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Antisense Transcription Occurs at the Promoter of a Mouse Imprinted Gene, *Commd1*, on the Repressed Paternal Allele

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The *Commd1* gene is imprinted in the adult mouse brain and is predominantly expressed from the maternal allele. A paternally expressing imprinted gene, *U2af1-rs1*, resides in the first intron of *Commd1* in an antisense orientation. We found that RNA polymerase II phosphorylated at serine 2 of the carboxyl-terminal domain repeats, a marker of transcription elongation, is enriched on the paternal allele than on the maternal allele in the *Commd1* promoter. The *Commd1* promoter harbours no allelic differences in DNA methylation and histone modifications. These results strongly suggested that imprinting of *Commd1* is generated by interference with paternal *Commd1* transcription by the oppositely directed *U2af1-rs1* transcription.

Key words: genomic imprinting, epigenetics, DNA methylation, histone modification.

Abbreviations: DMR, differentially methylated region; CTD, carboxyl-terminal domain; ICR, imprinting control region; ChIP, chromatin immunoprecipitation; Pol II, RNA polymerase II; H3-K4me3, histone H3 trimethylated at K4; H3-K9me2, histone H3 dimethylated at K9; H3-K27me3, histone H3 trimethylated at K27; H3-Ac, acetylated histone H3; pS2-Pol II, RNA polymerase II phosphorylated at S2; pS5-Pol II, RNA polymerase II phosphorylated at S5.

Imprinted genes are expressed from one of the two parental alleles, in a parent specific manner. Imprinted genes are generally linked to differentially methylated regions (DMR) that harbour allele-specific methylation (*1*). This differential methylation occurs in a CpG island, a genomic region highly enriched in CpG dinucleotides. Among the DMRs linked to imprinted genes, DMRs whose methylation is established during gametogenesis and is maintained throughout development have been described. Such DMRs coordinately regulate imprinting of imprinted genes linked to them and are termed imprinting control regions (ICR).

The murine imprinted gene *Commd1* (also known as *Murr1*) is predominantly expressed from the maternal allele in adult brain (*2*). This gene contains another imprinted gene, *U2af1-rs1*, in its intron 1. *U2af1-rs1*, an intron-less gene, is expressed exclusively from the paternal allele and is transcribed in the direction opposite to the *Commd1* gene (Fig. 1A). The genes surrounding *Commd1* are not imprinted and thus, *Commd1* and *U2af1-rs1* form a small imprinted domain on chromosome 11 (*3*). The *U2af1-rs1* promoter locates in a maternally methylated DMR. The maternal methylation is established during female gametogenesis, and is maintained throughout the development (*3*, *4*). To date, no other DMR has been identified in the genomic region of the *Commd1*/*U2af1-rs1* locus (*3*). Taken together, it is hypothesized that the DMR of *U2af1-rs1* is the ICR for this small imprinted domain. It is plausible that the paternal expression of *U2af1-rs1* is caused by the methylation of the maternal promoter in the DMR. However, the mechanism underlying *Commd1* imprinting is yet to be elucidated.

The *Commd1* promoter region has a CpG island, and thus allele-specific methylation was the most likely mechanism underlying *Commd1* imprinting. Bisulfite sequencing was undertaken on the promoter in adult brain to determine the methylation status in detail. In order to distinguish parental alleles with SNPs, we used adult brain tissue from an F1 mice resulting from the cross between C57BL/6 female and PWK male. The experiment on mice was approved by the Saga University Animal Care and Use Committee and was carried out according to the Regulation on animal experimentation at Saga University. On both parental alleles, all of the analyzed CpG sites within the region remained essentially unmethylated (Fig. 1B). This result suggests that allelic differences in *Commd1* expression do not depend on the differential methylation of the promoters.

Histone modifications are also important epigenetic determinants that influence the transcriptional activity of genes (*5*). Methylation of histone H3 at lysine 9 (K9) is associated with repression of gene expression. In contrast, methylation of histone H3 at K4 and acetylation of histone H3 are associated with active gene expression. A similar phenomenon is also observed for

