Establishing the epigenetic status of the Prader–Willi/Angelman imprinting center in the gametes and embryo

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The Prader–Willi/Angelman imprinted domain on human chromosome 15q11–q13 is regulated by an imprinting control center (IC) composed of a sequence around the SNRPN promoter (PWS-SRO) and a sequence located 35 kb upstream (AS-SRO). We have previously hypothesized that the primary imprint is established on AS-SRO, which then confers imprinting on PWS-SRO. Here we examine this hypothesis using a transgene that includes both AS-SRO and PWS-SRO sequences and carries out the entire imprinting process. The epigenetic features of this transgene resemble those previously observed on the endogenous locus, thus allowing analyses in the gametes and early embryo. We demonstrate that the primary imprint is in fact established in the gametes, creating a differentially methylated CpG cluster (DMR) on AS-SRO, presumably by an adjacent de novo signal (DNS). The DMR and DNS bind specific proteins: an allele-discrimination protein (ADP) and a de novo methylation protein, respectively. ADP, being a maternal protein, is involved in both the establishment of DMR in the gametes and in its maintenance through implantation when methylation of PWS-SRO on the maternal allele takes place. Importantly, while the AS-SRO is required in the gametes to confer methylation on PWS-SRO, it is dispensable later in development.

INTRODUCTION

The coordinated regulation of clustered imprinted genes is mediated by imprinting centers (ICs), which are responsible for the establishment of the imprint in the gametes, for its maintenance through embryo development and in the ultimate execution of differential expression programs for the two parental alleles (1).

One of the best-studied examples of such an IC is the one that coordinates expression of the imprinted genes located across a 2 Mb region of human chromosome 15 or its mouse ortholog on chromosome 7 (Fig. 1A). Failure to establish a proper imprint of this region in humans results in the neurobehavioral disorders, Prader–Willi syndrome (PWS) and Angelman syndrome (AS) (reviewed in 2,3).

Although the molecular mechanism by which this imprinting control works is still obscure, imprinting mutations caused by microdeletions focused attention to two sequences that constitute this center: a 4.3 kb sequence (PWS-SRO) located 35 kb upstream to the SNRPN transcription start site (5). Loss of AS-SRO affects the maternal imprint and causes inappropriate expression of the normally silenced paternal genes on the maternal allele. These genes include the UBE3A antisense transcript, which in the case of an imprinting defect is believed to silence the UBE3A gene on the maternal allele, resulting in Angelman syndrome (6). On the other hand, deletion of the PWS-SRO sequence results in inappropriate silencing of the genes that are normally expressed on the paternal allele, resulting in Prader–Willi syndrome.

In a recent study using lymphoblasts of PWS and AS patients, reciprocal chromatin structures at the PWS-SRO and AS-SRO have been observed (7). While the AS-SRO on the maternal allele is sensitive to DNase I, and wrapped on nucleosomes that are acetylated on histones H3 and H4, this epigenetic pattern is characteristic of the paternal allele at PWS-SRO. Genetic analysis shows that the maternal AS-SRO confers DNA methylation and closed chromatin structure upon the neighboring PWS-SRO on the maternal allele.
Figure 1. (A) Schematic map of the imprinted 2 Mb PWS–AS domain on chromosome 15q11–q13 and expansion of the IC region composed of the 1 kb AS-SRO sequence and 4.3 kb PWS-SRO with a 35 kb intervening sequence. (B) 1. Map of the AS–PWS transgene composed of 1 kb AS-SRO (nucleotides 5897–7138, accession no. AF148319), 4.6 kb PWS-SRO and 6 kb of the intervening sequence (nucleotides 25 066–14 745, accession no. U41384) and positions of the designated restriction sites. Horizontal arrow, SNRPN transcription start site. 2. Map of a PWS transgene composed of a 4.6 kb PWS-SRO and 6 kb intervening sequence with no AS-SRO sequence. 3. Map of a lox-AS–lox-PWS transgene, as in 1 with the AS-SRO sequence flanked by lox sequences. Probe used in Southern analysis as described in Figures 2 and 4. Lines below represent the restriction fragments that appear in the Southern gels. (C) List of the transgenic lines used in the present study and details. The non-informative transgenic line displays partial methylation of PWS-SRO on both alleles.
Nevertheless, PWS-SRO shows no influence on the epigenetic features of the AS-SRO. The results of this study suggested a unidirectional stepwise program in which the imprinted AS-SRO confers imprinting on the PWS-SRO. This hypothesis could not be tested in humans for ethical reasons and required an animal model. Such a model could reveal the molecular mechanism that governs the imprinting process in this 2 Mb imprinted domain and help to unequivocally define cis elements and trans factors that will shed light on the role of AS-SRO and PWS-SRO sequences in the process.

