Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 1102–1107, February 1998
Developmental Biology

Identification of the *Meg1/Grb10* imprinted gene on mouse proximal chromosome 11, a candidate for the Silver–Russell syndrome gene

(androgenetic embryos/prenatal growth deficiency/growth factor receptor-bound protein/insulin and insulin-like growth factors/signal transduction pathways)

NAOKI MIYOSHI*, YOSHIMI KUROIWA*†, TAKASHI KOHDA*, HIROSHI SHITARA‡, HIROMICHI YONEKAWA‡, TOHRU KAWABE§, HIDEAKI HASEGAWA§, SHEILLA C. BARTON¶, M. AZIM SURANI¶, TOMOKO KANEKO-ISHINO‖, AND FUMITOSHI ISHINO*,**,††

*Gene Research Center, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama-226; ‡Department of Laboratory Animal Science, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan; §Tokai University, School of Medicine, Bohseidai, Isehara-shi, Kanagawa 259-11, Japan; ¶Wellcome/Cancer Research Campaign Institute of Cancer and Developmental Biology, Tennis Court Road, Cambridge CB2 1QR, and Physiological Laboratory, University of Cambridge, United Kingdom; ‖Tokai University, School of Health Sciences, Bohseidai, Isehara-shi, Kanagawa 259-11, Japan; and **Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, 4-1-8 Hon-machi, Kawaguchi-shi, Saitama 332, Japan

Communicated by Mary F. Lyon, Medical Research Council, Oxon, United Kingdom, November 25, 1997 (received for review September 22, 1997)

ABSTRACT **In a systematic screen for maternally expressed imprinted genes using subtraction hybridization with androgenetic and normal fertilized mouse embryos, seven candidate maternally expressed genes (*Megs*) have been isolated, including the *H19* and *p57^{Kip2}* genes that are known to be maternally expressed. Herein, we demonstrate that an imprinted gene, *Meg1*, is apparently identical to *Grb10* (growth factor receptor-bound protein 10), which is located on mouse proximal chromosome 11. Grb10 protein was reported to bind to the insulin receptor and/or the insulin-like growth factor (IGF) I receptor via its src homology 2 domain and to inhibit the associated tyrosine kinase activity that is involved in the growth promoting activities of insulin and IGFs (IGF-I and -II). Thus, it is probable that *Meg1/Grb10* is responsible for the imprinted effects of prenatal growth retardation or growth promotion caused by maternal or paternal duplication of proximal chromosome 11 with reciprocal deficiencies (MatDp.prox11 or PatDp.prox11), respectively. In the human, it has been reported that the maternal uniparental disomy 7 is responsible for the Silver–Russell syndrome (SRS) whose effects include pre- and postnatal growth retardation and other dysmorphologies. The human homologue *GRB10* on chromosome 7q11.2–12 is a candidate gene for Silver–Russell syndrome.**

Genomic imprinting results in the preferential expression of either a paternal or maternal allele of imprinted genes that affect mammalian development, growth, and behavior (1–3). Furthermore, uniparental disomy or partial uniparental duplication of some chromosomal regions is associated with several human diseases (4–6), indicating involvement of imprinted genes in these cases. To elucidate the biological importance of genomic imprinting and its role in human diseases, it is important to isolate the remaining imprinted genes systematically and to establish what kinds of genes are involved in this phenomenon. In the mouse, more than 10 paternally expressed imprinted genes have been isolated, but only 4 maternally expressed genes located in two imprinted chromosomal regions, distal chromosome 7 [*H19* (7), *Mash2* (8), and *p57^{Kip2}* (9)] and proximal chromosome 17 [*Igf2r* (10)], have so far been reported. We have described a subtraction-hybridization method (11, 12) for systematic isolation of paternally expressed genes (*Pegs*) using parthenogenetic and normal fertilized embryos. From this screen, we have identified eight *Pegs* (*Peg1–8*) in five different chromosomal imprinted regions, including the previously reported imprinted genes *Igf2* and *Snrpn* (11–16), and then we have applied this method with further improvements for isolating *Megs* from normal fertilized and androgenetic embryos that have only paternal genomes.

In humans, paternal duplication of chromosome 11p15.5 causes Beckwith–Wiedemann syndrome that showed embryonal overgrowth and some dysmorphologies (17, 18). At least two imprinted genes, *p57^{Kip2}* (9, 19, 20) and *Igf2* (13, 21) that are located on distal mouse chromosome 7 and share syntenic homology to human chromosome 11p15.5, are implicated in Beckwith–Wiedemann syndrome. In another case, paternal duplication of 15q11–13 results in Angelman syndrome (AS) with severe mental retardation and seizure. Mutations of *UBE3A/E6-AP* (ubiquitin protein ligase 3A) that is located in the region responsible for this syndrome have been reported in AS patients (22, 23). Recently, it was demonstrated that *UBE3A/E6-AP* is imprinted in a very restricted region of the brain and it represents the best candidate gene for AS (24–26).

In this study, we report the identification of seven candidate *Megs* and demonstrate that an imprinted gene, *Meg1*, is identical to *Grb10* gene (27) that encodes growth factor receptor-bound protein 10, a negative regulator in the signal transduction pathways for insulin and/or insulin-like growth factors (IGFs). Because it is located on proximal chromosome 11 (27), it is highly probable that *Meg1/Grb10* is responsible for the imprinting effects of prenatal growth retardation or embryonal growth promotion by MatDp.prox11 or PatDp.prox11, respectively (3, 28). Moreover, chromosome location of human *GRB10* on chromosome 7p11.2–12 (29) suggests that it is also a candidate gene for the Silver–Russell syndrome (SRS) that shows pre- and postnatal growth retardation with some morphological abnormalities (30–32).

