The *Drosophila abrupt* gene encodes a BTB–zinc finger regulatory protein that controls the specificity of neuromuscular connections

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Motor axons make synaptic connections with specific muscles, and this specificity unfolds during development so that neuromuscular growth cones make specific pathway choices and ultimately recognize and synapse on their specific muscle targets. The *Drosophila* *clueless* mutation was identified previously in a genetic screen for mutations that disrupt neuromuscular guidance and connectivity. We show here that *clueless* is allelic to *abrupt*. The *abrupt* gene is required for the embryonic formation of specific synaptic connections between motor neurons and a subset of muscles. Mutations in abrupt also reveal its role in establishing and maintaining muscle attachments, sensory cell formation, and morphogenesis of adult appendages. The abrupt gene encodes a zinc finger protein with a conserved BTB domain. *Abrupt* is expressed in muscle nuclei but not motor neurons, suggesting that abrupt controls the expression of molecules required for correct motor neuron targeting, as well as molecules required for correct muscle attachments.

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Coordinated movement requires that muscles be properly innervated by the appropriate motor neurons. The specificity of neuromuscular connections unfolds during embryonic development as motor neuron growth cones extend into peripheral tissues, make a series of pathway choices that lead them into the correct region of mesoderm, and ultimately recognize and synapse onto their appropriate muscle targets. The *Drosophila* embryo provides a simple and convenient system for genetically dissecting the molecular mechanisms that control neuromuscular connectivity. In each A2–A7 abdominal hemisegment of a *Drosophila* embryo, ~50 motor neurons (H. Sink and R. Fetter, pers. commun.) innervate 30 individually identified muscles in a highly specific and stereotypic manner (Johansen et al. 1989a,b, Weisje et al. 1991; Sink and Whitington 1991). Evidence for specificity among connections is further revealed by experimental manipulations in which the number or positions of muscles are altered (for review, see Keshishian et al. 1993). These observations suggest a model in which specific synaptic connections between motor neurons and muscles in *Drosophila* are determined by surface and/or secreted recognition molecules expressed by muscles.

Muscle development occurs in a series of events (Jain 1995; Whitington 1993). Mesoderm-derived myoblasts predrake during embryonic stage 14, giving rise to muscle pioneers (founder cells), which prefigure the position and patterning of somatic muscles. Additional myoblasts fuse with the founder cells to form a muscle syncytium. Muscle fibers extend toward and attach to the underlying epidermis at stage 16. The muscles are innervated by motor neurons during stage 16. Each of the 30 individual muscle fibers in each abdominal segment (A2–A7) has a unique position, morphology, pair of attachment sites, and motor neuron innervation.

During development, the embryonic muscles are innervated by motor neurons that exit the central nervous system (CNS) in two main pathways: the segmental nerve (SN) and the intersegmental nerve (ISN) (Johansen et al. 1989a, 1989b; Sink and Whitington 1991). The ISN represents a single major nerve bristle, whereas the SN splits into four branches: SNa, SNb, SNc, and SNd. The SNa innervates dorsal muscles, SNb projects to lateral muscles, SNc innervates ventral lateral muscles, and SNc and SNd project to the most ventral muscles. Of particu-
ular interest are four motoneuron RPs: R1P, R3P, R5P, and R8P. Their cell bodies lie adjacent to each other near the midline of the CNS. Their axons fasciculate together, extend across the midline, and exit the contralateral side of the CNS. They extend out via the SNB and then leave the SNB in the SNC, where they enter and explore the ventral muscle region. The growth cones of these 

motoneurons form a stereotypical, three-branched terminal 

arc as they make specific synaptic connections onto 

ventral muscles 7 and 6 [29], 13 [R1P, R6P], and 12 

[R1P]. Other SNB motoneurons innervate these same 

muscles.

We would expect that the ability of individual mo-

toneurons to recognize specific muscles requires the in-

teractions between receptor molecules on the motoneu-

ron growth cones with recognition molecules either se-

creted or localized on the surface of muscle targets. The 

identity of the regulatory, signaling receptor, and down-

stream molecules that control this specificity of connec-

tions is largely unknown. However, a number of surface 

and secreted molecules have recently been implicated in 

playing a role in these events in Drosophila, including 

Consectin (Nose et al. 1994), fasciclin III (Chiba et al. 

1995), and Sephapordin I [Marthes et al. 1995].

Regulation of the identity of certain muscles in Dros-

ophila is under the control of the homoe box-containing 

proteins S59 and ateron (Dohrmann et al. 1990; Bour-

gnoult et al. 1992). It is not known whether these types of 

muscle identity genes on their own control the expres-

sion of specific surface and/or secreted muscle recogni-

tion molecules or, alternatively, whether other regula-

tory genes are expressed lower in the hierarchy to con-

trol the expression of these recognition molecules. In 

this paper we present evidence showing that a more 

ubiquitously expressed muscle regulatory gene, abrupt 

(ab) may be required for the expression of the surface 

and/or secreted muscle identities that allow the SNC 

motoneurons to recognize their specific ventral muscle 

targets.

Identification of molecules that mediate neuromuscu-

lar connectivity has been achieved by two main ap-

proaches. The first approach involves identifying genes 

by their patterns of expression, using either monoclonal 

antibody screens (e.g., Fasciclin III, Patel et al. 1987; 

Sephapordin I, Kolodkin et al. 1992) or enhancer trap 

screens (e.g., Connectin, Nese et al. 1992). Although 

none of these three genes have striking loss-of-function 

mutant phenotypes to this system, all three display 

striking neuromuscular phenotypes in gain-of-function 

conditions when the genes are ectopically expressed 

(Nose et al. 1992, 1994, Chiba et al. 1995; Marthes et al. 

1995). The second approach involves identifying genes 

by their mutant phenotypes (Vann et al. 1993). The 

initial mutant screen focused on the second chromo-

some and identified three genes affecting axon guidance 

[beaten path, short stop, and stranded] and two genes 

affecting neuromuscular connectivity [clueless (chla) and 

walkabout (wabg)].

