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Alternative splicing and expressivity of the *Axin*^{Fu} allele in mice

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Variable expressivity and incomplete penetrance are common for numerous mutations. In most cases the reasons behind these phenomena remain obscure. Caused by the insertion of a murine retrotransposon into intron 6 of the *Axin* locus, the *Axin*^{Fu} mutation induces alternative splicing and ultimately leads to abnormal tail development in mice. In this investigation RNase protection assay was used to investigate the potential connection between alternative splicing with the expressivity and penetrance of the mutant allele. The results reported here confirm previous observations that alternative splicing occurs in mRNA transcribed from the mutant *Axin*^{Fu} allele. However this investigation also shows that lower levels of alternative splicing commonly take place in the wild type transcript. Correlation analysis reveals a significant connection between tail abnormalities and the ratio of correct to alternatively spliced mRNAs. Overall this paper demonstrates that higher levels of alternatively spliced mRNAs correlate with stronger expression of the mutant trait.

Keywords: alternative splicing, expressivity, genotype and phenotype, mice, mutation, penetrance, RNase protection assay.

Introduction

It is commonly accepted that the majority of observed mutations demonstrate variable expressivity and incomplete penetrance. However, in most cases the causes of these widespread phenomena are unknown, and explanations usually suggest that differences in genetic background and environment are the major contributors. To further address the issues of variable expressivity and incomplete penetrance, mRNA profiles were studied in mice carrying the mutant *Axin*^{Fu} allele. In addition to other developmental abnormalities, this mutation shortens tail length due to kinking that results from vertebral fusions (Reed, 1937; Ruvinsky *et al*., 1991). The *Axin* gene is located proximally on chromosome 17 and encodes a component of the Wnt signalling pathway, an important regulator of embryonic axis formation in mammals (Zeng *et al*., 1997; Hart *et al*., 1998). The mutant *Axin*^{Fu} allele results from the insertion of an intracisternal-A particle (IAP), a murine retrotransposon within intron 6 of the *Axin* gene (Vasicek *et al*., 1997). Transcripts from the mutant allele are not always spliced alternatively, somewhere within intron 6 or the IAP insertion, but can also undergo correct splicing.

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It has been suggested that mutation of the *Axin*^{Fu} allele leads to the fused phenotype through a gain-of-function effect (Ruvinsky *et al*., 1991), possibly by encoding a competing form of Axin protein (Hsu *et al*., 1999). Furthermore the *Axin*^{Fu} allele is also involved in gene conversion-like events, which in turn lead to mosaicism resulting in an unusual pattern of inheritance for the mutation (Ruvinsky *et al*., 2000, 2001).

The expressivity and penetrance of this mutant trait varies significantly depending on genetic background and the direction of reciprocal crosses (Ruvinsky & Agulnik, 1990). Using RNase protection assay (RPA), the relative amounts of wild and alternatively spliced Axin mRNAs produced by different genotypes were measured. This information was utilized to determine whether variations in mRNA profiles correlate with expressivity and penetrance of the mutant allele. Currently there are indications that alternative splicing may occur in approximately 35% of mammalian genes (Brett *et al*. 2000), indicating the significance of this project.

Materials and methods

Mice

Strains 129/Rr *Axin*^{Fu}/+ and TF/Le (*tf*/*tf*) were obtained from the Jackson Laboratory (USA). *Axin*^{Fu}/+

mice were crossed to *tf*/*tf* mice. The resulting offspring were used in the following crosses. Cross 1:**m** *Axin*^{Fu}+/+ *tf* × **f** +*tf*/+*tf*; cross 2:**m** +*tf*/+*tf* × **f** *Axin*^{Fu}+/+*tf*; cross 3:**m** *Axin*^{Fu}+/+*tf* × **f** *Axin*^{Fu}+/+*tf*.

