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Domain mapping of tube, a protein essential for dorsoventral patterning of the *Drosophila* embryo

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The tube protein plays an essential role in the signal transduction pathway that establishes dorsoventral polarity in the *Drosophila melanogaster* embryo. Characterization of each of four *tube* mutants revealed a substitution or insertion in the amino-terminal half of the protein. This portion of the tube protein is also evolutionarily conserved, as demonstrated by isolation and sequencing of the *Drosophila virilis tube* gene. Moreover, RNA microinjection assays and germline transformation experiments demonstrated that the amino-terminal domain alone provides substantial levels of gene function: constructs encoding only the amino-terminal domain restore dorsoventral polarity to embryos lacking any maternal *tube* function. In the carboxy-terminal domain, sequence conservation is concentrated in the five octapeptide repeats. Although the repeat-containing domain by itself provides no rescue of the *tube* maternal effect phenotype, it is necessary for wild-type levels of *tube* activity. This domain is thus likely to play an ancillary role in axis formation.

Key words: dorsal/embryonic development/pattern formation/signal transduction/tube

Introduction

Dorsoventral polarity in *Drosophila melanogaster* is established by a concentration gradient of the dorsal protein in nuclei of the syncytial blastoderm embryo (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989). The concentration of dorsal in nuclei causes embryonic cells to adopt specific, position-dependent fates (for review see St Johnston and Nüsslein-Volhard, 1992). At present, 12 maternal effect loci are known to be required for the generation of the dorsal gradient (Nüsslein-Volhard, 1979; Anderson and Nüsslein-Volhard, 1984; Schüpbach and Wieschaus, 1989; Roth *et al.*, 1991). Recessive mutations in *dorsal* or 10 other dorsal group loci block import of dorsal into nuclei; all cells in the embryo adopt a position-independent fate and differentiate into a cell type normally reserved only for those cells resident at the dorsal surface of the embryo. Dominant mutations in the twelfth locus, *cactus*, also result in a dorsalized phenotype. In contrast, recessive mutations in *cactus*, as well as dominant mutations in several dorsal group loci, promote import of dorsal into nuclei everywhere, resulting in an opposite or ventralized phenotype.

Genetic and molecular studies have helped to specify the roles played by the dorsal group loci and *cactus*, as well as to order these genes in a functional hierarchy (Anderson *et al.*, 1985; Hashimoto *et al.*, 1988; Roth *et al.*, 1991; Stein *et al.*, 1991; Stein and Nüsslein-Volhard, 1992; P.Hecht and K.Anderson, personal communication). Data from these analyses support a model of signal transduction in which all components of the intracellular portion of the pathway, including dorsal, are initially distributed uniformly in the embryo. Signalling is triggered by activation of a transmembrane receptor, encoded by the *Toll* gene, at the ventral surface of the embryo. The products of three genes, *tube*, *pelle* and *cactus*, act downstream of *Toll* to transduce positional information intracellularly and to effect, consequently, the graded nuclear translocation of dorsal.

Homologies between *Toll* and the type I interleukin-1 receptor, between *dorsal* and the *rel*/NF-$\varkappa$B family of transcription factors and between *cactus* and the I$\varkappa$B family, indicate that the pathway regulating nuclear import is conserved between organisms as evolutionarily divergent as flies and mammals (Steward, 1987; Gay and Keith, 1991; Schneider *et al.*, 1991; Geisler *et al.*, 1992; Kidd, 1992). To understand better the mechanisms required for selective nuclear transport of dorsal and NF-$\varkappa$B, we are currently characterizing the two components of the *Drosophila* signal transducing mechanism, *tube* and *pelle*, for which counterparts in the NF-$\varkappa$B pathway have not yet been identified.

We recently cloned and sequenced cDNAs for both *tube* and *pelle*. The *pelle* locus encodes a protein kinase with greatest similarity to members of the *raf/mos* sequence subfamily. Kinase activity is required for *pelle* function, indicating that the transmission of information from an activated form of the *Toll* receptor requires the phosphorylation of one or more components of the signalling pathway (Shelton and Wasserman, 1993). In contrast, the role of *tube* in signal transduction is not yet clear. The carboxy-terminal half of the protein contains multiple copies of an 8-amino acid repeat, but neither the repeats nor any other portion of the protein exhibit significant sequence similarity to known proteins (Letsou *et al.*, 1991).

Here we explore tube protein function using three experimental approaches: (i) biochemical and molecular characterization of *tube* mutants to determine the nature and abundance of mutant forms of tube, (ii) sequencing of the *tube* gene from *Drosophila virilis* to identify evolutionarily conserved regions and (iii) microinjection assays and germline transformation of deletion constructs to probe the function of conserved sequences. These experiments indicate that the two halves of the tube protein have functionally distinct roles in signal transduction.

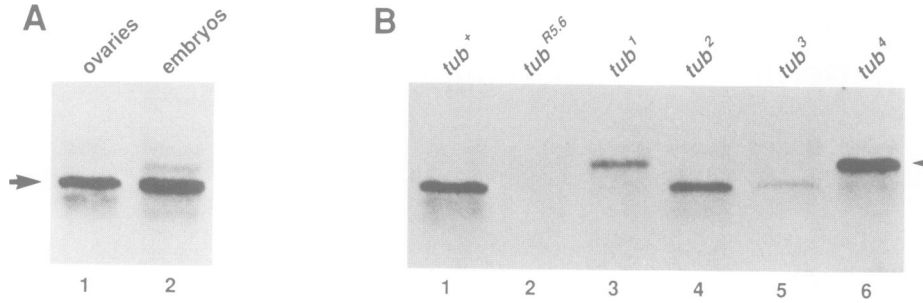

Fig. 1. Immunoblot analysis of tube protein in ovaries and embryos. Protein extracted from ovaries and from 0–3 h embryos was fractionated on a 10% SDS–polyacrylamide gel. Proteins were transferred onto a charged nylon membrane and detected with anti-tube polyclonal serum. Each lane contains 15 μg total protein; equivalency of loading was confirmed by silver staining of identical samples (not shown). (**A**) Lane 1, wild-type ovaries; lane 2, wild-type embryos. (**B**) Lane 1, *tube* hemizygous [*tub*$^+$*/Df(3R) XM3*] embryos; lane 2, *tube* null [*tub*$^{R5.6}$*/Df(3R) XM3*] embryos; lane 3, *tub*^{1}*/tub*$^{R5.6}$ embryos; lane 4, *tub*^{2}*/Df(3R) XM3* embryos; lane 5, *tub*^{3}*/Df(3R) XM3* embryos; lane 6, *tub*^{4}*/Df(3R) XM3* embryos. Arrow on the left points to tube species of ~51 000 MW; arrowhead on right points to tube species of 58 000 apparent MW.