The chla and wabg genes show similar mutant pheno-

types that affect only a subset of motoneuron connec-

tions: The SNB motoneurons fail to form their normal 

innervation on the ventral longitudinal muscles. In chla 

mutant embryos, the SNBs make correct pathway 

choices and reach the ventral muscle field. However, it 

fails to establish its typical three branch terminal arbor 

morphology, and instead, the growth cones wander and 

terminate at ectopic locations. Target muscle recogni-

tion of neighboring target branches are unaffected in 

chla 

mutant embryos, as both the SNB and SNAs form appro-

priate synapses. Thus, chla is not required for synapo-

sogenesis in general but rather appears to control the abil-

ity of a group of motoneurons to identify their specific 
targets from within a group of neighboring muscles.

In this paper we show that chla is allelic to the 

drosophila ab gene and that null mutations of ab 

display a motoneuron targeting defect similar to that 
described previously for the erythromycin-resistant 

(dm) induced chla mutations. We also describe additional 

defects in embryonic muscle attachment and in 

postembryonic morphology. Molecular analysis of the ab gene indicates that it encodes a zinc finger (ZF) transcription factor con- 

taining a conserved BTB domain. ab is expressed in the nuclei of muscles but not notomeurons, suggesting that 

ab controls muscle-specific gene expression required for 

target recognition by appropriate motoneurons.

Results

Two enhancer trap insertions identified the ab gene

Enhancer trap lines representing 1500 distinct P[acman] 

insertions were screened for expression of a galactosini-

dase in the embryonic CNS midline cells [Crews et al. 

1992]. The AB97 line showed strong embryonic CNS 

midline staining as well as epidermal and imaginal disc 

expression. The insertion was mapped by polytene 

diagram in situ hybridization to chromosomal interval 

33E2-3. The laboratories of Jose Campos-Craig (Univer-

sity of Cologne, Germany) and Elisabeth Kust (also 

identified an enhancer trap insertion [AB94] as 32E 

that revealed a similar pattern of expression and this was likely to be an insertion in the same gene.

The ab gene discovered by Bridges and colleagues 

around 1925 was located at 32E. Mutations in the AB97 

alleles are characterized by the failure of longitudinal 

wing vein L5 to extend to the wing margin [Fig. 1A, B, 

Lindley and Zimmer 1991]. The BR79 line is hyperfiliated 

and has a wild-type wing venation pattern. However, 

the homoe box 34F line reveals an L5 wing venation 

defect similar to ab [Fig. 1C]. The BR79 insertion lies in 

completion ab, indicating that the 34F line has an ab muta- 

tion (Fig. 1D). When the P element is excited by the 

introduction of transposase, the 34F wing defect reverts 

to wild type in multiple lines, indicating that the P ele-

ment insertion is the cause of the wing defect. These 

results suggested that the 924 insertion disrupts the ab 

gene and that the BR79 insertion may be adjacent to the 

ab gene.

chla is allelic to ab

The original chla mutation (chla) originated from an EMS

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screen for mutations that disrupt motor neuron guidance and connectivity [Van Vactor et al. 1993]. The clu1 mutation shows normal motor axon pathfinding but defective connectivity of the SNb motor neurons with their synaptic muscle targets. Genetic mapping placed the clu1 mutation in the 32E region. The clu1 allele fails to complement abh and shows the L5 wing venation defect (Fig. 1E). This indicates that clu1 is allelic to abh (clu1 is redesignated abh1). Two additional EMS alleles (abh2 and abh3) were identified in a screen for noncomplementation of clu1 lethality, and several克斯-induced alleles were identified in a screen for noncomplementation of the ab adult phenotype. The heteroallelic combinations of these new alleles indicate that abh is a lethal locus.

abh null mutants show motor neuron connectivity defects

Further genetic analysis of abh required the generation of abh null mutants. No preexisting deficiencies uncovered the 32E region, so we utilized both P-element transposase-induced excisions in P-element hopping and γ-ray mutagenesis to create chromosomal deletions that remove abh. Both abhD and abhE were found to be amorphic on the basis of genetic and molecular analysis (see below, Fig. 3). It is likely that abhD represents a null mutation, specific for abh, whereas abhE may uncover additional, unidentified genes.

Previous work on abh [Van Vactor et al. 1993] revealed a specific motor neuron connectivity defect in which SNb motor neurons fail to make proper synaptic connections with their ventral longitudinal muscle targets. Utilizing the new abh alleles, the abh connectivity defect was further investigated using monoclonal antibody (mAb-1D4) that stains all motor axons and terminal arbor, and mAb 2D5 that recognizes Fasciculus III and stains the RP neurons. The abhD and abhE null alleles and the three abh EMS alleles were analyzed as homozygotes and as heterozygotes over abhC. Staining with mAb 2D5 indicates that the RP neuron form and are located at their normal positions within the CNS. In addition, staining with mAb 1D4 shows that these axons make the appropriate pathway choices to reach the ventral muscle field. However, in abh mutant embryos, the SNb axon pause at the proximal edge of muscle 13 and form abnormal branches instead of forming their wild-type axonal extensions onto the muscle fibers. These aberrant branches wander over the prospective target muscles and occasionally form connections at ectopic sites, such as at or near muscle 8. At embryonic stage 17, abh mutants do not establish the normal three-branched terminal arbor of SNb axons on muscles 7, 6, 13, and 12 (Fig. 2A–C). The transverse nerve is often incomplete and invades the ventral muscle field. Ectopic endings from the transverse nerve are seen in other muscles that disrupt SNb innervation of the ventral muscles [I.A. Fambrough, D. Van Vactor, H. Sink, C. Kopczynski, and C.S. Goodman, unpubl.]. The transverse defects in clu mutants are limited to the ventral region of the embryo, the connectivity of the motor axons in the ESN and SNa is normal. The SNc and,
rarely, SNb show some morphological abnormality, but the small size and deep ventral position of these nerve branches make their connectivity more difficult to assess. All alleles and allelic combinations described here show similar defects to each other and to those observed previously for ab/h/2. However, weak adult viable alleles, such as ab/a, do not show motoneuron connectivity defects.

ab null mutants show muscle attachment defects

Analysis of the mature embryonic muscle pattern in ab mutants reveals that most muscles appear normal in their position, number, morphology, and expression. Muscle Myosin, which is expressed in all muscles, and Fascin 3 and Connectin, which are expressed on the surface of subsets of muscles.

