© 1993 Oxford University Press

Nucleic Acids Research, 1993, Vol. 21, No. 24 5577–5582

A new imprinted gene cloned by a methylation-sensitive genome scanning method

Izuho Hatada, Takako Sugama and Tsunehiro Mukai
National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan

Received September 21, 1993; Revised and Accepted November 1, 1993 DDBJ accession no. D17407

ABSTRACT

We cloned a new imprinted gene by searching for parental-origin-specific CpG methylations using methylation-sensitive two-dimensional genome scanning method. This gene encodes a putative 51 kDa protein with significant similarity to U2 small nuclear ribonucleoprotein auxiliary factor small subunits, an essential mammalian splicing factor, and is located on mouse chromosome 11, of which maternal duplication/ paternal deficiency results in a small body.

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 effects of particular chromosomal regions on 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.

So far, only few known murine genes have been found to be imprinted, such as *Insulin-like growth factor II, Insulin-like growth factor II receptor, H19, small nuclear ribonucleoprotein polypeptide N* (4–8). It is evident there still exist numbers of other imprinted genes. To totally understand the biological significance of genomic imprinting, further effort is required to isolate new imprinted genes. However, there has been no effective method for isolating unknown imprinted genes.

We have recently developed a new genome scanning method, restriction landmark genome scanning (RLGS) (9). In this study, using this method, we searched for parental-origin-specific CpG methylations which may be involved in regulation of genomic imprinting (10,11) and isolated a new imprinted gene.