MATERIALS AND METHODS

Mice and Embryos. Androgenetic embryos of 129/sv strain were prepared by pronuclear transplantation exactly as de-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: IGF, insulin-like growth factor; SRS, Silver–Russell syndrome; SSQA, sequence-specific quantitative amplification.

‡Present address: Central Laboratory for Key Technology, Kirin Brewery Co. Ltd., Yokohma, Japan.
††To whom reprint requests should be addressed at: Gene Research Center, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama-226, Japan. e-mail: fishino@bio.titech.ac.jp.

scribed (33). Androgenones were isolated on day 9.5 of gestation. Only viable embryos with 6–15 somites were collected; the rest were excluded from the pool used for preparation of cDNA libraries. All extraembryonic membranes were also excluded. Further day 9.5 and day 12.5 embryos from (C57BL/6 × *Mus spretus*)F_1 and (C57BL/6 × *M. spretus*)F_1 × C57BL/6 were produced by *in vitro* fertilization or obtained after natural mating.

Subtraction Hybridization Between Embryos from Fertilized and Androgenetic Eggs. Subtraction-hybridization experiments were carried out as described (11, 12) with modifications. Twenty-two androgenetic embryos (6–15 somite stage) and 26 stage-matched fertilized embryos of the same 129/sv genotype (10–15 somite stage) were used to prepare cDNA. The mRNA was purified via an oligo(dT)-cellulose method using the Fast Track mRNA isolation kit (Invitrogen). Five hundred nanograms of each mRNA was converted to first-strand cDNA according to the instructions provided in the λZAPII cDNA synthesis kit (Stratagene) except that Superscript II reverse transcriptase (GIBCO/BRL) and dT^{13-18} primer (Boehringer Mannheim) were used. After secnd-strand cDNA synthesis, cDNAs were blunted by treatment with cloned *Pfu* DNA polymerase and ligated with specific 20-mer oligonucleotide linkers on both ends. Two different sets of linkers (F linker and A linker) were used for the cDNA from normal and androgenetic embryos, respectively. One-fifteenth of the cDNAs was amplified by PCR using primers complementary to the F linker and A linker, respectively. cDNA was added to a 100-μl reaction mixture containing 20 mM Tris·HCl (pH 8.2), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 0.1% Triton X-100, 10 μg of BSA, all four dNTPs (each at 120 μM), 80 pmol of each oligonucleotide primer, and 2.5 units of *Pfu* DNA polymerase (Stratagene). Amplification consisted of a total of 30 cycles at 96°C for 5 sec, 65°C for 1 min, and 72°C for 10 min in a Perkin–Elmer GeneAmp PCR system 9600. For cDNA from androgenetic embryos, a 5′ biotinylated primer was used in all cycles of PCR. The cDNA from both the fertilized and androgenetic embryos were amplified for the same number of PCR cycles prior to subtraction hybridization. A linker primers were 5′-GATTACTCGAGACTAATATC-3′ and 5′-pGATATTAG-TCTCGAGTA-3′; F linker primers were 5′-TCGACTCGAGT-ATAGTTACA-3′ and 5′-pTGTAACTATACTCGAGT-3′.

After amplification, subtraction was carried out as described (11). Subtracted cDNA obtained after three subtractions and PCR amplifications was used as a probe for screening.

DNA Sequencing. Sequences were determined by the dideoxynucleotide method using the Thermo Sequenase fluorescent labeled cycle sequence kit (Amersham) for the Shimadzu automatic Sequencer DSQ-1000L.

Genomic PCR and Reverse Transcription-Coupled PCR by the Sequence-Specific Quantitative Amplification (SSQA) Method. DNA fragments 352 bp long from the two strains containing the polymorphic site were amplified first with a common primer set: 96°C for 15 sec, 65°C for 30 sec, and 72°C for 1 min for 30 cycles. Each PCR product (5 ng, about 10%) was then reamplified with strain-specific forward primers. The same reverse primer was used in both amplifications. Amplification consisted of 10 cycles at 96°C for 15 sec, 52°C for 30 sec, and 72°C for 1 min in a Perkin–Elmer GeneAmp PCR system 9600.

First primer sets were as follows: forward primer, 5′-AAATGACGACTCCGTGTAACC-3′; reverse primer, 5′-TTAACACCCTCTGCATTCCC-3′. Second strain-specific primers were as follows: B6-specific forward primer, 5′-TTTTAATAGGTAGACTCTGTTC-3′; *spretus*-specific forward primer, 5′-TTTTAATAGGTAGACTCTGTTA-3′. Only exo$^-$ *Pfu* DNA polymerase (Stratagene; used in this study) and delta *Tth* DNA polymerase (TOYOBO, Osaka) that lack 3′ → 5′ exonuclease activity gave satisfactory results with regard to specificity and quantitativeness. Recombinant *Taq* DNA polymerase and native *Pfu* DNA polymerase gave false bands derived from mismatched extension (data not shown) in both reciprocal reactions.

Genomic PCR and reverse transcription-coupled PCR were performed with Takara Ex *Taq* (Takara Shuzo, Kyoto). Fifty nanograms of genomic DNA was used for the genomic PCR. One microgram of total RNA was used to synthesize cDNA with the Superscript preamplification system (GIBCO/BRL) and 1% (day 9.5 embryos) or 10% (adult mouse samples) of the resulting material was used for reverse transcription-coupled PCR.

