

Control elements within the PWS/AS imprinting box and their function in the imprinting process

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A cluster of imprinted genes on human chromosome 15q11–q13 (the PWS/AS domain) and its ortholog on mouse chromosome 7c is believed to be regulated by an imprinting control center. Although minideletions in this region in Angelman syndrome (AS) and Prader–Willi syndrome (PWS) patients revealed that two elements, shortest deletion regions of overlap in AS families and PWS families (AS–SRO and PWS–SRO), respectively, constitute the IC, the molecular mechanism that governs this regional control remains obscure. To understand how this imprinting center works, a mouse model was sought. The striking similarity between the human and mouse sequences allowed the generation of a minitransgene (AS–SMP) composed of AS–SRO and the *Snrpn* minimal promoter (SMP) the mouse ortholog of PWS–SRO. This minitransgene carries out, in a highly reliable and reproducible manner, all steps of the imprinting process. In an attempt to decipher the molecular mechanism of the imprinting process, we generated and tested for imprinting five minitransgenes based on AS–SMP, in which various parts of the 160 bp SMP were deleted. These experiments revealed a set of five *cis* elements that carry out the various steps of the imprinting process. This set includes: (i) two copies of a *de novo* methylation signal (DNS) that establish the maternal imprint during oogenesis; (ii) an allele discrimination signal that establishes the paternal imprint; and (iii) two elements that act together to maintain the paternal imprint. Two functionally redundant sets of the five elements were found on the respective endogenous mouse sequence explaining the previously published contradictory results of targeted deletion experiments. Together with the fact that all five elements bind specific proteins that are presumably the factors acting in *trans* in the imprinting process, our observations set the stage for a comprehensive study of the molecular mechanism involved in the control of the imprinting process.

INTRODUCTION

Imprinting of the 2 Mb Prader–Willi/Angelman syndrome (PWS/AS) domain on human chromosome 15q11–q13 and its mouse orthologue on mouse chromosome 7c is believed to be regulated by a regional imprinting control center (IC). However, the mechanism by which this imprinting control works is unclear. The entire 2 Mb domain contains numerous paternally expressed genes and at least two maternally expressed genes (1) (Fig. 1A). A breakthrough in understanding the imprinting control mechanism was achieved by studying AS and PWS families with imprinting mutations caused by microdeletions of two sequences: a 4.3 kb sequence (PWS–SRO) that includes the *SNRPN* promoter/exon1 (2) and an 880 bp sequence (AS–SRO) located 35 kb upstream to the *SNRPN* transcription start site (3). Deletion of the AS–SRO sequence is implicated in AS and that of PWS–SRO is

implicated in PWS. It became clear that these sequences, although separated by a 35 kb intervening sequence, can communicate and constitute the imprinting center that regulates the imprinting of the entire 2 Mb AS/PWS domain.

In a recent study using lymphoblasts of PWS and AS patients, differential chromatin structures at the PWS–SRO and AS–SRO have been demonstrated (4). The results of this study suggested a unidirectional stepwise program in which the imprinted AS–SRO confers imprinting on the PWS–SRO. To better understand the molecular mechanism that governs the imprinting process in this 2 Mb imprinted domain, an experimental animal system was essential. Such an experimental system should allow the identification of control elements within the AS–SRO and PWS–SRO sequences and determine their function in the imprinting process at the molecular level. Fortunately, the PWS–SRO orthologue in the mouse includes the *Snrpn* promoter and exon 1 harbored within

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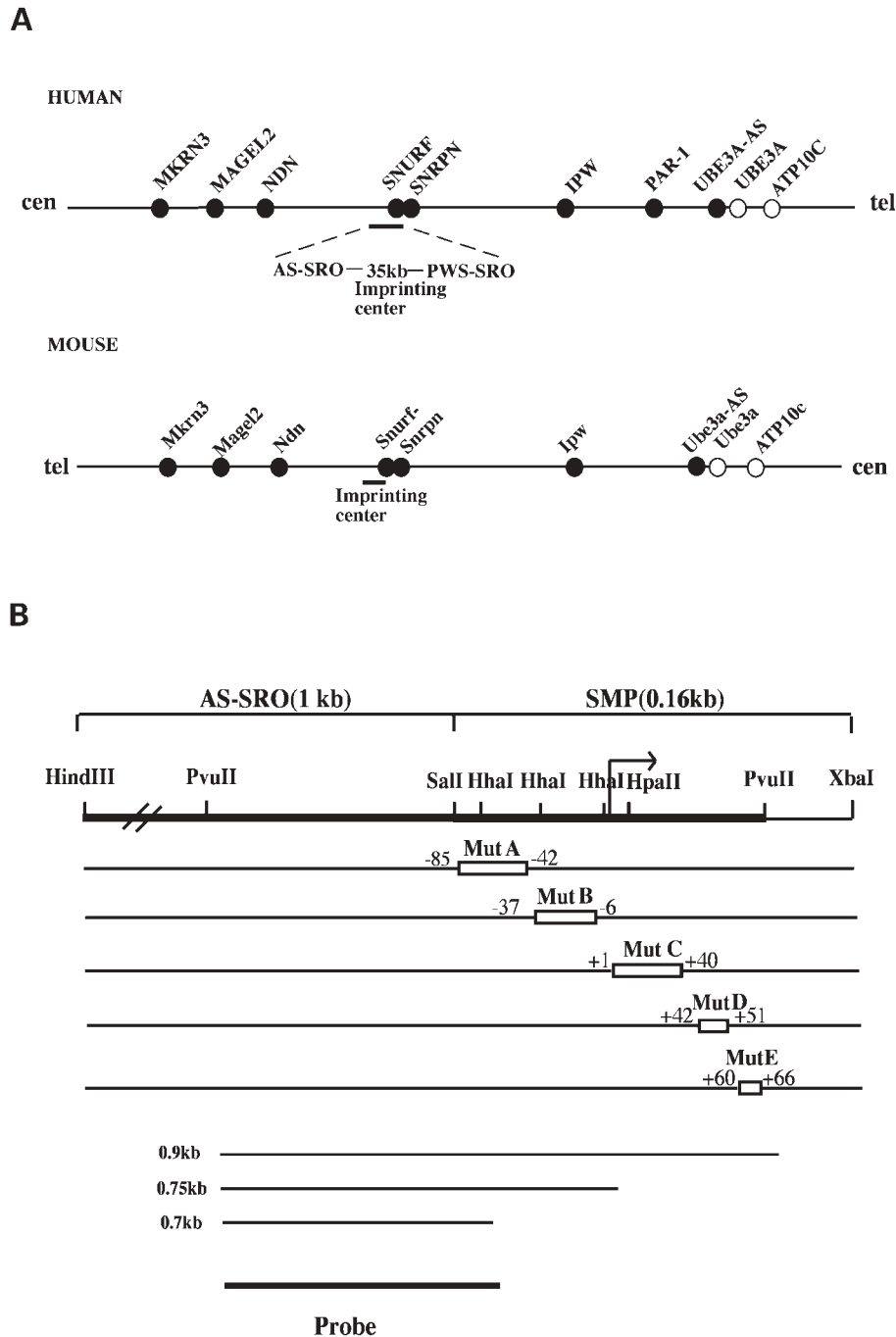


Figure 1. (A) Schematic map (not to scale) of the human AS/PWS domain and its mouse orthologue. Solid circles: paternally expressed genes. Open circles: maternally expressed genes. (B) Schematic presentation of DNA constructs representing the AS-SMP minitransgenes used in this study. The three *HhaI* sites and the *HpaII* site were used to monitor the methylation status of the transgene. The mutations were performed by replacements of the sequences designated by empty boxes. Numbers are positions on the SMP sequence with respect to the *Snrpn* transcription start site (horizontal arrow). The 0.9, 0.75 and 0.7 kb fragments are obtained by *PvuII*, *PvuII* + *HpaII* or *PvuII* + *HhaI* digestion, respectively. The probe is of the human AS-SRO sequence allowing distinction of the transgene from the mouse endogenous DNA.

a clearcut differentially methylated CpG island which has properties of an imprinting element. Not only is it differentially methylated on the maternal allele, but this methylation pattern is established during gametogenesis and maintained throughout development (for summary, see Fig. 5A) (5).

