Mutation of *twins* encoding a regulator of protein phosphatase 2A leads to pattern duplication in *Drosophila* imaginal discs

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The *Drosophila* gene *twins* was identified through a P-element-induced mutation that caused overgrowth in posterior regions of the wing imaginal disc. Analyses using position-specific markers showed that the inactivation of this locus induced the formation of extra wing blade anlagen in the posterior compartment of the disc. The duplication was mirror symmetrical, and the line of the symmetry did not correspond to any of the known compartment borders. We isolated the *twins* gene and found that it encoded one of the regulatory subunits of protein phosphatase 2A (PP2A). These results suggest a novel aspect of physiological roles of protein dephosphorylation; that is, the control of PP2A activity is crucial for specification of tissue patterns.

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To elucidate how tissue-specific patterns generate from initially homogenous cell masses is one of the central issues in developmental biology, and *Drosophila* imaginal discs have been providing attractive model systems for such studies (for reviews, see Bryant 1978; Whittle 1990). Imaginal disc precursors arise from the ectoderm soon after the cellular blastoderm stage, and the primordia continue rapid proliferation during larval stages until the late third instar. The mature imaginal disc is essentially a monolayered epithelial sac. Although cells comprising the disc epithelium look similar, their differentiation fates are already diversified so as to produce an organ with a specific pattern. At metamorphosis, the pattern becomes visible by terminal differentiation of the cells into the exterior structures of the adult body. Topological relationships between adult cuticular structures and their origins in discs have been established. However, in contrast to the well-documented story of determination of the embryonic body axes, we have fragmentary information about the mechanisms of the pattern formation in imaginal discs.

Molecular basis for the pattern formation in imaginal discs has been investigated through isolation and characterization of mutations that alter morphology of the adult body (for reviews, see Whittle 1990; Wilkins and Gubb 1991). Genes that play vital roles in establishment of the body axes of the embryo are often recruited for the

formation of adult structures. Many of the segment polarity genes belong to such a class. In imaginal discs, particular segment polarity genes function for cells located in distinct spatial domains to acquire positional identities. For example, at early developmental stages, imaginal discs are divided into two compartments, anterior and posterior, and the compartment borders are stably maintained by the action of *engrailed* (*en*) throughout disc development (Morata and Lawrence 1975; Lawrence and Morata 1976; Kornberg 1981; Lawrence and Struhl 1982).

Besides the genes mentioned above, many others are assumed to operate on the specification of imaginal disc patterns. Such genes could be identified by screening pupal lethal mutants, because severe morphological defects in discs may cause malformation of adult structures. Pupal lethal strains have been screened for imaginal disc abnormalities (for review, see Shearn 1978). Several genes that are necessary for wing development were found and cloned, including *apterous*, *vestigial*, *defective dorsal discs*, and *abnormal wing disc*. Mutations in these loci lead to extensive cell death or blocking of cell proliferation in the wing discs (Butterworth and King 1965; Fristrom 1969; Whittle 1979; Simcox et al. 1987; Dearolf et al. 1988a,b; Williams et al. 1991; Cohen et al. 1992). The present study was aimed at the identification of novel genes involved in the pattern formation of the imaginal disc. To isolate such genes efficiently, we took advantage of a collection of single P element-induced lethal lines (Cooley et al. 1988a,b; Bier et al. 1989). We

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were able to obtain mutants in which growth of imaginal discs was not impaired, but discs became morphologically abnormal. Among them, we chose a particular mutant, whose locus we named *twins*, for detailed analyses. In this mutant, parts of the wing imaginal disc were duplicated in a mirror image fashion. We cloned the *twins* gene and found that the cDNA encoded a *Drosophila* homolog of one of the regulatory subunits of protein phosphatase 2A (PP2A), one of the major classes of serine/threonine phosphatases (for review, see Cohen 1989). Thus, our results suggest that regulation of protein phosphatase activity may play a critical role in the specification of imaginal disc patterns.

Results

Identification of the *twins* locus

The wild-type wing disc consists of a large central pouch, which is the precursor of the adult wing blade, and peripheral regions that give rise to more proximal thoracic structures, the hinge and the notum (Fig. 1A). Using a collection of lethal strains, we performed a screen for mutants that form morphologically aberrant imaginal discs in larvae. One of the mutants obtained was *lethal(3)11C8*. Larvae homozygous for this mutation had

wing discs that exhibited unusual outgrowth in the posterior compartment (Fig. 1B). On a morphological basis, the overgrown region resembled the wing pouch. This phenotype was highly penetrant, as shown by the observation that ~70% of the mutant wing discs showed the extra structures recognizable under a stereo dissecting microscope. Like the wing discs, haltere discs showed similar outgrowth in the mutants. In contrast, no evidence has been obtained for pattern abnormality in eye-antenna and leg discs.

lethal(3)11C8 had a copy of the enhancer-trap vector P-*lacW* (Bier et al. 1989) inserted into the cytological position 85F. Reversion tests suggested that the insertion was responsible for both pupal lethality and the disc phenotype (see Materials and methods). Because the mutant produced duplicated patterns in wing imaginal discs, as described below, the locus inactivated by the P insertion was named *twins*, and the original allele was designated *twins^P*.

Pattern duplication in *twins^P* imaginal discs

To study how patterns in wing imaginal discs were altered in *twins^P*, we employed two types of position-specific molecular probes. One was β -galactosidase (β -gal) expressed in specific patterns in discs owing to the insertions of *lacZ* into particular loci. The individual chromosomes carrying these *lacZ* insertions were introduced into a *lacZ⁻* derivative of *twins^P*, and β -gal expression patterns were visualized by X-gal staining. The second approach was to use antibodies that bind to the products of marker genes such as *en*.

The *lacZ* insertion strain ryXho38 reproduces the pattern of *scabrous* (*sca*) RNA expression in discs (Mlodzik et al. 1990; C. Hama and M. Hoshino, pers. comm.). β -Gal is expressed as scattered patches, each of which contains sensory mother cells (SMCs). In wild-type wing discs, a characteristic double array of the stained cells was observed along the transverse line in the anterior compartment of the wing blade anlage (Fig. 2A). These β -gal-positive cells label the presumptive anterior wing margin (Mlodzik et al. 1990). The mutant wing disc had an extra set of the double array of cells, as well as other patchy signals, in the overgrown area (Fig. 2B). Most of these extra signals appeared to be arranged in a mirror symmetry to the original ones. These observations suggested that the wing pouch region of the disc was duplicated in the posterior compartment and that the additional wing pouch may have both anterior and posterior compartments. The presumptive boundary between the two pattern copies (broken line in Fig. 2B) did not correspond to any of the known compartment boundaries. Use of anti-*achaete* antibody (Skeath and Carroll. 1991) as an independent marker for SMCs also confirmed the duplication of the wing blade anlage (data not shown).