***In Situ* Hybridization.** RNA probes were generated by using the DIG RNA labeling kit (Boehringer Mannheim). Embryos and placental tissues were dissected at day 13, embedded in OCT compound, and frozen. *In situ* hybridization was carried out as described (11, 14) with some modifications. In brief, sections were fixed in 4% paraformaldehyde, acetylated with acetic anhydride/triethanolamine, and then dehydrated in ethanol. Digoxigenin-labeled RNA probes were hybridized to the sections at 65°C overnight in 50% formamide/5× SSC/1× Denhardt's solution/300 μg of tRNA/1 mM EDTA/10 mM DTT. Sections were washed with 50% formamide/2× SSC at 65°C and then incubated with RNase A at 37°C, followed by subsequent washes with 50% formamide in 2× SSC (twice) and in 0.1× SSC at 65°C. Next, anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) was added and expression was detected by using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) color reaction. Finally, sections were counterstained with methyl green.

RESULTS

Systematic Screening of *Megs*. Twenty-two androgenetic embryos (6–15 somite stage) (1, 33) and 26 stage-matched fertilized embryos (10–15 somite stage) were used to isolate mRNA (1.6 μg and 2.4 μg, respectively). Five hundred nanograms of each mRNA was converted to cDNA and ligated with specific linker as described (11, 12). Aliquots of the cDNA, representing only one-fifteenth of both cDNA pools, were used for the subtraction procedure. After amplification by PCR with primers that were complementary to their linkers, three cycles of subtractions were carried out and the resulting cDNA was

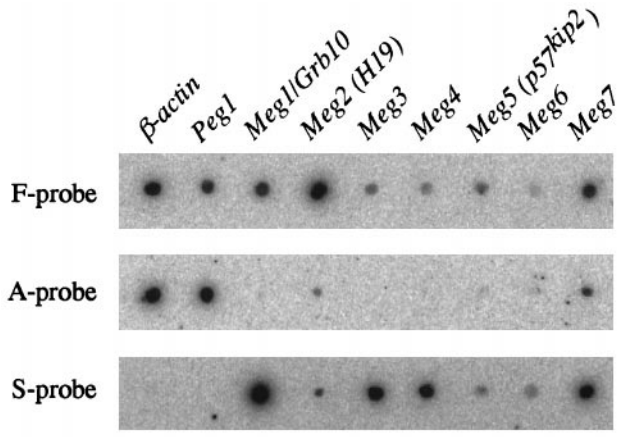

FIG. 1. Differential hybridization of candidate maternally expressed genes. The control β-actin gene that is biallelically expressed and the *Peg1/Mest* gene that is paternally expressed were removed after three cycles of subtraction. The positive control, *H19,* which is expressed maternally is shown as *Meg2* (*H19*). Thirty nanograms of each DNA from clones representing *Meg1* and *Meg3–7* were spotted onto filter membranes and hybridized with F, A, and S probes, respectively. Because of the very high level of expression of the *Meg2* (*H19*) gene, only 1.5 ng of DNA was spotted.

used as a subtracted probe to screen for *Megs*. Fifty thousand plaques of a cDNA library made from day 8 embryos were screened. Positive plaques were purified and isolated cDNAs were dotted onto three membrane filters and hybridized with three probes from normal fertilized (F probe), androgenetic (A probe), and subtracted cDNA (S probe), respectively. From this analysis, we finally selected seven candidate clones for *Megs*. As seen in Fig. 1, *Meg1, 3, 4,* and *6* were clearly detected by the F probe but not by the A probe. They were also highly concentrated in the subtracted cDNA. Clones representing *Meg2, 5,* and *7* were selected despite the fact that they were detected by the A probe, because their expression in the fertilized embryos seemed severalfold higher than in the androgenetic embryos. As a control, we used the β-actin and *Peg1/Mest* genes, and both of these were excluded as expected after three cycles of subtractions. However, the previously identified imprinted gene *H19* (identical to *Meg2*; see below) did not show efficient enrichment in the subtracted probe. This may be because a slight expression of *H19* (about 1% of the amount in the fertilized embryos) observed in the androgenetic embryos after PCR amplification (data not shown). This may explain the reduced enrichment of *H19* in the subtracted probe because a 100-fold excess of androgenetic cDNA was used in each subtraction step.

After DNA sequencing, *Meg2* and *Meg5* were found to be identical to *H19* (7) and $p57^{Kip2}$ (9), respectively; these genes were previously shown to be imprinted and maternally expressed. Leaky expression from the paternal allele of $p57^{Kip2}$ was reported previously, which is also consistent with the less-efficient enrichment of this clone, similar to the observations described above for *H19*. On the basis of the available evidence, it is probable that like *Meg1* (see below), the remaining candidates are likely to represent additional candidate imprinted genes. These results suggest that there are comparable numbers of *Megs* and *Pegs* in the mouse genome because we have isolated about seven *Megs* and a similar number of *Pegs* after screening only fifty thousand clones.