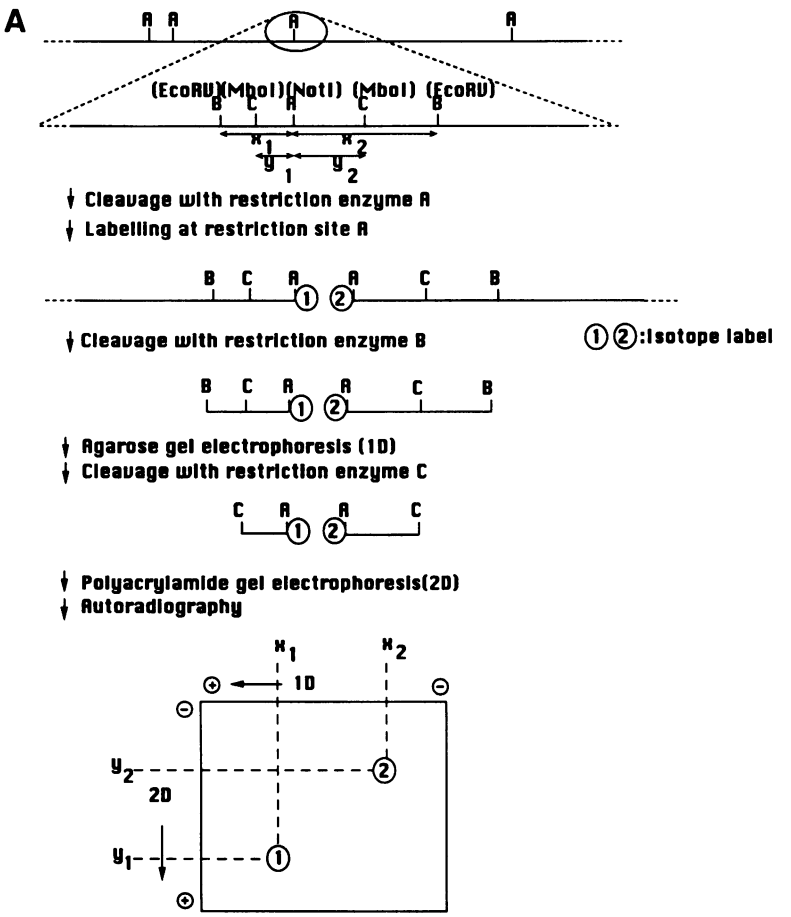
METHODS

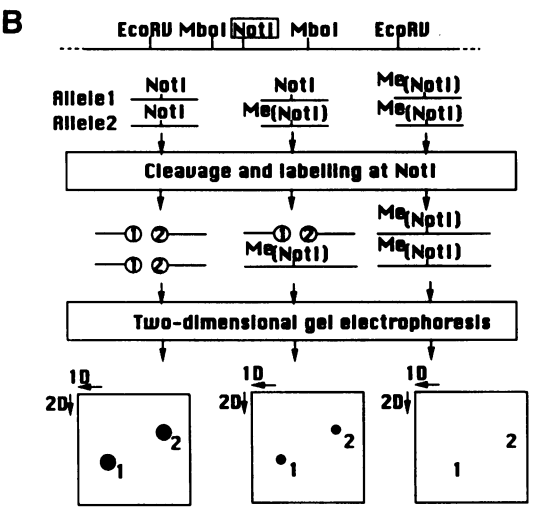
Restriction landmark genomic scanning

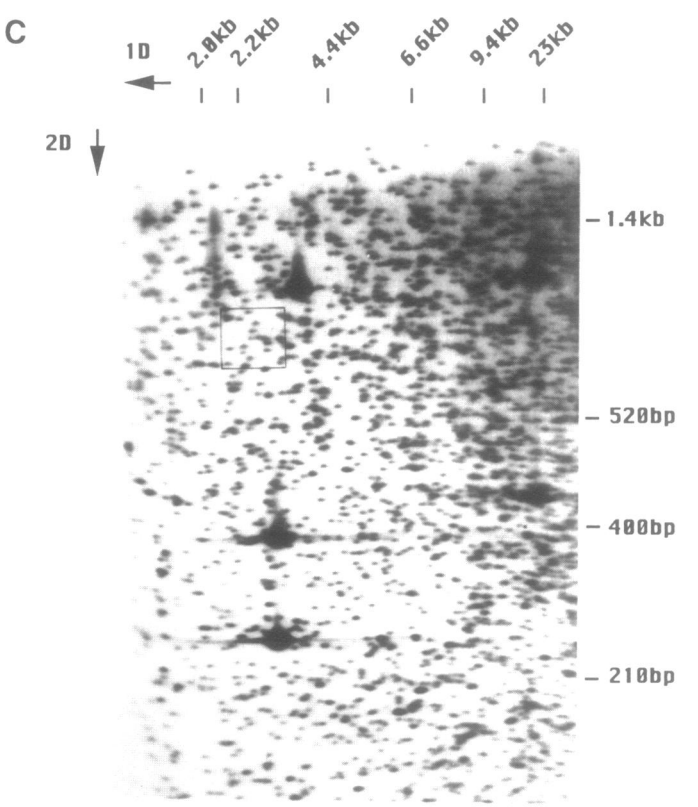
Ten micrograms of genomic DNA was allowed to react for 30 min at 37°C with 10 units of DNA polymerase I in 50 μl of 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.33 μM dGTPαS, 0.33 μM dCTPαS, 33 μM ddATP and 33 μM ddTTP. Incorporation of these nucleotide analogues to non-specific cleaved ends protects non-specific incorporation of radioisotope resulting in high background. Thereafter, the enzyme was inactivated at 65°C for 30 min. The treated DNA was then digested with 100 units of *Not*I (restriction enzyme A) for 1 hr in 85 ml of 50 mM Tris–HCl(pH7.5), 100 mM NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.01% Triton X-100 and 0.01% BSA. The cleavage ends were filled in with 20 units of Sequenase Ver.2.0Ö (USB & Co. Ltd.) in the presence of 0.33 μM [α-^{32}P] dGTP (3000 Ci/mM) and 0.33 μM [α-^{32}P] dCTP (6000 Ci/mM) for 30 min at 37°C in 100 ml of 50 mM Tris–HCl (pH7.5), 100 mM NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.16 μM dGTPαS, 0.16 μM dCTPαS, 33 μM ddATPαS and 33 μM ddTTPαS. To stop the reaction, excessive ddGTP and ddCTP (33 μM) were added. The labeled DNA was digested with *Eco* RV (restriction enzyme B), phenol-extracted and ethanol-precipitated. One microgram of the DNA was fractionated on a 50×20×0.1cm agarose gel (0.8% Seakem GTG agarose, FMC), then electrophoresed in 1×TAM buffer (50 mM Tris–acetate, pH 7.5/0.7 mM Mg acetate) at 4.5 V/cm for 12 hr. The DNA-containing portion of the gel was excised as a strip, and soaked for 30 min in the *Mbo*I digestion buffer (10 mM Tris–HCl, pH7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol). Thereafter, DNA was digested in the gel with 1500 units of *Mbo* I (restriction enzyme C) at 37°C for 2 hr. The gel was fused to a 50×50×0.1 cm polyacrylamide gel (5–6% polyacrylamide, acrylamide/bisacrylamide, 29:1) by adding melted agarose to fill up the gap. Second-dimensional electrophoresis was carried out in 1×TBE buffer at 8 V/cm for 6 hr. The gel was dried up. An area 35×43 cm of the appropriate region from the original gel was then excised and autoradiographed for 3–10 days on a film (XAR-5, Kodak) at −70°C using an intensifying screen (Quanta III™, Dupont).