PCR amplification

Genomic DNA was isolated by phenol/chloroform extraction from 0.5 cm tail cuttings following overnight digestion with proteinase K. Multiplex reactions consisted of primer P23 (5′ cggagctattccgagaacg 3′) which is specific for exon 6, G245.R1 (5′ gaccagagcccaagaaaaaccc 3′) which targets intron 6, and IAP-F (5′ gcgcatcactccctgattg 3′) which anneals the LTRs of the IAP insertion in the *Axin*^{Fu} allele. The final $MgCl_2$ concentration was 1.5 mM.

RNase protection assay

RNA was prepared from kidney samples using the RNaqueous RNA extraction system (Ambion). The probe used for the RNase protection assay was generated using the Thermoscript RT-PCR system (Life Technologies) with primers P1 (5′ ttccgagaacgcaggcaccac 3′) and P3 (5′ cccaggacgctcgatggacaag 3′) described in Vasicek *et al*. (1997); cDNA was cloned into pBluescript II using the TA cloning strategy (Marchuk *et al*., 1991).

Antisense RNA probe was transcribed from the cDNA template cloned in pBluescript II using the Maxiscript transcription system (Ambion). RNase protection assays were performed with the RPAIII system (Ambion). Assays were electrophoresed on a 5% polyacrylamide gel and exposed to Biomax MS autoradiography film (Kodak) with an intensifying screen at −80°C overnight.

Gel analysis

Autoradiographs from the RPAs were digitally captured using the Ultra-Violet Products (UVP) gel documentation system GDS 7600 with GRAB-IT annotating software (v. 2.55). Quantification of the bands was performed using PHORETIX 1D standard gel analysis software (v. 3.01; AGP Technologies) incorporating the rubber band method of background subtraction.

Statistical analysis

Microsoft Excel was used to perform the standard statistical analyses including calculation of mean values, standard deviations and correlation coefficients.

Results

The genotypes of all mice studied were confirmed by PCR analysis of the *Axin* locus. The recessive mutation *tufted*, which is tightly linked to the *Axin* gene, was used as a genetic marker to aid in genotyping (Belyaev *et al*., 1981). Segregation data obtained from the studied crosses were similar to those observed in previous investigations (Ruvinsky & Agulnik, 1990). The level of penetrance of the *Axin*^{Fu} mutation varied significantly between the crosses (cross 1 ≈ 88%, cross 2 ≈ 24%, cross 3 ≈ 45%). Furthermore the expression of tail abnormalities was noticeably different between reciprocal crosses 1 and 2. In cross 1 the majority of heterozygotes (*Axin*^{Fu}+/+*tf*) expressed varying degrees of tail deformities including shortening, nodes and kinking. Typically mice that expressed a mutant phenotype in crosses 1 and 3 had considerably shortened tails of around 40–50 mm in length (Tables 1 and 2), being about half that expected for wild type mice. In contrast, in most cases heterozygotes from cross 2 developed a tail phenotype that was close to normal.

The IAP insertion in intron 6 led to the *Axin*^{Fu} mutation, which in turn causes alternative splicing (Vasicek *et al*., 1997). Accordingly, the primary aim of this investigation was to determine if there is a connection between the level of wild and alternatively spliced RNAs with expressivity and penetrance of the mutant trait. To accomplish this, nine phenotypically normal *Axin*^{Fu}+/+*tf* mice were selected from cross 1 along with 10 *Axin*^{Fu}+/+*tf* mice with strong mutant phenotypes. In both cases a near equal number of males and females was used. In cross 2, mice with a strong mutant phenotype were lacking and almost all studied *Axin*^{Fu} mice developed tails that were barely different from normal. The mean tail length for mice that expressed slight tail abnormalities was 89 mm and the mean for mice that did not develop visible malformations was 93 mm. Correspondingly, there were no differences in the mean values of the amount of correct (0.66–0.63) and alternative (0.24–0.24) RNA transcripts between the two groups that were barely distinct in phenotype. Thus comparisons were impossible in cross 2 and the data were not used for further analysis. From cross 3 we selected nine *Axin*^{Fu}+/Axin^{Fu}+ mice that displayed a strong mutant phenotype, again with near equal representation of males and females. As weak expression of the *Axin*^{Fu} phenotype is rarer in homozygotes, we used all five available PCR genotyped mice from cross 3 that expressed a weak phenotype (three females and two males). One +*tf*/+*tf* mouse was sampled for each cross as a control.

