ* (GAGCCTGCTAAAGCAAAAAAGAAGTCACC and CGTAACCGTGCATCTGCCAGTTTGAGG). PCR was performed separately for *SNRPN* and *lacZ*. Assays then were pooled and samples were separated on a 1.8% agarose gel stained with ethidium bromide.

RESULTS

Generation of Transgenic Flies Carrying Fragments from the 15q11-q13 Imprinting Center. To identify putative silencing elements from the *SNRPN* exon 1 region (Fig. 1*A*), we made use of a GAL4 competition system established previously (ref. 24; Fig. 1*B*). Fragments of interest were subcloned in a vector containing the *lacZ* gene under the control of an *hsp70* minimal promoter. Activation of this promoter can be conferred by GAL4 protein that is supplied in trans from a strain expressing the *GAL4* gene under developmental control. The mini-*white* transformation marker, which is required for the deposition of the fly's red eye pigment, lies downstream of the *lacZ* gene (Fig. 1*B*). Using P element-mediated transformation, we generated several independent transgenic fly lines from the construct containing the PWSPX fragment (Fig. 1*A*) and a control construct containing a random fragment of nonimprinted human DNA.

Repression of *lacZ* and mini-*white* Expression. To determine the effect of these sequences on *lacZ* expression, we crossed

transgenic strains with flies expressing *GAL4* under developmental control. Third instar larvae from these crosses were homogenized, and β-gal activity was quantitatively determined by using chlorophenolred-β-D-galactopyranoside. This revealed a profound silencing effect in all of the strains transgenic for the PWSPX fragment compared with all of the strains transgenic for the control fragment (Fig. 1*C*). Silencing was found independently of the parental origin of the transgene (data not shown), and the PWSPX fragment was sufficient to mediate an ≈8-fold reduction in β-gal activity ($P < 0.001$).

From the appearance of the flies transgenic for the PWSPX fragment, it was evident that also the more distantly located mini-*white* transformation marker (Fig. 1*B*) was expressed at lower levels. Quantitative determination of red eye pigments revealed that pigment levels are reduced significantly in flies carrying the PWSPX fragment compared with control flies ($P = 0.01$; data not shown). Thus, the fragment appears also to be capable of long distance repression of the mini-*white* promoter. However, the effect was not as pronounced as for the *lacZ* gene (data not shown).

Additional controls were performed to demonstrate that the introduction of foreign DNA into *Drosophila* has no effect on the expression of neighboring reporter genes. We compared *white* expression in two groups of five strains each carrying either a transgene with DNA from the human *APP* locus (ref. 18; chromosome 21q21-q22) or a transgene with DNA from the *Drosophila Pc* locus (19). In these constructs, the mini-*white* gene is placed immediately 3′ to the *APP* and *Pc* sequences, respectively, as is the *lacZ* gene in the constructs described above. Eye pigment quantification of adult age-matched flies yielded an average value of 0.105 for the human-related transgene vs. 0.102 for the *Drosophila*-related transgene (Fig. 1*D*). Thus, mini-*white* expression is not affected by the presence of the human sequences. The difference between the two transgenes is not significant as determined by the *t* test ($P = 0.87$).

Characterization and Delineation of the Silencing Element. To initiate the characterization of the silencing element, we analyzed *lacZ* expression in GAL4-induced fly lines transgenic for P[UZP2] (Fig. 2*A*). This construct contains the PWSPX fragment in 3′–5′ orientation. Chlorophenolred-β-D-galactopyranoside assays performed under the same conditions as previously used revealed a reduced silencing activity compared with P[UZP1] (Fig. 2*B*). This result could be caused by a directionality effect in the sense that silencing is stronger in the 3′ direction of the PWSPX fragment. Alternatively, reduced silencing could be attributed to a distance effect indicating that the silencing element is close to the repressed *hsp70–lacZ* promoter in P[UZP1] but farther away in P[UZP2]. If the silencer were located to the immediate vicinity of *SNRPN* exon 1, it also should be present in the PWSDS fragment that shares with the PWSPX fragment 740 bp around *SNRPN* exon 1 (Fig. 1*A*). Therefore, we analyzed *lacZ* expression in GAL4-induced strains transgenic for P[UZP3], a construct containing the PWSDS fragment in 3′–5′ orientation (Fig. 2*A*). These strains showed a degree of silencing that was indistinguishable from P[UZP1], suggesting that silencing is bidirectional (Fig. 2*B*). Reduced silencing of the P[UZP2] *lacZ* gene could then be explained by a distance effect that would be consistent with the moderate repression observed for the more distant mini-*white* gene in P[UZP1].

