Aberrant Methylation of an Imprinted Gene U2af1-rs1(SP2) Caused by Its Own Transgene*

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Genomic imprinting refers to the parental allele-specific expression of genes. The precise mechanism underlying this phenomenon, which may involve DNA methylation, is not yet known. U2af1-rs1(SP2) is an imprinted gene expressed from the paternal allele and is methylated on the maternal allele. Here we report an artificial system in which expression and methylation of the endogenous imprinted gene U2af1-rs1 can be affected by interaction with its own transgene in the testis. We suggest that there is a mechanism in male gametogenesis by which the U2af1-rs1 gene is kept unmethylated to be expressed in the offspring in addition to a mechanism in female gametogenesis by which the U2af1-rs1 gene is methylated and is not expressed in the offspring.

The epigenetic phenomenon of genomic imprinting determines an inequality of the maternal and paternal genomes. In mammals, genomic imprinting ensures functional inequality of paternal and maternal genomes in the fertilized egg and causes developmental failure of embryos produced by parthenogenesis or by gonogenesis or androgenesis (1–6). The precise mechanism underlying this phenomenon, which may involve DNA methylation, is not yet known. Previously, we cloned an imprinted gene that is involved in genomic imprinting, and as a result the machinery would be disturbed. The XbaI fragment cloned from C57BL/6 was the 8.3-kb genomic fragment that contained the whole coding of U2af1-rs1 plus 2.9 kb upstream from the transcription initiation site and 2.0 kb downstream from the poly(A) signal. This fragment was injected into fertilized eggs of C57BL/6 by a previously described method (10).

Transgenic Mice—The insert of Xb5-sf was purified from agarose gel and microinjected into fertilized eggs of C57BL/6 by a previously described method (10).

Southern and Northern Analysis—Southern and Northern hybridization analysis was performed for 20 h at 65 °C in a solution of 6 × SSC (0.9 M NaCl and 0.09 M sodium citrate), 0.5% (w/v) of SDS, 5 × Denhardt’s solution (1% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 100 μg/ml heat-denatured herring-sperm DNA, and heat-denatured probes (1 × 10⁶ cpm/ml). Membranes were washed for 20 min at 65 °C in 0.1 × SSC, 0.1% SDS. The intensity of the band was measured by using a BAS2000 image analyzer (Fuji).

RT-PCR—Total RNA, prepared by the acid-phenol method (11), was applied to RT-PCR as described previously (12).

RESULTS AND DISCUSSION

To study the trans-acting mechanism of genomic imprinting, we made transgenic mice of an imprinted gene, U2af1-rs1 using the XbaI fragment (Fig. 1a) (9). We expected that the多 copy transgene would titrate the trans-acting factor which is involved in genomic imprinting, and as a result the machinery would be disturbed. The XbaI fragment cloned from C57BL/6 was the 8.3-kb genomic fragment that contained the whole coding of U2af1-rs1 plus 2.9 kb upstream from the transcription initiation site and 2.0 kb downstream from the poly(A) signal. This fragment was injected into fertilized eggs of C57BL/6. We obtained five transgenic F1 animals but only two transgenic F1 animals had offspring (Tg8 and Tg28). Both of these founder animals had 20–30 transgene copies, and the expression level was twice to three times that in the wild-type mouse (data not shown). We could distinguish endogenous and transgenic U2af1-rs1 by digestion with EcoRV because the transgene was constructed to have an additional EcoRV site in the 5'-noncoding region (Fig. 1a). There is a SmaI site at the 199-bp upstream position from the initiation site of transcription. This site on the maternal allele is methylated and then not digested by SmaI in the wild-type mouse. On the contrary, the site on the paternal allele is not methylated and is then completely digested by SmaI (Fig. 2).

The methylation status of the SmaI site of endogenous U2af1-rs1 was examined in the offspring of hemizygous transgenic females crossed with wild-type males and was found to be methylated as in the wild-type mouse (n = 138, data not shown). Next we examined the methylation status of the offspring of hemizygous transgenic males crossed with wild-type...
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Fig. 1. The methylation status of the endogenous U2af1-rs1 gene in the offspring of hemizygous transgenic males crossed with wild-type females. a, restriction map of endogenous and transgenic U2af1-rs1 gene. The transgene has an additional EcoRV site in the 5′-noncoding region. The bar indicates the probe used. The solid box indicates the transcribed region. b, methylation-sensitive Southern blot profile of the offspring of hemizygous transgenic males crossed with wild-type females. Genomic DNA was digested with BanHI, EcoRV, and Smal, size fractionated on a 1% agarose gel, and hybridized to the probe indicated. Tg8 and Tg28 indicate the offspring of Tg8 and Tg28, respectively. Asterisks indicate the mice in which the endogenous U2af1-rs1 on the paternal allele was methylated in addition to the maternal allele.

