36–41 Nucleic Acids Research, 1995, Vol. 23, No. 1

# Allele-specific methylation and expression of an imprinted U2af1-rs1 (SP2) gene

**Izuho Hatada*, Kazunori Kitagawa, Tetsuji Yamaoka, Xudong Wang, Yuji Arai, Kazuo Hashido, Sachiko Ohishi, Junichi Masuda, Jun Ogata and Tsunehiro Mukai**

National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565, Japan

Received October 19, 1994; Revised and Accepted December 1, 1994

DDBJ accession no. D26474

## ABSTRACT

**The mouse U2af1-rs1(SP2) gene, which was cloned by a two-dimensional genome scanning method, is expressed exclusively from the paternally inherited chromosome. This gene has significant similarity to U2AF and located in chromosome 11, of which maternal duplication/paternal deficiency results in a small body. In this report, we cloned genomic *U2af1-rs1(SP2)* and found its promoter was methylated in a maternal-allele-specific manner. This allelic methylation was not established in parental gametes, but established between 1.5 d.p.c. and 12.5 d.p.c. on the contrary, the allele-specific expression occurred in the two-cell stage when transcription initiates. Absence of the methylation of the upstream region in this stage indicates that methylation is not necessary for inactivation of the expression.**

## INTRODUCTION

Genomic imprinting is the parental-allele-specific expression of genes. In mammals, parental imprinting ensures functional inequality of paternal and maternal genomes in the fertilized egg and causes developmental failure of embryos produced by parthenogenesis or by gynogenesis or androgenesis (1). Parental effect on particular chromosomal regions involving embryo survival and gross phenotypic abnormalities were unequivocally documented by producing paternal or maternal disomies by means of Robertsonian and reciprocal translocations in the mouse (2,3). Such studies have established the fact that several autosomal chromosomes are concerned in imprinting.

In general, an inverse correlation between gene expression and DNA methylation at CpG dinucleotides has been established for a variety of vertebrate and viral genes (4). Therefore DNA methylation may be involved in regulation of genomic imprinting (5–8). We have recently cloned an imprinted gene *SP2* by searching for parental-origin-specific CpG methylation using restriction landmark genome scanning (RLGS) (9,10). This gene is methylated at *Not*I site on the maternal allele and expressed from the paternal allele. *SP2* encodes a putative 51 Kd protein with significant similarity to U2 small nuclear ribonucleoprotein auxiliary factor (U2AF), an essential mammalian splicing factor, and is located on mouse chromosome 11, of which maternal duplication/paternal deficiency results in a small body (2). *SP2* expressed predominantly in the brain (9), specially in the pyramidal neurons in the hyppocampus and dental gyrus (unpublished data). We renamed *SP2* as *U2af1-rs1* because several related genes have been isolated in mouse and human (unpublished data).

In this study, we cloned genomic *U2af1-rs1* to identify the promoter and study whether methylation occurred in an allele-specific manner. Furthermore, we examined the methylation in gamete and expression and methylation in the two-cell embryo.

## MATERIALS AND METHODS

### Cloning

Genomic DNA was isolated from mouse liver and digested with *Xba*I and electrophoresed on Seakem GTG (FMC). Fragments around 8.3-kb were cut out from the gel and purified by PREP-A-GENE kit (Bio Rad) to perform ligation with λZAP *Xba*I digest. This library was screened with the $^{32}$P-labeled insert of pSP2 (9). Recombinant clone Xb5-1S was obtained from this library.

### Sequencing

The insert of Xb5-1S was subcloned into pBluescript and the sequence around the mRNA coding region was determined by the primer walking method, employing the *Taq* dideoxyterminator kit (ABI).

### RACE

RACE (11) was performed by using the 5′-AmpliFINDER RACE kit (CLONTECH). The primer used for First-strand cDNA was 5′-CAGTCACCAGGTATCTGCA-3′ and that used for PCR was 5′-AGTTATCCGCAGTATGTCTG-3′.

* To whom correspondence should be addressed

### CpG island analysis

The sequence of Xb5-1S was analyzed by using the 'CpG Bio' program (9).

### Harrplot

Harrplot (12) was performed by using GENTYX-MAC HARR PLOT software.

### Plasmid construction

pDra-Cp-CAT was constructed by inserting a 398 bp *Dra*I–*Csp*I fragment of pXb5-1S, which contains from 346 bp upstream to 51 bp downstream of the transcription initiation site, into the *Hin*dIII site of O/A CAT (13).