The striking similarity of the human and mouse sequences (Fig. 1A) allowed the generation of a minitransgene (AS-SMP) that includes 1.0 kb of the human AS-SRO (the mouse ortholog has not been identified as yet) and a 160 bp sequence that constitutes the mouse *Snrpn* minimal promoter (SMP) (6).

The SMP sequence comprises the entire first exon of *Snrpn* and an 84 bp sequence located upstream to the transcription start site (7). The AS-SMP transgene apparently contains all *cis*-acting elements which are needed to establish and maintain the imprint (6). In this transgene, like in the genomic counterpart, the *Snrpn* promoter undergoes *de novo* methylation during oogenesis, while the paternal allele is protected from *de novo* methylation during spermatogenesis. Like the endogenous gene domain, the maternal allele is protected from the global demethylation that takes place in the preimplantation embryo, while the paternal allele remains unmodified during embryogenesis escaping the wave of *de novo* methylation that takes place at the postimplantation stage (8,9). Moreover, the AS-SMP sequence contains all elements required to direct allele specific expression and switch expression from the unmethylated expressed state in F₁ progeny of a paternally transmitted transgene to the methylated repressed state in F₂ progeny upon maternal transmission (for summary see Fig. 5A) (6).

RESULTS

As mentioned above, the AS-SMP minitransgene is capable of imprinting in a highly reliable and reproducible manner (6). We therefore used this system in the current study to identify *cis*-acting elements within SMP and study their role in the multistep imprinting process. We focused on SMP as it was absolutely essential in carrying out the imprinting process. Replacement of SMP in the AS-SMP transgene with a β globin sequence that is unrelated to the imprinting process resulted in loss of imprinting. When methylation of this sequence was analyzed, it was methylated upon maternal, as well as paternal, transmission (data not shown; see also Fig. 5C, construct 4).

AS-SMP was modified by replacing different parts of the 160 bp sequence of the SMP (6) with unrelated sequences of similar length (see Materials and Methods). These mutated constructs MutA, MutB, MutC, MutD and MutE, respectively (Fig. 1B) were used to generate transgenic lines. At least four lines of each mutated construct were established with male and female founders and numerous F₁ and F₂ progeny were produced by paternal or maternal transmission (Table 1). All lines were tested for the methylation status of the three *HhaI* sites and one *HpaII* site within SMP (Fig. 1B). Methylation was assayed on DNA samples from F₁ and F₂ adults, sperm (Sp), oocytes and blastocysts (for results of *HhaI* site methylation see Figs 2 and 4; data of *HpaII* site methylation not shown). The methylation data were reproducible with apparently no position or copy number effects. They revealed five *cis* elements and their function in the imprinting process (for summary, see Fig. 5).

MutA and MutC reveal elements involved in *de novo* methylation during oogenesis

Replacing the 43 bp (positions -85 to -42) of the 5' end of SMP (MutA in Fig. 1B) with an unrelated sequence of the same length (see Materials and Methods) resulted in loss of the capacity of the AS-SMP transgene to *de novo* methylate the maternal allele during oogenesis (Fig. 2A). Oocytes of the MutA transgene were unmethylated, suggesting that a *de novo*

methylation signal exists within the 43 bp sequence of the 5' end of SMP. Interestingly, this unmethylated state of the maternal allele persisted throughout development (data not shown) and prevailed in F₁ and F₂ adults of maternally transmitted transgenes. As expected, the transgene was unmethylated in sperm and in F₁ or F₂ adult paternally transmitted transgenes (Fig. 2A).

Replacement of 40 bp (positions +1 to +40) that constitute the 5' part of exon 1 (MutC in Fig. 1B) resulted in an identical loss of the *de novo* methylation capacity during oogenesis. Oocytes of MutC transgenes and the maternal allele of F₁ and F₂ adults were unmethylated (Fig. 2C). The results presented in Fig. 2A and C suggest, therefore, that two *de novo* methylation signals (DNS) map to the upstream sequence of SMP and of exon 1, respectively. The two elements (DNS1 and DNS2), although identical in sequence (see below), are not redundant in function. The presence of both elements is essential for successful *de novo* methylation during oogenesis.

An allele discrimination signal (ADS) is located upstream to the *Snrpn* transcription start site

Transgenes in which 30 bp (position -37 to -6) upstream to the *Snrpn* transcription start site were replaced by an unrelated sequence (see Materials and Methods; MutB in Fig. 1B) showed methylation in sperm, suggesting that an allele discrimination signal (ADS) that prevents methylation during spermatogenesis had been lost by this modification. Unexpectedly, the paternal methylation was erased post-fertilization and re-established post-implantation. The transgene in F₁ and F₂ adults was methylated (Fig. 2B). This observation suggests that the loss of ADS function impaired the establishment of the imprint and, as a result, this sequence was not recognized as an imprinted sequence. Consequently, the sequence was demethylated during the genome-wide demethylation event that takes place in the preimplantation embryo and underwent *de novo* methylation at the post-implantation stage. In contrast, the oocytes in the MutB transgenes were methylated as expected and this methylation persisted during embryo development and in adults (Fig. 2B).

Defining the *cis* elements using band shift experiments

To precisely specify the *cis*-acting elements embedded within the mutated sequences described above, we carried out band shift experiments with the respective labeled oligonucleotide probes using nuclear extracts of normal, parthenogenetic and androgenetic ES cells as source of proteins. Using the 5' sequence of SMP (MutA) and a series of mutated oligonucleotides derived from this sequence revealed a protein that binds specifically to a 7 bp element (AGGGAGC), designated DNS1, which is located at positions -44 to -37 (Figs 3A and 5B). Each mutation, such as A→G or C→T, designated by asterisks, abolished the binding of the protein (lane 2 in Fig. 3A). See also the list of oligonucleotides used for the band shift experiments in Table 2. Other mutations in the flanking sequence did not affect binding (lane 3 in Fig. 3A). The protein binding capacity of the direct repeat of DNS1 at positions +11 to +17 was verified by similar band shift experiments in the context of the sequence surrounding these positions (data not

