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nubbin encodes a POU-domain protein required for proximal-distal patterning in the *Drosophila* wing

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SUMMARY

The *nubbin* gene is required for normal growth and patterning of the wing in *Drosophila*. We report here that *nubbin* encodes a member of the POU family of transcription factors. Regulatory mutants which selectively remove *nubbin* expression from wing imaginal discs lead to loss of wing structures. Although *nubbin* is expressed throughout the wing primordium, analysis of genetic mosaics suggests a localized requirement for *nubbin* activity in the wing hinge. These observations suggest the existence of a novel proximal-distal growth control center in the wing hinge, which is required in addition to the well characterized anterior-posterior and dorsal-ventral compartment boundary organizing centers.

Key words: POU domain, wing development, pattern formation, growth control, imaginal disc, compartment, *Drosophila*

INTRODUCTION

Embryonic organizers have been identified in vertebrate embryos as groups of cells that can cause a global reorganization of spatial pattern when transplanted to an ectopic location. Cells from the dorsal blastopore lip of the amphibian embryo can organize a supernumerary dorsal-ventral axis when transplanted to an ectopic position (Spemann and Mangold, 1924; Smith and Slack, 1983). Cells from the posterior margin of the chick limb bud reorganize anterior-posterior pattern when transplanted to the anterior margin of a host limb bud (Saunders and Gasseling, 1968; Tickle et al., 1975). In both cases, cells from the organizer region graft have the ability to respecify the fates of the surrounding cells so as to organize a complete secondary axis, which consists of both host and graft tissue.

In *Drosophila*, groups of cells with organizer activity have been identified by production of genetic mosaics, which alter patterns of gene expression in situ, rather than by direct transplantation of cells from the organizer region (Struhl and Basler, 1993; Diaz-Benjumea and Cohen, 1993; Basler and Struhl, 1994; Diaz-Benjumea and Cohen, 1994). Using this approach it has recently been shown that the dorsal-ventral (D/V) and anterior-posterior (A/P) compartment boundaries each produce an organizing center responsible for generating spatial pattern in the developing Drosophila wing (Diaz-Benjumea and Cohen, 1993; Basler and Struhl, 1994). In both cases interaction between distinctly specified cells in adjacent compartments induces the formation of the organizer.

The initial asymmetry between compartments is established by localized expression of a selector gene, which assigns cells their compartment-specific identity (García-Bellido, 1975). The homeodomain protein Engrailed specifies the identity of cells in the posterior compartment (García-Bellido, 1975; Morata and Lawrence, 1975; Lawrence and Morata, 1976; Kornberg, 1981; Kornberg et al., 1985; DiNardo et al., 1985). Posterior cells communicate with nearby cells in the anterior compartment by serving as a localized source of the secreted signaling protein Hedgehog (Mohler and Vani, 1992; Lee et al., 1992; Tabata et al., 1992; Taylor et al., 1993), which induces anterior cells near the compartment boundary to form the A/P organizer (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Similarly, the D/V boundary organizer is established by interaction between dorsal cells, specified by localized expression of the selector gene *apterous*, and the adjacent cells in the ventral compartment (Diaz-Benjumea and Cohen, 1993; Williams et al., 1994). The inherent asymmetry of cell interaction across the compartment boundary is used to generate signaling centers, which serve to specify cell fate and to control growth in a symmetric manner on both sides of the compartment boundary (Diaz-Benjumea and Cohen, 1993; Basler and Struhl, 1994).

On the basis of common features in their mutant phenotypes, a number of genes have been implicated in mediating the activity of either the A/P or D/V organizers in the wing disc. In both cases the focus for the requirement of activity of these genes lies along the compartment boundary. Thus, mutations in D/V patterning genes typically cause scalloping of the wing margin (Butterworth and King, 1965; James and Bryant, 1981; Campbell et al., 1992; Diaz-Benjumea and Cohen, 1993; Williams et al., 1994; Speicher et al., 1994; Couso et al., 1994). By contrast, A/P patterning genes do not affect the margin directly, but may cause interstitial deletions of wing structures along the A/P compartment boundary (Fausto-Sterling, 1978; Posakony et al., 1991).

In this report we present evidence that the *nubbin* gene (*nub*) identifies a different class of mutations affecting wing development. We show that *nubbin* encodes a member of the POU

family of transcription factors (*pdm-1*, Billin et al., 1991; Lloyd and Sakunju, 1991; Dick et al., 1991) which is expressed in the primordium of the adult wing. Like strong mutations in the D/V or A/P systems (Cohen et al., 1992; Williams et al., 1993; Basler and Struhl, 1994), *nub* mutations cause almost complete deletion of the wing. The requirement for *nub* activity does not appear to be focused on either the A/P or D/V compartment boundaries. Rather, the phenotypic defects observed in *nub* mutant wings suggest a focus for *nub* activity in the hinge region of the wing disc. These findings suggest the existence of a previously unrecognized growth control center in the wing hinge which functions independently of the well characterized A/P and D/V organizers.

MATERIALS AND METHODS

nubbin alleles

Two previously identified alleles of *nub* and deletion uncovering the gene are described by Lindsley and Zimm (1992). nub^1 was isolated as a spontaneous wing mutant and nub^2 is thought to have been X-ray induced, although this is not certain. Df(2L)$prd^{1.7}$ deletes the interval 33B2-3; 34A1-2, which includes the *nub* locus. Df(2L)*Prl* deletes the interval 32F1-3; 33F1-2. The proximal breakpoint apparently directly affects the *nub* gene. Three new alleles named nub^{HX1}, nub^{HX2} and nub^{HX5} were generated by X-ray mutagenesis. Males from an isogenized line carrying the recessive markers *cn sca bw* were irradiated (115 kV, 5 mA for a total dose of 4000 Rad, using a Torrex 120/150D X-ray source) and mass mated to nub^2 *b*/CyO females. New *nub* alleles were identified by the wing phenotype in F_1 progeny from this cross. Since the nub^2 *b* tester allele is viable over a large deletion, alleles of all types, including nulls, can be recovered in this screen. HX1 behaves genetically as a hypomorph. HX5 is associated with a visible deletion (cytology Df(2L)33A;33F). The w^+ P-element cosmid 18cos402 was introduced into the germ line of w^{1118} flies by standard P-element mediated transformation (Rubin and Spradling, 1982).

Clonal analysis

Somatic mosaic clonal analysis was carried for both nub^1 and nub^2. To give the *nub* mutant clones a growth advantage, homozygous clones were induced in larvae heterozygous for *nub* allele (cytological map position 33F1-2) and a chromosome carrying the dominant Minute mutation M(2L)24F and marked with a f^+ duplication (located at 24F and 30B respectively; see Diaz-Benjumea and Hafen, 1994). Clones of cells in which recombination occurs proximal to 33F will be homozygous for the *nub* mutation and will lack both the recessive f^+ marker and the Minute. These cells will have a relative growth advantage (Morata and Ripoll, 1975). Clones were induced by irradiating larvae at 60±12 and 84±12 hours AEL, Minute time (corresponding to second instar; 115 kV, 5 mA for a total dose of 1000 Rad). Adult cuticle preparations were as described by Diaz-Benjumea and Hafen (1994).

