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A *Tirant* insertion in the alcohol dehydrogenase locus of *Drosophila melanogaster*

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An alcohol dehydrogenase allele Adh^{AAS44} isolated from an Australian natural population of *Drosophila melanogaster* is associated with low activity in adult flies. A 5.0 kb insertion in the first intron between the distal and proximal promoters has been partially sequenced and shown to be homologous to the retrotransposon *Tirant.* The insertion is the major change in the transcriptional unit of Adh^{AAS44}, but there is also a single nucleotide change in the adult *Adh* enhancer. The main phenotypic effect associated with the *Tirant* insertion is a reduction in alcohol dehydrogenase activity and transcript level in adult flies. There is a very much smaller reduction in 3rd instar larvae. The data indicate that *Tirant* affects transcription from the distal promoter. The possible mechanisms for this differential effect on transcription are discussed.

Keywords: ADH activity, *Adh* transcripts, alcohol dehydrogenase, *Drosophila melanogaster*, *Tirant*.

Introduction

The variety and genomic distribution of transposable elements (TEs) is well documented in *Drosophila melanogaster*. Over 40 families of TEs have been described (Finnegan, 1989), making up about 10 per cent of the genomic DNA. TEs are important structural components of *D. melanogaster* heterochromatin (Pimpinelli *et al*., 1995) and numerous studies have demonstrated that natural populations are highly polymorphic for TE insertions (Charlesworth & Langley, 1991). Studies of restriction fragment length polymorphism (RFLP) in the region of specific gene loci, for example *Adh* (Aquadro *et al*., 1986; Jiang & Gibson, 1992a), *Amy* (Langley *et al*., 1988), and *Gpdh* (Symonds & Gibson, 1992) have revealed extensive sequence length variation, most of which is caused by insertions or deletions of less than 200 bp, but some result from known TEs.

Assessing the phenotypic effects of TE insertions in natural populations is of evolutionary significance from at least two aspects. The site of insertion of a TE will determine whether there is a phenotypic effect, either on the equilibrium level of a protein or, in exceptional circumstances, by producing a new protein (Wilanowski *et al*., 1995). Secondly, the potential for further transposition of a TE from the region of the locus is relevant to the future stability of the gene. For these reasons it is important to identify TE insertions and to assess their phenotypic effects.

Jiang & Gibson (1992a) assessed restriction endonuclease variation in the 12 kb region surrounding the *Adh* locus in 194 chromosomes sampled from seven Australian and six Chinese populations of *D. melanogaster*. They found 10 different insertions (of 0.28, 0.35, 0.4, 0.48, 0.7, 1.0, 1.5, 3.0, 4.5 and 5.0 kb) which were subsequently cloned into λEMBL3 and probed with DNA sequences from each of seven known TEs. Insertion 0.28 kb had homology with *B104B* (Scherer *et al*., 1982) and insertion 0.4 kb had homology with *F101* (Dawid *et al*., 1981). Two of the insertions — the 1.5 kb inserted 2 kb from the 5′ end of the coding region and the 5.0 kb inserted in intron 1 — were associated with abnormal (low) ADH activity in adult flies (Jiang & Gibson, 1992b).

The first intron in the *Adh* gene in *D. melanogaster* separates two promoters which produce developmentally regulated transcripts, with the proximal transcript present in larvae until the mid to late

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third instar (Savakis *et al*., 1986). At this time transcription switches to the distal transcript until the beginning of the pupa stage, although the level of transcription is much lower than it had been for the proximal transcript (Corbin & Maniatis, 1989). In adult flies the distal transcript is the major form. Transcription of *Adh* is regulated by sequences 5′ to each promoter and by two major enhancers upstream from the distal promoter (Fig. 1).

The 5.0 kb insertion in the Adh^{AAS44} allele separates the two promoters and the enhancer regions from the proximal promoter. For this reason it was important to assess the effects of this insertion on ADH activity. We have identified the 5.0 kb insertion in Adh^{AAS44} and shown that it has differential effects on ADH activity in larvae and adult flies.