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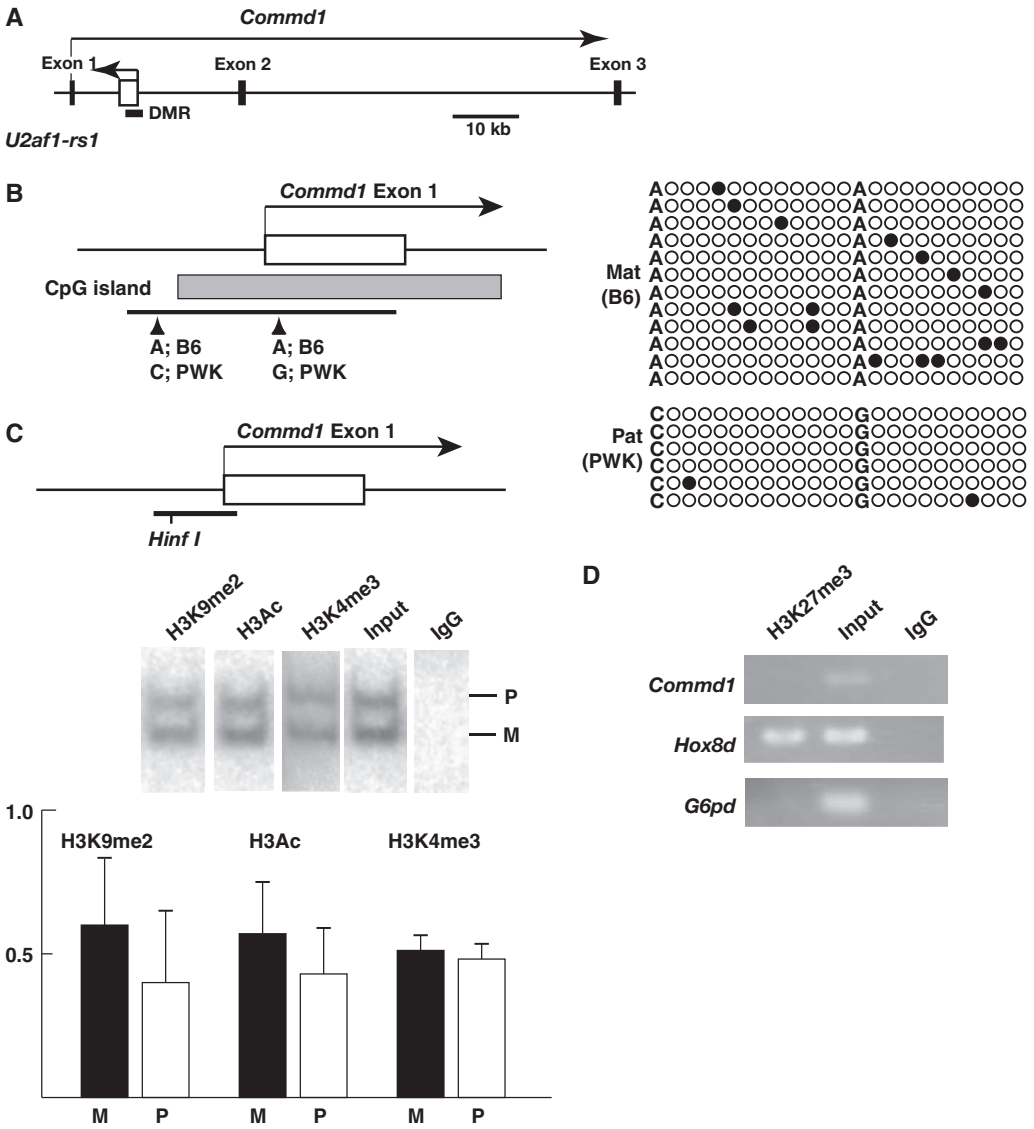