To further characterize and delineate the *SNRPN* silencing element, we generated strains transgenic for deleted derivatives of the original constructs. In P[UZP1Δ], the 740-bp region of P[UZP1] overlapping with P[UZP3] is deleted whereas in P[UZP2Δ] only the 740-bp region of P[UZP2] shared with P[UZP3] is retained (Fig. 3*A*). Chlorophenolred-β-D-galactopyranoside assays showed that β-gal activity in larvae transgenic for P[UZP1Δ] was drastically increased

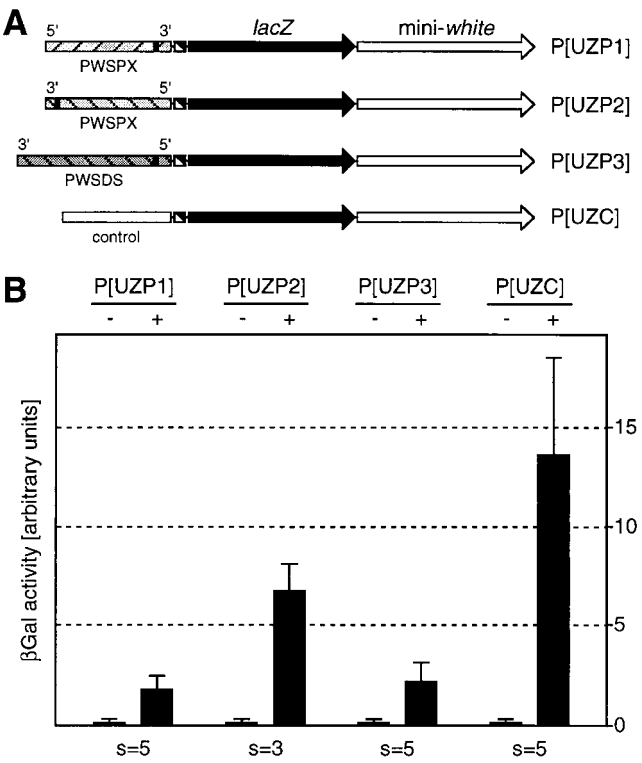

FIG. 2. Quantitative determination of *lacZ* expression in transgenic larvae carrying fragments from the PWS-imprinting control region. (*A*) Structure of transgenes. (*B*) Relative β-gal activity in the presence (+) or absence (−) of *GAL4*. The depicted values are mean values for all strains analyzed. s, number of strains; error bars indicate the SD.

compared with P[UZP1] (Fig. 3*B*). Furthermore, β-gal activity was moderately decreased from P[UZP2Δ] compared with P[UZP2] (Fig. 3*B*). Expression levels from P[UZP1Δ] were similar to P[UZC] controls confirming that the 740-bp fragment was required for silencing. Expression levels from P[UZP2Δ] are similar to P[UZP1] although repression is not as strong as from the latter transgene. These experiments thus confirmed the previous assumptions that silencing is bidirectional and shows a distance effect. In addition, the deletions uncovered a 740-bp fragment contained largely in the *SNRPN* CpG island (Fig. 1*A*) that is necessary and also is partially sufficient for silencing.