Materials and methods

Drosophila melanogaster *stocks*

The Adh^{AAS44} allele associated with low ADH activity was identified in a survey of restriction site variation

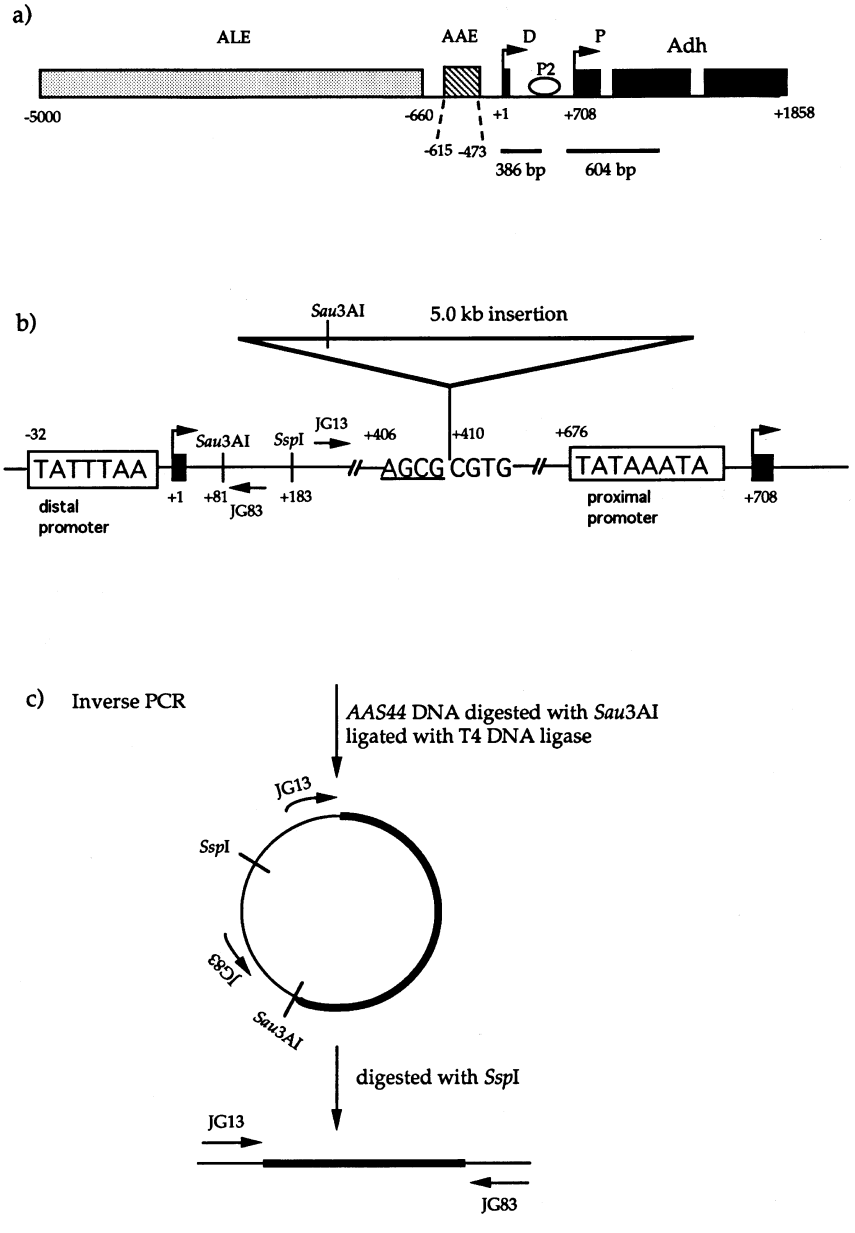

Fig. 1 The structure of the *Adh* gene in *Drosophila melanogaster*, the positions of its major regulatory elements and the location of the insertion. (a). The *Adh* exons are shown as black boxes with the two transcription start sites indicated by solid arrows. The *Adh* adult enhancer (AAE) is shown as a hatched box and the larval enhancer (ALE) as a dotted box. The binding site for P2 protein is shown as an oval. Nucleotide numbering follows Kreitman, 1983. The probes used for Northern analyses are indicated. (b). The site of the *Tirant* insertion is indicated in the first intron of Adh^{AAS44}. The 4-bp duplication target site is underlined. The two TATA boxes are shown. (c) Inverse PCR strategy used to amplify part of the insert in Adh^{AAS44}. The *Adh* gene, the site of the insertion and the positions of a number of restriction enzyme sites are shown. The structure of the ligated molecule, with the *Ssp*I site used for linearization is indicated. The positions of the two PCR primers are indicated with the insertion sequences represented as a thick black line.