To investigate the relative levels of wild type and alternative transcripts in all sampled mice, RNase

Table 1 Mouse tail lengths and densities of the mRNA bands from the RPA analysis

Genotype	Tail phenotype	Tail length (mm)	Density band 1	Density band 2A	Density band 2B
Axin^{Fu}+/Axin^{Fu}+	Strong†	32	0.3581	0.5035	0.1384
		35	0.4161	0.2290	0.3549
		35	0.3886	0.4840	0.1274
		40	0.1928	0.5756	0.2315
		40	0.1802	0.5808	0.2390
		40	0.2126	0.6144	0.1730
		45	0.6786	0.1624	0.1590
		45	0.4816	0.3853	0.1331
		45	0.4478	0.4238	0.1284
	Weak	78	0.4846	0.3944	0.1210
		95	0.4684	0.4091	0.1225
		100	0.6254	0.2140	0.1607
		100	0.6114	0.2457	0.1429
		103	0.4607	0.4071	0.1322
Axin^{Fu}+/+*tf*	Strong†	45	0.5237	0.3426	0.1338
		45	0.7182	0.1458	0.1359
		45	0.7000	0.1604	0.1397
		45	0.6736	0.2032	0.1232
		45	0.4435	0.4765	0.0800
		50	0.5124	0.3242	0.1634
		50	0.4978	0.3629	0.1393
		55	0.4156	0.4838	0.1006
		55	0.6628	0.2300	0.1072
		60	0.5764	0.2756	0.1481
	Weak	93	0.7123	0.1587	0.1290
		90	0.6368	0.2923	0.0709
		90	0.7061	0.1755	0.1184
		95	0.7894	0.1319	0.0787
		95	0.7199	0.1733	0.1068
		97	0.7528	0.1236	0.1236
		100	0.7670	0.1306	0.1023
		100	0.7067	0.2266	0.0667
		105	0.6589	0.1831	0.1580
+*tf*/+*tf*	Normal	100	0.6736	0.2081	0.1182
		100	0.8276	0.0780	0.0943
		105	0.7460	0.1703	0.0836

†Mice with tail length below 60 mm.

Protection Assay (RPA) was employed (Fig. 1A) with an antisense RNA probe transcribed from a cDNA template (Fig. 1B). This probe was used originally to determine that alternative splicing was prevalent in *Axin*^{Fu} transcripts (Vasicek *et al*., 1997).

Three protected fragments were observed in both hetero- and homozygotes (Fig. 1C). The expected 286 bp RNA fragment (band 1) corresponded to full-length, correctly spliced, wild type mRNA. This protected fragment included part of exon 6, all of exon 7 and part of exon 8. Two close migrating alternatively spliced fragments of 238 bp and 234 bp, bands 2A and 2B, respectively, were also found (Fig. 1C). Whereas the 238 bp fragment was anticipated, the smaller 234 bp fragment was not expected and the exact nature of this protected fragment (band 2B) is unknown. Unexpectedly wild type mice, in addition to a fully protected, correctly spliced mRNA, also displayed bands 2A and 2B (Fig. 1C, lane 3). However the density of the alternatively spliced mRNA fragments from wild type mice was noticeably lower (Table 1).