Results

Two classes of mutations in tube

We began our investigation of tube structure and function by determining the nature of the defect in four EMS-induced *tube* mutations. Protein extracts were prepared from wild-type ovaries, as well as from 0–3 h embryos produced by wild-type females, by females hemizygous or null for *tube*, and by females heterozygous for each of the four EMS-induced *tube* mutations (*tub*^{1}, *tub*^{2}, *tub*^{3} and *tub*^{4}) in *trans* to a deletion. Using a tube-specific antibody to probe protein blots of these extracts, we detected a protein species of MW ~51 000 in wild-type ovaries and embryos (Figure 1A, lanes 1 and 2). This band was of the size expected for full-length tube protein (calculated MW 50 000) and was absent from embryos produced by females *trans*-heterozygous for two deletions of the *tube* gene, *tub*$^{R5.6}$ and *Df(3R)XM3* (Figure 1B, lanes 1 and 2).

Extracts derived from EMS-induced *tube* mutants were examined by immunoblotting. The tube protein in embryos from females carrying the *tub*^{2} or *tub*^{3} alleles comigrated with the wild-type species (Figure 1B, lanes 4 and 5). In contrast, protein extracts from embryos produced by females carrying either the *tub*^{1} or *tub*^{4} mutant allele contain a more slowly migrating tube species of ~58 000 MW (Figure 1B, lanes 3 and 6). In extracts from the *tub*^{4} mutant, an additional species comigrating with the wild-type band was faintly visible. Levels of protein produced were comparable with wild-type for *tub*^{1}, *tub*^{2} and *tub*^{4}, but were greatly reduced in extracts prepared from the *tub*^{3} mutant (Figure 1B).

To determine the molecular nature of the *tube* mutant defects, we constructed genomic libraries from the mutant lines and isolated each of the *tube* alleles on a 10 kb *Eco*RI fragment. By identifying single-strand conformational polymorphisms (SSCP, Orita *et al.*, 1989), we were able to localize three of the four mutations (*tub*^{2}, *tub*^{3} and *tub*^{4}) to 200–400 bp regions in the *tube* gene (data not shown). In the case of *tub*^{2}, the mutation was localized precisely by a diagnostic restriction fragment length polymorphism (RFLP) resulting from disruption of a *Sac*I restriction site.

By sequencing the polymorphic regions in *tub*^{2}, *tub*^{3} and *tub*^{4}, as well as the entire *tub*^{1} transcription unit, we identified nucleotide changes that could be responsible for the observed mutant phenotypes. For the *tub*^{2} and *tub*^{3} alleles, we found nucleotide changes resulting in non-conservative substitutions in the amino-terminal third of the protein. In *tub*^{2}, position 418 in the DNA is converted from a G to an A, changing a glutamate residue to a lysine at amino acid 140. Similarly, mutation of a T to an A at position 386 in *tub*^{3} results in conversion of a valine to an aspartate at position 129 in the protein.

Table I. Sequence of the 5′ and 3′ splice sites from wild-type and *tube* mutant introns

Source	Intron sequence
Consensus	G:GTAAGTA...AG:A
D.melanogaster tub$^+$	G:GCAAGTA...AG:A
D.melanogaster tub^1	G:GCAAGTA...AA:A
D.melanogaster tub^4	G:GCAAGAA...AG:A
D.virilis tub$^+$	G:GTAAGTA...AG:A

The DNA sequence of the 5′ and 3′ splice sites are shown for an invertebrate consensus (Senapathy *et al.*, 1990), for *D.melanogaster* wild-type, for the *D.melanogaster tub*^{1} and *tub*^{4} mutations, and for *D.virilis* wild-type. Bases differing from the consensus sequence are shown in bold.

To confirm that the substitutions identified in the *tub*^{2} and *tub*^{3} alleles disrupt *tube* function *in vivo*, we used a rapid and sensitive microinjection assay to assess the function of altered *tube* genes. As previously reported, embryos derived from *tube* mutant females gastrulate symmetrically and form hollow tubes of dorsalized larval cuticle (Anderson and Nüsslein-Volhard, 1984). Such embryos can be fully rescued by microinjection of RNA transcripts from a cDNA, referred to here as ptub462, encoding the 462 amino acid wild-type tube protein (Letsou *et al.*, 1991). Eggs collected from *tub*^{3}*/Df(3R)XM3* females and injected with 1 mg/ml ptub462 RNA develop into embryos which all gastrulate in the asymmetric manner of the wild-type. These embryos form characteristic dorsal, lateral and ventral larval cuticular elements, and often hatch.

The *tub*^{2} and the *tub*^{3} mutations were introduced into the wild-type *tube* cDNA by site-specific mutagenesis. The altered cDNAs were transcribed *in vitro* and the transcripts were then injected into embryos derived from *tub*^{3}*/Df(3R)XM3* females. When transcripts from the cDNA representing the *tub*^{2} mutation were injected at a concentration of 1 mg/ml, 0/24 embryos were rescued. Similarly, 0/38 embryos were rescued by 1 mg/ml RNA generated from the *tub*^{3} construct. We conclude that the point mutations identified in *tub*^{2} and *tub*^{3} abolish maternal tube activity.

For the *tub*^{1} and *tub*^{4} mutations, DNA sequence analysis revealed single nucleotide substitutions in the SSCP-defined polymorphic region of the *tub*^{4} allele. Both mutations map to the single *tube* intron and disrupt consensus splice site sequences (Table I). The nucleotide change in *tub*^{1} destroys the consensus 3′ splice site in the intron. *In vivo* and *in vitro* studies of splice site requirements indicate that such a mutation should abolish splicing (Wierenga *et al.*, 1983). In the case of *tub*^{4}, the nucleotide change lies in the extended 5′ consensus sequence of the intron that is recognized by the U1 small nuclear ribonucleoprotein particle (Zhuang and Weiner, 1986). This mutation is expected to reduce, but not eliminate, splicing (Wierenga *et al.*, 1983).

Since the intron contains 66 nucleotides and lacks an in-frame stop codon, unspliced RNA encodes a protein 22 amino acids larger than that encoded by the wild-type cDNA. It is therefore likely that the more slowly migrating *tub*^{1} and *tub*^{4} protein species arise from translation of unspliced RNA (Figure 1B, lanes 3 and 6). Consistent with the behavior of splice site mutations in tissue culture studies (Wierenga *et al.*, 1983), some protein comigrating with wild-type tube is produced by *tub*^{4} (Figure 1B, lane 6), whereas the more slowly migrating species is the only detectable product of the *tub*^{1} allele (Figure 1B, lane 3).

To test further whether intron inclusion is responsible for the phenotypes of *tub*^{1} and *tub*^{4} mutants, we generated a construct (ptub▽22I) in which the wild-type *tube* intron was reinserted into the *tube* cDNA. The encoded protein differs from the spliced product by the presence of 22 extra amino acids inserted immediately after amino acid 154 in the wild-type sequence. When injected at an RNA concentration of 1 mg/ml, the ptub▽22I transcripts rescued 0/14 embryos derived from *tub*^{3}*/Df(3R)XM3* females. We conclude that splicing of the wild-type *tube* transcript must occur to yield wild-type activity in the pathway for dorsoventral axis formation.