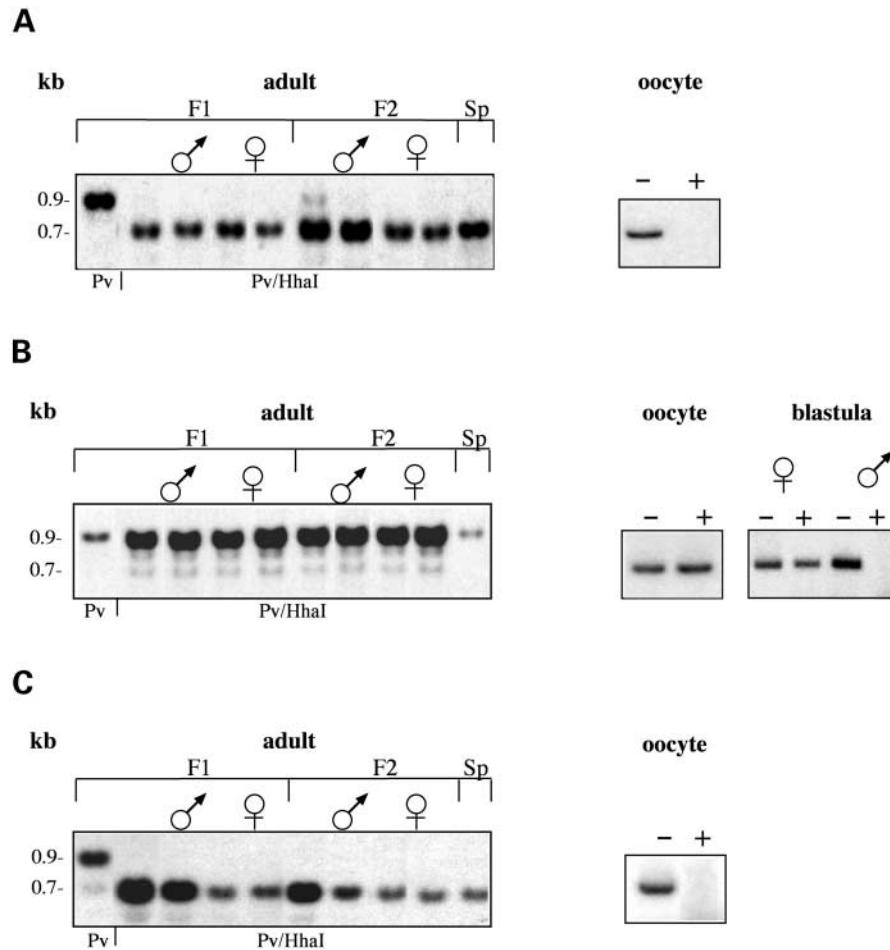


Figure 2. Effect of (A) MutA, (B) MutB and (C) MutC on the methylation of the transgene. Left panels represent Southern blot analyses of methylation in sperm (Sp) and tail DNA (adult). Bands 0.9 and 0.7 are as described in Figure 1. The results presented are of two adult offspring of each F₁ and F₂, respectively [(♂) paternal, (♀) maternal transmission], Pv, *PvuII*. The methylation status of the transgene in oocytes was determined by PCR as described in the Materials and Methods. (+) DNA digested with HhaI prior to PCR, (–) PCR product of undigested DNA.

Table 1. The transgenic lines used in the present study: summary. Of each mutated construct at least two male and two female founders were generated. Copy numbers of the transgene in each founder was estimated as described in Materials and Methods. F₁ and F₂ progeny were used for methylation analysis as described in Materials and Methods. Results of these analyses are summarized in Figure 5A. The results obtained with more than 90% of the lines were consistent and reproducible. Only one line of MutB and one of MutC were not informative. In both lines methylation was partial in both maternal and paternal transmission.

Transgene	Number of founders		Number of F ₁ transmission		Number of F ₂ transmission		Copy number	Non-informative lines
	♂	♀	♂	♀	♂	♀		
MutA	3	3	17	14	11	12	1–20	0
MutB	4	3	15	13	13	14	10–50	1
MutC	2	3	16	18	12	11	5–20	1
MutD	2	2	8	7	11	6	5–20	0
MutE	2	2	9	9	3	7	10–20	0

shown, and Fig. 5B). DNP, the protein that binds DNS, was found in normal, parthenogenetic and androgenetic ES cells (Fig. 3A, lanes 6 and 7, respectively). This protein, however, is absent in differentiated ES cells or in somatic cells such as F9, L8, MEL, HeLa and HEK293 cells (data not shown). These observations suggest that DNP is still required in the

preimplantation embryo, presumably to enforce the maintenance of the maternal allele.

Band shift analysis with the 30 bp sequence upstream to the *Snrpn* transcription start site (MutB) and the respective mutated derivatives revealed a 12 bp element (AAACCTGAGCCA) that specifically binds a protein factor. Mutations that abolished