In the wild-type wing disc, the *sca-lacZ* signals are considered to localize within the anterior compartment not only in the wing pouch but also in more proximal regions (Garcia-Bellido et al. 1976; Campuzano and Modolell 1992). Most of these anterior signals, if not all,

Figure 1. Morphology of wild-type and *lethal(3)11C8* mutant wing imaginal discs. Whole-mount photographs of wing imaginal discs. In this and subsequent figures, wing discs were isolated from wandering third-instar larvae and placed in such a way that the anterior compartment is to the left and the dorsal compartment is down. (A) Wing disc dissected from a wild-type (Oregon-R) larva. (W, H, and N) Approximate centers of the regions that give rise to the wing blade, hinge, and notum, respectively (Bryant 1978). (B) Wing disc isolated from a larva homozygous for the *l(3)11C8* mutation. The outgrowing region shown by the arrow looks like an extra wing pouch. This phenotype was induced by an insertion of P-element vector P-*lacW* which has the reporter gene *lacZ* (Bier et al. 1989). Both wild-type and mutant discs were stained with X-gal. In homozygous *twins^P* discs, cells in the overgrown area expressed *lacZ* more strongly than those in the original wing pouch. Bar, 100 μ m.

Figure 2. Duplication of the wing blade anlage as revealed with the *sca-lacZ* marker. An enhancer-trap line that reproduces *sca* expression was used to highlight the patterns of SMCs (Mlodzik et al. 1990; C. Hama and M. Hoshino, pers. comm.). Its *lacZ* expression was detected by X-gal staining. (A) Wild-type wing disc; (B) *twins* wing disc. Arrowheads indicate the *lacZ* expression pattern characteristic of the anterior compartment in the wing pouch; SMCs of the presumptive anterior wing margin are stained. The mutant produced an extra copy of the wing marker (arrow) that was placed in mirror symmetry to the original one. The broken line represents a hypothetical line of symmetry. Bar, 100 μ m.

appeared to be preserved in the mutant disc, although perfect assignment of individual signals was difficult (Fig. 2B). Thus, the formation of the anterior structures was probably not so much disturbed in the mutants, in contrast to the posterior compartment that was disorganized by the formation of the extra wing pouch.

To examine in more detail how the *twins* mutation disrupted imaginal disc patterns, we immunostained tissues for the *en* and *invected* (*inv*) products, as markers of the posterior compartment cells (Fig. 3A; Patel et al. 1989). The wing duplication in *twins* was associated with partial losses of the *en/inv* expression outside the wing pouch, as shown by two examples (Fig. 3B,C). In the case of Figure 3B, only a small area covering the posterior wing pouch expressed the marker genes, suggesting that the proximal domain of the posterior compartment disappeared. According to the fate map (Bryant 1978), the missing region should give rise to the ventral structures of the notum. In the sample of Figure 3C, another *en/inv*⁺ patch was observed in a posterior-proximal corner of the duplicated structure. This suggests that more complicated disorganization of the posterior compartment, such as triplication, may have taken place. Taken together, the *twins* phenotype could be interpreted as replacement of the proximal part of the posterior compartment with one or more additional copies of the wing pouch along with other associated anterior structures.

Another *lacZ* marker enabled us to detect small pattern perturbations in *twins* imaginal discs. *lacZ* on the PA49 chromosome is expressed in cells along the anterior and posterior compartment boundary (Fig. 4A; C. Hama and M. Hoshino, pers. comm.). In mutant wing discs whose morphology was not grossly affected, ectopic *lacZ* signals reproducibly appeared at a fixed posi-

tion in the posterior compartment (Fig. 4B). This result suggested that an initial action of the *twins* mutation could be to alter cell fates in a subset of the posterior compartment. The pattern of the PA49 β -gal expression was complicated in heavily distorted mutant discs (Fig. 4C), consistent with the notion that pattern duplications may occur at multiple sites in a single wing disc.

In some of previously identified mutations that lead to pattern duplication, cell death is thought to be a primary cause for the abnormal pattern (Bryant 1978, 1987). To detect dead cells in imaginal discs of *twins*^P third-instar larvae, we made and observed serial histological sections and also carried out whole-mount staining with acridine orange or trypan blue (see Materials and methods). In neither preparation did we find a higher incidence of cell death as compared with wild-type discs.

twins phenotypes at earlier developmental stages

To help in understanding the mechanisms underlying the *twins* duplication, we attempted to trace back the abnormal wing disc formation to earlier developmental stages. First, we examined wing disc precursor cells in *twins* homozygous embryos by immunostaining of the *vestigial* protein that is present in nuclei of disc primordia (Williams et al. 1991). We were unable to find any significant increase or reduction in the number of cells in the primordia. In all of the wild-type, heterozygous mutant and homozygous mutant embryos examined, ~20 cells were found in each wing disc anlage (data not shown). We then looked for ectopic PA49 β -gal signals in wing discs of younger mutant larvae. Under the sensitivity of our experimental technique, only larvae of in-

Figure 3. Visualization of cells belonging to the posterior compartment by immunostaining of the *en/inv* product. Wild-type (A) and mutant wing discs (B, C) were stained with anti-*en/inv* antibody (Patel et al. 1989). Cells belonging to the posterior compartments are assumed to express antigens. In A, a haltere disc and a leg disc remain attached to the wing disc. An immunoreactive proximal region (arrowhead in A) is missing in the mutant discs (B, C). Considering the folding pattern of the epithelium, a hypothetical boundary of the duplicated wing blade anlage was drawn (broken line in C). An extra patch of *en/inv*⁺ cells in a corner of the duplicated region could be produced by partial triplication of the wing pouch (arrow in C). Bar, 100 μ m.

intermediate to late third instar displayed extra β -gal-positive cells outside the compartment border.

Cloning of the *twins* locus

A stretch of 30 kb of genomic DNA flanking the site of insertion of *P-lacW* was cloned by the plasmid rescue and chromosomal walking methods (Fig. 5A). Various fragments of the genomic DNA were used as probes for Northern blot analysis of embryonic and larval poly(A)⁺ RNA to identify putative *twins* transcripts. *P-lacW* interrupted one transcription unit from which three mRNAs (3.0, 4.0, and 4.6 kb) were generated (Fig. 5B, probe II). All of the three messengers were undetectable in RNA isolated from homozygous *twins*^P larvae (Fig. 5B, probes II and III). On the other hand, messages of two neighboring transcription units (Fig. 5A, broken lines) were detected in the mutant RNA samples (data not shown). These results strongly suggest that the former set of transcripts derive from the *twins* locus. Using probes I and III, *twins* cDNA clones were isolated from cDNA libraries made from RNA of embryos, whole larval bodies, and eye-antenna discs. Comparison of the cDNA sequences with that of the genomic region surrounding the P insertion site revealed that the transposon was inserted very close to one of the exon-intron junctions, 7 bp downstream of the first initiation codon (arrowhead in Fig. 6, see also the legend to Fig. 6).

