Direct transcriptional control of the Dpp target *omb* by the DNA binding protein Brinker

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The gradient morphogen Decapentaplegic (Dpp) organizes pattern by inducing the transcription of different target genes at distinct threshold concentrations during *Drosophila* development. An important, albeit indirect, mode by which Dpp controls the spatial extent of its targets is via the graded downregulation of *brinker*, whose product in turn negatively regulates the expression of these targets. Here we report the molecular dissection of the *cis*-regulatory sequences of *optomotor-blind* (*omb*), a Dpp target gene in the wing. We identify a minimal 284 bp Dpp response element and demonstrate that it is subject to Brinker (Brk) repression. Using this *omb* wing enhancer, we show that Brk is a sequence-specific DNA binding protein. Mutations in the high-affinity Brk binding site abolish responsiveness of this *omb* enhancer to Brk and also compromise the input of an unknown transcriptional activator. Our results therefore identify Brk as a novel transcription factor antagonizing Dpp signalling by directly binding target genes and repressing their expression.

Keywords: Brinker/DNA binding protein/Dpp signalling/*omb*/transcription

Introduction

Members of the transforming growth factor- β (TGF- β) superfamily of cytokines elicit a wide array of cellular responses, including the regulation of cell division, survival and specification of developmental fates (Kingsley, 1994). The *Drosophila* TGF- β homologue Decapentaplegic (Dpp) has been shown to function as a morphogen, i.e. it can exert long-range patterning effects and specify positional identities in a concentration-dependent manner (Lecuit *et al.*, 1996; Nellen *et al.*, 1996; Podos and Ferguson, 1999). Dpp is required for multiple aspects of embryonic and adult development, such as specification of the dorsoventral axis in the embryo (Irish and Gelbart, 1987; Ferguson and Anderson, 1992; Podos and Ferguson, 1999), mesoderm and endoderm induction (Frasch, 1995; Bienz, 1997), tracheal cell migration (Vincent *et al.*, 1997) and patterning of the

adult appendages (Spencer *et al.*, 1982; Capdevila and Guerrero, 1994; Zecca *et al.*, 1995).

Dpp is expressed in the developing wing disc, in response to short-range signalling by Hedgehog, in a thin central stripe of cells along the anteroposterior compartment boundary (Basler and Struhl, 1994; Tabata and Kornberg, 1994). From this position, Dpp moves outwards and exerts a long-range organizing effect in both compartments. Three presumed Dpp target genes have been identified, namely *vestigial* (*vg*), *optomotor-blind* (*omb*) and *spalt* (*sal*), all of which are expressed in broad nested domains centred on the Dpp expression stripe (Kim *et al.*, 1996; Lecuit *et al.*, 1996; Nellen *et al.*, 1996). Expression of these targets appears to be triggered by distinct thresholds of Dpp concentration (Lecuit *et al.*, 1996; Nellen *et al.*, 1996).

In the past few years, several previously unknown, well conserved components of the Dpp signalling pathway have been discovered; this has led to the following paradigm of signal transduction. The binding of Dpp to a type II and type I receptor complex [consisting primarily of the serine threonine kinases encoded by *punt* and *thickveins* (*tkv*); Brummel *et al.*, 1994; Nellen *et al.*, 1994; Penton *et al.*, 1994; Letsou *et al.*, 1995; Ruberte *et al.*, 1995] leads to phosphorylation of receptor-specific Smads [such as the *Mothers against dpp* (*Mad*) gene product; Sekelsky *et al.*, 1995]. The phosphorylated Smad then associates with a general Smad partner, such as the *Medea* (*Med*) gene product (Das *et al.*, 1998; Hudson *et al.*, 1998; Inoue *et al.*, 1998; Wisotzkey *et al.*, 1998), and the Smad complex translocates to the nucleus where it functions together with other transcription factors to regulate expression of target genes (Massague and Wotton, 2000). *Drosophila* Mad was the first Smad demonstrated to possess direct DNA binding activity (Kim *et al.*, 1997). Results from work carried out on the Dpp response elements from *vestigial* and *tinman* strongly indicate that direct binding of Smad proteins to enhancer sequences is a critical feature of Dpp signal transduction and that, at least for these enhancers, Smad sites play essential roles in activating gene transcription (Kim *et al.*, 1997; Xu *et al.*, 1998).

The recent cloning and characterization of the *brinker* (*brk*) gene is a novel twist to the possible mode by which Dpp signalling controls target gene expression (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a,b; Minami *et al.*, 1999; Ashe *et al.*, 2000). *brk* is essential for proper patterning of both the embryonic ectoderm and the wing disc, indicating that it is an integral component of the Dpp system of positional information. In the wing disc, *brk* is expressed in the lateral-most regions of the disc adjacent to cells that express *omb*. Its expression is negatively regulated by Dpp signalling and this repression requires the zinc finger protein Schnurri (Marty *et al.*, 2000). *brk* mutant clones lead to outgrowths and pattern duplications

in the anterior and posterior regions of the wing, indicating that loss of *brk* results in ectopic activation of the Dpp pathway. *brk* clones outside the endogenous *sal* and *omb* expression domains but within the wing pouch show strong ectopic *omb* expression and weak *sal* expression in a strictly cell-autonomous manner. Epistasis experiments using double-mutant combinations of *brk* with either *tkv* or *Mad* showed that Dpp signalling is not required to activate Dpp target genes in cells lacking *brk* function (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a). However, direct activation of Dpp targets by Dpp/Mad has not been excluded, particularly in the case of *vg* and *sal* where the levels of ectopic expression are lower in *brk* mutant cells than in their endogenous expression domains. Although no *brk* orthologues have been identified to date, the fact that Brk is able to antagonize BMP signalling and induce dorsal cell fates in *Xenopus* embryos suggests that Brk function is conserved between arthropods and chordates (Minami *et al.*, 1999).