made on flies collected from All Saints (Vic.) (Jiang & Gibson, 1992a). The ADH produced by this allele is electrophoretically 'fast'. The chromosome bearing the allele is homozygous lethal and we have not been able to recombine the lethality from the *Adh* locus. Adh^{AAS44} is maintained heterozygous with *Df(2L)64j*, a deficiency that spans the *Adh* locus (Lindsley & Grell, 1968).

Heterozygotes between Adh^{nLA248} and Adh^{AAS44} were produced for experimental purposes. Adh^{nLA248} is an X-ray induced *Adh* null allele, which produces an *Adh* transcript 250 bp longer than normal (Chia *et al*., 1985b). The chromosome bearing Adh^{nLA248} also carries the markers *cn* and *bw* (Lindsley & Grell, 1968) and when homozygous gives white-eyed flies. Heterozygous *AAS44/nLA248* larvae were harvested from mass cultures set up on riboflavin-supplemented media (0.3 g/L). Under these culture conditions heterozygotes $++/cn\ bw$ can be distinguished from *cn bw/cn bw* homozygotes by the colour of the Malpighian tubules. Larvae were collected at 72–80 h and 88–96 h. Adult *AAS44/nLA248* flies were separated from the progeny of single-pair matings between *AAS44/nLA248* males and homozygous *nlA248* virgin females by virtue of their wild-type eye colour.

A normal activity *Adh* allele, *AC5*, was used as a control; *AC5* encodes electrophoretically fast enzyme.

Extraction of total DNA

Genomic DNA was extracted from whole flies according to the method described by Chia *et al*., 1985a.

Inverse PCR

The lambda clone containing Adh^{AAS44} (Jiang & Gibson, 1992a) lost titre, so to identify the 5.0 kb insertion in Adh^{AAS44}, the 5′ part of the insertion was amplified using inverse PCR (Triglia *et al*., 1988). The Adh^{AAS44} genomic DNA was digested with *Sau*3AI, as there were likely to be several *Sau*3AI sites along the 5 kb insertion fragment. There was a *Sau*3AI site in the first intron of *Adh* (Fig. 1). An *Ssp*I restriction site between the primers JG83 and JG13 was used to linearize the circularized DNA. The primer pair JG83 (position 104–184, 5′-GCCC-TTTTGCTACTTACTT-3′) and JG13 (position 288–307, 5′-CCTGTTCCGCATATTCCTCT-3′), which are located between the *Sau*3AI site and the insertion site, were used for PCR amplification (Fig. 1).

PCR amplification and sequencing

The rest of the Adh^{AAS44} gene was amplified with appropriate primers described by Gibson *et al*. (1992). The PCR amplification protocol and the purification of amplified DNA for sequencing has been described by Reed & Gibson (1993).

ADH enzyme activity assays

Flies used for ADH assays were raised from single-pair cultures on a high-protein medium (10 g agar, 5 g yeast, 15 g sucrose, 50 g malt, 30 mL karo and 10 g hi-pro per L) at 25°C. Male progeny were collected and aged for 7–10 days at 20°C before assaying. Flies were frozen in liquid nitrogen and stored at −90°C for up to 7 days. Assay extracts were prepared and ADH activities were measured as previously described (Gibson & Wilks, 1988).

