Imprinting analysis in the Acrodysplasia region of mouse chromosome 12

Erin N. McMURRAY, Eric D. ROGERS and Jennifer V. SCHMIDT

Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL 60607, U.S.A.

Synopsis

The insertional mouse mutation Adp (Acrodysplasia) confers a parent-of-origin developmental phenotype, with animals inheriting the mutation from their father showing skeletal abnormalities, whereas those inheriting the mutation from their mother are normal. This parental-specific phenotype, along with mapping of the insertion to a region of chromosome 12 proposed to contain imprinted genes, suggested that disruption of genomic imprinting might underlie the Adp phenotype. Genomic imprinting is the process by which autosomal genes are epigenetically silenced on one of the two parental alleles; imprinting mutation phenotypes manifest after inheritance from one parent but not the other. Imprinted genes typically occur in dense clusters that contain few non-imprinted genes and therefore representative genes from the Adp critical region could be assayed to identify any imprinted domains. None of the genes analysed were found to be imprinted, however, suggesting that other explanations for the Adp phenotype must be considered.

Key words: Acrodysplasia, craniofacial development, genomic imprinting, limb development, mouse

INTRODUCTION

Genes subject to genomic imprinting are expressed from only one of the two parental alleles. Nearly 80 genes to date have been shown to be regulated by genomic imprinting, and many of these play important roles in embryonic growth and development [1] (http://www.geneimprint.com; http://www.mgu.har.mrc.ac.uk/research/imprinting). One characteristic of imprinted genes is that they are organized in large genomic clusters; this clustering is believed to reflect the use of shared regulatory elements among multiple genes. This co-regulation is illustrated by mutations within imprinted regions of the mouse that affect the expression of all or many of the genes in that region [2–4]. In fact, many imprinted gene clusters were first identified by the mapping of mutations that displayed parent-of-origin-specific phenotypes [5–7]. A striking parent-of-origin mouse phenotype, Adp (Acrodysplasia), was described in 1990 by DeLoia and Solter [8]; Adp is the result of an insertional mutation of a transgene carrying the human growth hormone releasing factor gene under the control of the mouse metallothionein promoter. Adp mice show varying degrees of skull and limb pathology at midgestation, with shortening of the skull and a loss, reduction and/or fusion of the bones of the wrist/ankle and digits [8]. As the Adp transgene is not expressed during embryogenesis, the phenotype likely results from altered expression of one or more endogenous genes at the integration site. The Adp phenotype is manifest only when the transgene is inherited from the father [AdpPat (paternal inheritance of Adp)], but not when inherited from the mother [AdpMat (maternal inheritance of Adp)]. This pattern of inheritance suggested that the Adp transgene may have integrated into an imprinted region of the genome, such that expression of a paternally expressed gene involved in skeletal development was altered. Although Adp animals are no longer available (T. Watanabe and D. Solter, personal communication), examination of genes located near the integration for allele-specific expression could still reveal an imprinted domain if it were present.

MATERIALS AND METHODS

RNA isolation and SNP (single nucleotide polymorphism) identification

RNA was extracted from control B (C57BL/6 mouse strain) and C (Cast/Ei mouse strain) embryos, and from B × C and C × B crosses, at E12.5 (embryonic day 12.5) using TRIzol® reagent according to the manufacturer’s instructions (Invitrogen). RNA

Abbreviations used: Adp, Acrodysplasia; AdpPat, maternal inheritance of Adp; AdpMat, paternal inheritance of Adp; B, C57BL/6 mouse strain; C, Cast/Ei mouse strain; E12.5, embryonic day 12.5; HDAC, histone deacetylase; Mb, megabases; RT, reverse transcription; SNP, single nucleotide polymorphism.

1To whom any correspondence should be addressed (email jvs@uic.edu).
was treated with TURBO DNA-free DNase (Ambion), and oligo-dT-primed RT (reverse transcription) reactions were carried out using 2 μg of total RNA and Superscript III (Invitrogen). RT reactions were diluted 1:10, and PCR amplification was performed using 2 μl of the dilution. Control reactions carried out in the absence of reverse transcriptase were negative. The resulting PCR products were cloned using the pCRII-TOPO kit (Invitrogen), and sequenced to identify SNPs between the B and C sequences; two independent amplifications were analysed for each gene to rule out Taq-induced mutations.