Here we are concerned with the mechanisms by which Brk regulates Dpp target genes. The lack of obvious structural motifs in the Brk protein sequence has prevented a clear prediction of how Brk functions. Since Brk appears to be a nuclear protein, we explored the prospect that it might be a DNA binding protein. Even as a transcriptional regulator, however, Brk could function indirectly to repress Dpp targets, e.g. by transcriptionally repressing a general activator or by inducing the expression of a repressor of Dpp target genes. To discriminate between these possibilities, we set out to isolate the regulatory sequences of *omb*, which appears to be the clearest case of a Dpp target gene whose regulation is primarily under Brk control. We report here that Brk is a sequence-specific DNA binding protein that interacts strongly with a site within the minimal *omb* wing-specific enhancer. We also provide combined genetic and biochemical evidence that Brk binding to the *omb* enhancer is responsible for its repression in lateral regions of the wing imaginal disc. Our results identify Brk as a novel transcription factor antagonizing Dpp signalling by directly binding target genes and repressing their expression.

Results

Isolation of the *omb* imaginal disc enhancers

The *omb* gene was localized within a genomic walk of 340 kb (Pflugfelder *et al.*, 1990). By mapping chromosome breakpoints and neighbouring loci, the minimal and maximal extents of the *omb* gene were determined to be ~80 and 160 kb, respectively (Pflugfelder *et al.*, 1990). We systematically scanned 100 kb of the *omb* locus for regulatory elements; this region comprises 45 kb of upstream sequences and the first four introns (see Figure 1A). Restriction fragments from genomic lambda phages were cloned into a *lacZ* reporter P-element and assayed for enhancer activity in imaginal discs upon germline transformation (see Materials and methods). Three of these constructs showed reproducible, tissue-specific enhancer activities (Figure 1), each mimicking a subset of those of the endogenous *omb* gene. *pomb7* is located 27 kb upstream of the *omb* transcription unit and drives *lacZ* expression in the wing imaginal disc. *pomb19* [a subfragment of which has been used as a marker for

polar photoreceptor axons by Newsome *et al.* (2000)] and *pomb23* comprise intronic sequences and cause *lacZ* transcription in the eye and leg disc, respectively (Figure 1A). Although all three enhancers are Dpp responsive (not shown), they are well separated from each other and do not show significant overlap in tissue specificity. Below we focus solely on the wing enhancer.

Identification of a minimal Dpp-responsive *omb* wing enhancer

The 6 kb *pomb7* wing enhancer was analysed further by establishing a restriction map and deriving five non-overlapping and four overlapping subfragments for *in vivo* assays (Figure 1B). The 1.5 kb *XhoI*–*HindIII* fragment (*pomb33*) showed a *lacZ* expression pattern indistinguishable from that of wild-type *omb*. To narrow down further the sequences that are required and those that are sufficient for *omb* wing enhancer activity, four series of *pomb33* derivatives were generated. In the first series, terminal deletions were introduced from each end (WF13–WF18; Figure 1B). The second series consists of nine 300 bp subfragments, each one overlapping by 150 bp with its neighbouring fragment (WF4–WF12; Figure 1B). This redundancy was applied to avoid disrupting vital transcription factor binding sites as a result of the way in which adjacent fragments were divided. Of these, WF12 was the only fragment that could drive *lacZ* expression in the wing disc. In the third series, internal deletions within WF14 (which still mimics endogenous *omb* expression) were generated (Figure 1B, inset to the right).

Our results from the analysis of subfragments of *pomb33* and derivatives of WF14 suggested to us that there might be considerable redundant regulatory information encoded within the larger fragments. We therefore focused on WF12 as the minimal enhancer that still recapitulates important aspects of *omb* expression in the wing pouch. Unlike *omb*, the WF12-driven *lacZ* reporter is not expressed in the prospective notum region (Figure 2A and C). However, we ascertained that WF12 is Dpp responsive by examining its activity in cells expressing *Tkv^{QD}*, a constitutively active form of *Tkv* (Nellen *et al.*, 1996). We also confirmed that WF12 activity is still subject to Brk control. WF12–*lacZ* expression was ectopically induced, in a cell-autonomous fashion, by gain of *Tkv^{QD}* activity (Figure 2D) or by loss of *brk* activity (Figure 2E). Thus, the 284 bp fragment WF12 represents a long-sought-after Dpp response element within the *omb* gene and is an ideal tool for dissecting the Brk-mediated regulation of a Dpp target.

