Different forms of *Ultrabithorax* proteins generated by alternative splicing are functionally equivalent

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Communicated by G.Morata

The *Ubx* gene of *Drosophila* normally produces several forms of *Ubx* proteins through alternative splicing of two microexons. We describe here two new viable *Ubx* mutations that show similar and almost wild-type adult phenotypes. Molecular characterization has shown that one of them, *Ubx*^{MX17}, is an inversion within the *Ubx* transcription unit including one of the microexons involved in alternative splicing. This results in mutant flies possessing a very abnormal array of *Ubx* proteins, probably including spliced forms not present in wild-type flies. Yet these protein products successfully substitute for the normal ones and allow virtually normal *Ubx* function. We argue that the different *Ubx* proteins are developmentally equivalent and that the slight mutant phenotype observed in *Ubx*^{MX17} flies is not due to the abnormal set of *Ubx* proteins but to a breakpoint in a *cis*-regulatory region.

Key words: alternative splicing/*Ubx* functions/*Ubx* microexons

Introduction

Alternative splicing is a phenomenon occurring in many species (Breitbart *et al.*, 1987) by which a single gene can produce different protein forms. In most cases its functional significance is not known although there are instances, like the sex determining process of *Drosophila* (Nagashi and Baker, 1990), in which alternative splicing plays a key role.

Ultrabithorax (*Ubx*) is a homeotic gene, belonging to the bithorax complex (BX-C) of *Drosophila* (Lewis, 1978; Sánchez-Herrero *et al.*, 1985), which, alone or in combination with other homeotics, specifies the characteristic development of parasegments 5–12.

Molecular analyses (Beachy *et al.*, 1985; O'Connor *et al.*, 1988; Kornfeld *et al.*, 1989) have shown that *Ubx* possesses one transcription unit, which through alternative splicing of two microexons produces distinct protein forms according to the incorporation of one, two or none of the microexons in the mature RNAs. The different *Ubx* proteins have different sequences near the homeobox (O'Connor *et al.*, 1988; Kornfeld *et al.*, 1989) suggesting that they may have different recognition properties and hence different developmental roles. This possibility is supported by the observation that the distribution of the different *Ubx* proteins is tissue specific (O'Connor *et al.*, 1988; Kornfeld *et al.*, 1989). It is further supported by the phenotype of the mutation

*Ubx*¹⁹⁵, produced by a nonsense codon in the second microexon, therefore inactivating all protein products encoded by mRNAs that contain this microexon (Wienzierl *et al.*, 1987). RNAs without microexon 2 are predominantly produced in the nervous system (O'Connor *et al.*, 1988; Kornfeld *et al.*, 1989) and this is less affected than other tissues in *Ubx*¹⁹⁵ mutants (Wienzierl *et al.*, 1987).

We have tested the functional significance of the different *Ubx* proteins by studying a mutation that produces a very abnormal array of spliced forms of *Ubx* products. Our results strongly suggest that different *Ubx* proteins are functionally equivalent.

Results

Genetic characterization of the mutations *Ubx*^{MX17} and *Ubx*^{MX19}

The mutation *Ubx*^{MX17} was X-ray induced in our laboratory. It is homozygous viable and shows (Figure 1B) a slight but clear transformation of the haltere (the appendage of the third thoracic segment of the adult) towards wing (the corresponding appendage of the second thoracic segment). The transformation is of the same type but stronger in hemizygous (*Ubx*^{MX17}/*DfUbx*) flies. Only the distal part of the appendage is altered; the proximal regions of the third thoracic segment remain unaffected. In addition, there is a partial transformation of the first abdominal segment (A1) into thorax as indicated by the reduced number of bristles (WT = 116 ± 8, MX17 = 72 ± 15). These two phenotypic changes indicate a partial loss of *Ubx* function in adult cells.

In contrast, the embryonic phenotype of homo- or hemizygous *Ubx*^{MX17} embryos appears entirely normal; the ventral denticle belts of the third thoracic (T3) and the A1 segments, where the *Ubx* phenotype is more strongly expressed, and which in *Ubx*[−] embryos are like that of segment T2, retain the wild-type pattern. We also fail to find any sign of *Ubx* transformation (Hayes *et al.*, 1984) in the dorsal side of the same segments.

