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Zim1, a maternally expressed mouse Kruppel-type zinc-finger gene located in proximal chromosome 7

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In analysis of a conserved region of proximal mouse chromosome 7 and human chromosome 19q, we have isolated a novel mouse gene, *Zim1* (imprinted zinc-finger gene 1), encoding a typical Kruppel-type (C2H2) zinc-finger protein, located within 30 kb of a known imprinted gene, *Peg3* (paternally expressed gene 3). Our studies demonstrate that *Zim1* is also imprinted; the gene is expressed mainly from the maternal allele and at high levels only during embryonic and neonatal stages. In contrast to most tissues, *Zim1* is expressed biallelically in neonatal and adult brain with slightly more input from the maternal allele. *Zim1* produces multiple transcripts that range in size from 7.5 to 15 kb. The 7.5 kb transcript is expressed at highest levels and appears to be embryo specific. Whole mount *in situ* hybridization analysis indicates that *Zim1* is expressed at significant levels in the apical ectodermal ridge of the limb buds during embryogenesis, suggesting a potential role of *Zim1* in limb formation. We have identified the potential human ortholog of *Zim1* near *PEG3* in a conserved, gene-rich region of human chromosome 19q13.4. The close juxtaposition of reciprocally imprinted genes has also been seen in other imprinted regions, such as human 11p15.5/Mmu7 (*H19*/*Igf2*) and suggests that the two genes may be co-regulated. These and other data suggest the presence of an unexplored, conserved imprinted domain in human chromosome 19q13.4 and proximal Mmu7.

INTRODUCTION

Genomic imprinting is an epigenetic process in mammals in which two parental alleles are marked during gametogenesis, resulting in parent-of-origin-dependent monoallelic expression or repression of certain genes during development. Approximately 30 different imprinted genes have been identified in human and mouse (1) and as many as 100 such genes are estimated to exist in the mammalian genome (2). Most of the known imprinted genes are clustered in chromosomal domains, indicating that genomic imprinting is a long-range phenomenon that affects relatively large chromosomal regions. Several studies have provided evidence to suggest that relatively small sequence elements, called imprinting centers (3,4), may regulate the imprinting of a whole domain and orchestrate coordinated expression of individual imprinted genes in each region (5,6).

Phenotypic studies of mice bred to carry partial uniparental disomies have permitted the identification of nine major imprinted domains located on seven different chromosomes (7). Similar observations drawn from surveys of human uniparental disomies (8) indicated that imprinting is conserved within mammals (9). Mouse chromosome 7 (Mmu7) contains at least three different imprinted domains, located in the proximal, central and distal portions of the chromosome, respectively. Two of the three known imprinted Mmu7 domains are syntenically homologous to human regions that are also associated with imprinted genetic disorders. The central Mmu7 domain is related to human chromosome 15q11–q13 (H15q11–q13), which contains genes associated with Prader–Willi and Angelman syndromes (3). The distal Mmu7 imprinted domain is related to H11p15.5 and contains genes related to those associated with Beckwith–Wiedemann syndrome (10). Although no clear human imprinted disorder has been mapped to chromosome 19q, maternal disomy (paternal deficiency) of the related proximal Mmu7 region is associated with late embryonic lethality in mice (11). Since animals disomic only for the proximal region have not been studied, the effects of paternal disomy of this region are uncertain. No imprinted genes had been identified within the proximal Mmu7 imprinted region until recently, when *Peg3* (paternally expressed gene 3) was isolated and mapped (12). The human homolog of *Peg3* is located in the telomeric portion of H19q13.4, a gene-rich segment that harbors several families of clustered Kruppel-type zinc-finger (ZNF) genes (13,14)

Since imprinted genes are often clustered, we predicted that additional imprinted loci would be found in the region of conserved homology of H19q13.4 and proximal Mmu7. Using the well-defined physical map of human chromosome 19 as a guide (13), we have localized several known and novel genes within the interval surrounding human and mouse *PEG3*/*Peg3* (14; J. Kim, unpublished data). In this paper, we describe the isolation and characterization of one gene, *Zim1* (imprinted zinc-finger gene 1), a novel ZNF gene located within 30 kb of *Peg3*. *Zim1* is expressed mainly from the maternal allele during the embryonic and neonatal stages and is expressed at high levels only in embryonic tissues. These studies indicate that an

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uncharacterized imprinted domain surrounds *Peg3* in proximal Mmu7 and suggests that conserved genes in H19q13.4 may also be imprinted.