Summary of results from the nub^1 clonal analysis: 42 clones include the hinge region; 32 clones do not include the hinge region; 7 clones without *nub* phenotype. Summary of results for nub^2 clonal analysis: 22 clones include the hinge region; 11 clones do not include the hinge region; 3 clones without *nub* phenotype. Clones in the hinge region are small in size, compared to wild-type clones, and show non-autonomous reduction of the wing. All of the clones in the hinge region show a non-autonomous effect on wing size. Clones not in the hinge region are also small in size, but do not affect wing size or shape. Approximately 10% of *f* mutant clones appear to be wild-type for *nub*. Since the meiotic separation between the f^+ transgene and *nub* is 12%, this class probably represents mitotic recombination between the two loci. Clones in this class are on average larger in size than *nub* mutant clones and do not affect wing development.

Histochemical methods

In situ hybridization to imaginal discs using DNA and RNA probes was carried out as described by Tautz and Pfeifle (1989) and according to manufacturers instructions (Boehringer Mannheim). To compare labeling of wild-type and mutant discs, fixation, hybridization and labeling reactions were carried out in one sample containing animals of both genotypes. The wild-type larvae were dissected leaving the proventriculus attached. The proventriculus was removed from the mutant larvae, providing a reliable means to distinguish larval genotype after the reactions when the discs were dissected and mounted for microscopic examination. Acridine orange staining was done as described by Masucci et al. (1990).

Molecular methods

cDNA clones were isolated from an imaginal disc cDNA library (Nellen et al., 1994). Complete DNA sequence of the nubα cDNA, partial sequence of nubβ and genomic sequence of nub^1 are available on request. Genomic libraries were made from 3 *nub* alleles: Df(2L)*Proxless*/CyO, nub^1 *b pr*, and nub^2 *b* using standard methods (Ausubel et al., 1994). Phage clones were isolated from the *Proxless* library using a DNA probe from phage F4.5 from the *spalt* walk (Frei et al., 1988). To distinguish between clones deriving from the *Proxless* chromosome (which span the breakpoint) and those deriving from CyO, the isolated phage were tested for hybridization to a DNA probe from P1 phage no. 20-43 (mapped to 33F1-2 by the Drosophila Genome Center). Since the nub^1 and nub^2 libraries are from homozygous flies, all clones derive from the mutant chromosome. All other molecular cloning was carried out following standard procedures (Ausubel et al., 1994).

RESULTS

The adult wings of *Drosophila* develop from imaginal discs. The disc primordia are established as small clusters of cells straddling the parasegment boundary of the embryonic ectoderm, from which they inherit the subdivision into anterior and posterior compartments (reviewed in Cohen, 1993). A second subdivision establishes the dorsal and ventral compartments during the second larval instar (García-Bellido et al., 1973, 1976; Morata and Lawrence, 1979) and is required for continued growth and patterning of the wing (Diaz-Benjumea and Cohen, 1993; Williams et al., 1994). By the third instar, the disc has grown to about 50,000 cells and the spatial pattern of the adult wing has been established. The organization of the wing disc and its relationship to the structure of the adult wing is presented schematically in Fig. 1. The presumptive adult wing blade forms from the central fold of the disc epithelium, called the wing pouch (black shading, Fig. 1), while the wing hinge forms from the surrounding folds.

The *nubbin* mutant phenotype

The *nubbin* gene (*nub*) was originally identified as a viable spontaneous mutation which resulted in a dramatic reduction in the size of the wings and halteres, but which did not otherwise affect adult morphology, viability or fertility (Lindsley and Zimm, 1992). Fig. 2 presents a phenotypic series of defects observed in *nub* mutant alleles. In flies homozygous for the strongest viable allele, nub^1, the wing blade is reduced to a tiny stump and the wing hinge is partially deleted (Fig.

2E). A small amount of residual wing tissue is fused to the proximal hinge. Although the wing is reduced to a fraction of its normal size, a portion of the wing margin is still present (small arrows). The severity of the homozygous *nub*^{1} phenotype is increased slightly when *nub*^{1} is heterozygous with a deletion, suggesting that the *nub*^{1} allele retains some residual *nub*$^{+}$ function (not shown). *nub*^{2} homozygous flies show a weaker version of the same phenotype (Fig. 2D). *nub*^{2} wings show a similar deletion of the hinge region as *nub*^{1}, but a less severe reduction of the wing blade. The wing margin is reproducibly interrupted at the tip of the wing. Weaker mutant combinations lack the same set of hinge structures, but show still less severe reduction in the size of the wing blade and a relatively normal wing margin (Fig. 2C).

The *nub* phenotype is distinctly different from the wing scalloping observed in flies mutant for D/V patterning genes, including *apterous*, *vestigial* and *scalloped*, where wing structures are preferentially lost from the margin without affecting the internal pattern of wing veins (see e.g. Butterworth and King, 1965; James and Bryant, 1981; Campbell et al., 1992). In support of this view, *nub* mutants do not show any genetic interaction with genes in the D/V patterning system, including *apterous, Beadex, cut, Lyra, Notch, scalloped, Serrate, spade, vestigial*, and *wingless* (*apterous, cut, scalloped, spade* and *vestigial* were tested as double homozygotes with *nub. Beadex, Lyra, Notch, Serrate* and *wingless* were tested as heterozygotes, data not shown). *nub* mutant wings also differ from those of A/P patterning mutants including *fused*, where structures are preferentially lost along an axis parallel to the A/P compartment boundary (Fausto-Sterling, 1978). The *nub* phenotype is more consistent with a requirement for *nub* activity in the wing blade, which does not depend directly on the activity of either the A/P or D/V compartment boundaries.

Molecular cloning of *nubbin*

As a first step toward analyzing *nubbin* function, we made use of a chromosomal rearrangement to isolate the *nub* locus (see Fig. 3A). Flies heterozygous for Df(2L)*Proxless* show a dominant phenotype involving loss of structures from the wing hinge and reduction of the wing blade (Fig. 2B). The dominant *Proxless* phenotype exhibits a mixture of the features of a strong *nub* hypomorphic phenotype in the hinge region and a weak hypomorphic phenotype in the wing blade. When crossed to viable alleles of *nub*, the *Proxless* deletion behaves genetically as a simple lack of function mutation at the *nub* locus (Fig. 2F). Since another large deletion, which uncovers *nub*, Df(2L)*prd*$^{1.7}$, does not show any dominant phenotype, we conclude that (1) the *nub* gene is not haploinsufficient and (2) the breakpoint of the *Proxless* deletion must directly affect the *nub* transcription unit.