The second mutation, *Ubx*^{MX19}, was also X-ray induced. It is hemizygous viable but as it appeared in a lethal balancer (TM1) chromosome it cannot be studied in homozygous form in adult flies. Nevertheless, the phenotype of *Ubx*^{MX19}/*DfUbx* adults is very similar to that of *Ubx*^{MX17}/*DfUbx* and the *trans* heterozygous *Ubx*^{MX17}/*Ubx*^{MX19} flies are like homozygous *Ubx*^{MX17} ones.

The morphological alterations associated with these mutations indicate that they both produce a partial loss of *Ubx* function. We note that the adult phenotype of both *Ubx*^{MX17} and *Ubx*^{MX19} in the haltere appendage is very similar to that described (Casanova *et al.*, 1985a) for the mutation *Contrabithorax* (*Cbx*¹). The *Cbx*¹ chromosome has a breakpoint within the *Ubx* transcription unit (Bender *et al.*, 1983) which is probably responsible for the recessive phenotype (Casanova *et al.*, 1985a).

Fig. 1. (A) and (B). Dorsal view of the adult third thoracic segment in wild-type (A) and homozygous *Ubx*^{MX17} (B) flies. The haltere (H) appendage of the mutant is bigger and contains some bristles (arrow) like those normally seen in the wing. The wild-type haltere is devoid of bristles. (C) and (D). Peroxidase immunostaining of *Ubx* proteins in the haltere disc of wild-type (C) and *Ubx*^{MX17} (D) larvae. The monoclonal antibody Fp 3.38 which recognizes all *Ubx* protein products was used. In the wild-type disc there is an overall, uniform staining while in *Ubx*^{MX17} there is a reduction in the amount of antigen present in the central region of the disc (arrow). This region differentiates the portion of the adult appendage which is morphologically altered in B.

As it is in the haltere segment (T3) where the mutations show the transformation more clearly, we have studied the distribution of *Ubx* proteins in *Ubx*^{MX17} and *Ubx*^{MX19} haltere imaginal discs. The monoclonal Fp3.38 (White and Wilcox, 1984), able to recognize all *Ubx* protein products, was used. We find in both mutant discs a reduction in the amount of antigen in the centre of the disc (Figure 2D). This region corresponds in the fate map (Ouweneel and Van de Meer, 1973), to the distal part of the appendage, just where the mutant phenotype is observed. This finding suggests that the mutant phenotypes of *Ubx*^{MX17} and of *Ubx*^{MX19} are due to a local defect in the distribution of the protein in adult cells. Because of the similarities with the recessive phenotype of *Cbx*¹, we have also studied the *Ubx* protein distribution in *Cbx*¹/*DfUbx* haltere discs and found the same alteration as in *Ubx*^{MX17} and *Ubx*^{MX19}.

Molecular characterization of *Ubx*^{MX17} and *Ubx*^{MX19}

Preliminary characterization of *Ubx*^{MX17} DNA indicated a breakpoint in the region of -46 kb according to the map of Bender *et al.* (1983). For further studies we constructed a genomic library of the mutant DNA as well as one of the parental chromosome (called *mm*) on which the mutation was induced. Restriction maps of *Ubx* DNA from *Ubx*^{MX17} and *mm* were obtained (see details in Material and methods). Comparison of restriction maps and Southern hybridizations with probes spanning the -65 to -42 kb region (coordinates as in Bender *et al.*, 1983) revealed that in *Ubx*^{MX17} a fragment of ~18 kb, from -64 to -46 kb, is in reverse orientation (Figure 2). This alteration inverts the second microexon of the *Ubx* transcription unit, located at around -50 kb (Beachy *et al.*, 1985), as well as flanking intronic sequences, placing the codon sequence and splice sites of

this microexon on the non-transcribed strand. Therefore, *Ubx*^{MX17} mutants will not be able to produce any *Ubx* transcript containing microexon 2 and, consequently the class of *Ubx* proteins including the 17 amino acids encoded by this microexon will not be present in mutant flies. We have shown that this is the case by using a monoclonal antibody directed against this 17 amino acid sequence (generously provided by Javier López and David Hogness); the *Ubx*^{MX17} products are not recognized by this antibody while wild-type *Ubx* products are.

