Two Dominant Mutations in the Mouse Fused Gene Are the Result of Transposon Insertions

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ABSTRACT

The mouse Fused locus encodes a protein that has been implicated in the regulation of embryonic axis formation. The protein, which has been named Axin to distinguish it from the product of the unrelated Drosophila melanogaster gene fused, contains regions of similarity to the RGS (regulators of G-protein signaling) family of proteins as well as to dishevelled, a protein that acts downstream of Wingless in D. melanogaster. Loss-of-function mutations at Fused lead to lethality between days 8 and 10 of gestation. Three dominant mutations result in a kinked tail in heterozygotes. Two of the dominant mutations, Fused and Knobby, result from insertions of intracisternal A particle retrotransposons into the gene. The insertion in Fused, within the sixth intron, creates a gene that produces wild-type transcripts as well as mutant transcripts that initiate at both the authentic promoter and the 3'-most long terminal repeat of the insertion. Knobby, an insertion of the retrotransposon into exon 7, precludes the production of wild-type protein. Thus the Fused homozygote is viable whereas Knobby is a recessive embryonic lethal. In both mutants the dominant kink-tailed phenotype is likely to result from the synthesis of similar amino-terminal fragments of Axin protein that would contain the RGS domain, but lack the dishevelled domain.

The mouse Fused locus (Fu) was identified 60 years ago as the result of a dominant mutation whose phenotype is a kinky tail (Reed 1937). Although homozygotes of the original allele, Fu<sup>fused</sup> (Fu<sup>fu</sup>), are viable, they sometimes display deafness and other neurological and urogenital defects, in addition to the kinked tail (Reed 1937; Dunn and Gluecksohn-Waelsch 1954; Theiler and Gluecksohn-Waelsch 1956; Deol 1966). Two additional alleles of Fu were subsequently identified, Fu<sup>knob</sup> (Fu<sup>kn</sup>) and Fu<sup>knob</sup> (Fu<sup>knb</sup>) (Caspari and David 1940; Jacobs-Cohen et al. 1984). While sharing the kinked tail phenotype of Fu<sup>fu</sup> as heterozygotes, Fu<sup>kn</sup> and Fu<sup>knb</sup> homozygotes die in utero, between 8 and 10 days post coitum (Gluecksohn-Schoenheimer 1949; Jacobs-Cohen et al. 1984). The embryos display complete or partial duplication of axial structures, neural tube malformations and overgrowth of embryonic ectoderm, suggesting that the Fu gene may play a role in axis specification.

More recently a fourth allele of Fu was identified as a transgene insertion into the gene, based on its failure to complement the embryonic lethality of Fu<sup>kn</sup> (Perry et al. 1995). Unlike the other alleles, Fu<sup>Tgl</sup> is fully recessive and displays no tail kinks. However Fu<sup>Tgl</sup> homozygous embryos are indistinguishable from Fu<sup>kn</sup> homozygotes or Fu<sup>Tgl</sup>/Fu<sup>kn</sup> compound heterozygotes. These observations suggest that the dominant kinked tail phenotype is caused by a gain-of-function mutation at Fu, whereas the embryonic lethality is due to a loss-of-function of the gene. This conclusion is consistent with an earlier observation that a chromosome deletion that removes Fu has no heterozygous phenotype and fails to complement the embryonic lethality of Fu<sup>kn</sup> and Fu<sup>knb</sup> (Greenspan and O'Brien 1986). Furthermore Fu<sup>kn</sup> +/- trisomic mice have kinked tails, despite the presence of two wild-type copies of the gene (Ruvinsky and Agulnik 1991). Thus it seems most likely that the three dominant alleles express the normal gene product inappropriately or express a mutant form of the protein.

Aided by the molecular access provided by the transgene insertion, the Fu gene was cloned and was shown to be broadly expressed during embryogenesis and adulthood (Zeng et al. 1997). The gene encodes several mRNAs, the longest of which contains the potential coding capacity for a 992-amino acid protein. To avoid confusion with the unrelated Drosophila melanogaster gene fused, we have renamed the mammalian Fu gene Axin, for axis inhibition.