DNA from the *nub* locus was isolated by cloning the *Proxless* deletion breakpoint. The DNA fragment spanning the deletion breakpoint was shown to hybridize to a cosmid which includes the *pdm-1* gene, a member of the *Drosophila* POU-domain gene family (Fig. 3; Billin et al., 1991; Lloyd and Sakunju, 1991; Dick et al., 1991; Affolter et al., 1993). *pdm-1* is the only transcription unit in the cosmid that is expressed in a spatially restricted pattern in the wing (see legends to Figs 3 and 4). Characterization of cDNA clones revealed two alternate forms of the *pdm-1* transcript, which differ in their first exon, and which are predicted to produce proteins that differ at their amino termini (α and β; summarized in Fig. 3B). In situ hybridization using exon specific probes showed that both transcript forms are expressed in the wing disc (not shown).

molecular characterization of *nubbin* mutations

Detailed analysis of genomic DNA from the *Proxless* chromosome indicated that the mutation is associated with a complex rearrangement of the structure of the *pdm-1* tran-

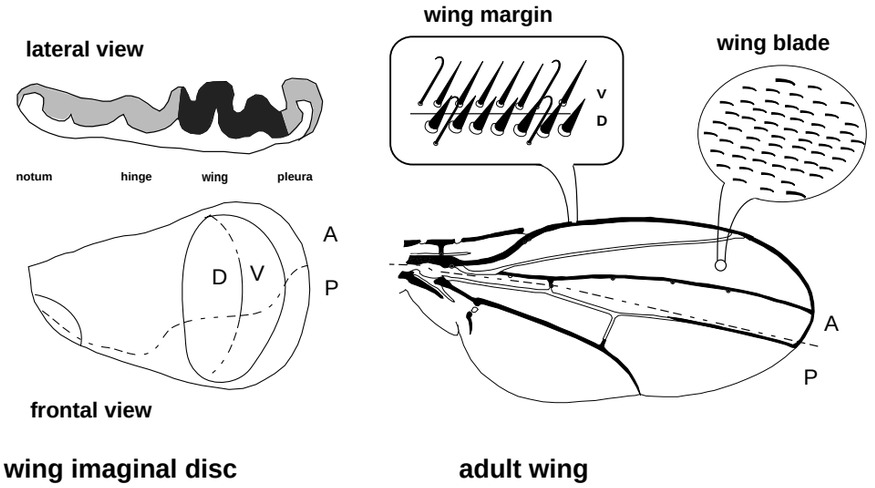
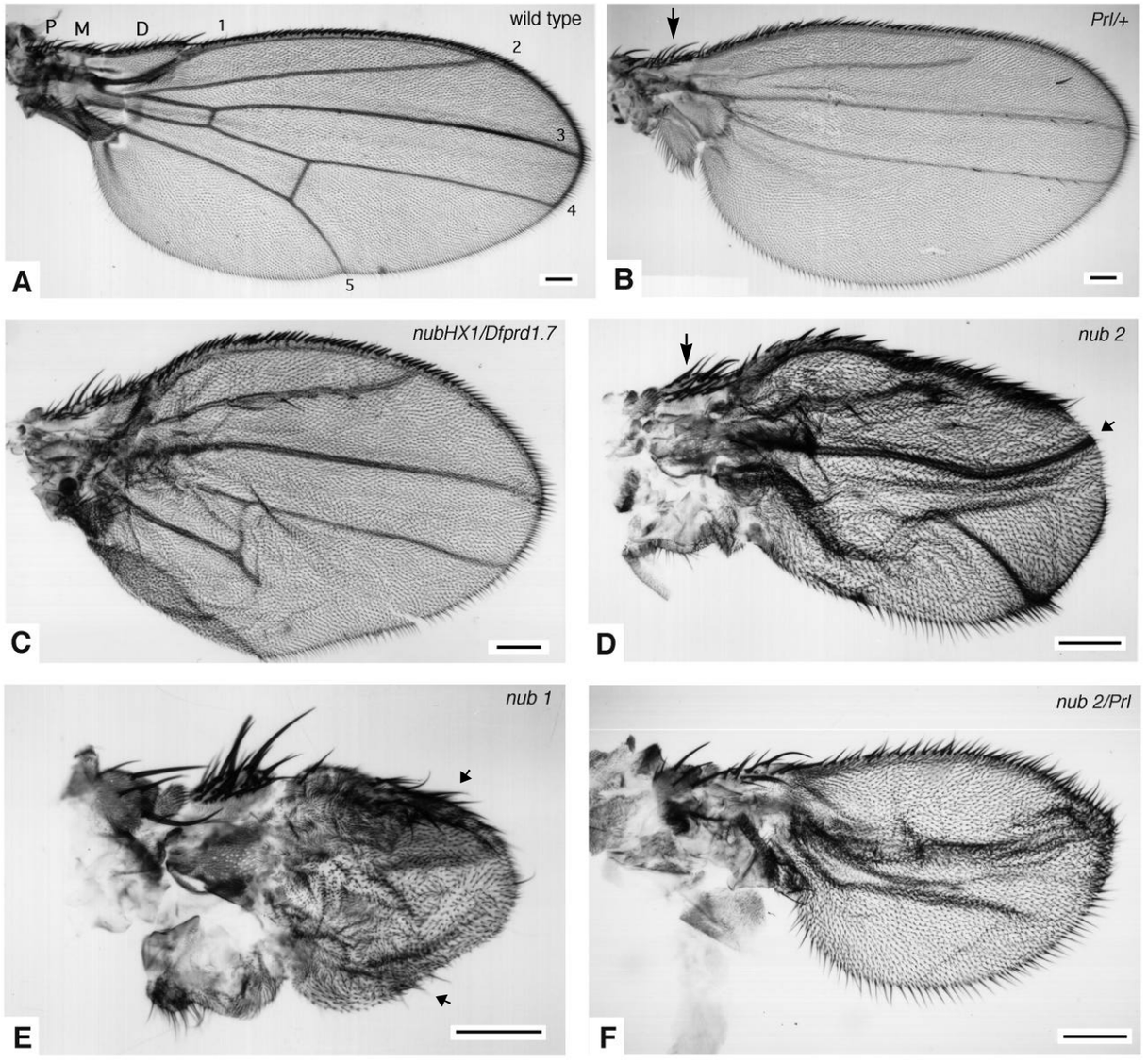


Fig. 1. Schematic representation of the wing imaginal disc and its relationship to the adult wing. The mature wing imaginal disc is a single layered epithelial sac. By the third larval instar the two sides of the sac have differentiated to form a thick pseudostratified epithelium on one side (light shading in lateral view) and a thin squamous epithelium on the other (depicted as a thin line). During growth of the disc the pseudostratified epithelium is thrown into a series of folds which project into the lumen of the disc. These folds will form the wing blade, the hinge and most of the thoracic body wall. The squamous layer is not shown in the frontal view. The central fold of the disc, called the wing pouch (dark shading), corresponds to the adult wing blade (depicted at right), while the surrounding region forms the hinge. The boundary between the dorsal and ventral compartments (D and V) bisects the wing pouch (indicated by a dashed line). This boundary forms the wing margin. The position of the anterior and posterior compartment boundary is also shown (A and P). The A/P boundary bisects the adult wing between the 3rd and 4th veins, but does not correspond to a morphologically distinct landmark (dashed line at right). The convention for numbering wing veins is indicated on the diagram. Vein L1 runs along the anterior edge of the wing, adjacent to the wing margin, and so is not shown as a separate structure. The wing fate map is drawn according to Bryant (1975). For a more detailed description of wing development see (Cohen, 1993; Fristrom and Fristrom, 1993).

scription unit. DNA has been deleted both upstream and downstream of the *pdm-1* transcription unit, and the remaining portion of the *pdm-1* transcription unit in the *Proxless* chromosome is inverted with respect to wild-type (summarized in Fig. 3). As a consequence of this rearrangement, the entire protein coding region of the nubβ form of the transcript (which begins in the second exon) is still present in the *Proxless* chromosome, but both of the identified alternative first exons, and presumably the transcription start sites have been deleted. Consistent with the nature of this rearrangement, *Proxless* behaves as a loss of function mutation when crossed to recessive *nub* mutants (see Fig. 2F). The *Proxless* mutant also has a dominant phenotype. This property might result from a gene fusion which links the coding region of *pdm-1* to another transcription unit. Rearrangements of this sort have been associated with a dominant mutant of *Antennapedia* (Frischer et al., 1986; Schneuwly et al., 1987). DNA sequence analysis of the junction fragment showed that the *Proxless* breakpoint is also associated with insertion of a B104 retrotransposon into the 3′-untranslated region of the *pdm-1* transcription unit (Fig. 3B; Scherer et al., 1982). B104 transposons have been implicated in the generation of dominant mutations associated with chromosomal rearrangements (Swaroop et al., 1985; Tsubota et al., 1989).