Identification of *Meg1* and Verification of its Imprinting. We isolated several *Meg1* cDNA clones, the longest of which was 5.4 kb. The homology search using this cDNA sequence showed that it was identical to *Grb10* but with a 75-bp deletion compared with the reported sequence (Fig. 2*a*). Ooi *et al.* (27) initially reported a 2.4-kb DNA sequence of *Grb10* that contained the entire coding region of Grb10 protein (Fig. 2*a*). Their Northern blot hybridization of *Grb10* suggested that the full-length cDNA was 5.5 kb. A splicing variant that has a 75-bp deletion (25-aa deletion) in the coding frame of the Grb10 protein was also recently reported and shown to be the major form of the *Grb10* transcript (34). The longest *Meg1/Grb10* cDNA in this study is 5.4 kb long and has the 75-bp deletion, indicating that this is probably almost the full-length clone of the major transcript (Fig. 2*a*). To verify maternal expression of *Meg1/Grb10*, we identified a point mutation polymorphism between C57BL/6 (B6) and *M. spretus* mice. However, this DNA polymorphism was not located in any known restriction enzyme recognition sites. Furthermore, single-strand conformational polymorphism analysis of fragments from the two strains did not give a clear result. We therefore used a PCR detection method with some modifications, using primers that have the point mutation site at the 3′ end (SSQA). This method was based on previously reported methods, such as the amplification refractory mutation system (35), allele-specific PCR (36), or mutant-allele-specific amplification (37), for detecting human genetic diseases. However, in our experiments using the primers shown in Fig. 2*a*, amplification specificity did not seem sufficient to avoid the production of false bands (mismatched amplification from reciprocal primers) under the usual reaction conditions (data not shown). Thus, we chose exo$^-$ *Pfu* enzyme for amplification because it lacks a 3′ → 5′ exonuclease proofreading activity. Its application much improved the specificity and quantitativeness of allele-specific amplification between B6 and *M. spretus* genomic DNAs (Fig. 2 *b–d Left*). With this SSQA method, maternal expression of *Meg1/Grb10* was confirmed in day 10 and day 13 whole embryos and skeletal muscles from day 1 neonates and adults of (B6 × *spretus*)F_1. Thus, only the B6-specific primer gave the expected band in all samples (Fig. 2*d Upper Right*). On the other hand, only the *spretus* allele was detected in the skeletal muscle of neonatal progenies of the backcross that are heterozygous for the *Meg1/Grb10* allele, produced by mating of (B6 × *spretus*)F_1 females to B6 males (Fig. 2*d Lower Right*).

In situ hybridization experiments showed that *Meg1/Grb10* was expressed in almost all tissues of embryos and placentas (Fig. 3 *a* and *c*) at the day 13 stage. The expression analysis by *in situ* hybridization was repeated and the time allowed for the color reaction was altered. These studies indicated that *Meg1/Grb10* was widely expressed. However, there were no detectable differences in expression between different tissues (see below). Expressions of *Meg1/Grb10* has been observed in heart, kidney, lung, liver, and brain in adult mice, and expression of human *GRB10* has been observed in adult human skeletal muscle and pancreas by Northern blot analysis (27, 38). These expression patterns appear to be consistent between embryos and adults. It is noteworthy that all the imprinted genes, including *Meg1/Grb10*, examined so far show expression in the placenta (unpublished results).

DISCUSSION

***Meg1/Grb10* in Insulin and IGFs Signaling Pathways May Affect Embryonal Growth.** Mouse *Grb10* has been reported to map to proximal chromosome 11 (27) (near *Egfr*). Genetic studies have shown that the maternal duplication and paternal deficiency of chromosome 11 proximal to the translocation breakpoint T30H (MatDp.prox11.T30H) caused prenatal growth retardation and, conversely, PatDp.prox11.T30H caused enhanced growth in both embryos and placentas (3, 39). In humans, the GRB10 protein binds to the insulin receptor and IGF-I receptor through its src homology 2 domain and negatively regulates the growth-promoting effects of insulin and IGFs (IGF-I and -II), by inhibiting the tyrosine kinase activities of these receptors toward their substrates, such as IRS-1 (insulin receptor substrate 1) and phosphatidylinositol 3-kinase (40). It has also been demonstrated that microinjection of human GRB10 src homology 2 domain inhibited growth at the cellular level (38). There have also been reports that deletion of *IGF1R* on chromosome 15 caused SRS with severe pre- and postnatal growth deficiency (41). In the mouse, knockouts of *Igf1* (42), *Igf2* (43), and their receptor, *Igf1r* (44), demonstrated that all these genes substantially affected embryonal growth. Furthermore, the *Irs-1* knockout demonstrated that this gene was also very important for embryonal growth (45, 46). Although no direct evidence has been obtained to demonstrate that Meg1/Grb10 interacts with Irs-1, it is probable that overexpression of Meg1/Grb10 inhibits the tyrosine phosphorylation of Irs-I, directly or indirectly, causing growth retardation in the MatDp.prox11.T30H embryos. Recently, the region has been narrowed down to between the T57H breakpoint and the centromere (47). In this imprinted region, another paternally expressed imprinted gene, *U2af1-rs1* (U2 small nuclear ribonucleoprotein auxiliary factor related sequence 1), has been reported (48, 49). Because the *U2af1-rs1* knockout mouse showed no apparent phenotype even in homozygotes,‡‡ *Meg1/Grb10* is now a good candidate

‡‡Sunahara, S., Nakamura, K., Nakao, K., Gondo, Y., Shibata, H., Hayashizaki, Y., and Katsuki, M., Annual Meeting of the Molecular Biology Society of Japan, August 26–30, 1996, Sapporo, Hokkaido, Japan.