### DNA transfection and CAT assay

L cells were cultured in minimum essential medium with 10% fetal calf serum. For each 3.5 cm dish, 1 mg of DNA was transfected by using LIPOFECTAMINE (Gibco-BRL). 48 h after transfection, 20 mg of total protein was assayed for CAT activity by the method described by Sleigh (14). Each assay was repeated at least three times.

### Southern analysis

Hybridization of Southern 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) SDS, 5× Denhardt's solution (1% each BSA, Ficoll and polyvinlpyrolidone), 100 mg/ml of heat-denatured herring-sperm DNA and heat-denatured probes ($1 \times 10^6$ c.p.m./ml). Membranes were washed for 20 min at 65°C in 0.2× SSC, 0.1% SDS.

### Isolation of sperm, oocytes and embryos

Standard techniques were used for obtaining sperm eggs and zygotes (15). Sperm were collected by squeezing resected epididymides.

Female mice were injected with 5 IU of pregnant mares' serum (PMS), followed by an injection 44–48 h later of 5 IU of human chorionic gonadotropin (hCG). After injection, females were housed overnight with PWK males. Embryos were collected at the one-cell (0.5 d.p.c.) and two-cell (1.5 d.p.c.) stages. Unfertilized eggs were flushed from the oviducts of superovulated females 18 h after hCG injection and then treated with hyaluronidase to remove adherent cumulus cells. Embryos were flushed from the oviducts of mice into the medium and then treated with Acid Tyrode to remove the zona pellucida. Eggs and embryos were collected by micropipette and washed several times with the medium.

### *Hha*I sensitive PCR assay

DNA was obtained from a small number of eggs, and embryos by the guanidine–HCl extraction method (16). A group of 30 eggs or 30 embryos was incubated in lysis solution (6 M guanidine–HCl, 140 ml; 7.5 M ammonium acetate, 10 ml; 20% Sarkosyl, 10 ml; 20 mg/ml Protenase K, 2 ml) at 60°C for 3 h. The lysate was passed through a 26-gauge hypodermic needle 10 times to shear the DNA to a minimal length. DNA was precipitated with ethanol after addition of 1 ml of ethachinmate (Nippongene) and 1 mg of pBluescript. The precipitate was collected by centrifugation, washed with 70% ethanol and dissolved in TE.

DNA was first cleaved at *Xba*I sites in order to reduce the DNA size and thus to facilitate the PCR reaction. One half of the digested DNA was further digested with *Hha*I and the other half was used as a non-digested control. To monitor completion of digestion, one tenth of the sample was electrophoresed in a 5% polyacrylamide gel. Polymerase chain reaction (PCR) was performed by using *Hha*I-digested and non-digested samples. Sequence of the primers used were 5′-CGTAGTTAC-CCGGTTATCCA-3′ and 5′-CCCCTTACTTTCCACAAG-AG-3′. The amplified DNA was detected by Southern hybridization.

### Semiquantitaive RT-PCR assay

Semiquantitative RT-PCR assay was based on the method described by Latham *et al.* (17). 20 mg of *Escherichia coli* tRNA as carrier and 0.25 pg of rabbit globin mRNA as internal control for RNA recovery and efficiency of the RT-PCR reaction was added to the 20 embryo, which were then lysed in 100 ml of guanidine isothiocyanate (GTC) solution [4 M GTC, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1 M β-mercaptoethanol]. Sodium acetate [10 ml of 0.2 M (pH 4)] was added, and the sample was extracted with 100 ml of unbuffered phenol and 25 ml of chloroform: isoamylalcohol (49:1). The resulting aqueous phase was collected, and the RNA was precipitated by the addition of 240 ml of ethanol. The precipitate was dissolved in water and treated with RQ1 DNase in the presence of RNasin. The RNA was extracted with phenol and ethanol-precipitated.

Reverse transcription was conducted on 8 embryos equivalents. The reaction was carried out in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 M dNTP, 10 mM dithiothreitol, 0.037 mg/ml $pd(N)_6$ and 400 U of SuperScript II (Gibco-BRL) at 42°C for 1 h. In case of negative control, reaction was carried out without SuperScript II. The samples were then boiled for 5 min and placed on ice.