RESULTS

Isolation of the *Zim1* gene

Previous studies had positioned *PEG3/Peg3* within human and mouse regions also known to contain large numbers of tightly clustered ZNF genes (14–16). To test whether genes adjacent to human *PEG3* are ZNF genes we hybridized a conserved Kruppel-associated box (KRAB)-positive genomic fragment (25670Krab), isolated from the *ZNF134* gene cluster [located 1 Mb distal to human *PEG3* (14)], to restriction digests of cosmid 14378 containing human *PEG3*. Subsequently, we detected and isolated a positively hybridizing fragment in cosmid 14378 (herein referred to as 14378Krab). To search for potential Kruppel-type ZNF genes near *Peg3* in mouse, a series of bacterial artificial chromosome (BAC) clones containing mouse *Peg3* (Fig. 1) were hybridized with the human 14738Krab probe. The human KRAB sequence detected a 2.0 kb fragment in *Eco*RI-digested BAC DNA, indicating the presence of related ZNF gene sequences near mouse *Peg3*. We screened a BAC 588F20 subgenomic library with the human 14378Krab sequence and also with the ZNF consensus oligonucleotide probe to isolate these potential ZNF sequences. Sequence analyses of positively hybridizing fragments indicated the presence of a novel, uncharacterized Kruppel-type ZNF gene. To obtain a full-length cDNA for the predicted gene, several rounds of RT–PCR and 5′- and 3′-RACE were performed with 14.5 day embryo and adult brain cDNA templates, using primers derived from the sequence of positively hybridizing BAC fragments. These experiments yielded a 3.0 kb nucleotide sequence containing one ORF, 579 amino acids in length (Fig. 2A). The predicted ORF of this gene, called *Zim1*, is composed of the KRAB domain (A and B box) at the N-terminus, 11 ZNF units at the C-terminus and a spacer domain positioned between the KRAB and finger domains. This organization, KRAB, spacer and finger region, represents the typical structure of KRAB-containing, Kruppel-type ZNF genes (16,17). Eleven finger units of *Zim1* are typical C2H2 type and some of these finger units are also connected by a conserved linker sequence (HTGEKPY).

To determine the exon–intron structure of *Zim1*, we employed a long-distance PCR strategy using oligonucleotides derived from the *Zim1* cDNA sequence (Fig. 2B). The cDNA sequence of *Zim1* is comprised of four different exons which are distributed over a genomic interval of ~13 kb. Most exon–intron boundary sequences of *Zim1* are in good agreement with the consensus sequence (AG/GT) of the exon–intron joining region. 5′-RACE experiments also yielded two other minor forms of *Zim1* (Fig. 2B). One alternative 5′-RACE clone obtained from adult brain contained the intron located between the KRAB A and B exons as a part of its transcript. Other 5′-RACE clones contained a different alternative exon–intron boundary; the intron located immediately 5′ of the KRAB A sequence was included in the cDNA products. This region is composed largely of a simple dinucleotide repeat, $(CACA)_n$, and does not contain an ORF. Although both unusual transcript forms may simply represent unusually stable splicing intermediates, imprinted genes such as *Igf2*, *Igf2r* and *Ube3a* are known to be transcribed in both the sense and antisense directions. The antisense products are believed to serve important functions despite containing no obvious ORF (18–20). Therefore, although the significance of these observations remains uncertain, the minor forms of *Zim1* cDNA we have detected may also represent functional forms of RNA produced from this locus. Inspection of 3′-RACE clones also indicated that *Zim1* may utilize multiple alternative polyadenylation sites (Fig. 2).

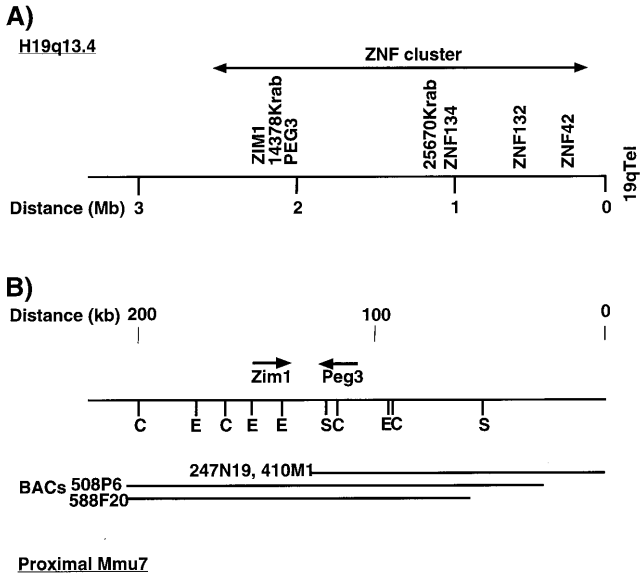

Figure 1. Comparative physical map of the human and mouse *Peg3/Zim1* regions. (**A**) The relative physical locations of *PEG3* and previously identified ZNF genes are shown in the diagram of human chromosome 19qtel. (**B**) A physical map of a 200 kb region of proximal Mmu7 gives the relative locations and transcription direction of *Zim1* and *Peg3*, as well as the restriction enzyme sites used in map construction, indicated by E (*Eag*I), C (*Cla*I) and S (*Sal*I). Mouse BAC coverage for this region is also shown.