Definition of conserved sequence elements in tube

To identify additional sequences important for *tube* function, we examined the evolutionary conservation of the *tube* gene. For these experiments we analyzed a *tube* gene from *D.virilis*, a species distantly related to *D.melanogaster* (estimated divergence time 62 million years; Beverley and Wilson, 1984). We first confirmed that *D.virilis* has *tube* activity by complementing the *D.melanogaster tube* mutant phenotype with cytoplasm from *D.virilis* embryos. Cytoplasm was withdrawn from precellular *D.virilis* embryos and microinjected into embryos from *D.melanogaster tube* mutant females. Although the efficiency of rescue was not as high as that observed when donor cytoplasm was obtained from *D.melanogaster* embryos, *D.virilis* cytoplasm rescued the *tube* maternal defect and restored asymmetric patterns of wild-type gastrulation (Table II).

We next isolated a *D.virilis tube* genomic clone by screening of a *D.virilis* library at low stringency with a probe containing 1.2 kb from the *D.melanogaster* tube cDNA. Sequence analysis of the *D.virilis tube* genomic clone and comparison to genomic sequence derived from *D.melanogaster* revealed that *tube* gene structure is conserved between these two species (Figure 2). A single short intron is situated at the same position in the two genes. The *D.melanogaster tube* intron contains an unusual 5′ splice site (GC in place of GT) that has been observed only rarely in functional introns (Lear *et al.*, 1990; Haviland *et al.*, 1991). However, neither this feature nor the capacity to encode a larger protein is conserved in *D.virilis* since the 60 nucleotide long *D.virilis* intron contains both a consensus GT 5′ splice site (see Table I) and an in-frame termination codon.

Table II. Rescue of the *D.melanogaster* maternal effect *tube* phenotype by microinjection of cytoplasm

Source of donor cytoplasm	Number of embryos injected	Number of embryos rescued
D.melanogaster	39	32
D.virilis	47	10

Embryos from *tub*^{3}*/Df(3R)XM3* were injected prior to pole cell formation with cytoplasm from the indicated wild-type donor. Embryos exhibiting many or all of the asymmetric features of wild-type gastrulation, including cephalic and ventral furrows, posterior and anterior midgut invaginations, pole-cell migration and germ band extension, were scored as rescued.

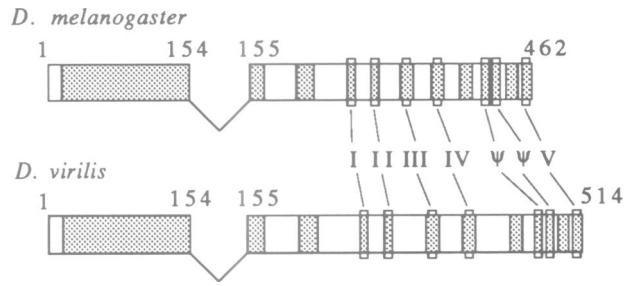

Fig. 2. Scale representations of the *tube* genes from *D.melanogaster* and *D.virilis*. Position along the amino acid sequence is indicated above the schematic for each gene. Exons are shown as boxes; the single intron in each gene is represented as a V-shaped line. Filled boxes represent regions sharing >70% sequence identity; unshaded regions share <40% identity. The five bracketed *tube* repeats are numbered I-V; the bracketed pseudorepeats, degenerate forms of the repeat consensus (Letsou *et al.*, 1991), are designated by the ψ symbol.

Overall, the proteins encoded by the two *tube* genes share a high degree of sequence identity (57%) and similarity (73%) (Figure 3). An extensive region of very high sequence conservation (72% identity) is located in the amino-terminal half of the protein, amino acids 25−173 in the *D.melanogaster* protein sequence. Only a single amino acid gap is required to align this portion of the two tube proteins. Moreover, the amino acid substitutions responsible for the *tub*^{2} and *tub*^{3} mutant phenotypes map to this conserved domain, alter conserved residues and fall within a stretch of 28 amino acids in which the two proteins differ at only a single position.

The carboxy-terminal half of the tube protein shows much less conservation. However, whereas the regions between the five 8-amino acid repeats in this region vary significantly in both composition and length, the repeats themselves are highly conserved in sequence and number. As in *D.melanogaster* (Letsou *et al.*, 1991), each *D.virilis* tube repeat contains asparagine at position 1, proline at position 3, serine or threonine at position 6 and leucine at position 8, as well as hydrophobic amino acids at positions 2 and 5. Furthermore, ordinal conservation of the octapeptide

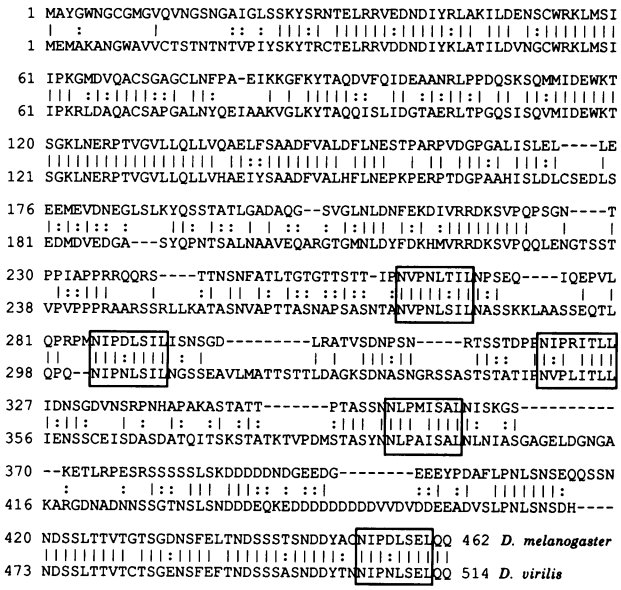

Fig. 3. Alignment of the deduced *tube* amino acid sequences. Sequences were aligned with the BestFit program (Devereux *et al.*, 1984). Dashes represent gaps introduced during alignment. Vertical bars indicate amino acid identities; colons mark conservative amino acid substitutions. The following substitutions were considered to be conservative: A ~ G ~ P ~ S ~ T; I ~L ~ M ~ V; D ~ E ~ N ~ Q; K ~ R. The five *tube* repeats are boxed; degenerate (pseudo) repeats begin at positions 406 and 420 in the *D.melanogaster* sequence.

repeats is apparent. For example, position 2 is a leucine and position 6 is an alanine in repeat IV in both *D.melanogaster* and *D.virilis*, but in no other repeat in either species. This evolutionary conservation suggests that the carboxy-terminal repeat elements are important for *tube* gene function.

Comparative sequence analysis of the *D.melanogaster* and *D.virilis tube* genes has thus revealed two regions of conservation at the amino acid level. The first, covering much of the first half of the protein, contains sequences disrupted in each of the four *tube* point mutants. The second, comprising the carboxy-terminal half of the protein, contains five copies of an amino acid motif that are highly conserved both within and between the two tube proteins.