PCR was conducted on 4 embryo equivalents. The sequence of primers used for *U2af1-rs1* were 5′-AGTACATAGGC-CTGCCCATG-3′ and 5′-AGATAACCACGGATACCTGG-3′, and for α-globin were 5′-GCAGCCACGGTGGCGAGTAT-3′ and 5′-GTGGGACAGGAGCTTGAAAT-3′. For each set of primers, semilog plots of the amount of radiolabeled amplicon product as function of cycle number was used to determine the range of cycle number over which amplicon production was linear on such plots (data not shown). The number of cycles was 40 and 30 for *U2af1-rs1* and α-globin, respectively. The amplified DNA was detected by Southern hybridization.

## RESULTS

### Genomic structure of U2af1-rs1 gene

Only a 8.3 kb fragment was detected in a *Xba*I digest of mouse genomic DNA by Southern analysis using a mouse cDNA probe (data not shown, 9). A mouse genomic library was then constructed from the *Xba*I digested genomic DNA fragment around 8.3 kb, and screened with the same cDNA probe yielding three putative genomic U2af1-rs1 clones. Three clones showed the same profile with restriction enzyme digestion. Therefore, we subcloned one of these clones, named Xb5-1S, into pBluescript.

```
      10        20        30        40        50        60        70        80        90       100
CTTCCAGGCATCTAGGATGTGGGTCTTAAAGCCAATGACACACTTACTCCAGCAAGGCCACAGCTTCTAACAGTGCCACTCCCTATACAAACCATCACAT
     110       120       130       140       150       160       170       180       190       200
TCTACTCCCTGGCCCCTATAGGCTTGTGCAAGCATAAGAGCCTATGGGGATCATACCTAAACATAGCATGATGCAAGGCCAGTCTAGTCTACAGAGCAAG
     210       220       230       240       250       260       270       280       290       300
TTCCAGGACAGCCACAGCTACACAGTAAAACCCTTGTCTCTGAAAAAACAAACAAACAAACAAAAATAAAAGATTTTTTTCTTGGCTTAAACACTT
     310       320       330       340       350       360       370       380       390       400
GTCTGAGCAGTCTTCTCTGGTGAATACCCCACAAAACTACATTACAGAACAAATGAAAGGAATGTGTTTGCAAAAAACAAAACACATTCAATTTTTTATT
     410       420       430       440       450       460       470       480       490       500
CCCTTGGTTTTTCGAGACAGGGTTTCTCTGTGTAGCCCTGGCTGTCCTGGAACTCACACTGTAGACCAGGCTGGCCTCGAACTCAGAAATCCGCCTGCCT
     510       520       530       540       550       560       570       580       590       600
TTGCCTCCCGAGTGCTGGGATTAAAGGCGTGCGACACCACACCAGGCCCACATTCAATATTTAAAAAACAAAACAACAACAAAACCAAAACCCCTTACTT
     610       620       630       640       650       660       670       680       690       700
TCCACAAGAGTGAGGAGCTGAAGCCAGAAAAACGGTTTACCTGGTAAAGCCCTTGACAAACAAATATAAGAACTTAATTTCTATTCCCAGCACCCACATA
     710       720       730       740       750       760       770       780       790       800
AACAAGCCCGGGAAGGTGAGCTGCCAGCACCGGCATGGAGGGGTGCCCTGCAGTGAGACAAGCCTGCCCCGGCTCAGGGCTGAGCATTCTTACTGCAGGA
     810       820       830       840       850       860       870       880       890       900
GCACTCCCACTGCAGGGGGCGGAGAACAAAAAAGCAGTAAATCGTACTGCGCAGGCCTGCCTTTCCCGGAGTAAAGACGTAACTGCAGATTACAGGGTA
     910       920       930       940       950       960       970       980       990      1000
ACTGCAGATCAGACATACTGCGGATAACTGCAGATACCTGGATGACTGCGCAGGCGGACCGTACTGCAGATAACCACCCGCAACTGCGCAGGCCGGTC
    1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
GGTAAGGCAGATAACCACTGTTCCAGCTGTACCATGGATAACCGGGTAACTACGTAGGCTGATCATACTACGGATAACCAGGTAACTGCGCAGGCCGGCC
    1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
ATGCCACGGAGGCTGGCCTTAAAGCGGATAACCGGGTAACTGCACAGGCCAGCTGTGCCATGCATAACCAGTAACTGCATAGGCTGGCCGTACCACAGAT
```

**Figure 1.** Nucleotide sequences of the region around the initiation site of the mouse genomic U2af1-rs1 gene. Transcription initiation site identified by 5′ RACE is boxed. The putative TATA box is underlined.