However, a few muscles show variable penetrance defects in their attachment in the epidermis. Some of the lateral longitudinal muscles (3, 5, 11, and 20) also show variable penetrance defects in the locations of their muscle attachments (Fig. 2A). It should be noted that the muscles in which we observe the connectivity defect (7, 6, 13, and 12) are distinct from the muscles in which we observe the attachment site defect (3, 5, 11, and 20).

In addition, several muscles that initially attach at the proper locations by stage 16, at later stages (after innervation and muscle contraction) begin to display spheroid phenotypes in which the muscles pull out of their insertion sites and rounded up into spheres. This is seen most commonly with muscles 7 and 12, with a lesser extent, with muscle 2, although in both cases it is highly variable (Fig. 2F). This phenotype could be owing to either a muscle or a perineural defect that impinges on the strength and quality of the muscle insertions. The defect is observed most strongly in ab alleles (ab/2, ab/a, ab/4, and ab/12), although expressivity is low with only a few hemisegments in an affected embryo. Weak alleles such as ab/a do not show either muscle phenotype. Interestingly, the ab/12 mutation does not show the spheroid muscle defect, ab/a shows does show the incorrect muscle attachment site defect, although at a weaker penetrance than in the strong allele. The absence of the spheroid phenotype in ab/12 may be attributable to the nature of the molecular lesion in this allele, which encodes in a truncated protein lacking the ZF DNA-binding domain (see below).

Adult phenotypes of ab mutants

Examination of viable (weak) ab alleles and viable combinations of lethal (strong) ab alleles over viable alleles
reveals that ab function is required for the development of numerous adult structures. For example, ab1 and P(194) are recessive viable mutations that show wing venation and macrochaetae (bristle) defects. More severe defects can be seen in combinations of other alleles. Combining the two pupal lethal mutations, ab(m1) [a P-element insertion derivative of P(199)] and ab(z8) [t-g allele from P(150)], results in adult escapers that display a broad range of defects. Similar defects are also observed in combinations of a viable allele (ab6, P(194)) with a lethal allele (ab12, ab10), and ab10/ab12.

Wing venation and bristle defects in ab(m1) ab(z8) and ab10/ab12 adult flies show more severe defects than those in ab1 and P(194). The wing veins are completely absent between the margin and posterior cross vein (PCV) (Fig. 1F), in contrast to ab1 and P(194) homoeogotes and heterozygotes, which are missing only a smaller section of L5 (Fig. 1B-D). There is also a more severe loss of the thoracic and wing homoeochromatoid bristles in the transheterozygote combinations. Two posterior scutellar bristles and two upper humeral bristles are invariably missing, and the super-alateral bristles are usually fewer in number than wild-type. Occasionally, one of the campaniform sense organs on wing vein L3 is absent.

In addition, legs of ab(m1) ab(z8) flies are severely gnarled, and the more distal segments are shortened in length or absent. The antennal arista are also deformed, and there is a furrow at the midline of the dorsal thorax with polarity altered of the remaining bristles. Males of ab(m1) ab(z8) and ab10/ab12 genotypes are sterile, and their external genitalia are rotated 180° relative to wild type. Many of these additional phenotypes are described for the ab6 allele [Lindsley and Zimm 1992]. This broad range of phenotypes indicates that ab is involved in multiple developmental processes.

The fact that ab1 (ab10) displays stronger and additional defects as compared with ab1 suggests that ab function is dosage dependent. Both penetrance and expressivity of wing venation and bristle defects correlate with the following alliastic strength: ab1 (ab1 = P(194)/P(194); ab1/ab1 (ab1/ab1) ab1/ab1 > ab1/ab1 > ab1/ab1. Less than 20% of homozygotes (ab1) and ab1/ab1 flies exhibit phenotypes, and the phenotypes are weak. Fifty percent of ab1/ab1 flies display an intermediate phenotype. One hundred percent of ab1/ab1/ab1 flies have severe defects. In addition, ab1/ab1/ab1 displays leg and antennal defects that are not observed in ab1 alone. One simple explanation is that ab functions in a concentration-dependent manner, with each process requiring a threshold level of ab expression.

Molecular cloning of the ab locus

Molecular cloning of the ab gene was initiated using inverse PCR to clone the genomic DNA adjacent to the P(199) insertion. Genomic DNA representing 33-kb was isolated by screening a wild-type genomic clone library followed by chromosomal walking (Fig. 3). Restriction fragments covering the entire DNA interval were hybridized in situ to whole-mount embryos to identify the ab transcription unit. The presence of a transcription unit was found adjacent to the P(199) insertion (probe d) and extends to the end of the walk (probes c-k, >21 kb).

Figure 3. Structure of the ab gene. The extent of the ab gene chromosomal walking is shown in a 33-kb DNA interval. The scale is marked with 1-kb intervals, and the orientation indicated "0" denotes the 5' side of the longest CDS. Numbers increase in the 5' to 3' direction with respect to ab DNA sequences. Below the scale is the restriction enzyme map for the enzymes: EcoRI (E), XbaI (X), and Bln (B). The positions of the MAX and N2 P-element insertions are indicated by the vertical lines extending from the inverted triangles, and the position of lacZ transcription is indicated by arrows. Fragments used for whole-mount experiments in in situ hybridization experiments are listed in alphabetical order below the restriction map. The presence (+) or absence (-) of hybridization is shown. Probes a-c failed to detect embryonic transcription, whereas probes d-k revealed the same embryonic expression pattern. Probe b was particularly intense (+ +) and was used to isolate ab DNA clones. The extent of the puc clones is illustrated. (O) Translated regions, (g) coding sequence. The I sequences are located outside the cloned genome region, and thus, the introduction structure of the gene is incomplete. Genomic clones ab-1, ab-2, and ab-3, which define the walk, are shown. The location and extent of two deletion mutants were mapped by single-embyro PCR and Southern blot analysis. The locations of the primers used for PCR are shown at bottom. The ab12 m position of P(194) contains an 8.7-kb deletion removing exon 1. The P-element has reassorted in the opposite orientation to P(194). The ab12 m strain is absent for all primer pairs except pair 5. This indicates that the rightward extent of the deficiency lies within the ab transcription unit (broken line between vertical lines), but the leftward extent of the deletion is unknown (broken line). Thus, additional genes may be absent in ab12 m.
The embryonic expression pattern of this transcript is identical to the lacZ expression pattern from the BLR7 and 94 enhancer trap lines. Fragments upstream of the P[BD] insertion failed to detect embryonic transcripts by whole-mount in situ hybridization or Northern analyses. Thus, no other detectable embryonic transcript lies within the 12 kb of the identified transcription unit. Sequences of maternal alleles (see below) show that this is the correct transcript.