The only functionally characterized element within the 740-bp fragment is the *SNRPN* promoter region (16). To determine the effect of this region on silencing, we deleted the region from −203 bp to +12 bp relative to *SNRPN* exon 1 (Fig. 3*A*, P[UZPPΔ]). The deletion resulted in a clear loss of silencing with *lacZ* expression levels being similar to the P[UZC] control construct and the P[UZP1Δ] deletion construct (Fig. 3*B*). Because the remaining DNA of P[UZPPΔ] is entirely derived from the *SNRPN* CpG island (9, 16), this result demonstrates also that the presence of CpG-rich DNA *per se* is not sufficient to induce silencing in the fly.

***SNRPN* and *lacZ* Promoter Activity in Transgenic Flies.** Because the *SNRPN* promoter is contained in all repressive fragments, silencing could have resulted from a competition between the *SNRPN* and the *lacZ* promoters for GAL4-mediated activation. If this were the case, *SNRPN* transcription should be induced strongly upon *GAL4* introduction. To determine the state of activity of the *SNRPN* and the *lacZ* promoters, we performed reverse transcription-PCR (Fig. 4*A*). We isolated total RNA from the same larval stage that was used for the determination of β-gal activity. A control PCR from pUZP1 plasmid yielded a strong signal for *lacZ* and a very

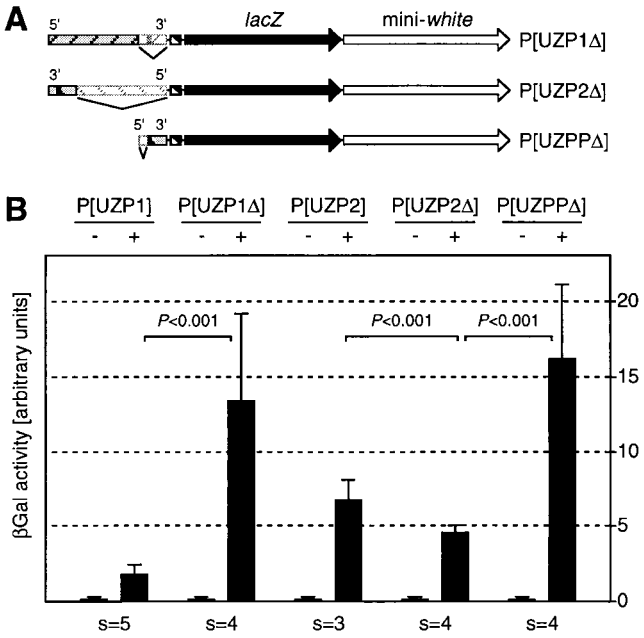

FIG. 3. The effect of deletions on PWSPX-dependent silencing. (*A*) Structure of deleted transgenes. P[UZP1Δ] is derived from P[UZP1] and contains a 740-bp deletion of the region around *SNRPN* exon 1. P[UZP2Δ] is derived from P[UZP2] and contains a 2.4-kb deletion leaving the 740-bp region around *SNRPN* exon 1. P[UZPPΔ] is derived from P[UZP2Δ] and contains a 215-bp deletion of the *SNRPN* promoter region. (*B*) Quantitative determination of *lacZ* expression in transgenic larvae carrying deleted constructs. Relative β-gal activity is shown in the presence (+) or absence (−) of *GAL4*. The depicted values are mean values for all strains analyzed (s, number of strains). Error bars indicate the SD, and *P* values were determined by the *t* test.