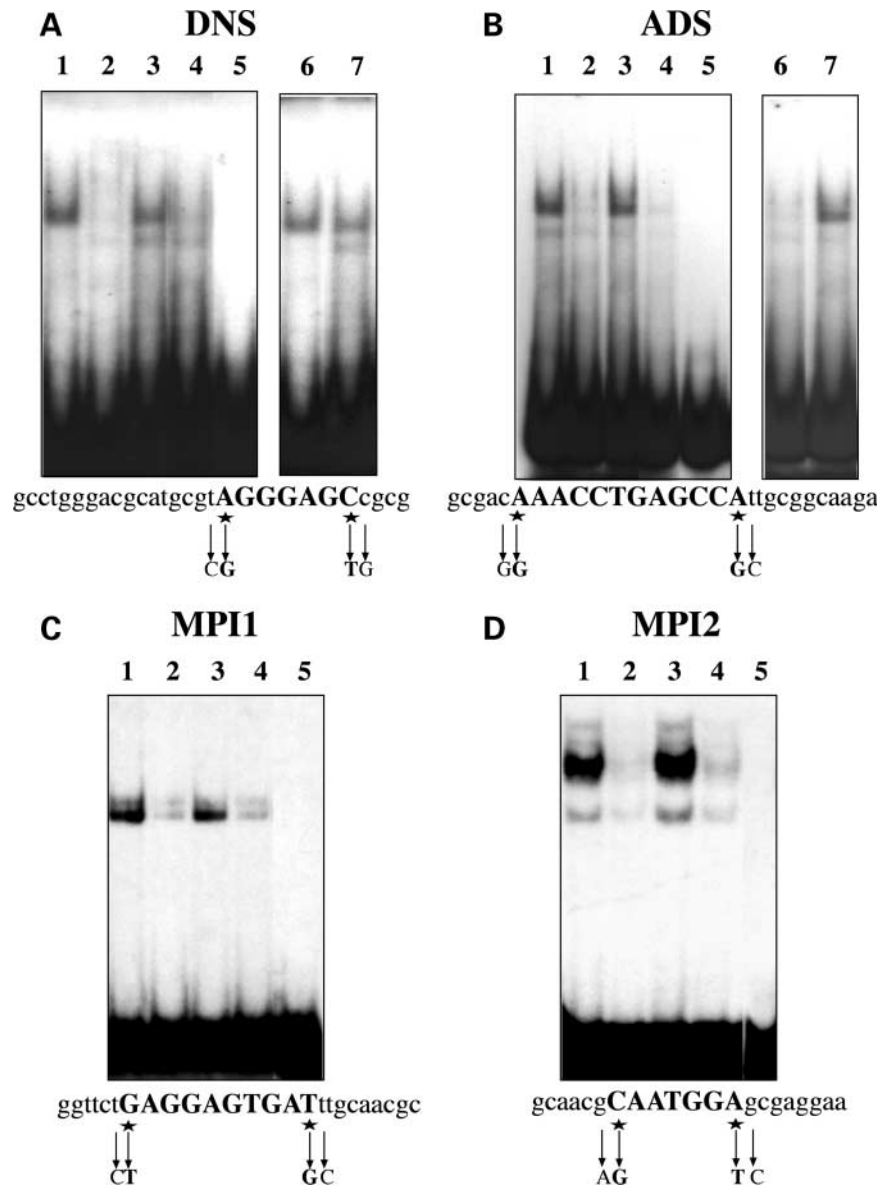


Figure 3. Band shift experiments. Nuclear extracts from ES cells were incubated with the corresponding labeled oligonucleotides (Table 2) containing the DNS, ADS, MPI1 and MPI2 sequences as described in the Materials and Methods (lane 1). Numerous single base mutations were used in this assay to determine the exact sequence required for binding. Mutations designated by asterisks abolished binding (lane 2). Mutations outside the binding element (no asterisk) did not affect binding (lane 3). Excess oligonucleotide competed on the binding (lane 4) and lane 5 represents the no extract control. The results are of representative experiments with (A) the *de novo* methylation signal (DNS). (B) The allele discrimination signal (ADS). (C, D) The maintenance of paternal imprint signals (MPI1 and MPI2). These band shift experiments led to the identification of the precise sequences of the corresponding elements (in bold letters). Lanes 6 and 7 in A and B represent band shifts with parthenogenetic and androgenetic ES cells, respectively.

protein binding are designated by asterisks (lane 2, Fig. 3B; see also list of oligonucleotides in Table 2). Other mutations in the flanking sequence did not affect protein binding (lane 3, Fig. 3B). Together with the results obtained in transgenic experiments in which the sequence juxtaposed to the transcription start site was replaced (Fig. 2B), the binding experiments suggest that the protein factor that binds this sequence may be the allele discrimination protein (ADP) that acts to prevent *de novo* methylation during spermatogenesis. This conclusion is strengthened by the observation that ADP is absent in parthenogenetic ES cells and present in androgenetic

ES cells (Fig. 3B, lanes 6 and 7, respectively). ADP, like DNP, is not present in differentiated ES cells or in any other somatic cells as listed above. Once again, this may suggest that ADP is required in the preimplantation embryo to support maintenance of the paternal imprint.

Two elements within the 3' part of *Snrpn* exon 1 control the maintenance of the paternal imprint

Band shift analyses with the 3' part of exon 1 revealed two different proteins that bind specifically to this sequence. Further

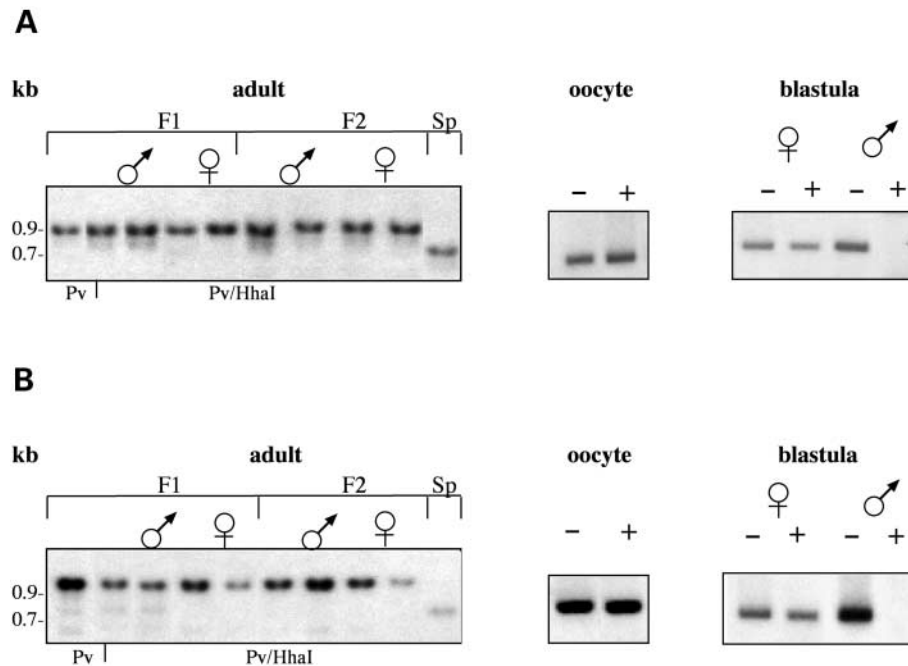


Figure 4. Effect of MutD and MutE on the methylation status of the transgene (see legend to Fig. 2).

band shift experiments with mutated oligonucleotides as described above for ADP and DNP revealed that each of the two proteins bind specifically to one of the two *cis* elements: a 10 bp element (GAGGAGTGAT) located at positions +42 to +51 and a 7 bp element (CAATGGA) located at positions +59 to +66 (Fig. 3C and D).

Identification of these two elements within the 3' part of exon 1 enabled us to generate two additional transgenic lines mutated in these sequences as described above. When the 10 bp sequence (positions +42 to +51) was replaced by an unrelated sequence (MutD in Fig. 1B), the capacity to maintain the unmethylated state of the paternal allele had been lost. Although sperm DNA (Sp) was unmethylated, and the paternal allele was still unmethylated in the blastocyst, it underwent methylation post implantation (Fig. 4A). These results strongly suggest that the 10 bp sequence is a signal responsible for the maintenance of the paternal imprint (MPI). Similar results were obtained when the 7 bp sequence (positions +59 to +66) was replaced by an unrelated sequence (MutE in Fig. 1B). The transgene was found to be unmethylated in sperm and blastocysts of the paternally transmitted transgene, but became methylated post implantation (Fig. 4B). In contrast, the maternal imprint was normal in both MutD and MutE, namely, the oocyte was methylated and this methylation persisted during development and in the adult. The results presented in Figure 4 suggest that both the 10 bp and 7 bp elements are required for the maintenance of the undermethylated state of the paternal allele, therefore designated MPI1 and MPI2, respectively. The fact that mutation of one of these two elements is sufficient to abolish this function suggests that the two factors that bind to these elements must work in concert possibly as a heterodimer, to maintain the unmethylated state of

the paternal allele. In contrast to DNP and ADP, the two proteins that bind MPI1 and MPI2 are present in differentiated ES cells and in somatic cells as mentioned before (data not shown). This may suggest that these two proteins are responsible for the maintenance of the paternal imprint primarily post implantation.

Expression of the mutated transgenes

A long-standing question is how the imprinting process relates to gene expression. Since SMP has been found here to harbor a set of *cis* elements that function in imprinting, we asked how the various mutations in these elements affect *Snrpn* promoter activity (Fig. 5A). Transcription constructs of the mutated transgenes (MutA–MutE) were fused to the gene encoding chloramphenicol acetyltransferase. The corresponding transgenic lines were subjected to RT–PCR assays using brain RNA of F₁ or F₂ progeny (see Materials and Methods). None of the mutated transgenes seem to synthesize transcription products (see summary of the results in expression *in vivo*, Fig. 5A). To test whether the failure of these transgenes to transcribe *in vivo* is a result of the replacement of sequences at the transcription start site which are critical for transcription initiation, we have transfected HEK293 cells with the mutated constructs fused to the CAT gene and assayed CAT activity as described in Materials and Methods (7).

