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Identification of a New Imprinted Gene, *Rian*, on Mouse Chromosome 12 by Fluorescent Differential Display Screening[1]

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Systematic screening of differentially expressed genes among androgenetic, parthenogenetic, and normal embryos by means of fluorescent differential display revealed five imprinted genes. One of them, named *Rian*, was expressed exclusively from the maternal allele and was closely linked to an imprinted gene, *Meg3(Gtl2)*, mapped to the distal end of chromosome 12. The *Rian* transcript did not have any apparent open reading frame, and its transcript was exclusively localized to the nucleus.

Key words: androgenetic embryo, imprinting, imprinted gene, parthenogenetic embryo.

Genomic imprinting refers to the differential expression of the two alleles of a gene, depending on their parental origins. In mammals, parental imprinting ensures the functional inequality of paternal and maternal genomes in the fertilized egg, and causes developmental failure of embryos produced by parthenogenesis or androgenesis (*1, 2*).

To elucidate the function of imprinted genes and the molecular mechanisms of imprinting, it is important to identify and characterize additional imprinted genes. Thus far, and by several approaches, a total of approximately 45 imprinted genes have been identified in the mouse (*3*). There are two types of systematic screening for imprinted genes. The first type is based on methylation differences, and includes restriction landmark genome scanning (*4*) and representational difference analysis (*5*). The second type is based on expression differences, and includes subtraction and differential display. Hagiwara et al. reported fluorescent differential display screening of the allelic expression status of multiple polymorphic transcripts in two reciprocal F1 hybrids (*6*). Here we report the systematic identification of imprinted genes among differentially expressed genes in androgenetic, parthenogenetic, and normal embryos by fluorescent differential display screening. This method was shown to be very effective; all peaks displayed could be utilized for screening, in contrast with the previous method involving differential display with which only polymorphic bands can be utilized for screening (*6*).

For fluorescent differential display (*6*), we used androgenetic E9.5, parthenogenetic E9.5, normal E8.5 (as a control for androgenetic E9.5 to fit the developmental stage), and normal E9.5 (as a control for parthenogenetic E9.5). Each combination of arbitrary and anchor primers provided at least 50 peaks, and 30 primer combinations were used for screening, *i.e.* 1,500 peaks were screened. We searched for peaks showing unique profiles for imprinted genes. Maternally expressed genes (Megs) should give no signal in androgenetic embryos, and their signal intensity in parthenogenetic embryos should be twice that in normal embryos. On the other hand, paternally expressed genes (Pegs) should give no signal in parthenogenetic embryos, and their signal intensity in androgenetic embryos should be twice that in normal embryos. We cloned four Megs and a candidate (*H19*, $p57^{KIP2}$ × 2, *Meg1*, and the clonederived from peak J312), and a Peg (*Peg3* × 2). The number of imprinted genes identified by our method was smaller than expected. There are two possible explanations for this. The first explanation involves developmental stage–dependent differences in the expressed gene population; there are some developmental stage–specific imprinted genes (*7*). Therefore, it is necessary to screen imprinted genes in other stages The second explanation is the disruption of imprinted expression in parthenogenetic embryos. This phenomenon has been reported for an imprinted gene, *U2af1-rs1* (*8*).

We further analyzed the clone derived from peak J312 (Fig. 1) because it exhibited no sequence similarity to any known imprinted genes. The expression profile of the gene in embryos was confirmed by RT-PCR (Fig. 2a). Expression was not detected in androgenetic embryos, and the expression level in parthenogenetic embryos was higher than that in normal embryos. These data suggest it is a good candidatefor a Meg. To determine whether or not the gene was maternally expressed, we sequenced RT-PCR products derived from C57BL/6(B) and PWK(P) newborn mice RNAs. We identified a single nucleotide polymorphism in the gene, the sequence AAATACTCATAAA being present in B animals and the sequence AAATACTCGTAAA in P animals (Fig. 2b). Direct sequencing of RT-PCR products of newborn mice RNA from reciprocal F1 crosses was performed (Fig. 2b). In BPF1 hybrids, only B type products were detected. In contrast, in PBF1, only P type products were detected. These results indicate exclusive expression of the maternal