The sequence around the mRNA coding region was determined by the primer walking method employing the *Taq* dideoxy-terminator kit (ABI) (Fig. 1, DDBJ accession number D26474). The transcription initiation site was determined by 5′ RACE. For construction of 1st strand cDNA, an anti-sense primer derived from residues 26–45 of 2916-bp cDNA was used for reverse transcription of brain polyA$^+$ RNA. Amplification was performed with the anti-sense primer derived from residues 7–26 of 2916-bp cDNA and linker primer. The amplified fragment was cloned into the *Eco*RV site of pBluescript and 6 independent clones were sequenced. All clones started at the location 906th from the beginning of the genomic sequence (Fig. 1). An TATA-like sequence (TAAAGAC) was located 33 bp upstream of the starting site. Comparison of the genomic sequence with cDNA sequence revealed that the *U2af1-rs1* is an intronless gene with an unusually long 5′ non-coding sequence (1168 bp).

To identify CpG island in this sequence, we plotted Obs/Exp CpG and %(G+C) using a 100-bp window moving along the sequence at 1 bp intervals (Fig. 2) There is a big CpG island spanning from upstream of the transcription initiation site to upstream of the translation initiation site. The *NotI* site, which was previously shown to be methylated in a parental-specific manner, was included in this region.

Harrplot analysis (data not shown) revealed a highly repeated region spanning from upstream of the transcription initiation site to upstream of the translation initiation site. The unit of this repeated sequence is the *FoK* I family, which is one of those possessing a variable number of tandem repeats (VNTR).

## Identification of the promoter region

Before examining the differential methylation and expression of this gene, the promoter activity was measured by using the CAT construct carrying the upstream region. pDra-Cp-CAT plasmid was transfected into mouse L cell. High activity was observed as shown in Figure 3, suggesting that plasmid is sufficient for basal expression.

## Parental specific methylation in promoter region

To study whether the upstream region of transcription initiation site is methylated in a parental specific manner, we first searched for a polymorphic restriction enzyme site in this region by Southern blot. A *Ban*I site located 164 bp upstream of the