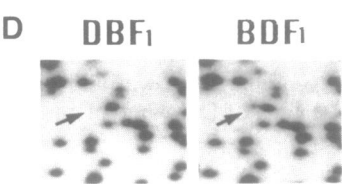
Cloning of spot2

Genomic DNA was digested with *Not*I and *Eco*RV followed by size separation by agarose gel. The spot2-containing region (1.8–2.4 kb of the lane) was cut out from the gel and DNA was electro-eluted and cloned into the *Not*I and *Eco*RV sites of pBluescript. Five micrograms of this boundary library DNA was


A
(EcoRV)(MboI)(NotI) (MboI) (EcoRV)
Cleavage with restriction enzyme A
Labelling at restriction site A
①②:Isotope label
Cleavage with restriction enzyme B
Agarose gel electrophoresis (1D)
Cleavage with restriction enzyme C
Polyacrylamide gel electrophoresis(2D)
Autoradiography
1D
2D


B
EcoRV MboI NotI MboI EcoRV
Allele1
Allele2
Cleavage and labelling at NotI
Two-dimensional gel electrophoresis
1D
2D


C
1D
2.0kb
2.2kb
4.4kb
6.6kb
9.4kb
23kb
2D
1.4kb
520bp
400bp
210bp


D
DBF1
BDF1

digested with *Not*I plus *Eco*RV and was applied to two-dimensional electrophoresis of RLGS (restriction enzyme A, *Not*I; restriction enzyme B, *Eco*RV; restriction enzyme C, *Mbo*I) along with *Not*I labeled genomic DNA. Spot2 was cut out from the gel and *No* I−*Mbo*I DNA fragment (0.7 kb) was electro-eluted for cloning into *Not*I and *Bam*HI sites of pKF3 (12,13; designated as pSP2NM). For cloning the *Not*I−*Eco*RV fragment (2.1 kb) of spot2, we screened boundary library using the insert of pSP2NM as a probe and obtained pSP2. *E. coli* TH2 was used as host cell for pKF3.

Southern and Northern analysis

Hybridization of Southern and Northern analysis was performed for 20 hr 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 BSA, Ficoll and polyvinylpyrrolidone), 100 μg/ml of heat-denatured herring-sperm DNA and heat-denatured probes (1×10^6 cpm/ml). Membranes were washed for 20 min at 65°C in 0.2×SSC, 0.1% SDS.

cDNA cloning

SP2 cDNA was isolated from mouse brain λgt10 library by hybridizaion with the insert of pSP2 and sequenced (2916 nucleotides; DDBJ, Genbank and EMBO accession no. D17407).

RT-PCR

Total RNA, prepared using the acid−phenol method (14) was applied to RT-PCR as described (15).

Mapping of *SP2*

The *Rsa*I digested DNA of BXD inbred strains was run in a long agarose gel (50 cm) to detect polymorphism between C57BL/6 and DBA2. Southern hybridization was performed using *Hind*III−*Eco*RI fragment of cDNA (corresponding to nucleotides 974−1409 of cDNA) as a probe. Using *Rsa*I polymorphism, we determined SDPs of BXD inbred strains.

RESULTS

Searching for parental-origin-specific CpG methylation by genome scanning

RLGS is a genome scanning method based on end-labeling of genomic DNA at rare restriction sites and its separation by two-dimensional gel electrophoresis (Fig.1A and ref. 9). Genomic DNA is cleaved and radioactively labeled at rare restriction sites (restriction enzyme A, e.g., *Not*I). The labeled DNA is then cleaved with restriction enzyme B (e.g., *Eco*RV) to reduce average molecular weight to several kilobase and size-fractionated in one dimension on agarose gel. The fractionated DNA is further digested with restriction enzyme C (e.g., *Mbo*I) and separated in the second dimension on acrylamide gel. This procedure gives a two-dimensional profile with thousands of scattered spots corresponding to sites for restriction enzyme A (Fig. 1C).

This method was used for searching for parental-origin-specific CpG methylation which may be involved in regulation of genomic imprinting (10,11), by using methylation-sensitive restriction endonucleases, such as *Not*I, that have CpG sites in their recognition sequences and that are not cleaved when 5 cytosine is methylated. It is expected that the intensity of any spot varies in proportion to the population of methylated DNA molecules (Fig. 1B). Demethylation of both alleles (left), methylation of one allele (middle) and methylation of both alleles (right) will give a full intensity (left), half-intensity (middle) and no spot (right), respectively. To identify parental-origin-specific methylation, we compared the spot profiles of F_1 progeny of reciprocal crosses between two inbred strains. If there is no parental-origin-specific methylation, the RLGS profiles of F_1 progeny should be completely identical, irrespective of one parental strain being male or female. However, we found 4 spots appeared in one cross while not appearing in another. These 4 spots are polymorphic between C57BL/6 and DBA2. One of these spots, spot2 (specific to DBA2), appeared in one cross (C57BL/6×DBA2)F_1, while not appearing in the opposite cross (DBA2×C57BL/ 6)F_1 (Fig. 1D). This suggests the DBA2 allele of spot2 is demethylated in (C57BL/6×DBA2)F_1, while methylated in (DBA2×C57BL/ 6)F_1.