In these transient transfection experiments only the MutD and MutE constructs showed CAT activity (see expression *in vitro* in Fig. 5A). These results suggested that, in addition to MutA that eliminated the 7 bp element that has previously been shown to be critical for *Snrpn* promoter activity (7), the

A

Transgene	Methylation						Expression			Imprint defect		
	F1		F2		Sp	Oo	Bl		in vivo		in vitro	
	♂	♀	♂	♀			♂	♀	♂	♀		
AS-SRO (1 kb) SMP(160bp)	-	+	-	+	-	+	-	+	+	-	+	None
MutA	-	-	-	-	-	-	-	-	-	-	-	Loss of maternal methylation
MutB	+	+	+	+	+	+	-	+	-	-	-	Loss of allele discrimination
MutC	-	-	-	-	-	-	-	-	-	-	-	Loss of maternal methylation
MutD	+	+	+	+	-	+	-	+	-	-	+	Loss of maintenance of paternal imprint
MutE	+	+	+	+	-	+	-	+	-	-	+	Loss of maintenance of paternal imprint

B

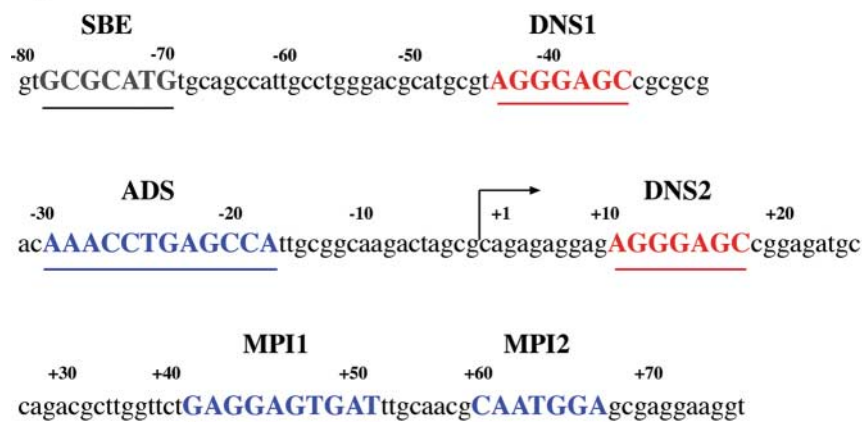


Figure 5. (A) Summary of methylation and Snrpn expression results and the implications on the imprinting process. The AS-SMP transgene is composed of AS-SRO (1.0 kb) and SMP 160 bp. DNAs analyzed were of F₁ and F₂ paternal (♂) and maternal (♀) transmission, respectively; sperm (Sp), oocytes (Oo) and blastocyst (Bl) DNA. The sequences replaced in MutA–E are designated by red arrows. The replacing sequences are listed in legend to Figure 1. Horizontal arrows mark the Snrpn transcription start site. Solid box, Snrpn exon 1; open boxes designate replaced sequences. No methylation of the transgene (–); methylated transgene (+). Imprint defect is a loss of a specific function of the imprinting machinery. (B) The SMP sequence and location of the cis elements which were observed and characterized in the present study. Numbers represent positions on the SMP sequence with respect to the Snrpn transcription start site (horizontal arrow). SBE, the 7 bp element required for Snrpn promoter activity (7). DNS1 and DNS2, de novo methylation signals, ADS, allele discrimination signal; MPI1 and MPI2, elements responsible for maintenance of paternal imprint. Elements are in bold, those controlling the maternal imprint in red and those controlling the paternal imprint in blue. (C) Sequences required for conferring imprinting: (1). P1 clone that includes 25 kb of the Snrpn gene and 50 kb upstream sequence (6); (2). The AS-SMP minitransgene construct (6); (3) SMP sequence alone (6); (4) minitransgene in which SMP was replaced by 900 bp of β globin sequence. Sequence confers imprinting (+). Sequence fails to confer imprinting (–). Horizontal arrows designate Snrpn transcription start site. (D) Location of the cis elements on the mouse endogenous region and the location of the published knockouts of the respective genomic regions. The elements DNS, ADS, MPI1 and MPI2 are clustered in two regions: set 1 at the SMP; and set 2, a downstream sequence at positions +740 to +6024. The open boxes (not to scale) represent sequences removed by targeting deletion experiments. Deletion spanning 42 kb (23 kb upstream and 19 kb downstream to the Snrpn transcription start site) abolished imprinting (–) (11). A 4.8 kb deletion (*HindIII–EcoRI*) that includes both set 1 and part of set 2 abolished imprinting (–), as well (12). Deletions of 0.9 (12) or 1.2 kb (R. Shemer *et al.*, unpublished data) that include only set 1 did not affect imprinting (+). (E) The sequences of the elements in set 1 are compared with those of the elements in set 2.

sequences replaced in MutB and MutC around the transcription start site are essential for the assembly of the transcription initiation complex. The successful transcription observed in the transfection experiments with the MutD and

MutE constructs suggests that the failure of these constructs to express *in vivo* is not because these sequences were replaced, but rather a result of the post-implantation methylation observed at the promoter region of these

