Repression of Dpp targets in the *Drosophila* wing by Brinker

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Summary

Patterning along developing body axes is regulated by gradients of transcription factors, which activate or repress different genes above distinct thresholds. Understanding differential threshold responses requires knowledge of how these factors regulate transcription. In the *Drosophila* wing, expression of genes such as *omb* and *sal* along the anteroposterior axis is restricted by lateral-to-medial gradients of the transcriptional repressor Brinker (Brk). *omb* is less sensitive to repression by Brk than *sal* and is consequently expressed more laterally. Contrary to previous suggestions, we show that Brk cannot repress simply by competing with activators, but requires specific repression domains along with its DNA-binding domain. Brk possesses at least three repression domains, but these are not equivalent; one, 3R, is sufficient to repress *omb* but not *sal*. Thus, although *sal* and *omb* show quantitative differences in their response to Brk, there are qualitative differences in the mechanisms that Brk uses to repress them.

Key words: Brinker, *Drosophila* wing, Repression, Imaginal disc, Dpp

Introduction

During animal development, spatial patterns of gene expression can be established along one body axis by a single transcription factor if the concentration or activity of this factor is graded along that axis, and if target genes are differentially sensitive to activation or repression by that factor. The best examples of this are found in the early *Drosophila* embryo. For example, the transcription factor Hunchback is graded along the anteroposterior (AP) axis and establishes distinct domains of gap and pair-rule gene expression along that axis (Rivera-Pomar and Jackle, 1996). Along the dorsoventral (DV) axis, there is a nuclear-cytoplasmic localization gradient of the Dorsal transcription factor, which is exclusively nuclear in the ventral region and cytoplasmic dorsally. The distinct expression domains of several genes along the DV axis can be explained by their sensitivity to either activation or repression by Dorsal (Stathopoulos and Levine, 2002).

Our understanding of how a single transcription factor can activate or repress a gene at one concentration, but is required at higher levels to influence another, is still fairly rudimentary. Again, some of the best insights into how threshold responses to transcription factor gradients are deciphered at the level of cis-regulatory elements have come from studies of transcription factors acting during early *Drosophila* embryogenesis. The most obvious mechanisms involve modifying the number of binding sites or the affinities of those sites in enhancers to vary their sensitivity to a transcription factor, and these mechanisms appear to be used in enhancers regulated by Dorsal, Hunchback, Kruppel and Knirps (Clyde et al., 2003; Jiang et al., 1992; Langeland et al., 1994). However, sensitivity to activation or repression may be achieved in other ways; for example, sensitivity to repression by Giant can to be modified by the positioning of binding sites in relation to a promoter (Hewitt et al., 1999).

Gradients of transcription factors are used to generate spatial patterns of gene expression in many other systems, including in the *Drosophila* wing, where the transcriptional repressor Brinker is expressed in a lateral-to-medial gradient in the anterior and posterior halves (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999; Muller et al., 2003). This pattern of expression is established by the morphogen Decapentaplegic (Dpp, a TGF-β), which is expressed at the center of the AP axis and, following secretion, becomes distributed in medial-to-lateral gradients in the anterior and posterior compartments (Blackman et al., 1991; Entchev et al., 2000; Masucci et al., 1990; Teleman and Cohen, 2000). Dpp, acting through the intracellular signal transducer Mad (a Smad) and Schnurri (a nuclear zinc finger protein), represses *brk* expression, so that the graded expression of *brk* mirrors that of the Dpp protein (Marty et al., 2000; Muller et al., 2003).

Brk functions to repress the expression of genes in the wing that were originally classified as Dpp targets. In fact, in the absence of both Dpp and Brk, these targets are still expressed, indicating that Dpp regulates their expression largely indirectly, through the repression of Brk. These targets include *spalt* (*sal*), *optomotor-blind* (*omb*; *bifid*, *bi* – FlyBase) and the vestigial quadrant enhancer (vg-QE, an enhancer recapitulating a portion of the expression of the *vestigial* gene) (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Kim et al., 1996; Marty et al., 2000), and have nested expression domains centered on the *dpp* stripe, so that the vg-QE domain is wider than that of *omb*, which is wider than that of *sal* (Kim et al., 1997; Lecuit et al., 1996; Nellen et al., 1996). As all three genes appear to be targets of Brk, this pattern of expression can be explained by a

differential sensitivity to Brk, with *sal* being repressed by very low levels of Brk, but vg-QE requiring higher levels.