Fig. 1. **Epigenetic analyses of the *Commd1* promoter.** (A) A schematic diagram of the *Commd1* and *U2af1-rs1* genes. The thin lines with an arrow head indicate the direction of transcription. The bar underlining the *U2af1-rs1* gene outlines the DMR of the gene. The gene diagrams are to scale. (B) Bisulfite sequencing of the *Commd1* promoter. The *Commd1* exon 1 (205 bp) and the CpG island (480 bp) are shown schematically to scale. The horizontal bar underlining the CpG island indicates the region PCR-amplified for sequencing. The SNPs used for the discrimination of the alleles are indicated underneath. Genomic DNA was isolated from mouse brain using the QIAamp DNA mini kit (Qiagen, USA) and was modified with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Germany). PCR was carried out with the modified DNA and the primers, BS-MurrA: 5′-GGAGTGAAGAAGGAAATAAT-3′ and BS-MurrB; 5′-AACTCCTCCGATAACACTTCPCR-3′. Amplification was performed under the condition of initial denaturation at 95°C for 12 min; followed by 45 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 1 min. PCR products were cloned and were sequenced. A total of 22 CpG sites are identified in the region. Each CpG site is represented by an open circle (unmethylated) or filled circle (methylated). Each row of circles illustrates the result of one PCR product clone. SNPs are also outlined. (C) The histone modifications at the *Commd1* promoter. ChIP analyses were performed with antibodies directed against H3-K9me2 (ab7312, Abcam, USA), H3-Ac (06-599, Upstate, USA), or H3-K4me3 (ab8580, Abcam, USA). Control experiments included the input chromatin used for ChIP (Input) and the chromatin precipitated with nonimmune-antisera (IgG). The analyzed region is highlighted with a horizontal bar under the promoter. The *Hinf* I site is located on the maternal allele and was used to discriminate from the paternal alleles. Brain tissue was treated with EpiQuik Tissue ChIP kit (Epigentec, Inc., USA) and the extract was immunoprecipitated with antibodies directed against each of the modified histones. PCR was performed under the condition of initial denaturation at 95°C for 12 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min. PCR primers are Murr9, 5′-TGCACATCGGAGTGAAGAAG-3′ and Murr10, 5′-TGTCCGTGAAAGGCGTTCTG-3′. End-labelled PCR products were obtained by the addition of one primer end-labeled with ^{32}P to the final amplification cycle (Hot-stop PCR) (*9*, *10*). PCR products were digested with *Hinf* I and electrophoresed. Representative electrophoregram of ChIP analyses on H3-K9me2, H3-Ac and H3-K4me3 was shown. ^{32}P-labeled DNAs in the electrophoregram were quantitated with a BAS-2000 (Fujifilm Co., Japan) and normalized with input values. The experiments were repeated six times on H3-Ac and four times on H3-K9me2 and H3-K4me3. Quantification results are summarized in the graph and are presented as mean with SD. M, maternal allele; P, paternal allele. (D) ChIP analyses on H3-K27me3 at the *Commd1* promoter. The ChIP experiments were performed same as in (C) with the antibody against H3-K27me3 (07-449, Upstate, USA). PCRs were done with unlabeled primers and PCR products were electrophoresed without *Hinf* I digestion. *Hox8d* and *G6pd* served as the positive and negative controls, respectively. The analyses were performed four times and produced consistent results.

imprinted genes (*6–8*). Thus, we performed chromatin immunoprecipitation (ChIP) on adult brain of the F1 mice to see allelic differences in dimethylated histone H3 at K9 (H3-K9me2), trimethylated histone H3 at K4 (H3-K4me3) or acetylated histone H3 (H3-Ac) at the *Commd1* promoter. We found that there were no significant differences in the histone modifications at the promoter for the two parental alleles (Fig. 1C). On the *U2af1-rs1* promoter, we have obtained the results consistent with the previous report by Fournier *et al*. (*8*), which indicated the enrichment of H3-K4me3 and H3-Ac on the active paternal allele and the enrichment of H3-K9me2 on the repressed maternal allele (data not shown).

Imprinted genes that lack allele-specific DNA methylation also exist in mice. Some of these genes show allelic differences in H3-K27me3 (*11*). As DNA methylation does not occur at the *Commd1* promoter, we next analyzed H3-K27me3 levels. As shown in Fig. 1D, H3-K27me3 is not detected in the promoter. These epigenetic analyses indicate that the imprinting of *Commd1* is not the result of allelic differences in DNA methylation and histone modification at the promoter.

In the previous study, we have found that the relative expression level of *U2af1-rs1* against *Commd1* increased about 10-fold during brain development from the embryo to the adult. We also detected antisense transcripts in the *Commd1* promoter only on the paternal allele in the adult brain (*2*). The paternal allele-specific antisense transcript was also detected further upstream of the *Commd1* gene (Supplementary Figure). The presence of the paternal allele-specific antisense transcripts indicates that transcription elongation, presumably originated from *U2af1-rs1*, occurs via the paternal *Commd1* promoter in the adult brain.

In order to determine whether RNA Polymerase II (Pol II) elongation complexes exist in the *Commd1* promoter on the paternal allele, we analyzed the binding of phosphorylated Pol II to the promoter region. The largest subunit of Pol II harbours a characteristic seven amino acid repeat (YSPTSPS) in the carboxy-terminal domain. Phosphorylation at Ser 5 (S5) in the repeat occurs upon the formation of an active pre-initiation complex at the promoter and thus, phosphorylated Pol II at S5 (pS5-Pol II) is mainly detected in promoter regions. In contrast, Pol II at a promoter is not phosphorylated at Ser 2 (S2) in the repeat, as the modification only occurs and then increases, when the enzyme proceeds to transcription elongation stages. In fact, pS2-Pol II is predominantly detected in the body of a gene (*12*, *13*).