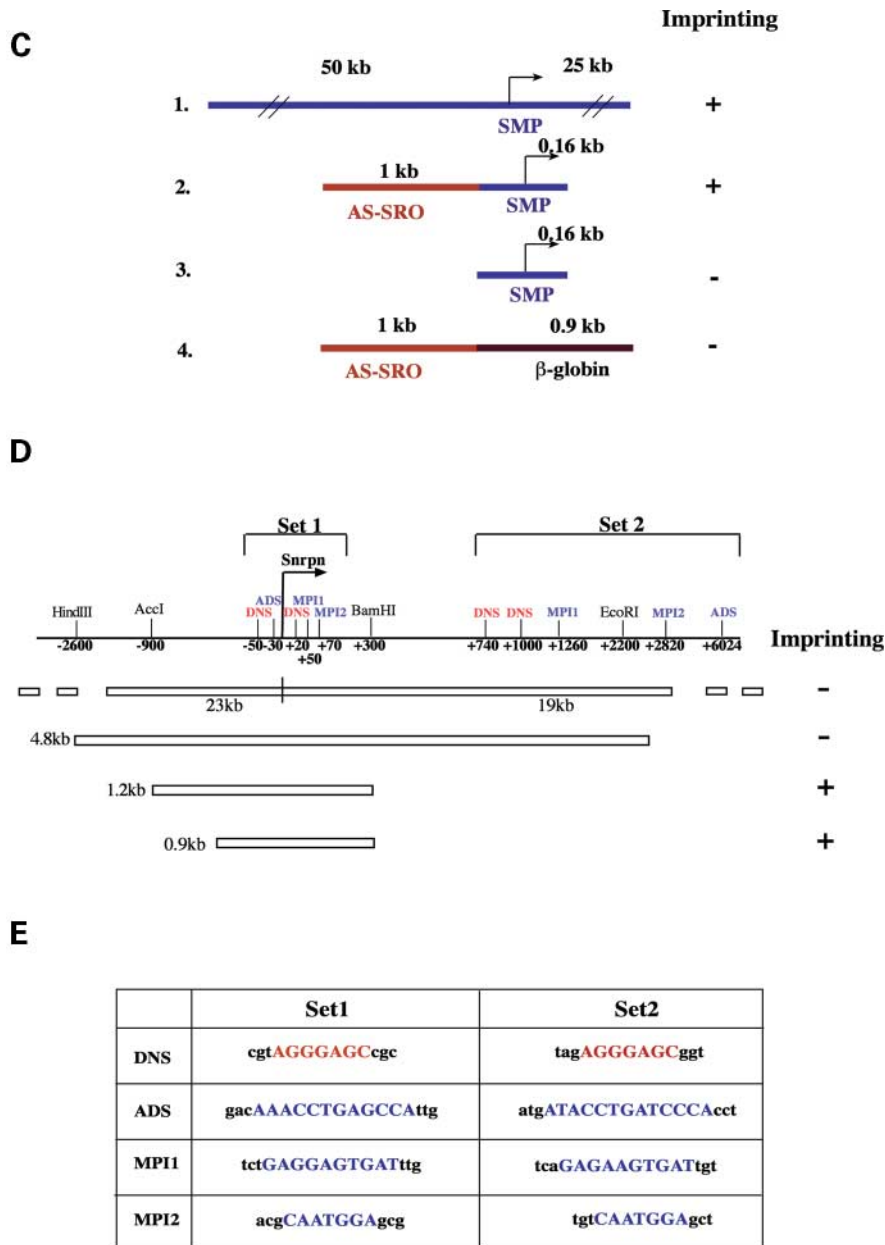


Figure 5. Continued.

constructs (see methylation in F₁ and F₂ progeny of these transgenic lines, Fig. 5A). It should be noted that, while methylation of SBE (Fig. 5B) was shown previously to inhibit Snrpn promoter activity *in vitro* (7), here we show the same inhibitory effect in an *in vivo* experiment. Taken together, the results of the transcription experiments with the mutated constructs imply that while transcription can go on (in MutD and MutE), imprinting is ablated. This implies that expression is secondary to imprinting, meaning that the expression capacity is dictated by the status of imprinting and not vice versa. This conclusion is in accord with results of experiments published previously (7).

DISCUSSION

The 2 Mb PWS/AS imprinted domain is believed to be regulated by an IC composed of two sequences AS-SRO and PWS-SRO. This was first suggested on the basis of imprint mutations observed in AS and PWS families with minideletions that include the AS-SRO or PWS-SRO, respectively (10). In a recent study using lymphoblasts of AS and PWS patients in which either the AS-SRO or PWS-SRO sequence were deleted, the epigenetic status of these two elements and their interrelationships were elucidated (4). While the AS-SRO determines the epigenetic features of PWS-SRO, the reverse

is not true. Therefore, a stepwise, unidirectional program in which structural imprinting at the AS-SRO brings about allele-specific repression of the maternal PWS-SRO had been suggested (4). Targeted deletions of orthologous sequences in the mouse (11,12) suggested that the imprinted genes on the mouse chromosome 7C region are regulated by an IC as well.

The striking similarity between the human and mouse imprinting control of this domain, and the need for a mouse model to study the molecular mechanism of this control system, prompted us to construct a minitransgene that contains all elements necessary to carry out the entire process of imprinting. Following the rationale that imprinting control elements are embedded within differentially methylated regions (DMRs), and the previous observation that Igf2r DMR2 can act as an imprinting box (13), led us to test whether Snrpn DMR1 that includes the Snrpn promoter and exon 1 would be sufficient to confer imprinting. Snrpn DMR1 proved to be insufficient in this regard but a 75 kb P1 clone that includes the entire Snrpn gene and 50 kb of upstream sequence was able to confer imprinting (6) (Fig. 5C). We then argued that a sequence orthologous to the human AS-SRO must be present within the Snrpn upstream domain.

Since we were unable to detect a sequence orthologous to AS-SRO in the mouse, we constructed the 1.2 kb minitransgene (AS-SMP) which was composed of AS-SRO and the SMP (6) (Fig. 1B). This minitransgene proved capable of supporting all steps of the imprinting process. Both parts of this minitransgene, the AS-SRO sequence and SMP, were absolutely required in driving the imprinting process (Fig. 5C). Replacing SMP with a 'naive' sequence, such as β globin, ablated the establishment of the imprint and the SMP in itself could not confer imprinting when AS-SRO was absent (Fig. 5C). It should be emphasized that the AS-SMP minitransgene is composed of authentic endogenous sequences of an imprinting center, unlike the unspecific sequences that have previously been reported to confer imprinting in transgenic experiments (14).

To understand the molecular mechanism that governs the imprinting process at the PWS/S domain, we embarked on a study intended to decipher the *cis* acting elements that operate within the framework of the PWS/AS imprinting center. Towards this goal we used the robust and reliable mouse system based on the minitransgene AS-SMP. As a first step in this endeavor we deciphered the *cis* elements which are embedded within the SMP sequence. A set of five *cis* elements that bind specific proteins and represent steps in the imprinting process had been identified in the 160 bp sequence of SMP. We identified two copies of a 7 bp sequence, DNS1 and DNS2, that apparently work together to establish a methylated state of the maternal allele during oogenesis (Fig. 5A and B). We have defined a 12 bp element located juxtaposed to the transcription start site element (ADS) and show that this element is responsible for the establishment of the paternal imprint by preventing *de novo* methylation during spermatogenesis. Finally, two elements were observed, MPI1 and MPI2, which map to the 3' part of exon 1 and apparently act together to maintain the unmethylated state of the paternal allele (Fig. 5A and B).

ADS and DNS elements have been observed before at the DMR2 region of the Igf2r imprinted gene (13). Although their sequences differ from the ADS and DNS sequences described here, they are clearly similar functionally. In fact, in both

imprinting systems the protein that binds DNS is present in parthenogenetic and androgenetic ES cells, while ADP that binds ADS is found only in androgenetic ES cells. It appears, therefore, 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 to SMP DNS or ADS could not be competed with its counterpart from Igf2r (data not shown). In this regard, it should be added that a similar mechanism of imprint establishment and maintenance including *cis* elements and *trans* acting factors had been described for the Igf2/H19 locus (15).

The observations made in the present study may suggest that the 160 bp SMP sequence includes all *cis* elements that are required for the imprinting process. However, as mentioned above, SMP is not sufficient, and without AS-SRO imprinting cannot be achieved. It is clear that additional *cis* elements that take part in the imprinting process are present in AS-SRO. Obviously, a complete understanding of the molecular mechanism that drives the imprinting process in the PWS/AS domain should await identification of the *cis* elements within AS-SRO. This, together with the data presented here, will allow a full understanding of how these elements interact with the elements which were found in SMP to carry out the imprinting process.

The AS-SMP sequence contains all elements required to direct allele specific expression of Snrpn and switch expression from the unmethylated expressed state in F₁ progeny of a paternally transmitted transgene to the methylated repressed state in F₂ progeny upon maternal transmission (6). We therefore asked how mutations in SMP affect expression of the Snrpn promoter. *In vivo* and *in vitro* Snrpn promoter activity assays were carried out with the transgenes mutated in SMP. These experiments revealed that, while Snrpn promoter activity is determined by the status of imprinting, the opposite is not true. Differential methylation of the Snrpn promoter on the maternal allele is achieved and maintained by the *cis* elements and *trans* acting factors described here. This differential methylation determines the monoallelic expression of Snrpn (Fig. 6).