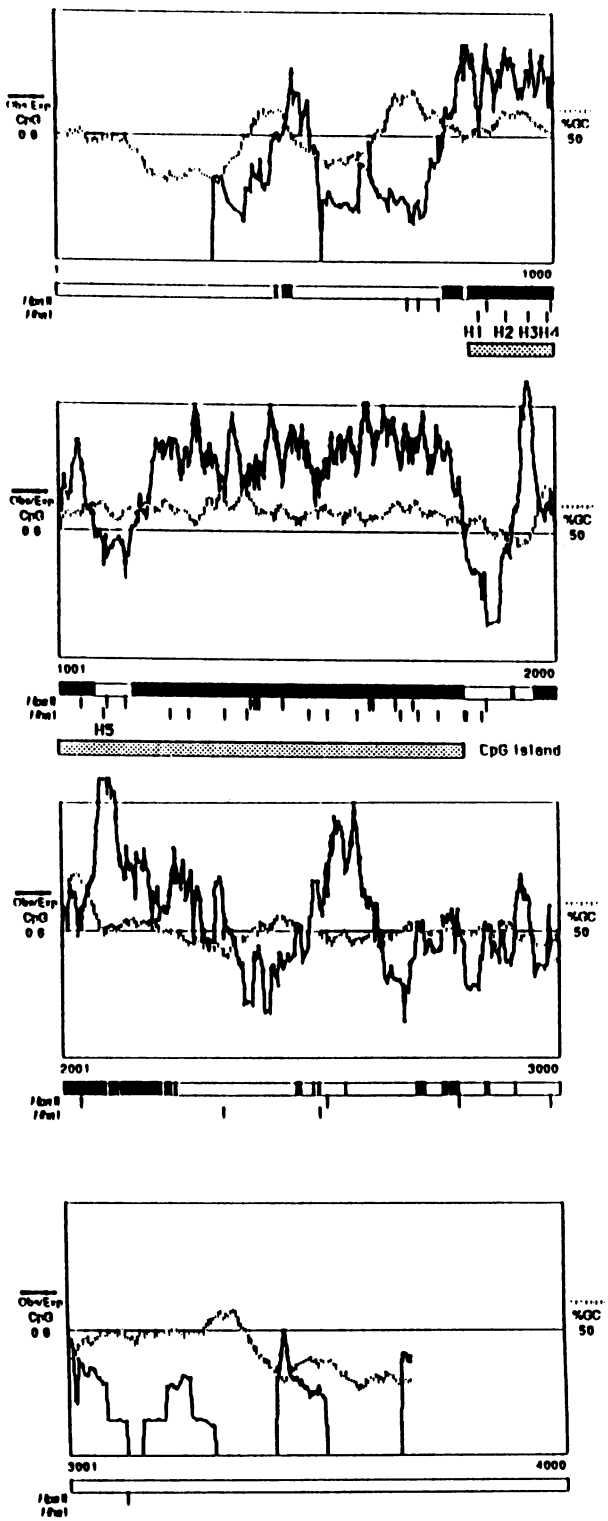


**Figure 2.** Analysis of the distribution of CpG dinucleotides in mouse U2af1-rs1 gene. The (G+C) percentage (dotted line) and the observed/expected CpG ratio (smooth curve) for 1st to 3691th of the nucleotide of U2af1-rs1 gene is plotted using a program 'CpG Bio' (9). These values are calculated for each sequence, using a 100-bp window moving along the sequence at 1 bp intervals. The regions with a (G+C) percentage above 50 and a observed/expected CpG ratio greater than 0.6 are indicated as bold bars. A CpG island is indicated as a dotted bar. H1, H2, H3, H4 and H5 represent *Hha*I sites at residues 850, 904, 950, 988 and 1088.

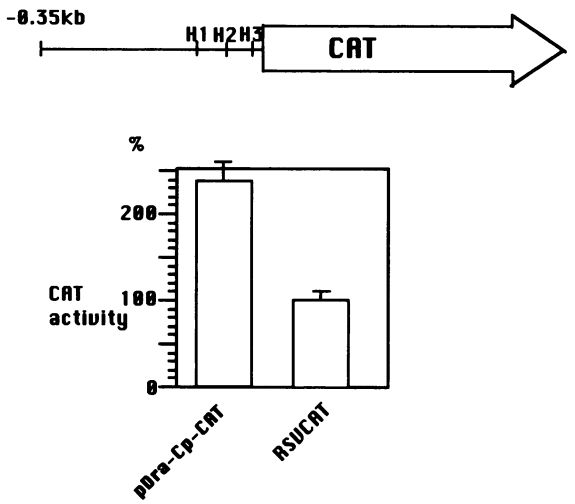


**Figure 3.** Promoter activity of the upstream region. From 346 bp upstream to 51 bp downstream of transcription site was fused to CAT gene. This region contains three *Hha*I sites (H1, H2, H3). The CAT activities expressed in L cells were normalized with an amount of protein. CAT activities are presented relative to that from RSVCAT in L cells.