Of the two other mutations with similar recessive phenotypes, *Cbx*¹ and *Ubx*^{MX19}, the former has been previously characterized by Bender *et al.*, 1983 at the molecular level; it is caused by a 17 kb fragment of DNA of the *postbithorax* region inserted at position -44 kb, very close to one of the two breakpoints of *Ubx*^{MX17}. In the case of *Ubx*^{MX19}, we have been unable to find any alteration in the -60 to -40 kb DNA region by Southern blot analysis (see Figure 2).

Expression of *Ubx* proteins in *Ubx*^{MX17} and *Ubx*¹⁹⁵ embryos

The comparison of the protein expression in the two mutations is of interest because in *Ubx*¹⁹⁵ the nonsense codon in the second microexon (Wienzierl *et al.*, 1987) produces the inactivation and probably the elimination of the proteins encoded by RNA classes I and II, while those of class IV are normal. In *Ubx*^{MX17}, and because of the structure of transcription unit, the proteins encoded by RNA classes I and II are also lacking. Yet, the former is an embryonic lethal with a strong phenotype (Wienzierl *et al.*, 1987), whereas *Ubx*^{MX17} is viable and only shows a very weak adult phenotype. This suggests that in *Ubx*^{MX17} the

Fig. 2. Comparison of the restriction maps of the mutation *Ubx*^{MX17} and the parental *mm* chromosome in the region near the 5'-end of the gene. Coordinates as in Bender *et al.* (1983). *Eco*RI (E), *Hind*III (H) and *Bam*HI (B) restriction sites are indicated. The restriction map of *mm* is like that described for Canton S (Bender *et al.*, 1983) except for a *Hind*III site (asterisk in the figure). The positions of the 5' and 1 and 2 microexons are indicated in the bottom row, together with the localization of the *Ubx*¹⁹⁵ and *Cbx*¹ mutations.

proteins normally encoded by RNA classes I and II are replaced by a new form(s), probably the hypothetical class III (Kornfeld *et al.*, 1989, see Table I), or some other novel variant, that functionally substitutes for the missing forms.

This suggestion is supported by the comparison of the patterns of Fp3.38 staining of *Ubx*^{MX17} and *Ubx*¹⁹⁵ mutant embryos which are illustrated in Figure 3. In the *Ubx*^{MX17} homozygous stock all embryos show a distribution of *Ubx* antigen like the wild-type (White and Wilcox, 1985a) and, as far as it can be judged from the intensity of stain, they have a normal amount of *Ubx* protein. By contrast, in *Ubx*¹⁹⁵ the *Ubx* antigen is restricted to the nervous system; in all other tissues there is much less *Ubx* protein than in *Ubx*^{MX17} or the wild-type. Furthermore, the little amount of antigen found in these tissues does not appear confined to nuclei, suggesting that although a truncated version of *Ubx* protein is synthesized, it cannot reach the nucleus and is subsequently degraded.

These results clearly indicate the lethality of *Ubx*¹⁹⁵ is due to a gross reduction in the amount of *Ubx* product in most tissues, whereas in *Ubx*^{MX17} the absence of classes I and II proteins is compensated by other *Ubx* protein forms.

Discussion

Our results bear on the problem of the functional significance of the different *Ubx* protein forms generated by alternative splicing. The functional products of the *Ubx* gene are encoded by the homeobox containing transcription unit, which in addition to the two exons located at the 5' and 3' ends, contains two 51 bp microexons. In the wild-type (O'Connor *et al.*, 1988; Kornfeld *et al.*, 1989) three classes of mature transcripts and hence of proteins are generated (Table I): class I utilizes the two microexons, class II uses only the second microexon, while transcripts encoding class IV include none. Classes I and II are further subdivided into *a* and *b* forms according to which of two splice donor sites of the 5' exon are used (Beachy *et al.*, 1985; Hogness *et al.*, 1985). An hypothetical class III, containing only amino acids

Table I. Normal forms of *Ubx* mRNAs in wild-type animals and possible forms in *Ubx*^{MX17} assuming that no new splice sites are used in the mutants

Exon composition	mRNA classes	Wild-type	MX17
5'123'	I,a,b	Present (86%)	Absent
5' 23'	II,a,b	Present (11%)	Absent
5'1 3'	III	Absent	Present?
5' 3'	IV	Present (3%)	Present

Abundance of different mRNA classes in the wild-type, as in Kornfeld *et al.* (1989) is shown in parenthesis. In *Ubx*^{MX17} class I is probably transformed into class III, and class II into class IV, which is increased with respect to wild-type. Classes I and II appear in *a* or *b* forms according to the usage of splice donor sites in the 5' exon (Beachy *et al.*, 1985).

encoded in the first microexon, does not occur in normal flies (Kornfeld *et al.*, 1989).