Here, we investigate how Brk represses gene expression, to further our understanding of why some targets are more sensitive to Brk than others. Previous studies have suggested that Brk may repress different targets by different mechanisms. First, Brk-binding sites in the cis-regulatory regions of some embryonic targets, including *zen* and *Ubx*, overlap with those of an activator, namely Mad (which can function both to activate and repress at different loci), and in vitro studies indicate that Brk and Mad can compete for binding to the same region of DNA (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001). Second, Brk possesses interaction motifs for the co-repressors Groucho (Gro) (Chen and Courey, 2000) and CtBP (Chinnadurai, 2002), indicating that it may use more active mechanisms to repress targets (Hasson et al., 2001; Saller and Bienz, 2001; Zhang et al., 2001). Loss of Gro or CtBP does result in derepression of some Brk targets, such as the vg-QE, but not others, such as *omb*, indicating that Brk may use different mechanisms to repress different genes (Hasson et al., 2001).

We show that Brk requires its DNA-binding domain (DBD) plus a repression domain to act as a repressor, the DBD alone is insufficient to repress targets, even those that have overlapping Brk- and Mad-binding sites. This poses the question of whether competition is a real phenomenon in vivo. Brk possesses at least three independent repression domains, the Gro and CtBP interaction motifs (GiM and CiM, respectively) and one other domain, defined here as 3R. However, these domains are not equivalent; 3R is sufficient for repression of *omb* but not *sal*, and this difference may be related to the spacing of Brk-binding sites in relation to activator sites. Thus, although *sal* and *omb* show quantitative differences in their response to Brk, this may actually be based more on qualitative differences in the mechanisms that Brk uses to repress them.

Materials and methods

Sequence comparisons

Sequences for *brinker* from *Anopheles gambiae* (accession number AY578799), *Drosophila pseudoobscura* (included in AADE01000981) and *Bombyx mori* (composite of AU004448 and AV402267) were identified through BLAST searches of NCBI databases.

Fly strains and mutational analysis

Flies carrying the following existing alleles or transgenes were used: brk^{F124}, brk^{E427}, brk^{F138}, brk^{M68}, $CtBP^{87De\text{-}10}$, gro^{E48}, vg-QE (P{vg(806)-lacZ}), hs-GFP ($Avic\backslash GFP^{hs.T:Hsap\backslash MYC}$), hs-flp (P{hsFLP}22), FRT18A (P{ry[+t7.2]=neoFRT}18A), FRT82B (P{neoFRT}82B), Ubi-GFP (P{Ubi-GFP(S65T)nls}3R), omb-lacZ (P{lacW}$bi^{Pol\text{-}1}$), C765 (Scer\GAL4$^{C\text{-}765}$), en-Gal4 (P{en2.4-GAL4}e16E), UbxB (P{Bhz}) and 24B-Gal4 (P{GawB}how24B). Unless indicated otherwise in parentheses, all genotypes are as denoted in FlyBase (http://flybase.bio.indiana.edu), where more information on each can be found. To molecularly characterize mutants, the *brk* gene was amplified by PCR from genomic DNA from hemizygous embryos and was sequenced.

Generation of in vitro mutated/modified UAS-brk transgenes

The UAS-brk^{A438} line, which produces an unmodified, untagged wild-type Brk protein, was generated by cloning a *brk* cDNA into a modified pUAST vector, which had the white gene removed. A >y+> flp-out cassette was inserted between the UAS sequences and the cDNA. Transgenic flies were generated by standard procedure and the flp-out cassette was removed from transformants using hs-flp, to generate UAS-brk^{A438}. All other transgenes were cloned into the standard pUAST vector and included a sequence to introduce two copies of the HA tag to the C terminus, the sequence of this was (GS/EF/RS)MAGNIYPYDVPDYAGYPYDVPDYAG (the HA sequence is underlined).

The exact locations of mutations and deletions in different transgenes are indicated in Fig. 2. C-terminal deletions were generated by PCR using an external primer, and internal primer flanked by *Kpn*I and either *Eco*RI, *Bgl*II or *Bam*HI sites, respectively. Mutations and internal deletions were generated by inverse PCR followed by re-ligation using two internal primers flanked by restriction sites generating the following changes to sequence. CM mutation (CiM), PMDLSLG to AMAAALA (*Not*I); GM mutation (GiM), FKPY to FAAA (*Not*I; similar mutations were shown to result in loss of CtBP and Gro binding to Brk); NA deletion (3R), residues 148-200 to RS (*Bgl*II); ΔA17 deletion, residues 173-189 to RS (*Bgl*II). The NLS and NLSW proteins were generated by PCR using an internal primer flanked by sequences encoding the following, PPKKKRKV (matching the NLS sequence from SV40 T antigen (Kalderon et al., 1984) plus WRPW for NLSW. The EC construct was generated by amplifying residues 151-228 and 383-452, respectively, by PCR and cloning onto NLS. More details on each construct and methods of construction are available on request.