A faint 2.5-kb band was seen in the mutant RNA after long exposures of the Northern blots (Fig. 5B, probes II

and III). This finding may be consistent with our result of genetic analysis that the *P-lacW* insertion did not appear to inactivate the gene completely (see Materials and methods). In the exons that were located downstream of the insertion site, the entire open reading frame (ORF), except the first two amino acids, was preserved. If cryptic transcription starts within the P element or an intron flanking the insert, it might produce a message that encodes a functional product. Another possible explanation of the faint 2.5-kb band is that it could be a minor splicing variant and the *P-lacW* insertion did not block its formation.

To obtain more information about how the *P-lacW* insert affected transcription of *twins*, the mutant RNA was probed on a Northern blot with a genomic fragment I that was located 5' upstream relative to the insertion point (Fig. 5A). Probe I detected multiple RNA bands ranging from 4.0 to 10 kb (Fig. 5B, probe I, lane *twins*^P). These bands were *twins-lacZ* fusion transcripts, as demonstrated by reprobing with a *lacZ* probe; many of the RNA bands hybridized with probe I exactly comigrated with those detected with the *lacZ* probe (Fig. 5B, probe *lacZ*, lane *twins*^P).

The *twins* locus encodes a regulator of PP2A

Nucleotide sequences of the overlapping cDNA clones were determined, and an ORF of 1329 nucleotides was found. The predicted translation product of this reading frame was a 51-kD protein with 443 amino acid residues

Figure 4. Altered expression of the compartment boundary marker in *twins* discs. (A) Normal wing disc. Cells located along the border of the anterior and posterior compartments are marked by β -gal expression of the PA49 chromosome (C. Hama and M. Hoshino, pers. comm.). (B) *twins* wing disc that had little morphological defects. The arrow shows an ectopic expression of the marker in the posterior compartment. (C) *twins* wing disc with a severe phenotype. Multiple patches of extra signals are observed in this grossly distorted mutant disc. Bar, 100 μ m.

(Fig. 6). The protein showed extensive similarity to the regulatory B subunits of human PP2A (Fig. 7). PP2A consists of three subunits: two classes of regulatory subunits, termed A and B; and a catalytic subunit, termed C (Cohen 1989). The human has two B subunits, hPR55 α and hPR55 β , encoded by separate genes (Mayer et al. 1991). The *twins* product was 78% and 75% identical to hPR55 α and hPR55 β in amino acid sequences, respectively. The budding yeast *Saccharomyces cerevisiae* has a homologous gene, *CDC55* (Healy et al. 1991). Four gaps had to be introduced into the alignment of the *twins* and the *CDC55* proteins, and they showed 50% identity.

Expression of *twins*

The P-*lacW* construct used for the enhancer-trap screen contains the *lacZ* reporter gene (Bier et al. 1989). The spatial expression pattern of β -gal in *twins*^P was studied to obtain information about the expression pattern of β -gal in *twins* mRNAs. The expression of *twins* transcripts was directly studied by whole-mount in situ hybridization of wild-type imaginal discs. The β -gal reporter gene was expressed ubiquitously in wing discs of heterozygous *twins*^P larvae, and this β -gal expression was consistent with results of in situ hybridization (data not shown). *twins* expression was not restricted in wing discs; eye-antenna and leg discs also expressed the gene. The transcript level was higher in imaginal discs than in the central nervous system.

To detect the *twins* protein, polyclonal antibodies

against T7 phage *gene 10*-*twins* fusion proteins were prepared. We detected a single band of the predicted size (51 kD) in immunoblots of crude extracts from various developmental stages (Fig. 8A). The gene product was abundant in embryonic lysates, particularly in those made from blastoderm embryos (0–2 hr). This implied the presence of maternal messages, which agreed with strong signals in blastoderm embryos hybridized with a *twins* cDNA probe (data not shown). The amount of the protein then gradually decreased during development. Although the 51-kD band was not clearly detected in the extracts made from whole bodies of third-instar larvae or pupae, the protein was enriched in fractions of larval central nervous system and imaginal discs, and of salivary glands. This spatial distribution was similar to those of mRNAs of A and C subunits of *Drosophila* PP2A (Mayer-Jäkel et al. 1992). Within the limit of detection of our Western analysis, the 51-kD band was missing in extracts prepared from homozygous *twins*^P larvae (Fig. 8B), demonstrating that this protein is a product of the *twins* gene.

Discussion

Pattern duplication in the *twins*^P wing disc

We identified a *Drosophila* gene, *twins*. Its mutation caused a mirror-symmetrical pattern duplication of specific regions in the wing imaginal disc. The homozygous mutant wing disc had an extra wing blade anlage in the

Figure 5. Molecular map of the *twins* locus and Northern blot analyses. (A) An isolated 30-kb genomic region and its *Eco*RI restriction sites. The insert of P-*lacW* (12 kb) is shown by a large open triangle above the line. (I, II, III) Genomic DNA fragments that were used as probes for Northern analyses in B. The *twins* gene produced three mRNAs of 3.0, 4.0, and 4.6 kb (see B). P-*lacW* was inserted into one of the exon/intron junctions. Boxes represent ORFs of cDNA clones. Size variation among the three messages seemed to be generated at least partially by alternative splicing in noncoding regions. This is because genomic probes I, II, III and the isolated cDNAs hybridized all of the mRNAs, and the nucleotide sequences of the cDNAs were identical except in 5'- or 3'-untranslated regions. Broken lines show two neighboring transcription units encoding 3.6- and 4.3-kb mRNAs, and 1.9-kb mRNA, respectively. End points of those units were not precisely determined. (B) Northern blot analyses. Poly(A)⁺ RNA was prepared from wandering third-instar larvae, either of the wild-type or of the *twins*^P homozygote.

Pairs of each RNA (~3.0 µg) were run in a formaldehyde gel, blotted, and hybridized with various probes. Three messages (3.0, 4.0, and 4.6 kb) are present in the wild-type RNA (probe II, lane +), but they are missing in the mutant sample (probe II, lane *twins*^P). Much longer exposure detected a faint 2.5-kb band in the mutant RNA (probe II or III, lane *twins*^P). Transcripts of the 5C actin gene (Fryberg et al. 1983) are present in similar abundance in the wild-type and the mutant. Among the three *twins* messages, the 3.0-kb band was most abundant in embryonic RNA (0–22 hr; data not shown); and in larvae intensity, the 4.0-kb mRNA was the strongest. The *twins*^P allele produced *twins-lacZ* fusion transcripts as demonstrated by use of probe I or the *lacZ* probe (lane *twins*^P).

posterior compartment. The additional wing pouch itself seemed to have both anterior and posterior compartments; thus, two almost complete pattern copies arose within a single disc. The mode of the *twins* duplication contrasts with a phenotype of the *en*¹ mutant, in which the posterior wing blade structures are replaced by the anterior ones, resulting in the formation of an anterior–anterior mirror image wing (Garcia-Bellido and Santamaria 1972; Brower 1984). Triplication seemed to occur occasionally in the posterior compartment of *twins* wing discs, resulting in more complicated disc patterns.