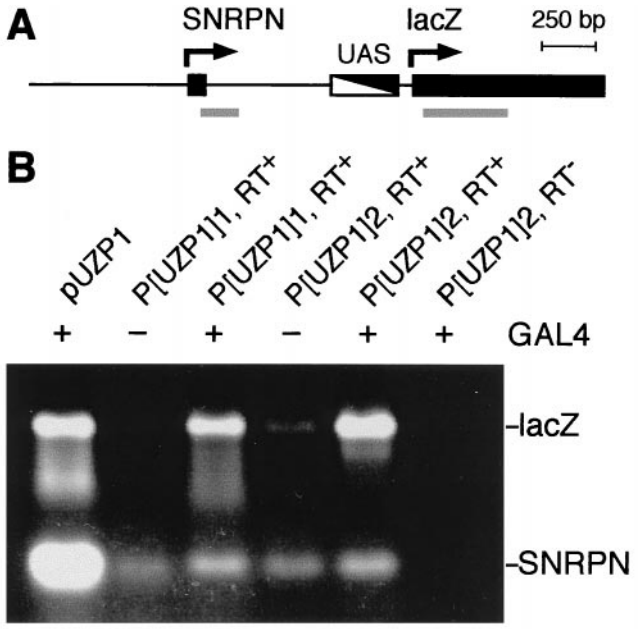

FIG. 4. Determination of the transcriptional status of the *SNRPN* and the *lacZ* promoters in transgenic larvae carrying the PWSPX fragment. (*A*) Schematic illustration of the central part of the P[UZP1] transgene. *SNRPN* exon 1 and the 5′ portion of the *lacZ* gene are depicted as black boxes, and the GAL4 binding sites (UAS) are depicted as a black and white box. Arrows indicate the direction of transcription. Gray lines designate the sequences amplified by PCR. (*B*) Result of reverse transcription-PCR from two different strains transgenic for P[UZP1] in the presence (+) or absence (−) of *GAL4*. PCR products were separated on an agarose gel stained with ethidium bromide. Lane 1 shows control PCR products from a plasmid template, and lane 6 shows the absence of reverse transcription-PCR products when reverse transcriptase was omitted.

strong signal for *SNRPN* (Fig. 4*B*, lane 1). In the absence of *GAL4*, no signal, or a very faint signal, was detectable for lacZ mRNA. In contrast, a strong signal was observed in the presence of *GAL4* indicating a substantial GAL4-mediated induction of *lacZ* expression (Fig. 4*B*). The signal for SNRPN mRNA was only fractional compared with the signal amplified from the plasmid template. This signal always could be detected and depended only a little on GAL4 induction (Fig. 4*B*). Thus, we concluded that promoter competition can hardly account for the observed silencing effect. We also noticed that low levels of SNRPN mRNA were present even in uninduced and silenced larvae. This result may point to the requirement of DNA methylation for vertebrate genomes to achieve a more stringent repression of transcription. Because the gene number of the unmethylated (25, 26) *Drosophila* genome is relatively small compared with vertebrates, a certain level of "transcriptional noise" may be tolerated and may explain the dispensability of DNA methylation in the fly (27).

DISCUSSION

The recent identification of a comparatively small element encompassing a presumptive imprinting center on human chromosome 15q11-q13 (11) presented the opportunity to use the advantages of *Drosophila* transgenesis to dissect further and characterize this element. Using an established GAL4 competition system (24), we demonstrated here that an isolated sequence from the human *SNRPN* locus was able to confer strong repression on a minimal *hsp70* promoter. The degree of silencing was comparable with the repression induced by strong endogenous *Drosophila* silencers (24) and also with the silencing element previously identified in the mouse *H19* upstream region (15).

The results obtained with the deleted constructs show that the region from −203 bp to +12 bp relative to *SNRPN* exon 1 is essential for silencing. Yet, the isolated region from −203 bp to +538 bp (Fig. 3, P[UZP2Δ]) revealed only partial silencing. This result might indicate that additional sequences, e.g., farther upstream and downstream to the *SNRPN* promoter, are required to achieve the full extent of repression as observed with the large fragments (PWSPX and PWSDS).

Several lines of evidence demonstrate the specificity of the observed silencing effect. (*i*) Silencing was observed only with fragments from the PWSSRO region and not with a randomly chosen control fragment from a nonimprinted region (chromosome 8q24). In addition, various transgenic strains carrying fragments from the human *APP* locus (chromosome 21q21-q22) did not reveal any silencing effects. (*ii*) Small deletions that leave a considerable amount of DNA from the 15q11-q13 region on the transgenes resulted in complete loss of silencing. Because the expression levels of these deletion constructs (P[UZP1Δ] and P[UZPPΔ]) were highly similar to the control construct, this result establishes also the "neutrality" of the control DNA. (*iii*) A role of CpG islands *per se* in the observed silencing is highly unlikely because the strongly expressed P[UZPPΔ] construct retains >500 bp of the *SNRPN* CpG island. In addition, the basic construct used for the generation of all P elements contains several additional CpG islands (Fig. 1*B*) without any incidence of silencing (24). And, (*iv*) silencing cannot be explained by promoter competition because the *SNRPN* promoter appears to be expressed at low levels from our transgenes and not to be induced by GAL4. Therefore, we concluded that specific signals in the *SNRPN* promoter region mediated the observed silencing effects.