Physical mapping experiments demonstrated that *Zim1* is located within 30 kb of *Peg3* and that *Zim1* and *Peg3* are transcribed in opposite directions (Fig. 1B). To find the potential human homolog of mouse *Zim1*, the KRAB and finger regions of *Zim1* were hybridized to high density filter arrays of the human chromosome 19-specific library (13,21). The *Zim1* finger probe detected a single strong positive cosmid, located next to *PEG3*, and the same cosmid was also strongly positive with the KRAB probe. These hybridization experiments indicate that the human homolog of mouse *Zim1* or a related gene is a near neighbor of human *PEG3* and also suggest conservation of the *Peg3/Zim1* genomic interval in both human and mouse (Fig. 1).

Zim1 is maternally expressed

To test the imprinting status of *Zim1*, we analyzed mRNA isolated from tissues of *Mus musculus* (C3Hf)×*M.spretus* hybrid animals. Comparison of the DNA sequence of ZNF regions of *Zim1* transcripts identified the presence of a single base substitution that distinguishes *M.spretus* from C3Hf mRNA. The result of this single base change is that cDNA produced from C3Hf transcripts contains a recognition site for the restriction enzyme *Msp*I, whereas the *Zim1* cDNA from *M.spretus* does not. Since ZNF sequences of *Zim1* are encoded by a single exon, amplification of

A)

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1    MKRSVSEVKTKAPVNEASPSPSPDQTANQISEKKRRIPITSLPTWYK   5'UTR

48   EPVIFKDVAVYFSQKEWQLLEPAQKDLYKDVMLENYENLISV        KRAB A

90   EYYIFKPKLITRLEQGVDLFAKENDVPGDPQQGEAGVSRSDTSA      KRAB B

134            GKKTGDNSTKAEIRKPDN
     SKITSLEKQKAADQGRGSQSLRAEKTSK
     SDDRPSQNKEKCASTSTTEASKTSIPGN                      Spacer
     KENESAIPGTSSGQTSAATTTPQSAPSE
     KPTSGKDVEGKPQTMRSSSTNPKRKPAR
264  QGKNHFKCKECGKTFNQTLHLVEHERIH
     TGEKPHKCDTCGKSFRHLSYFLTHYRIH
     TGVRPYKCKECGKAFNSSSTLNNHCRIH
     SGEKPFKCDECGKTFKQSTKLTRHQRIH
     TGEKPYKCGECNKCFGRSSSLREHKRIH
     TGEKPYCCQVCGKTFRVNSHLSEHQRLH                      Finger
     LKVKPYKCDKCGKHFRNSSYLTEHKQIH
     VPGARVDCPECGKVFACKVALLKHQKRH
     EANSRYRCKGCEKTFRCKSSIQRHERLH
     AGEKPFVCHKCDKGFTDKTTLNNHLKIH
     SGDRPDPCAQCGRTFKKLATLLIHQKKH
572  IKKKPTDV
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B)

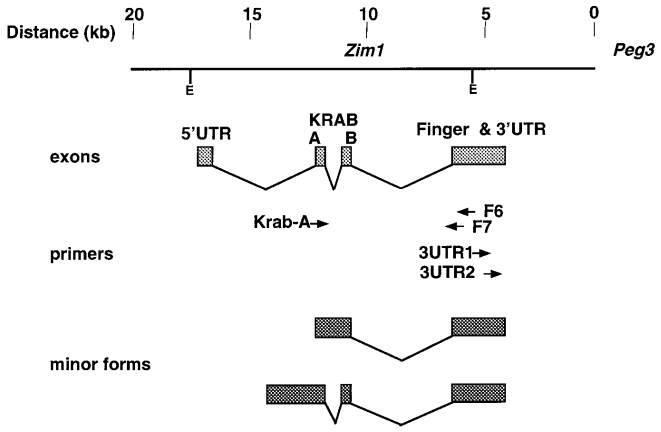

Figure 2. (**A**) The amino acid sequence of *Zim1*. The 5′-UTR, KRAB A and B, spacer and finger domains are shown and the two single-letter codes, C (cysteine) and H (histidine), in the finger domain are in bold. (**B**) The genomic organization of *Zim1*. The relative positions of four exons are shown on the map. E, position of the *Eag*I restriction enzyme sites. The positions of primers used for the 5′- and 3′-RACE are indicated and the minor forms of the *Zim1* cDNAs are also depicted.

contaminating genomic DNA presented a potential complication to these studies. To decrease and monitor the presence of genomic DNA, all templates were treated with RNase-free DNase I before cDNA generation and each PCR reaction was conducted alongside a parallel reaction containing RNA that had not been treated with reverse transcriptase.