Brk is a DNA binding protein

Based on its amino acid sequence, it has been proposed that Brk is a DNA binding protein containing a possible helix–turn–helix (HTH) motif (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a). We tested the DNA binding properties of Brk using *in vitro* synthesized full-length protein in an electrophoretic mobility shift assay (EMSA). As a DNA target, we used a double-stranded oligonucleotide derived from the *omb* WF12 enhancer (254–282, 284–257, see below). Brk protein is able to produce a retarded protein–DNA complex, indicating binding of Brk to the DNA (Figure 3C). Since the putative HTH motif is located at the N-terminus of the protein

Fig. 1. Identification of a minimal *omb* wing enhancer. (A) Molecular dissection of the *omb* regulatory region. The *omb* locus, indicating genomic positions according to Pflugfelder *et al.* (1990), is represented below the scale bar (a segment of the region from –130 to –20 kb is shown). The *omb* mRNA is depicted above the scale bar. All sites used for cloning (indicated as lines going below the line representing the genomic sequence) and all endogenous *HindIII* sites (indicated as lines that stop at the line representing the genomic sequence) are indicated. Phages from the *omb* walk are represented as lines and carry their original numbers (Pflugfelder *et al.*, 1990). Fragments used for *lacZ* reporter constructs to scan the region for enhancer elements are shown as filled rectangles with the names indicated above. B, E, H and S represent *Bam*HI, *Eco*RI, *Hind*III and *Sal*I sites, respectively. ND represents constructs whose expression pattern has not been determined. The *lacZ* expression patterns of the three enhancers (wing, eye and leg) are shown below the corresponding constructs. (B) Wing enhancer break-up. A restriction map of the 6 kb pomb7 enhancer fragment is shown. Constructs pomb31–pomb35 are non-overlapping reporter constructs and pomb36–pomb39 are overlapping reporter constructs that were generated using available restriction sites. The 1.5 kb pomb33 fragment is enlarged below, with the positions of the terminal deletion constructs WF13–WF18 and the 300 bp overlapping constructs (WF4–WF12) marked. pomb33, pomb37 and pomb38 led to identical expression patterns. pomb34 and pomb39 showed only a very weak expression in the wing pouch. The other derivatives in this series showed no expression. WF13 and WF14 showed expression identical to pomb33. WF15 leads to a strong expression along the dorsoventral boundary and very weak expression in the rest of the pouch. WF16 showed narrower expression than pomb33, resembling that of the *spalt* gene. WF17 showed a pattern similar to that of WF16, but the expression was very patchy. The 900 bp WF14 fragment is shown enlarged on the right, with, beneath it, a depiction of the internal deletion constructs derived from it. In each case the deleted sequence is replaced by a single *Eco*RI site. None of the internal deletions had any effect on the wild-type expression pattern.

(amino acids 44–99), we produced a series of C-terminally truncated versions of Brk (Figure 3A). These derivatives are correctly synthesized (Figure 3B) and still bind to target DNA (Figure 3C, lanes 3–5) as confirmed by EMSA. The above experiments demonstrate that Brk does indeed contain a DNA binding domain, which is located within its N-terminal 175 amino acids.

Brk binds to a specific site in the *omb* wing enhancer

On the basis of the results described above, we produced in *Escherichia coli* a glutathione *S*-transferase (GST) fusion protein containing the N-terminal 263 amino acids of Brk and used it in DNase I footprinting assays to scan the WF12 *omb* wing enhancer for potential Brk binding sites. GST–Brk protein strongly protected a single region of 17

nucleotides (nt) from DNase I digestion (Figure 4A). This sequence is located in the 3' part of the enhancer (nt 266–282). Another, weakly protected region located more 5' (nt 149–173) was not investigated further (data not shown). In a complementary assay, we scanned the WF12 fragment with a series of nine overlapping double-stranded oligonucleotides for Brk binding sites using EMSA (data not shown and Materials and methods). Only oligonucleotide 254–282/284–257 showed significant Brk binding, confirming the footprinting experiment.

To identify a consensus sequence for Brk target sites, we analysed another Dpp-responsive enhancer, *lab550* (Tremml and Bienz, 1992; Grieder *et al.*, 1997), using the same footprinting strategy (data not shown). All the sites protected by GST–Brk in the two enhancer elements are listed in Figure 4B. From this alignment we derived a

Fig. 2. Expression of reporter constructs in wild-type and mutant situations. (A–C) *lacZ* expression patterns of *omb-lacZ*, *brk-lacZ* and *WF12-lacZ*, respectively (*WF12-lacZ* expression was similar to that of *omb-lacZ* in the pouch, but lacked dorsal hinge expression). Since the expression levels driven by *WF12* were far stronger than those of *omb-lacZ* and *brk-lacZ*, the disc shown in (C) had to be recorded at a different exposure level. (D) *WF12* is ectopically active in clones of cells expressing the constitutively activated *Tkv^{QD}* receptor (marked by the absence of the green *CD2* marker). The expression levels in the clones were comparable to those in the endogenous domain (not apparent here since the endogenous domain is at a different focal plane). The upregulation of *WF12* activity was strictly cell autonomous, i.e. ectopic activation was seen only within the clone and was observed only in clones lying within the wing pouch. The yellow rings around the mutant clones in the merged panel are caused by the difference between the subcellular localization of the surface *CD2* marker and that of the nuclear β -Gal markers. (E) *WF12* is ectopically active in cells mutant for *brk* (marked by the absence of the green *GFP* marker). The levels of *WF12*-driven expression in the *brk* loss-of-function clones were commensurate with those in the centre of the discs.