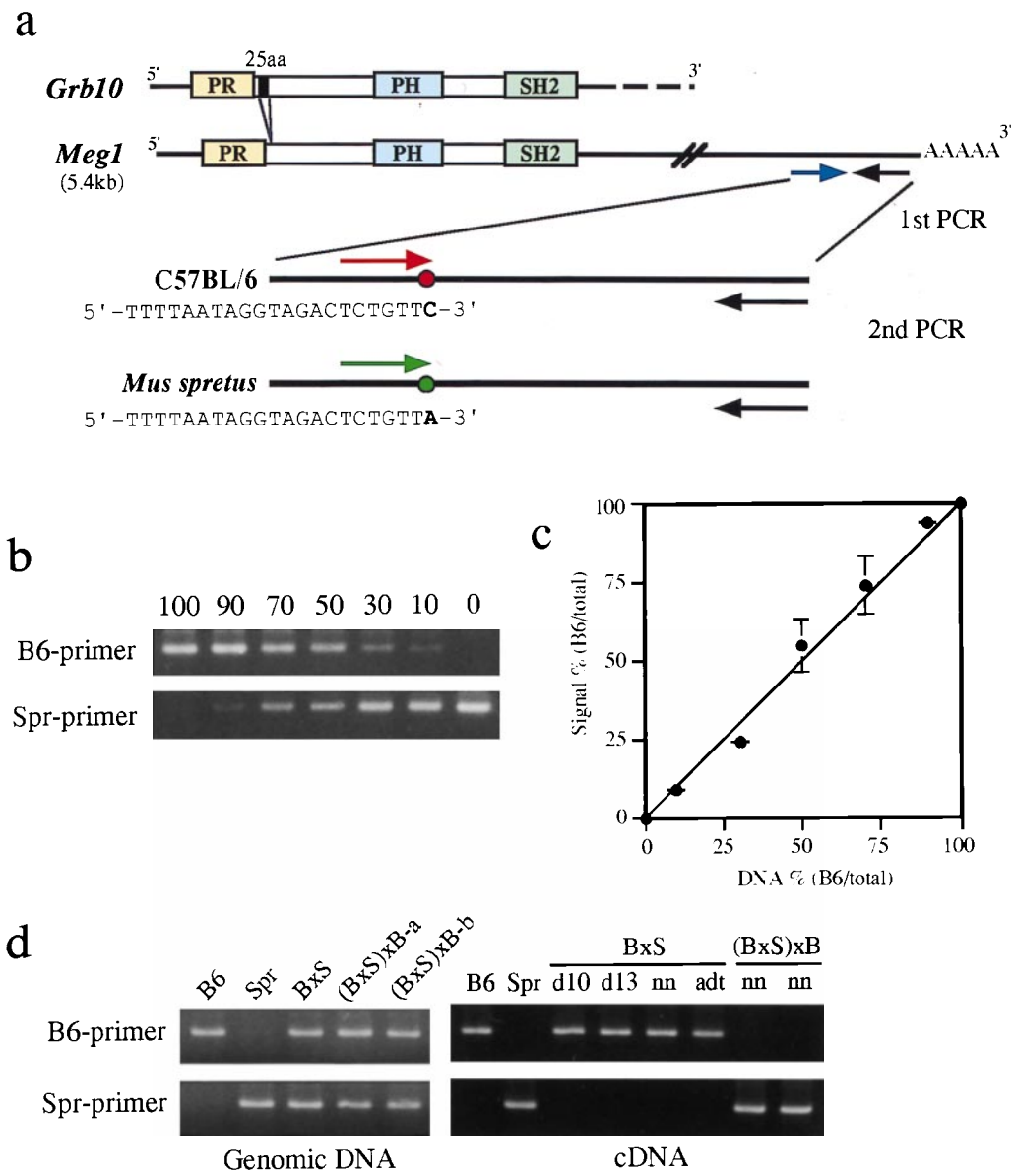

FIG. 2. (*a*) Gene structure of *Meg1/Grb10.* Protein coding regions are shown as open boxes with a three-domain structure, including PR (proline-rich sequences), PH (Pleckstrin homology domain), SH2 (src homology 2 domain), as described by Ooi *et al*. (27). The position of the first and second primer sets used for the SSQA method are shown by arrows (1st, blue and black; 2nd, red or green and black). A base substitution (C → A) occurred in *M. spretus* compared with the B6 strain in the 3′ untranslated region of the *Grb10* transcript. After 30 cycles of the first amplification with common primers, 5 ng of each PCR product was reamplified with a second set of primers. (*b*) Assessment of quantitativeness of SSQA method. Genomic DNAs isolated from B6 and *spretus* were mixed together in the proportions indicated. 100, 100% B6 and 0% *spretus* DNA; 90, 90% B6 and 10% *spretus* DNA, etc. SSQA was performed. DNAs (total 5 ng) amplified with the first PCR were reamplified with the second sets of primers for 10 cycles. (*c*) The results from three independent experiments of SSQA as shown in Fig. 2*b* were calculated and plotted. Calculation of the relative proportions of B6 DNA (*y* axis) was performed with the data measured by the PDI Bioimage analyzer, the discovery series. (*d*) Verification of *Meg1/Grb10* imprinting. (*Left*) Genomic DNAs from B6, *spretus,* (B6 × *spretus*)F_1 and two examples of (B6 × *spretus*)F_1 × B6 backcross progenies. (*Right*) Lanes: B6 and Spr, cDNA from adult skeletal muscle; BxS d10, d13, nn, and adt, cDNA from day 10 and day 13 whole embryos, skeletal muscle from day 1 neonate, and adult (B6 × *spretus*)F_1; (BxS)xB, two examples of cDNA from skeletal muscle of the progeny of (B6 × *spretus*)F_1 × B6 backcross (nn, day 1 neonates) that were heterozygous (as shown *Left* as (BxS)xB-a and -b).

for the gene responsible for the growth effects of this imprinted region.