Analysis of *Zim1* transcripts produced in seven different tissues isolated from *M.spretus*×C3Hf hybrid neonates indicated that *Zim1* is expressed monoallelically in most tissues (Fig. 3A). *Zim1* transcripts are derived mainly from the maternal allele in neonatal liver, lung, kidney, testis, heart and spleen. In contrast, *Zim1* appears to be expressed from both maternal and paternal alleles in neonatal brain, with slightly more input appearing to be derived from the maternal chromosome. To confirm this result, a reciprocal analysis was conducted using interspecific backcross progeny (the offspring of an interspecific hybrid female and a C3Hf male; Fig. 3B). The results of this reciprocal analysis were consistent with those obtained with hybrid mice, except that brain expression appeared to be monoallelic in the backcross animals. Although this difference was consistent throughout several independent trials, it is uncertain whether the reason for this discrepancy might be due to differences in genome imprinting between different subspecies, as shown in another case, *Kvlqt1* (22). It is also possible that this discrepancy might be caused simply by an unknown artifact of our imprinting test.

Analysis of *Zim1* transcripts produced by mouse embryos at different stages of gestation confirmed monoallelic expression. In fact, *Zim1* imprinting appears to be more pronounced during embryonic stages, with no trace of paternal *Zim1* transcripts detected in 9.5 and 14.5 d.p.c. embryos (Fig. 3C). *Zim1* expression was also examined in tissues isolated from 1-month-old hybrid mice, but these studies were not conclusive since *Zim1* transcripts were present at very low levels in tissues isolated from these older mice (data not shown).

Zim1 expression during embryonic development

To examine the tissue-specific expression of *Zim1*, we hybridized the *Zim1* cDNA probe to a northern blot carrying mRNA isolated from 16.5 d.p.c. whole mouse embryos and a selection of adult tissues (Fig. 4A). High levels of *Zim1* expression were detected in embryos; in agreement with RT–PCR results, very low levels of the transcript were detected in tissues of 2-month-old mice. The major *Zim1* transcript detected in embryonic tissues is ~7.5 kb in length. Long exposure (1 week) of the same northern blot indicated that the 9 kb *Zim1* transcript, but not the 7.5 kb species that predominates in embryos, is present at low levels in adult brain. Additional northern analyses indicated that other adult tissues, including heart and fat, also contain low levels of the 9 kb transcript (data not shown). To obtain more detailed information about the embryonic expression of *Zim1*, we hybridized two different *Zim1* probes, derived from the KRAB and 3′-UTR regions of the gene, to the embryo northern blot (Fig. 4B). Two different probes produced exactly the same result, as shown in Figure 4B; there are four different *Zim1* transcripts detected in embryos ranging in size from 7.5 to 15 kb and two major transcripts, 7.5 and 9 kb in size, show the highest expression levels in 14.5 d.p.c. embryos. The collection of different *Zim1* transcripts may be products of alternative splicing or, as suggested by their tissue-specific expression, may be produced through the use of alternative *Zim1* promoters. Alternative use of different polyadenylation sites, suggested by RACE studies (see above), may also contribute to length differences in transcripts produced from the *Zim1* gene.

Spatial expression pattern of *Zim1* during development

To study the spatial expression pattern of *Zim1* during development, we performed whole mount *in situ* hybridization on embryos isolated at different embryonic stages (Fig. 5). Significant levels of *Zim1* expression were detected in the apical ectodermal ridge (AER) of limb buds throughout the 9.5–14.5 d.p.c. period (Fig. 5A). As shown in Figure 5A, *Zim1* expression is detected in the AER region of forelimb buds and also the future AER region of hindlimb buds, indicating that *Zim1* expression in AER precedes limb bud formation. The dark signals observed in the otic vesicles of these embryos were also observed with sense probes, although the AER signals were not (data not shown). The coloration of the otic vesicles is therefore due to non-specific trapping of the probe, as confirmed by several control experiments. The *Zim1* expression patterns in AER were