The densities of the three protected mRNA fragments were compared and for every individual sample, measurement of each band was adjusted as a fraction of the sum of the densities of all three bands (Table 1). This

Table 2 Mean values of tail length in mice, relative densities of mRNA bands and correlation coefficients

	Genotype				
	Axin^{Fu}+/*Axin*^{Fu}*tf*		*Axin*^{Fu}+/+*tf*		+*tf*/+*tf*
Expressivity of mutant tail phenotype	Strong†	Weak	Strong†	Weak	Normal
Number of mice	9	5	10	9	3
Tail length	39.67 ± 4.85	95.1 ± 10.25	49.5 ± 5.50	96.11 ± 4.96	101.67 ± 2.89
Relative density of correctly spliced mRNA, band 1	0.37 ± 0.16	0.53 ± 0.08	0.57 ± 0.11	0.72 ± 0.05	0.75 ±0.08
Relative density of alternatively spliced mRNA, band 2A	0.44 ± 0.16	0.33 ± 0.10	0.30 ± 0.12	0.18 ± 0.05	0.15 ±0.07
Relative density of alternatively spliced mRNA, band 2B	0.19 ± 0.08	0.14 ± 0.02	0.13 ± 0.02	0.11 ± 0.03	0.10 ±0.02
Correlation coefficient: correctly spliced mRNA, band 1/tail lengths	0.55, $P < 0.05$		0.62, $P < 0.01$		—
Correlation coefficient: alternatively spliced mRNA, band 2A/tail length	−0.41, $P < 0.10$		−0.54, $P < 0.05$		—

†Mice with tail length below 60 mm.

adjustment removed any potential influence of sample variation between individuals.

Analysis revealed a significant correlation between tail length and the relative densities of band 1 and 2A in homo- and heterozygotes (Table 2). A few conclusions can be drawn from this observation. Firstly, there is a significant positive correlation (0.55–0.62) between tail length and the amount of wild type *Axin* transcript (band 1) in both *Axin*^{Fu}+/*Axin*^{Fu}+ and *Axin*^{Fu}+/+*tf*. Secondly, there is a negative correlation (−0.41 to −0.54) between tail length and the amount of alternative transcript (band 2A) in both compared genotypes. In homozygotes this correlation was just short of being statistically significant. These negative correlations indicate that competition may exist between the Axin proteins produced by wild and alternatively spliced mRNAs. Accordingly this suggests that the ratio between correct and alternatively spliced mRNAs may be an essential factor in expression of the mutant phenotype.

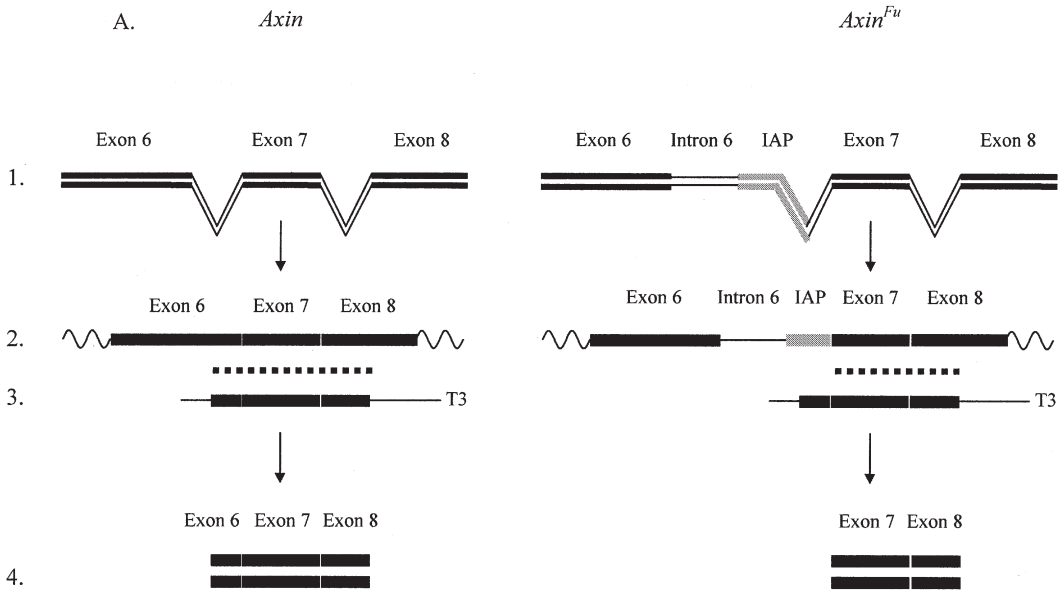
Discussion

This investigation was aimed at determining whether the ratio between wild type and alternative transcripts correlated with expressivity and penetrance of the *Axin*^{Fu} mutation. Essentially this study was devoted to narrowing the gap in the current understanding of the pathway leading from genotype to phenotype, in carriers of the *Axin*^{Fu} allele.