Imprinted Genes in Insulin and IGFs Signaling Pathways. It has been suggested that the growth-promoting effects of IGF-II (Igf2) are mediated via its binding to Igf1r (50). Thus, two kinds of paternally expressed genes, *Ins* (*1* and *2*) (51) and *Igf2* (13, 43), and two maternally expressed genes, *Igf2r* (10) and *Meg1/Grb10*, are involved in the same growth controlling pathway, which is consistent with the parental conflict hypothesis for the evolution of genomic imprinting in mammals (52). This theory postulates growth promoting effects of paternally expressed genes and growth inhibiting effects for maternally expressed genes. Although recent analysis of imprinted genes from our systematic screening of *Pegs* and from other researchers showed that not all imprinted genes support the conflict hypothesis, it is noteworthy that four imprinted genes involved in the insulin and IGF pathways function as proposed by this hypothesis.

***GRB10* Is a Candidate Gene for SRS.** Human *GRB10* is located on chromosome 7q11.2–12 (29), the region that is known to show conservation of synteny with mouse proximal chromosome 11 (53), whereas human *U2AF1-RS1* has been mapped to 5q22–31 (54, 55). Maternal disomy of human chromosome 7 has been reported to cause SRS, which has

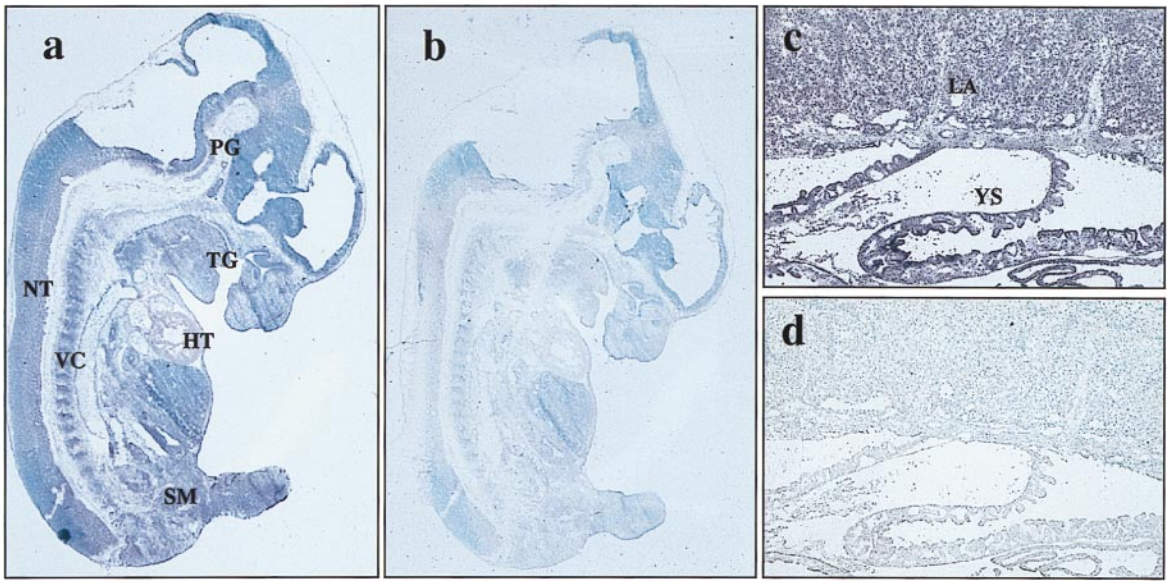

FIG. 3. Expression of *Meg1/Grb10* in day 13 embryo and placenta by *in situ* hybridization. A 0.7 kb of the 3′ untranslated region of *Meg1/Grb10* was cloned into pBluescript II SK(−). Antisense RNA probes (*a* and *c*) and control sense probes (*b* and *d*) were produced from the T7 or T3 promoters by RNA polymerase. The control sense RNA probe did not give any positive signal. HT, heart; NT, neural tube; PG, pituitary gland; SM, skeletal muscle; TG, tongue; VC, vertebral cartilage; LA, labyrinth; YS, yolk sac.

symptoms including pre- and postnatal growth retardation and some morphological disorders as described above (30–32). There have been reports that in one patient who had paternal duplication of short arm and maternal duplication of long arm of chromosome 7 showed only postnatal growth retardation. Thus, because genes on both the short and long arms of chromosome 7 may be involved in this syndrome and the phenotypes observed in duplication of the chromosomes in question in both human and mice are very similar, it is probable that they are induced by a deficiency or gain of function of imprinted gene(s) in both cases. The involvement of this gene in SRS is being examined by experiments to show imprinting of human *GRB10* and in experiments using *Meg1/Grb10* in transgenic mice. These studies are expected to reveal that one of the main causes of SRS is disruption of the insulin and IGF signaling pathways. We have previously suggested that the imprinted gene *PEG1/MEST* on 7q31–34 is another candidate gene for SRS (56).