Here we show that a transgene composed of the sequences of AS-SRO, SNRPN promoter/exon 1 and 10 kb upstream to the SNRPN transcription start site (Fig. 1B) is capable of carrying out successfully all steps of the imprinting process. This implies that the human sequences are recognized and acted upon by the mouse imprinting machinery. The epigenetic features of the IC on this transgene resemble those previously observed on the human endogenous locus (7), therefore allowing for the first time a detailed study of the epigenetic features of the IC in the gametes and early embryo.

RESULTS

The AS–PWS transgene composed of AS-SRO, the SNRPN promoter/exon 1 and 10 kb upstream to the SNRPN transcription start site (Fig. 1B) carried out successfully all steps of the imprinting process. It is not clear why in a previous experiment a P1 human transgene that was claimed to contain the complete IC was not imprinted (8). It should be noted, however, that we used a shorter transgene that misses a long stretch of sequences between AS-SRO and PWS-SRO, presumably allowing more efficient interactions between the two parts of the IC. In any event, we show that PWS-SRO in adult F1 and F2 progeny of male and female founders of five transgenic lines (Fig. 1C) were methylated upon maternal transmission of the transgene and unmethylated upon paternal transmission (Fig. 2A), implying that the human sequence is acted upon by the mouse imprinting machinery mimicking the human in vivo endogenous situation. Therefore, our transgenic lines provided a reliable and convenient experimental tool to determine the stage in embryo development when PWS-SRO methylation on the maternal allele takes place.

The NotI sites (positions +10 and −80) and the HpaII sites (position +80 and +220) (Fig. 1B) were unmethylated in both sperm and oocytes and stayed unmethylated in blasto-cysts upon both maternal and paternal transmission. Maternal methylation was first seen at the post-implantation embryonic stage (E 7.5), while the paternal allele remained unmethylated (Fig. 2).

Contradictory results on the methylation status of the SNRPN promoter in human oocytes were reported in the literature. El-Maarri et al. (9) found the SNRPN promoter in oocytes to be unmethylated, whereas Geuns et al. (10) reported that human oocytes are methylated. Our results obtained by PCR methylation analysis (Fig. 2B) and the bisulfite method (Fig. 2C) were in accordance with those obtained by El-Maarri et al. The data on the methylation of the SNRPN promoter in the adult, which are presented in Figure 2A, are consistent with previously published results in humans (7), indicating that the transgenic sequence recapitulates the endogenous situation. The methylation of Snrpn in the pre-implantation embryo is unknown in humans and can be implied from the transgene shown here (Fig. 2B).

DNase I sensitivity at PWS-SRO and AS-SRO was consistent, although not as striking, with the human endogenous situation (7). At the PWS-SRO paternal allele was more DNase I sensitive than the maternal allele, whereas the maternal allele was more sensitive at the AS-SRO (Fig. 3A). Acetylation of histone H3 was enriched on the paternal allele of PWS-SRO and at the maternal allele of AS-SRO (Fig. 3B). Methylation of H3(K4), as expected, was also enriched on the paternal allele in PWS-SRO and on the maternal allele in AS-SRO (Fig. 3C). Taken together, the epigenetic features of AS-SRO and PWS-SRO in the transgene appear to follow the trend seen previously for the endogenous situation (7). We believe that the reason why the observations described here are not as striking as those described earlier in the endogenous situation (7) is a result of the multicopy nature of the transgene.

We have previously suggested that AS-SRO is a key element in conferring methylation upon PWS-SRO. This is now confirmed using the AS–PWS transgene in which AS-SRO was deleted. PWS-SRO in both F1 and F2 progeny was unmethylated upon maternal and paternal transmission (Fig. 4A). We then asked whether AS-SRO is essential only during gametogenesis or whether it plays also a critical role during embryogenesis. To answer this question we used animals in which AS-SRO in the transgene was flanked by lox sequences. These transgenes were mated with a cre-expressing mouse and the progeny tested for the requirement of the AS-SRO sequence during embryo development. To ensure that the lox-containing construct functions like the parental transgene, methylation of the PWS-SRO sequence was analyzed in the progeny of this transgene crossed with a wild-type mouse. As expected, progenies showed differential methylation of PWS-SRO on the maternal allele (Fig. 4B).

