Disruption of Imprinted Expression of U2afbp-rs/U2af1-rs1 Gene in Mouse Parthenogenetic Fetuses*

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Yusuke Sotomaru‡, Yosuke Kawase‡‡, Takayuki Ueda†, Yayoi Obata†§, Hiroshi Suzuki**
Ikuo Domeki†, Izuo Hatada‡‡, and Tomohiro Kono¶¶

From the ‡Department of Animal Science, Tokyo University of Agriculture, 1737, Funako, Atsugi-shi, Kanagawa 243-0034, Japan, the §Research Laboratory for Molecular Genetics, Niigata University, 757 Ichiban-chou, Asahimachidori, Niigata 951-8510, Japan, the **Pharmaceutical Technology Laboratory, Chugai Pharmaceutical Co., Ltd., 1-135, Komakado, Gotemba, Shizuoka 412-8513 Japan, the ¶¶Gene Research Center, Gunma University, 3-39-22 showa-machi, Maebashi-shi, Gunma 371-8511, Japan and the §§Department of BioScience, Tokyo University of Agriculture, 1-1-1, Sakauragaoka, Setagaya-ku, Tokyo 156-8502, Japan

The present study shows that the U2afbp-rs gene, a paternally expressed imprinted gene, is activated and expressed in a biallelic manner from maternal alleles in parthenogenetic mouse fetuses on day 9.5 of gestation. The mean expression was detected to be 88% (51–134%) of that in control biparental fetuses, using real-time quantitative reverse transcription and polymerase chain reaction. This disrupted expression of the gene was associated with changes in the chromatin structure but not with the methylation pattern in the regulation region. The present results show that parental specific expression of imprinted genes is not always maintained in uniparental embryos. This suggests that both parental genomes are necessary to establish parental specific expression of the U2afbp-rs gene.

Genomic imprinting is characterized by parental specific expression of a group of genes. This expression is thought to be established according to the specific markers imposed on the genome during gametogenesis (1–5). It is well known that imprinted genes are usually accompanied by differential methylation levels on CpG islands in the promoter region (6–9). This leads to definitive functional differences between male and female gametes, causing parthenogenetic/gynogenetic and androgenetic embryos, which consist, respectively, of two sets of male and female gametes, causing parthenogenetic/gynogenetic and androgenetic embryos, which consist, respectively, of two sets of maternal or paternal genomes. These embryos die by day 10 of gestation (10–12). Molecular analyses of imprinted genes showed that parthenogenetic/gynogenetic and androgenetic fetuses express only maternally expressed and paternally expressed imprinted genes, respectively (13).

Since insulin-like growth factor II (Igf2)1 (14) and its receptor (Igf2r) (15) genes were first identified as imprinted genes in 1991, around 40 of these types of genes have been identified. However, the genes identified thus far constitute only a small portion of the number of imprinted genes, which is estimated to be 1–2% of all genes. Recently, an inclusive attempt was made to identify imprinted genes by the subtraction method using parthenogenetic and androgenetic mouse embryos (16, 17). Some imprinted genes, such as Peg1/Mest (16), Peg3 (18), and Meg/MegR10 (17) were successfully identified, but the number is smaller than expected. This limited success in identifying genes may suggest that the expression of some imprinted genes is unregulated in uniparental embryos.

The U2afbp-rs gene is a paternally expressed imprinted gene located on mouse chromosome 11 (19–21). This gene was identified by a systematic screening performed using a restriction landmark genome scanning procedure for loci subject to imprinted methylation (19–21). The gene is expressed in all tissues throughout development, with expression restricted to the paternal allele and predominantly in the brain of the adult mouse (21), but its function is unknown. In this study, we show that the paternally expressed U2afbp-rs gene is expressed abundantly from maternal alleles in parthenogenetic mouse fetuses. This suggests that the parental genome is necessary for establishing and/or maintaining the paternal expression of the gene.