Being aware of the possibility that differences may exist between transgenic systems and endogenous loci, we searched for the elements that we have observed in the transgenes by screening a 200 kb endogenous sequence around the Snrpn promoter. This screen revealed that the five elements described here appear in the endogenous mouse sequence in two complete sets. One cluster of imprinting elements is the one described here located within the SMP region (set 1). A second complete set of the five *cis* elements is found clustered in a 2 kb sequence located downstream to the Snrpn transcription start site at positions +740 to +6024 (set 2; Fig. 5D). While the sequences of DNS and MPI2 are identical in the two sets, MPI1 and ADS in set 2 differ from ADS and MPI1 in set 1 by one and two nucleotides, respectively (Fig. 5E). Nevertheless, ADS and MPI1 in set 2 bind specifically and efficiently the corresponding proteins (data not shown). A second putative ADS sequence mapped to set 2 at position +1324 that differed from ADS in set 1 by three nucleotides did not show any binding capacity (data not shown).

Previously reported results of targeted deletion experiments (Fig. 5D; R. Shemer *et al.*, unpublished data) (11,12), together with

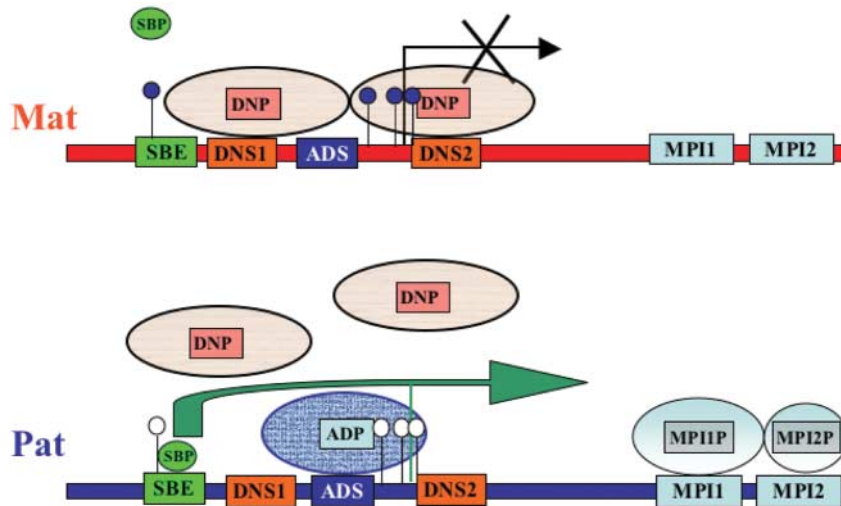


Figure 6. The imprinting elements affect *Snrpn* promoter activity. A homodimer protein complex of DNP binds on the maternal allele (Mat) to DNS1 and DNS2 and induces *de novo* methylation of the CpG island at the *Snrpn* promoter/exon 1 sequence including the enhancer element SBE (7). Methylated CpGs are represented by closed lollipops. The unmethylated state of the paternal allele (open lollipops) is established by replacing DNP from DNS1 and/or DNS2 with the allele discrimination protein (ADP) that binds to ADS. The unmethylated state on the paternal allele (Pat) is maintained by the heterodimers that bind to MPI1 and MPI2. Thus, SBE on the paternal allele is protected from *de novo* methylation throughout adult life and *Snrpn* is expressed preferentially from the paternal allele (horizontal arrow).

the findings described here, clearly imply that the two sets of five elements on the mouse genome are functionally redundant. Deletions around the endogenous *Snrpn* promoter sequence failed sometimes to impair imprinting. This happened when only one of the two sets of elements was removed. When an 0.9 kb sequence that includes the *Snrpn* exon 1 and 600 bp of upstream sequence was removed by homologous recombination, imprinting was normal (12). Similar results were obtained when 1.2 kb of this region were deleted (R. Shemer *et al.*, unpublished data). In both cases, only set 1 of the imprinting signals was removed, leaving the downstream set of elements (set 2) intact (Fig. 5D). In contrast, the more extended knockouts of 4.8 (12) or 42 kb (11) that include both sets of elements in fact ablated imprinting (Fig. 5D). While our transgenic experiments clearly demonstrate that set 1 is sufficient to carry out the imprinting process, the knockout experiments indicate that set 2 is sufficient to do so *in vivo*. The observations presented here of the imprinting signals and their location within the *Snrpn* promoter region give for the first time a logical explanation for the contradictory results obtained in the various knockout experiments. Above all, results of the targeted deletion experiments strongly support the relevance of the findings described here to the *in vivo* situation on the endogenous sequence.

MATERIALS AND METHODS

Preparation of DNA for microinjection

The mutated transgene DNA fragments were cloned in pBLCAT3 and linearized by restriction with *Hind*III. For MutA, MutB and MutC the sequences were replaced with the pBLCAT3 polylinker (5'-CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCC-3'). MutD and MutE sequences were replaced with *Bam*HI sites 5'-GGATCC-3'; which were introduced prior to ligation. The linearized DNA was electro-

phoresed 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 a final concentration of 15 ng/ μ l and injected into the male pronucleus of a fertilized egg derived from (C57Black \times 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 tail DNA. Lines were derived from female or male founders for each mutated construct. Copy numbers of each line were estimated by Southern blot analysis, comparing bands of the transgene with the endogenous sequence (Table 1).

Isolation of oocytes and pre-implantation mouse embryos

Three- to 4-week-old (C57Black \times 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 a second injection from superovulated females into M2 medium and purified from contaminating granulosa cells as described before (8). For the production of pre-implantation mouse embryos, superovulated transgenic F₁ females were mated overnight with (C57Black \times BALB/c) F₁ males (maternal transmission), or transgenic F₁ males were mated with (C57Black \times BALB/c) F₁ females (paternal transmission). Zygotes were collected from female oviducts as described above for oocyte isolation and incubated at 37°C in M16 medium (Sigma) for 4 days, covered with paraffin oil and 5% CO₂ to obtain pre-implantation embryos at the blastula stage. DNA preparations from oocytes, sperm, preimplantation embryos and tail tissues were as described before (8).