**Imprinting analysis**

A second set of primers was then designed to amplify a small region of each gene containing the SNP (Table 1). PCR conditions were as follows: Fkbp3, Fancm, Pole2, Arid4a and Trim9 were amplified at 95 °C, 30 s, 61 °C, 30 s and 72 °C, 1 min 15 s for 35 cycles. Dact1, Mia2, Daam1, Tgfb3, Lrfn5, Sav1 and Mdga2 were amplified at 95 °C, 30 s, 62 °C, 30 s and 72 °C, 1 min for 35 cycles. PCR reactions were purified using the QIAquick PCR Purification kit (Qiagen), and sequenced directly using one of the amplification primers. Chromatograms from B and C animals confirmed each previously identified SNP, and chromatograms from B × C and C × B animals were analysed for the expression of one or both alleles of each gene.

### RESULTS AND DISCUSSION

**Selection of candidate genes**

Initial mapping of the Adp mutation was performed using an interspecific backcross panel that localized the integration to mouse chromosome 12, between D12Mit54 and D12Mit4 [8].

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**Table 1 Gene characteristics, primers used to amplify SNPs and SNP locations**

The name of each gene tested is shown and the characteristics each has in common with other imprinted genes are noted in the column of each gene Primer Primer sequence Location Sequence

- **Arid4a** F, 5C
  - Primer: AnF 5’-AGAGGGACATTTCTCCACAGC-3’
  - Location: Exon 13, nucleotide 8
  - Sequence: B: TACTCCCATCACC
  - Primer: AnR 5’-TGCCAGACGCAGTATCTTCAAAA-3’
  - C: TACTCCCATCACC

- **Daam1** X, 5C
  - Primer: DaaF 5’-AGCCAGTGATTCTCCACAGGTCA-3’
  - Location: Exon 26, nucleotide 31
  - Sequence: B: CGGAGATGAGGA
  - Primer: DaaR 5’-ATGATGTCGCTGCAGAGCAGC-3’
  - C: CGGAGATGAGGA

- **Dact1** F, 5C, 3C
  - Primer: DacF 5’-ACAGACAATTCCTGACCATCCT-3’
  - Location: Exon 4, nucleotide 491
  - Sequence: B: CGGGCCTTTCCT
  - Primer: DacR 5’-ACTGAGTTCCTACACAGGGCTTT-3’
  - C: CGGGCCTTTCCT

- **Fancm** X, 5C
  - Primer: FanF 5’-GGCTTCATCTTGATAGACGTG-3’
  - Location: Exon 21, nucleotide 254
  - Sequence: B: TAAAGATGCTCA
  - Primer: FanR 5’-CTTTCGATCTTCCTGACAGC-3’
  - C: TAAAGATGCTCA

- **Fkbp3** X, 5C
  - Primer: FkbF 5’-GCCAGACATCGTCTGACAGC-3’
  - Location: Exon 3, nucleotide 69
  - Sequence: B: TGCAGACAAACC
  - Primer: FkbR 5’-AGTGCTCTCTGTACACAGGCTG-3’
  - C: TGCAGACAAACC

- **Lrfn5** 5C
  - Primer: LrfF 5’-AGACGTTGCTCTTCTCGACATTT-3’
  - Location: Exon 3, nucleotide 971
  - Sequence: B: ACCTCCCATCAT
  - Primer: LrfR 5’-AATCTGGATAGCAACCCAGACTTT-3’
  - C: ACCTCCCATCAT

- **Mdga2** 5C
  - Primer: MdgF 5’-TGCAAGATCCAGATGGTCCTCACC-3’
  - Location: Exon 15, nucleotide 2
  - Sequence: B: TATGAGGTCTTA
  - Primer: MdgR 5’-ATCGAGATAGCAGTCGTA-3’
  - C: TATGAGGTCTTA

- **Mia2** 5C
  - Primer: MiaF 5’-ACCTGGAGATATTCAACAGTGCC-3’
  - Location: Exon 4, nucleotide 542
  - Sequence: B: AGCTGAGACTG
  - Primer: MiaR 5’-ATCTGGATAGCAACCCAGACTTT-3’
  - C: AGCTGAGACTG

- **Mnat1** 5C, 3C
  - Primer: MnatF 5’-TACGAGCTCAAGGACAGTGCC-3’
  - Location: Exon 6, nucleotide 87
  - Sequence: B: GCCAGACATCTAG
  - Primer: MnatR 5’-AGAGAAGAGATAGCAGCACGC-3’
  - C: GCCAGACATCTAG

- **Pole2** X, 5C, 3C
  - Primer: PoleF 5’-CCAGACTACCAAGCAGAGGTCC-3’
  - Location: Exon 15, nucleotide 46
  - Sequence: B: AAGGATCCGGTT
  - Primer: PoleR 5’-AGGAGCCATGTCCTGAGATAA-3’
  - C: AAGGATCCGGTT