It is generally expected that we can find other parental-origin-specific methylation by using other restriction enzyme A, B or C because we only searched here the methylation of NotI sites in combination with *Eco*RV and *Mbo*I as restriction enzyme B and C.

Cloning of a parental-origin-specific methylated spot

The *Not*I boundary library was constructed and applied to RLGS (restriction enzyme A, *Not*I; restriction enzyme B, *Eco*RV; restriction enzyme C, *Mbo*I) to clone spot2. Two-dimensional gel electrophoresis of this library was performed together with *Not*I labeled genomic DNA. Spot 2 was cut out from the gel and *Not*I−*Mbo*I DNA fragment (0.7 kb) of this spot was electro-eluted for cloning into pKF3 (12,13; designated as pSP2NM). To further clone the longer *Not*I−*Eco*RV fragment (2.1 kb) of spot2, we screened the boundary library, using the insert of pSP2NM as a probe. As a result, we isolated the pSP2 clone.

We then used it as a probe to examine the methylation status at the *Not*I site between two reciprocal crosses [(DBA2× C57BL/6)F_1 and (C57BL/6×DBA2)F_1] by Southern

Figure 1. (**A**) Procedure for genome scanning by two-dimensional gel electrophoresis (RLGS). A, B and C represent sites for restriction enzyme A, B and C, respectively. (x_1 or x_2) and (y_1 or y_2) represent the distance from a site for restriction enzyme A to the neighboring site for restriction site B and C, respectively. See reference 9 for strategy and detailed description of RLGS. (**B**) Detection of difference of methylation in genomes by RLGS. Maps show DNA-methylation state at the *Not*I site which is used as restriction enzyme A. Three types of methylated state are expected depending on which allele is methylated. No allele is methylated (left), one allele is methylated (middle) and both alleles are methylated (right). ^{Me}(*Not*I) represents the methylated site of this enzyme. As the CpG methylation of *Not*I site affects the cleavage of this site, RLGS profile would result in the change in the spot intensity. Each sample would give a full intensity spot (left), half-intensity spot (middle) or no spot (right). (**C**) The RLGS profile of C57BL/6. *Not*I, *Eco*RV and *Mbo*I were used as restriction enzyme A, B and C, respectively. Size markers are shown in kbp (kilobasepairs) or bp (basepairs). (**D**) Detection of the difference of spot intensity in F_1 progeny of reciprocal crosses. (DBA2×C57BL/6)F_1 (DBF_1) and (C57BL/6×DBA2)F_1 (BDF_1) DNA from 14.5 day P.C. embryos were applied to RLGS and boxed area in (C) is shown. Arrows indicate spot 2. Spot 2 did not appear in DBF_1, but appeared in BDF_1.

hybridization (Fig.2). A double digest with *Not*I and *Eco*RV revealed a 2.09 kb band (corresponding to demethylated fragment) in DBA2, which is absent in C57BL/6 (Fig. 2b). This is due to DNA polymorphism between the two species. In (DBA2×C57BL/6)F_1, the 2.09 kb band was completely undetectable, whereas in (C57BL/6×DBA2)F_1 this band was detectable, indicating that the *Not*I site is completely methylated on the maternal allele and demethylated on the paternal allele.