Table 2. Identification of elements

Oligonucleotides used for band shift experiments to define	Binding capacity
<i>The DNS element</i>	
5'-gcctgggacgcatgctgAGGGAGCcgcg-3'	+
5'-gcctgggacgcatgctgAGGGAGCgcatc-3'	+
5'-gcctgggacgcatgctgAGGGAGTgcatc-3'	-
5'-gaggatccacgcatgctgAGGGAGCgcatc-3'	+
5'-gaggatccgacacaagttAGGGAGCgcatc-3'	+
5'-gaggatccgacacaagtcgGGGAGCgcatc-3'	-
5'-gaggatccgacacaagtcAGGGAGCgcatc-3'	+
5'-gcctgggacgcatgctgGGGAGCcgcg-3'	-
5'-gcctgggacgcatgctgAGGGAGTcgcg-3'	-
5'-gcctgggacgcatgctgAGGGAGCcgcg-3'	+
5'-gcctgggacgcatgctgAGGGAGCggcg-3'	+
5'-gaggagAGGGAGCcgagat-3'	+
5'-gaggaggGGGAGCcgagat-3'	-
<i>The ADS element</i>	
5'-gcgacAAACCTGAGCCAttgccgcaaga-3'	+
5'-gcgacAAACCTGAGCCGctagaagtccg-3'	-
5'-gcgacAAACCTGAGCCActagaagtccg-3'	+
5'-gaaggAACCTGAGCCActagaagtccg-3'	-
5'-gaaggAAACCTGAGCCActagaagtccg-3'	+
5'-gcgacAAACCTGAGCCAttgccgcaaga-3'	-
5'-gcgacAAACCTGAGCCgttgccgcaaga-3'	-
5'-gcgagAAACCTGAGCCAttgccgcaaga-3'	+
5'-gcgacAAACCTGAGCCActgcccgaaga-3'	+
5'-gccttgAACTCTGAGCCAttgat-3'	-
5'-gaaatgATACCTGATCCCAcctta-3'	+
<i>The MPI1 element</i>	
5'-ggttctGAGGAGTGATttgcaacgc-3'	+
5'-ggttctGAGGAGTGAgcagatgtag-3'	-
5'-ggttctGAGGAGTGATcgatgtag-3'	+
5'-gaagcactAGGAGTGATcgatgtag-3'	-
5'-gaagcacGAGGAGTGATcgatgtag-3'	+
5'-ggttcttAGGAGTGATttgcaacgc-3'	-
5'-ggttctGAGGAGTGAgttgcaacgc-3'	-
5'-ggttctGAGGAGTGgTttgcaacgc-3'	-
5'-ggttccGAGGAGTGATttgcaacgc-3'	+
5'-ggttctGAGGAGTGATctgcaacgc-3'	+
5'-gcaagtcaGAGGAGTGATgtgtccaggt-3'	+
<i>The MPI2 element</i>	
5'-gcaacgCAATGGAgcgaggaa-3'	+
5'-gcaacgCAATGGAgatattgc-3'	+
5'-gcaacgCAATGGTcatattgc-3'	-
5'-gcaacgCAATGGAcataattgc-3'	+
5'-gagccgagAATGGAcataattgc-3'	-
5'-gagccgcaAATGGAcataattgc-3'	+
5'-gcaacgAATGGAgcgaggaa-3'	-
5'-gcaacgCAATGGTgagaggaa-3'	-
5'-gcaacaCAATGGAgcgaggaa-3'	+
5'-gcaacgCAATGGAccgaggaa-3'	+

Methylation analysis

By PCR. DNA prepared from 10–20 pooled blastocysts or 20–40 pooled oocytes was digested with *PvuII*, *PvuII* + *HhaI* or *PvuII* + *HpaII* and subjected to PCR using 10 μ Ci/ μ l of [α - 32 P-dCTP] and primers as described below. 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 when the site is methylated and thus refractory to digestion by the methylation-sensitive restriction endonuclease *HpaII* or *HhaI*. The PCR primers used for the MutA–MutE transgenes were upper, 5'-CTCTCAGAACTGTTCC

TGC-3', and lower: 5'-GCACTTTCCTACTACTAGAATCC-3'. All DNA samples were tested for complete digestion by the restriction enzyme using ApoAI primers (8).

By Southern blotting. Samples of 1–5 μ g DNA prepared from mouse tail and sperm as described above were digested with *PvuII*, *PvuII* + *HhaI* or *PvuII* + *HpaII*. Digested DNA was resolved by gel electrophoresis, Southern blotted and probed with a labeled fragment produced by PCR using 5'-GCAAGAGCAGTCATTTCC-3' as upper and 5'-CGTCC-CAGGCAATGGCTGC-3' as lower primer. The PCR product was digested with *PvuII* and end labeled with [α - 32 P]dCTP and RediPrime labeling system (Amersham).

Band-shift assays

Nuclear extracts were prepared from undifferentiated, differentiated, parthenogenetic and androgenetic ES cells, as well as from F9, L8, Hela, HEK293 and MEL cells, as described before (16). Aliquots of 3–5 \times 10⁷ ES cells grown in DMEM–Ham's F12 medium were supplemented with 20 pg/ml LIF (Gibco). Labeled oligonucleotides as listed in Table 2 were prepared by end filling using Klenow DNA polymerase I (Roche Industries). The radioactive probe (10⁴ cpm/20–50 pg) was incubated at 30°C for 30 min, with nuclear extract (~10 μ g protein) in a buffer containing 12 mM HEPES, 60 mM KCl, 0.6 mM Na₂EDTA, 0.6 mM DTT, 5 mM MgCl₂ and 1 μ g poly (dI–dC) in a final volume of 20 μ l. The reaction mixtures were electrophoresed on a 4% polyacrylamide gel. The normal and mutated oligonucleotides used are described in the legend to Figure 3.

Transfections and CAT assay

Human embryonic kidney HEK-293 were grown in DMEM supplemented by 10% heat-inactivated fetal calf serum. The cultures were split into 35 mm dishes, 24 h prior to transfection. We used DOTAP Liposomal Transfection Reagent (Roche Industries), to introduce into the cells 2 μ g of the pBLCAT3 plasmid that contains the constructs mutated as described above, together with 1 μ g of a standard RSV–LacZ construct. The cells were harvested 48 h after transfection and cell extracts were prepared in 100 μ l of 0.25 M Tris pH 8 buffer by three cycles of freezing and thawing. Transfection efficiency was determined in each sample by the β -gal assay in a reaction mixture containing 415 μ l of 0.1 M phosphate buffer pH 7.5 and 85 μ l of *o*-nitrophenyl β -D-galactopyranoside in 0.1 M phosphate buffer. Following 30 min of incubation at room temperature the reaction was terminated by the addition of 144 μ l of 1 M Na₂CO₃. The CAT assay was performed in a 100 μ l reaction mixture consisting of 25 μ l of 1 M Tris pH 8, 20 μ l acetyl coenzyme A (3.5 mg/ml) and 2.5 μ l D-threo-(dichloroacetyl-1-¹⁴C) chloramphenicol (Amersham, UK). Following 1 h of incubation at 37°C, chloramphenicol was extracted in 1 ml ethyl acetate followed by evaporation and resuspension of the samples in 25 μ l ethyl acetate. TLC silica gel 20 \times 20 sheets (Merck) were used for ascending chromatography in chloroform–methanol (190 : 10 v/v). The dried sheets were exposed to X-ray film overnight (Kodak).

RT-PCR assay

Brain RNA from the AS-SMP-CAT mutated transgenes were prepared using TriPure isolation reagent (Roche Industries) according to the manufacturer's instructions. RNA was reverse transcribed using oligo (dT) primer. The RT-PCR products were further amplified by PCR using the following primers from the CAT gene sequences: CAT upper, 5'-CCTTACAAG-CCGCTGAACAA-3''; CAT lower, 5'-CTTGGCGCAAATG-TTGCTT-3'. To test the RNA samples for absence of DNA contamination primers flanking Snrpn intron 6 were used: upper, 5'-TTGGACTTCCCCCTGCTCGTC-3'; and lower, 5'-GCAGTAAGAGGGGTCAAAGC-3'.

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