In the next experiment (Fig. 4C) a male carrying the AS–PWS transgene where AS-SRO is flanked by lox sequences was mated with a female that harbors the cre gene. An F1 female progeny of this mating lost the AS-SRO sequence (data not shown), and methylation of PWS-SRO, as expected, did not take place. This F1 female, when mated with wild-type male, yielded progenies with an unmethylated PWS-SRO (Fig. 4C). This is in contrast to the normal maternal transmission described in Figure 4B. Note that the transmission of the AS-SRO deletion described in Figure 4C is reminiscent of the situation in AS families. When a female harboring a construct containing AS-SRO flanked by lox sequences (Fig. 4D) mated with a male harboring cre, AS-SRO was removed by the blastula stage (data not shown); nevertheless, PWS-SRO became methylated (Fig. 4D). These results indicate that while AS-SRO is essential for PWS-SRO methylation during gametogenesis and early embryogenesis (Fig. 4C) it is dispensable later in development toward the time when PWS-SRO becomes methylated (Fig. 2B). PWS-SRO is probably marked by AS-SRO at an early stage to be methylated after implantation.

Having shown that AS-SRO is essential for PWS-SRO methylation, we studied the other epigenetic features of
PWS-SRO in a mouse carrying a transgene in which AS-SRO was deleted. Although the differential DNase I sensitivity was lost in the AS-SRO deleted transgene (Fig. 5A compared with Fig. 3A), the histone H3 acetylation pattern did not change (Fig. 5B). These observations are consistent with the endogenous situation observed in AS patients (7). We therefore took advantage of this transgenic system to study the methylation status of AS-SRO in the gametes and during development.

One CpG cluster (I) (positions 65–183 on AS-SRO) was unmethylated in the gametes, throughout embryonic development and in the adult. Another cluster of CpG sites (II) (positions 322–339) was methylated in sperm and unmethylated in oocytes (Fig. 6). The human sperm endogenous AS-SRO was also methylated at cluster II (data not shown). This differential methylation was maintained through implantation (E 7.5) and lost in the adult (Fig. 6). It should be mentioned that the entire AS-SRO sequence was reported before and confirmed by us to be methylated in lymphoblasts (11) (and our data not shown). However, the methylation status of AS-SRO in human oocytes and the early embryo was not reported previously. Results of band shift experiments, as described subsequently, suggest that cluster II and not cluster I plays a role in the imprinting process.
The band shift experiments revealed that a protein present in parthenogenetic ES cells (Fig. 7A, lane 1), to a lesser extent in normal ES cells (lane 2) and absent from androgenetic ES cells (lane 3), binds to the CpG cluster II (oligo I-ADS). The methylated cluster II (oligo Ila-ADS) failed to bind the protein (lane 4). Also, oligo IIb-ADS in which one CpG site, designated by star, was mutated (lane 5), and oligo III representing cluster I (data not shown) did not bind the protein. These combined band shift experiments suggest that the differentially methylated CpG cluster II in AS-SRO may have a function presumably as an allele discrimination signal (ADS) that is recognized specifically by an allele discrimination protein (ADP), which is probably expressed in oocytes but not in sperm.

A 9 bp sequence, of which the core 7 bp is identical with the sequence of the de novo methylation signals (DNS) that were identified recently in the mouse Snrpn promoter region (12), is present in the AS-SRO sequence upstream to the ADS described earlier (data not shown and Fig. 7D). Band shift assays with a DNS containing oligo (oligo I-DNS) revealed a protein (DNP) that binds this sequence in extracts of ES cells, parthenogenetic (Fig. 7B, lane 1), normal (lane 2) and androgenetic (lane 3) ES cells, suggesting that unlike ADP, DNP is synthesized in both gametes, sperm and oocyte. This protein did not bind to a mutated oligo (oligo II-DNS) (lane 4).