Subtraction-Hybridization Method. To date, 11 imprinted genes (*Peg1–8*, *Meg1, 2,* and *5*) have been identified on six chromosomal regions by the subtraction-hybridization method using combinations of parthenogenetic and control fertilized embryos for *Pegs* (11) and androgenetic and fertilized embryos for *Megs* (this study). These findings demonstrate that our method for the identification of imprinted genes is very efficient and requires very small amount of biological material (12). The method could therefore be used in other areas of research on development on cell differentiation.

We thank J. Itoh (Tokai University) for assistance with photographs. This work was supported by grants from the Kanagawa Academy of Science and Technology and the Ministry of Education, Science and Culture [Grant-in-Aid for Scientific Research (C) 08680703] to T.K.-I.; PRESTO, a research program of the Japan Science and Technology Cooperation and the Ministry of Education, Science and Culture [Grant-in-Aid for Scientific Research (B) 08458216] to F.I.; Wellcome/CRC to A.S.; Japan–U.K. Research Cooperation, a research program of the Royal Society, British Council, and the Japan Society for the Promotion of Science to A.S. and F.I.

1. Surani, M. A., Barton, S. C. & Norris, M. L. (1984) *Nature (London)* **308,** 548–550.
2. McGrath, J. & Solter, D. (1984) *Cell* **37,** 179–183.
3. Cattanach, B. M. & Kirk, M. (1985) *Nature (London)* **315,** 496–498.
4. Engel, E. (1980) *Am. J. Med. Genet.* **6,** 137–143.
5. Reik, W. (1989) *Trends Genet.* **5,** 331–336.
6. Engel, E. (1997) *Ann. Genet.* **40,** 24–34.
7. Bartolomei, M. S., Zemel, S. & Tilghman, S. M. (1991) *Nature (London)* **351,** 153–155.
8. Guillemot, F., Caspary, T., Tilghman, S. M., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Anderson, D. J., Joyner, A. L., Rossant, J. & Nagy, A. (1995) *Nat. Genet.* **9,** 235–242.
9. Hatada, I. & Mukai, T. (1995) *Nat. Genet.* **11,** 204–206.
10. Barlow, D. P., Stoger, R., Herrmann, B. G., Saito, K. & Schweifer, N. (1991) *Nature (London)* **349,** 84–87.
11. Kaneko-Ishino, T., Kuroiwa, Y., Miyoshi, N., Kohda, T., Suzuki, R., Yokoyama, M., Viville, S., Barton, S. C., Ishino, F. & Surani, M. A. (1995) *Nat. Genet.* **11,** 52–59.
12. Kaneko-Ishino, T., Kuroiwa, Y., Kohda, T., Surani, M. A. & Ishino, F. (1997) *Frontiers in Molecular Biology: Genomic Imprinting*, eds. Reik, W. & Surani, A. (Oxford Univ. Press, Oxford, U.K.), pp. 146–164.
13. DeChiara, T. M., Robertson, E. J. & Efstratiadis, A. (1991) *Cell* **64,** 849–859.
14. Kuroiwa, Y., Kaneko-Ishino, T., Kagitani, F., Kohda, T., Li, L. L., Tada, M., Suzuki, R., Yokoyama, M., Shiroishi, T., Wakana, S., Barton, S. C., Ishino, F. & Surani, M. A. (1996) *Nat. Genet.* **12,** 186–190.
15. Leff, S. E., Brannan, C. I., Reed, M. L., Ozcelik, T., Francke, U., Copeland, N. G. & Jenkins, N. A. (1992) *Nat. Genet.* **2,** 259–264.
16. Kagitani, F., Kuroiwa, Y., Wakana, S., Shiroishi, T., Miyoshi, N., Kobayashi, S., Nishida, M., Kohda, T., Kaneko-Ishino, T. & Ishino, F. (1997) *Nucleic Acids Res.* **17,** in press.
17. Reik, W., Brown, K. W., Schneid, H., Le Bouc, Y., Bickmore, W. & Maher, E. R. (1995) *Hum. Mol. Genet.* **4,** 2379–2385.
18. Morison, I. M., Becroft, D. M., Taniguchi, T., Woods, C. G. & Reeve, A. E. (1996) *Nat. Med.* **2,** 311–316.
19. Zhang, P., Liegeois, N. J., Wong, C., Finegold, M., Hou, H., Thompson, J. C., Silverman, A., Harper, J. W., DePinho, R. A. & Elledge, S. J. (1997) *Nature (London)* **387,** 151–158.
20. Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W. & Elledge, S. J. (1995) *Genes Dev.* **9,** 650–662.
21. Sun, F. L., Dean, W., Kelsey, G., Allen, N. & Reik, W. (1997) *Nature (London)*, in press.
22. Matsuura, T., Sutcliffe, J. S., Fang, P., Galjaard, R. J., Jiang, Y. H., Benton, C. S., Rommens, J. M. & Beaudet, A. L. (1997) *Nat. Genet.* **15,** 74–77.