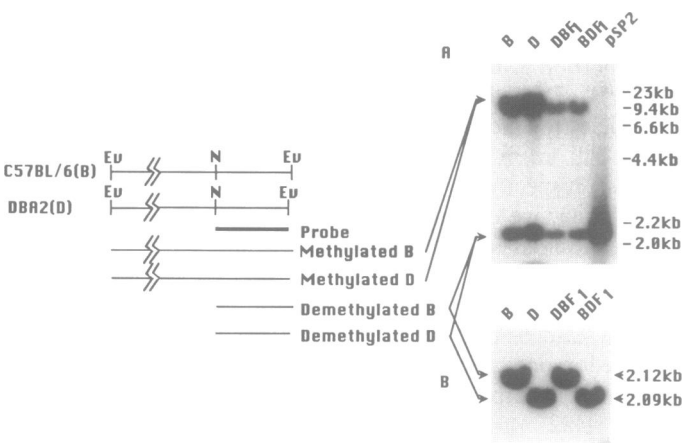


Figure 2. Differential methylation of *SP2* in F_1 progeny of reciprocal crosses analyzed by Southern blot. C57BL/6 (B), DBA2 (D), (DBA2×C57BL/6)F_1 (DBF_1), and (C57BL/6×DBA2)F_1 (BDF_1) DNA from 14.5 day P.C. embryos were digested with *Not*I plus *Eco*RV and hybridized with pSP2. Maps show *Eco*RV site neighboring to the *Not*I site in C57BL/6 and DBA2. An *Eco*RV site is located 2.12 kb downstream from *Not*I site in C57BL/6 and 2.09 kb in DBA2. Therefore, we can theoretically distinguish the demethylated alleles of these two mice. Lines represent the length of the DNA fragments detected by the probe. Restriction sites shown are: Ev, *Eco*RV; N, *Not*I. **(A)** Southern blot profile of ordinary-sized gel. It is difficult to distinguish slight mobility difference of demethylated C57BL/6 (2.12 kb) and DBA2 (2.09 kb) fragments by ordinary-sized gel. The pSP2 DNA digested with *Not*I plus *Eco*RV is in the right end lane. **(B)** Southern blot profile of 50 cm gel. Digested DNA was size-fractionated on 50 cm agarose gel. The portion of the gel which contains DNA sized around 2 kb was cut out and transferred to nylon membrane to be hybridized by the probe. The allele from DBA2 is completely methylated at *Not*I site in DBF_1 while the allele from C57BL/6 is completely methylated in BDF_1. Same results were obtained by using whole bodies of new born mice (data not shown).

Parental imprinting of *SP2*

SP2 DNA encodes 3.1 kb mRNA as shown by Northern blotting. It was expressed ubiquitously but was most abundant in the brain (Fig. 3A). We cloned cDNA from the mouse brain library and determined its entire sequence (2916 nucleotides; DDBJ, Genbank and EMBO accession number D17407). To clarify whether this gene is expressed only from one allele or from both, we performed RT-PCR followed by digestion with a restriction enzyme which could distinguish the *SP2* alleles of each parental strain in F_1 progeny of reciprocal cross between C57BL/10 and *Mus musculus molossinus* (*M.m.molossinus*; Fig. 3B). *SP2* was expressed exclusively from the paternal allele which was demethylated at *Not*I site, but not from the maternal allele which was methylated.

There is a *Not*I site in cDNA (located at 399 from the beginning of the cDNA sequence). To clarify whether this *Not*I site being derived from the parentally-origin-specific-methylated *Not*I site of *SP2* genomic DNA, we performed DNA sequencing around *Not*I site of pSP2(data not shown). As a result, the *Not*I site of *SP2* was found to be transcribed. This *Not*I site is located in a CpG island (Fig.4).

cDNA sequencing and mapping study reveals *SP2* encodes a new protein located on chromosome 11

SP2 cDNA sequence revealed a 720 nucleotide open reading frame corresponding to an encoded protein of 240 amino acids and a predicted M_r = 51,364 (Fig. 5). Homology search revealed this protein has significant similarity (31%) to U2 small nuclear ribonucleoprotein auxiliary factor small subunits (Fig. 5), an essential mammalian splicing factor.

To determine the map position of *SP2*, we carried out a recombinant inbred (RI) strain analysis using a set of 26 RI strains derived from C57BL/6 and DBA2 mice (BXD strains). The strain distribution pattern (SDP) of the *SP2* alleles was determined by Southern blot using *the Hind*III−*Eco*RI fragment probe which detects *Rsa*I polymorphism. A 0.97 kb fragment was present in DNA from C57BL/6 and a 0.95 kb fragment in DNA from DBA2. Among BXD SDPs data (16), the most similar ones