MATERIALS AND METHODS

Production of Uniparental Embryos—B6CBF1 (C57BL/6N X CBA) mice were used as oocyte and sperm donors to produce embryos. Oocytes at metaphase II were collected from superovulated mice and artificially activated with SrCl2 (Sr2+) (22, 23). To produce diploid parthenogenetic embryos, during the culture in Sr2+ medium and following the first 4 h of culture in M16 medium, 5 μg/ml cytochalasin B was added to these media to inhibit a second polar body extrusion. Androgenetic embryos were produced by in vitro fertilization of enucleated oocytes according to the procedure reported previously (24). When required, pronuclear transfer (25) was performed to produce diploid androgenetic monosomes in monospermic embryos. Control biparental embryos were produced by in vitro fertilization. The obtained blastocysts were transferred to the uterine horns of CD-1 females on day 3 of pseudopregnancy (2.5 days postcoitum), and fetuses were recovered at day 9.5 of gestation.

Expression Analysis—Total RNA was isolated from the fetuses at day 9.5 of gestation using an SV Total RNA Isolation System (Promega). The first strand of cDNA was synthesized from 1 pmol of each primer, 1.5 mCi MgCl2, and 250 μM dNTPs. The amplification consisted of a total of 35 cycles at 95, 63, and 72 °C, respect-
**Imprinted Gene in Mouse Parthenogenones**

**RESULTS**

To confirm that the paternal expression of U2afbp-rs gene is maintained in uniparental fetuses, we first examined the expression of the U2afbp-rs gene in each parthenogenetic and androgenetic fetus at 9.5 days by semi-quantitative RT-PCR.

**Analysis of Methylation in the Promoter and Open Reading Frame (ORF) Regions**—In an upstream Cpg island (regions I, II, and IV) and the ORF (region III) of the U2afbp-rs gene (26) in parthenogenetic and normal biparental fetuses, the methylation pattern was analyzed by Southern hybridization using genome DNAs that had been digested by methyl-sensitive restriction enzymes (Fig. 1). Extracted genome DNAs were digested by DraI and HindIII to obtain DNA fragments containing the Cpg islands or by EcoT14I and PshI for ORF. Then the DNA fragments were digested by HpaII or HhaI and subjected to electrophoresis (1.5% NuSieve:agarose (3:1)). The DNAs were transferred to a nylon membrane (Hybond-N+), and the hybridized membrane was detected by autoradiography. The probes used were derived from PCR products using primer sets of the ORF and antisense reverse transcription and polymerase chain reaction (RT-PCR).

Quantitative analysis of gene expression was performed by means of real-time quantitative reverse transcription and polymerase chain reaction (RT-PCR). By adding a fluorescently labeled probe (5'-TGGATAACTGGATAGACG-3') to the PCR reaction mixture, the quantity of PCR products was detected by monitoring the luminous intensity with an ABI PRISM 7700 Sequence Detection System (PerkinElmer Life Sciences).

**Imprinted Gene**—In parthenogenetic and control biparental fetuses, the methylation levels in the promoter regions (6–9) were determined by the DNase-I hypersensitivity assay (Fig. 5). After digestion of the RT-PCR products (243 bp) with HpaII, two bands (194 bp for B6CBF1 and 177 bp for JF1 alleles) were detected showing that the gene was expressed from both alleles. It was also confirmed that, in the control biparental fetuses, the gene was not expressed from the maternal allele. These results suggest that the regulation mechanism for paternal specific expression of the U2afbp-rs gene is not established in parthenogenetic fetuses. The quantitative analysis in androgenetic fetuses, which have two paternal alleles, showed that expression of the U2afbp-rs gene was almost equal (mean 83%, 45–109%) to that in controls (without RT), indicating that both alleles were expressed from the paternal allele. However, in the parthenogenetic fetuses, two bands (194 bp and 177 bp for JF1 alleles) were detected, suggesting that only the maternal alleles were possibly hemimethylated. However, in the parthenogenetic fetuses, no sequence was digested in the HpaII sites in the HhaI site, indicating that both alleles were entirely hypermethylated despite considerable expression of the gene. This suggests that other regulation factors may be involved in the disruption of the regulated expression of the U2afbp-rs gene in the parthenogenetic mouse fetuses.