Interestingly, band shift cross-competition experiments (Fig. 7C) that were performed with oligo I-ADS and oligo I-DNS (Fig. 7A and B) revealed that while 100× excess of unlabeled oligo I-ADS competes with itself (Fig. 7C, lane 2), unlabeled oligo I-DNS did not compete with oligo I-ADS on the binding of ADP (lane 3). The non-relevant oligo III (representing CpG cluster I) did not compete with oligo I-ADS as well (lane 4). In the reciprocal competition experiment, unlabeled oligo I-DNS competed efficiently with itself on the binding of DNP (lane 6). However, strikingly, in these experiments unlabeled oligo I-ADS could compete efficiently with oligo I-DNS (lane 7), while a non-relevant oligonucleotide failed to compete with oligo I-DNS (lane 8). These results may suggest that ADP binds strongly to ADS and presumably prevents binding of DNP to DNS. Moreover, these two proteins may interact and participate in a multiprotein complex as proposed subsequently (Fig. 8).
**Figure 4.** Requirement of the AS-SRO sequence to confer methylation upon the maternal allele of PWS-SRO. (A) Southern blot analysis of methylation of PWS-SRO in F1 and F2 progeny of a transgene lacking AS-SRO (2 in Fig. 1B; for band identification see Fig. 1B) (maternal and paternal transmission). (B) AS–PWS transgene in which AS-SRO is flanked by lox sequences (3 in Fig. 1B). Female F1 inherited the transgene from her father (F0 males) that mated with a CB6 F1 female. PWS-SRO in this F1 female was practically unmethylated (Southern insert). This female mated with a CB6 F1 male yielded a transgenic F2 mouse in which PWS-SRO was methylated (Southern insert). (C) AS–PWS transgenic male in which AS-SRO is flanked by lox sequences mated with a female harboring the cre gene. A female progeny of this mating that lost AS-SRO was unmethylated on PWS-SRO (Southern insert). This female mated with a CB6 F1 male yielded a progeny that inherited the transgene that lacks AS-SRO. PWS-SRO in this F2 progeny was not methylated (Southern insert). (D) A female, F1 progeny of a lox–AS–lox–PWS male mated with a CB6 F1 female inherited the transgene for her father and was not methylated on PWS-SRO (Southern insert). However, mating this female with a cre-harboring male yielded the F2 progeny that lost AS-SRO after fertilization, yet conferred methylation on PWS-SRO (Southern insert).
transgene. Histone H3 acetylation (Ac-H3) of the PWS-SRO in the AS-SRO deleted maternally transmitted progeny that possess AS-SRO (Fig. 4B). (Figure 5. A) DNase I sensitivity of the BglII/EcoRI sequence of the PWS-SRO was analyzed as described in Materials and Methods. Maternally transmitted progeny that lost AS-SRO as described in Fig. 4C are compared with paternal transmission of the transgene (Fig. 4A). Furthermore, an unmethylated state of PWS-SRO upon both maternal and paternal transmission results in PWS-SRO (13,14). Here we prove unequivocally that AS-SRO confers imprinting upon PWS-SRO later in development (7).

The present study provides evidence to support the hypothesis that AS-SRO acquires the primary imprint in the gametes and confers imprinting upon PWS-SRO later in development (7).

Here we use a transgene that includes both the AS-SRO and PWS-SRO sequences. This transgene carries out the entire imprinting process and resembles the epigenetic features of the corresponding endogenous sequences in the adult. Thus, this experimental system could serve as a reliable tool to study the epigenetic features of AS-SRO and PWS-SRO, the interrelationship between these important elements and the establishment of their imprint in the gametes and in the early embryo. This is of particular importance as questions concerning the steps in imprinting that take place in the gametes and early stages of embryo development cannot be addressed in humans.

On the basis of methylation analysis of PWS-SRO in AS patients with microdeletions of sequences that include AS-SRO, it was assumed that AS-SRO confers methylation on PWS-SRO (13,14). Here we prove unequivocally that removal of AS-SRO from the transgene sequence results in an unmethylated state of PWS-SRO upon both maternal and paternal transmission of the transgene (Fig. 4A). Furthermore, using a transgene in which AS-SRO was flanked by lox sequences, we could remove AS-SRO prior to gametogenesis or after fertilization by arranging the mating with a pre-expressing mouse. This set of experiments clearly demonstrates that while AS-SRO is absolutely essential during gametogenesis and probably in the early embryo, it is dispensable later in development (Fig. 4D).