Clonal analysis and ectopic expression

Homozygous mutant clones were generated in imaginal discs by hs-flp/FRT-induced mitotic recombination. Clones were generated in the second or early third instar of larvae with the following genotypes:

y omb-lacZ brk^{F138} FRT18A/hsGFP FRT18A; hs-flp (and similarly for brk^{M68}, brk^{E427} and brk^{F124});

y brk^{F138} FRT18A/hs-GFP FRT18A; vg-QE; hs-flp (and similarly for brk^{M68}, brk^{E427} and brk^{F124}); and

hs-flp; FRT82B $CtBP^{l(3)87De\text{–}10}gro^{E48}$/FRT82B Ubiq-GFP (and similarly for single mutant clones).

Clones in discs were identified by the loss of GFP.

Ectopic expression of UAS-transgenes was achieved by independently crossing transformant lines to two Gal4-expressing lines: en-Gal4 (expressed in the posterior) and C765 (ubiquitous expression in the wing). For assigning activity level as observed in adult wings, the following criteria were used.

++++, wild-type level (no modified/mutated protein achieved this level). No adults were obtained with en-Gal4 even when reared at 17°C. With C765 at 20°C, there was an almost complete loss of wing blade.

+++, some adults were obtained with en-Gal4 at 20-25°C, but showed substantial loss of posterior wing tissue and veins. With C765 at 25°C, there was an almost complete loss of wing blade.

++, adult flies were obtained with en-Gal4 at 25-30°C. Their wings had a loss of tissue or a fusion of veins IV and V and loss of the posterior crossvein. With C765 at 30°C, the wings were slightly smaller and had vein defects, including extra crossveins.

+, adult flies obtained with en-Gal4 at 25-30°C, with loss of the posterior cross vein. With C765 at 30°C there was little or no effect on the wings.

–, no activity. No abnormal phenotype under 25°C; at 30°C there was often some disruption to wing venation, such as extra small veins around the posterior crossvein and vein V. This was distinct from the other phenotypes above and may be caused by weak dominant-negative activity.

At least three lines of each construct were tested apart from F124 (one line). Although there was some variability in the level of activity from line to line, in general most lines from any one construct fell

into the same category of activity level. To be assigned to one of the above categories, at least two lines from a construct had to have a similar level of activity; in fact, for most constructs, at least three lines had similar levels of activity.

Generation of mutations in UAS-brk^{A438}

A homozygous strain of UAS-brk^{A438} was mutated with ethylmethane sulphonate (EMS) using standard procedure (Grigliatti, 1986) and crossed to the ubiquitous Gal4 line C765. The progeny were raised at 25°C, which normally results in flies with almost no wing blade (Fig. 3B); flies with larger wings were selected. The UAS-brk transgene was amplified from potential mutants by PCR and sequenced.

Immunostaining, X-gal staining and analysis of adult wings

Dissection and staining of imaginal discs was carried out by standard techniques. *omb* expression was detected using a *lacZ* enhancer trap. The following antibodies were used: anti-Sal (rabbit, 1:50) (Kuhnlein et al., 1994); anti-βgal (rabbit, 1:2000, Cappell; and chicken, 1:2000, Abcam); anti-HA (mouse, 1:1000, Covance). Embryos carrying the UbxB reporter were stained with X-gal by the standard protocol (Lawrence and Johnston, 1986); all embryos were fixed and stained along side each other. Wings from adult flies were mounted in GMM.

Results

Comparison of Brk homologs in other species identifies the DBD and the GiM and CiM

As an initial approach to identifying the regions of the Brk protein required for it to repress gene expression, a comparison was made between Brk proteins from four different insect species: the Dipterans, *Drosophila melanogaster*, *D. pseudoobscura* and *Anopheles gambiae*, and the more distantly related Lepidopteran, *Bombyx mori*. This identified a single region of extended similarity consisting of 60 amino acids (Fig. 1) that coincides with the helix-turn-helix (HTH) motif predicted from the original sequence and the DBD identified by in vitro studies (Rushlow et al., 2001; Saller and Bienz, 2001; Sivasankaran et al., 2000; Zhang et al., 2001). The size of this domain and the positioning of the HTH are consistent with a previous suggestion (Jazwinska et al., 1999) that the Brk DBD has similarity to a homeodomain, which is the same size and is composed of three helices, the last two comprising a HTH. Outside of the DBD, a single shorter region with significant similarity was identified, centered on the GiM (Fig. 1). The CiM is also conserved amongst all four species (Fig. 1). However, outside of these motifs and the DBD, there are no significant regions of similarity shared by all four proteins.