Although the data presented in this paper confirm alternative splicing in the *Axin*^{Fu} gene, as previously reported (Vasicek *et al*., 1997), several pieces of additional information were found. It was shown that alternative splicing occurs in wild type mice, although to a lesser degree than that observed for *Axin*^{Fu} transcripts. The RPA approach does not provide information beyond the region of protection, hence it is not possible to indicate whether the alternatively spliced mRNAs from wild and *Axin*^{Fu} alleles were identical or whether they were the products of different alternative splicing events.

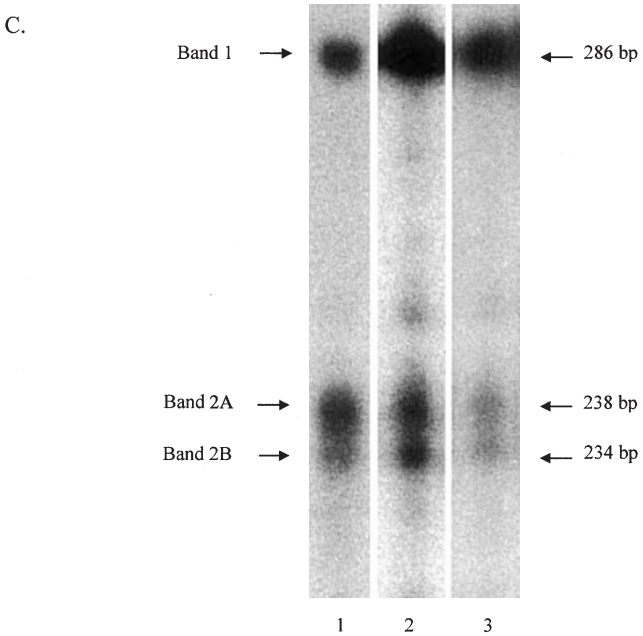
Most importantly, an inverse relationship between tail length and the levels of correctly and alternatively spliced mRNA was observed in *Axin*^{Fu} heterozygotes and particularly in homozygotes. Collectively the data demonstrate that this shift in the levels of wild and mutant mRNAs correlates with expressivity and eventually penetrance of the mutant allele.

Although a connection between mutations and alternative splicing has been established, there is little data concerning the involvement of alternative splicing in variable expressivity and incomplete penetrance of mutations. However it has been shown that among cystic fibrosis sufferers with a particular genotype, the severity of the disease depends on the levels of correct and alternatively spliced mRNA transcripts (Chiba-Falek *et al*., 1998). In another example, low penetrance of the retinoblastoma-predisposition gene (*RB1*) may be caused by alternative splicing that leads to the production of a less severely disrupted protein (Onadim *et al*., 1992). Finally, extreme variation of phenotype in sufferers of familial adenomatous polyposis (FAP) has been attributed to alternative splicing, specifically within the adenomatous polyposis coli (*APC*) gene. However, in this case



B.

5′TTCCGAGAACGCAGGCACCACCCTCAGTGCTGGGGATTTGCCCTTTGGTGGTAAAACTAGTGCACCTTCCA
AAAGAAACACCAAGAAGGCTGAATCTGGGAAGAATGCCAATGCTGAGGTACCCAGTACCACAGAGGACGCTGA
GAAGAACCAGAAGATCATGCAGTGGATCATTGAGGGAGAGAAGGAGATCAGTAGACACCGGAAGGCAGGCCAT
GG*GTCTTCTGGGTTGAGGAAGCAGCAGGCCCATGAAAGCTCCAGGCCCTTGTCCATCGAGCGTCCTGGG* 3′



it was not ruled out that unidentified modifying genetic factors might influence penetrance (Rozen *et al.*, 1999).