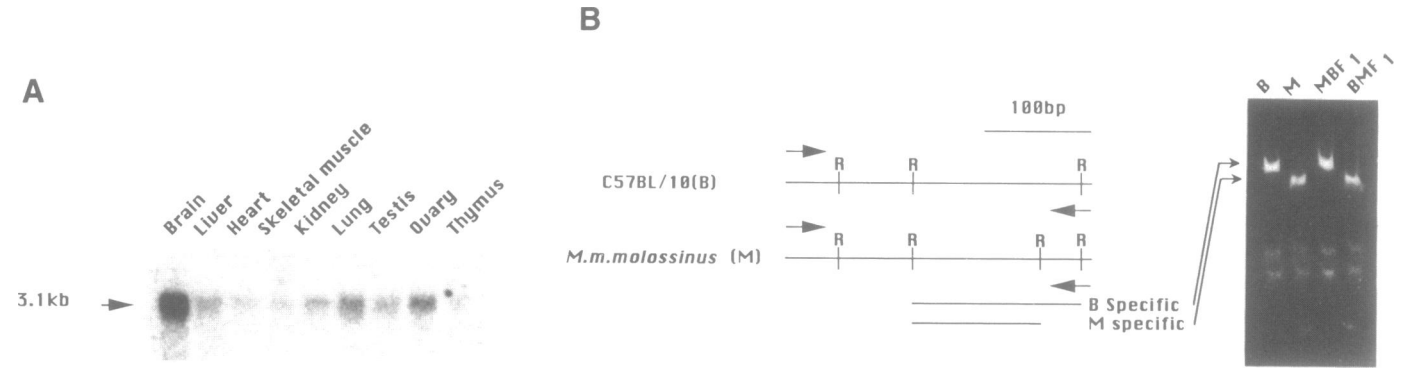


Figure 3. **(A)** Expression of *SP2* in mouse tissues. A northern blot of RNA from mouse brain, liver, heart, skeletal muscle, kidney, lung, testis, ovary and thymus was probed with pSP2. **(B)** Differential expression of *SP2* in F_1 progeny of reciprocal crosses. Poly(A)$^+$ RNA from whole bodies of newborn mice C57BL/10 (B), *M.m.molossinus* (M), (*M.m.molossinus*×C57BL/10)F_1 (MBF_1), (C57BL/10×*M.m.molossinus*)F_1 (BMF_1) was subjected to the RT-PCR followed by cleavage with *Rsa*I. As one of the *Rsa*I sites in the amplified region is polymorphic between C57BL/10 and *M.m.molossinus*, we can distinguish C57BL/10 specific fragment (long line) and *M.m.molossinus* specific fragment (short line). *SP2* is expressed from the allele of C57BL/10 in MBF_1 and expressed from the allele of *M.m.molossinus* in BMF_1. The primers were 5′-TGTGGTACGGCCAGCCTATG-3′ and 5′-GATCAGACATACTGCGGATA-3′. R represents site for *Rsa*I.

occurred for the chromosome 11 markers *Glns* (2 of 26 strains are recombinants) and *Hba* (3 of 24 strains are recombinants). Using the method of Silver (17) for estimating confidence intervals for linkage estimates based on RI analysis, these results placed *SP2* 2.1cM distal to *Glns* (95% confidence intervals of 0.2−10.1 cM) and 3.85 cM proximal to *Hba* (95% confidence intervals of 0.7−15.7 cM).