Pattern duplication and deficiency were reported in wings of the mutant *tetraaltera* (*tet*), whose genetic map position is 3-48.5 (James and Bryant 1981). Although *tet* is located very closely to *twins* or within the same chromosome band (85F), our complementation test did not support the possibility that *tet* and *twins*^P are allelic (see Materials and methods).

PP2A is involved in pattern duplication

Molecular analyses of the *twins* locus demonstrated that the product is one of the regulatory subunits of PP2A. The subunits of PP2A have been divided into three groups: the catalytic subunit, C, and two classes of regulatory subunits, A and B (Cohen 1989). *twins* was found to code for a *Drosophila* homolog of the B subunit. Biochemical analyses of vertebrate enzymes suggest that

the B subunit interacts with the AC heterodimer and suppresses the activity of this enzyme toward a variety of substrates in vitro (Imaoka et al. 1983; Usui et al. 1988).

Serine/threonine protein phosphatases have been divided into four major classes, 1, 2A, 2B, and 2C, on the basis of their dependence on divalent cations and response to specific inhibitors. PP2A has been implicated in many cellular events, including glycolysis, lipid metabolism, cell proliferation, and replication of viral DNA (Cohen 1989; Cohen et al. 1990; Mumby and Walter 1991; Thomas 1992). Genetic analyses of yeast mutants revealed physiological roles of PP2A in cell division cycle, cell growth, and/or bud morphogenesis (Kinoshita et al. 1990; Sneddon et al. 1990; Healy et al. 1991; Ronne et al. 1991). Although *Drosophila* genes encoding A and C subunits were cloned, investigation of their functions in vivo has been hampered by the lack of mutations of those genes (Orga et al. 1990; Mayer-Jäkel et al. 1992). Our characterization of the *Drosophila twins* mutant highlights a new aspect of PP2A function in multicellular organisms, namely, regulation of the activity of this enzyme may be crucial for pattern formation.

Possible mechanisms of pattern duplication in *twins*^P

One of the well-known mechanisms to generate duplicated patterns in imaginal discs is cell death and subse-

1	GAATTCACGGCAATTGGTAGCTTGGAACTCTTATTGTAATCTTCTTCCCTCTACATGCTTAAGTCGTGTAACACACAAAAAAT	91
92	ACACAATTAAACAAAAGATCAATGCATTAACAAACACGGAACCAAGTGGTTAATCAACGAATAGTGAAGAGCGTATGAAGAAGAGT	187
188	GTAATAATCGCGCTCAACTTTTGAACCTTTAAATCGGCAGCTACACAAACACACCCCGTGAGAAAGAGAGCGAGCTAGAGAGCGCGAGC	283
284	GCAGCTCCTTTGGCCTCTCTCTTTGTTGTTGTTATTTTGTGCTGCTCGCTTTTGGTGTCTAGTGTGTTGTTGTTTGTAGCTGCGGC	379
1	* M A G N G E A S W C F S Q I K G A L D D D	21
380	TCCTTAAATCGACGCAAACTGTCGGTAAACATGGCGGTAATGGAGAGGGCTTGGTGCTTCTACAGATTAAAGGCGCCCTAGACGATGAT	475
22	V T D A D I I S C V E F N H D G E L L A T G D K G R V V I F Q	53
476	GTCACGGATGACAGATCATCTCGCTGGAATTCATCAGATGGCGAGCTGCTGGCCACTGGTGACAAAGGCGGTGCGCTGTCATCTTTCAG	571
54	R D P A S K A A N P R R G E Y N V Y S T F Q S H E P E F D Y L K	85
572	CGTGATCTGCCTCAAAAGCGCCCAATCGAGAGCGGGAATACAATGCTATTTCGACATTCCAATGGCAGAGCCGAAATCGACTACCTCAAG	667
86	S L E I E E K I N K I R W L Q Q K N P V H F L L S T N D K T V K	117
668	TCCTGGAGATTGAGGAGAAGATAAACAGATCGGTGGCTGCAACAAAAGAAATCCCGTGCACTTCTGCTCTCGACCAACGACAGACAGTAAA	763
118	L W K V S E R D K S F G G Y N T K E E N G L I R D P Q N V T A L	149
764	TTGTGGAAGTCACTGAGCTGACAAGTCTGTCGGCGGTACAACACGAAGGAGGAGAAGGCTGATCGGGATCCACAGAATGTAACGGCCCTC	859
150	R V P S V K Q I P L L V E A S P R R T F A N A H T Y H I N S I S	181
860	CGAGTGCCATCCGTGAAGCAGATACCACTGCTGGTGGGCGCTCCCAACGGCGCACCTTCGCCAATGCACACCTATCAGATCAATCAATAAGC	955
182	V N S D Q E T F L S A D D L R I N L W H L E V V N Q S Y N I V D	213
956	GTTAATTCGGATCAGGAGACCTCTTGTGCGCCGACGCTCGGGATCAATCTGTGGCACTGGAGGTGGTCAACCAGAGCTAACAACTGTTCGAC	1051
214	I K P T N M E E L T E V I T A A E F H P T E C N V F V Y S S S K	245
1052	ATCAAGCCAAACCAATGAGGAGCTAACAGAGGTGATACCGCGCGCAATTCATCCGACGGATGCAATGTGTTGTCTACTCGAGCTCAAG	1147
246	G T I R L C D M R S A A L C D R H S K Q F E E P E N P T N R S F	277
1148	GGCACAATCAGATTATGTGATATGCGTTGGCGGGCTATGCGACCGGCAGCAACAGTTGAGGAGCCGAGAATCCAACGAATCGCAGCTTT	1243
278	F S E I I S S I S D V K L S N S G R Y M I S R D Y L S I K V W D	309
1244	TTACGGAAATAATCAGCTCCATCAGCGATGTGAAGCTAAGCAACTCGGTCGCTAGATGATCTCCAGGATTACTTGAGCATCAAGTGTGGGAT	1339
310	L H M E T K P I E T Y P V H E Y L R A K L C S L Y E N D C I F D	341
1340	CTGCATATGGAACAAAGCCCAATGAGACCTATCGGTTTCATGAATACCTGCGGCCAAGCTGTGTTGGCTGACAGGAATGACTGCATCTTCGAC	1435
342	K F E C C W N G K D S S I M T G S Y N N F F R V F D R N S K K D	373
1436	AAGTTCGAGTGTGTTGGAACGGCAAGGACAGCTCAATATGACCGGAAGCTACAACAACTTCTTCGCGCTTTCGATCGCACTCGAAAAGGAT	1531
374	V T L E A S R D I I K P K T V L K P R K V C T G G K R K K D E I	405
1532	GTGACGCTAGAGCGCTCCAGGACATCATCAACCGAAACGGTGCTTAAGCCACGGAAAGTTGCACTGGCGCAACGAAAGAGGATGAGATC	1627
406	S V D C L D F N K K I L H T A W H P E E N I I A V A A T N N L F	437
1628	AGCGTGAGCTGTCTAGATTCAACAAGAGATCTCGACACCGCTGGCACCAGGAAATATCATCGCGTGGCTGGCAACATAACCTCTTC	1723
438	I F Q D K F *	443
1724	ATATTTCAGGATAAATTTAGCCAACTCTCTCAAGAACCACACCATACGCCCAATACACAGCACTATATCTACAGAAACACCAAGAAT	1819
1820	ACGATCAGCGCTCATCTTATGTTAAATTAATCGGTTGTTTCAAAATAGTTTCAAAATTCACCTGCAATCAAAATGTTTGCCTAACACATTAC	1915
1916	CTCAGGCCACCTTATCCCTATCCCATCCATGTTCTGCTTCCGCCCCCAACAAATACATCATCAATGCCATAGCAAAAGCTTTGTAATGT	2011
2012	ATTTCTCGCGTGTATGCGTAAACGAGAAACAAATACCAAGAACTTAAGAGATGGAACAAATTTTATACATTACATTATAAATAGTGAAGCGTA	2107
2108	TATTGATTTCATGTAGCGCAATCAAAACCCCAATAGAGTAATATATTATGTTAGCTATTGATTCGCCGAAATTTTGTGATAATTAGCGC	2203
2204	CAGCGCGAAGTATTGTATAAAAGATAAATAATACAGATAAAAAAATAAATAAATGCAATGTTGTAACATTTTTCGTTGCGCTGTT	2299
2300	AAGTTACGATGAAAGTCAAGAAACATATGCGCCCTGTTTGTCTACTTTATAAATATAGACTAAACAGGAATTTCCGATTGTTTATTCGTTGA	2395
2396	CAACGTTATTCCATGCAAAAGAGAGCATAGGCGCGGGAGCTTGTGATCGACTATGTGCTCATTTTAACTCATCAGCAAGCTCAGCAATC	2491
2492	GAACAGCGGAAGTTGCTCAGTCAACCAATTTAAATGCTCAATTAACACTTAATAATTCACCTAAGAACCCAACTGCTAGCTAGCCAGAAAG	2587
2588	CAGAACTCAAGAGATGAGCAACCGGGAAGAGTATAAATAGATGCATTGATAACGTATGCATCGCAGTTAATTGATACCTACATATACATAT	2683
2684	AAAAAAGCAGCGCTAAGTTTAAATACAAATAGTGTCTCAATTCGTTTATGAAAGTCTATCTGAAATGAGAGAAACAGAAAAAACA	2779
2780	ATAATAAGAAATACTATTTTATACGCTACATTTAAAGCTAAGTATTGCTACTTTTAGTTCAACTATGCGTGGTGGTCAAGACCAATTTCTCA	2875
2876	CATATAGTCTATTGCCCCCTGGCCCCCGCCCTTTTTCATCGGTCAACAACTTTCCAGCGAGTGTACTGTTTGTAAAGTTTCTGTATTCA	2971
2972	TTGTGATGTTTCCATAAAGAACTATAAACAAACCAACCTTTTATAGGTTATCTAGTGTCTAGTGTAAACAAATAAAAAACAAACAAAT	3067
3068	TATAAAACGCAACCATAAAGGAATTC	3095