Generation and analysis of mutated/modified Brk protein

To determine whether the DBD, GiM, CiM or other domains of Brk are required for repression of targets in the wing, three approaches were taken to identify or generate mutant/modified forms of Brk protein in which one or more of these domains/motifs were mutated or deleted. (1) The molecular lesions in three previously reported mutations in the endogenous gene, *brk^{F124}*, *brk^{F138}* and *brk^{E427}* (Lammel et al., 2000; Lammel and Saumweber, 2000), were characterized (Fig. 2B). (2) Five new mutations were generated in a wild-type *brk* transgene carried in line UAS-brk^{A438} (Fig. 2C). This screen was performed by driving ubiquitous expression of this transgene in the wing using the Gal4 line C765: this results in an almost complete elimination of the wing blade at 25°C (Fig.

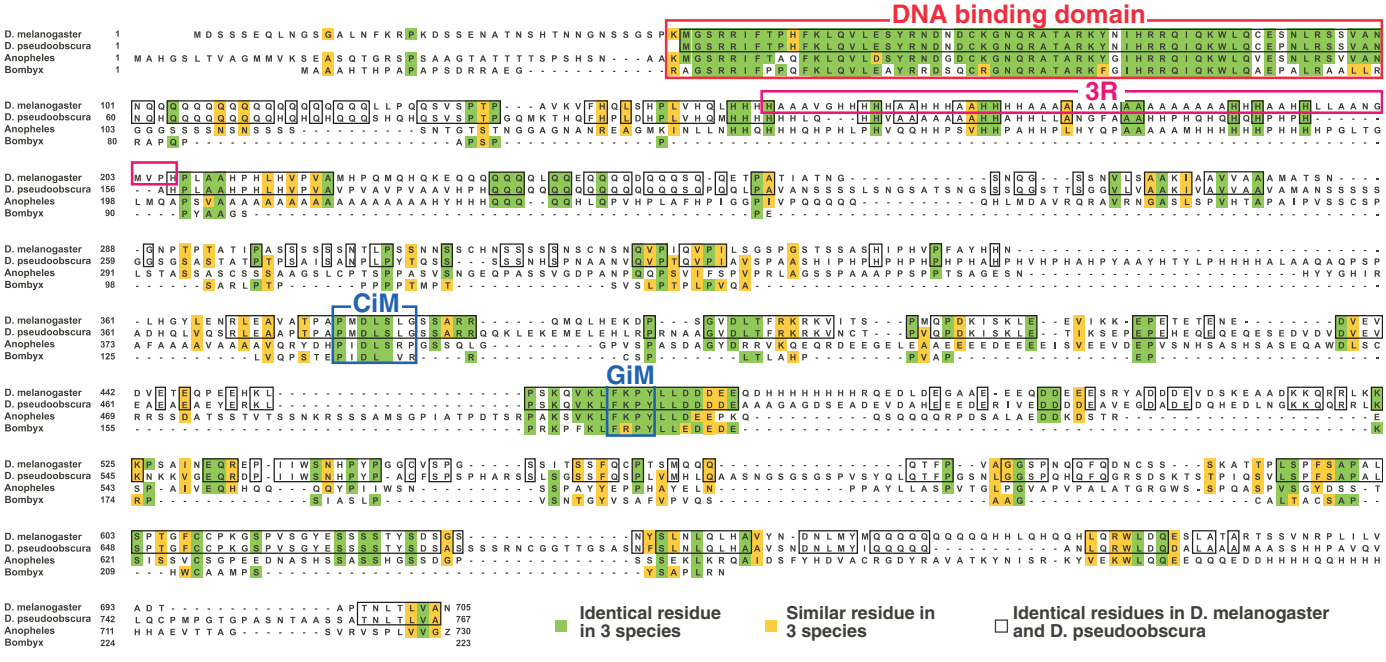

Fig. 1. Sequence comparison of Brk from *Drosophila melanogaster* and from three other insects, *D. pseudoobscura*, *Anopheles gambiae* and *Bombyx mori*. The DNA-binding domain (boxed in red) is strongly conserved in all four species. Outside of the DNA-binding domain there is only one other region of significant similarity, centered on the Groucho interaction motif (GiM, blue), although the short CtBP interaction motif (CiM, blue) is also conserved. The third repression domain, 3R, of *D. melanogaster*, identified by functional studies described here, is outlined in pink. This domain shares some limited sequence identity with *D. pseudoobscura*, but this does not extend to the other two species, although it is rich in Ala and His residues in *Anopheles*, as well as in the two *Drosophila* species.