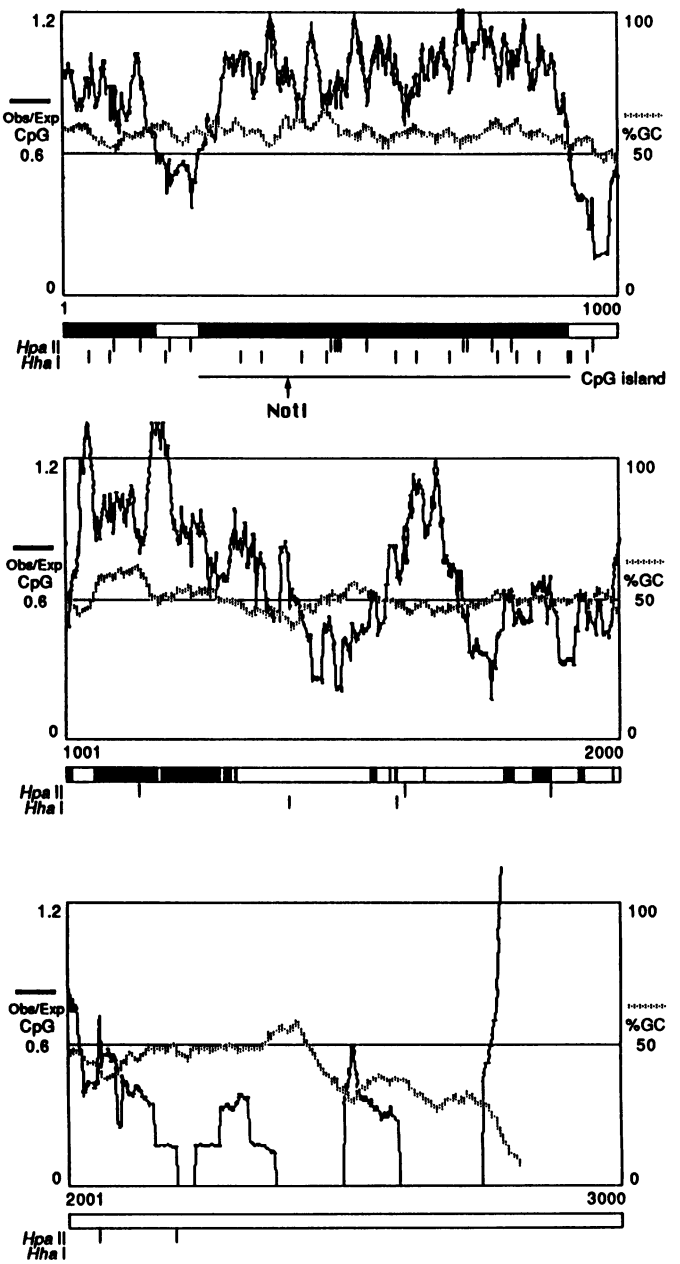


Figure 4. Analysis of the distribution of CpG dinucleotides in SP2. The (G+C) percentage (dotted line) and the observed/expected CpG ratio (smooth curve) for nucleotides 1−2816 of cDNA is plotted using a program 'CpG Bio' (K. Hidaka, unpublished). These values are calculated for each sequence, using 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 line. *Not*I site analyzed here is also indicated in the figure.

DISCUSSION

We cloned a new imprinted gene by searching for parental-allele-specific methylation by restriction landmark genomic scanning. This is the only general method for isolating new imprinted genes. Therefore, it will be of great use for identifying other new imprinted genes.

The differentially methylated restriction site is located in a CpG island like other imprinted genes(Fig. 4, refs 10,11). Therefore it is possible to speculate methylation of CpG island is involved in the regulation of expression of imprinted genes.

SP2 cDNA encodes a putative 51 kDa protein with significant similarity (31%) to U2 small nuclear ribonucleoprotein auxiliary factor small subunits (Fig. 5), an essential mammalian splicing factor. Using recombinant inbred strains, this gene was mapped on the proximal region of chromosome 11 (Table 1), in a region that is parentally imprinted (2). Mice carrying maternal duplication/paternal deficiency for proximal chromosome 11 are

```
SP2    1  MASRQTAIPEKLSRKQYKAAMKKEKRKKRRQKMARLRALEAPPEEDDDVSANEELAERLL
SP2   61  EIERQRLHEEWLLREEKAQEEFRIKKKKEEAARKQKEEQERQIKAEWEEQQKKQREEEEQ

SP2  121  KLQEKREREEAVQKMLDQAENERIWQNPEPPKDLRLEKYRPSCPFYNKTGACRFGNRCSR
                                                 **    * ** * **** * ****
U2AF   1                                  MAEYLASIFGTEKDKVNCSFYFKIGACRHGDRCSR

SP2  181  KHDFPTSSPTLLVKSMFTTFGMEQCRRDDYDSDANLEYSEEETYQQFLDFYHDVLPEF-K
           *  ** * *                                    * *       * * * *
U2AF  36  LHNKPTFSQTIALLNIYRN----PQNSSQSADGLRCAVSDVEMQEHYDEFFEEVFTEMEE

SP2  240  NVGKVIQFKVSCNLEPHLRGNVYVQYQSEEECQAALSLFNGRWYAGRQLQCEFCPVTRWK
            * *    *  **  ** *****    **    *    * ** *    *   ***
U2AF  92  KYGEVEEMNVCDNLGDHLVGNVYVKFRREEDAEKAVIDLNNRWFNGQPIHAELSPVTDFR

SP2  300  VAICGLFEMQKCPKGKHCNFLHVFRNPNNEFREANRDIYMSPPAWTGSSGKNSDRRER-K
           * *   **  * *  *** *       * *   * *         *      ** * *
U2AF 152  EACCRQYEMGECTRGGFCNFMH-LKPISRELR---RELYGRRRKKHRSRSRSRERRSRSR

SP2  359  DHHEEYYSKSRSYHSGSYHSSKRNRESERKSPHRWKKSHKQTTKSHERHSSRRGREEDSS
          *              *      * *  **
U2AF 208  DRGRGGGGGGGGGGGGRERDRRRSRDRERSGRF

SP2  419  PGPQSQSHRT
```

Figure 5. Comparison of amino acid sequences of putative polypeptide encoded by *SP2* cDNA (*SP2*) and U2 small nuclear ribonucleoprotein auxiliary factor small subunits (U2AF) (21). The asterisks indicate conserved amino acids. We observed a 31% homology between these two polypeptides.