In most offspring, endogenous U2af1-rs1 transmitted from the transgenic fathers was not methylated like that in the wild-type mouse (110/123). However, in 11% (13/123) of the offspring, the endogenous U2af1-rs1 transmitted from the transgenic fathers was methylated unlike that in the wild-type mouse (Fig. 1b). This aberrant methylation is not restricted to the Smal site because we can also detect it with NolI, HpaII, and HhaI (data not shown). Therefore, most of the allelic methylation sites may be related with this phenomenon. This methylation of the endogenous gene was not only observed in the transgene-positive offspring, but also in the transgene-negative offspring (Fig. 1b). Therefore, this aberrant methylation may be caused by the transgene in the father, rather than in the offspring. The aberrant methylation could not be due to any mutations affecting the methylation of the endogenous U2af1-rs1 gene. If a mutation occurred in the father's germ cell and was transmitted to the offspring to cause aberrant methylation, this mutation would be further transmitted to cause aberrant methylation in their offspring. However, no aberrant methylation was observed in the offspring of the transgene-negative males with the aberrant methylation crossed with wild-type females (data not shown). This aberrant methylation is not a naturally occurring phenomenon because we could not detect it in any wild-type mice (n = 130). This phenomenon does not seem to be a special effect caused by integration of the transgene into a special site because it was observed in the offspring of two independent founder animals Tg8 and Tg28. Southern blot analysis of Tg8 and Tg28 also supports this speculation (data not shown).

To examine whether the aberrant methylation of the endogenous gene is related to the expression of the U2af1-rs1 gene, a transgene-negative mouse with the aberrant methylation was analyzed by Northern blot analysis. We could not detect any signal in this mouse in contrast to the strong signal in the wild-type and normal offspring (Fig. 3). Despite the absence of U2af1-rs1 expression, no gross abnormality was observed in the mouse. A related gene, U2af1-rs2 (13), may compensate U2af1-rs1. RT-PCR using a radioisotope was performed to detect the weak expression of U2af1-rs1. There was no signal in the mouse in contrast to the strong signal of paternal alleles of wild-type and normal offspring (Fig. 4, short exposure). A long exposure revealed that both paternal and maternal alleles were equally expressed at a low level (Fig. 4). This level was equal to the level expressed from the maternal alleles in wild-type and normal offspring. Therefore, the aberrant methylation is related to the suppression of the endogenous U2af1-rs1 gene.

The methylation status was also examined in the testis and sperm of the transgenic father. In the testis and sperm, the endogenous gene was mostly methylated in the transgenic father in contrast to complete demethylation in the wild-type mouse (Fig. 5). This confirms that the aberrant methylation was caused by a transgene in the testis of the father. However, the population of methylated endogenous U2af1-rs1 is different between the sperm of the father and its offspring. This may be due to the demethylation of the gene in the remaining offspring after fertilization or the disadvantage to survival of the sperm with methylated U2af1-rs1.

Our findings suggest the existence of a trans-acting factor(s) that interacts with the U2af1-rs1 gene and prevents it from being methylated in the testis. In the testis of the father with the transgene, this factor seems to be titrated by the multicopy transgene. It is intriguing to think that a large CpG island in the U2af1-rs1, which has a very high GC and CpG content and contains a multiple tandem repeat of short sequence (8, 9), is involved in the titration. The possibility that the overproduction of U2af1-rs1 protein by the transgene elicits some kind of response to cause aberrant methylation cannot be denied. However, this seems to be unlikely because the U2af1-rs1 expres-
the transgenic father also should be kept methylated in all offspring. However, only 11% of the offspring had an aberrant methylation. Therefore, the machinery involving this transacting factor(s) should be different from that making a gametic imprint in female gametogenesis. In other words, U2af1-rs1 seems to have two mechanisms for regulating the methylation status in gametogenesis. By one mechanism in female gametogenesis, the U2af1-rs1 gene is methylated and is not expressed in the offspring. By another mechanism in male gametogenesis, the U2af1-rs1 gene is kept unmethylated to be expressed in the offspring. Here, we observed that the latter mechanism was affected by a multicopy transgene. Whether this mechanism can be similarly applied to other imprinted genes remains to be determined.

This is the first observation that the endogenous methylation of an imprinting gene was affected by its own transgene. This artificial system would be useful to study the imprinting mechanism.

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FIG. 3. Expression of U2af1-rs1 in transgene-negative offspring. Brain RNA from wild-type mouse and transgene-negative offspring of Tg8 was subjected to Northern blotting. Ethidium bromide staining indicates an equal amount of RNA in each lane. No signal could be detected in the transgene-negative offspring, which had methylation on the paternal allele in contrast to a strong signal in both the wild-type mouse and transgene-negative mouse, which had no methylation on the paternal allele. The same result was obtained for Tg28 offspring. The presence (+) or absence (−) of methylation on the paternal allele is indicated.

FIG. 4. Analysis of allelic expression in transgene-negative offspring. Brain RNA from wild-type mouse and transgene-negative offspring of Tg8 was subjected to RT-PCR followed by digestion with MspI. By digestion with MspI, paternal (C57BL/6) and maternal (PWK) alleles could be distinguished. The presence (+) or absence (−) of methylation on the paternal allele is indicated. RT-PCR was performed with (+) or without (−) reverse transcriptase.

FIG. 5. The methylation status of the endogenous U2af1-rs1 gene in sperm and testis. Genomic DNA from a Tg28 parent was digested with BamHI, EcoRV, and Smal, size fractionated on a 1% agarose gel, and hybridized to the probe indicated in Fig. 1. The endogenous gene in the testis and sperm was mostly methylated in the transgenic father in contrast to complete demethylation in the wild-type mouse. The same result was obtained for Tg8 parents.
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