The most striking aspect of the predicted amino acid sequence of Axin is a 130-amino acid-long domain in the amino terminal half of the protein that shares similarity to proteins known or predicted to be negative regulators of G-protein signaling. Thus the domain is referred to as the RGS domain, for regulators of G-protein signaling (Koelle and Horvitz 1996; Dohl-
MAN and THORNER 1997). Proteins containing the RGS domain have been identified in eukaryotes from yeast to humans, though the sequence similarity among them rarely extends beyond the RGS domain itself. Two well characterized RGS-encoding genes are egl-10 in Caenorhabditis elegans (KOELLE and HORVITZ 1996) and SST2 in S. cerevisiae (CHAN and OTTE 1982; DIETZEL and KURJAN 1987; DOHLMAN et al. 1995). The egl-10 mutation affects the timing of periodic behaviors such as egg laying and writhing and genetic analysis suggests that it does so by modulating the activity of Gα-1. The yeast recessive mutation sst2 results in supersensitivity to mating pheromone via overstimulation of pheromone signaling through its G-protein-coupled seven-transmembrane domain receptor.

By analogy to egl-10 and sst-2, the axis duplications characteristic of loss-of-function mutations at Axin might be explained if the function of the protein is to suppress the formation of the primitive streak throughout the circumference of the egg cylinder except at the position that is destined to become the primitive streak. This model has been experimentally tested by injecting wild-type mouse Axin mRNA into the dorsal side of Xenopus laevis embryos, where it inhibited axis formation by interfering with signaling through the Wnt pathway (ZENG et al. 1997). A potential role for Axin in signaling in the Wnt pathway during gastrulation is also suggested by a region in its carboxy terminus that displays significant homology to the intracellular protein, dishevelled. dishevelled was originally identified in D. melanogaster as a gene that acts positively downstream in the wingless signaling pathway (KLINGENSCHMIT et al. 1994; NOORDERMEER et al. 1994; THEISEN et al. 1994).

In this article, the molecular nature of the mutations in two Axin dominant alleles, Axin\(^{fu}\) and Axin\(^{kb}\), is described. Both mutations result from the insertion of an intracisternal A particle (IAP) transposon into the Axin gene, resulting in very similar mRNA fusions between IAP RNA and Axin sequences. The precise locations of the two insertions explain the difference between the viabilities of Axin\(^{fu}\) and Axin\(^{kb}\) homozygotes and imply that both alleles result in the production of very similar amino terminal fragments of Axin that contain the RGS domain, but lack the dishevelled homology.

**MATERIALS AND METHODS**

**Mice:** BTBR Fu\(^{fu}\)/+ animals, originally reported as BTBR Fu\(^{kb}\)/+ (ROSSI et al. 1994), were obtained from Dr. WILLIAM DOVE at the University of Wisconsin. C3H Fu\(^{kb}\)/+ animals were obtained from Dr. KAREN ARTZT at the University of Texas at Austin. 129/Rr Fu\(^{fu}\)/Fu\(^{fu}\) mice were obtained from the JACKSON Laboratory.

**DNA analysis:** For large preparations of DNA, tissues were homogenized for 45 sec with a polytron (Kinematica, Luzern, Switzerland) in 100 mM NaCl, 10 mM Tris, HCl pH 8.0, 50 mM EDTA at a concentration of 1.2 ml/g of tissue. The suspension was adjusted to 0.5% SDS and 0.2 mg/ml proteinase K and incubated at 55° for 4 hr. The solution was extracted with an equal volume of phenol, precipitated with 2 vol ethanol, and dissolved in H\(_2\)O. One-centimeter tail fragments were treated identically, except that the homogenization step was omitted and replaced with occasional vortexing during the incubation.