We performed ChIP analyses of pS2-Pol II and pS5-Pol II at the *Commd1* promoter with antibodies directed against pS2-Pol II or pS5-Pol II. As shown in Fig. 2A, pS2-Pol II, which is the enzyme generally absent from promoter regions, was detected in the *Commd1* promoter enriched on the paternal allele. This result indicates that transcription elongation occurs in the *Commd1* promoter on the paternal allele and supports the notion that paternal allele-specific transcription originating from *U2af1-rs1* passes through the *Commd1* promoter. pS2-pol II was also detected in the maternal promoter. Given that the ChIP method detects protein-binding just outside of the PCR-amplified region in addition to that within the region, the pS2-Pol II in the maternal promoter may represent the enzyme bound to exon 1 of *Commd1* and involved in transcription of *Commd1* (Fig. 2B).

Although one can expect that pS5-Pol II exists at the *Commd1* promoter with enrichment on the active maternal allele, pS5-Pol II was detected equally on both alleles (Fig. 2A). Part of the pS5-Pol II on the paternal allele may be Pol II doubly phosphorylated at S2 and S5 (pS2/pS5-Pol II) involved in the elongation, originating from

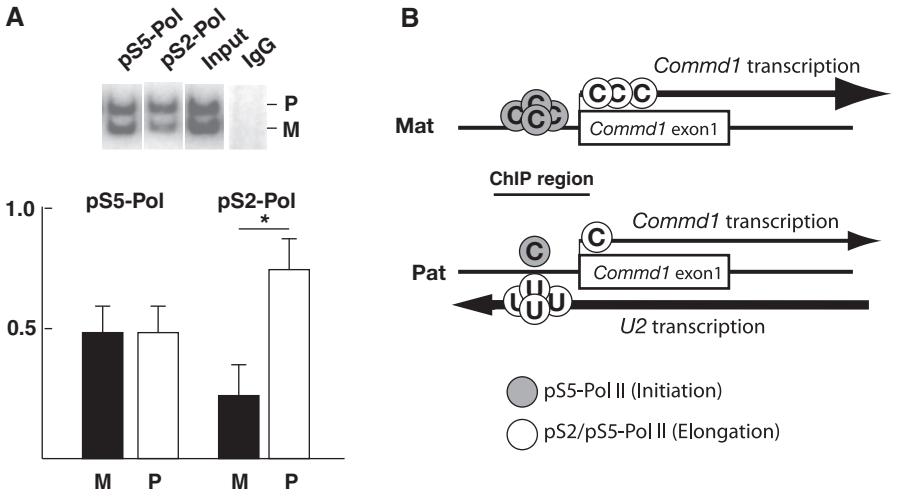

Fig. 2. **The phosphorylated RNA polymerase II at the *Commd1* promoter.** (A) ChIP analyses were performed in the same region as Fig. 1C and D with an antibody directed against pS5-Pol II (ab5131, Abcam, USA) or pS2-Pol II (ab5095, Abcam, USA). ChIP analysis with each antibody was repeated ten times. The asterisks indicate a significant difference ($P < 0.001$ by paired *t*-test). (B) A model of the distribution of phosphorylated Pol II at the promoter. Pol IIs labelled with C are for *Commd1* transcription and those labelled with U are for the transcription of the *U2af1-rs1* gene. The Pol IIs shown in the *Commd1* exon 1 are conjectured to explain the ChIP results in (A) (see text).

U2af1-rs1 (Fig. 2B). The phosphate molecule located at S5 is not dephosphorylated during transcription elongation and thus pS2-Pol II undergoing elongation retains phosphorylated S5 in mammals (*12*). pS5-Pol II and pS2-Pol II are also enriched on the paternal allele in the region ~1,000 bp upstream of the *Commd1* gene (data not shown), where a paternal antisense transcript was detected (Supplementary Figure).

In the current study, we report the detection of Pol II involving in transcription elongation at the *Commd1* promoter on the paternal allele. As allelic differences were not detected in DNA methylation and histone modification in the *Commd1* promoter, transcriptional interference is hypothesized to be the cause of *Commd1* imprinting as follows. In this case, maternal allele-specific methylation at the *U2af1-rs1* promoter resulted in the paternal allele-specific expression of the gene. The transcription of the gene extends to the *Commd1* promoter on the paternal allele only. This antisense transcription interferes with *Commd1* transcription and reduces the expression level of the paternal *Commd1* gene in the adult brain where the relative expression level of *U2af1-rs1* against *Commd1* has increased.