RNA extraction and quantitative Northern analysis

The methods used for total RNA extraction from adult flies for Northern analyses have previously been described (Freeth *et al*., 1988). Total RNA was also extracted from 72 to 80 h and 88–96 h larvae using the same procedure. The filters loaded with the adult RNA were hybridized with a nick-translated labelled pSAF-2 probe (Goldberg, 1980) and the filters loaded with the larval RNA were hybridized with a nick-translated labelled 604 bp *Bam*HI/*Hin*dIII fragment (+651 to+1256) cloned into M13mp9 (Fig. 1, Lockett & Ashburner, 1989). Filters were also probed with a 386 bp *Sal*I/*Hpa*I fragment (−63 to +326), which is specific for the distal transcript (Fig. 1).

The hybridization intensities of the transcript bands were measured on the PhosphorImager instrument (Molecular Dynamics). The signal intensity of each band observed was measured separately and the relative intensity for each sample was calculated.

Results

The insertion in Adh^{AAS44} *is* Tirant

Southern analyses (Jiang & Gibson, 1992a) had shown that the 5.0 kb insertion in Adh^{AAS44} was located in a *Hpa*I (−386)/*Hin*dIII (−219) fragment in the first intron between the two *Adh* promoters (see Fig. 1). This analysis also revealed a 200 bp insertion in a 1.45 kb *Eco*RI fragment, 1320 bp 5′ to the distal promoter. This region contains the *Adh* larval enhancer (ALE) (Corbin & Maniatis, 1990),

but as we have observed a similar insertion in this position in alleles with normal activity we have not investigated it any further.

The 5.0 kb insertion in Adh^{AAS44} could not be amplified directly by PCR using primers in the *Adh* gene. Instead we used inverse PCR, with the protocol outlined in Fig. 1, to amplify part of the insertion. A 150 bp PCR product was subcloned into pBluescript SK(+) and sequenced. The sequence was found to be 97 per cent homologous (EMBL data bank) to the 5′ end of the 5.7 kb *Tirant* TE, first described as an insert in the extramacrochaetae (*emc*) locus in *D. melanogaster* (Garrell & Modolell, 1990) — *Tirant* was named after a medieval Castilian Knight. The restriction map of the insert (Jiang & Gibson, 1992a) strongly supports the conclusion that the insert is derived from *Tirant*, although the resolution of the map was insufficient to detect the region deleted compared to the 5.7 kb element. Later, using an *Adh* primer and one based on the *Tirant* sequence described by Garrell & Modolell (1990), we amplified by PCR and sequenced the 3′ end of the insertion in Adh^{AAS4}. These data further confirmed that the insertion was *Tirant*, that it was inserted in *Adh* at position +406 to +409, i.e. AGCC, and that this target site was duplicated. The target site differs at two nucleotides from the target site (CCCG) previously described (Garrell & Modolell, 1990). *Tirant* is inserted in the same transcriptional orientation as the *Adh* gene.

In order to be confident that any phenotypic effects on ADH activity associated with the *Tirant* insertion were not confounded by nucleotide changes elsewhere resulting in amino acid substitutions, the Adh^{AAS44} gene was sequenced in the coding regions from the larval leader at +650 to the 3′ flanking region at +1697. In addition, intron 1 was sequenced from +288 to +409 and the region from −640 to −460, which spanned the 142 bp *Adh* adult enhancer (AAE) (Falb & Maniatis, 1992), was also sequenced (Fig. 1).

The DNA sequence confirmed that Adh^{AAS44} is a fast electrophoretic allele with C at +1490 (Kreitman, 1983). There were no other changes that would give rise to amino acid substitutions, and other differences found in comparison to the consensus sequence for Adh^{F} had previously been described in normal activity Adh^{F} alleles (Kreitman, 1983; Laurie *et al*., 1991). There were two nucleotide changes in the AAE sequence (−615 to −473) and one of these (A to T) at position −523 was in the central core region at the binding site for the transcription factor AEF-1. It is possible that this change might affect the function of the AAE.