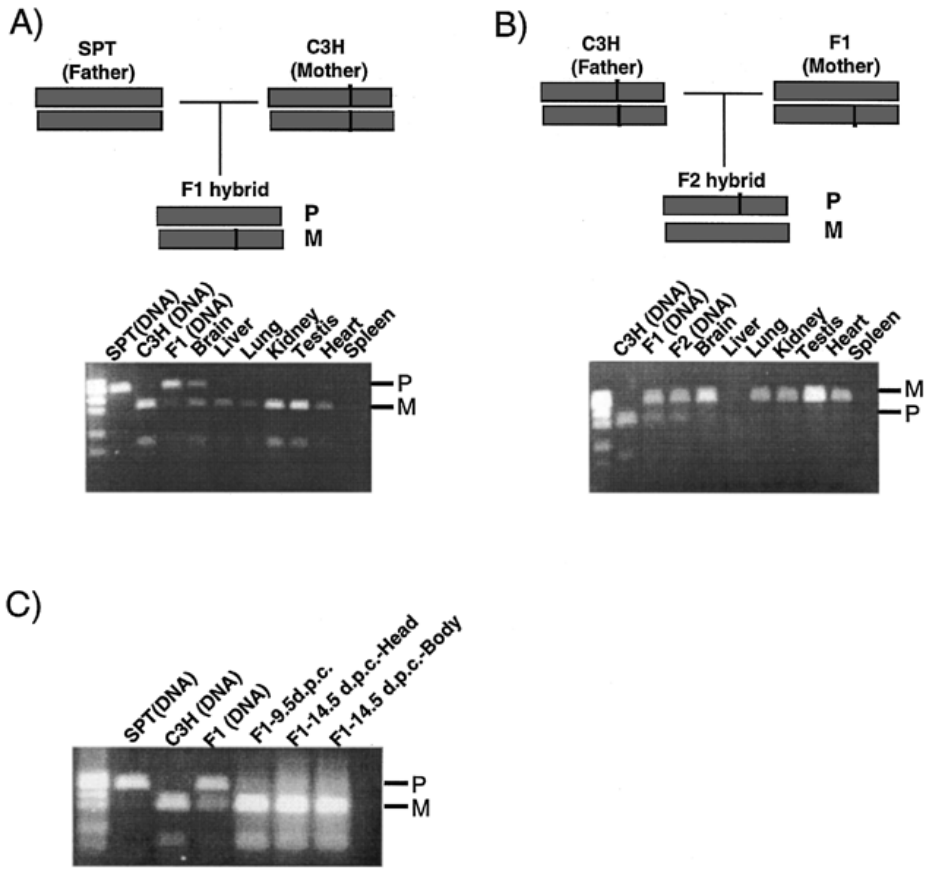

Figure 3. Monoallelic expression of *Zim1*. The schematic diagrams depict interspecific crosses used to determine parental alleles of imprinted loci. Rectangles represent parental alleles, with *Msp*I restriction enzyme site polymorphism indicated by an internal line. (**A**) Imprinting test result using RNAs derived from F_1 neonatal tissues of an interspecific cross, male *M.spretus* (SPT)×female *M.musculus* (C3H). (**B**) Result of reciprocal imprinting test using neonatal tissues derived from F2 offspring of the backcross, male *M.musculus* (C3H)×female F_1. (**C**) Imprinting test result using day 9.5 and 14.5 embryos. Products of RT–PCR were digested with *Msp*I and separated on 1.8% agarose gels to differentiate paternal (P) and maternal (M) transcripts.

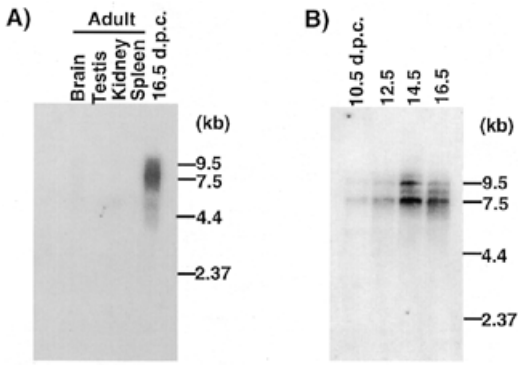

Figure 4. Northern blot analyses of *Zim1*. Each lane contains 2 μg of poly(A)$^+$ RNA. The 3′-UTR of *Zim1* was used as a probe. (**A**) Comparison of adult organ-specific expression with day 16.5 whole embryo expression. (**B**) Embryonic expression profile. RNAs were isolated from embryos at 10.5–16.5 d.p.c.

compared with those of fibroblast growth factor 8 (*Fgf8*) (Fig. 5B and C), which has been shown to be expressed in the AER and is thought to be involved in limb formation (23). *Zim1* expression in the AER appears to be much more restricted spatially than does that of *Fgf8*; *Zim1* is expressed at the tip of the AER whereas *Fgf8* is expressed in the whole AER region. *Zim1* expression is also consistently observed to be greater in the anterior region of the AER than in the posterior region while *Fgf8* is expressed throughout the anterior–posterior axis of the AER. We also detected lower levels of *Zim1* expression in other tissues, including somites and primordial gut, but the significance of these low levels of expression is unclear at present and needs to be studied more thoroughly before conclusions can be drawn.