It is known that the major morphogenetic events responsible for vertebral development in mice occur in the embryo between the 9th and 10th days of development (Beddington, 1987). Though the Axin protein is detected around the same stage of development (Zeng *et al.*, 1997), differences in tail structure are not observed earlier than 12–13 days after fertilization. Furthermore, weak abnormalities may develop much later, even 7–10 days after birth. Thus it is not possible to study putative connections between alternative splicing and tail phenotype during the critical stage in early develop-

Fig. 1 A. RPA strategy. 1. Structure of the region covering exons 6, 7 and 8 in *Axin* and *Axin*^{Fu} DNA. 2. Wild type and alternative transcripts from *Axin* and *Axin*^{Fu}, respectively. Wavy lines represent exons beyond the region of interest. As the exact features of the alternative transcript for *Axin*^{Fu} are unknown, the alternative transcript described here represents only one possibility. 3. Antisense RNA probe transcribed from the cDNA template T3 promoter. Area of hybridization between probe and target indicated by dashed lines. 4. Double stranded hybrids protected from RNase digestion. When the antisense probe hybridizes wild type transcript, it protects a full length 286 bp fragment, being the sum of the regions of exons 6, 7 and 8 covered by the probe. When the antisense probe hybridizes an alternative transcript where exon 6 is no longer adjacent to exons 7 and 8, the probe protects a 238-bp fragment, being the sum of exons 7 and 8 only. A 48-bp fragment is also expected from the alternative transcript representing a protected fragment from exon 6 only (not shown). B. Sense strand of the cDNA template used for transcription of probe in RPA. Standard font, 3′ portion of exon 6 (48 bp); *underlined*, the entire exon 7 (171 bp); *italic*, 5′ portion of exon 8 (67 bp). C. 5% polyacrylamide gel of RNase protection assay. Lane 1, *Axin*^{Fu}+/*Axin*^{Fu}+ homozygote; Lane 2, *Axin*^{Fu}+/+*tf* heterozygote; Lane 3, +*tf*/+*tf*.

ment. For this reason only adult mice were used in this initial study.

Our data reveal that the ratio between wild and alternatively spliced mRNAs and the corresponding proteins may be critical in the determination of tail phenotype. Hence it is likely that competition between the different forms of Axin protein ultimately determines tail phenotype. It is suggested that once the amount of protein translated from alternatively spliced transcript exceeds a particular level, a morphogenetic barrier may be overcome, causing tail development to deviate from the normal pathway. Accordingly the ratio of competing proteins would establish a threshold for development of the mutant character, thus determining expressivity and penetrance. Assuming this scenario, one may expect that a mouse may occasionally appear which does not have the mutant allele but develops non-inheritable tail abnormalities resembling the classical fused phenotype.

One case was found where the relative densities of bands 1 and 2A were almost equal in mice with weak (cross 3) and strong (cross 2) tail abnormalities, respectively (Table 2). This observation may not appear to fit well with the explanation proposed in this paper, but it must be taken into consideration that the genetic background of these two groups was not identical. Particularly, the proportion of alleles inherited from the +*tf*/+*tf* stock differs significantly in crosses 1 and 3. For this reason it was decided to use comparisons only within a group of mice with the same genotype and age to eliminate possible effects of genetic background.

The correlation data (Table 2) show that the ratio of alternative and correctly spliced mRNAs contributes around 50–60% to the overall determination of tail phenotype. It is likely that variation in genetic background and environmental factors may affect the frequency and severity of tail abnormalities in mice carrying the *Axin*^{Fu} allele. Drastic differences in expressivity and penetrance observed between reciprocal test crosses are typical for the *Axin*^{Fu} mutation and support this view.

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