Using a probe derived from fragment k that gave the strongest signal in the hybridization, multiple cDNA clones were isolated. Northern analysis with a fragment common to all cDNA clones revealed a single 5.1-kb transcript. The longest cDNA clone we sequenced is 5 kb and thus near full length. Sequence and Southern blot hybridization analyses of both cDNA and genomic clone DNA placed the 5' end of the longest cDNA clone at 4 kb downstream from the P[BD] insertion site and 3.2 kb away from the P[BL7] insertion site. Genomic DNA containing the 3' end of the ab gene was not cloned.

**Temporal and spatial expression of ab**

We determined the pattern of ab expression using three different methods, all of which yielded similar results: in situ hybridization with ab cDNA probes, staining with a rat polyclonal Ab antiserum, and anti-β-galactosidase staining of the ab enhancer trap lines. The specificity of the polyclonal Ab antiserum was confirmed by absence of staining in ab+null mutant embryos (Fig. 4F). In all tissues in which it is expressed, the Ab antiserum indicates that the Ab protein is expressed in the nucleus.

**Nervous system expression**

Initial ab expression is within the CNS midline cells (Fig. 4A-C). Beginning at stage 9, uniform CNS midline precursor staining is observed and expression continues in these cells through stage 13. Stained cells include the precursors to the VUM cells, some of which contribute axons to the SNs. Importantly, the CNS midline cells are the only embryonic CNS cells to express ab. Neither the lateral neuroectodermal precursor cells nor mature lateral CNS neurons express ab. In particular, the RP3, RP4, RP4', and RPS motorneurons, whose muscle connectivity is affected in ab mutant embryos, do not exhibit detectable levels of ab as assessed by in situ hybridization, lacZ, and anti-Ab staining (Fig. 4C). Additional nervous system expression is observed in the precursors and mature cells that constitute the stomatogastric nervous system.

**Epididymal expression**

Segmentally repeated stripes of ectodermal expression appear at stage 11 (Fig. 4A), and the expression becomes uniform throughout the entire embryo by stage 12 (Fig. 4E) with the exception of the tracheal cells where it is absent. Strong epidermal expression continues throughout embryogenesis and is uniform except for stronger expression in the specialized segmental border cells.

**Figure 4. Embryonic and imaginal expression of ab.** (A) Ventral view of stage 11 embryo stained with Ab antiserum showing strong CNS midline precursor staining (arrow) and segmentally repeated cuticular stripes (arrowheads). Anterior is at left in this and subsequent panels. (B) Sagittal view of stage 11 embryo stained with Ab antiserum indicating CNS midline precursor [center] and stomatogastric nervous system precursor cells (arrowheads) expression. (C) High-magnification view of the CNS of a stage 14 dissected embryo showing CNS midline expression of Ab and absence of Ab expression in lateral CNS. The BLR2 line was stained with Ab-β-galactosidase, identical results were obtained by staining with the Ab antiserum and in situ hybridization with an ab cDNA probe. These are three gastrula, and the brackets indicate the lateral borders of the CNS. (D) Nuclear Ab expression in segmental muscle fibers. Prominently shown are the ventral muscles in a unfilled stage 17 embryo. The epidermal staining in the background obscures the muscle staining. However, it is apparent that all muscles express Ab including muscles 7, 6, 13, and 12 that show connectivity defects in ab mutants. Arrows indicate nuclear staining within muscles 7 and 6, muscles 13 and 12 are not in focus in this photograph. (E) Uniform embryonic expression shown in a sagittal view of a stage 13 embryo stained with Ab antibody. (F) Absence of Ab antibody expression in homozygous ab117 mutant stage 13 embryo (G) X-gal staining of eye-antennal disc from the BLR8 line. Expression is widely distributed throughout the disc. (H) The wing-mirioptum disc of BL7 shows widespread expression of lacZ as assayed by X-gal staining. Scale bar, 70 μm (A, B, E, F) 25 μm (C), 30 μm (D), and 75 μm (G, H).

**Muscle expression**

During myoblast fusion and syncytial muscle formation at stage 14, ab mRNA can be detected in the somatic muscle cells. By stage 16, all 30 abdominal muscles ex-
press Ab, and staining with the Ab antisera indicates that all muscle nuclei express Ab [Fig. 4D]. Although quantitative analysis is complicated by the adjacent epidermal staining, it is apparent that the levels of Ab in individual muscles varies. The highest concentrations are seen in the ventral longitudinal [7, 6, 13, and 12], ventral oblique [15, 16, and 17], and segmental border [8] muscles. Interestingly, the ventral longitudinal muscles are the ones that display the connectivity defects observed in ab mutants.