Our results show that splicing in *Ubx*^{MX17} mutant is very different from the wild-type. Although the overall amount of *Ubx* protein appears to be normal, classes I and II that normally make >90% of the total are lacking. If no new splice sites are used in *Ubx*^{MX17}, then only protein class IV and maybe III can be generated (Table I). In any case the array of spliced forms of *Ubx*^{MX17} proteins must be very different from the normal one, as illustrated by the fact that an antibody directed against the 17 amino acid sequence encoded by microexon 2 does not recognize any *Ubx* protein in *Ubx*^{MX17} embryos.

Which are the developmental consequences of this dramatic alteration in *Ubx* protein composition? Our results argue that these are minor or non-existent. This is supported by the following arguments. The first is that the larval epidermal pattern of *Ubx*^{MX17} is like the wild-type, indicating that *Ubx*^{MX17} proteins (probably III and IV variants, see Table I) successfully substitute for the normal ones in establishing embryonic *Ubx* function. It is noteworthy that the embryonic epidermis contains in wild-type embryos the *Ubx* forms which must be missing in *Ubx*^{MX17}

Fig. 3. Pattern of expression of Ubx proteins in (a) *Ubx*^{MX17} and (b) *Ubx*¹⁹⁵ homozygous mutant embryos of stage 13. In (a) the distribution of Ubx proteins is indistinguishable from the wild-type. In (b) Ubx proteins are essentially restricted to the nervous system, although some faint stain can also be observed in the epidermis.

(O'Connor et al., 1988; Kornfeld et al., 1989). In support of the same view, Mann and Hogness (1990) have shown that Ubx proteins encoded by both Ubx RNA classes I and IV can determine similar larval epidermal patterns.

Our second argument is based on the comparison of the adult phenotype of *Ubx*^{MX17} with those of *Ubx*^{MX19} and *Chx*¹. At a first glance, the partial adult transformations may indicate that *Ubx*^{MX17} proteins do not provide an entirely normal Ubx function. However, our finding that the amount of Ubx protein is reduced in the central portion of the disc, precisely where the mutant phenotype is observed, suggests that this is caused by a defect on the normal distribution of the protein. This would not be expected if the adult phenotype was due to the abnormal functioning of *Ubx*^{MX17} proteins. Furthermore, we note the same phenotype and the same alteration on the distribution of Ubx proteins in the haltere discs of *Ubx*^{MX19} and *Chx*¹ and in neither of these mutants alterations in the DNA including the second microexon have been detected (Bender et al., 1983; our own results).

In our opinion the adult phenotype of *Ubx*^{MX17} is most probably caused by the breakpoint in the -(44-46) kb region, where *Chx*¹ also has a break. These breaks may disrupt an intronic region necessary to ensure normal distribution of Ubx products along the proximo-distal axis of the haltere imaginal disc. In Ubx it is known that other intronic regions play important regulatory roles (Casanova et al., 1985b; Peifer and Bender, 1986) and affect the distribution of Ubx protein in the haltere imaginal disc (White and Wilcox, 1985b; Cabrera et al., 1985).

It has been suggested (Mann and Hogness, 1990) that some Ubx protein variants may have a specific role in the peripheral nervous system. We have not examined the internal tissues (mesoderm, nervous system) of *Ubx*^{MX17} adult flies, but the fact that the mutation is homozygous viable and fertile makes it unlikely that these tissues are grossly altered.

Our results indicate the functions of Ubx protein forms I and II, the most abundant of Ubx proteins (O'Connor et al., 1988; Kornfeld et al., 1989), can be performed by other variants, very likely types III and IV. This implies that all Ubx proteins are functionally equivalent and that the presence or absence of the second microexon has little effect on their developmental properties. Thus, in the case of Ubx we argue that protein diversity achieved through alternative splicing is of little importance, although there are well documented examples, like the establishment of male or female developmental pathways (Nagashi and Baker, 1990) in which it plays a critical role. It is possible that the different Ubx protein forms generated in wild-type animals arise from differences in the splicing machinery of different tissues which tend to favour the appearance of specific protein products.