Effect of Tirant *on ADH activity and* Adh *transcripts*

As *Tirant* was inserted between the two *Adh* promoters we assayed ADH activity in 3rd instar larvae and adult flies. The second chromosome bearing Adh^{AAS44} was homozygous lethal so assays were made on larvae and adults heterozygous Adh^{AAS44}/Adh^{nLA248}, which are also heterozygous for *cn* and *bw* carried on the *nLA248* second chromosome. Larvae of the genotype + +/*cn bw*, when cultured on media supplemented with riboflavin, are distinguished by the dark yellow colour of their Malpighian tubules, in contrast to the pale yellow colour of the tubules in *cn bw*/*cn bw* homozygotes. These assay data (Table 1) show that, compared to the Adh^{F} control allele, Adh^{AAS44} has about one-third the activity in adult flies but more than 75 per cent of the normal level in larvae. Early third instar larvae (72–78 h) have significantly ($P<0.05$) lower relative activity than the control, compared to late (90–96 h) third instar larvae.

The relative transcript levels between Adh^{AAS44} and the control (Fig. 2 and Table 2) paralleled the

Table 1 ADH activity of Adh^{AC5}/Adh^{nLA248} and Adh^{AAS44}/Adh^{nLA248} heterozygotes of *Drosophila melanogaster*. Each value is the mean of assays of the progeny from single-pair crosses for adults or from a mass cross for larvae. Standard errors in parenthesis

Genotype	Developmental stage	Number of assays	ADH activity	Relative activity
AC5/nLA248	Adults	5	229.6 (±9.7)	1
AAS44/nLA248	Adults	5	78.5 (±7.6)	0.34
AC5/nLA248	72–78 h larvae	4	71.7 (±17.5)	1
AAS44/nLA248	72–78 h larvae	5	53.7 (±3.5)	0.75
AC5/nLA248	90–96 h larvae	2	67.5 (±20.1)	1
AAS44/nLA248	90–96 h larvae	2	60.3 (±9.1)	0.89

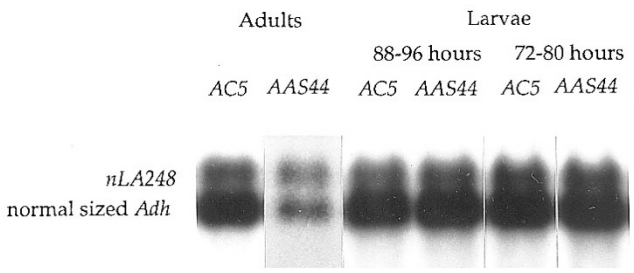

Fig. 2 Northern analyses for adults and larvae of Adh^{AAS44}/Adh^{nLA248} and Adh^{AC5}/Adh^{nLA248} heterozygotes. From left to right, samples were loaded as: Adh^{AC5} adults, Adh^{AAS44} adults, Adh^{AC5} 88–96 h larvae, Adh^{AAS44} 88–96 h larvae, Adh^{AC5} 72–80 h larvae and Adh^{AAS44} 72–80 h larvae. The adult filters were probed with the nick-translated labelled pSAF-2 and the larval filters were probed with the nick-translated labelled *BSBH6*. The upper band represents the *nLA248*-specific RNA. The lower band represents the normal sized *Adh*-specific RNA.

ADH activity differences, although for each developmental stage the difference was less. Thus, in adults, Adh^{AAS44} produced about 55 per cent of the transcript level of the control compared to 34 per cent of the ADH activity.

Discussion

The sequence data for the 5′ region, together with the restriction map, indicate that the insertion in Adh^{AAS44} is *Tirant* (Garrell & Modolell, 1990). This insertion, located in the first intron of *Adh* is the first example of a TE insertion in the *Adh* transcriptional unit in alleles sampled from natural populations. Previously Aquadro *et al*. (1986) found a low-activity *Adh* allele (R142) with a 5.2 kb *copia* retrotransposon inserted 243 bp 5′ to the distal transcription start site. Dunn & Laurie (1995), using P element transformation of deletion constructs, showed that excision of *copia* from the cloned R142 gene caused a threefold increase in ADH activity.