Figure 6. Sequence of *twins* cDNAs. The sequence of larval *twins* cDNAs is shown along with the predicted translation of the longest ORF. Asterisks represent stop codons that flank the ORF. The second amino acid of the ORF is methionine, which is most likely an ATG initiator codon. Sequence AAC preceding this start codon matches well the *Drosophila* translation start consensus (Cavener 1987). Three splicing junctions in the ORF were determined at the base pair level. Between nucleotides 419 and 420 (arrowhead) one junction is located, which corresponds to the center of an 8-bp sequence duplicated on insertion of *P-lacW*; the second one is positioned between nucleotides 571 and 572; and the third one is between 1375 and 1376 (arrows). Nucleotides 1–1100 represent the sequence of cDNA clone 13, and the rest of the sequence is derived from clone 51.

quent regeneration (Bryant 1978, 1987). The polar coordinate model predicts that when >50% of an imaginal disc is removed by either cell death or in situ microsurgery, the surviving fragment undergoes duplication (French et al. 1976; Bryant et al. 1981). However, we do not think that the *twins* duplication can simply be the result of cell death, because we did not detect extensive cell death in the mutant imaginal discs during the third instar, although this does not rule out the possibility that many cells die earlier.

Another possible mechanism is that the transformation of cell fate took place in *twins* mutants. In the *twins*

wing disc, a group of cells showed ectopic expression of the PA49 marker in the posterior compartment; in the wild-type, this marker was expressed only along the anterior–posterior boundary. It should be emphasized that these ectopic expressions were observed even in mutant discs with morphology that was almost normal. This indicates that some intracellular changes preceded the appearance of morphological defects. These observations support the possibility that local cell fate changes trigger the formation of extra pattern copies. If some cells in the posterior compartment are transformed into precursor cells of the wing pouch, they would give rise to extra