To further understand how AS-SRO confers imprinting on PWS-SRO, we studied the epigenetic features of AS-SRO in the gametes and early embryo using the same transgenic experimental system. On the basis of our previous observation that the epigenetic features at AS-SRO are allele specific (7), we predicted that AS-SRO must have a differentially methylated region (DMR) that is established in the gametes to allow allele discrimination in the embryo (7). In fact, we demonstrate here a short DMR present in AS-SRO that is methylated in sperm and unmethylated in oocytes. This DMR is maintained in the pre-implantation embryo until PWS-SRO undergoes its methylation specifically on the maternal allele (Figs 2B and 6). Interestingly, the differential methylation of AS-SRO is lost post-implantation and the DMR is almost equally methylated on both alleles in the adult progeny of the transgenic mice (Fig. 6). A similar loss of the endogenous DMR was observed using human lymphoblast cells carrying deletions of the AS-SRO either on the paternal or the maternal allele (data not shown). These methylation changes during development strongly suggest that the differential methylation of AS-SRO plays a critical role in the establishment of a DMR at the PWS-SRO sequence. This observation is another example of a DMR that plays a regulatory role in the imprinting process in accordance with our previous suggestion that regulatory elements in the imprinting process reside within DMRs (15).

The possibility that the newly discovered DMR in AS-SRO plays a role in conferring imprinting on PWS-SRO gains support by the identification of a protein that binds to this DMR on the unmethylated maternal allele. This protein is found in parthenogenetic ES cells and is absent from androgenetic ES cells (Fig. 7A). Our assumption is, therefore, that this protein is present in oocytes and absent in sperm (Fig. 8A). Its absence in sperm allows a nearby DNS that shares its sequence with the previously described DNS sequences in the mouse Snrpn promoter (12) to bind DNP and facilitate methylation of ADS. In contrast, the presence and binding of ADP to ADS in oocytes may prevent binding of a de novo protein (DNP) to DNS. As a result ADS is not methylated (Fig. 8A).

The DMR being maintained throughout the pre-implantation stages of development suggests that ADP binding to its site prevents methylation on the maternal allele in the early embryo as well. This conclusion is strengthened by the observation that ADP is present in embryonic cells such as F9 and HEK293 (data not shown), and by the fact that ADP failed to bind to a methylated ADS (Fig. 7A, lane 4).

In Figure 8B we describe a possible mechanism by which, based on our observations, AS-SRO communicates and confers the imprinting of PWS-SRO.

As mentioned earlier, signals for allele discrimination and de novo methylation have been previously observed at the DMR2 region of the Igf2r imprinted gene (16). Although these elements differ in sequence from the ADS and DNS sequences described here, they clearly show the
same mode of function. Interestingly, these elements in Igf2r and in AS-SRO are positioned similarly within 110 bp. It appears, therefore, that these sequences represent an imprinting box. In both imprinting systems, DNP seems to be synthesized in both gametes, whereas ADP is expressed differentially. It can, therefore, be concluded that each imprinting control system operates with its own specific cis elements and trans acting factors, albeit by a similar mechanism. This conclusion was corroborated by band shift experiments in which binding of DNP to DNS or ADP to ADS could not be competed with its counterpart from Igf2r (data not shown).

MATERIALS AND METHODS

Preparation of DNA for microinjection

The AS–PWS, PWS and lox-AS–lox-PWS constructs (respectively, 1, 2 and 3 in Fig. 1B) were cloned in the pT7T3 vector and restricted with PvuI. For insertion of the lox sequences, the parental transgene was cut with HindIII and SalI (Fig. 1B1) and the HindIII/HindIII fragment 5′-ATAACTTCGTATAATGTATGCTATACGAAGTTATATAGCTT-3′ and the SalI/SalI fragment 5′-ATAACTTCGTATAGTATGCTATACGAAGTTAT3′ (Fig. 1B3) were introduced into both ends of HindIII-AS-SRO-SalI prior to ligation. The PvuI restricted DNA was electrophoresed on 1% agarose gel and the isolated band purified on an ion exchange column (Elutip-d column, Schleicher and Schuell). The purified fragment was ethanol-precipitated, dissolved in the injection buffer (7.5 mM Tris, 0.2 mM EDTA, ph 7.5) to final concentration on 15 ng/μl and injected into the male pronucleus of a fertilized egg derived from (C57Black/C2 BALB/c) F1 mice. Embryos were transferred at the two-cell stage to the oviducts of pseudopregnant F1 females. Founders were identified by Southern blot analysis of the tail DNA. Lines were derived from female or male founders for each of the earlier-described constructs. Copy numbers of each line were estimated by Southern blot analysis, comparing bands of the transgene with the endogenous sequences (Fig. 1C).