DISCUSSION

We have identified and characterized a novel imprinted gene, *Zim1*, located near *Peg3* in the proximal Mmu7, and have confirmed the presence of a closely related human gene in the syntenically homologous region of H19q13.4. Like *Peg3*, *Zim1* contains regions that are predicted to encode Kruppel-type (C2H2) ZNF domains. However, the two genes differ markedly in structure. In addition to the ZNF segment, *Peg3* contains additional protein coding motifs, including proline-rich domains that are not commonly found in genes of the Kruppel-type (12). In contrast, the sequence and organization of *Zim1* are that of a typical Kruppel-type gene, predicted to encode a protein with N-terminal KRAB domains linked through a spacer sequence to

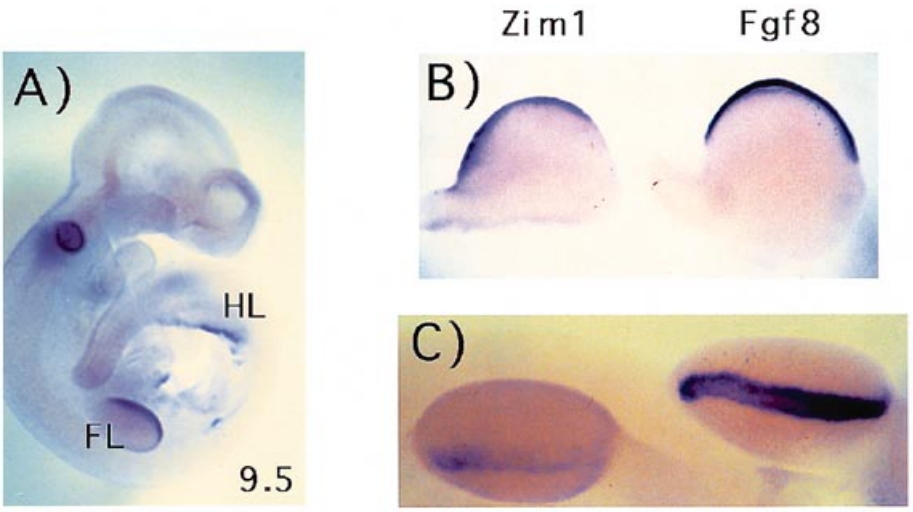

Figure 5. *Zim1* expression in embryos by whole mount *in situ* hybridization. **(A)** Day 9.5 embryo, lateral view. *Zim1* expression is detected in the forelimb buds (FL) and hindlimb buds (HL). Note that the hindlimb buds have not fully formed in this stage embryo but *Zim1* expression is still detected in the future hindlimb bud region. (**B**) Forelimb buds of day 9.5 embryos, lateral view. (**C**) Forelimb buds of day 9.5 embryos, frontal view. Comparison of *Zim1* and *Fgf8* expression patterns in the AER.

C-terminal finger regions. Most KRAB-containing Kruppel-type ZNF genes are expected to act as transcriptional repressors based on the evidence that the KRAB domain shows a repressing activity when joined to other DNA-binding modules (24,25) and also that the ZNF domains of C2H2-type ZNFs exhibit DNA-binding capability (26).

Zim1 is expressed at high levels only in embryonic tissues and is transcribed primarily from the maternal allele during embryonic and neonatal stages. The gene is expressed at significant levels in the AER of the developing limb buds. The AER is known to be a major signaling center for the developing limb; the AER controls the growth and differentiation of cells located underneath the AER to form proper limbs (27). The high expression levels in AER might suggest a potential role of *Zim1* in AER and also a possible link to other genes that are involved in limb formation, such as *Fgf8*, Sonic hedgehog (*Shh*) and *Wnt7a*.

In contrast to the function of other known imprinted genes, *Zim1* is unique in that the predicted function of this Kruppel-type ZNF gene is transcriptional control of other downstream genes. Developing embryos are expected to require multiple layers of transcriptional regulation to coordinate precise timing of expression for many genes and it is interesting to speculate that *Zim1* might play a role in regulating genes which must be repressed during normal development. Many imprinted genes, including *Igf2*, *Ins2*, *Igf2r*, *Grf1* and *Gnas1*, are known to be involved in cell growth and signal transduction. Therefore, the imprinting effects of these genes on growth and development is easily envisioned. However, the effects of silencing or overexpressing a transcription factor gene would be more complicated and indirect and manifested in the function of unknown downstream genes. Considering the hypothesis that parent–offspring conflict is one of the major reasons for imprinting in placental mammals (28,29), it is possible that *Zim1* might be part of a genetic cascade involved in fetal and/or neonatal growth. Although the function of *Zim1* remains to be proven, imprinting of a potential transcription factor adds a potentially interesting twist to our understanding of this complicated genetic phenomenon.

Animals carrying partial paternal disomy (uniparental disomy) including proximal Mmu7 exhibit reduced postnatal growth rates and viability (30). Since the breakpoint of the translocation used to produce these partial disomies is located below the central imprinted domain, these animals are disomic not only for the *Zim1–Peg3* but also for the *Snrpn–Ube3a* region (31). The phenotype associated with partial disomy of proximal Mmu7 may therefore be associated with the absence of transcripts from more than one maternally expressed gene. The imprinting status of *Ube3a*, which represents the only maternally expressed gene discovered so far in the central Mmu7 imprinted region (4), is restricted spatially and temporally, with imprinted expression documented only in a specific region of the adult brain (32). Therefore, the extent to which *Ube3a* influences neonatal growth may be limited. With the discovery of *Zim1*, it is possible to imagine that the loss of regulatory control of a downstream gene(s) may in fact be a contributing factor to the retardation of postnatal growth in disomic mice.