Table 1. BXD SDP of SP2 and flanking markers

	BXD strain number																														
	1	2	5	6	8	9	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	3
Chr. 11							1	2	3	4	5	6	8	9	0	1	2	3	4	5	7	8	9	0	1	2
Glns	B	D	B	D	B	D	B	B	D	D	D	B	D	B	D	B	B	D	D	B	D	D	D	D	D	D
SP2	B	D	B	D	B	D	B	D	D	D	D	B	D	D	D	B	B	D	D	B	D	D	D	D	D	D
Hba	B	D	B	D	B	B	B	B	D	D	D	B	D	D	D	B	B	B	D	B	D	D	D	D	U	U

The letters B and D are used as genetic symbols for alleles inherited from progenitor strains C57BL/6 and DBA2, respectively. U represents an unknown allele. X represents a recombination event between flanking markers.

smaller than normal mice. Likewise, paternal gene deficiencies in human chromosome 15q11-13 is associated with Prader–Willi syndrome (PWS) (18,19), whose common clinical features include short stature (20) in addition to other pleiotrophic features. In this region, small nuclear ribonucleoprotein polypeptide N, which is shown to be imprinted in mouse (7,8), is located. Therefore, it is intriguing to speculate that *SP2* may function in the regulation of body size, perhaps as a trans-acting factor promoting production of an alternative spliced product which encodes the active form of some growth promoting gene. It still remains possible, however, that *SP2* may also be involved in a phenotype, the manifestation of which is difficult to observe in mouse such as mental disorder as observed in PWS.

ACKNOWLEDGEMENTS

We thank Drs K.Moriwaki for providing *M.m.molossinus*, Dr K.Hidaka for helping in homology search analysis and providing 'CpG Bio' and Applied Biosystems Japan for helping in sequence analysis. This work was supported by 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. Barlow, D.P., Stoger, R., Herrmann, B.G., Saito, K. and Schweifer, N. (1991)*Nature*, **349**, 84–7.
5. DeChiara, T.M., Robertson, E.J. and Efstratiadis, A. (1991)*Cell*, **64**, 849–59.
6. Bartolomei, M.S., Zemel, S. and Tilghman, S.M. (1991)*Nature*, **351**, 153–5.
7. Leff, S.E., Brannan, C.I., Reed, M.L., Ozcelik, T., Francke, U., Copeland, N.G. and Jenkins, N.A. (1992)*Nature Genet.*, **2**, 259–64.
8. Cattanach, B.M., Barr, J.A., Evans, E.P., Burtenshaw, M., Beechey, C.V., Leff, S.E., Brannan, C.I., Copeland, N.G., Jenkins, N.A. and Jones, J. *et al.* (1992)*Nature Genet.*, **2**, 270–4.
9. Hatada, I., Hayashizaki, Y., Hirotsune, S., Komatsubara, H. and Mukai, T. (1991)*Proc. Natl. Acad. Sci. U. S. A.*, **88**, 9523–7.
10. Ferguson, S.A., Sasaki, H., Cattanach, B.M. and Surani, M.A. (1993)*Nature*, **362**, 751–5.
11. Stoger, R., Kubicka, P., Liu, C.-G, Kafri, T., Razin, A., Cedar, H. and Barlow, D.P. (1993)*Cell*, **73**, 61–71.
12. Toda-Minowa M. and Hashimoto-Gotoh T. (1992)*Gene*, **121**, 25–33.
13. Hashimoto-Gotoh, T., Tsujimura, A., Kuriyama, K. and Matsuda, S. (1993)*Gene*, in press.
14. Chomczynski, P. and Sacchi, N. (1987)*Anal. Biochem.*, **162**, 156–9.
15. Veres, G., Gibbs, R. A., Scherer, S. E. and Caskey, C. T. (1987)*Science*, **237**, 415–7.
16. Taylor, B.A. (1989)In Lyon, M.F. and Searle A.G. (eds),*Genetic Variants and Strains of the Laboratory Mouse*, 2nd edn., Oxford University Press, pp773–96.
17. Silver, J. (1985)*J. Hered.* **78**, 436–40.
18. Ledbetter, D.H. (1981)*New Engl. J. Med.*, **304**, 325–9.
19. Nicholls, R.D., Knoll, J.H., Butler, M.G., Karam, S. and Lalande, M. (1989)*Nature*, **342**, 281–215.
20. Prader, A., Labhart, A. and Willi, H. (1956)*Schweiz. Med. Wochenschr.* **86**, 1260–1.
21. Zhang, M., Zamore, P.D., Carmo, F.M., Lamond, A.I. and Green, M.R. (1992)*Proc. Natl. Acad. Sci. U. S. A.*, **89**, 8769–73.