Unlike *copia* (Mount & Rubin, 1985), the *Tirant* TE is less well studied, with only two *Tirant* insertions previously described. The limited data suggest that *Tirant* has a number of structural features common to *Drosophila* retrotransposons. For example, the 4 bp target site is duplicated on insertion, the element contains moderately repetitive DNA and has 5 bp inverted terminal repeats (Garrell & Modolell, 1990). Recently, Thomas *et al*. (1995) have described a spontaneous dominant mutation at the *Serrate* locus (Ser^D) caused by a 505 bp *Tirant* insertion. From comparison of the sequence with that described by Garrell & Modolell (1990), the authors conclude that *Tirant* has 416 bp LTRs that terminate with 5 bp inverted repeats and is thus a typical retrotransposon. There are no data on the genomic copy number.

The *Tirant* associated with the *Ach* mutation at the *extramacrochaetae* locus is inserted such that it truncates the *emc* ORF. The functional mutant protein encoded lacks 42 carboxy-terminal amino acids present in the wild-type protein (Garrell & Modolell, 1990). In Ser^D, *Tirant* is inserted in the 3′ untranslated region of the gene. The truncation of the *Ser* RNA within the *Tirant* LTR eliminates putative RNA degradation signals downstream, which leads to increased stability of *Ser* RNA, higher levels of *Serrate* protein and a complex phenotype (Thomas *et al*., 1995).

The main phenotypic effect associated with Adh^{AAS44} is an $\approx$70 per cent reduction in ADH activity in adult flies but less reduction ($\approx$20 per cent) in 3rd instar larvae, and these changes are paralleled by changes in transcript levels. The probe used in the Northern blots identified transcripts both from the distal and proximal promoters. Our attempts to quantify transcripts in larvae from the distal promoter only using a 386 bp distal transcript specific probe (Fig. 1, Lockett & Ashburner, 1989) were not successful, so we cannot differentiate transcripts from the two promoters in the late 3rd instar larvae.

The sequence data indicated that only the *Tirant* insertion in the first intron and a single nucleotide change in the AAE region at position 523 differed from normal-activity alleles. The change in the AAE might affect the fidelity of the binding site and thus reduce adult activity, but this can only be gauged by further experiments, using P element transformation of appropriate constructs. As the reduction in ADH activity and transcript levels is substantial in adults, it seems more likely that *Tirant* is the causative factor. The *Tirant* insertion is 28 bp 5′ to the binding site of the P2 protein (see Fig. 1), which is a transcriptional regulatory factor of the proximal promoter (Heberlein *et al*., 1985), and this may be the cause of the small effect in larvae. *Tirant* also distances the larval enhancers (ALE) from the proximal promoter and this could also lead to a reduction in proximal transcript levels. Nevertheless, the major phenotypic effect seems to be brought about by the effect of *Tirant* on transcription from the distal promoter. It is possible that this effect is mediated by a lowered rate of transcription or by the lower stability of the much larger primary transcript that will be initiated from the distal promoter.

There is, however, no known way of deleting the *Tirant* insertion from Adh^{AAS44} to test directly for an effect on *Adh* transcription. As occurs with Ser^{D}, it is possible that the poly(A) signal (AATAAA) 100 bp downstream of the 5′ termini of *Tirant*, might be functional in *Adh* transcription initiated from the distal promoter, given that *Tirant* is in the same transcriptional orientation as *Adh*. However, the Northerns did not reveal such a truncated transcript (of about 500 bp) that would be expected if transcription terminated within *Tirant*. Finally it should be considered whether sequences within *Tirant* are binding sites for factors which normally enhance *Adh* transcription, possibly modifying transcript levels in different tissues, as occurs with the *gypsy* insertion in the *yellow* locus (Geyer & Corces, 1992).

Whatever the mechanism turns out to be, the *Tirant* insertion in the *Adh* locus is associated with a differential effect on ADH activity with a much larger reduction in adult flies. This phenotypic effect on ADH may be associated with reduced fitness (Van Delden, 1982), although there is some evidence that larval ADH levels are more relevant to fitness than are adult levels (Heinstra *et al*., 1989). The Adh^{AAS44} allele provides another example of the kind of effects that transposable elements can induce in natural populations and hence contribute to the variation on which selection might act.

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