Definition of domain structure by RNA injection assay

To address whether the conserved elements in each half of tube could be distinguished functionally, we used the RNA microinjection assay to test deleted forms of the *D.melanogaster* gene for complementation of the *tube* maternal phenotype. We constructed a series of amino-terminal and carboxy-terminal deletions in ptub462, each of which retained the 5′ and 3′ untranslated regions of the wild-type cDNA. The three amino-terminal deletions (ptubΔN17, ptubΔN34 and ptubΔN256) were each generated by fusion of coding sequences from the 3′ end of the *tube* cDNA to the *Nco*I site that maps to the initiator methionine in *tube* (Figure 4). Deletion of as few as 17 residues from the amino-terminus of *tube* (ptubΔN17) completely abolished its ability to rescue embryos from tub^3/*Df(3R)XM3* females.

A series of carboxy-terminal deletions was also assayed for rescuing activity. We constructed five progressive deletions that removed from 167 to 282 amino acids (ptubΔC167, ptubΔC205, ptubΔC221, ptubΔC254 and ptubΔC282). Surprisingly, all of these constructs retained rescuing activity, despite the fact that four of them lacked all five repeat elements (Figure 4). Injection of 1 mg/ml RNA generated from any of these constructs restored wild-type gastrulation patterns to >80% of the embryos from tub^3/*Df(3R)XM3* females and in many cases produced hatching larvae. Further deletion from the carboxy-terminus, however, eliminated rescuing activity; transcripts generated from ptubΔC308, encoding a tube protein only 154 amino acids in length, failed to rescue the *tube* mutant phenotype (Figure 4).

To determine whether rescue seen with deletion constructs lacking all repeat elements reflected wild-type levels of *tube* activity, we titrated the activity of ptub462 and ptubΔC205 RNA in embryos derived from *tube* null [$tub^{R5.6}$/*Df(3R)XM3*] females. Injection of RNA generated from ptub462 at 100 or 1000 μg/ml restored dorsoventral polarity and often rescued these embryos to hatching. Substantial restoration of polarity was also seen when transcripts from the deletion construct ptubΔC205 were injected at 100 and 1000 μg/ml, as evidenced by the appearance of a range of dorsolateral and ventral cuticle structures (Table III). RNA transcribed from ptubΔC205 did not, however, fully rescue the ventral cuticle pattern and did not produce any hatching larvae. Furthermore, rescue was weaker than that seen with wild-type RNA at all concentrations tested.

The RNA injection experiments thus define amino acids 1 and 180 as the approximate boundaries of a protein domain that is necessary and sufficient to provide substantial levels of tube activity.

Definition of domain structure in germline transformants

To assay the maternal role of each domain under conditions more closely approximating those found in the developing *Drosophila* embryo, we conducted germline transformation experiments. We first defined a genomic fragment that could fully complement the *tube* maternal-effect phenotype. Sequences spanning 7.8 kb from the *tube* genomic locus were subcloned into the w^+ P element vector pCaSpeR4; the resulting plasmids were then used for germline transformation. Two independent transformants of the P[w^+, tub462] construct were isolated and each was introduced into the *tube* null background. Both integrants rescued the null maternal phenotype of *tube*, restoring fertility to $tub^{R5.6}$/*Df(3R)XM3* females (Table IV).

We next generated germline transformants for P element constructs in which the *tube* gene had been truncated from either the carboxy- or amino-terminal end. The P element constructs P[w^+, tubΔN256] and P[w^+, tubΔC205] encode deletion derivatives of the *tube* gene identical to those encoded by the plasmids ptubΔN256 and ptubΔC205 described above. As was observed in the RNA-microinjection assay, deletion of residues from the amino-terminus of the tube protein abolished rescuing activity: P[w^+, tubΔN256]; $tub^{R5.6}$/*Df(3R)XM3* females were sterile and laid only dorsalized eggs (Table IV). In contrast, each of three independent stable integrants of P[w^+, tubΔC205] provided substantial rescuing activity in a *tube* null background. At 25°C, the embryos from transformed females produced larval cuticles with both dorsolaterally derived filzkörper and ventrally derived denticle belts. Furthermore, at 18°C the P[w^+, tubΔC205] transgene restored partial fertility to *tube* null females (Table IV). The

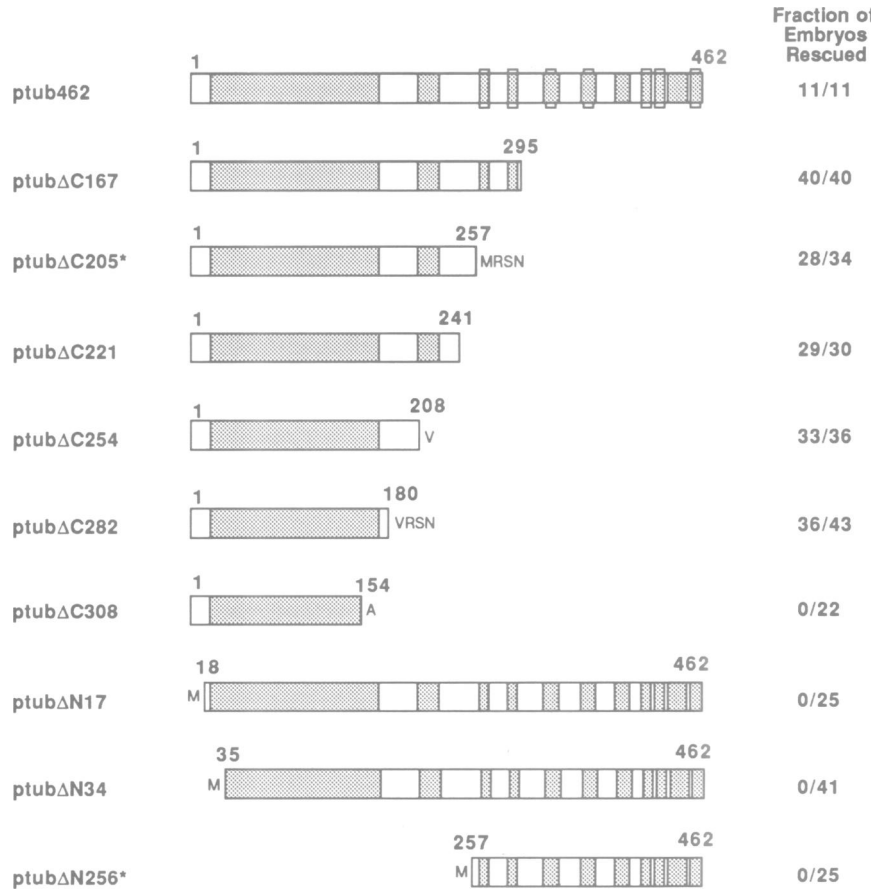

Fig. 4. *tube* deletion constructs used in the RNA microinjection assay. Position in the wild-type protein sequence is indicated by numbers above each bar. Regions conserved between *D.melanogaster* and *D.virilis* are represented as filled boxes. The wild-type *D.melanogaster tube* cDNA is shown at the top, with the position of the *tube* repeats and pseudorepeats marked with brackets. Shown below are schematic, scale representations of the six C-terminal *tube* deletions (ΔC) and the three amino-terminal *tube* deletions (ΔN). The two constructs marked with an asterisk contain the two halves of the tube protein encoded by the truncated *tube* P element constructs described in the text. The number in each deletion construct designation indicates the number of amino acids deleted, beginning with the amino-terminal methionine or the carboxy-terminal glutamine. Amino acids appended to the protein in the course of construction are designated by the single letter code. For all amino-terminal deletions, the amino-terminal methionine was restored during plasmid construction. The fraction of embryos rescued is the number of injected embryos that were visibly rescued at gastrulation (see Table II) divided by the total number of embryos that survived injection.