Isolation of oocytes and pre- and post-implantation mouse embryos

Three- to four-week-old (C57Black × BALB/c) transgenic F1 females were superovulated by an injection of 10 units of pregnant mare’s serum gonadotropin (PMSG) followed by an injection of 10 units of human chorionic gonadotropin (hCG) 48 h later. Oocytes were collected 17 h after the second injection into M2 medium where granulosa cells
Figure 7. Band shift assays (see Materials and Methods) were performed with the CpG cluster II (oligo I-ADS) using parthenogenetic ES cell nuclear extract (lane 1), normal ES cell nuclear extract (lane 2) and androgenetic ES cell nuclear extract (lane 3). Nuclear extract from parthenogenetic ES cells was incubated with methylated CpG oligo IIa-ADS (lane 4) and mutated CpG oligo IIb-ADS (lane 5). Lane 6: no extract added. (B) Band shift assays were performed with the oligo I-DNS containing the DNS sequence (underlined). Using parthenogenetic ES cell nuclear extracts (lane 1), normal ES cell nuclear extract (lane 2) and androgenetic ES cell nuclear extract (lane 3). Nuclear extract from normal ES cell was incubated with single base mutated (encircled) oligo II-DNS (lane 4). Lane 5: no extract added. (C) Nuclear extract from parthenogenetic ES cells was incubated with the CpG cluster II (oligo I-ADS) (lane 1) and 100× excess of competitors: non-labeled oligo I-ADS (lane 2); non-labeled oligo I-DNS (lane 3); non-labeled oligo III (lane 4). In the reciprocal competition experiment the nuclear extract from parthenogenetic ES cells was incubated with labeled oligo I-DNS (lane 5) and competed with 100× excess of non-labeled oligo I-DNS (lane 6); CpG cluster II (oligo I-ADS) (lane 7); non-labeled oligo III (lane 8). (D) Presentation of the position of CpG cluster I, DNS and cluster II (ADS) on the 1.2 kb AS-SRO sequence.
were removed as described before (17). Superovulated transgenic F1 females mated with (C57Black × BALB/c) F1 males (maternal transmission) or transgenic F1 males mated with (C57Black × BALB/c) F1 females (paternal transmission) were the source of pre-implantation embryos. Zygotes collected from female oviducts as described earlier for oocyte isolation were covered with paraffin oil and 5% CO₂ and incubated at 37°C in M16 medium (Sigma) for 4 days. Superovulated transgenic F1 females were mated with (C57Black × BALB/c) F1 males (maternal transmission) or transgenic F1 males were mated with (C57Black × BALB/c) F1 females (paternal transmission) and 7.5 dpc embryos dissected. The cre gene under the PGK promoter was constitutively expressed (18). Cre-harboring females were mated

Figure 8. A proposed model suggesting the mode of functional interaction between AS-SRO and PWS-SRO. (A) ADP, a protein that binds to the differentially methylated CpG cluster II (ADS), being present in parthenogenetic ES cells and absent in androgenetic ES cells, is suggested to be present in oocytes and absent in sperm. Its absence in sperm allows a nearby DNS that shares its sequence with the previously described DNS sequences in the mouse (12) to bind DNP and facilitate methylation of ADS (closed lollipops). In oocytes, ADP binds strongly to ADS, prevents DNP from binding to DNS, therefore ADS does not undergo de novo methylation (open lollipops). On the basis of band shift competition experiments we proposed that in this case DNP interacts with ADP that is bound to the DNA. (B) Since the differential methylation of ADS persists in the early embryo we speculate that that the complex ADP/DNP that is anchored to the unmethylated ADS on the maternal allele interacts with a second DNP molecule bound to DNS that is found in the SNRPN promoter, forming a DNP/DNP dimer that is capable of methylating in cis the DMR1 sequence that is located within the SNRPN promoter region (closed lollipops) (21,22). Such a DNP dimer was recently suggested to be required to confer methylation upon PWS-SRO (12). It should be noted that the DNS in the SNRPN promoter and the DNS in AS-SRO are the only DNS sequences found across a 200 kb DNA stretch in and around the IC domain. The interaction between the AS-SRO and PWS-SRO most likely requires the synthesis of other, yet uncharacterized, proteins (yellow circle, blue rectangle, and gray pentagon). These proteins form a multiprotein complex that facilitates the communication between AS-SRO and PWS-SRO and promotes methylation of PWS-SRO. This may explain why PWS-SRO is not methylated in the gametes but rather undergoes methylation later in embryo development. On the paternal allele (Pat) the methylated status of ADS prevents ADP binding to ADS (Fig. 7A) and DNP binding to DNS as well (data not shown). Thus the de novo methylation multiprotein complex cannot be assembled, leaving the PWS-SRO unmethylated (open lollipops).
with males carrying the AS–PWS transgene where AS-SRO is flanked by lox sequences as described earlier. F1 female progeny of this mating were analyzed by PCR using the following primers to determine transmission of the cre gene as well as deletion of AS-SRO sequence: upper primer for cre, 5′-GACAGTTCACCGGCATCAAC-3′; lower primer for cre, 5′-CGATGCAACGAGTGATGAGGTTC-3′; upper primer for AS-SRO, 5′-CACCTTGAGTGTCATTAG-3′; lower primer for AS-SRO, 5′-CCTCAATTGGACACATTAC-3′.