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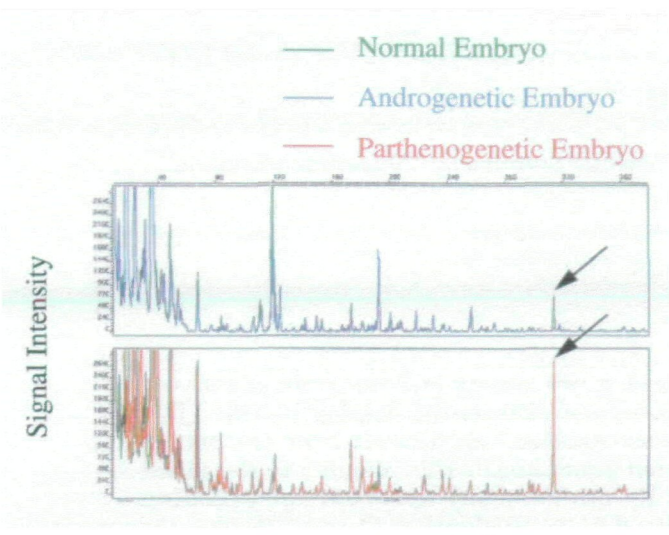


Fig 1 **Identification of a cDNA peak displayinga maternal expression pattern.** Typical fluorescent differential display patterns of RNAs from androgenetic (E9 5, blue line in the upper column), parthenogenetic (E9 5, red line inthe lower column), and normal embryos (green lines, E8 5 in the upper columnand E9 5 in the lower column) with a capillary DNA sequencer (ABI310) The peak (J312) indicated by the arrow in each column represents the Meg *Rian*, which was detected using HEX labeled GTTTTTTTTTTTTTTTV and CACCAGGTGA Other arbitrary primers used were TTCGAGCCAG, GTGAGGCGTC, GGGGGTCTTT, CCGCATCTAC, GATGACCGCC, GAACGGACTC, GTCCCGACGA, TGGACCGGTG, CTCACCGTCC, TGTCTGGGTG, ACCGCGAAGG, GGACCCAACC, GTCGCCGTCA, TCTGGTGAGG, TGAGCGGACA, ACCTGAACGG, TTGGCACGGG, GTGTGCCCCA, CTCTGGAGAC, GGTCTACACC, CCCAAGGTCC, GGTGCGGGAA, CCAGATGCAC, GTGACATGCC, TCAGGGAGGT, AAGACCCCTC, AGATGCAGCC, TCACCACGGT, and CTTCACCCGA. The fragment length of peak J312 was 312bp PCR products were subjected to polyacrylamide gel electrophoresis and bands around 312 bp were excised The recovered DNAs were subjected to reamplification by PCR The reamplified products were subcloned into the TA-cloning vector Several clones were sequenced and their sizes were determined The correct clone was selected as to the size estimated from the sequence

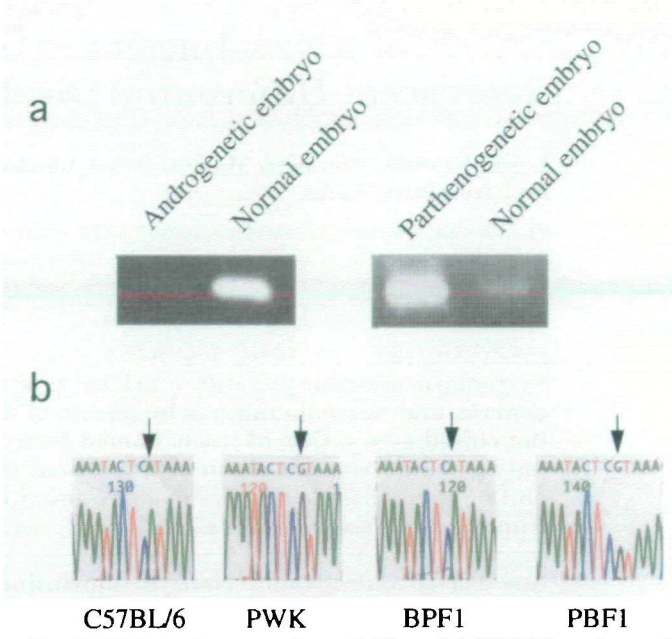


Fig 2 **Maternal expression of *Rian*.** (a) RT-PCR analysis of RNAs from androgenetic (E9 5), parthenogenetic (E9 5), and normal embryos (E8 5 as a control for androgenetic embryos, and E9 5 as a control for parthenogenetic embryos). The primers used were GCTTTTTAATGGCTGTGCATATAC and AGCAATTAATCTCACTAGTGCCT (b) Direct sequencing was performed for RT-PCR products from C57BL/6, PWK, BPF1, and PBF1 The polymorphic base is indicated by arrows. BPF1 mice were produced by natural mating between C57BL/6 females and PWK males. PBF1 mice were producedby natural mating betweenPWK females and C57BL/6 males

allele of the gene.