- **Sav1** X, 5C
  - Primer: SavF 5’-AACATCTCCAGCGAGGACTCT-3’
  - Location: Exon 2, nucleotide 335
  - Sequence: B: TGAGAGACAGAG
  - Primer: SavR 5’-ATGTTGATATAGAGGGAGGAC-3’
  - C: TGAGAGACAGAG

- **Sstr1** 5C
  - Primer: SstrF 5’-ACTACTTGACCTGATCTGAGAG-3’
  - Location: Exon 2, nucleotide 1618
  - Sequence: B: GAAGCTCATTG
  - Primer: SstrR 5’-GGACTCTCAGGGAGGAGCAGCT-3’
  - C: GAAGCTCATTG

- **Tgfb3** X, F
  - Primer: TgfF 5’-ATGGTGTACCCACCTAGCTTAT-3’
  - Location: Exon 7, nucleotide 27
  - Sequence: B: CTCGGACACAGAG
  - Primer: TgfR 5’-GCAAGAGATGTGACGAGTCAAA-3’
  - C: CTCGGACACAGAG

- **Trim9** 5C, 3C
  - Primer: TrimF 5’-GGGTCTCCTCAGACATACAGGA-3’
  - Location: Exon 14, nucleotide 202
  - Sequence: B: CTCTGAGTGCTAC
  - Primer: TrimR 5’-ACATGGAGGGTCTTGTGAGT-3’
  - C: CTCTGAGTGCTAC
Subsequent analysis of a larger backcross panel refined the Adp interval to the region between D12Mit36 and D12Mit34 [9]. The use of Robertsonian translocations to identify imprinted regions of the mouse genome suggested that imprinted genes may lie within the Adp interval of chromosome 12 [10] (http://www.mgu.har.mrc.ac.uk/research/imprinting). On the current mouse genome sequence (Ensembl Build 37, April 2007), the Adp interval represents a region of 9 Mb (megabases) (62–71 Mb), well beyond the limits of typical genetic analysis using a candidate gene approach. The genetic organization of the region, however, displays several dense gene clusters with large regions devoid of genes (Figure 1). The gene clusters are reminiscent of imprinted domains, and suggested that analysis of representative genes within each cluster would likely demonstrate imprinting if it were present.

A select group of genes was chosen for analysis based on four characteristics common to imprinted genes: (i) a predicted non-coding RNA product, (ii) a known role in growth control, (iii) the presence of a CpG island near the gene and (iv) expression in embryo or placenta (Table 1). No bioinformatics-based prediction strategies were used in the present study. Genes selected for imprinting analysis were Arid4a, Daam1, Dact1, Fancm, Fkbp3, Lrfn5, Mdga2, Mia2, Mnat1, Pole2, Sav1, Sstr1, Tgf3 and Trim9, all of which show one or more of these characteristics (Figure 1). Fkbp3 and Fancm reside in a proximal gene cluster centred at 66 Mb. The Fkbp3 gene encodes an FK506-binding immunophilin that interacts with the HDACs (histone deacetylases) HDAC1 and HDAC2 and the transcription factor YY1 (Yin and Yang 1), itself a regulator of imprinting [11]. The Fkbp3 gene is expressed throughout embryonic development and in many tissues of the adult animal (all gene expression data are from Unigene and additional references as indicated). Fancm encodes a DNA helicase involved in DNA repair, and is expressed from gastrulation through adult stages [12,13]. A CpG island is present near the promoters of both Fkbp3 and Fancm. The Fkbp3/Fancm gene cluster is separated from the next more proximal and distal genes by an approx. 4 Mb region on each side that is largely devoid of genes. Only Lrfn5 lies in the proximal gene desert, whereas Mdga2 is found in the distal gene desert. Lrfn5 belongs to the leucine-rich repeat and fibronectin III domain-containing family of proteins, and is expressed in the nervous system of the developing mouse embryo [14]. Mdga2 encodes a transmembrane protein belonging to the Ig superfamily and is expressed in the developing rat nervous system [15]. CpG islands are present at the promoters of both the Lrfn5 and Mdga2 genes [14,15].