consensus sequence, GGCGC/TC/T, for high-affinity Brk binding. To assess the importance of each nucleotide, we scanned the *WF12* Brk binding site with single point mutations (Figure 4C) and tested the ability of these mutants to bind *in vitro* synthesized full-length Brk protein. Mutations in the five central nucleotides of the Brk site (marked with asterisks in Figure 4B) abolished Brk binding (Figure 4D), indicating that these residues form the core recognition motif and that Brk is a DNA binding protein with a high target site specificity.

The Brk binding site in *WF12* is also a target of an essential transcriptional activator

Loss of Brk activity *in vivo* results in ectopic activation of the *WF12* enhancer, so one would predict that mutations in *WF12* that abolish Brk binding should also lead to ectopic enhancer activity. To test this hypothesis, we mutated the core of the Brk binding site from GGCGCC to GATATC (mut4X, Figure 4C). As expected, this change completely abolished Brk binding in EMSA analysis (Figure 4D). We introduced the same 4 bp mutation into the *WF12-lacZ* reporter transgene. Unexpectedly, this mutation com-

pletely abolished *lacZ* expression rather than expanding it. We interpret this result as an indication that the Brk site overlaps with that of an activating input. Next we introduced into *WF12-lacZ* single base pair mutations that interfere with Brk binding (see above), anticipating that at least some of these mutations would still allow the unknown activator to bind, resulting in an uncoupling of the two inputs.

Brk binding to the *omb* enhancer is essential for lateral repression

Two of the five point mutations that prevent Brk binding (see Figure 4D), mut271 and mut272, completely abolished *WF12* enhancer activity as observed with the 4 bp mutation. However, mutations mut273, mut274 and mut275 still expressed *lacZ*, albeit only in a narrow stripe along the dorsoventral boundary (Figure 5). This pattern unravels a hitherto masked, strong input into *WF12* from a dorsoventral patterning system (see Discussion). Although these mutant enhancers show a reduced extent of expression along the dorsoventral axis, all of them exhibit a clear expansion in expression along the anteroposterior axis (see

Fig. 3. Brk is a DNA binding protein. **(A)** Schematic representation of the Brk protein and the C-terminal deletions generated from it. The coloured rectangular inserts correspond to stretches of repetitive amino acids. Blue represents glutamine, orange serine and brown histidine/alanine. The pink rectangle represents the region of putative homeodomain (HTH) homology. The putative repression domain (PMDLSLG) and the nuclear localization signal (RRLKKK) are also indicated (Jazwinska *et al.*, 1999a). For the deletion constructs, the approximate positions of the restriction sites used for generating the deletions are indicated. The dark green boxes at the C-termini of the truncated constructs represent additional amino acids generated by the cloning procedure. **(B)** SDS-PAGE of [³⁵S]methionine-labelled proteins corresponding to full-length Brk (lane 1) and the three C-terminally deleted proteins indicated in (A) (lanes 2–4). The positions of the molecular weight markers (MW) are indicated on the left. **(C)** EMSA analysis of *in vitro* synthesized Brk full-length protein (lane 2) and C-terminally deleted proteins (lane 3, 1–515; lane 4, 1–266; lane 5, 1–175) binding to a labelled double-stranded oligonucleotide derived from the *omb* wing enhancer. Lane 1 is the free probe and lane 6 corresponds to 4 μ l of control reticulocyte lysate. On the left, the arrows indicate the positions of the corresponding retarded bands generated by the proteins bound to the probe. ns, non-specific binding.

Figure 5). This latter property corresponds to the behaviour expected from the loss of a functional Brk binding site in the WF12 enhancer. We interpret these observations to indicate that mut273–mut275 represent mutations that completely abolish Brk binding but only partially prevent input by the activator. We further found that mutations flanking the Brk binding site (mut269, mut270, mut276 and mut277) abolish expression of WF12–*lacZ* (data not shown), indicating that the binding site of the activator extends beyond that of Brk.

Genetic support for the loss of Brk responsiveness

We sought to validate our assumption that mut273–mut275 exhibit an extended expression along the dorsoventral boundary due to loss of Brk-mediated repression. Both the wild-type WF12 enhancer and the mutant derivatives were examined in cells that ectopically express Brk protein from a *tub>CD2>brk* flip-out transgene. While Brk potently repressed the transcriptional activity of WF12 (Figure 6A), it did not repress the mutant enhancers (Figure 6B). Thus, the ability to bind Brk *in vitro*, the lateral repression by endogenous Brk and the responsiveness to ectopic Brk *in vivo* all correlate with single nucleotide exchanges in the Brk core binding site. Together, these results are taken as evidence that the

wild-type *omb* WF12 enhancer is a direct target of Brk repression.