The DNAs were digested with restriction endonucleases according to the conditions provided by New England Biolabs and analyzed on 1.0% native agarose gels. The DNAs were transferred to Hybond-N+ nylon membranes (CHOMCZYNSKI and SACCHI 1987 and crosslinked to the membranes with ultraviolet light (Stratalinker, Stratagene, La Jolla, CA). The filters were hybridized to the following probes generated by RT-PCR: an exon 2 probe that spans bases 363–812 in Axin CDNA, using the numbering system in ZENG et al. (1997) (primers 5'CATGTGTTCACGTGATCGAGTCCG'3' and 5'TGCGG ATCGACGGCTTCTTCC3''), an exon 6 probe containing bases 1681–2164 (primers 5'CCGGTACGCGAGGAGGA G3' and 5'CAACAAAAAGGCCAATCCCCAG3''), and an exon 10 probe containing bases 2904–3627 (primers 5'GAG GAAGTTGAGGAGGATGAG3' and 5'CATGTTAAAGATCCG GTCCCG'). The three probes were labeled by random-priming using DNA polymerase (FEINBERG and VOGELSTEIN 1984). The filters were washed under conditions of high stringency (0.1% SDS, 1.5 mM sodium citrate, 15 mM NaCl at 65°) and exposed to X-ray film.

DNA sequencing reactions were performed with Perkin-Elmer cycle sequencing TaqFS reagents and analyzed on a Perkin-Elmer-Applied Biosystems 373 automated sequencer. Long segments of genomic DNA were amplified from 1.0-μg samples of mouse genomic DNA with the Perkin Elmer (Branchburg, NJ) XL PCR Kit in a 9600 thermal cycler. DNA primers flanking the IAP insertion sites were P1 in exon 6 (5'TTCCGAGAACGCCAGGACCAC3') and P3 in exon 8 (5'CCAGAGACGCCTGATGAGA3'). The P2 primer within the IAP long terminal repeats (LTRs) was 5'CCATCT TGTGAGGCGGAATGTG3').

**RNA analysis:** RNA was prepared by LiCl/urea extraction (AUFFRAY and ROUGEON 1980) and analyzed on 0.8% agarose gels in the presence of 0.22 M formaldehyde with constant buffer recirculation (TSANG et al. 1992). The gels were blotted to Hybond-N+ nylon membranes (BIO-RAD, Hercules, CA) (CHOMCZYNSKI and SACCHI 1987). The filters were hybridized to the probes indicated above, along with an α-actin cDNA probe, to control for RNA loading.

**cDNA cloning and RT-PCR:** The Marathon cDNA Amplification kit (Clontech, Palo Alto, CA) was used to amplify the mutant cDNAs with 0.67 μl of a 60:1 mixture of KlenTaq (Ab Peptides Inc., St. Louis, MO) in 50-μl reactions. Nested 5' amplifications were done with the Marathon adaptor primer AP1 and the reverse primer beginning at base 2760 (5'CCCCCACAGAAATAGTAGCACAAC3') and then AP2 plus the reverse primer beginning at base 2720 (5'ACTCCGGCCACCTGCGTCTTCC3'). The resulting fragments were purified from agarose gels with QIAEXII (Qiagen, Chatsworth, CA) and cloned into the TA cloning vector (Invitrogen, San Diego, CA). Further RT-PCR was performed with primers P1, P2 and P3 derived from the Axin CDNA and IAP sequences.

**RNase protection:** Uniformly labeled RNA probes were prepared according to the Ambion protocols using RNA polymerases and RNasin from Promega (Madison, WI) and [\(\beta\)P]CTP (800 or 300 Ci/mmol) from Dupont/NEN (Wilmington, DE). Hybridizations and RNase digestions were performed according to the Ambion (Austin, TX) RPA II\(^{\beta}\) kit except that after digestion the 200-μl reactions were treated with 0.15 μg/μl proteinase K and 0.5% SDS for 20 min at 37°, extracted with an equal volume of phenol/chloroform (3:1), and precipitated with ethanol. The reaction products were resolved on 5% acrylamide gels.
Insertions in the Mouse *Fused* Gene

**RESULTS**

Transposon insertions in the Axin gene: To screen for alterations in the structure of the *Axin* gene in the three *Axin* alleles, probes derived from exon 2 (5'), exon 6 (M) and exon 10 (3') were hybridized to liver genomic DNA prepared from *Axin*/*Axin*, *Axin*/+ and *Axin*/*+ animals (Figure 1). The patterns of bands observed with the 5' and 3' probes were identical in all mutant samples, and identical to 129/Rr, BTBR and CSH DNAs, the genetic backgrounds on which the mutations are maintained. However with the exon 6 probe, a novel band was evident in each mutant sample (Figure 1B).