23. Kishino, T., Lalande, M. & Wagstaff, J. (1997) *Nat. Genet.* **15,** 70–73.
24. Albrecht, U., Sutcliffe, J. S., Cattanach, B. M., Beechey, C. V., Armstrong, D., Eichele, G. & Beaudet, A. L. (1997) *Nat. Genet.* **17,** 75–78.
25. Rougeulle, C., Glatt, H. & Lalande, M. (1997) *Nat. Genet.* **17,** 14–15.
26. Sun, H. & Nathans, J. (1997) *Nat. Genet.* **17,** 12–13.
27. Ooi, J., Yajnik, V., Immanuel, D., Gordon, M., Moskow, J. J., Buchberg, A. M. & Margolis, B. (1995) *Oncogene* **10,** 1621–1630.
28. Cattanach, B. M. & Beechey, C. V. (1996) *Mouse Genome* **94,** 96–99.
29. Jerome, C. A., Scherer, S. W., Tsui, L. C., Gietz, R. D. & Triggs-Raine, B. (1997) *Genomics* **40,** 215–216.
30. Spotila, L. D., Sereda, L. & Prockop, D. J. (1992) *Am. J. Hum. Genet.* **51,** 1396–1405.
31. Eggerding, F. A., Schonberg, S. A., Chehab, F. F., Norton, M. E., Cox, V. A. & Epstein, C. J. (1994) *Am. J. Hum. Genet.* **55,** 253–265.
32. Kotzot, D., Schmitt, S., Bernasconi, F., Robinson, W. P., Lurie, I. W., Ilyina, H., Mehes, K., Hamel, B. C., Otten, B. J., Hergersberg, M., Werder, E., Schoenle, E. & Schinzek, A. (1995) *Hum. Mol. Genet.* **4,** 583–587.
33. Barton, S. C. & Surani, M. A. (1993) in *Manipulations of Genetic Constitution by Nuclear Transplantation*, Vol. 225, pp. 732–744.
34. Laviola, L., Giorgino, F., Chow, J. C., Baquero, J. A., Hansen, H., Ooi, J., Zhu, J., Riedel, H. & Smith, R. J. (1997) *J. Clin. Invest.* **99,** 830–837.
35. Newton, C. R., Graham, A., Heptinstall, L. E., Powell, S. J., Summers, C., Kalsheker, N., Smith, J. C. & Markham, A. F. (1989) *Nucleic Acids Res.* **17,** 2503–2516.
36. Wu, D. Y., Ugozzoli, L., Pal, B. K. & Wallace, R. B. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2757–2760.
37. Takeda, S., Ichii, S. & Nakamura, Y. (1993) *Hum. Mutat.* **2,** 112–117.
38. O'Neill, T. J., Rose, D. W., Pillay, T. S., Hotta, K., Olefsky, J. M. & Gustafson, T. A. (1996) *J. Biol. Chem.* **271,** 22506–22513.
39. Cattanach, B. M., Beechey, C. V., Rasberry, C., Jones, J. & Papworth, D. (1996) *Genet. Res.* **68,** 35–44.
40. Liu, F. & Roth, R. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10287–10291.
41. Tamura, T., Tohma, T., Ohta, T., Soejima, H., Harada, N., Abe, K. & Niikawa, N. (1993) *Clin. Dysmorphol.* **2,** 106–113.
42. Baker, J., Hardy, M. P., Zhou, J., Bondy, C., Lupu, F., Bellve, A. R. & Efstratiadis, A. (1996) *Mol. Endocrinol.* **10,** 903–918.
43. DeChiara, T. M., Efstratiadis, A. & Robertson, E. J. (1990) *Nature (London)* **345,** 78–80.
44. Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J. & Efstratiadis, A. (1993) *Cell* **75,** 59–72.
45. Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., Sekihara, H., Furuta, Y., Ikawa, Y., Kasuga, M., Yazaki, Y. & Aizawa, S. (1994) *Nature (London)* **372,** 182–186.
46. Araki, E., Lipes, M. A., Patti, M. E., Bruning, J. C., Haag, B. R., Johnson, R. S. & Kahn, C. R. (1994) *Nature (London)* **372,** 186–190.
47. Beechey, C. V. & Cattanach, B. M. (1997) *Mouse Genome* **95,** 161.
48. Hatada, I., Sugama, T. & Mukai, T. (1993) *Nucleic Acids Res.* **21,** 5577–5582.
49. Hayashizaki, Y., Shibata, H., Hirotsune, S., Sugino, H., Okazaki, Y., Sasaki, N., Hirose, K., Imoto, H., Okuizumi, H., Muramatsu, M., Komatsubara, H., Shiroishi, T., Moriwaki, K., Katsuki, M., Hatano, N., Sasaki, H., Ueda, T., Mise, N., Takagi, N., Plass, C. & Chapman, V. M. (1994) *Nat. Genet.* **6,** 33–40.
50. Czech, M. P. (1989) *Cell* **59,** 235–238.
51. Giddings, S. J., King, C. D., Harman, K. W., Flood, J. F. & Carnaghi, L. R. (1994) *Nat. Genet.* **6,** 310–313.
52. Haig, D. & Graham, C. (1991) *Cell* **64,** 1045–1046.
53. Buchberg, A. M. & Camper, S. A. (1993) *Mamm. Genome*, S164–75.
54. Kitagawa, K., Wang, X., Hatada, I., Yamaoka, T., Nojima, H., Inazawa, J., Abe, T., Mitsuya, K., Oshimura, M., Murata, A., Monden, M. & Mukai, T. (1995) *Genomics* **30,** 257–263.
55. Kalcheva, I., Plass, C., Sait, S., Eddy, R., Shows, T., Watkins-Chow, D., Camper, S., Shibata, H., Ueda, T., Takagi, N., Hayashizaki, Y. & Chapman, V. M. (1995) *Cytogenet. Cell Genet.* **68,** 19–24.
56. Kobayashi, S., Kohda, T., Miyoshi, N., Kuroiwa, Y., Aisaka, K., Tsutsumi, O., Kaneko-Ishino, T. & Ishino, F. (1997) *Hum. Mol. Genet.* **6,** 781–786.