Discussion

Dpp regulates numerous cellular events during development. Most prominently, gradients in Dpp activity control cell fates along the dorsoventral axis of the early embryo and along the anteroposterior axis of imaginal discs (Podos and Ferguson, 1999). All of the known responses to Dpp can be attributed to the transcriptional induction or repression of target genes. Major interest is devoted, therefore, to the mechanisms by which Dpp signalling controls gene expression. Important advances have recently been made in *Drosophila* by the discovery that a significant aspect of Dpp target gene control involves the repressive action of Brk, whose expression itself is regulated by Dpp (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a,b; Minami *et al.*, 1999). Here we addressed the molecular mechanism by which Brk might exert its influence on Dpp target genes. Our experiments demonstrate that Brk can (i) bind DNA in a sequence-specific manner and (ii) act directly on Dpp target genes by repressing their transcription.

Fig. 4. Definition of the Brk target site. **(A)** DNase I footprinting analysis of the *omb* wing enhancer with increasing amounts of affinity-purified GST-Brk fusion protein. The left-most lane represents the standard G+A Maxam-Gilbert sequencing reaction. The GST lanes correspond to the control reactions with 2 µg of affinity-purified GST protein. The protected region with the corresponding nucleotide sequence is indicated in brackets to the right. On the left, one hypersensitive site at the 3' border of the protected region is also indicated by an arrow marked 'hs'. **(B)** Alignment of the single sequence protected by GST-Brk in the *omb* wing enhancer with four regions protected in the lab550 enhancer. The bottom sequence represents the consensus sequence derived for Brk DNA binding. The stars above the *omb*-derived sequence indicate nucleotides in the putative consensus core that, when mutated, abolished Brk binding; the dashes represent nucleotides that, when mutated, did not affect Brk binding [see (C)]. **(C)** Sequence of the oligonucleotides derived from the *omb* wing enhancer carrying single point mutations; these oligonucleotides were used in the EMSA shown in (D). The grey box is delimiting the putative consensus core for Brk binding. The mutated oligonucleotides (mut275-mut271 and mut4X) that abolish Brk binding are grouped separately. **(D)** EMSA of the binding of *in vitro* synthesized full-length Brk protein to the different oligonucleotides carrying point mutations as indicated in (C). To each double-stranded oligonucleotide (indicated at the bottom), either Brk (4 µl of reticulocyte lysate) or control reticulocyte lysate (RRL; 4 µl of reticulocyte lysate without DNA template) were added. For wild type, mut274, mut271, mut268 and mut4X, the extra lane on the right represents the control probe. The arrows on the left indicate the position of the band generated by Brk or by the free probe. In addition to the probes shown in this figure, we also tested probes of mut270 and mut276, both of which bound Brk normally.

The *omb* wing enhancer

Key to our advance was the identification of regulatory elements of *omb*, one of a few genes that are positively regulated by Dpp in the wing imaginal disc. *omb* is particularly suited for the analysis of Brk function, because it is the only known Dpp target gene whose expression along the Dpp-regulated axis appears to be controlled by Brk alone. This conclusion is derived from the finding that *brk* single-mutant or *brk tkv* double-mutant cells express *omb* at equivalent levels (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a). Therefore, normal *omb* activation could be achieved by simple relief from Brk repression without invoking direct input from the Mad-Medea signalling complex. By contrast, ectopic expression of *sal* in *brk* mutant clones that additionally lack either *tkv* or *Mad* is lower than the normal level of expression of *spalt* in the centre of the disc (Campbell *et al.*, 1999; Jazwinska *et al.*, 1999a; Marty *et al.*, 2000). Hence, for genes such as *sal*, a higher complexity of regulatory input is expected. To circumvent the fact that the *omb* locus is particularly large (Pflugfelder *et al.*,

1990), we identified the *omb* wing enhancer and isolated a subfragment that was <300 bp and hence well suited for molecular and biochemical analysis.

Brk is a DNA binding protein

It has been proposed that *brk* encodes a nuclear protein with features of a transcriptional repressor (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a). These features include several putative nuclear localization signals, potential repression domains and a stretch of amino acids in the N-terminus of Brk with a high probability of folding into a HTH structure. Within the putative recognition helix, several amino acids are identical to residues found at the equivalent position in the recognition helix of many homeodomains, suggesting a DNA binding function. Our biochemical analyses demonstrate that the N-terminal part of the Brk protein binds DNA with high affinity and selectivity. In the *omb* wing-pouch enhancer, we identified a single high-affinity site. The *dpp*-dependent enhancer of the *lab* gene contains four Brk binding sites and is repressed by ectopic Brk in the endoderm (data not

Fig. 5. *In vivo* effects of point mutations in the Brk footprint. The sequence from position 254 to 285 of the WF12 enhancer is shown. The sequence stretch protected by Brk (positions 266–282) in DNase I footprinting analysis is represented as a filled box shown above the sequence. The five nucleotides presumed to form the Brk core are shown in bold and have stars above them. The five mutations (mut271–mut275) that were introduced are represented. As can be seen from the *lacZ* staining obtained with each of the mutations, mut273, mut274 and mut275 result in expanded expression domains while mut271 and mut272 abolish expression.

shown). Alignment of the five high-affinity sites identified in *lab* and *omb* allowed us to derive a consensus for Brk binding: GGCGC/TC/T. In contrast to homeodomain binding sites, which are rich in AT (Gehring *et al.*, 1994), the Brk site exhibits a high GC content, suggesting that the DNA contacting residues in these proteins differ. Structural studies will be required to elucidate the details of the DNA binding mode of Brk and to find out whether the Brk DNA binding domain folds into a three-dimensional structure similar to that of homeodomains. In addition, structure–function analyses using reverse genetics combined with biochemical studies will be necessary to determine important regions in the

protein that make Brk such a potent repressor for Dpp target genes.