Long-range PCR of genomic DNA with primers derived from exons 6 and 8 was utilized to characterize the nature of the change in genomic DNA. In all wild-type DNAs, a 1900-bp product was amplified, corresponding to parts of exons 6 and 8, all of exon 7 and two introns 352 and 1263 bp in length. In both *Axin*/*Fused* and *Axin*/*+* DNAs, a novel ~7.5-kb band was amplified as data not shown). The band was cloned from each mutant sample and partially sequenced.

As shown in Figure 2B, the *Axin*/*Fused*-specific 7.5-kb band contained an insertion of an intracisternal A particle...
(IAP) element, a member of the abundant family of murine transposable elements. The insertion began 35 bp downstream of exon 6, within intron 6. The IAP was of the [\(\Delta\)] type (Kuff and Lueders 1988), having an internal 1.9-kb deletion relative to the full length IAP retrotransposon. However the 344-bp LTRs were intact and predicted by sequence comparison to known LTRs to contain bidirectional promoters (Christy and Huang 1988). The IAP genome was present in the opposite transcriptional orientation to the Axin gene and exhibited the typical 6-bp duplication of host genomic sequence at its ends.

The Axin\(^{Kb}\)-specific band also contained an [\(\Delta\)] type IAP element, in this case inserted at 140 bp into exon 7 (Figure 2C). As was the case with Axin\(^{Ku}\), the transcriptional orientation of the IAP was opposite to that of the wild-type Axin gene, and the LTRs were highly homologous to the consensus IAP sequence.

A surprising result was obtained when long-range PCR was performed on samples of DNA from our colony of Axin\(^{Kb}\) mice, which we obtained in 1990 from Dr. Karen Artzt. The Axin\(^{Kb}\) mutation was indistinguishable from that of Axin\(^{Ku}\), a result that was confirmed by allele-specific PCR analysis with primers that uniquely amplified Axin\(^{Kb}\) DNA (data not shown). The apparent identity of Axin\(^{Kb}\) and Axin\(^{Kb}\) was also consistent with the Southern blot data in Figure 1, where Axin\(^{Kb}\) and Axin\(^{Kb}\) exhibited the same novel band with the exon 6 probe. Fearing that we had inadvertently contaminated the Axin\(^{Kb}\) stock with Axin\(^{Kb}\) animals, we obtained Axin\(^{Kb}\) DNA from Dr. Mary Lyon at Harwell, who identified the original mutation. In these samples, the molecular character of the mutation was indistinguishable from Axin\(^{Kb}\). Further discussions with Dr. William Dove, from whom we had obtained Axin\(^{Kb}\), resolved the issue. His Axin\(^{Kb}\) stock, obtained from Dr. Jean-Louis Guenet, had actually been Axin\(^{Kb}\). Thus the genetic mapping and the complementation studies that we previously reported (Rossi et al. 1994; Perry et al. 1995) had been performed with Axin\(^{Kb}\), not Axin\(^{Kb}\).

Transcription of Axin\(^{Ku}\) and Axin\(^{Kb}\): The impact of the IAP insertions at Axin on transcription was investigated by Northern blotting of RNAs isolated from adult tissues of Axin\(^{Kb}\)/+ and Axin\(^{Ku}\) homozygous animals (Figure 3). With all probes, a 3.9-kb mRNA corresponding to the size of +/+ Axin RNA was detected in samples of brain, liver, kidney and spleen RNAs, reflecting the broad pattern of expression of the gene in adult (Zeng et al. 1997). The appearance of abundant wild-type transcript in Axin\(^{Ku}\) homozygous RNA suggested that the intronic IAP element did not preclude correct RNA splicing between exons 6 and 7.