Postembryonic expression

Ab is expressed in all of the imaginal discs, a result consistent with the panoply of adult defects observed in ab mutations. The expression in each disc is generally strong and dispersed as shown in the eye-antennal disc [Fig. 4G] and thoracic wing-mesonotum disc [Fig. 4H]. Expression in the thoracic disc resides in regions that give rise to the scutellum and a broad section of the wing including the vein area, cell types that show defects in ab mutants.

ab encodes a BTB-ZF protein

The ab mRNA sequence was determined by complete sequencing of a 4.5 kb cDNA clone, pcalb3 (Fig. 5). The longest open reading frame predicts a protein of 904 amino acids. The sequence surrounding the ATG codon, TTAATCTGG, perfectly matches the consensus translation start sequence [G/AA/AG/ATGC]. A second in-frame ATG, located at nucleotide 751, has a perfect match [CCAAAATATG] with the consensus and could serve as an alternative start codon. Sequence analysis of different cDNA clones reveals the presence of two alternatively

Figure 5. cDNA sequence and predicted amino acid sequence of Ab. Shown is the complete nucleotide sequence of the 4.5-kb pcalb3 cDNA clone. An open reading frame of 904 amino acids is shown using the one letter code. The BTB domain is boxed (amino acid residues 76–109), and the two putative Cys2-His2 ZFs are also boxed (amino acid residues 543–600). The potential poly(A) site in the untranslated region is underlined. The tegumented 1241–1310 (underlined) are absent in several other cDNA clones and probably represent an alternatively spliced form of abmRNA. The 10-amino-acid protein conserved prominently features these Am-Po repeats. Because sequence analysis is multiple cDNA clones and amplified PCR products provide multiple examples of each mRNA form, the difference is unlikely to be a cloning artifact. Two other cDNA clones partially sequenced were longer than pcalb3 with the additional sequence attributable to longer 5' untranslated regions.
spliced forms of ab mRNA. An alternative splice is used in the first exon such that different mRNAs start at the same nucleotide but are spliced either 45 bases or 31 bases downstream. In addition, embryonic mRNAs differ in the presence or absence of a 30 base insertion within the coding sequence from nucleotide position 1181 to 1210. Interestingly, this region encodes a repeated asparagine-proline motif.

The predicted Ab protein sequence behaves to the recently described family of BTB-ZF proteins (Harrison and Travass 1990, Godt et al. 1993). The Ab protein possesses two Cys-His, ZFs and a BTB domain (Fig. 5 and 6). ZFs are usually DNA-binding domains associated with transcriptional regulatory proteins, and the BTB domain is a highly conserved 215-amino-acid region, shared most often with ZF transcription factors. Staining with an Ab antiserum revealed nuclear localization, consistent with a role as a transcription factor (Fig. 4D).

According to the degree of similarity between members of the BTB family, they can be subdivided into two groups: a high identity group, which includes Ab, and a low identity group (Fig. 7). Zollman et al. (1994). The high-identity group is composed of more than a dozen Drosophila genes including Broad-Complex (BR-C), tsk, bric-a-brac (bbr), longitudinals lacking (lola), and arthrocon-servative (arc). The average identity within the high-identity group is 56%. Other BTB domains are more distantly related, average identity between the two groups and among other BTB domains is 24-30%. Drosophila kelch, human LAZ3, murine ZES, and a number of putative genes fall into this group. Within the BTB domain family, Ab falls into the high-identity group and is a BTB domain in most closely related to BR-C, with 59% identity: Ab is 54% identical with Bab, 48% with Tsk, 50% with Caga, and 47% with Lola. With lower average identity, Ab is related to ZFS (31%), Kelch (28%), and LAZ3 (27%).

Molecular analysis of ab mutations

We used Southern blot analysis and PCR to map deficiency breakpoints in the genomic region of the Ab gene. The PCR approach involved creating primer pairs from sequenced regions of genomic DNA and cDNA clones and performing PCR on individual homozygous mutant embryos (Fig. 9). Analysis of two mutants (ab107 and ab107) indicated that they were deficient for portions of the ab gene (Fig. 8). All of the PCR primer pairs except pair 1 failed to amplify DNA from ab107, suggesting that ab107 is a deletion of at least 26 kb of DNA. Because the deletion removes colinear sequences and the probable start site of transcription, ab107 is predicted to be genetically null for ab. However, the upstream breakpoint remains unknown, and it is possible that ab107 removes additional genes on chromosome 2.

In contrast, ab101 is likely to be a null, single gene ab mutation. This mutation was generated by transposase-induced P-element excision. Analysis of ab101 indicates that the original P-element has reinserted at the same site in an inverted orientation and that an adjacent 0.7 kb deletion was generated. This deletion begins at the P-insertion site and extends 0.5 kb past the first insertion, thus removing exon 1. Given the location of the ab101 deletion, it is unlikely that it uncovers additional genes. Both strains were analyzed for the presence of ab transcripts and Ab protein by whole-mount embryo in situ hybridization and antibody staining. The results showed that one quarter of the embryos derived from heterozygous crosses lacked ab transcripts and protein, consistent with these ab mutants being null (Fig. 4E).

Portions of the ab gene of the EMS-induced mutations ab107 and ab107 were sequenced using a single-embryo reverse transcription-PCR method (Fig. 6). The ab101 gene contains a single nucleotide pair change (CGC to TGC) in the region encoding the second ZF, resulting in an amino acid substitution of arginine to cysteine. The presence of the novel cysteine could conceivably interfere with the structure of the ZF, which depends on its structure and function by the coordination of Zn2+ between cysteine and histidine residues. The ab107 mutation has a transversion mutation (CAG to TGA) in the second codon of the ZF domain, arguing to stop 12 amino acids before the ZF and is predicted to generate a protein unable to bind DNA. These results prove that the BTB-ZF gene described here encodes the ab locus. Moreover, they suggest that the zincingers are required for normal ab function.

Discussion
ab encodes a BTB-ZF transcription factor

We have shown here that the ab gene encodes a BTB-ZF transcription factor by deficiency breakpoint mapping of

Figure 6. Sequence structure of the Ab protein and location of ab mutations. Shown is a boxed representation of the Ab protein, which is 904 amino acids in length. The amino terminus is shown at left, and amino acid residue numbering is shown to the right. (A) Two Cy's:Hsp ZF's (amino acid residues 545-600) with their amino acid sequence shown below. The consensus ZF motif is KxCy-Cx-Cy, CysXn-HisXn-CysXn, and the essential Cy's are underlined. The ab101 mutation introduces a termination codon at residue 581 predicting a transcribed protein lacking the ZF DNA-binding domain. The ab107 mutation changes Arg585 in the second ZF to a Cys residue.