Fig. 2. Mutated/modified Brk protein and activity. (A, part i) Domains/motifs in the Brk protein. DBD, DNA binding domain; Q, poly-glutamine; H, histidine rich; A, poly-alanine; 3R, independent repression domain. CiM and GiM, interaction motifs for the co-repressors CtBP and Gro, respectively. (A, parts ii-v) Properties and effects of modified/mutated Brk proteins shown in B-D. (A, part ii) Domains present: present (+), deleted (–) or mutated (M). (A, part iii) Nuclear (N) or cytoplasmic (C) localization (blank spaces, here and in other columns, indicates that they were not tested). (A, part iv) Activity level assessed by effect on the phenotype of adult wings ('–', no repressor activity; '++++', maximal activity; see Materials and methods for details on assigning activity level). (A, part v) The ability of each protein to repress the endogenous *sal* gene, an *omb-lacZ* line, and two reporters, vg-QE and UbxB. Y, repressed; N, not repressed; U, we were unable to detect reliable differences in UbxB expression between wild-type and mutant embryos of any genotype. (B) EMS point mutants in the endogenous *brk* gene. A protein truncated within the DBD, brk^{M68}, or immediately after it, brk^{E427}, has no activity, whereas a truncation producing a longer protein, but which still does not include the CiM or GiM, brk^{F138}, has significant activity and can repress *omb*, but not the other targets. An amino acid substitution within the DBD, brk^{F124}, also abolishes activity. (C) Point mutants in a UAS-brk transgene, A438. Four result in amino acid substitutions in the DBD and reduce activity. The fifth mutation, A438-53, results in a truncation immediately after the DBD and has no activity. (D) In vitro mutated/modified UAS-brk transgenes. There are two basic requirements for these transgenes to repress gene expression: the DBD and a repression domain/motif. In addition to the CiM and GiM, there is another independent repressor domain, 3R, located between the DBD and CiM. The DBD plus any one of the three repressor domains/motifs is sufficient to repress some Brk targets, although there is some variability in the ability of individual repressor domains to repress different targets.

3B). Five point mutants in UAS-brk^{A438} were identified as flies with a significantly increased wing size in this assay (Fig. 3). (3) Modified UAS-brk transgenes were generated that produced HA-tagged proteins in which different regions were included, modified, mutated or fused to other domains, including nuclear localization sequences (NLS) and repression motifs (Fig. 2D).

The activity of these mutated/modified Brk proteins was compared with that of the wild-type protein by analyzing the expression of known Brk targets: *sal*, *omb* and the vg-QE in wing discs, and a Ubx reporter, UbxB, in embryos (Kirkpatrick et al., 2001; Saller and Bienz, 2001). For endogenous mutants (brk^{F124}, brk^{F138} and brk^{E427}), expression of these targets was examined in marked homozygous mutant clones. For null mutants, such clones show misexpression of Brk targets in lateral regions (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999).

The UAS-brk transgenes were misexpressed in the posterior of wing discs using en-Gal4, and the expression of Brk targets in the posterior was compared with that in the anterior. Larvae were raised at different temperatures to vary the amount of protein ectopically expressed (Gal4 is cold sensitive). At 20°C, wild-type Brk^{3PF3} completely repressed *sal* and *omb*, and almost completely repressed the vg-QE (Fig. 2D, Fig. 5A,J). At 17°C, sal is still completely repressed, but some omb expression can be detected, indicating that wild-type Brk is more effective at repressing *sal* than *omb* (Fig. 5B). In embryos, UAS-brk transgenes were misexpressed in mesoderm using the 24B driver; at 25°C, Brk^{3PF3} completely repressed UbxB (Fig. 6B).

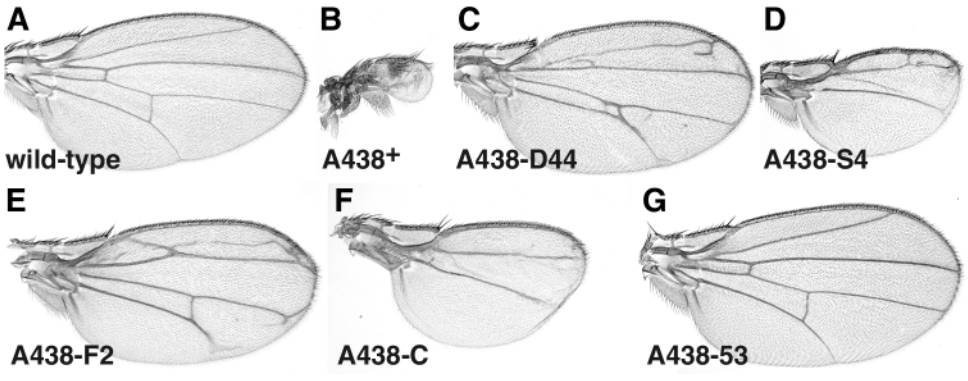

Fig. 3. Mutations in UAS-brk^{A438}, which originally contained a wild-type brk transgene. (A) Wild-type adult wing. (B) Misexpression of wild-type A438 results in a dramatically reduced wing. (C-G) Misexpression of mutated forms of A438, however, results in larger wings: from completely wild-type in the case of A438-53, indicating a complete loss of activity, to small in the case of A438-S4 and A438-C, indicating significant, but less than wild-type, activity. Wings produced with A438-D44 and A438-F2 are almost wild type in size, but have venation defects indicating that some activity still remains.