We screened the E12.5 cDNA library and got a cDNA of 5.4 kb in length. 5′ RACE, and comparison of the cDNA and the RACE products sequence revealed that the cDNA was full-length (DDBJ accession number, AB063319). A search with its sequence revealed that this gene exhibited no significant similarity to any sequence in the database. Weak similarity was detected with the Bsr gene (*9*), which was identified as non-coding RNA expressed in the rat brain The cDNA sequence contains multiple small open readingframes (ORFs, Fig. 3b); however, none of the ATGs is in the context of a Kozak consensus sequence. The longest ORF comprises only 76 amino acids Neither this, nor any of the shorter ORFs, encodes an amino acid sequence exhibiting homology to proteins with known functions. The subcellular location of the transcripts was analyzed to assess whether or not this gene was associated with the translational machinery in the cytoplasm. In contrast with a translated gene such as *G3PDH*, RNA FISH analysis showed the transcripts were predominantly localized in the nucleus (Fig. 4a). Therefore, we named the gene derived from peak J312 *Rian* (RNA Imprinted and Accumulated in Nucleus) RT-PCR analysis of various tissues revealed that Rian was only detected in the brain (Fig 4b).

Genetic mapping of *Rian* was carriedout by analysis of a radiation hybrid panel (The Jackson Laboratory RH mapping panel) of 100 mice. *Rian* was mapped to a 5.91 cR region between D12Mit279 and D12Mit133, where *Meg3/Gtl2* (*10, 11*), a maternally expressed imprinted gene, is located. This region is syntenic to rat6q32, where Bsr is located (*9*). Rian exhibits weak homology to the *Bsr* gene, which was identified as non-coding RNA expressed in the rat brain The paternal duplication of mouse distal chromosome 12 leads to late embryonal/neonatal lethality and growth promotion, whereas maternal duplication leads to late embryonal lethality and growth retardation (*12*). This indicates the presence of imprinted genes on chromosome 12 that are essential for normal growth and development. Consistent with this, a transgene insertion, *Gtl2*^{lacZ}, mapping to the distal portion of chromosome 12, results in growth retardation on paternal inheritance. This insertion is located 3kb upstream of the *Meg3/Gtl2* gene (*10*). The lethality accompanying the array of mutant phenotypes identified for maternal and paternal duplication of mouse distal chromosome 12 indicates that imprinted genes on chromosome 12 play roles in multiple lineages that are not affected in the *Gtl2*^{lacZ} mutant. A reciprocally imprinted gene, *Delta-like* (*Dlk*), exhibiting homology to genes involved in the Notch signaling pathway, was identified 80 kb upstream of *Meg3/Gtl2* (*13–16*). *Rian* and/or *Dlk* could be involved in the lethality accompanying the array of mutant phenotypes.