In the reciprocal experiment when females harboring a construct containing AS-SRO flanked by lox sequences were mated with males harboring cre, deletion of AS-SRO was analyzed by PCR in 16–26 cell and blastocyst stage single embryos, as well as in adult progeny of this mating by using the same primers for cre (as described earlier), and the following primers for AS-SRO: upper, 5′-CCTCTCGCTATTACGCCA-3′; lower, 5′-CAGGTACCTACCATTGCAC-3′.

DNA extraction

Oocytes and pre-implantation embryos collected as described earlier were incubated for 5 h at 55 °C in 200 μl TNE solution (10 mM Tris pH 7.5, 100 mM NaCl and 10 mM EDTA), 0.5% SDS, 0.5 mg/ml Proteinase K. One microliter of 20 mg/ml glycogen was added as carrier. This incubation was terminated by 1.5 mM NaCl (final concentration) and samples centrifuged at 14,000 r.p.m. in an Eppendorf bench-top microcentrifuge. DNA was phenol extracted and ethanol precipitated. Mouse tail tissue as well as 7.5 dpc embryos were incubated overnight at 55 °C with 0.5 mg/ml Proteinase K in 600 μl of 50 mM Tris pH 7.5, 100 mM EDTA, 0.5% SDS. DNA was extracted by phenol and phenol/chloroform/isoamylalcohol (25:24:1 v/v/v) followed by ethanol precipitation. Sperm DNA was prepared as described before (17).

Methylation assays

Methylation of the CpG sites was assayed by PCR, Southern blot analysis and the bisulfite methods.

PCR analysis. DNA prepared from 10 to 20 pooled blastocysts or 20 to 40 pooled oocytes was digested with EcoRI/BglII, EcoRI/BglII + NotI or EcoRI/BglII + HpaII and subjected to PCR using 10 μCi/μl of [α-32P]dCTP and primers as described subsequently. The PCR products were electrophoresed on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4% polyacrylamide gels. Gels were dried and exposed to an X-ray film. A PCR product is observed only on 4%

DNA samples (1–5 μg) were digested with EcoRI/BglII or EcoRI/BglII + NotI. Restriction fragments were resolved by gel electrophoresis, Southern blotted and probed with a labeled fragment produced by PCR using 5′-CTCCCCAGGC TGTCTTGGAGAA-3′ as upper and 5′-GGCTCTCAT ACTTAGATCTAGG-3′ as lower primer. The PCR product was end labeled with [α-32P]dCTP and RediPrime labeling system (Amersham).

Bisulfite method. DNA samples from mouse tails, sperm, 10–20 pooled blastocysts or 20–40 pooled oocytes, and embryos (E 7.5) prepared as described earlier were subjected to bisulfite treatment. DNA was amplified using primer sets that encompass two CpG clusters present in the AS-SRO sequence (clusters I and II) (Fig. 6) and one region of PWS-SRO (Fig. 2C). The primers used were: cluster I upper 1, 5′-TTTTGAGTGTGTTATTAGTATATTG-3′ and lower 1, 5′-TTACTCCCCAACCCTTAAAAATTTA-3′; cluster II upper 1, 5′-CTAAAAACATTTTTACATTAAAAATTTTTA A-3′ and cluster II lower 1, 5′-AAATATGATGTGTGTTGTT GTTTTATTGT-3′; PWS upper, 5′-TTAAACCTTAAAA TCTTTTTTACAAA-3′ and PWSI lower, 5′-ATTTA TTATTTATTTTGGTTTTTTATTTT-3′. For the AS-SRO CpG clusters a second round of semi-nested amplification was carried out using the upper primers listed earlier, and the following lower primers: cluster I lower 2, 5′-ACTCTA TTCACATATTAAAACAACTTAAAAAATTTG-3′ and cluster II lower 2, 5′-TGGAGTAATTTGTGTTTTATTTTTTTTT-3′.