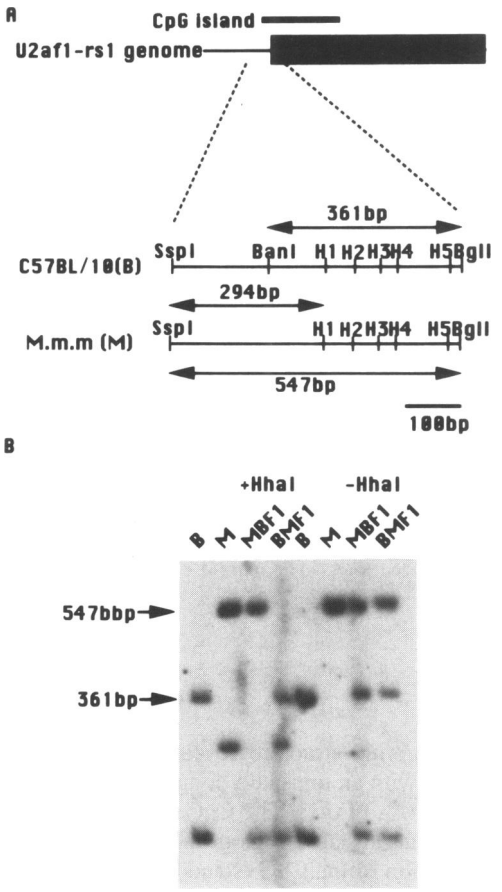


**Figure 4.** Analysis of DNA methylation of U2af1-rs1 gene. (**A**) Map of upstream and 5′ region of U2af1-rs1 gene. A *Ban*I site (742) is present in C57BL/6 but not in *Mus musculus molossinus* (M.m.m.). *Ssp*I, *Bgl*I represents those sites present in 556 and 1103. H1, H2, H3, H4 and H5 represent *Hha*I sites at residues 850, 904, 950, 988 and 1088. H1 and H2 are in the upstream region. The black box represents the mRNA coding region. (**B**) Methylation-sensitive Southern blot. C57BL/10(B), *M.m.molossinus* (M), (*M.m.molossinus* × C57BL/10)$F_1$ (MBF1) and (C57BL/10 × *M.m.molossinus*)$F_1$(BMF1). DNA from whole body of newborn mice was digested with *Ssp*I, *Ban*I, *Bgl*I and *Hha*I (+HhaI) or *Ssp*I, *Ban*I and *Bgl*I(-HhaI). The probe is the 547 bp *Ssp*I–*Bgl*I fragment. 361-bp band is the methylated C57BL/10 fragment and 547-bp band is the methylated *Mus musculus molossinus* fragment.

transcription initiation site was found to exist in C57BL/10, but not to exist in *Mus musculus molossinus* (*M.m.molossinus*). Therefore, we were able to detect a 361-bp fragment in C57BL/10 and a 547-bp fragment in *M.m.molossinus* when genomic DNA was digested with *Ban*I, *Ssp*I and *Bgl*I (Fig. 4). When digestion was performed with *Hha*I in addition to *Ban*I, *Ssp*I and *Bgl*I, we found 547-bp *M.m.molossinus* specific fragment in (*M.m.molossinus* × C57BL/10)$F_1$ and 361-bp C57BL/10 specific fragment in (C57BL/10 × *M.m.molossinus*)$F_1$. This indicates methylation of the *Hha*I sites (H1 to H5, H1 and H2 are in the upstream region) was exclusively on the maternal allele, which is consistent with a functional role for DNA methylation, the repressed allele being methylated.

### Analysis of methylation in gamete

Having shown that the upstream region of *U2af1-rs1* is differentially methylated, we then set about to determine whether this pattern is established in gamete or not. If modification of this locus serves as an imprinting signal for identifying the parental alleles, these sites should be unmethylated in the sperm while methylated in the oocyte. In order to test this hypothesis, we first analyzed sperm by methylation-sensitive Southern blot using the same probe used for detection of parental specific methylation in the promoter region. We found that the upstream region is almost unmethylated in sperm (Fig. 5A). DNA from mature oocytes was assayed for methylation-sensitive PCR analysis. In contrast to our expectations, we could not amplify the upstream region from the *Hha*I-digested DNA (Fig. 5B). Thus, the differential methylation pattern found in somatic cells cannot be derived directly from the pre-fertilization parental gametes and must therefore be established at some stage during post-fertilization embryonic development.

### Analysis of expression in pre-implantation embryo

By using semiquantitative RT-PCR assay (17) we determine whether *U2af1-rs1* is expressed in pre-implantation. U2af1-rs1 RNA was not detected at the one-cell stage, but started to express at the two-cell stage when transcription initiates (18, Fig. 6A). We can distinguish the maternal and paternal allele in the embryos by cleaving the RT-PCR product with a polymorphic enzyme because embryos are F1 of C57BL/6 female and PWK male (BPF1). *U2af1-rs1* was expressed from the paternal allele and not from the maternal allele (Fig. 6B). We expected that methylation in the upstream region begins at this stage. However, we could not amplify the upstream region from the *Hha*I-digested two-cell (1.5 d.p.c.) DNA (Fig. 6C). Thus, methylation in the upstream region seems not necessary for repression of the maternal allele in the two-cell embryo. We detected the parental specific methylation in the upstream region at 12.5 d.p.c. (Fig. 6D). Therefore, methylation is established between 1.5 d.p.c. and 12.5 d.p.c.