The Pole2, Sav1, Trim9, Arid4a, Dact1 and Daam1 genes are members of a large gene cluster that spans the distal Adp region marker at 71 Mb. Pole2 encodes the small subunit of DNA polymerase epsilon [16]. It is expressed in embryonic and extraembryonic tissues, and is flanked by CpG islands. Sav1 encodes a Salvador homologue protein, which has been shown to be important for epithelial development in the mouse. In mice lacking Sav1, some organs display hyperplasia and the mice exhibit retarded growth and perinatal lethality [17]. This gene carries a CpG island across its promoter and is expressed in both embryonic and extraembryonic tissues. Sav1 was predicted to be regulated by genomic imprinting in a genome-wide bioinformatics screen [18]. Trim9 is expressed in the nervous system of both the embryo and the adult, and is also flanked by CpG islands [19]. Trim9 is a member of the N-terminal RBCC (RING finger/B-box/coiled coil; RING stands for really interesting new gene) protein family, whose members bind microtubules and are believed to function as ubiquitin-protein isopeptide ligases [20]. The Arid4a/Rbbp1 gene encodes a retinoblastoma-binding protein that interacts with the regulatory imprinting centre at the Prader–Willi syndrome/Angelman syndrome domain [21,22]. Deletion of the Arid4a and Arid4b gene products alters the pattern of epigenetic modifications at the imprinting centre, and the expression of genes in the region. Arid4a is broadly expressed during development and in the adult, and the gene carries a CpG island across its promoter. Dact1 codes for the protein Dapper 1, is flanked by CpG islands and is expressed in the limb bud mesenchyme and the central nervous system of the mouse embryo [23,24]. In Xenopus embryos, deletion of maternal Dapper 1 RNA results in the disappearance of normal head structures and the notochord [23]. Daam1 belongs to the formin group of proteins [25]. It is expressed in the central nervous system and limb bud mesenchyme of the early mouse embryo and carries a CpG island across its promoter [26,27].

The Sstr1, Mia2, Mnat1 and Tgf3 genes lie outside the mapped Adp critical region, at 59, 60, 74 and 87 Mb respectively, but were included in the analysis because they have known roles
Imprinting analysis of candidate genes

The mouse genome sequence was used to design primers within each gene, and these regions were amplified by RT–PCR from B and C E12.5 mouse embryo RNA. This time point was chosen because it is the first day on which the Adp phenotype is visible [8]. The amplified fragments were cloned and sequenced, and SNPs were identified between the B and C alleles. Once each SNP was identified, additional primer sets were generated to more closely span the SNP (Table 1). RT–PCR was performed using parental E12.5 embryo RNA, along with RNA from F1 crosses in both directions, B × C and C × B. The amplified fragments were purified and sequenced directly, to examine expression from either one or both of the parental alleles. In all cases, analysis demonstrated biallelic expression of the gene examined, as indicated by the presence of both B and C alleles at the SNP position (Figure 2 and results not shown).

The most probable interpretation of the data presented here is that the Adp critical region does not contain clusters of imprinted genes. Alternate explanations for the parent-of-origin phenotype of the Adp mutation must therefore be considered. It may be that the integration disrupts an imprinted gene farther outside the critical region than those analysed. The distances involved exceed those of any known imprinted domain, however, making this scenario unlikely. What is more plausible is that the Adp mutation causes changes in epigenetic modifications to nearby
non-imprinted genes, altering their expression. Such epigenetic changes would need to be germline-specific to accomplish the paternal Adp phenotype. Interestingly, subsequent analysis of DNA from Adp mice has shown that the transgene is highly methylated in phenotypically normal Adp<sup>Mat</sup> mice, and undermethylated in affected Adp<sup>Pat</sup> mice (T. Watanabe and D. Solter, personal communication). The maternally applied methylation is erased only after two passages through the male germline. As the transgene itself is not expressed in affected animals, these results suggest that methylation of the transgene alters local or regional gene expression, perhaps through the known propensity of DNA methylation to spread to adjacent sequences. As the Adp phenotype is manifest in the heterozygous state, the gene(s) altered would have to be haploinsufficient. It remains formally possible that there is imprinting of Adp-region genes not examined, or of genes examined at different developmental stages or in only certain tissues. As most imprinted gene clusters carry only a small number of non-imprinted genes, however, it is probable that our analysis of 14 genes would have localized any such cluster. Rather than a result of altered genomic imprinting, the parental specificity of the phenotype may result from unique treatment of the Adp transgene by the methylation machinery of the female germline. Identification of the causative gene(s) for the Adp phenotype promises to uncover a gene with a currently unknown role in skeletal development.

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REFERENCES

10 Beechey, C. V. and Cattanach, B. M. (1997) Genetic and physical imprinting map of the mouse. Mouse Genome 95, 100–105

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