hPR55α	MAGAGGGNDIQWCFQVQKGAVDVDAEADIISTVEFNHSGELLATGDKGGRVVFQOEQE	60
hPR55β	MEEDIDTRKINNSFLRDHSYATEADIISTVEFNHTGELLATGDKGGRVVFQOEQE	56
twins	MAGNGEASWCFQKIGALDDVTDADIISCFEFNHDGELLATGDKGGRVVFQORDPA	57
CDC55	MAQNNFDFKFSQCFGDKADIVVTEADLITAVEFDYDTGNLATGDKGGRVVFERSNS	57
hPR55α	NKIQSHSRGEYNVYSTFQSHEPEFDYLSLEIEEKINKIRWLPQKNAQFLISTNDKTIK	120
hPR55β	SKNQVHRRGEYNVYSTFQSHEPEFDYLSLEIEEKINKIRWLPQKNAQFLISTNDKTVK	116
twins	SKAANPRRGEYNVYSTFQSHEPEFDYLSLEIEEKINKIRWLPQKNPVHLLSTNDKTVK	117
CDC55	-----RHCEYKFLTEFQSHDAEFDYLSLEIEEKINEIKWLRPTQRSHELLSTNDKTIK	111
hPR55α	LWKISERDKRPEGYNLKEED-----GRYRDPPTVTTLRVEVFRPMDLMV	164
hPR55β	LWKVSEDRDKRPEGYNLKEE-----GRLRDPATITTLRVEVLRPMDLMV	160
twins	LWKVSEDRDKSFGGYNTKEEN-----GLIRDQPNVTALRVPVSKQIPLLV	161
CDC55	LWKVVEKNKLVLSQNNLTGVTFAKKGKPDNHNRRGGSVRAVLSLQSLKLPQLSQHDKII	171
hPR55α	EASPRRIFANAHTYHINSISINSYETILSADDLRINLWHLIEITDRSFNIVDIKPNMEE	224
hPR55β	EATPRRVFANAHTYHINSISVNSDYETYSADDLRINLWNEFITNQSFNIVDIKPNMEE	220
twins	EASPRRTFANAHTYHINSISVNSDQETFLSADDLRINLWHLVNVQSYNIVDIKPNMEE	221
CDC55	AATPKRIYSNAHTYHINSISLNSDQETFLSADDLRINLWNLDPDSFNIVDIKPNMEE	231
hPR55α	LTEVITAAEFHPHSCNTFVYSSSGKTIRLCDMRASALCDRHSKLFEPEPDSNRSFFSEI	284
hPR55β	LTEVITAAEFHPHHCNTFVYSSSGKTIRLCDMRASALCDRHTKFFPEPDSNRSFFSEI	280
twins	LTEVITAAEFHPTECNFVYSSSGKTIRLCDMRSAALCDRHSKQFEPEPNTRSFSEI	281
CDC55	LTEVITSAEFHPQECNLFMYSSSGKTIRLCDMRQNSLCDNKTKTFEEYLDPIHNEFTETI	291
hPR55α	ISSISDVKFSHSGRYMMTRDYL SVKIWDLNENRPVETVQVHEYLRSKLSLYENDCIFD	344
hPR55β	ISSISDVKFSHSGRYIMTRDYLTVKVDLNENRPIETVQVHDYLRSKLSLYENDCIFD	340
twins	ISSISDVKLSNGRYMISRDYLSIKVDLHMETKPIETVQVHEYLRAKLSLYENDCIFD	341
CDC55	TSSISDIKFSPNGRYIASRDYLTVKIWDVNMNPKFLKTIINIEQLKERLSDTYENDAIFFD	351
hPR55α	KFECCWNGSDSVVMTGSYNNFF-----	366
hPR55β	KFECCWNGSDSVIMTGSYNNFF-----	362
twins	KFECCWNGKSSIMTGSYNNFF-----	363
CDC55	KFEVNFSGDSSSVMTGSYNNFMIPNVVTSGDNDNGIVKTFDEHNAFNSNSKNIHNSI	411
hPR55α	-----RMFDRNTKRDITLEASRENNKPR-----T	390
hPR55β	-----RMFDRNTKRDVTLEASRENSKPR-----A	386
twins	-----RVFDRNSKKDVTLEASRDIIKPK-----T	387
CDC55	QNKDSSSSGNSHKRRSNGRNTGMVGSSNSRSSIAGGEGANSEDSGTEMEIVLQADKTA	471
hPR55α	VLKPRKVCASGKRKKDEISVDLDFNKKILHTAWHPKENIIAVATNNLYIFQDKVN	447
hPR55β	ILKPRKVCVGGKRRKDEISVDLDFSKKILHTAWHPSENIIVAATNNLYIFQDKVN	443
twins	VLKPRKVCVGKRRKDEISVDCLDFNKKILHTAWHPENIIIVAATNNLYIFQDKF	443
CDC55	FRNKRYGSLAQRSARNKDWGDDIDFKKNIHLFHSWHPRENISIAVAATNNLYIFSAL	526

Figure 7. Similarity of the *twins* product to regulatory B subunits of PP2A. Amino acid sequences of the two B subunits of human PP2A (hPR55α and hPR55β; Mayer et al. 1991), the *S. cerevisiae* CDC55 protein (Healy et al. 1991), and the putative *twins* product are aligned. Amino acids that are identical among the four proteins are shaded. The *twins* product is 78% and 75% identical to hPR55α and hPR55β, respectively, and 50% identical to the CDC55 protein.

copies of this structure. Our preliminary analysis of adult lethal alleles of *twins* favors for the cell transformation hypothesis; during the formation of sensory organs, cell type identity was altered in these alleles (K. Shiomi and T. Uemura, unpubl.). In previous work, pattern duplications or triplications in imaginal discs were ascribed to interruption of intercellular communications by some mechanisms other than cell death (Simpson and Grau 1987; Santamaria et al. 1989). It is thus possible that in *twins* imaginal discs, initial fate change occurring

in a restricted population of cells might disrupt their interactions with the surrounding cells, leading to the formation of ectopic pattern elements.

We observed discrepancies between the *twins*^P phenotype and the spatiotemporal expression pattern of the *twins* product. Although *twins* was expressed in embryos, developmental defects in the mutants were observed only in imaginal discs at late larval stages. This may be at least partly the result of the presence of maternal messages. By rapid cell proliferation during larval

Figure 8. Immunoblot analyses of the *twins* protein at various developmental stages. (A) Samples were prepared from wild-type Oregon-R animals at the indicated developmental stages, and immunoblots were probed with the anti-*twins* antibody, anti-TW-1. The expression of the 51-kD protein was maximum in 0- to 2-hr embryos and decreased gradually. The blot of embryonic samples was exposed for a much shorter time than other samples. In third-instar larvae, the protein was enriched in fractions of the central nervous system (CNS), imaginal discs (Disc), and salivary glands (SG). (B) Immunoblot test for the *twins* protein in *twins*^P homozygotes. Samples enriched with larval central nervous system and discs were prepared from the wild-type (WT) or *twins*^P homozygotes. The 51-kD protein was undetectable in the mutant sample. In A and B, 100 μg of protein was run in each lane.

periods, the gene products derived from the maternal messages and zygotic *twins^P* mRNAs should be diluted; finally, the mRNA level may become lower than that necessary for establishing cell identities in imaginal discs. Another discrepancy was that although *twins* expression took place in the entire regions of a wing disc and in other imaginal discs such as the eye–antenna disc, the *twins^P* effect was obvious only in a particular domain of the wing disc. This could be explained by restricted localization of the presumptive target molecules of PP2A, although other possible mechanisms cannot be ruled out.