The close proximity of *Zim1* (maternally expressed) and *Peg3* (paternally expressed) is reminiscent of gene organization observed in other imprinted domains, such as those containing *Igf2* and *H19* (Beckwith–Wiedemann syndrome region) and *Snrpn* and *Ube3a* (Prader–Willi and Angelman syndrome region). Although this close juxtaposition of pairs of reciprocally imprinted genes cannot be generalized as a common feature of imprinted domains, this trend is compatible with one recent hypothesis. The enhancer competition model (6) hypothesizes that two reciprocally imprinted genes compete *in cis* for a single, shared enhancer, with the result that only one gene can be expressed at a given time from one parental allele. In support of this model, *Igf2* and *H19* have been shown to rely upon the shared endoderm-specific enhancer. Although it remains to be studied in future whether *Peg3* and *Zim1* share unidentified enhancers and also whether the imprinting of *Peg3*/*Zim1* can be explained by the enhancer competition model, the identification of a second imprinted gene near a known imprinted gene in proximal Mmu7 suggests that the imprinting of *Peg3* and *Zim1* might be related

to each other and also that this region might have more imprinted genes.

MATERIALS AND METHODS

Mouse BAC clone isolation and analysis

To obtain large-insert clones containing mouse *Peg3*, we screened high density mouse 129/Sv BAC library filters (Research Genetics, Huntsville, AL) with a pool of three mouse genomic fragments corresponding to the transcribed regions of *Peg3* (12,14). BAC DNA samples were prepared with the alkaline lysis protocol (33), digested with several rare-cutting enzymes, including *Cla*I, *Eag*I, *Sal*I and *Not*I, and separated on pulsed-field gels (run in a Chef Mapper instrument; Bio-Rad, Hercules, CA). DNA was transferred to nylon membranes (Hybond; Amersham, Uppsala, Sweden) and hybridized according to standard protocols (34). BAC end clones were isolated using a single primer PCR approach (35). Subgenomic libraries were generated from one *Peg3*-containing BAC clone (588F20) by ligating *Sau*3AI digests of BAC DNA into λZAP-II (Stratagene, La Jolla, CA) and M13 phage vectors.

Imprinting tests

To test the expression of *Zim1* from maternal and paternal alleles, we isolated tissues from hybrid offspring produced by crossing *M.musculus* (C3Hf) females with *M.spretus* male mice. To confirm monoallelic expression of *Zim1*, we analyzed tissues from offspring of *M.musculus*×*M.spretus* hybrid females, tested to carry *M.spretus* alleles of *Zim1*, which were backcrossed to C3Hf males. Embryos at two different stages (E9.5 and E14.5) and tissues from 7-day-old and 1-month-old animals were collected from each cross. RNA was isolated using a commercially available kit (rapid total RNA isolation kit; 5′–3′, Boulder, CO). RNA samples were treated with RNase-free DNase I (Stratagene) for 30 min at 37°C and 50 µg were used for the synthesis of cDNA (using the cDNA synthesis module; Amersham). The final volume of each reverse transcription reaction was 40 µl, and 1 µl of this material was taken for each PCR reaction. For imprinting tests of *Zim1*, two oligonucleotides were used, Zim1 F5 (5′-GAGAAGCCGTACTGCTGTCA-3′) and Zim1 F2 (5′-CTTGCACCGGTACCTGGAGT-3′). PCR amplification of the *Zim1* finger region was performed using the following program in a Perkin Elmer-Cetus (Foster City, CA) GeneAmp 9600 instrument: 95°C for 30 s, 60°C for 1 min, 72°C for 1 min for 30 cycles; 72°C for 5 min for 1 cycle. PCR reactions were carried out in a 50 µl reaction mixture containing 300 ng of each primer, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTPs, 1% Triton X-100 and 1.25 U *Taq* DNA polymerase. The quality of PCR reactions was checked on 1.8% agarose gels. cDNA templates were checked for genomic DNA contamination by performing parallel PCR reactions using templates that had not been treated with reverse transcriptase. Ten microliters of each PCR reaction mixture was digested with 10 U *Msp*I in a 30 µl reaction and the digests examined after separation on 1.8% agarose gels.