Table III. Microinjection assay of *tube* wild-type and carboxy-terminal truncation constructs in *tube* null background

Injected RNA (μg/ml)	Number of embryos scored	Number of embryos not rescued	Number of rescued cuticles (unhatched)			Number of hatched larvae
			FK	VD + FK	WT	
No RNA (uninjected)						
	60	60	0	0	0	0
Wild type (ptub462)						
1000	102	0	1	22	30	49
100	74	0	8	33	24	9
10	49	0	14	24	11	0
*ptub*ΔC205						
1000	117	0	52	65	0	0
100	54	12	40	2	0	0
10	39	32	7	0	0	0

Embryos from *tube* null [$tub^{R5.6}/Df(3R)XM3$] females were injected with transcripts from the constructs listed, at the indicated concentrations. Unhatched embryos were scored as unrescued if they lacked all dorsolateral, lateral or ventral cuticle elements; and as rescued if they produced only filzkörper (FK), ventral denticles and filzkörper (VD + FK) or a wild-type pattern of cuticle elements (WT). Embryos which hatch as larvae are considered fully rescued.

P[w^+, tubΔC205] element also restored partial fertility at 25°C in the non-null, $tub^1/tub^{R5.6}$, background (data not shown).

To determine whether the activity of the conserved amino-terminal domain could be augmented in *trans* by the presence of the carboxy-terminal domain, a strain was constructed

Table IV. Rescue of *tube* maternal and zygotic null phenotypes by germline transformants

Insert	Maternal phenotype		Zygotic viability	
	Fertility	Cuticle	Percent expected	Number scored
No insert	Sterile	D_0	14%	648
P-tub462	Fertile	WT	146%	525
P-tubΔN256	Sterile	D_0	32%	1078
P-tubΔC205	Sterile	D_2	104%	660
P-tubΔC205 (18°C)	Semi-sterile[a]	D_2−WT	ND	ND

Transgenes for each construct were crossed into the *tube* null [$tub^{R5.6}/tub^{R5.6}$] background and the listed phenotypes scored at 25°C, except where noted. WT, wild-type; D_0 eggs are completely dorsalized; D_2 eggs develop ventral denticles and filzkörper in addition to dorsal hairs, but never hatch. For zygotic viability the percent expected *tube* homozygotes was calculated as the number of *tube* homozygotes divided by one half the number of heterozygotes. Number scored = total number of flies counted.
[a]15% of embryos hatch. ND, not determined.

with both P[w^+, tubΔC205] and P[w^+, tubΔN256] in the null background. The phenotype of the double transformant at 25°C was identical to that of siblings carrying only the P[w^+, tubΔC205] construct (data not shown), indicating that the amino-terminal and carboxy-terminal domain constructs could not together provide wild-type *tube* function.

The results of the germline transformation experiments confirm that the amino-terminal domain is necessary for *tube* function. Moreover, they demonstrate that under some circumstances, the amino-terminal domain is also sufficient to provide complete phenotypic rescue of an embryo derived from a *tube* null female.

Zygotic function of full-length and truncated tube transgenes

Several genes in the dorsoventral signal transduction pathway exhibit zygotic as well as maternal phenotypes (Gerttula *et al.*, 1988; Letsou *et al.*, 1991; Roth *et al.*, 1991), suggesting that components of the signalling pathway act more than once in the *Drosophila* life cycle. In the case of *tube*, loss-of-function mutations not only affect the morphology of pupae, reducing their axial ratio (length/width) by ~20%, but also decrease zygotic viability (Letsou *et al.*, 1991; P.Hecht and K.Anderson, personal communication). Having found that the amino-terminal *tube* domain was both necessary and sufficient for maternal gene function in axis formation, we wished to determine which portions of the tube protein are required for zygotic function.

We first assayed the ability of a *tube* transgene to rescue the phenotypic effects of *tube* mutations on pupal morphology. In the absence of the transgene, pupae homozygous for the null mutation $tub^{R5.6}$ had a mean axial ratio of 2.5 (± 0.1; n = 61), whereas the mean for $tub^{R5.6}/+$ heterozygotes was 3.0 (± 0.1; n = 183). Introduction of the full-length P[w^+, tub462] construct into the null background resulted in a mean axial ratio of only 2.7 (± 0.1; n = 60). Thus, the genomic sequences in the P[w^+, tub462] element provide insufficient zygotic tube expression to rescue fully the visible pupal phenotype.

Next, we assayed the effect of the full-length P[w^+, tub462] construct on the viability of *tube* null flies. Null *tube* mutations reduce zygotic viability, although the degree of lethality is somewhat variable. In the absence of the transgene, flies homozygous for the $tub^{R5.6}$ null mutation were underrepresented >7-fold compared with $tub^{R5.6}/+$ siblings. In the presence of the P[w^+, tub462] element, such flies were found at a frequency 1.5-fold greater than expected (Table IV); the overrepresentation presumably reflects the deleterious effects of the balancer chromosome in the heterozygotes. Thus, the full-length transgene provided substantial rescue of the zygotic semi-lethal *tube* phenotype.

Transgenes for the truncated forms of tube were assayed similarly. The amino-terminal deletion P[w^+, tub462ΔN256] by itself had little effect on viability; transformed flies homozygous for the *tube* null mutation were still underrepresented >3-fold relative to heterozygous siblings. Given the variability in the semi-lethal phenotype, we could not be certain whether or not this deletion construct provided zygotic *tube* function. In contrast, homozygous $tub^{R5.6}/tub^{R5.6}$ flies harboring the carboxy-terminal deleted P[w^+, tub462ΔC205] transgene appeared at a frequency equal to that of heterozygous siblings. The amino-terminal domain of the tube protein is therefore clearly sufficient to provide significant rescue of the *tube* semi-lethal zygotic phenotype.