Molecular characterization of the adult viable alleles, *nub*^{1} and *nub*^{2} shows that both are associated with rearrangements in the DNA near the 5′-end of the *pdm-1* transcription unit (Fig. 3B). *nub*^{1} is a spontaneous mutation associated with an insertion of a 412 retrotransposon near the first exon of the nubα transcription unit (within the first intron of the nubβ transcription unit). Insertion of mobile elements is often associated with spontaneous mutations in *Drosophila* (Ashburner, 1989). The *nub*^{2} mutation is associated with a rearrangement in the genomic DNA, approx. 5 kb upstream from the first exon of



the nubβ cDNA. These observations raised the possibility that *nub*^{1} and *nub*^{2} might be regulatory mutations, which affect the expression of the *pdm-1* transcription unit (see Fig. 4).

The *pdm-1* transcription unit encodes *nub* gene function

The *pdm-1* transcription unit is expressed in the developing wing primordium beginning in the second larval instar (not shown). In the third instar the domain of expression fills the wing pouch (Fig. 4A). In the everted pupal wing disc, it is apparent that *pdm-1* expression fills the wing blade and at least part of the hinge region of the wing (Fig. 4B). This domain of expression correlates well with the regions of the wing that are affected in *nub* mutants. *pdm-1* is also expressed in the corresponding region of the haltere (not shown) and in a series of faint rings in the leg disc (Fig. 4C).

To determine whether *pdm-1* expression was affected in *nub* mutants, discs from homozygous *nub*^{1} and *nub*^{2} mutant larvae were labeled by in situ hybridization. In order to permit a direct comparison, the wild-type wing disc in Fig. 4A was labeled together in a single reaction mix with the *nub*^{1} mutant discs shown in 4E. *pdm-1* expression is specifically down-regulated in the wing and haltere discs of the *nub*^{1} mutant (using expression in the legs disc as an internal control). We were unable to detect *pdm-1* transcript in the wing pouch, indicating a strong reduction in the level of expression of the transcript in the *nub*^{1} mutant wing discs. The mutant wing pouch appears flatter and less folded due to the decrease in the size of the presumptive wing blade and hinge in the disc. There is some residual *pdm-1* expression in the *nub*^{2} discs (Fig. 4E), consistent with the observation that *nub*^{2} shows a weaker mutant phenotype than *nub*^{1} (Fig. 2). However the level is clearly reduced compared to the control. In both cases, the intensity of labeling of the mutant leg discs is comparable to that to the wild-type control (Fig. 4C).

As an independent test to ask whether the *pdm-1* transcription unit encodes *nub* gene function, we undertook to rescue the *nub* mutant phenotype by introducing the *pdm-1* gene into *nub* mutant flies as a P-element cosmid. Transgenic flies carrying one copy of the *pdm-1* cosmid show significant rescue of the *nub*^{1} and *nub*^{2} mutant phenotypes (Fig. 5). The size of the wing blade is substantially increased compared to those of the homozygous mutant without the transgene. There is also a significant rescue of the wing hinge structures.

In summary, three lines of evidence suggest that the *pdm-1* transcription unit encodes *nub* gene function. First, the cosmid containing *pdm-1* rescues the *nub* mutant phenotype. Second *pdm-1* expression is specifically down-regulated in the wing blade in two adult viable *nub* mutations. Third, three independent *nub* alleles show chromosomal rearrangements which involve the *pdm-1* transcription unit. Taken together these observations indicate that *nub* mutations are caused by defects in the *pdm-1* transcription unit. We refer to this transcription unit as *nub*.

Localized requirement for nub function in the wing hinge

A number of wing mutants, including *scalloped* and *vestigial*, show loss of wing tissue due to abnormal cell death (James and Bryant, 1981; Williams et al., 1993). Acridine orange staining shows a local concentration of dying cells in the wing hinge region of the *nub* mutant discs, but no substantial increase in cell death throughout the wing blade (Fig. 4H). Localized cell death in the hinge region is correlated with a reduction in the intensity of the rings of *wingless* expression in the hinge in the recessive allele *nub*^{1} (Fig. 4F,G) and in the dominant *Proxless* allele (not shown). A phenotypically similar deletion of hinge structures can be caused by late removal of *wg* function in the third larval instar, using a temperature sensitive allele of *wg* (Couso et al., 1994). These observations suggest that the deletion of structures in the hinge reflects a requirement for *nub* function mediated through localized activation of *wg* expression.

Fig. 2. The *nubbin* mutant phenotype. (A) Cuticle preparation of a wild-type wing. The wing blade is a large flat structure composed of two apposed epithelial cell layers (dorsal and ventral). The wing blade is subdivided by veins (numbered 1-5), which provide mechanical support and serve as pathways for neurons. At this magnification the first vein cannot be seen as a distinct structure since it runs along the anterior edge of the wing adjacent to the dorsal-ventral boundary. In the anterior compartment the D/V boundary is marked by a distinctive triple row of sensory bristles (see diagram in Fig. 1). The wing hinge lies at the base of the wing wing blade. The epithelial sheet is highly folded in the hinge region. At the anterior edge of the wing the hinge region is subdivided into three domains called the proximal, medial and distal costa (indicated by P, M, D, nomenclature follows Bryant, 1975). The scale bars show the size of the proximal costa region to give an indication of the relative magnification of the wings in each panel. (B) Wing from a fly heterozygous for the dominant *nub* allele *Proxless* (*Prl*). Medial and distal costa structures, as well as other structure of the wing hinge have been deleted, producing a wing that is 'spliced' along the axis running from the body wall to the wing tip (proximal-distal; arrow = proximal costa). The size of the wing is slightly reduced, vein L5 is missing and vein L2 is interrupted distally. The region of the wing hinge affected in *Prl* is essentially identical to that observed in the hypomorphic alleles *nub*^{1} and *nub*^{2} (described in detail below), but the extent of reduction in the wing blade is less severe. The *Prl* phenotype demonstrates that the defect leading to the 'spliced' phenotype in the wing hinge can be partially separated from the reduction in size of the wing blade. (C) Hypomorphic mutant phenotype produced by the allelic combination of *nub*^{HX1}/Df(2L)*prd*$^{1.7}$ Note the intermediate reduction of the wing blade coupled with a strong 'spliced' phenotype, indicating loss of hinge structures. (D) *nub*^{2} homozygous mutant wing showing strong reduction of the wing blade and hinge region. Internal wing structures are reduced. Vein L5 is missing. Veins L2 and L4 are reduced, but vein L3 is present (small arrow). The wing margin is present, but reproducibly shows a small gap at the tip of the wing. The entire medial and distal costa are missing. The humeral crossvein and an associated dorsal sensilla campaniforma, a pair of dorsal sensillae campaniforma, which mark the vein L1 and the sc12 group of sensilla are missing, but the sc25 group is present. The alula is missing. The proximal costa is unaffected, as indicated by the characteristic arrangement of bracted bristles (arrow). The details described for the mutant phenotype in the hinge are not visible in the figure at this magnification. (E) *nub*^{1} shows an identical deletion of structures from the hinge, but a more severe reduction of the wing blade. (F) Flies heterozygous for *Proxless* and *nub*^{2} show a more severe phenotype that *nub*^{2} homozygotes. The severity of the *nub*^{2}/*Proxless* phenotype is equivalent to that obtained when *nub*^{2} is place over another large deletion (Df(2L) *prd*$^{1.7}$), which completely removes the *nub* region (not shown), suggesting that in addition to the dominant phenotype the *Proxless* deletion behaves as a lack of function mutant for *nub*.