Another insertion of the P element into *twins* has been independently isolated in other laboratories, and the locus was called *abnormal anaphase resolution¹* (*aar¹*; Mayer-Jäkel et al. 1993). In larval neuroblasts of *aar¹*, chromosome segregation during anaphase is aberrant. Similar abnormal mitotic figures were observed also in *twins^P* neuroblasts, and both the mitotic phenotype and the disc pattern defects could be rescued by germ line transformation with the wild-type *twins* gene (H. Ohkura and D. Glover, pers. comm.). These results suggest pleiotropic effects of the *twins^P* mutation on different cell types. Neither *twins^P* nor *aar¹* appears to be a complete loss-of-function mutation. To grasp the in vivo roles of *twins* more accurately, it would be important to isolate and characterize null mutations, although abundant maternal messages might still obscure the null phenotype even in the complete absence of zygotic gene activity.

A critical question that remains to be answered is how PP2A is involved in cell fate determination or other possible mechanisms inducing pattern duplication. This has to be explained at the level of regulation of protein dephosphorylation. It would be necessary to narrow down the physiological targets of PP2A and to eventually identify the key substrates that are involved in pattern specification. It would be also intriguing to identify protein kinases that share the same target molecules. These studies should shed light on our question about a link between protein dephosphorylation and tissue pattern formation.

Materials and methods

Isolation of imaginal disc mutants

Two collections of third-chromosome lethal lines (Cooley et al. 1988a; Bier et al. 1989) were used for screening. Both collections were made through insertional mutagenesis with either of the two P-element vectors. These plasmids were pUCHsneo (Steller and Pirrotta 1985) and P-lacW (Bier et al. 1989). The mutant chromosomes had been kept as balanced stocks over TM3, *Stubble*. To determine lethal periods of individual strains, those chromosomes carrying mutations were rebalanced over TM6B, *Tubby*. Of a total of 292 lethals examined, 69 pupal lethal strains were isolated. Late third-instar larvae homozygous for each lethal mutation were dissected to observe their imaginal disc morphology under a dissecting microscope. Seven lethal mutants showed imaginal disc defects. In four of those seven lines, the size of discs was normal or even larger, but their overall shapes were quite different from those of the wild type.

One of them was *lethal(3)11C8*, which had a single copy of P-lacW inserted into the chromosome band 85F or the boundary of 85F/86A. This chromosome was designated as *twins^P* in this study. Stocks were grown at 25°C, unless mentioned otherwise.

twins^P homozygous mutants exhibited extended larval periods (~2 days longer than heterozygous mutants or wild-type animals), and wandering mutant third-instar larvae were larger than the wild-type animals. Under uncrowded conditions, the frequency of the homozygous mutant pupae from heterozygous parents (*twins^P/+*) was >90% of that expected on the basis of the number of heterozygous siblings. Thus, most of the *twins^P* homozygotes were able to reach the pupal stage. The mutant pupae did not develop into pharate adults.

Excision of P-lacW from *twins^P*

The P-lacW element in *twins^P* was remobilized by mating to flies carrying the P transposase source Δ2-3 (Robertson et al. 1988). Seventy independent lines were established that had lost the *miniwhite⁺* marker gene in P-lacW. Of these, 22 jump-outs were homozygous viable, and the mutant phenotype had reverted to the wild-type in all 22 lines. The genomic DNA of each chromosome was analyzed by Southern blot hybridization. All of the excisions appeared to be precise. Therefore, the pupal lethality and the disc phenotype of *twins^P* was solely the result of the P-lacW insertion. The strain Δ2-3 was obtained from T. Tanimura (Kyushu University, Japan).

The other 48 lethal jump-outs were classified into three groups:

1. Fifteen pupal lethal lines that showed disc duplication phenotype. All were associated with internal deletions of P-lacW. *twins⁶⁰* and *twins⁴⁷* were two examples of this group. The level of *lacZ* expression was reduced in *twins⁶⁰*, and this was used for pattern analyses with molecular markers (see below). *twins⁴⁷* had a much stronger activity of β-gal than *twins^P*.
2. Twenty-two adult lethal strains. By genetic criteria, they were hypomorphic alleles of *twins* (K. Shiomi and T. Uemura, unpubl.).
3. Eleven lethal lines that acquired mutations unrelated to the *twins* locus.

In this excision experiment, none of imprecise jumpers appeared to delete the genomic sequence under the resolution of Southern analyses.

Complementation test with a deficiency chromosome for the 85F region

To know whether *twins^P* is a complete loss-of-function mutation or not, we examined the phenotype of *twins^P* carried over a deficiency chromosome for the 85F region. *Df(3R)by62*, provided by K. Matthews (Bloomington Stock Center) and M. Michinomae (Konan University, Japan), uncovers a chromosomal region from 85D11-14 to 85F16 (Kemphues et al. 1980, 1983). Animals of *twins^P/Df(3R)by62* were pupal lethal like *twins^P* homozygotes; however, all imaginal discs tended to show retarded growth, and the typical *twins^P* duplication phenotype was rarely found. Although *Df(3R)by62* presumably affects dosage of many genes, *Df(3R)by62/+* larvae produce normal imaginal discs. These observations indicated that (1) *twins^P* was not a null mutation but retained a low level of activity, and (2) the amount of the gene products made from only one copy of *twins^P* may not be sufficient for proliferation of disc cells.

Complementation test with tet

Because of the following two reasons, it was possible that *twins*

might be allelic to *tet* (Lindsley and Grell 1968). First, a cytological location of *twins* (85F) was very close to the genetic map position of *tet* (3-48.5). Second, in the background of the *outstretched small eye–small eye* (*os^s*) mutation that is located on the X chromosome, morphology of *tet* wing discs is similar to that of *twins^P* (James and Bryant 1981). These workers reported that 11% of the *os^s* and *tet* double mutant adults showed some defects in derivatives of the wing disc.

The *os^s*, *tet* double mutant was obtained from the Bloomington Stock Center. Either at 17°C or at 25°C, no obvious disorganization of adult cuticles was found in males of *os^s/Y; tet/twins^P*. Thus, our complementation test did not provide positive evidence that *tet* and *twins* were allelic. We confirmed that both *tet* and *twins* were included in the same genomic region uncovered by the *Df(3R)by⁶²* chromosome.

lacZ marker chromosomes

Three strains expressing β -gal in imaginal discs were used in this study. All have inserts of P elements containing *lacZ* (*P-lacZ*) on the second chromosome. ryXho38 appears to have a *P-lacZ* insert into the *sca* locus (Hama et al. 1990). PA49 expresses *lacZ* along the boundary of the anterior and posterior compartments. Both ryXho38 and PA49 were characterized by C. Hama and M. Hoshino (National Center of Neurology and Psychiatry, Japan). The *lacZ* expression patterns in the *twins⁻* background were examined histologically in homozygous *twins⁶⁰* mutant larvae that contained at least one copy of the *P-lacZ* insertion.