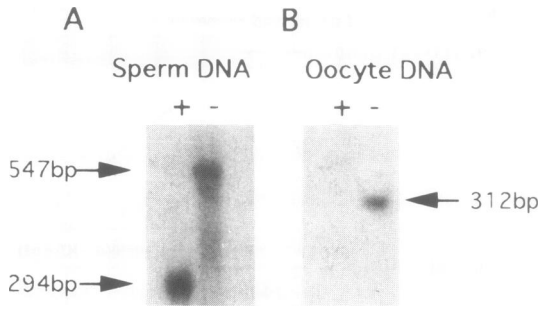


**Figure 5.** Analysis of methylation in gametes. (**A**) Methylation sensitive Southern blot. DNA from C57BL/6 sperm was digested with *Sspl*, *Bgll* and *Hhal*; (+), or *Sspl*, *Bgll*; (–). The probe is the same one used in Figure 4. 574-bp band is the completely methylated fragment which contains H1–H5 and 294-bp band is the unmethylated fragment. Refer to Figure 4A. (**B**) Methylation-sensitive PCR analysis of the upstream region in oocytes. *Hhal* sites (H1–H4) were located between the two PCR primer. PCR was performed either with DNA digested with *Xbal* and *Hhal* (+) or *Xbal* (–).

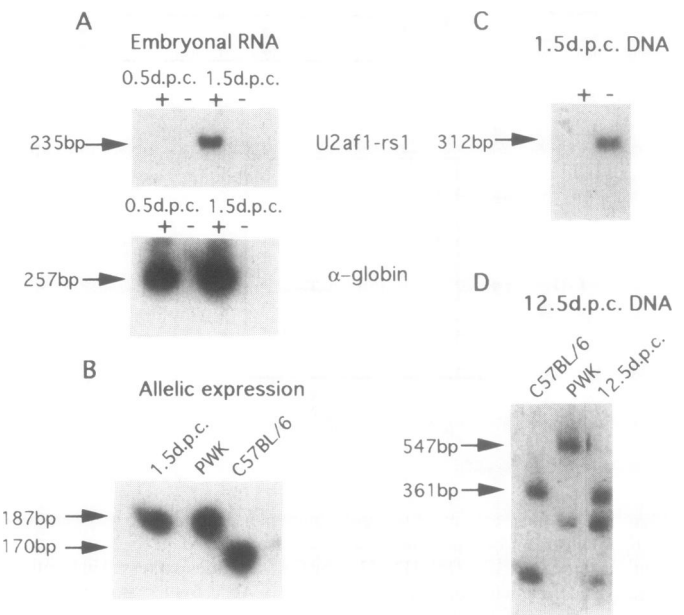


**Figure 6.** Analysis of expression and methylation in the embryo. (**A**) Expression of *U2af1-rs1* in one-cell and two-cell embryos. Embryos from one-cell (0.5 d.p.c.) and two-cell (1.5 d.p.c.) stages were used for RT-PCR and followed by Southern blot as shown in Materials and Methods. Reverse transcription was performed either with using SuperscriptII (+) or without using one (–). (**B**) Allelic analysis of U2af1-rs1 RNA in one-cell and two-cell embryos. Amplified DNA from two-cell embryos were digested with *Mspl*. 187-bp band can been seen from PWK RNA and 170-bp band can been seen from C57BL/6 RNA. (**C**) Methylation sensitive PCR analysis of the upstream region in two-cell embryos. *Hhal* sites (H1–H4) were located between the two PCR primer. PCR was performed with DNA being digested either with *Xbal* and *Hhal* (+) or *Xbal* (–). (**D**) Methylation sensitive Southern blot of 12.5-d.p.c. (C57BL/6 × PWK)$F_1$ embryo. C57BL/6, PWK adult liver DNA and (C57BL/6 × PWK)$F_1$ 12.5-d.p.c. embryo (12.5 d.p.c.) DNA was digested with *Sspl*, *Banl*, *Bgll* and *Hhal* . The probe is the one used in Figure 4. 361-bp band is the methylated C57BL/6 fragment and 547-bp band is the methylated PWK fragment.

## DISCUSSION

Analysis of genomic structure revealed that *U2af1-rs1* is an intronless gene with an unusually long 5′ untranslated sequence (from 906 to 2073, Fig. 1). The CpG island of U2af1-rs1 gene consists of 5′ untranslated region and a part of the upstream region. The region of the CpG island corresponds to the highly repeated region revealed by Harrplot analysis. The unit of this repeated sequence is *Fok*I family, which is one of those possessing a variable number of tandem repeats (VNTR). B. Neumann and D. P. Barlow reported that imprinted genes and transgenes like the *Igf2*, the *Xist* and the TG.A transgene contain iterated repeats and have a high GC density (18). Therefore, it is intriguing to think that the CpG island of *U2af1-rs1* which consists of iterated repeats, may be involved in some part of the imprinting mechanism.