Although reduction of *wg* activity can explain the requirement for *nub* in the hinge (Couso et al., 1994; Phillips and Whittle, 1993), removing *wg* activity during third instar does not lead to the global reduction in the size of the wing observed in *nub*^{1} and *nub*^{2} mutants. This is consistent with the observation that the severity of the *nub* phenotype in the wing blade does not correlate with the hinge phenotype (see Fig. 2). Since *nub* encodes a predicted transcription factor expressed throughout the wing pouch, we performed a genetic mosaic analysis to address whether there is a cell autonomous requirement for *nub* activity in growth of the wing (Fig. 6). *nub* mutant clones are considerably smaller than wild-type control clones, consistent with a defect in either cell proliferation or cell viability within the clone (cell sizes appear normal). However, the autonomous reduction in clone size is not sufficient to explain the overall reduction in

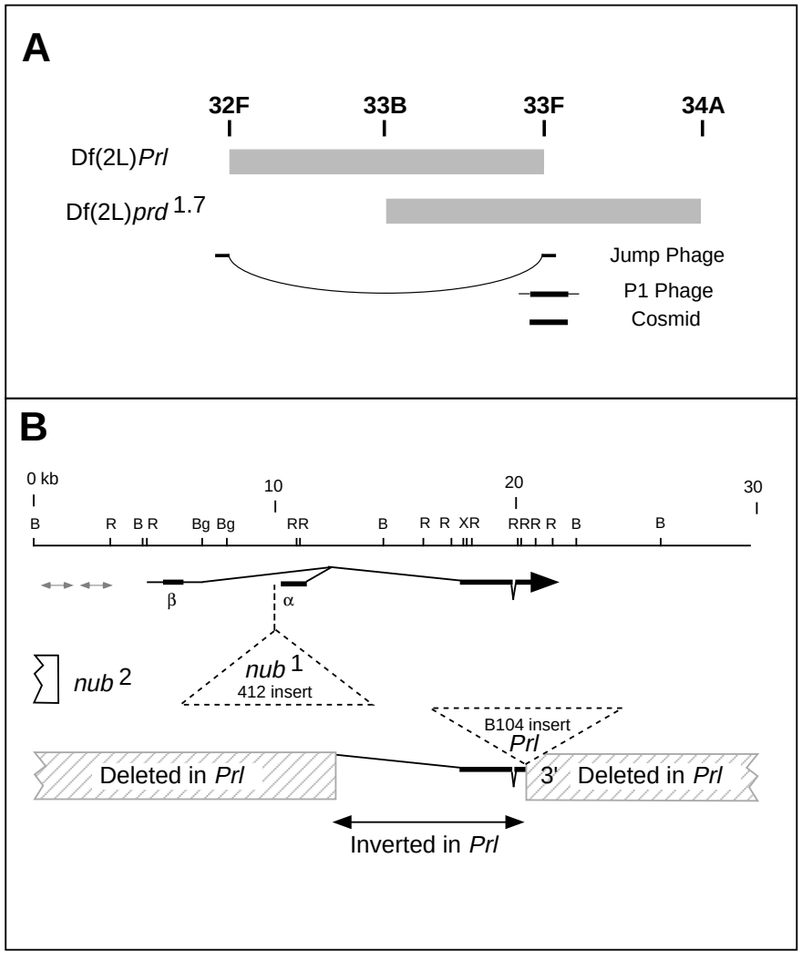


Fig. 3. Physical organization of the *nubbin* locus. (A) *nub* has been mapped genetically to the interval 33D-F on the basis of its inclusion in Df(2L)*Prl* and Df(2L)*prd*$^{1.7}$ and on its exclusion from Df(2L)64j (not shown; Lindsley and Zimm, 1992). The dominant phenotype of the *Proxless* deletion suggests that the *nub* locus is located at the deletion breakpoint. The *Proxless* deletion spans the cytogenetic interval from 32F1-3 to 33F1-2. The distal breakpoint of *Proxless* lies in a previously cloned region near the *spalt* gene (Frei et al., 1988). Using a DNA fragment from the *spalt* region we isolated genomic clones that span the deletion breakpoint in the *Proxless* mutant chromosome. The identity of the DNA from the proximal side of the *Proxless* deletion was confirmed by hybridization to a P1 phage clone covering the region 33F1-2, and to a cosmid from 33F1-2 that includes the *pdm-1* transcription unit (cosmid 18cos402) (Billin et al., 1991; Lloyd and Sakunju, 1991; Dick et al., 1991). (B) Molecular lesions associated with *nub* alleles. The structure of the *pdm-1* transcription unit is indicated below the physical map of the cosmid. The locations of the chromosomal rearrangements associated with *nub*^{1}, *nub*^{2}, Df(2L)*Prl* are shown. *nub*^{1} is associated with the insertion of 412 retrotransposon in the first intron of the nubβ transcription unit, near the first exon of nubα. Since the sequence of the genomic DNA from exons 2 and 3 of the *pdm-1* transcription unit from *nub*^{1} showed no significant alteration in the coding capacity of the transcript (data not shown), we infer that the insertion is responsible for the *nub*^{1} mutation. *nub*^{2} is associated with an undefined lesion located approx. 5 kb upstream from the first exon of the nubβ transcription unit (within the first kb of DNA at the left end of the cosmid). Df(2L)*Prl* contains a complex rearrangement. In addition to the large cytologically visible deletion, there has been an insertion of a B104 transposable element in the 3′-UTR of the transcript and an inversion (double-headed arrow). Genomic DNA 3′ to the insertion site is also deleted. The positions of exons 2 and 3 were confirmed by sequencing the genomic DNA from the *nub*^{1} mutant allele. The locations of exon 1 from nubα and from nubβ were determined by hybridization and by DNA sequencing of genomic DNA (nubα lies within 500 bp upstream from the *Eco*RI site at approx. 11 kb, near the 5′ end of the *pdm-1* cDNA; Exon 1 of nubβ is located within the indicated 2.2 kb R-Bg fragment; not shown to scale). Based on the sequence of genomic DNA, we find no evidence for the existence of the small 2nd exon described by Cockerill et al. (1993). Both classes of transcript use a common splice acceptor site for exon 2. Although the open reading frame begins with an in-frame methionine codon in the first exon of the embryonic cDNA (Cockerill et al., 1993), this is not the case for the nubβ class of imaginal disc cDNAs. The first possible translation start in nubβ is located in the 2nd exon. Thus it is possible that two different forms of the protein product are made in the discs. The *Bam*HI fragments of the cosmid were used as probes to screen for transcripts expressed in the wing disc. The 8 kb *Bam*HI fragment containing most of the *pdm-1* transcription unit (coordinates +15 to +23) hybridizes strongly to the wing disc. The adjacent 11 kb fragment (coordinates +4 to +15) containing the first exons of nubα and nubβ hybridizes weakly in the same pattern (data not shown). Small fragments containing the first exon of nubβ or of *pdm-1* each hybridize to the wing disc, indicating that both forms of the transcript are expressed in the disc. None of the other fragments show significant expression in the wing disc. Screening of cDNA libraries revealed the presence of two additional small transcription units in the 4.5 kb *Bam* fragment upstream from *pdm-1*. Complete restriction maps are available on request. Abbreviations: (B) *Bam*HI; (Bg) *Bgl*II; (R) *Eco*RI; (X) *Xho*I.