Unexpected complexity at the Brk binding site

Surprisingly, reverse genetics at the *omb* WF12 Brk binding site did not mimic the results obtained from forward genetics. Substitution of the Brk binding site did not result in ectopic expression of WF12–*lacZ*, but rather in its extinction. This result can be best explained by postulating that additional proteins bind to the Brk site, proteins that must confer an activation input on WF12 enhancer activity. However, some of the single point mutations in the Brk core site did allow residual activating

Fig. 6. Effect of ectopic Brk expression on the enhancer activity of wild-type and mutant WF12. (A) WF12-driven reporter gene expression is potently repressed by low levels of Brk. Using a *tub>CD2>brk* construct, clones were generated (marked by the absence of green CD2 staining) that express *brk* under the control of the *tubulin* promoter. (B) *mut274-lacZ* is not repressed in clones of cells expressing Brk ectopically, irrespective of whether these clones are located at the centre or at the periphery of the disc.

input to occur while still preventing Brk binding. These mutations (*mut273–mut275*) are key for our conclusion that direct binding is relevant for the lateral repression of WF12 activity by Brk.

What could be the nature of the positive input at the Brk site? The simplest view is to propose a Dpp signalling input at this site, e.g. direct binding of a Mad–Medea complex. However, from genetic experiments described above, we know that Dpp input is dispensable in a situation in which Brk is absent, so it should also be possible to dispense with Dpp input in the equivalent situation in which Brk is prevented from binding. We envisage two scenarios that are compatible with our results. In the first scenario, it is assumed that, in addition to Brk, two proteins bind to the core Brk site. One would be a general wing-pouch activator that can only bind to an intact Brk site. The other protein would be a margin activator that requires a GC at positions 271 and 272 for binding, but does not exhibit a requirement for positions 273–275. Hence, mutations at 271 and 272 lead to a complete extinction of expression, while mutations at 273–275 still allow activation of expression along the presumptive wing margin. In the second scenario, only a single activator is proposed. This activator would be most abundant or most active in the vicinity of the margin. While mutations at 271 and 272 would be incompatible with binding, mutations at 273–275 would only reduce, but not obliterate, binding to this activator. In the presence of these latter mutations, the WF12 enhancer would therefore only be activated at sufficient levels near the margin. For both scenarios, the activity or expression of the presumed activator is likely to be under the control of wingless (Wg) signalling. Indeed, it has been noted previously that *omb* expression is dependent on Wg input (Grimm and Pflugfelder, 1996). Moreover, we have observed that WF12-*lacZ* expression

is upregulated in cells expressing a constitutively active form of Armadillo (data not shown). However, we have not been able to detect binding sites for Pangolin (van de Wetering *et al.*, 1991; Brunner *et al.*, 1997) or Vestigial/Scalloped (Halder *et al.*, 1998; Simmonds *et al.*, 1998).

Mode of action of Brk

Our discovery that an intact Brk binding site is required for the activity of the presumed activating factors opens the possibility that these factors compete with Brk for DNA binding. Thus, in a simple, yet speculative, model, Brk could antagonize expression of *omb* by preventing access of these transcriptional activators to DNA. Although the identity of these factors and their mode of action are still unknown, our main conclusion is that Brk represses *omb* transcription directly by its ability to bind to *omb* enhancer sequences, and not via the transcriptional control of secondary repressors. An intriguing aspect of Brk that deserves exploration is its possible role in shaping the Dpp morphogen gradient and in refining the amplitudes of target gene expression. The challenge for the future will be to determine how Brk interacts with other transcriptional factors, be it on the *omb* enhancer or on the *cis*-elements of Dpp targets, which appear to be controlled by even more complex mechanisms.

Materials and methods

Reporter constructs

Phages spanning the *omb* walk are described by Pflugfelder *et al.* (1990). Phage DNA was extracted using standard protocols and the fragments used for making initial reporter constructs were obtained based on available restriction site information (Pflugfelder *et al.*, 1990). Inserts derived from *pomb33* and all subsequent derivatives were generated by PCR. The WF series of constructs were cloned as *XbaI–BamHI* fragments using primers with appropriate site extensions. The terminal-most derivatives were cloned as *Asp718–XbaI* or *XbaI–XhoI* fragments.