Many previous studies have reported that a gene can be repressed in *cis* by active neighbouring genes through transcriptional interference in yeast (*14*, *15*), *Drosophila* (*16*) and mammals (*17*). In some cases, transcriptional interference plays an important role in the regulation of gene expression (*15*, *16*). *Commd1* is the first imprinted gene described whose imprinted expression is suggested to be regulated by transcriptional interference.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

REFERENCES

1. Reik, W. and Walter, J. (2001) Genomic imprinting: parental influence on the genome. *Nat. Rev. Genet.* **2**, 21–32
2. Wang, Y., Joh, K., Masuko, S., Yatsuki, H., Soejima, H., Nabetani, A., Beechey, C.V., Okinami, S., and Mukai, T. (2004) The mouse *Murr1* gene is imprinted in the adult brain, presumably due to transcriptional interference by the antisense-oriented *U2af1-rs1* gene. *Mol. Cell. Biol.* **24**, 270–279
3. Zhang, Z., Joh, K., Yatsuki, H., Wang, Y., Arai, Y., Soejima, H., Higashimoto, K., Iwasaka, T., and Mukai, T. (2006) Comparative analyses of genomic imprinting and CpG island-methylation in mouse *Murr1* and human *MURR1* loci revealed a putative imprinting control region in mice. *Gene* **366**, 77–86
4. Hatada, I., Nabetani, A., Arai, Y., Ohishi, S., Suzuki, M., Miyabara, S., Nishimune, Y., and Mukai, T. (1997) Aberrant methylation of an imprinted gene *U2af1*-rs1(SP2) caused by its own transgene. *J. Biol. Chem.* **272**, 9120–9122
5. Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science* **293**, 1074–1080
6. Fournier, C., Goto, Y., Ballestar, E., Delaval, K., Hever, A.M., Esteller, M., and Feil, R. (2002) Allele-specific histone lysine methylation marks regulatory regions at imprinted mouse genes. *EMBO J.* **21**, 6560–6570
7. Higashimoto, K., Urano, T., Sugiura, K., Yatsuki, H., Joh, K., Zhao, W., Iwakawa, M., Ohashi, H., Oshimura, M., Niikawa, N., Mukai, T., and Soejima, H. (2003) Loss of CpG methylation is strongly correlated with loss of histone H3 lysine 9 methylation at *DMR*-LIT1 in patients with Beckwith-Wiedemann syndrome. *Am. J. Hum. Genet.* **73**, 948–956
8. Soejima, H., Nakagawachi, T., Zhao, W., Higashimoto, K., Urano, T., Matsukura, S., Kitajima, Y., Takeuchi, M., Nakayama, M., Oshimura, M., Miyazaki, K., Joh, K., and Mukai, T. (2004) Silencing of imprinted *CDKN1C* gene expression is associated with loss of CpG and histone H3 lysine 9 methylation at *DMR*-LIT1 in esophageal cancer. *Oncogene* **23**, 4380–4388
9. Uejima, H., Lee, M.P., Cui, H., and Feinberg, A.P. (2000) Hot-stop PCR: a simple and general assay for linear quantitation of allele ratios. *Nat. Genet.* **25**, 375–376
10. Uejima, H., Lee, M.P., Cui, H., and Feinberg, A.P. (2001) Correction: hot-stop PCR: a simple and general assay for linear quantitation of allele ratios. *Nat. Genet.* **28**, 97
11. Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., Higgins, M., Feil, R., and Reik, W. (2004) Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. *Nat. Genet.* **36**, 1291–1295
12. Morris, D.P., Michelotti, G.A., and Schwinn, D.A. (2005) Evidence that phosphorylation of the RNA polymerase II carboxyl-terminal repeats is similar in yeast and humans. *J. Biol. Chem.* **280**, 31368–31377
13. Hirose, Y. and Ohkuma, Y. (2007) Phosphorylation of the C-terminal domain of RNA polymerase II plays central roles in the integrated events of eucaryotic gene expression. *J. Biochem.* **141**, 601–608
14. Prescott, E.M. and Proudfoot, N.J. (2002) Transcriptional collision between convergent genes in budding yeast. *Proc. Natl Acad. Sci. USA* **99**, 8796–8801
15. Martens, J.A., Laprade, L., and Winston, F. (2004) Intergenic transcription is required to repress the *Saccharomyces cerevisiae SER3* gene. *Nature* **429**, 571–574
16. Petruk, S., Sedkov, Y., Riley, K.M., Hodgson, J., Schweisguth, F., Hirose, S., Jaynes, J.B., Brock, H.W., and Mazo, A. (2006) Transcription of bxd noncoding RNAs promoted by trithorax represses *Ubx in cis* by transcriptional interference. *Cell* **127**, 1209–1221
17. Martianov, I., Ramadass, A., Serra Barros, A., Chow, N., and Akoulitchev, A. (2007) Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript. *Nature* **445**, 666–670