cDNA isolation and 5′- and 3′-RACE

Hybridization of a human cosmid containing *PEG3* sequences (cosmid 14378) with a probe corresponding to the conserved KRAB motif associated with many ZNF genes (36) indicated that a Kruppel-type ZNF gene was located near *PEG3*. The KRAB-containing fragment was subcloned from the cosmid and used to screen the subgenomic library generated with BAC 588F20. Fragments isolated from the BAC contained a conserved KRAB sequence representing portions of a novel mouse gene. ZNF encoding sequences were isolated from the same mouse BAC by screening the subgenomic library with a degenerate ZNF oligonucleotide probe [CA(C/T)AC(A/T)GG(A/T/G)GA-(A/G)AA(A/G)CC(T/C/A)TA (37)]; this probe corresponds to the conserved amino acid sequence motif HTGEKPY, which is unique to Kruppel-type ZNF genes. The nucleotide sequence linking the KRAB A and finger regions of *Zim1* was obtained by analyzing RT–PCR products generated from a 14.5 day embryo cDNA template (mouse 14.5 day embryo Marathon cDNA template; Clontech, Palo Alto, CA) with two oligonucleotide primers, KRAB A (5′-AGGAACCAGTGATCTTCAAA-3′) and F2 (5′-CTTGCACCGGTACCTGGAGT-3′). To obtain the 5′- and 3′-ends of *Zim1*, we employed the RACE technique (38). Two different cDNA templates derived from 14.5 day embryo and adult brain RNA were used. The sequences of oligonucleotides used for the 5′- and 3′-RACE of *Zim1* are as follows: F6 (5′-GCTTTGGATTTGTGGAACTG-3′) and F7 (5′-TTGA-GATGGCCTGTCATCAC-3′) for 5′-RACE; UTR1 (5′-CCCT-ACTTGGAGCATTTGTG-3′) and UTR2 (5′-CTTGCCTTC-CAATAACTAAG-3′) for 3′-RACE. Amplified RACE products were separated on 0.8% agarose gels and the major fragments in terms of mass were isolated from the gels using a gel extraction column (QIAquick gel extraction kit; Qiagen, Valencia, CA). The fragments were subcloned into the TA cloning vector (TA cloning kit; Invitrogen, Carlsbad, CA).

Sequencing and sequence analysis

Subcloned genomic and cDNA fragments were sequenced from both directions using a fluorescence-based cycle sequencing DNA sequencing kit (dye terminator sequencing core kit; PE Applied Biosystems, Warrington, UK) and reactions analyzed on an ABI 373 automated sequencer. Sequence alignments and database searches were analyzed using GCG software v.8 (Genetics Computer Group, Madison, WI).

Northern blot analysis

Poly(A)$^+$ RNA was isolated from embryos and from adult tissues using oligo(dT) columns [rapid poly(A)$^+$ mRNA isolation kit; 5′–3′]. Two micrograms of purified poly(A)$^+$ RNAs were separated on a 1.0% formaldehyde–agarose gel, transferred to a nylon membrane (Hybond; Amersham) and cross-linked to the blot by UV irradiation. Northern blots were hybridized with probes representing different *Zim1* transcribed regions: (i) the KRAB A region or (ii) the 3′-UTR region of the gene. Procedures and conditions for generating probes and for performing hybridizations were as previously described (34).

Whole mount *in situ* hybridization

To generate an antisense and a sense riboprobe for the *Zim1 in situ* hybridization, one *Eco*RI fragment of *Zim1* corresponding to the finger region of *Zim1* (Genbank accession no. AF111101, nt 1354–2495) was subcloned into predigested pBluescript and used as a template DNA for *in vitro* transcription reactions. The

template DNA for an antisense probe was generated by PCR using two oligonucleotide primers, T3 (5′-ATTAACCCTCACTAAAG-3′) and F3 (5′-GGTTTCACTGACAAAACTAC-3′), and the template DNA for a sense probe with T7 (5′-TAATACGACTCACTATAG-3′) and F2 (5′-CTTGCACCGGTACCTGGAGT-3′). Amplified PCR products were treated once with phenol–chloroform, washed with TE on a microcon-100 (Amicon, Beverly, MA) and concentrated to 1 µg/µl concentration. One microgram of each template DNA was used for each *in vitro* transcription reaction with T7 and T3 RNA polymerases. To generate an antisense probe for mouse Fgf8, two different oligonucleotides, Fgf8a (5′-GCTGGGCAGGGAGCCCACTT-3′) and Fgf8b (5′-CTTCTGCCATGGCGTTGATG-3′), were used to amplify exons 2 and 3 of *Fgf8* (GenBank accession no. Z46883; 39) and then this PCR product was used for the second round PCR to prepare the template DNA for *in vitro* transcription with two primers, Fgf8a and Fgf8c (5′-TAATACGACTCACTATAGGGCTTCTGCCATGGCGTTGATG-3′). For the detection of probes, antisense and sense probes were labeled with digoxigenin-UTP with an RNA labeling kit (DIG RNA labeling kit; Boehringer Mannheim, Indianapolis, IN) and anti-DIG-alkaline phosphatase from sheep and BM Purple AP substrate (Boehringer Mannheim) were used according to the manufacturer's instructions. Whole mount *in situ* hybridization experiments were performed according to a standard protocol (40) with minimum modification.

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