Internal deletions in WF14 were generated using primers flanking the deletions such that an *EcoRI* site replaced the deletion in each case. All constructs were sequenced to confirm that no unwanted mutations had been introduced. Constructs carrying point mutations in the Brk footprint of WF12 were generated using the initial primer used to generate WF12 and a second primer derived from the end of the fragment and carrying a single mismatch at the relevant position. These primers were (substitution in bold):

mut275 CGGGATCCAAGCTTGGT**T**GGCGCTCAAGTAGC;
mut274 CGGGATCCAAGCTTGGT**T**GCGCTCAAGTAGC;
mut273 CGGGATCCAAGCTTGGT**G**AGCCTCAAGTAGC;
mut272 CGGGATCCAAGCTTGGT**G**CTCCTCAAGTAGC;
mut271 CGGGATCCAAGCTTGGT**G**CGACTCAAGTAGC.

The *lacZ* reporter vector used for generating the transformant lines was pX27 (Segalat *et al.*, 1994). It contains a minimal *hsp70* promoter with a canonical TATA box.

Brk expression plasmids

The cDNA for *brk* (kindly provided by S.Roth) was cut with *DraI* and *HindIII*, blunted with Klenow and cloned into the mammalian expression vector pSG5 (Green *et al.*, 1988), which had been cut with *EcoRI* and filled in with Klenow. All C-terminal deletions were generated from pSG-brk as follows: 1–515 was cut with *EagI* and *Eco47III*, filled in with Klenow and re-ligated, generating upon translation a protein of 534 amino acids; 1–266 was cut with *BamHI* and re-ligated, generating a protein of 269 amino acids; 1–175 was cut with *PstI* and re-ligated, generating a protein of 230 amino acids. The GST fusion construct was generated by cutting a *SlyI* fragment from pSG-brk, which contains amino acids 19–282, blunting with Klenow and ligating it into pGEX2T (Pharmacia), cut with *SmaI*.

Protein production

Full-length Brk was produced *in vitro* using the Promega TNT T7 Coupled Reticulocyte Lysate System, according to the manufacturer's instructions. The GST–Brk fusion protein was prepared from a fresh culture of bacteria containing pGex-brk, induced with 1 mM isopropyl- β -D-thiogalactopyranoside for 4 h. Cells were harvested by centrifugation, resuspended in MTPBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄ pH 7.3) containing the proteinase inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated for 2×1 min, at 50% maximal power with a Branson sonifier. After addition of Triton X-100 to 1%, the lysate was pelleted by centrifugation and glutathione–Sepharose 4B resin was added to the supernatant. After 2 h of incubation on a rotating wheel at 4°C, the resin was washed once with MTPBS, three times with MTPBS containing 0.1% NP-40 and 0.5% Triton X-100, and once with MTPBS containing 0.5% Triton X-100 and 1% Tween-20. The fusion protein was then eluted with 10 mM reduced glutathione in 50 mM Tris–HCl pH 8, 100 mM NaCl and dialysed overnight at 4°C in 10 mM Tris–HCl pH 8, 1 mM dithiothreitol (DTT), 0.1 mM PMSF, 20% glycerol.

Protein–DNA binding assays

EMSA. Double-stranded oligonucleotides corresponding to sequences in the *omb* wing enhancer were end-labelled by standard Klenow fill-in and purified over a Sephadex G25 spin column (Roche). Approximately 20 000 c.p.m. of labelled probe were incubated on ice for 30 min with 4 μ l of reticulocyte lysate containing either full-length Brk protein or the empty expression vector, in a buffer containing 10 mM Tris–HCl pH 7.5, 50 mM KCl, 5 mM DTT, 1 mM EDTA, 4% Ficoll and 0.5 μ g poly(dI–dC). The reaction mixtures were loaded on a 5% polyacrylamide/0.5× Tris–borate–EDTA (TBE) native gel and electrophoresed for ~2 h. The gel was dried and exposed to XAR Kodak film with an intensifying screen at –70°C.

DNase I footprinting. A subcloned 344 bp fragment of the *omb* wing enhancer was 3' end-labelled by standard Klenow fill-in, purified on polyacrylamide gel and incubated with 0.5–5 μ g of affinity-purified GST–Brk protein and 1 μ g of poly(dI–dC) for 10 min at 20°C in a binding reaction containing 50 mM MgCl₂, 20 mM Tris–HCl pH 7.9, 1 mM DTT, 25% glycerol. Two hundred nanograms of DNase I (Roche) were added to the mixture and incubation was continued for 90 s at room temperature. The reaction was stopped by addition of 0.2 M NaCl, 30 mM EDTA, 1% SDS, 100 μ g/ml yeast tRNA, extracted with phenol–chloroform and precipitated in ethanol. Pellets were resuspended in 9 μ l of standard 80% formamide dye, denatured for 5 min at 95°C and run on a 6% urea–polyacrylamide gel. The gel was dried and exposed to XAR Kodak film with an intensifying screen at –70°C.