The activity of UAS-brk transgenes was also assessed in adult wings using both en-Gal4 and the weaker C765 driver. UAS-brk^{3PF3}; en-Gal4 animals did not survive to adults, even at 17°C, whereas UAS-brk^{3PF3}; C765 adults emerged at 20°C with an almost complete loss of the wing blade (Fig. 7B). The modified/mutated Brk proteins showed a wide range of activity levels based on the wing phenotype they induced (Fig. 7C-F). Activity level was classified into five categories ('–', no repressor activity to '++++', full, wild-type activity; see Materials and methods for criteria).

The results of these studies are summarized in Fig. 2 and are described in detail below.

Mutations in the DBD reduce or completely eliminate Brk activity

Mutations in the DBD were identified in one of the endogenous mutants, *brk^{F124}*, and four of the UAS-brk^{A438} mutants, D44, S4, C and F2 (Fig. 2). *brk^{F124}* has an amino acid substitution in the recognition helix R82W (Fig. 2B), and resulted in a protein with little or no activity: *brk^{F124}* mutant clones in wing discs were indistinguishable from those of null alleles, showing autonomous misexpression of *sal*, *omb* and the vg-QE (Fig. 4A,B). The mutations in UAS-brk^{A438} were located in different regions of the DBD (Fig. 2C) and had varying effects on the activity of the resulting Brk protein, as assessed by the size of the wing produced with C765 (Fig. 3), although none appeared to completely eliminate activity. We also generated UAS-*brk^{F124}*, which generated a full-length, tagged protein with the same mutation that is found in *brk^{F124}*. This also had no repressor activity, as assessed by *sal* and *omb* expression, although when misexpressed at high levels it did result in the formation of ectopic veins in adult wings, which may be due to an abnormal activity of this protein; for example, it may have a modified DNA-binding specificity (Fig. 2D, Fig. 7F).

The DBD of Brk alone has no repressor activity

If Brk represses simply by binding-site competition, a protein consisting primarily of the DBD should be capable of doing this. Residues 44-99 have previously been shown to be sufficient to bind DNA and compete with activators in vitro (Saller and Bienz, 2001). Two mutants were identified that should produce proteins consisting primarily of the DBD, *brk^{E427}* and UAS-brk$^{A438-53}$ (truncated at residues 102 and 115, respectively; Fig. 2). However, both mutants had no detectable activity in vivo (Fig. 2B,C; Fig. 3G). One possible reason for their inactivity was that the mutant proteins lack an NLS and were predominantly cytoplasmic. However, their localization could not be characterized with available antibodies, so an equivalent tagged protein, Brk^{53} (Fig. 2D), was expressed in wing discs and was shown to be primarily cytoplasmic (data not shown). Consequently, we attached a NLS to produce the protein Brk^{NLS}. This was localized in the nucleus (Fig. 5E) but still had no detectable activity (Fig. 2D), having no effect on *sal* or *omb* expression (Fig. 5C,D), or on the size of adult wings (Fig. 7F).

The in vitro studies suggesting that Brk may use binding site competition as a mechanism for repression were carried out with genes expressed in the embryo (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001), and it is possible that Brk does not use this mechanism in the wing. Consequently, we tested one of these embryonic targets, UbxB (Thuringer et al., 1993), which is expressed in the midgut mesoderm, but found that it too was not repressed by Brk^{NLS} (Fig. 6C).