the size of the mutant wings, since *nub*^{1} mutant clones typically grow to occupy a territory larger than the entire wing of a homozygous *nub*^{1} mutant (compare Fig. 6A,C with Fig. 2E).

In spite of the fact that *nub* is expressed throughout the presumptive wing, we observed a region-specific difference in the ability of mutant clones to grossly perturb wing development. Clones which occupy a portion of the wing hinge region cause a non-autonomous reduction in the size of the wing (e.g. Fig. 6B-D). Although it is difficult to define a precise boundary line between regions in which the clones behave autonomously and non-autonomously, clones which include a portion of the region delimited by the proximal, medial and distal costa on the anterior side and the alula on the posterior side cause non-autonomous phenotypes (indicated by brackets in Fig. 6A). Even relatively small mutant clones in the hinge region can cause non-autonomous loss of wing tissue (Fig. 6B). However, larger clones that include the hinge but that also fill a portion of the wing blade produce a stronger reduction of the wing (compare Fig. 6B with C and D). By contrast, relatively large clones, which do not enter the hinge region, do not cause any obvious reduction of the wing blade (Fig. 6A), even though such clones may cross the D/V boundary. The extent of the non-autonomous reduction caused by clones in the hinge region can be quite substantial (compare the affected wing in Fig. 6D with the bottom edge of a normal wing at the top of the frame).

Comparison with other mutations that affect clone size indicates that the non-autonomous effects of *nub* mutant clones cannot simply be attributed to under-proliferation in the hinge region. Typically clones that have a growth disadvantage do not affect the overall size or shape of the wing (e.g. clones of cells mutant for the EGF receptor; Diaz-Benjumea and García-Bellido, 1990). Growth of the surrounding wild-type cells is increased in proportion, to compensate for the small size of the mutant clone, producing a wing of normal size and shape. Similarly, heterozygous Minute cells typically under-proliferate to compensate for the large size of Minute^{+} clones, which have a relative growth advantage (Morata and Ripoll, 1975; Simpson and Morata, 1981).

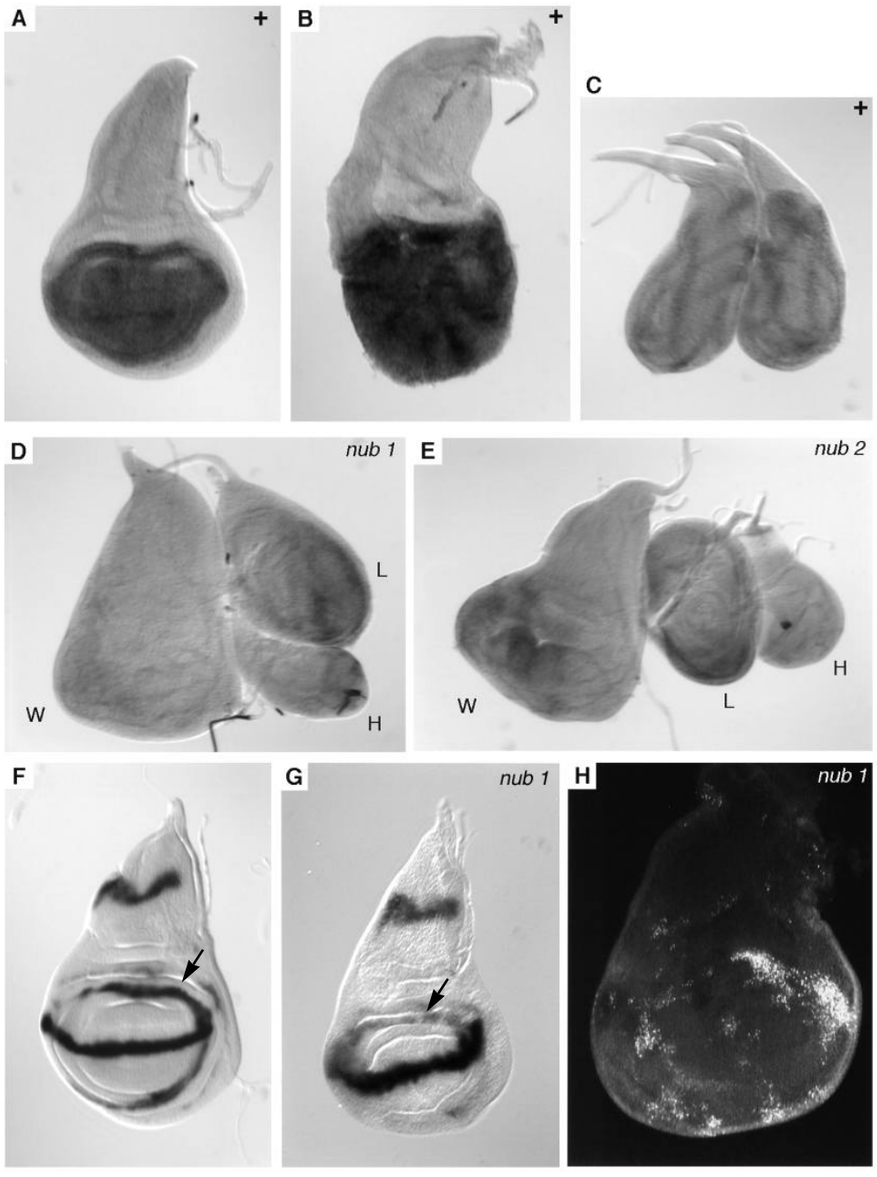


Fig. 4. Expression of the *pdm*-1 transcription unit in imaginal discs. (A) Wing imaginal disc from a wild-type third instar larva. (B) Early pupal wing disc, wild-type. (C) Leg discs from wild-type larva. (D,E) Leg, wing and haltere discs from larvae homozygous mutant for *nub*^{1} and *nub*^{2} respectively. Note the absence of labeling in the wing disc of *nub*^{1} in D and the reduced level in the wing disc of *nub*^{2} in E. In both cases the low level of labeling in the leg discs is unaffected. To permit a direct comparison, the fixation, hybridization and labeling reactions were carried out with mutant and wild-type discs processed together in a single sample. The wild-type wing in A and the legs in C were processed together with the *nub*^{1} mutant discs shown in D (see Materials and methods for details). (F,G) *wg-lacZ* expression in wild-type (F) and *nub*^{1} mutant wing discs (G). Note the strong reduction of *wg* expression in the peripheral ring that outlines the wing hinge (arrows in F and G). (H) Acridine orange staining of a *nub*^{1} mutant wing disc reveals abnormally high cell death in the region of the wing hinge (brightly stained cells), but only isolated dying cells in the wing pouch.