The complete sequence of pomb33 has been submitted to DDBJ/EMBL/GenBank (accession no. AF291717) and is numbered from 1 to 1474. WF14 extends from nt 590 to 1474 and WF12 from nt 1191 to 1474 of pomb33. An extended version of WF12 was used for the footprint analysis. The following oligonucleotide pairs were used to scan the entire WF12 fragment for Brk binding in EMSA (oligonucleotides are numbered according to their starting and ending nucleotide positions):

1–46 CCCTCGTTTTGATGTTGCTCGCTGGCTGCCAACGCGCTTT-TGTCTT;
48–3 TTAAGACAAAAGCGCGTTGGCAGCCAGCGAGCAACATC-AAAACGAG;
25–71 GCTGCCAACGCGCTTTTGTCTTAAAGCTCTGGAAGTGG-CTGGCTGGC;
72–28 AGCCAGCCAGCCAGTTCCAGAGCTTTAAGACAAAAGCG-CGTTGGC;
55–98 GGAAGTGGCTGGCTGGCTGGCTGTTGCTGTTGCTTTTGT-TGCCG;
99–57 TCGGCAACAAAAGCAACAGCAACAGCCAGCCAGCCAG-CCAGTT;
81–128 CTGTTGCTTTTGTGGCGATGTTGTACCGATGTTGTTGC-TGGGCATTG;
129–83 TCAATGCCCGACGAACAACATCGGTACAACATCGGCAACAAAAGCAAC;
112–155 GTTGTGCTGGCGATTGAAGACGTTGCTTATAAACGT-TGATCAG;
156–110 TCTGATCAACGTTTATAAGCAACGTTCTTCAATGCCCA-GCAACA;
175–222 AGTGAAGTTAGTGCTCCGAGTCTTTTGGCCGTTTCG-GTTTTATTTT;
223–177 TAAAAATAAAAACCGAACCGCCAAAAGGACTGCGGAG-CACTAACTCA;
207–253 GTTCGGTTTTTATTTTATTTGTTTCTCCTTGTTTTTT-GTATTTT;
254–209 TGAAATACAAAAACAAGGAGAAAAACAAATAAAAT-AAAAACCGA;
150–178 GATCAGAAGCCGGTGCCGCCGCTCAGTG;
184–154 TAACCTCACTGAGGCGGCGCCACCGGCTTCT;
254–282 ATACCCGGCTACTTGAGGCGCCACCAAGC;
284–257 TAAGCTTGGTGGCGCCTCAAGTAGCCGGG.

The following point mutant oligonucleotide pairs were used to test for Brk binding in EMSA:

mut280a ATACCCGGCTACTTGAGGCGCCACCATTGC;
mut280b TAAGCATGGTGGCGCCTCAAGTAGCCGGG;
mut278a ATACCCGGCTACTTGAGGCGCCACAAAGC;
mut278b TAAGCTTTGTGGCGCCTCAAGTAGCCGGG;
mut277a ATACCCGGCTACTTGAGGCGCCACAAAGC;
mut277b TAAGCTTGTGGCGCCTCAAGTAGCCGGG;
mut276a ATACCCGGCTACTTGAGGCGCCTCAAGC;
mut276b TAAGCTTGCAGGCGCCTCAAGTAGCCGGG;
mut275a ATACCCGGCTACTTGAGGCGCAACCAAGC;
mut275b TAAGCTTGGTGGCGCCTCAAGTAGCCGGG;
mut274a ATACCCGGCTACTTGAGGCGACCAACAGC;
mut274b TAAGCTTGGTGGCGCCTCAAGTAGCCGGG;
mut273a ATACCCGGCTACTTGAGGCTCCACCAAGC;
mut273b TAAGCTTGGTGGAGCCTCAAGTAGCCGGG;
mut272a ATACCCGGCTACTTGAGGAGCCACCAAGC;
mut272b TAAGCTTGGTGGCTCCTCAAGTAGCCGGG;
mut271a ATACCCGGCTACTTGAGTCCGCCACCAAGC;
mut271b TAAGCTTGGTGGCGACTCAAGTAGCCGGG;
mut270a ATACCCGGCTACTTGATGCGCCACCAAGC;
mut270b TAAGCTTGGTGGCGCATCAAGTAGCCGGG;
mut269a ATACCCGGCTACTTGCGGCGCCACCAAGC;
mut269b TAAGCTTGGTGGCGCCGCAAGTAGCCGGG;
mut268a ATACCCGGCTACTTTAGGCGCCACCAAGC;
mut268b TAAGCTTGGTGGCGCCTAAAGTAGCCGGG.

Clonal analysis and histochemistry

Tkv^{QD}-expressing clones were generated as described in Nellen *et al.* (1996) and *brk* mutant clones as described in Campbell and Tomlinson (1999). *tub>CD2>brk* clones were induced 48 h before dissection by giving a 30 min heat shock at 36°C. Imaginal discs were fixed and stained using standard protocols. Antibodies used were rabbit polyclonal anti- β -galactosidase, mouse monoclonal anti-CD2 (Serotec), rabbit polyclonal antibody against green fluorescent protein (GFP; Clontech), mouse monoclonal anti- β -galactosidase and Alexa fluorescent secondary antibodies. β -galactosidase activity was detected by fixing third instar larval

discs and subjecting them to a standard X-gal colour reaction for 2 h at 37°C. In the case of *pomb7*, *pomb19* and *WF12* reporters, the staining reaction was stopped after 30 min.

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