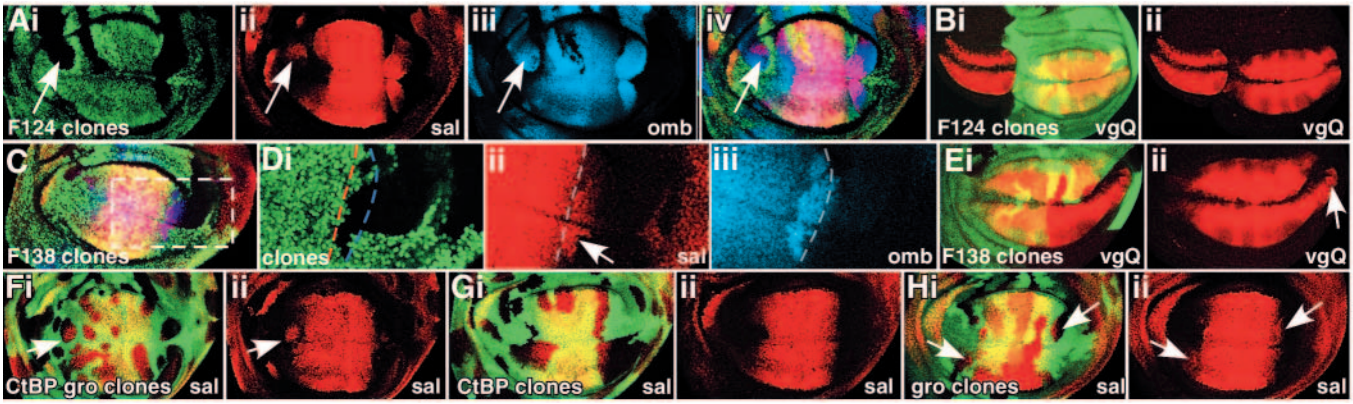

Fig. 4. Phenotype of *brk* point mutants and *CtBP* and *gro* mutants. Third instar wing discs containing mutant clones, marked by the loss of a ubiquitous GFP transgene, and stained for *omb* (*lacZ*, anti-βgal), Sal (antibody) and/or vg-QE (*lacZ*, anti-βgal) expression. (A,B) *brk^{F124}* clones show ectopic expression of Sal and *omb* within the wing pouch (arrow), and expansion of the vg-QE domain. (C) *brk^{F138}* clones. (D) Detail from boxed area in C, showing a large clone in the posterior compartment. The edge of the normal Sal and *omb* domains are shown in red and blue, respectively, in part i, and in white in parts ii and iii (these are approximations). If this were a null mutant clone, Sal and *omb* would be expressed in all of the mutant cells in the wing pouch, and *omb* would extend outside of the pouch (see A). However, there is no ectopic *omb* expression in *brk^{F138}* mutant cells, apart from possibly an expansion to one or two cells wider than normal (note, *omb* is on the same chromosome as *brk*, so that *omb* expression is upregulated in *brk* mutant cells within its endogenous domain because these cells are now homozygous for the *omb* enhancer trap). There is some ectopic Sal expression, but only in mutant cells within the endogenous *omb* domain (arrow in part ii) and not more laterally. (E) vg-QE expression is expanded laterally in some *brk^{F138}* clones (arrow). (F-H) *CtBP gro* double mutant clones are similar to those of *brk^{F138}*, only showing ectopic Sal expression (F, arrow) immediately adjacent to the endogenous domain (when located in the *omb* domain, not shown). By contrast, Sal is not ectopically expressed in any CtBP single mutant clones (G), whereas there is an occasional, minor deregulation of Sal in *gro* clones (H, arrow).