Discussion

The *Toll, tube, pelle, dorsal* and *cactus* gene products are required intracellularly to transmit maternal information specifying the dorsoventral axis of the *Drosophila* embryo. The Toll protein is thought to be a transmembrane receptor that is activated by binding of a ventrally localized ligand in the extracellular perivitelline space. Activated Toll then triggers transduction of an intracellular signal that overcomes the inhibitory effect of cactus on dorsal protein nuclear import. The transmission of this signal requires both *tube* and *pelle*. It has recently been demonstrated that *pelle* encodes a protein kinase (Shelton and Wasserman, 1993) and that dorsal protein phosphorylation *in vivo* is regulated directly or indirectly by *pelle* (S.Gillespie and S.Wasserman, unpublished results). In studying the dorsoventral signalling pathway we have focused here on *tube*, a component for which a biochemical function has not yet been ascribed.

tube gene structure

In analyzing the genomic DNA for the *D.melanogaster* tube, we have found a single intron. Like the majority of introns in this species (Mount *et al.*, 1992), the *tube* intron is <80 nucleotides in length. This intron is unusual, however, in having a non-consensus 5′ splice site, as well as the capacity to encode 22 amino acids in frame with the flanking exonic sequences. We therefore considered the possibility that the

protein encoded by unspliced message might represent an alternative *in vivo* gene product (Shapiro and Senapathy, 1987). If so, our data show that it is unlikely to function in the maternal dorsoventral pathway. First, we did not detect the translation product of unspliced message in extracts from wild-type embryos. Second, the tub^1 and tub^4 mutations, which block splicing and yield substantial amounts of protein from unspliced message, are defective for maternal *tube* function. Third, unspliced *tube* RNA fails to provide rescuing activity in the microinjection bioassay. The open reading frame in the intron is thus most likely a chance consequence of the intron's short length.

We have noted previously that both tub^1 and tub^4 act as hypomorphic, rather than amorphic alleles, and that tub^4 is by far the weaker allele of the two (Letsou *et al.*, 1991). It may be that the unspliced *tube* RNA produced by these alleles encodes a protein with residual activity; such activity might be observed in injections of greater numbers of embryos or at higher RNA concentrations than were assayed in our experiments. Alternatively, it may be that splicing of tub^1 RNA does occur, albeit at levels too low to be readily detected, and that the hypomorphic phenotypes of both alleles reflect only the levels of spliced RNA produced.

Interspecific sequence comparison of the tube gene from D.melanogaster and D.virilis

Sequence comparisons between distantly related species provide a useful tool for the identification of functionally important protein domains (Wilson *et al.*, 1977; see for example Yao and White, 1991). For this reason, we employed the microinjection rescue assay to demonstrate the existence of a functional *tube* gene in *D.virilis* and then cloned and sequenced this homolog. The estimated time of divergence for these two species, ~60 million years (Beverley and Wilson, 1984), is sufficiently long to permit correlative predictions of protein domain conservation and functional significance. Comparison of the two tube sequences revealed that the *tube* genes have a similar structure and that the tube protein sequence is composed of both conserved and divergent blocks of amino acids.

The intron−exon structure of the *D.virilis tube* gene was deduced by comparison to the *D.melanogaster* sequence. Both genes contain a single short intron that disrupts the coding sequence at the same position. However, the two introns are not similar at the level of either nucleic acid or amino acid sequence. Whereas the *D.melanogaster* tube gene has the potential to encode an unspliced protein of greater than wild-type length, the *D.virilis* intron includes termination signals in all three reading frames. Thus, splicing is clearly required to produce a full-length *tube* protein in *D.virilis*.

Substantial conservation (72% identity, 84% similarity) was found within the amino-terminal portion of the tube protein. In this region, a stretch of 149 amino acids in the *D.melanogaster* sequence (residues 25−173) is virtually colinear with the corresponding region in the *D.virilis* sequence; only a single amino acid gap is required to align the two sequences. This highly conserved region coincides well with the effector domain of tube defined in functional assays (see below).

In contrast, considerable amino acid divergence is apparent in the carboxy-terminal two-thirds of the tube protein sequence. Insertions and deletions in this region account for >95% of the length difference between the tube proteins from *D.melanogaster* (462 amino acids) and *D.virilis* (514 amino acids). Despite significant sequence divergence, the carboxy-terminal region from *D.melanogaster* and *D.virilis tube* can be readily aligned. Embedded in this large region are the octapeptide repeats, each of which is conserved. In addition, two other stretches of sequence similarity are evident: one, falling between amino acids 406 and 450 in the *D.melanogaster* sequence, contains at least two degenerate copies of the repeat motif; the other, encompassing amino acids 388−404 in *D.melanogaster* tube, is divergent in sequence and in length, but is highly acidic in both proteins.

Much of the tube protein that is highly divergent maps in the central region of the amino acid sequence (residues 174−262), between the conserved amino-terminus and the repeat-containing region. This organization suggests that the central region of tube may act as a hinge or connector between two functional domains. Similarly, the substantial divergence in composition and length of sequences between each of the octapeptide repeats prompts us to suggest that these intervening amino acids function as spacers within the protein.

We considered the possibility that portions of the *D.virilis* tube protein dissimilar to corresponding regions in the *D.melanogaster* protein might comprise functional motifs or domains absent in the latter. As with the *D.melanogaster* protein, however, we found that no portion of the *D.virilis* tube protein exhibited significant sequence similarity to proteins in the NBRF database (Devereaux *et al.*, 1984). Nevertheless, it may be that the variable subregions are required for species-specific protein interactions. Consistent with this hypothesis, we observed that embryonic cytoplasm from *D.virilis* was substantially less efficient than that from *D.melanogaster* in rescuing the maternal-effect *tube* phenotype in *D.melanogaster* embryos.

Function of the amino-terminal tube domain

By comparing the deduced amino acid sequence for tube from *D.melanogaster* and *D.virilis*, we identified a conserved region that spanned ~150 amino acids in the amino-terminal half of the protein. Since evolutionary comparisons are only predictive of function, we further investigated domain structure by means of an *in vivo* assay for *tube* function.

An amino-terminal deletion removing residues 2−17 of the tube protein, ptubΔN17, abolished rescuing activity in the RNA microinjection assay. Although the amino acid sequence of the deleted region is dissimilar between *D.melanogaster* and *D.virilis*, we note that in both tube proteins there are exactly 23 residues between the initiator methionine and the conserved amino-terminal domain. Since differences in sequence length are found in all other divergent regions of tube, length, more than composition, may be the critical determinant of function disrupted by the deletion in ptubΔN17.

The carboxy-terminal boundary of the amino-terminal domain defined by sequence conservation lies at amino acid 173. This corresponds well with the boundary defined in the RNA microinjection experiments: deletion of amino acids 181−462 leaves domain function intact, whereas deletion of residues 155−462 eliminates rescuing activity. The functionally defined amino-terminal domain thus comprises the first third of the tube protein and encompasses the region most highly conserved.

All four tube point mutations disrupt the amino-terminal domain. Two mutations, *tub*^{1} *and tub*^{4}, block splicing and thereby introduce 22 extra amino acids immediately following amino acid 154. The other two mutations, *tub*^{2} and *tub*^{3}, change the charge at single positions in the amino acid sequence and cause loss-of-function phenotypes. The *tub*^{3} mutation also diminishes the amount of tube protein. However, since the level of protein from spliced RNA is greater in embryos from *tub*^{3}/*Df* females than in embryos from *tub*^{4}/*Df* females (see Figure 1), but *tub*^{3} is much more severe in phenotype than *tub*^{4}, the point substitution in *tub*^{3} must reduce protein activity. In conjunction with the deletion experiments, the mutant analyses demonstrate that sequence elements in the amino-terminal domain are strictly required for *tube* function.