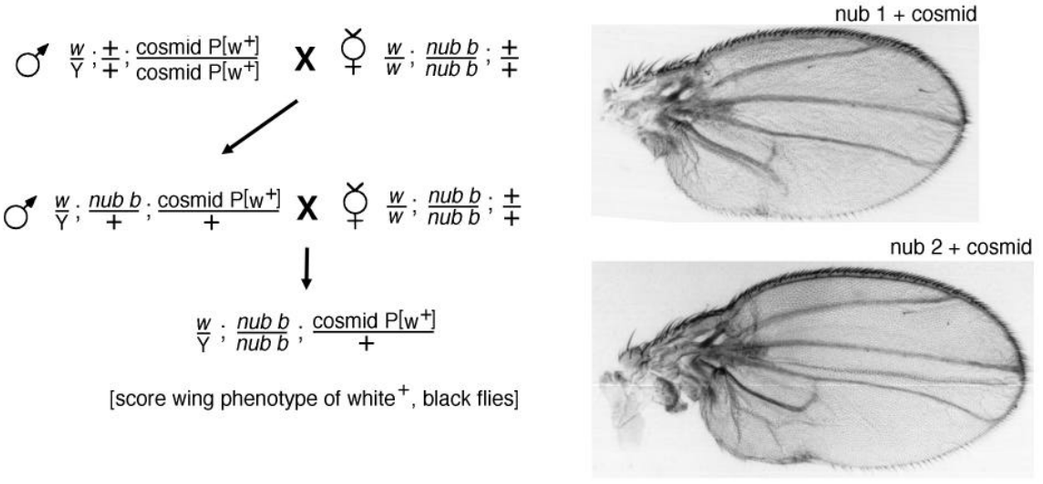


Fig. 5. The *pdm-1* cosmid rescues the *nub* mutant phenotype. The scheme outlining the genetic crosses used to introduce the cosmid transgene into the *nub* mutant background is shown on the left. nub^1 and nub^2 mutant phenotypes are significantly rescued by one copy of the cosmid (compare with nub^1 in Fig. 2E and nub^2 in Fig. 2D). Introducing an additional copy of the cosmid does not improve the rescue (data not shown), suggesting that regulatory elements may be missing from the cosmid. This suggestion is consistent with the location of the molecular lesion in the nub^2 chromosome at the extreme end of the cosmid (see Fig. 3B).

Although *nub* transcript is widely distributed throughout the wing pouch, these observations suggest a localized requirement for *nub* activity near the base of the wing. *nub* activity is apparently required in the hinge region to control the growth and patterning of the entire wing. These findings suggest the existence of a previously unrecognized growth control center located in the proximal region of the wing, which is not related to either the A/P or D/V compartment boundary organizers.

DISCUSSION

The relationship between mutant phenotypes and the patterning activity of compartment boundaries

On the basis of a common mutant phenotype several genes have been implicated in mediating the activity of the D/V organizer system. The D/V boundary of the imaginal disc corresponds to the future wing margin, located at the perimeter of the adult wing (illustrated in Fig. 1). Weak mutations in the *apterous* gene cause loss of structures from the outer edges of the wing without perturbing the internal organization of the wing blade, while stronger lack of function mutations delete the entire wing (Butterworth and King, 1965; Cohen et al., 1992). Mutations in the *vestigial*, *scalloped*, *Serrate*, *cut* and *wingless* genes can produce a comparable range of phenotypes under appropriate conditions (James and Bryant, 1981; Campbell et al., 1992; Williams et al., 1993; Speicher et al., 1994; Couso et al., 1993, 1994). Taken together with the observation that the domains of expression of these genes are defined with reference to the D/V boundary (Baker, 1988a; Campbell et al., 1992; Williams et al., 1993, 1994; Couso et al., 1993, 1994; Speicher et al., 1994), the similarity in mutant phenotypes suggests that the spatial focus for the activity of these genes is located at the D/V boundary.

Mutations in genes that mediate the activity of the A/P compartment boundary organizer produce a distinctly different range of phenotypes, in which the defects are focused on the A/P compartment boundary. Removing *hedgehog* activity from the posterior compartment of the wing disc leads to almost complete loss of the adult wing (Basler and Struhl, 1994). The tiny wing remnant has a complete wing margin, suggesting that structures are lost from the internal region of the wing, rather than from the D/V boundary. Consistent with this interpretation, *fused* mutant wings lack structures near the A/P compartment boundary, producing an interstitial deletion (Fausto-Sterling, 1978). *fused* is thought to mediate the activity of *hedgehog* signaling in the embryo (Ingham, 1993) and its adult mutant phenotype suggest that it performs a similar function in the wing disc. Localized expression of *decapentaplegic* is thought to mediate the patterning activity of the A/P boundary organizer (Basler and Struhl, 1994; Capdevila et al., 1994; Capdevila and Guerrero, 1994; Diaz-Benjumea et al., 1994). Clones of cells lacking *dpp* activity, which abut the A/P boundary from the anterior side, also cause interstitial deletion of wing structures, consistent with a focus of requirement along the A/P boundary (Posakony et al., 1991).

Evidence for a proximal-distal organizing center in the wing imaginal disc

The defects observed in *nub* mutant wings cannot be easily explained in terms of the activity of either the A/P or D/V organizer systems. Analysis of genetic mosaics has emphasized that the focus of requirement for genes mediating the activity of the A/P and D/V patterning systems is located near the respective compartment boundaries. Clonal analysis of *vestigial* and *scalloped* mutants shows a localized requirement for the activity of these genes at the wing margin (Simpson et al., 1981). Clones of cells that do not meet the margin do not cause wing scalloping. Similarly, *fused* gene activity is only required in anterior cells located near the A/P compartment