Histology

To search for dead cells in the mutant imaginal discs, histological serial sections of *twins^P* third-instar larvae were prepared. All fixative solutions were made in 0.1 M sodium phosphate buffer (PB, pH 7.2). Larvae were torn into two halves, and the anterior portions containing imaginal disc complexes were fixed in a 2.5% glutaraldehyde solution. After 1 hr, the buffer was gradually replaced with *n*-butanol. The samples were then transferred into a 1 : 1 mixture of *n*-butanol and paraffin, embedded in paraffin, and serially sectioned at 4 μ m. The sections were stained with methylene blue/azure II and mounted in Eukitt (O. Kindler). To detect dead cells in whole-mount preparations, imaginal discs were stained with acridine orange or trypan blue as described by Spreji (1971). The number of dead cells was as small as that in the wild-type tissue and much smaller than that in the cell death mutant *lethal(3)9C3*, which was isolated from the screening of this study (K. Shiomi. and T. Uemura, unpubl.).

X-gal staining was performed according to Bier et al. (1989), with slight modifications. Embryos were fixed for 20–30 min in 3.5% formaldehyde in PB and incubated with X-gal in the presence of Triton X-100 (Bellen et al. 1989). Anterior half-larva were everted entirely (Ursprung 1966) and fixed for 15 min in 0.5% glutaraldehyde in PB or in PBS. Triton was not added in staining larval tissues.

Immunohistochemistry

We basically followed the protocol described by Bodmer and Jan (1987). Embryos or larval tissues were fixed in 3.5% formaldehyde in PB. All antibody incubations were done in PBT (0.3% Triton X-100 in PB) containing 2% bovine serum albumin (Miles), and washes were in PBT. Rabbit anti- β -gal antibodies (Capell, diluted 1 : 200) were preabsorbed against Oregon-R samples. The embryos were processed with solutions of the

Vectastain ABC kit according to the manufacturer's instructions. Then 0.5% NiCl₂ was added to the peroxidase reaction. To visualize disc primordia, embryos were collected from *twins^{47/+}* parents, stained first with X-gal and then with anti-vestigial antibody provided by J. Williams and S. Carroll (Williams et al. 1991). We chose lateral views of stage 17 embryos and counted numbers of the wing disc primordia in dark blue embryos (*twins^{47/twins⁴⁷}*), light blue embryos (*twins^{47/+}*), and *lacZ⁻* embryos (*+/+*). Primordia of >30 embryos of each genotype were studied.

Dissected imaginal discs were incubated with the anti-*en/inv* monoclonal antibody 4D9 (Patel et al. 1989) or with the anti-*achaete* antibody 984A11C1 (Skeath and Carroll 1991) overnight at 4°C and washed several times. Biotinylated anti-mouse immunoglobulin and fluorescein–streptavidin (both purchased from Amersham) were used for secondary and tertiary reactions, respectively, at a dilution of 1 : 100. The samples were mounted in 90% glycerol containing 1 mg/ml of paraphenylenediamine.

Cloning and analysis of the *twins* gene

The *twins* gene was isolated using the plasmid rescue procedure (Steller and Pirrotta 1986). The *twins^P* genomic DNA was digested with *Bgl*II, *Bam*HI, *Pst*I, or *Eco*RI and self-ligated before transformation into *Escherichia coli* MC1061. An Oregon-R genomic library (L. and Y.N. Jan, University of California, San Francisco) was screened to isolate longer genomic DNA from this region. Total RNA was extracted by the CHAOS method (Jonas et al. 1985), and the selection of poly(A)⁺ RNA was done batchwise using Oligotex (Takara). The RNA was run on formaldehyde gels, blotted onto Hybond N (Amersham) and UV cross-linked (Stratalinker, Stratagene).

cDNA libraries of 9- to 12-hr embryos (K. Zinn, University of California, Los Angeles), 3- to 12-hr embryos (L. Kauvar and T. Kornberg, University of California, San Francisco), late third-instar larvae (L. Kauvar), and eye–imaginal discs (G. Rubin, University of California, Berkeley) were screened with genomic DNA fragments as probes. Two overlapping cDNA clones (13 and 51) covering 3.1 kb were sequenced with the Sequenase II system (U.S. Biochemical). Clone 13 was isolated from the eye disc library, and 51 was obtained from the late third-instar larval cDNA library. A precise insertion site of *P-lacW* in *twins^P* was determined by partial sequences of the rescued plasmids flanking the P-element ends. A corresponding genomic region of Oregon-R was also sequenced, and it was confirmed that the *P-lacW* insertion was not accompanied by deletion and/or rearrangement of the neighboring genomic DNA. Sequence manipulations and data base searches were done using DNASIS (Hitachi) and IDEAS (Kanehisa 1982).

In situ hybridization

Whole-mount in situ hybridization to embryos and imaginal discs was carried out according to Tautz and Pfeifle (1989) and to protocols written by M. Mlodzik (EMBL) and N. Patel (Carnegie Institute of Washington). Single-stranded DNA probes were prepared by polymerase chain reaction. A *twins* cDNA fragment (nucleotides 1101–1998 in Fig. 6) was used as a template.

Preparation of antibodies

The T7 *gene10–twins* fusion genes were constructed by cloning either an *Eco*RI–*Eco*RI 0.45 kb [nucleotides 651–1100 in Fig. 6] or an *Eco*RI–*Hind*III 0.9 kb (nucleotides 1101–1998) fragment into pGEMEX-1 (Promega). Fusion proteins (TW-1 and TW-2,

respectively) were overexpressed in *E. coli*, and inclusion bodies were run on SDS–polyacrylamide gels. The fusion proteins were electroeluted with a Biotrap elution chamber (Schleicher & Schuell). SDS was removed by passage over Ampure DT (Amersham). Recovered protein solutions were used for immunization of rabbits. We followed the protocol of Inuzuka et al. (1991) for affinity purification of the antibodies.

Western blot analysis

Dechorionated embryos, larvae, pupae, and adults were homogenized in 1× Laemmli's sample buffer containing 1 mM phenylmethylsulfonyl fluoride (but not bromphenol blue), sonicated, and immediately boiled for 5 min. After centrifugation, the supernatants were stored at –80°C. To enrich the larval central nervous system and imaginal discs, extraneous tissues such as trachea, guts, fat bodies, and salivary glands were removed from everted anterior half-larvae and processed as described above. The protein concentration of each sample was determined using the Lowry method (Lowry et al. 1951) with BSA as standard. Proteins in the extracts were separated by SDS-PAGE and transferred to nitrocellulose. To detect the *twins* protein, the ECL kit (Amersham) was used according to the manufacturer's instructions. We could not detect significant signals in whole-mount staining of embryos and larval tissues with either anti-TW-1 or anti-TW-2.

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Note

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence data bases under accession number D13004.

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