The deletion experiments indicate that the amino-terminal domain is not only necessary, but also sufficient, to confer high levels of *tube* gene function. A construct encoding only the amino-terminal tube domain (ptubΔ282) provided substantial rescuing activity in the RNA injection experiments. Furthermore, a transformant encoding the amino-terminal half of tube completely restored dorsoventral polarity in a fraction of embryos produced by *tube* null females at 18°C. Lastly, the amino-terminal transformant construct sufficed to provide significant rescue of the zygotic semi-lethality of a null *tube* mutation. In no experiment, however, was the activity of the amino-terminal domain equal to that of the full-length tube protein.

What role might the amino-terminal domain of *tube* play in regulating dorsal nuclear import? If all of the components required for the dorsoventral signalling pathway have been identified, previously established genetic and molecular hierarchies limit the possibilities. One possibility is that the amino-terminal tube domain interacts with Toll and pelle, fulfilling a role analogous to that of the SH2 and SH3 domain-containing proteins such as *Caenorhabditis elegans* sem-5 and human GRB2 (Clark *et al.*, 1992; Lowenstein *et al.*, 1992) or the bacterial cheW protein (Bourret *et al.*, 1991; Stock *et al.*, 1992). Alternatively, the amino-terminal domain could be required to transmit information from pelle to the complex of cactus and dorsal.

Function of the carboxy-terminal tube domain

Assays of both maternal and zygotic function indicate that the carboxy-terminal half of the tube protein is not strictly required for *tube* activity. However, the evolutionary conservation of the octapeptide repeats provides substantial support for the idea that the carboxy-terminal portion of tube comprises a second functional domain. What role might the the repeat-containing domain play in dorsoventral axis formation? This domain might exert its influence on tube function by modulating the activity or stability of the amino-terminal domain. Alternatively, it might serve to localize the amino-terminal domain. In the absence of the repeats, tube might diffuse across the cytoplasm of the syncytial embryo. Such diffusion would be decreased at lower temperatures, consistent with the fact that a fraction of embryos from *tube* null females transformed with the amino-terminal tube domain are rescued to hatching at 18°C, but not 25°C. Further investigation of the tube protein may therefore provide insight into both the spatial regulation and mechanism of signal transduction in the developing embryo.

Materials and methods

Drosophila strains

The wild-type *D.melanogaster* strain was Oregon R. Balancers, marker mutations, the *tube* deficiency *Df(3R)XM3* and the *tube* alleles *tub*^{1}, *tub*^{2}, *tub*^{3} and *tub*^{4}, have been described previously (Anderson and Nüsslein-Volhard, 1984; Ashburner, 1989; Letsou *et al.*, 1991). The null allele *tub*$^{R5.6}$, which removes the entire *tube* transcription unit, was the generous gift of Kathryn Anderson (UC Berkeley). The *D.virilis* strain was provided by Allan Spradling (Carnegie Institution of Washington). Flies were maintained at 25°C unless otherwise stated.

Production of anti-tube polyclonal antibodies

Expression of a *tube*−glutathione-S-transferase fusion protein (GST−tube) in a derivative of the *Escherichia coli* strain JM109 utilized the pGEX inducible vector system. Plasmid pGEX-KG-tube was constructed by (i) using linkers to convert the *Pvu*II site in the *tube* cDNA, pNB448, into a *Xho*I site and then (ii) ligating the *Nco*I−*Xho*I fragment into the *Nco*I and *Xho*I sites of the pGEX derivative pGEX-KG supplied by D.Endress. The majority of the GST−*tube* fusion protein expressed after induction with 0.1 mM IPTG was insoluble; inclusion bodies were purified as described by Harlow and Lane (1988). The fusion protein was purified further by preparative SDS denaturing polyacrylamide gel electrophoresis. A gel fragment that contained ~500 μg of the *tube* fusion protein was homogenized in PBS (50 mM NaCl, 5 mM KCl, 14 mM NaH_2PO_4 and 36 mM Na_2HPO_4) and mixed with an equal volume of Freund's complete adjuvant (Gibco). Rabbits were injected subcutaneously, boosted after 2 weeks and then injected on a 4 week schedule with 250 μg material prepared with Freund's incomplete adjuvant (Gibco) as above. Rabbits were routinely bled 10 days post-injection.

Immunoblot analysis

Protein extracts were prepared from 0−3 h embryos produced by *tub*$^{R5.6}$/*Df(3R)XM3*, *tub*^{1}/*tub*$^{R5.6}$, *tub*^{2}/*Df(3R)XM3*, *tub*^{3}/*Df(3R)XM3*, *tub*^{4}/*Df(3R)XM3*, *Df(3R)XM3/TM3* and +/+ females. Dechorionated frozen embryos were homogenized with a pestle in 25 vol of Laemmli sample buffer prepared without reducing reagent (Laemmli, 1970). Samples were incubated for 5 min at 100°C and undissolved material was sedimented by centrifugation. Protein concentrations were determined by the bicinchoninic acid (BCA) assay (Pierce). After protein concentration was determined, β-mercaptoethanol was added to 5% and samples were again heated to 100°C for 5 min. Protein extracts from Oregon R females were prepared similarly except that following homogenization with a pestle, ovaries were subjected to sonication (three 30 s pulses with a fine-tip probe) to ensure complete disruption of the tissue.

Protein extracts were separated on a standard 10% SDS−polyacrylamide gel. Immunoblotting and chemiluminescent detection were performed essentially as described by Gillespie and Hudspeth (1991). Primary antibody (tube-specific polyclonal antiserum) was used at a dilution of 1/10 000. Alkaline phosphatase-conjugated goat anti-rabbit IgG (CalBiochem) was used at a dilution of 1:40 000.

Isolation of genomic clones

Two genomic clones encoding a wild-type *D.melanogaster* tube allele were used in this study. The first is described in Letsou *et al.* (1991); the second was isolated from a genomic library constructed in our laboratory (C.Shelton and S.Wasserman, unpublished results). To construct genomic libraries from flies carrying each of the *tube* alleles, DNA was isolated from *tub*^{1}/*Df(3R)XM3*, *tub*^{2}/*Df(3R)XM3*, *tub*^{3}/*Df(3R)XM3* or *tub*^{4}/*Df(3R)XM3* adult flies (Ashburner, 1989), digested with *Eco*RI and subcloned into the bacteriophage λ DASH (Sambrook *et al.*, 1989). Genomic clones for the *tube* gene from *D.virilis* were isolated from a library generously supplied by J. Tamkun (University of California, Santa Cruz), using as probe the *Hin*dIII fragment from pNB448 (Letsou *et al.*, 1991) that contains 1.2 kb from the 5′ end of the cDNA insert. Hybridizations and washes were performed using low stringency conditions: hybridizations proceeded overnight at 50°C in 6 × SSPE, 0.5% SDS. Filters were subsequently washed for 10 min at room temperature in 6 × SSPE, 0.5% SDS and twice for 15 min at 50°C in 1 × SSPE, 0.5% SDS.