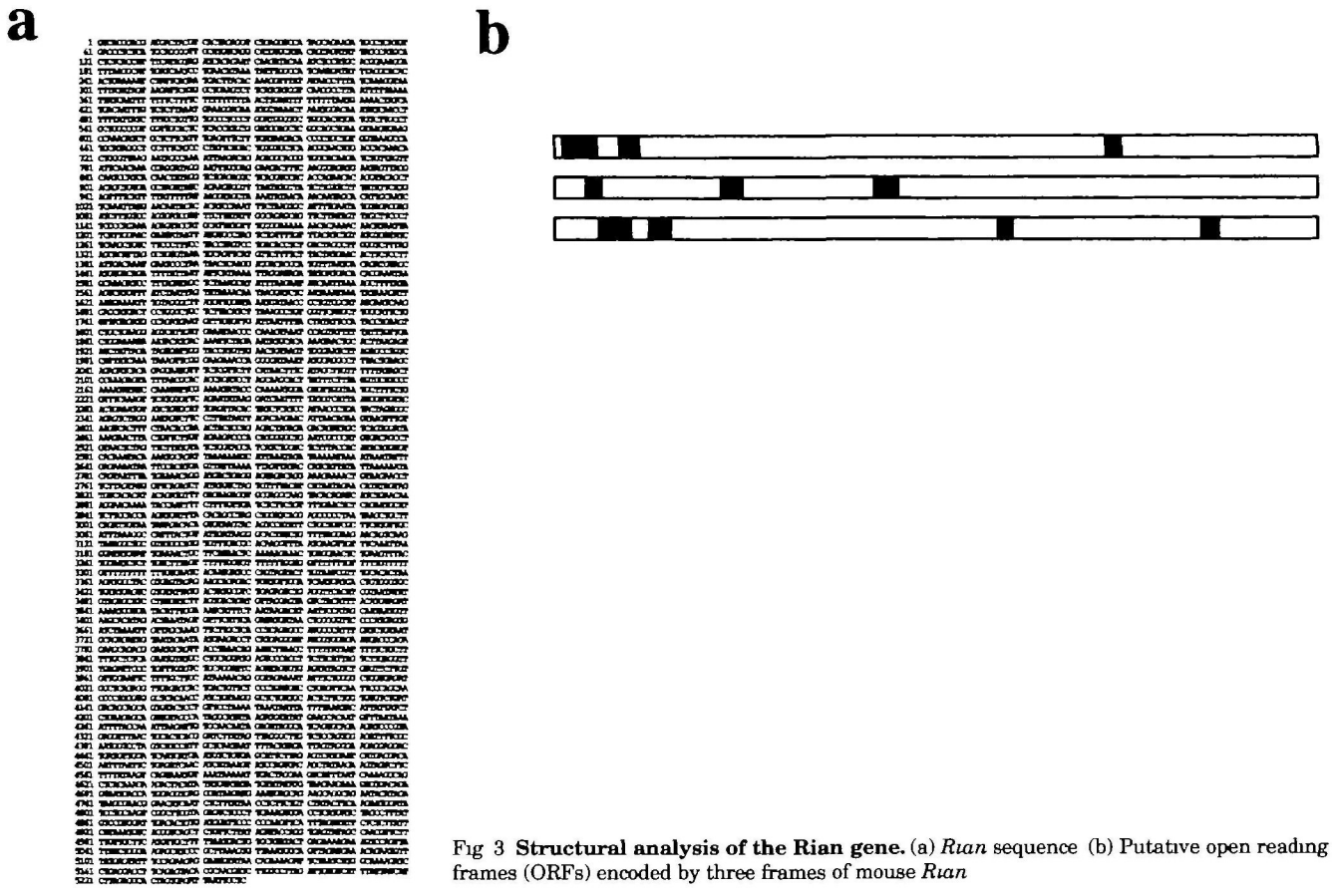


Fig 3 **Structural analysis of the Rian gene.** (a) *Rian* sequence (b) Putative open reading frames (ORFs) encoded by three frames of mouse *Rian*

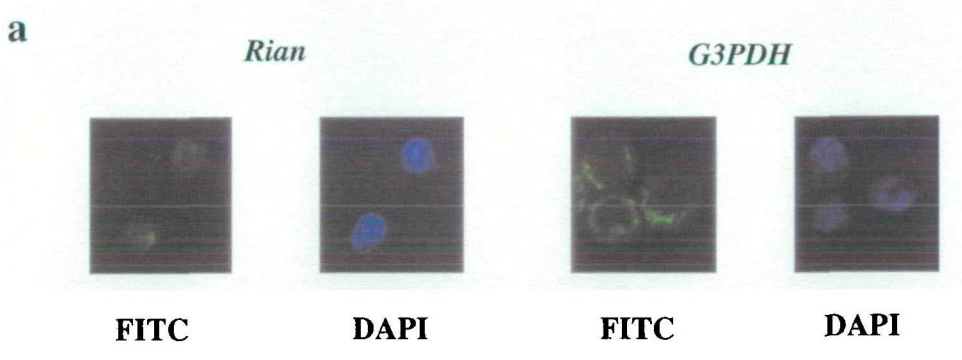
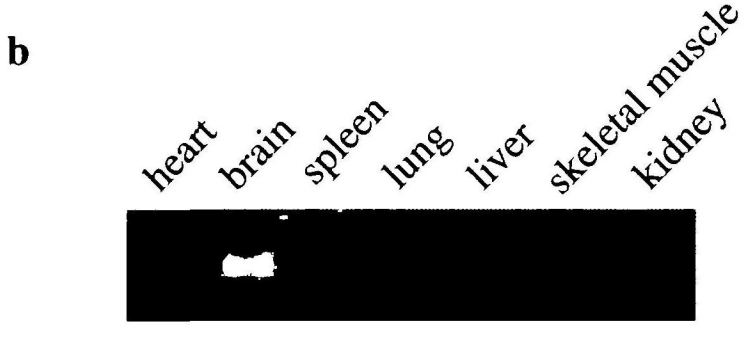


Fig 4 **Tissue distribution of *Rian* transcripts.** (a) *Rian* RNA localization in murine cells RNA FISH of *Rian* and *G3PDH* was performed (b) RT-PCR was performed on RNA from heart, brain, spleen, lung, liver, skeletal muscle, and kidney samples. The primers used were GCTTTTTAATGGCTGTGCATATAC and AGCAATTAATCTCACTAGTGCCT



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