Because chromatin structure is also related to gene expression (27, 28), chromatin conformation of the U2afbp-rs gene was determined by the DNase-I hypersensitivity assay (Fig. 5).
The DNase-I hypersensitive sites in the DraI-EcoRI fragment were detected in both control and parthenogenetic fetuses (arrowheads in Fig. 5) when the fragments were digested by more than 20 units/ml DNase-I. The status of DNase-I hypersensitive sites of parthenogenetic fetuses was similar to that of the controls, suggesting that the chromosomes of the parthenogenetic fetuses have an open conformation. This perhaps caused the unregulated expression of the U2afbp-rs gene in the parthenogenetic fetuses.

**DISCUSSION**

We reported previously that expressions of mouse paternally expressed imprinted genes Peg1/Mest (16), Igf2 (14), Peg3 (18), and Snrpn (29) are not properly detected by RT-PCR in mouse

parthenogenetic fetuses at day 9.5 of gestation (30). Further analysis by real-time quantitative RT-PCR confirmed parental specific expression of the Igf2 gene in both the parthenogenetic and androgenetic fetuses (31). These previously published observations show that parental expression of imprinted genes is generally maintained in uniparental fetuses. The current study demonstrated, however, that anti-theoretical gene expression of the paternally expressed imprinted gene U2afbp-rs is caused in the parthenogenetic fetuses. The expression reached an av-
methylated at 88%, ranging from 31 to 134% of that in biparental fetuses. Notably, the expression occurred at a level very different from that of leakage, suggesting that both maternal and paternal alleles are necessary for the regulated parentally specific expression of imprinted genes. U2afbp-rs was identified by the restriction landmark genome scanning procedure based on loci subject, but the subtraction method (16, 17) failed to trap the U2afbp-rs gene, perhaps because the expression of the gene originates from the maternal alleles of parthenogenones.

During oocyte growth, the maternal genome is epigenetically modified, and maternally specific expression patterns of imprinted genes are established as a result of the primary imprinting. To demonstrate this, we obtained parthenogenetic mouse fetuses developed from constructed oocytes that contained nongrowing and fully grown oocyte-derived genomes that were derived from immature oocytes at diplonte stage and mature oocytes at metaphase II, respectively. The experiments clearly demonstrated that these fetuses develop to day 13.5 of gestation (32), and paternally expressed imprinted genes are expressed from the nongrowing oocyte alleles (30). It has also been found that U2afbp-rs is active in the nongrowing oocyte allele in such parthenogenetic fetuses2. Before fertilization, the maternal allele of the U2afbp-rs gene has been imprinted to be repressed by the methylation of the CpG island in specific sites of its promoter regions (regions II and IV). A wide range of methylation occurs in the promoter region (region I) at the two-cell stage. This methylation may play a crucial role in determining allele-specific expression, although maternal expression was maintained at later stages (26). The question raised by the present results is whether the expression of U2afbp-rs from maternal alleles is associated with the demethylation of the regulatory region. Analysis of methylation status by Southern hybridization and HpaII-PCR (data not shown) showed that the region was hypermethylated and that expression of the U2afbp-rs gene was from the maternal allele in the control fetuses. A previous report (33) suggested that methylation levels in imprinted genes (Igf2r, Igfr2, Peg1Mest, Peg3, and H19) failed to trap the U2afbp-rs gene, perhaps because the expression of the gene originates from the maternal alleles of parthenogenones.

Many imprinted genes, such as Igf2, Igf2r, Peg1Mest, Peg3, and H19, maintain their parental specific expression in uniparental embryos (13, 16, 18, 31, 35), that is, in parthenogenetic/gynogenetic and androgenetic embryos. However, the present results show that this is not the case for the U2afbp-rs. This suggests that both parental alleles are necessary for the establishment and maintenance of the parentally specific expression pattern. The molecular mechanisms underlying the disruption of imprinted status in parthenogenetic embryos remain to be clarified. Further investigation into this kind of disruption could provide greater insight into the characteristics of imprinted genes.

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