In addition to the wild-type transcript, the samples from the mutant animals also contained RNAs ranging in size from 1.7 to >12 kb. The sizes of these novel bands were very similar between the two mutant alleles, although the relative intensities of these bands differed among the tissue samples. With the 3' exon 10 probe, an additional strongly hybridizing 1.7-kb RNA was detected in all Axin\(^{Kb}/+\) tissues, most prominently in spleen RNA. A very faint band of slightly larger size was also observed in Axin\(^{Ku}\) RNA, while no RNA in this size range was evident in +/+ RNA.

To characterize these novel RNAs in more detail, an RNAse protection assay with a wild-type RNA probe generated from a cDNA clone that spanned the IAP insertion site in both alleles was utilized (Figure 4). The probe protected a full length 393-bp fragment in both +/+ and Axin\(^{Ku}\) homozygous RNAs, confirming the conclusion that the Axin\(^{Ku}\) IAP element did not prevent splicing of wild-type Axin transcripts. However, an additional band of 338 bp was also detected in Axin\(^{Ku}\) samples. This size corresponds to the sum of the lengths of protected regions of exons 7 and 8, and implies that in addition to the normal splice, an aberrant splice to the
The radiolabeled antisense probe in the Northern analysis (Figure 3) shows that transcripts initiated from the antisense promoter and excluded or included the alternative exon, respectively. A 290-bp fragment identifies transcripts that initiated at the antisense promoter at the 3' end of the LTR. The 3'-specific transcript presumably corresponds to the abundant 1.7-kb RNA that hybridized exclusively to the 3' probe in the Northern analysis in \( \text{Axin}^{Kb} \) RNA (Figure 3).

RT-PCR analysis of the \( \text{Axin}^{Fu} \) transcripts using exon 6 and 8 primers identified two major transcripts: the correctly spliced wild-type transcript (band A in Figure 5, A and C), as expected from RNase protection analysis (Figure 4), and an aberrant transcript in which the 3' splice site of exon 6 was ignored and transcripts continued into intron 6 and the IAP itself to position 5596 (band B). The latter would generate an RNA 5.0 kb in
To ask whether the Axin" allele also generated a transcript initiated from the 3' LTR, an RNase protection probe was generated that spanned the 3' end of the insertion site (Figure 6B). While the wild-type RNA protected a band of 283 bp, corresponding to exons 7 and 8, the Axin" RNA protected two additional bands, a fully protected probe suggesting transcription through the 3' LTR and a smaller band consistent with initiation at the 3' end of the LTR. The low intensity of the latter band is consistent with the low intensity of the ~1.8-kb 3'-specific band on Northern blots (Figure 3).

Thus both Axin" and Axin" generate fusion transcripts that initiate at the 5' end of the Axin gene and proceed through the IAP element, as well as 3' transcripts that initiate within the LTR of the IAP itself.

**Putative protein products of Axin" and Axin":** Conceptual translation of the Axin" transcripts predicts the synthesis of two novel proteins. By skipping exon 7, an internally deleted RNA missing the 57 amino acids encoded by exon 7 will be generated (transcript C in Figure 5). Additional transcripts that initiate at the authentic Axin promoter (transcripts D–F) will generate a truncated version of the protein containing the first 637 amino acids of Axin protein [based on the use of the AUG codon at base 391 of the cDNA (ZENG et al. 1997)] followed by 65 amino acids derived from the translation of IAP sequence before a termination codon is encountered. The 3' Axin" transcript identified in Figure 6A is unlikely to generate a protein containing the carboxy terminus of Axin, as the two potential in-frame AUGs are preceded by two out-of-frame AUG codons, one of which is contained within a close match to the Kozak consensus sequence for optimal translation initiation (GGCATGG) (KOZAK 1986; 1989) and would generate a five-amino acid peptide. Nevertheless, there is the potential for the 3' transcript to generate either a 168- or a 148-amino acid carboxy-terminal Axin protein.