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the abl allele [induced by P-element excision] and sequence analysis of two EMS alleles. The abl allele is transcript null and removes 0.7 kb of DNA including the first exon. The abl allele has a missense amino acid substitution in the second Z7 and the abl allele possesses a nonsense mutation that truncates the protein and deletes the ZF.

ab controls the specificity of neuromuscular connections

Genetic analysis indicates that ab is required for SNS motor axons to recognize and synapse on their muscle targets; the ventral longitudinal muscles 7, 6, 13, and 12. The ab gene is expressed during the stages of motor axon outgrowth and innervation in muscle cells but not in the relevant ectomesoderm. This is consistent with a model in which ab functions within muscle cells by controlling the expression of genes involved in muscle recognition by the CNS. The sex-specific synapse phenotype is most easily explained owing to lack of ab expression in muscles, it remains possible that the loss of ab CNS muscle or epidermal expression results in the connectivity phenotype. This issue can be resolved by using phenotypic rescue with ab mutant fly strains that express ab specifically in the different cell types (Brand and Perrimon 1993). Because the recognition events between several motoneurons and muscles are affected, it is probable that several distinct genes involved in the recognition process may be regulated by ab. Muscle cell formation, differentiation, and identity are normal in ab mutants, with the exception of muscle attachment defects in a few muscles (see below). This result implies that the function of ab is not as a regulator of the initial steps of muscle development, differentiation, or identity per se but rather as a regulator operating downstream in the hierarchy and controlling the expression of muscle genes that encode specific surface or secreted proteins.

One surprising feature of ab expression is that it is present in the nuclei of all somatic muscle cells; not just the ventral longitudinal muscles that show the altered connectivity in ab mutants. Thus, it remains unclear how the restriction of ab function to the ventral longitudinal muscles is generated. Several explanations are possible. The ab gene may function in a concentration-dependent mode such that different concentrations exert different effects on gene transcription. Ab has the ability to form multimers, both in homotypic and heterotypic modes (S.H. and S.T. Crews, unpublished), and this may be relevant because different concentrations of ab could result in functionally different monomeric or multimeric configurations. Interestingly, ab is expressed at higher levels in the ventral muscle group than in the lateral and dorsal groups, although the significance remains ambiguous because high levels are present in both the ventral longitudinal muscles that show connectivity defects and the ventral oblique muscles that do not. Preliminary analysis of ab and ab phenotype indicates that penetrance and expression are sensitive to ab gene dosage. Dosage dependency has been observed for other BTB proteins. The ab gene is expressed as a gradient along the limb proximal–distal axis, and ab mutations result in homotypic transformations of the vasa in a dosage-dependent fashion (Gould et al. 1993). GAGA protein can also distinguish high and low-affinity DNA-binding sites in a concentration-dependent manner (O'Brien et al. 1995).

Spatial restriction of ab function could also arise through combinatorial interactions between ab and other transcription factors. The BTB domain of other proteins is capable of influencing protein-protein interactions as well as other functions (Hardwicke and Treisman 1994). Interactions with non-BTB-ZF transcription
factors mediated by other regions of the proteins are also possible. It is estimated that the BTB-ZF protein family contains at least 40 Drosophila members, only a few of which have been identified (Zholovitz et al. 1994). It is possible that multiple BTB-ZF proteins act in concert to define regional muscle specificity by selectively regulating the expression of appropriate genes. One candidate might be the Drosophila Hulc gene. This gene encodes a BTB-ZF protein related to Ab and is expressed in muscle cells as well as the nervous system (Cimler et al. 1994). Phenotypes of hulc mutants are complex, showing effects on axonal growth and guidance (Seager et al. 1993; Cimler et al. 1994). Another BTB-ZF protein is Ttk that shows absence of all thoracic muscles in performal mosaic patches (Wadsworth et al. 1994; Gao et al. 1995). Interestingly, there is evidence for an all-bric combination to show a reduction in thoracic bristles.

Null mutations of pbr revealed a spinal cord muscle defect in the dorsal muscles. During stage 17, muscle 1, and to a lesser extent muscle 2, was observed to pull away from its attachment site and form a spherical mass. This phenotype is reminiscent of, but less severe than, myoblastoid and masquerade mutations (Wright 1986; Massell et al. 1988; Murauge-Otia et al. 1995). The establishment and maintenance of muscle attachment requires proper development of muscles, extracellular matrix, and specialized epidermal segment border cells (Bate 1990; Voelk and Vasilatkhavan 1994). Because pbr is present in both muscle and segmental epidermal border cells, it cannot be present in the overexpressed muscle attachment defect. The ab muscle attachment defect poses a question concerning whether the ab transduction connectivity phenotype is a secondary defect. Two lines of evidence suggest it is not. First, the embryonic muscle domains in which the two defects occur do not overlap. The spherical and attachment muscle defects are observed in the dorsal and lateral muscles, whereas the motomuscon connection defect is restricted to the ventral muscle field. Second, the EMS-induced allele ab(1)6mos has a motomuscon connectivity phenotype identical to null ab alleles but does not show a spherical muscle defect. Thus, it is likely that the connectivity and spherical muscle defects reflect two independent functions of ab, one of which is disrupted in ab(1)6mos mutant embryos.

Properties of the ab transcription factor

The presence of two Cys-His-ZFs in the ab conceptual protein sequence suggests that Ab functions as a DNA-binding transcription regulatory protein. This role is reinforced by the observation that ab is localized to embryonic nuclei when stained with an Ab antibody. Observations made by Kalonis and O'Farrell (1993), B. Kalonis, pers. comm. [further show that Ab binds DNA in vitro. They independently cloned the ab gene (IK238) in a screen for Drosophila proteins that recognize the consensus binding sequence of the homeo domain protein Engrailed (En) (Kalousis and O'Farrell 1995). The Ab ZF binds variants of the En binding site with a slightly different preference from that of En. The similarity of the binding site of Ab and that of homeodomain proteins causes the possibility that Ab may compete or combine with homeodomain-containing proteins to regulate target gene expression.