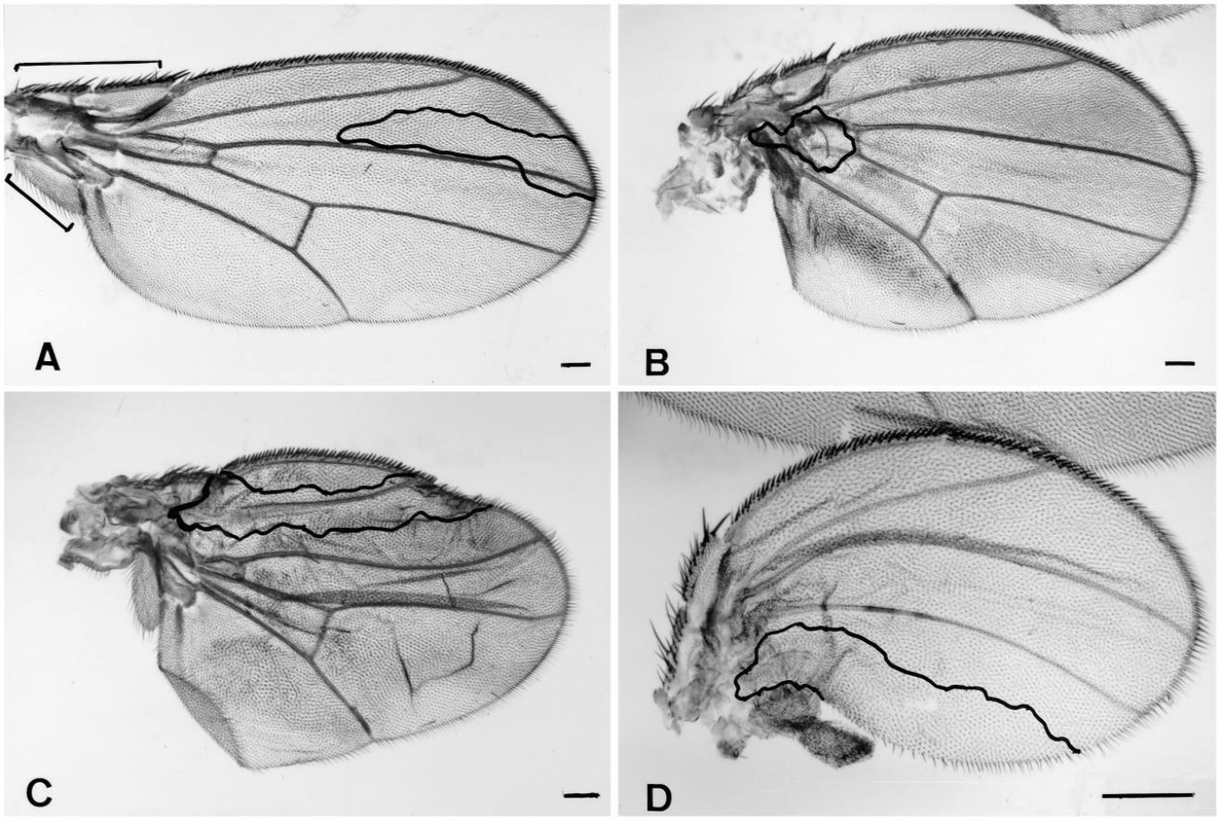


Fig. 6. Clones of *nub* mutant cells in the wing. (A-C) Clones of cells homozygous mutant for nub^1. (D) Clone of cells mutant for nub^2. Clones of mutant cells were genetically marked with the cell autonomous marker *forked*. The location of the clones are outlined, since the difference between mutant and wild-type cells cannot be seen at this magnification. Scale bars are provided to allow a comparison of the relative sizes of the wings. Details on clone frequency are in materials and methods. (A) Large clone in the dorsal anterior compartment which does not enter the hinge region. This clone does not affect the growth or pattern of the wing. Clones of this type are smaller than wild-type clones and often show minor abnormalities, including extra bristles along the wing veins and wing margin and thicker veins. Comparable minor defects in bristle pattern and vein structure are seen in the wings of homozygous *nub* mutants (see Fig. 2). (B) Small clone in the dorsal compartment of the wing hinge causes a substantial reduction in the size of the wing. (C) Large clone including the hinge region of the anterior compartment. The clone crosses the D/V boundary and covers roughly comparable areas in the dorsal and ventral compartments (only dorsal is outlined). The clone causes an extensive reduction of the anterior compartment. (D) Large nub^2 mutant clone including a portion of the hinge region in the posterior compartment. The clone covers roughly comparable areas in the dorsal and ventral compartments (only dorsal is outlined).

boundary (Fausto-Sterling, 1978). By contrast, the clonal analysis reported here shows a localized requirement for *nub* gene activity near the base of the wing. Clones of *nub* mutant cells in the hinge region cause a non-autonomous loss of tissue from the wing blade, while clones in other regions do not grossly perturb wing development. The combination of a localized requirement for *nub* activity together with a non-autonomous phenotype suggests that the wing hinge region may serve as a growth control center, required in addition to those generated by the A/P and D/V compartment boundaries.

Possible roles of *nubbin* in wing development

A number of parallels with the D/V patterning system suggest that *nub* may be involved in the activity of the proposed proximal-distal growth control center in the wing hinge region. The D/V compartment boundary is established in the second instar wing disc (García-Bellido et al., 1973, 1976; Morata and Lawrence, 1979) by localized expression of the selector gene *apterous* in the dorsal compartment (Diaz-Benjumea and Cohen, 1993). Interaction between dorsal and ventral cells directs expression of *vestigial* and *scalloped* at the wing margin (Williams et al., 1993, 1994). At roughly the same stage another boundary of cell lineage restriction subdivides the wing disc along the proximal-distal axis into compartments that correspond to notum and wing blade (García-Bellido et al., 1973, 1976). Localized expression of *nub* begins in the second instar wing disc, in a domain which corresponds to the wing pouch.

In this context we can consider two possible roles for *nub*: (1) nub might function as a downstream mediator of the activity of a wing hinge organizing center, analogous to *vestigial* and *scalloped* in the D/V system or (2) *nub* might serve as a selector gene for the wing (as opposed to notum). Although the behaviour of nub^1 mutant clones favors the first alternative, we cannot formally exclude the second alternative.

Clonal analysis using a complete lack of function mutation of *nub* could, in principle, distinguish between these possibilities. If the proximal-distal patterning system functions like the D/V system, such clones would be expected to cause a cell autonomous transformation in fate between compartments and to lead to formation of an ectopic organizing center (see Diaz-Benjumea and Cohen, 1993; Blair et al., 1994; Williams et al, 1994). Unfortunately, a null allele of *nub* is not available. In addition, *nub*, like *engrailed*, has a closely related homolog, *pdm-2* (or *miti-mere*) located nearby (Coleman et al., 1987; Lloyd and Sakonju, 1991; Billin et al., 1991; Dick et al, 1991; Cockerill et al., 1993; Bhat and Schedl, 1994), which is also expressed in the wing disc (not shown). Although *engrailed* is generally accepted to function as a selector for the posterior compartment, clones of cells lacking *engrailed* do not make an ectopic A/P organizer (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Lawrence and Struhl, 1982). It is possible that clones of cells simultaneously lacking the activity of both *engrailed* and *invected* or the activity of both *nub* and *pdm-2* would have a more dramatic effect.

We gratefully acknowledge the technical contributions of Christine Pfeifle, who participated in cloning the breakpoint of the *Proxless* deletion and in isolating the *nubbin* cDNAs, and of Sandra Sciani-manico who generated the transgenic flies carrying the *pdm-1* cosmid. We thank Reinhard Schuh for providing DNA clones from the *sal* region which were used to clone the breakpoint of the *Proxless* deletion. We thank Shige Sakonju for communicating unpublished results and for providing the *pdm-1* P-element cosmid (18cos402) used in the rescue experiment, and the Drosophila Genome Center at LBL for providing the P1 library to the EMBL.

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Note added in proof

Recent results using anti-nubbin antibody show a low, but detectable level of nub protein in the nub^1 mutant wing disc. This level of nub expression was below the detection level of our in situs.