Aside from the wild-type Axin protein, initiation of transcription at the Axin" promoter (transcript B in Figure 5) is predicted to generate an amino terminal peptide that is 47 amino acids shorter than the Axin" fragment, as the result of a termination codon immediately after the exon 6 junction. As in the case of Axin", it is unlikely that the 3' transcript produces a fragment of Axin protein, as the first potential in-frame AUG is preceded by four out-of-frame AUG codons, one of which has a good Kozak consensus sequence (GTGACATGG), and predicts a different five-amino acid peptide than the one predicted in Axin".

**DISCUSSION**

The visible kinky-tailed phenotype of the dominant mutations in Fu, now renamed Axin, allowed the gene to be identified 60 years ago (REED 1937). The kinked tail was attributed to duplications in the posterior neural tube, resulting in transient fetal tailbud bifurcations and asymmetrical fusions of ribs and vertebrae, leading to kinking and shortening of the tail (GLUECKSOHN-
Schoenheimer 1949; Jacobs-Cohen et al. 1984). Two lines of evidence had supported the proposal that the mutation was a gain-of-function: the absence of kinked tails in animals heterozygous for a deletion of a large chromosomal region surrounding Axin (Greenspan and O’Brien 1986) or a transgene insertion into the gene (Perry et al. 1995), and the retention of a kinked tail in Axin<sup>lo</sup> animals with two copies of the wild-type gene (Ruvinsky and Agulnik 1991). The elucidation of the structure of the Axin gene in two semi-dominant alleles, Axin<sup>lo</sup> and Axin<sup>fu</sup>, provides a plausible molecular explanation for the gain-of-function model.

Both mutations result from the insertion of an IAP retrotransposon into the gene, within 456 bp of one another. In the case of Axin<sup>lo</sup>, the insertion does not disrupt the production of wild-type Axin protein, as the insertion occurs within the sixth intron. Thus the viability of Axin<sup>lo</sup> homozygotes is readily explained. In the case of Axin<sup>fu</sup>, the insertion occurs within exon 7, thereby preventing the synthesis of the normal protein product. Axin<sup>fu</sup> homozygotes, in fact, are indistinguishable in phenotype from animals homozygous for the recessive transgene insertion allele Axin<sup>lo</sup>, which eliminates the production of the 3.9-kb Axin RNA (Zeng et al. 1997).

A third allele of Axin, Kinky, was described in 1940 (Caspari and David 1940; Dunn and Gluecksohn-Waelsch 1954). In the course of this study we discovered that our stock of Axin<sup>lo</sup> mice were, in fact, Axin<sup>lo</sup> mice, the confusion having arisen some years ago during a transfer of the mice to the United States from France. Greenspan and O’Brien (1986) conducted an extensive comparison of the survival of the three dominant alleles as homozygotes and concluded that Axin<sup>lo</sup> was the most severe allele, with no homozygous progeny recovered, Axin<sup>lo</sup> was intermediate in severity with very small numbers of animal surviving to birth (19% of expected) and Axin<sup>lo</sup> was fully viable. However in our experience as well as that of others, both the tail kink and embryonic phenotypes of Axin are sensitive to genetic background, which was not uniform in that study. Using two probes tightly linked to Axin, Axin<sup>lo</sup> and Ax-
in$^{K_0}$ could not be distinguished in that study. Unfortunately no DNAs have survived from this study, so it is not possible to examine the molecular nature of the Axin$^{K_0}$ allele and to confirm that it was distinct from Axin$^{K_0}$.

The very similar tail phenotypes exhibited by Axin$^{Fu}$ and Axin$^{K_0}$ suggest that they have the same underlying molecular explanation. This proposal is consistent with the very similar positions of the IAP insertions, which imply that either the site is a hot-spot for retrotransposition or the phenotype is manifested with only a subset of potential integration sites. Such site-specific effects have been observed with the clustered sites of transgene insertions and recombination breakpoints in the mouse limb deformity (ld) gene that result in the same recessive malformation of the limb (Vogt et al. 1992).