BTB-ZF proteins are required for a wide range of developmental events. They can be either transcriptional activators or repressors. Ttk is a transcriptional repressor of segmentation genes (Brow et al. 1991; Brown and Wu 1993; Xiong and Manelli 1995; ZFE) is a mammalian transcriptional repressor of the c-myc protooncogene, a property that maps to the amino-terminal ZF amino acid containing the BTB domain (Nimura et al. 1995). Other in vitro experiments have shown that the BTB domain from Ttk, Ttk, and GAGA inhibits the DNA-binding activity of their ZFs, as well as heterologous DNA-binding motifs, including POU-homeo domain, MAD5 domain, and Cys-His-ZF (Barbier and Treisman 1994). The GAGA factor acts as an activator of Kruppel and Ulx transcription through an inhibition mechanism (Kerrigan et al. 1991). BTB C-globin activates and represses different aspects of the cytochrome response during metamorphosis. It is unknown whether Ab acts as a transcriptional activator or repressor. Proper motor axon guidance and connectivity require not only the presence of attractive molecules, but also the absence of repulsive factors (Nose et al. 1994; Chiba et al. 1995; Matthews et al. 1995). Thus, Ab may function by activation or repression. Future genetic experiments are required to identify the gene targets of ab.

Recent evidence has suggested that BTB-containing proteins may influence transcription by modulating chromatin structure. Two BTB protein encoding genes, Tl and Elav1-93D, are enhancers of position-effect variegation, suggesting a role in chromatin condensation (Forre et al. 1994). GAGA factor is associated with specific regions of heterochromatin and generates nucleosome-free promoter regions both in vitro and in vivo (Raff et al. 1994; Tsuchiya et al. 1995). The promoter of the Drosophila hop-70 gene, GAGA protein occupies high-affinity binding sites prior to heat shock. Upon heat shock induction, the distance between DNA protein spreads over the length of the transcript in a 3' to 5' manner, probably owing to the binding of GAGA protein to its low affinity binding sites. The effect of GAGA protein to hop-70 displays a similar kinetics to that of RNA polymerase (O'Brien et al. 1995). Because BTB domains mediate protein-protein interactions, they may interact with chromosomal complexes to open and maintain chromatin structure or interact with additional transcription factors to control transcriptional activity.

Materials and methods

Drosophila stocks

The ab strain was acquired from the Bloomington Stock Center, Indiana University, Bloomington, IN. The BRP enhancer trap line was created in a large-scale screen done at ICLA (Crews et al. 1993). The 94-enhancer trap line was generated.
using pblw as an enhancer trap detector in the lab of Jose Cam-
pos-Ontuaga and Elisabeth Knust. The \( \text{ab}^{\text{sh1}} \) mutation was iso-
lated originally in a motoneuron connectivity and guidance
screen (Van Vactor et al. 1993). The following \( \text{ab} \) lethal muta-
tions were generated in this study: (1) \( \text{ab}^{\text{sh2}} \) and \( \text{ab}^{\text{sh3}} \) are EMS-
induced mutations, alleles identical by their failure to complement \( \text{ab}^{\text{sh1}} \)
lethality. (2) \( \text{ab}^{\text{sh4}} \) was generated as a heterozygous lethal mu-
tation by P-element-mediated excision of \( \text{P}(\text{BE23})/ \text{CyO} \). (3) \( \text{ab}^{\text{sh5}} \) was recovered by P-element-mediated excision/local hopping of \( \text{P}(\text{BE23}) \), based on its severe pleiotropic effect when crossed in (strain in \( \text{P}(\text{BE23}) \), and (4) \( \text{ab}^{\text{sh6}} \) and \( \text{ab}^{\text{sh7}} \) were isolated from the 94 strain as w-
revertants from progeny of 94 males irradiated with 4-5 krad of 
\( \text{Co}^{60} \). Chromosomes bearing lethal mutations were maintained as \( \text{CyO}, \text{CyO} \text{F}^{1} \), and \( \text{act}^{\text{gala2}}, \text{leu2}^{\text{gala2}} \), or \( \text{CyO} \text{F}^{1} \), \text{leu2}^{\text{gala2}} \) balanced stocks.

Molecular analysis of \( \text{ab} \) gene

Genomic DNA corresponding to the \( \text{ab} \) gene was initially iso-
lated by cloning DNA adjacent to the \( \text{BE23} \) element. Reverse
PCR (Ochman et al. 1988) was performed on \( \text{BE23} \) DNA using primers corresponding to the 3′ end of the P-element.
Wild-type 
\( \text{Drosophila} \) genomic DNA cloned into the 
\( \Phi 11 \) bacteri-
ophage vector (constructed by S.T. Crews) was screened using
\( \Phi 11 \)-labeled PCR products to isolate overlapping genomic clones.
Genomic DNA was extracted with a chromosomal walk by
screening a \( \Phi 11 \)-derived \( \Phi 11 \)-genomic library (Stratagene)
using a \( \Phi 11 \)-derived \( \Phi 11 \)-genomic DNA fragment located at
fragment 1 (Fig. 3). The DNA walking probe was labeled with
\( \text{[32P]} \text{dGTP} \) and hybridization was detected using anti-
\( \text{5'-alkaline phosphatase} \) (\( \text{1:20,000} \)) and the chemilumi-

tescence substrate Lumigen PTO (Genius system, Boehringer
Mannheim).

Three \( \text{Cl} \) clones were isolated from an embryonic 2- to
12-hr \( \text{Ag} \) library (constructed by Kai Zimn, California Insti-
tute of Technology) using a \( \Phi 11 \)-derived \( \Phi 11 \)-genomic DNA probe (Fig. 3).
The three clones (A01b, A01c, and A01d) contained a common 0.6-kb fragment region
sequence, and this fragment was radioactively labeled and used to isolate
three \( \text{Cl} \) clones (A01c, A01d, and A01e) from embryonic 4- to
6-hr \( \text{Cl} \) clone \( \Phi 11 \)-plasmid library (Brown and Keynes 1988). Clones A01c, A01b, A01d, and A01e were
subjected to DNA sequencing. The predicted reading frames were gener-
at d with consensus-internal III (\( \text{Koziol} \) Pretogae K-base set on both strands and sequenced using \( \text{[32P]} \text{dATP} \) on a double-
strand template (U.S. Biological/Chemical sequins kit). Additional
primers corresponding to the \( \text{ab} \) sequence were synthesized and used to complete the DNA sequence where required.