Reaction conditions for the first PCR were five cycles of 95 °C 1 min, 52 °C 3 min, 72 °C 3 min followed by 35 cycles of 95 °C 45 s, 52 °C 1 min, 72 °C 1 min. Conditions for the second PCR were 50 cycles of 95 °C 45 s, 52 °C 1 min, 72 °C 1 min followed by 7 min at 72 °C. PCR products were then cloned into easy-pGEM vectors using the manufacturer’s instructions (Promega). Clones containing the relevant products were sequenced and analyzed for methylation status of individual CpGs.

DNase I sensitivity analysis

Splenic cells of transgenic mice were dispersed and lymphocytes were cultured for 3 days in RPMI supplemented with 20% FCS in the presence of 10 μg/ml LPS (Sigma L4005). The cells (2–3 × 10⁶) were resuspended in nuclei buffer (20 mM Tris pH 7, 3 mM CaCl₂, 2 mM MgCl₂, 0.3% NP-40) and incubated on ice for 10 min. Following homogenization using a Dounce homogenizer, nuclei were resuspended in RSB (10 mM Tris pH 7, 10 mM NaCl, 3 mM MgCl₂) to a concentration of 10⁵ nuclei/ml. DNase I (Sigma D4263) was added to final concentrations of 1–30 units/ml in 150 μl aliquots, and reactions were incubated at 37 °C for 12 min. Extracted genomic DNA (10 μg) was then digested with the appropriate restriction enzymes (HpaII for AS-SRO; BglII + EcoRI for PWS-SRO) and Southern blotted, as previously described.

Chromatin immunoprecipitation

Chromatin Immunoprecipitation was performed as described by Simon et al. (19) with slight modifications. Splenic cells
of transgenic mice were dispersed and lymphocytes were cultured for 3 days in RPMI supplemented with 20% FCS in the presence of 10 μg/ml LPS. Lymphocytes were then cross-linked with 1% formaldehyde solution [1% formaldehyde, 10 mM NaCl, 100 mM EDTA, 50 mM EGTA, 5 mM HEPES (pH 8)] and quenched with 125 mM glycine. Cells were lysed with LB1 buffer [100 mM HEPES–KOH (pH 7.5), 280 mM NaCl, 2 mM EDTA, 20% glycerol, 1% NP-40, 0.5% Triton X-100] washed with LB2 buffer [400 mM NaCl, 2 mM EDTA, 1 mM EGTA, 20 mM Tris (pH 8)] and resuspended in LB3 buffer [2 mM EDTA, 1 mM EGTA, 20 mM Tris (pH 8)]. Lysates were sonicated 12× for 30 s in Cell disruptor (Heat systems. Ultrasonic 350 with output power 4). Debris were pelleted and supernatants were incubated with anti-acetylated H3 or anti-dimethyl K4 H3 (Upstate Biotechnology) preincubated with protein A agarose beads. Before incubation a 1/50 input fraction was withdrawn. Beads were washed 7× with RIPA buffer [50 mM HEPES (pH 7.6), 1 mM EDTA, 0.7% DOC, 1% NP-40, 0.5 mM LiCl] and once with TE buffer [50 mM Tris (pH 8), 10 mM EDTA]. Beads were resuspended in elution buffer [50 mM Tris (pH 8), 10 mM EDTA, 1% SDS], incubated at 65°C for 15 min with continuous shaking and spun down. Supernatant (bound fraction) and input fraction were incubated overnight at 65°C for reverse cross-linking. DNA was purified by phenol/chloroform extractions and two aliquots 1 and 4 μl were amplified by PCR (94°C 4 min, 55°C 2 min, 72°C 2 min and then 94°C 30 s, 55°C 30 s, 72°C 30 s for 28 s up to 35 cycles) in the presence of 10 μCi/μl of [α-32P]dCTP. Primers for GAPDH were as follows: upper, 5′-TTCATCCAAGCGTGTAAGGG-3′; lower, 5′-TGGTTCCCAGGACTGGACTGT-3′. Primers for AS-SRO were as follows: upper, 5′-CCGTCAGTGACGGATGGAGCGG-3′; lower, 5′-CTTGAGGGGTTTGAGTGTA-3′. PCR products were run on acrylamide gels and exposed to Phosphoimager (BAS-IIIs FUJI).

**REFERENCES**