The integration of IAP elements through retrotransposition into endogenous genes has been associated with the silencing of the host genes as well as with their inappropriate activation through the action of the strong transcriptional elements within the LTRs (for review see Kuff and Lueders 1988). For example, IAP insertions within the 5' region of the mouse agouti gene result in ubiquitous overexpression of the wild-type protein that generates the yellow coat color, obesity, diabetes and tumorigenesis associated with dominant mutations at the locus (Duhl et al. 1994; Michaoud et al. 1994). In these instances the IAP LTR overrides the normal transcriptional regulation of the gene. In the case of Axin$^{K_0}$, overexpression or ectopic expression of the wild-type Axin protein is ruled out by the fact that the insertion occurs within an exon.

The insertion of an IAP can also result in the production of chimeric proteins containing fusions of IAP- and host gene-derived segments, the most plausible explanation for the kinked tails in Axin$^{Fu}$ and Axin$^{K_0}$. Both Axin dominant alleles are predicted by RNA analysis to synthesize an amino terminal fragment of Axin containing the RGS domain coded by exon 2, but lacking the homology to disheveled, found in exons 9 and 10. The proteins would differ in the extent of Axin sequence at their carboxy termini, as well as the inclusion in Axin$^{K_0}$ of additional IAP-coded amino acids.

An alternative possibility comes from the observation that both alleles generate a 3' distal transcript that initiates at the bidirectional promoter in the 3' LTR and transcribes through the 3' end of the Axin gene. Zeng et al. (1997) have recently shown that a deletion of the RGS domain in the amino terminal end of the protein creates a dominant negative form of Axin that can act alone to generate a secondary axis in the ventral region of the Xenopus embryo, presumably by interfering with the inhibitory activity of wild-type Axin in the frog embryo. However given the arguments cited above in favor of the mutations at Axin acting as gain-of-function mutations, it seems unlikely that the mutant Axin$^{K_0}$ and Axin$^{Fu}$ proteins act in this manner in the mouse. Further more conceptual translation of the potential 3' transcripts indicates that the translational machinery would encounter multiple out-of-frame AUG codons before an in-frame AUG is reached. Hence although we cannot rule out the possibility that the dominant mutation is caused by the synthesis of a small peptide or a carboxy-terminal fragment of Axin, we view the synthesis of similar amino terminal fragments as a more likely possibility.

It is also worth noting that we cannot rule out the existence of transcripts that initiate within the 5'-most (relative to Axin) LTR of the IAP and transcribe through the 3' end of the gene. Such transcripts would be impossible to distinguish from those that initiate at the Axin promoter, but they would be predicted from RNA analysis to splice in the same manner within the IAP. Finally the bidirectional nature of the promoter in the IAP LTRs (Christy and Huang 1988) leaves open the possibility that the dominant phenotype is generated by antisense (relative to the Axin gene) transcription initiated at either the 5' or 3' LTRs. The Northern analysis of adult tissues (Figure 3) failed to identify any transcripts that contain Axin sequences 5' to the insertion site but not 3' of the site, as this alternative would require, and a sense (relative to Axin transcription) RNA probe was not protected from RNase digestion (data not shown). However it is possible that such transcripts are of low abundance, masked by other transcripts, or silenced in adult tissues, as IAP transcription is known to be more widespread early in development than in later stages (Poznanski and Calarco 1991).

Whatever the nature of the neomorphic protein produced by Axin$^{Fu}$ and Axin$^{K_0}$, the kinked tails of the mice are consistent with a perturbation in the development of the most posterior somites, leading to vertebral fusions. Analysis of skeletons of both Axin$^{Fu}$ homozygotes and Axin$^{K_0}$/+ heterozygotes revealed no defect in the axial skeleton other than in the tail (T. J. Vasicek, unpublished results).