Analysis of \( \text{ab} \) embryonic transcripts was determined by Northern blot analysis. Samples containing 3 \( \mu \)g of embryonic 6- or 12-hr \( \text{Ag} \) RNA were fractionated on an 0.7% \( \times 2.3 \) M
formaldehyde-agarose gel, blotted onto a Nytran membrane, and
hybridized with a \( \Phi 11 \)-labeled 6.6-kb BamHI \( \text{ab} \) DNA frag-
ment.

Generation of \( \text{ab} \) aneuploidy

Ab protein for immunostaining was synthesized in bacteria fused to a 6× histidine tag. The fusion genes were constructed by inserting a 691-bp BamHI fragment encoding alanine amino acids 53-
279 containing the \( \text{B} \) region and a 651-bp BamHI-NcoI frag-
m ent (encoding alanine amino acids 278-498) of the \( \text{ab} \) DNA into the BamHI sites of the \( \text{B} \) region and the BamHI-PstI sites, respectively, of the \( \Phi 11 
\) plasmid 
\( \Phi 11 \). Fusion proteins were prepared under dena-
turing conditions using Ni-NTA resin following the Magenta

nuces recommendations (Günter). For immunization of rats, \( \text{100} \) \( \mu \)g of protein was emulsified in Ribi adjuvant (Immuno-
tech Research). Rats were injected at 2-week intervals. One
week after the fourth and subsequent injections, the serum was
collected and used for analysis. Prior to use, the antiserum
was preabsorbed against 5- to 15-hr embryos, which do not show de-
tectable ab aneuploids. An antiserum generated using both fusion
proteins gave identical staining patterns.

Antibody staining

Embryos were collected, fixed, and stained as described previ-
ously (Perel et al. 1987; Yan Yuctur et al. 1993). Primary anti-
bodies were incubated with embryos at 25°C for 1 hr or over-
night at 4°C. Antibodies were used at the following dilutions:
monoclonal anti-galactosidase (PrepGest at 1:100), monoclo-
nal anti-FasCII at 1:8, anti-myosin at 1:500, and anti-
ab at 1:200. Stained whole-mount embryos were mounted in
100% methylcyanoide. Muscle staining was visualized in
stage 13 to stage 17 embryos that were dissected with tungsten
needles and mounted in 80% glycerol in PBS. Embryos were
virtualized and reconstructed with a Zeiss Apotom microscope.

Polytenic chromosomes and embryonic whole mount in situ
hybridization

Hybridization of DNA probes to polytenic chromosomes was carried out according to Langer-Salter et al. (1982). Salivary
glands were dissected from third instar larvae in saline, fixed, and hybridized to biotin-dUTP-labeled DNA fragments at 42°C. Hybridization was detected using HRP-conjugated streptavidin and diaminobenzidine.

Embryo whole-mount in situ hybridization experiments were performed according to Tasca and Piccallo (1985). Hybridiza-
tions were carried out at 48°C for DNA probes or at 60°C for 
RNA probes. Digoxygenin-labeled DNA and RNA probes were labeled according to the manufacturer (Boehringer Mannheim). Hybridiza-
tion was visualized by in situ hybridization and washed embryos for 2 hr with anti-DIG-alkaline phosphatase (1:5000), followed by an X-phosphate/BTX reaction. Embryos were exam-
ined and photographed as whole mounts or filters in 80% glyc-

erol/sodium-borate saline (PBS).

Mapping chromosomal deficiencies by Southern blot hybridization and single-embryo PCR

Breakpoints of chromosomal deficiencies within the \( \text{ab} \) gene region were determined by Southern blot hybridization and sin-
gle-embryo PCR. The PCR procedure utilizes primers to amplify 
diverse genomic DNA regions, followed by gel electropho-
estores to determine whether the amplified fragments are
present in mutant embryos. PCR primers were designed using
\( \text{DNA} \) sequence information. Lethal \( \text{ab} \) de-
elation mutants were microinjected with a \( \text{CyO} \) or \( \text{CyO} \text{F}^{1} \text{leu2}^{\text{gala2}} \) balancer. Heterozygous flies
were allowed to lay eggs, and 12 embryos were randomly picked and crushed in 0.5-10\-
\( \mu \)l lysis buffer (50 mM Tris-HCl at pH 8.0, 5 mM NaCl, 1 mM EDTA, 200 \( \mu \)g/ml of proteinase K). After incubation for 30 min at 37°C, the proteinase K was inactivated at 95°C for 3 min. One
microliter of the embryonic DNA sample was added to 15 \( \mu \)l of 
PCR mix (Perkin-Elmer) and subjected to PCR amplification in a Perkin-Elmer 480 thermal cycler. Typical PCR parameters were:
25 cycles at 94°C for 30 sec, 55°C for 45 sec, and 72°C for
30 sec; followed by 1455° anneal gel electrophoresis. The sample
contained the primer pair from the ab locus and a control
primer pair derived from an unrelated gene, enu/\( \text{d76} \). Hua and

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Sequence analysis of ab mutants using reverse transcription (RT)-PCR

Embryos were collected from 

relative abundance of the ab fragment is much higher than the other products, which suggests that a second primer set is deleted in the mutant.

Southern blot analysis utilized restriction-endonuclease DNA from the ab mutant embryos indicated that the position of the band is consistent with the results obtained from the PCR analysis. The probes used were 32P-labeled probes specific for the ab fragment. All of the probes hybridized with the ab fragment, indicating that the ab fragment is transcribed in the mutant.

The results of the RT-PCR analysis indicate that the ab fragment is transcribed in the mutant. However, the relative abundance of the ab fragment is much higher than the other products, which suggests that a second primer set is deleted in the mutant.

References

Add the following references to the list:

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