DNA sequence analysis

Analysis of single-strand conformational polymorphisms (SSCP) was performed essentially as described by Orita *et al.* (1989). Nucleotide sequencing was carried out by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the T7 Sequenase kit 2.0 (US Biochemicals) and synthetic oligonucleotides as primers on double-stranded

DNA templates. The nucleotide sequence of the *D.virilis tube* genomic clone and of the *D.melanogaster tube* intron have been deposited in the GenBank data base (accession nos L20448 and L20449, respectively).

Construction of mutated tube cDNAs

Six carboxy-terminal deletions were constructed by fusion of coding sequences from the 5′ end of the *tube* cDNA (pNB448) to the unique *Nsi*I site in its 3′ untranslated region. To construct ptubΔC167, ptubΔC205, and ptubΔC221, pNB448 was cleaved at a unique restriction site, *Bst*BI (nt 880), *Sal*I (nt 768) or *Sca*I (nt 721), respectively, and double-stranded ends were synthesized using the Klenow fragment of DNA polymerase I. To complete these constructions, plasmids were subsequently cleaved at the unique *Nsi*I site (nt 1488 in pNB448) and 5′ overhangs were eliminated using nuclease S1. Finally, digested DNA was ligated under conditions that promoted intramolecular reaction and transformed into the bacterial strain XL1-Blue (Stratagene).

To construct the remaining carboxy-terminal truncations, restriction sites were introduced into the pNB448 sequence using synthetic oligonucleotides complementary to the coding strand of the cDNA to prime DNA synthesis: for ptubΔC254, a *Hpa*I site was introduced at position 625 with the oligonucleotide GTCTTTTTAAAGTTAACCAGGTTCAGGC (bases differing from wild-type are shown in bold). Similarly, the *Msc*I site at position 539 in ptubΔC282 and the *Nsi*I at position 461 in ptubΔC308 were generated with the oligonucleotides GCCCCTCGTTGGCCACTTCCATTTCC and GGGGTGGATGCATTTAGGAAGTC, respectively.

To complete construction of the remaining carboxy-terminal deletion plasmids, the modified pNB448 plasmids containing the restriction site polymorphisms described above were digested by the corresponding restriction enzymes. For ptubΔC254 and ptubΔC282, plasmids were also cleaved at the unique *Nsi*I site (nt 1488) and 5′ overhangs were eliminated using nuclease S1. Lastly, plasmids were circularized using T4 DNA ligase and introduced into bacteria. For ptubΔC308, sequence between the newly constructed *Nsi*I site (nt 461) and the endogenous *Nsi*I site (nt 1448) was deleted by *Nsi*I restriction, ligation, and transformation.

Three amino-terminal deletions were generated by fusion of coding sequences from the 3′ end of the *tube* cDNA (pNB448) to the *Nco*I site that maps to the initiator methionine in *tube*. Each deletion construct thus maintains the wild-type reading frame and contains both the endogenous initiator methionine and the endogenous 5′ regulatory sequences. Each amino-terminal deletion construct also contains an identical 3′ non-coding region that includes the endogenous polyadenylation site. To construct ptubΔN17, pNB448 was cleaved at its unique *Nco*I site and a strand complimentary to the 5′ overhang was synthesized with the Klenow fragment of DNA polymerase I. A subsequent round of cleavage at nucleotide 55 by *Nar*I was followed by treatment with the Klenow enzyme in the presence of dCTP only. To construct ptubΔN34 and ptubΔN256, pNB448 was restricted at nucleotides −2 and 102 with *Nco*I and *Mlu*I or at nucleotides −2 and 768 with *Nco*I and *Sal*I. The 5′ overhangs generated by each of these cleavages were made double stranded using the Klenow polymerase. Subsequent ligation of blunt ends was performed as described above.

To reintroduce the wild-type intron into the *tube* cDNA, a 3.3 kb *Bam*HI fragment f310 (Letsou *et al.*, 1991), which contains genomic sequence from the *tube* locus, was subcloned into pBluescript(SK+) (Stratagene) to generate pBS576. The intronless 946 bp *Nco*I−*Bam*HI from pNB448 was replaced by the 1012 bp intron-containing *Nco*I−*Bam*HI fragment from pBS576 to generate the intron-containing cDNA, ptub▽221. The molecular nature of the newly constructed plasmid was confirmed by restriction analysis.

Plasmids carrying the point mutations found in tub^2 and tub^3 were constructed *in vitro* using the Altered Sites site-directed mutagenesis system as directed by the supplier (Promega). The *Eco*RI−*Sal*I cDNA fragment was subcloned into the pSELECT vector and site-directed alterations were introduced with the GGTGCAGGCAAAGCTCTTCAG oligonucleotide for tub^2 and with CGGCCCACGGATGGGGTATTG for tub^3. For each mutation, a restriction fragment from the mutated region of the *tube* gene was then used to replace the equivalent region in pNB448.

Both restriction analysis and DNA sequence analysis were employed to confirm the molecular nature of all altered *tube* cDNA constructs.

Microinjection rescue assay

Cytoplasmic transfer was performed as described by Anderson and Nüsslein-Volhard (1984). For RNA microinjections, wild-type and mutant *tube* cDNAs were linearized with *Not*I at a site 3′ of the *tube* insert (Brown and Kafatos, 1988). SP6 transcripts were then generated *in vitro* and injected into dechorionated embryos prior to pole-cell formation at 18°, as previously described by Wieschaus and Nüsslein-Volhard (1986) and Letsou *et al.* (1991). Injected embryos were incubated at 22°C; rescued and non-rescued maternal phenotypes were scored as described (Letsou *et al.*, 1991).

P element construction and transformation

P[w^+, tub462] was constructed by inserting a 7.8 kb *Bam*HI−*Eco*RI fragment, comprising genomic fragments f310 and f307 from the *tube* locus (Letsou *et al.*, 1991), into the polylinker of the w^+ P element vector pCasper 4 (Pirrotta, 1988) using standard techniques (Sambrook *et al.*, 1989). P[w^+, tubΔC205] and P[w^+, tubΔN256] are analogous to P[w^+, tub462] except that P[w^+, tubΔC205] encodes a truncated form of *tube* in which carboxy-terminal amino acid residues 258−462 have been deleted and P[w^+, tubΔN256] encodes a truncated form of *tube* in which amino-terminal amino acid residues 2−256 have been deleted. P element-mediated germline transformation into a w^{1118} background was performed as described by Spradling (1986), except that a coinjected plasmid carrying P[ry$^+$, Δ2-3] (Robertson *et al.*, 1988) served as the transposase source.

Scoring of zygotic phenotypes

Pupal axial ratios (length/width) were determined as described by Letsou *et al.* (1991). Pupae were sorted according to differences of 0.1 in axial ratio. Adults emerging from each class were genotyped on the basis of visible markers.

For viability tests, virgin crosses of lines homozygous for the P(w^+)tub462 insert in a w^{1118}; $tub^{R5.6}$/*TM3* background were set up in bottles at 25°C. Emerging adults were sorted as $tub^{R5.6}$/*TM3* or $tub^{R5.6}$ homozygotes and the number of flies in each genotypic class were counted.

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