The somites in the tail bud arise from a different set of mesodermal precursors than the rest of the axial skeleton (Tam and Tan 1992), leaving open the possibility that genes could have specific effects in posterior somites. The restriction of the dominant phenotype of Axin to the tail is reminiscent of the selective sensitivity of the tail to heterozygous loss-of-function mutations in the Brachyury (T) gene. The product of this gene is a T-box-containing transcription factor that is required for the development of all trunk mesoderm (Herrmann et al. 1990). Homozygous T/T embryos die early in development from a failure of posterior development, but in heterozygotes the only structure affected is the tail, which is shortened or kinked. The tail defect has been attributed to a requirement for higher concentrations of T gene function in the posterior notochord (MacMurray and Shin 1988; Stott et al. 1993). A simi-
lar sensitivity of the tail to reduced dosage of a gene product is evident with hypomorphic alleles of Wnt-3A (GRECO et al. 1996). Whether the limited effect of the neomorphic Axin proteins is the consequence of a restricted domain of expression of the proteins in the tailbud, or differential sensitivity of the tail to the proteins remains to be determined.

The presence of IAP insertions in both Axin\(^{k0}\) and Axin\(^{Ia}\) may help to explain a curious property of the mutations: their variable penetrance that is influenced by the parent-of-origin of the mutation. The penetrance of the tail kinks in Axin mutant animals is known to be influenced by genetic background, for example varying between 61% and 88% for two large backcrosses with Axin\(^{k0}\) (ROSSI et al. 1994; SUTHERLAND et al. 1995). In several studies using both Axin\(^{k0}\) and Axin\(^{Ia}\) it has been noted that the penetrance of the kinked tail phenotype was lower in offspring from female mutant parents than male parents (REED 1937; DUNN and CASPARI 1945; DUNN and GLUECKSOHN-WAELSCH 1954; THEILER and GLUECKSOHN-WAELSCH 1956; RUVINSKY and AGULNIK 1990; SUTHERLAND et al. 1995). This is reminiscent of the parent-of-origin effects on the severity of the phenotype in the agouti IAP insertion alleles (DUHL et al. 1994; MICHAUD et al. 1994). In the case of both dominant IAP insertion alleles Aty and Asipty, female mutant mice produce almost exclusively severely affected offspring whereas male Aty mice have between 10% and 34% pseudo-agouti progeny (WOLFF 1971, 1978). The severity of the phenotype was correlated with differences in the DNA methylation of the activating IAP LTR, that is, the more methylated the IAP LTR, the lower the expression of the protein and the less severe the phenotype. Furthermore males were more likely to transmit the IAP in a methylated state than females. This parental difference is consistent with observations that IAPs are more methylated during spermatogenesis than oogenesis (SANFORD et al. 1987), and that DNA methylation has a negative effect on LTR activity (LAMB et al. 1991).

In the case of Axin, the parental bias is in the opposite direction to that in agouti, that is, males are more likely to have penetrant offspring. The parent-of-origin effect on the penetrance of Axin has been used to suggest that the gene is regulated by genomic imprinting (RUVINSKY and AGULNIK 1990), to the extent that Axin is listed on the current map of genomic imprint regions in the mouse genome (BEECHY and CATTANACH 1996). The recessive behavior of complete deletions (GREENSPAN and O’BRIEN 1986) and insertions in the Axin gene (PERRY et al. 1995) now rule out this possibility. However the lower penetrance of Axin mutations upon female inheritance, coupled with the observation that the penetrance from Axin mothers is reduced even further in the C57BL/6J background (RUVINSKY and AGULNIK 1990) is reminiscent of the effects observed with transgene imprinting (HADCHOUEL et al. 1987; REIK et al. 1987; SAPIENZA et al. 1987; ALLEN et al. 1990; SASAKI et al. 1991; UEDA et al. 1992). In these examples, foreign transgenes were more heavily methylated when inherited from mothers than fathers, and this effect was influenced by genetic background. Whether the parent-of-origin effect on penetrance of the kinked tails can be explained by a modifier that affects the levels of DNA methylation remains to be determined.

In conclusion two dominant mutations in the Axin gene result from the insertions of IAP retrotransposons at sites just 456 bp apart. The insertions most probably result in the production of similar neomorphic proteins whose expression affects the development of the most posterior somites, either because of the expression of the neomorphic proteins is restricted to the tailbud, or the most posterior somites are the only tissues sensitive to their presence.

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