In order to understand the relationship between parental imprinting and DNA methylation, we have evaluated the methylation in the upstream region of U2af1-rs1 gene (Fig. 4). The upstream region of this gene is differentially methylated in an allele-specific manner in the newborn mouse. However, this modification pattern is not directly inherited from the gametes, but is rather added to the DNA at some stage between 1.5 d.p.c. and 12.5 d.p.c. Figs 5, 6). A similar result is observed in the *Igf2r* region 1 which overlaps the gene promoter, and which is allelically methylated in adult tissues like *U2af1-rs1* (8,20).

We expected that U2af1-rs1 gene would express in the early embryonic stages because this gene is a housekeeping gene, expressing in every tissue (9). This gene is actually expressed in the two-cell stage when transcription initiates (Fig. 6). This expression is from the paternal allele. This is different from *Igf2r* and *Igf2* which express biallelically in preimplantation embryos (17). The component required for inactivating the imprinted allele of *U2af1-rs1* seems to be present at the two-cell stage. Demethylation of the upstream region at the two-cell stage indicates methylation is not essential for inactivation of the maternal allele of the U2af1-rs1 gene at this stage. The methylation in upstream regions seems not to inactivate the expression directly, but rather stabilizes the inactivation because inactivation precedes the methylation.

## ACKNOWLEDGEMENTS

We thank Dr J. Forejt for providing PWK, Dr K. Hidaka for her assistance in CpG-islands analysis. This work was supported by Special Coordination Funds for promoting Science and Technology (Encouragement System of COE) from the Science and Technology Agency of Japan, a Research Grant for Aging from the Ministry of Health and Welfare and by a Grant-in-Aid for Creative Basic Research (Human Genome Program) from the Ministry of Education, Science and Culture in Japan.

## REFERENCES

1 Solter, D. (1988) *Annu. Rev. Genet.*, **22**, 127–46.
2 Cattanach, B. M. and Kirk, M. (1985) *Nature*, **315**, 496–8.
3 Searle, A. G. and Beechey, C. V. (1990) *Genet. Res.*, **56**, 237–44.
4 Cedar, H. (1988) *Cell*, **53**, 3–4.
5 Bartolomei, M. S., Webber, A. L., Brunkow, M. E. and Tilghman, S. M. (1993) *Genes Dev*, **7**, 1663–73.

6 Brandeis, M., Kafri, T., Ariel, M., Chaillet, J. R. , McCarrey, J. , Razin, A., and Cedar, H. (1993) *EMBO J.*, **12**, 3669–3677.
7 Ferguson, S. A., Sasaki, H., Cattanach, B. M. and Surani, M. A. (1993) *Nature*, **362**, 751–5.
8 Stoger, R., Kubicka, P., Liu,C. G. , Kafri, T. , Razin, A., Cedar, H. and Barlow, D. P. (1993) *Cell*, **73**, 61–71.
9 Hatada, I., Sugama, T. and Mukai, T. (1993) *Nucleic Acids Res.* ,**21**, 5577–5582.
10 Hatada, I., Hayashizaki, Y., Hirotsune, S., Komatsubara, H., and Mukai, T. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 9523–9527.
11 Frohman, M.A., Dush, M. K., and Martin, G.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8998–9002.
12 Harr, R., Hagblor, P., and Gustafsson, P. (1982) *Nucleic Acids Res.*, **10**, 365–374.
13 Joh, K., Takano, K., Mukai, T. and Hori, K. (1991) *FEBS lett.*, **292**, 128–132.
14 Sleigh, M. J. (1986) *Anal Biochem*, **156**,251–6.
15 Hogan, B., Constantini, F., and Lacy, E. (1986) *Manipulation the mouse embryo*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
16 Jeanpierre, M. (1987) *Nucleic Acids Res.*, **15**, 9611.
17 Lathan, K. E. (1994) *Genes & Dev.*, **8**, 290–299.
18 Flach, G., Johnson, M. H., Braude, P. R., Taylor, R. A. S., and Bolton, V. N. (1982) *EMBO J.*, **1**, 681–686.
19 Neumann, B. and Barlow, D. P. (1993) Abstr. LVIII Cold Spring Harbor *Symposium on Quantitaive biology DNA and Chromosomes*, **165**.
20 Barlow, D. P., Stoger, R., Hermann, B. G., Saito, K. , and Schweiger, N. (1991) *Nature*, **349**, 84–87.