Previous experiments with *Drosophila* transgenes derived from the mouse *H19* locus uncovered a 1.2-kb silencing element in the upstream region of this imprinted gene (15). This fragment is highly overlapping with the sequence required

for imprinting of *H19* transgenes (28). Because deletion of the element on mouse transgenes resulted in parent-of-origin-independent *H19* expression, involvement of silencing in the allele-specific inactivation of the mouse *H19* gene was suggested (15). The results presented in this study support the notion that *Drosophila* is a system in which mammalian imprinting signals can elicit a molecular response. Silencing elements identified in *Drosophila* and imprinting signals identified in mammals would thus be highly overlapping sequences. In addition, the similarities between silencing and imprinting imply that genomic imprinting makes use of mechanisms that are evolutionarily conserved between fly and mouse. The availability of elaborated genetic tools in *Drosophila* should allow us to identify trans-acting factors involved in the process.

It has been proposed previously that the region around *SNRPN* exon 1 contains an imprint switch element required for the establishment of both the paternal and the maternal epigenotypes on human chromosome 15q11-q13 (11). The silencing we observed in *Drosophila* would reflect one direction of the switch, i.e., the generation of a repressed chromosomal environment on 15q11-q13 (maternal imprint). Erasure of this imprint in primordial germ cells must then require an activating factor that interacts with the silencer and displaces the silencing proteins. A maternally derived deletion of this target sequence, as observed in fathers of PWS imprinting mutation patients, would therefore result in a failure to erase the maternal imprint in the male germ line and, consequently, in the transmission of a paternal chromosome with a (grand)-maternal imprint. The failure to observe, in mothers of AS patients, a block of the paternal to maternal imprint switch by a paternally derived deletion of the silencer can be explained by the fact that *SNRPN* is likely to play a role in PWS (29) and therefore cannot be deleted in normal individuals. Deletions in AS imprinting mutation families never affect *SNRPN* exon 1 but affect exon BD3 or an element close by (Fig. 1*A*, ASSRO). This region appears to encode a factor that may interact with the silencer to establish the maternal imprint (11). In this respect, it is also interesting to notice that the ASSRO did not reveal a silencing activity comparable with the PWSSRO in transgenic flies (unpublished results).

A striking feature of imprinting mutation patients is the finding that the deletion of the imprinting control element affects methylation and expression of genes over a domain of ≈2 Mb (8, 9, 12). The silencing effect we describe here, however, functions probably not over distances greater than a few kilobases. This effect could be explained by a limited degree of evolutionary conservation. On the other hand, silencing could be reinforced by additional elements dispersed over the 2-Mb imprinted domain that would not be present in our transgenes. A precedent for this kind of silencing can be found in the 350-kb *Drosophila* bithorax complex where a few chromosomal elements nucleate silencing complexes of the Polycomb group. The stability of silencing appears to require an interaction of the silencing proteins with additional secondary sites along the entire complex (30, 31). It must be noted, however, that instead of being involved in the long range repression of the 15q11-q13 imprinted domain, the silencer could act also as a local repressor of *SNRPN* transcription from the maternal allele.

Our results further substantiate the notion that an evolutionary conserved silencing mechanism is involved in the local or long range monoallelic repression of imprinted genes (15). In addition, the precise delineation of the silencing element to a region of a few hundred base pairs should serve as a starting point for the molecular characterization of the imprint switch element (11) deleted in PWS patients.

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