Materials and methods

Fly mutants and stocks

The mutations *Ubx*^{MX17} and *Ubx*^{MX19} were isolated in a F2 mutagenesis experiment irradiating (~3500 rads) males of genotype *mm/TM1*. *mm* is an abbreviation of a *mwh jv st red sbd² e ca* chromosome that we routinely use for mutagenesis experiments (Sánchez-Herrero et al., 1985). For genetic analysis we used a number of stocks with deletions or mutations of the BX-C; *Df(3R)P9* is a deletion of the whole BX-C used as a chromosome totally lacking Ubx activity. The chromosomes *Ubx*¹⁹⁵ and *Chx*¹ have been previously described (Wienzierl et al., 1987; Casanova et al., 1985a).

Characterization of the mutant DNAs

In the case of *Ubx*^{MX17} preliminary characterization of mutant DNA by Southern blots using lambda phages covering the whole of the Ubx gene as probes indicated abnormal restriction fragments when phages λ 2261 and λ 2229 (Bender et al., 1983) were used. Further Southern blot analysis using several plasmids covering the region gave abnormal restriction patterns with p3130 and p3118 indicating an alteration at around -46 kb.

For a more precise characterization, genomic libraries of *Ubx*^{MX17} and the parental *mm* chromosome were constructed in EMBL 3 vector (Kaiser and Murray, 1986). Both libraries were screened using plasmid p3130 as probe. After three rounds of selection four *mm* and two *Ubx*^{MX17} phages were isolated. Their restriction maps were constructed by single and double digestions with *Eco*RI (E), *Hind*III (H) and *Bam*HI (B). The four *mm* phages turned out to be identical and covered the -(49-35) kb region of the gene. The restriction map is like that described by Bender et al. (1985) for Canton S, except for the presence of a *Hind*III site around -46 kb.

Of the two *Ubx*^{MX17} phages, one of them, MX17III, covers the -(43-28) kb region and its pattern of restriction fragments is like that of Canton S in the same region. The restriction map of the other *Ubx*^{MX17} phage, λ MX17I, was found to be different from *mm*. The approximate length of the insert was 18 kb. To determine if this DNA came from other regions of Ubx, we transferred by dot blot 1 μ g of DNA of several plasmids covering the whole Ubx transcription unit and hybridized them with nick-translated phage DNA. Only plasmids p3124, p3142 and p3130 gave positive hybridization.

We carried out Southern analysis of digestions of λ MX17I using DNA from plasmids p3124, p3142 and p3130 as probes. In the -(41-46) kb region we find no difference with the *mm* map. From around -46 there is an *Eco*RI fragment which hybridizes with both p3130 and p3124 probes. The region which hybridizes with p3124 was determined to correspond to the -(62.5-64) kb segment and is in reverse orientation with respect to *mm*. The next segment only hybridizes with p3124 and its restriction map corresponds to this plasmid in reverse orientation. These results together with the previous Southern analysis of *Ubx*^{MX17} DNA using the same plasmids as probes, establish the existence of an inversion with breakpoints around -46 and -64.

The DNA of *Ubx*^{MX19} was assayed by Southern blot analysis using the λ phages covering the whole of Ubx DNA. The pattern of restriction fragments found was like that of the parental *TM1* chromosome. The mutant DNA was also assayed with plasmids p3124, p3142 and p3130 covering the -(40-60) kb region. Again no difference was found with respect to the wild-type.

Antibody staining

The Fp3.38 anti-Ubx antibody (White and Wilcox, 1984) was used following standard procedures for peroxidase stain (ABC kit, Vector Lab) for

both embryos and imaginal discs. The monoclonal 5C2A, that recognizes an epitope in the second microexon, was used following the instructions of Javier López (in preparation).

Larval and adult cuticle preparations

For the larval cuticle we used the standard method of Van der Meer (1977). For mounting adult cuticle, pertinent pieces were cut, digested with hot KOH, washed in propanol and mounted in Euparal for light microscope examination.

Acknowledgements

We thank Javier López and David Hogness for their generous gift before publication of a sample of their microexon 2 specific antibody. We also thank Michael Akam, Ernesto Sánchez-Herrero and Miguel A. Penalva for illuminating discussions and comments on the manuscript, and to Dr Roberto Marco for advice and encouragement. The financial support of the Fundación Ramón Areces and the Dirección General de Investigación Científica y Técnica is also acknowledged.

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Received on June 6, 1990; revised on July 18, 1990