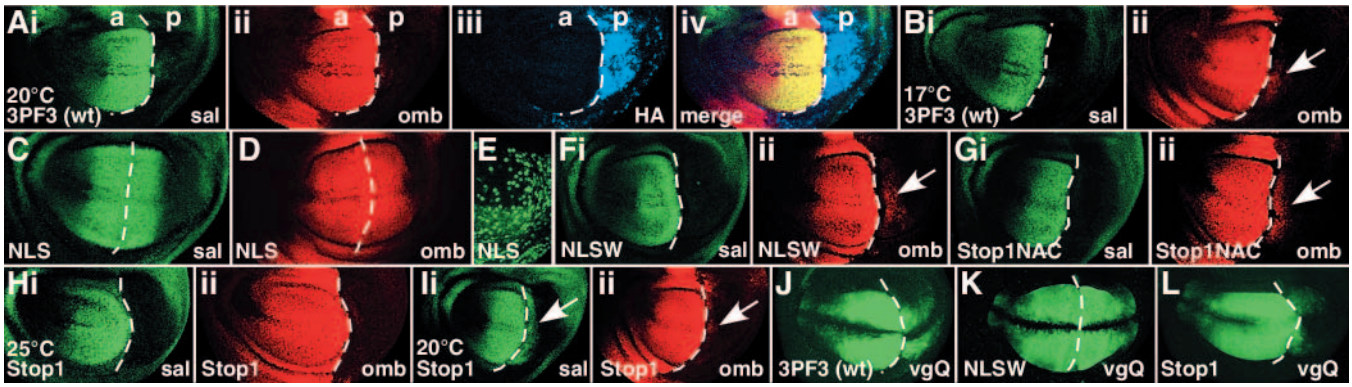

Fig. 5. Repression of *sal*, *omb* and vg-QE following misexpression of modified/mutated forms of Brk in the posterior of wing discs using en-Gal4. The interface between posterior (p) and anterior (a) is marked by a dashed line (i.e. transgene expression was driven in cells to the right). (A) Wild-type Brk^{3PF3} at 20°C completely represses *sal* (green) and *omb* (red). Brk^{3PF3} is tagged with the HA epitope, shown in blue in part iii. (B) At lower temperatures (resulting in lower levels of transgene expression), wild-type Brk^{3PF3} still completely represses *sal* (green) but some *omb* expression remains (arrow). (C,D) Brk^{NLS} (at 30°C) has no effect on the expression of *sal* or *omb*. (E) Higher magnification image focused on the large peripodial membrane cells, stained for HA, showing that expression of Brk^{NLS} is exclusively nuclear. (F) By contrast, Brk^{NLSW} (at 30°C) can repress *sal* completely and *omb* (arrow) almost completely. (G) $Brk^{Stop1NAC}$ (at 25°C), which possesses only a CiM, completely represses *sal* and almost completely represses *omb* (arrow). (H) At 25°C, Brk^{Stop1}, which lacks both the GiM and CiM, represses *sal* and *omb* completely. (I) However, at 20°C, although the lower levels of Brk^{Stop1} are sufficient to almost completely repress *omb* (there is some residual expression, arrow in ii), Sal is still expressed at high levels in the posterior (arrow in i). (J-L) Repression of vg-QE expression. (J) Brk^{3PF3} drastically reduces the level and width of the vg-QE expression domain. (K) Brk^{NLSW} has no effect on vg-QE expression. (L) Brk^{Stop1} almost completely represses vg-QE.

Addition of a minimal repressor motif to the DBD restores activity

One possible reason for the inactivity of the Brk^{NLS} protein is that it cannot actually bind DNA in vivo. To test this, we designed an identical construct, UAS-brk^{NLSW}, with the addition of a minimal repressor motif consisting of just four amino acids, WRPW (Fig. 2). WRPW functions as a repression motif by recruiting Gro (Aronson et al., 1997; Fisher et al., 1996). Brk^{NLSW} protein has considerable activity; it significantly reduced the size of adult wings (but is not as effective as wild-type protein), and it could repress *sal* completely and *omb* almost completely (Fig. 5F). In this regard it behaves similarly to wild-type protein, i.e. it represses *sal* more effectively than *omb*. It had no effect, however, on vg-QE expression (Fig. 5K), but could substantially repress UbxB in the embryo (Fig. 6D).

The CiM and GiM are required for Brk to repress some, but not all targets

If the DBD alone is unable to repress gene expression, other regions of the protein must be required, the obvious candidates being the CiM and GiM. However, analysis of the brk^{F138} mutant, which lacks both motifs, revealed that Brk could repress at least one target, *omb*, in their absence. This mutant is predicted to produce a protein truncated at residue 332, i.e. before these two motifs (Fig. 2B). In contrast to null clones, *omb* was not derepressed in brk^{F138} clones (Fig. 4C,D), although we cannot rule out the possibility that there may be a very minor expansion of expression of one or two cell diameters (the edge of the endogenous domain is difficult to resolve precisely, because it is not completely straight). However, *sal* and the vg-QE were ectopically expressed in some brk^{F138} clones (Fig. 4C,D,E). For *sal*, this ectopic expression was restricted to the *omb* domain, i.e. expansion of the *sal* domain did not extend to the edge of the wing pouch, as it would in *brk* null mutant clones.

A recent study showed that *sal* expression is dependent upon Omb (del Alamo Rodriguez et al., 2004), suggesting that the lack of ectopic *sal* expression in lateral brk^{F138} clones (i.e. outside of the *omb* domain) could be explained by the absence of ectopic *omb* in these clones, rather than by Brk^{F138} directly repressing *sal*. In support of this, *sal* is not misexpressed in *omb brk* null double-mutant clones (data not shown). del Alamo Rodriguez et al. also suggested that vg-QE expression is dependent upon Omb (del Alamo Rodriguez et al., 2004). However, vg-QE expression actually shows expansion into lateral brk^{F138} clones (Fig. 4E), i.e. in the absence of any ectopic *omb* expression. Possible explanations for these conflicting results are that vg-QE expression may not be dependent upon Omb in all situations, or that there is some ectopic *omb* expression in these clones but it cannot be detected with the *omb* enhancer trap used.

CtBP or Gro is required for repression of *sal*

Brk^{F138} protein appears to be able to repress *omb* without recruiting either CtBP or Gro, but either one or both may be required for repression of *sal* and the vg-QE. This is consistent with previous studies showing that neither CtBP nor Gro is required for the repression of *omb*, and that Gro is required for repression of the vg-QE (Hasson et al., 2001). We have extended these studies, and have found that, in contrast to *omb*, Gro is required for the full repression of *sal*, whereas CtBP can partially substitute for the loss of Gro [this is in contrast to previous claims that neither Gro nor CtBP is required for repression of *sal* (Hasson et al., 2001)]. *CtBP*/*gro* double mutant clones in the wing had the same phenotype with respect to *sal* expression as brk^{F138} clones, i.e. ectopic expression, but only within the *omb* domain (Fig. 4F). By contrast, *sal*

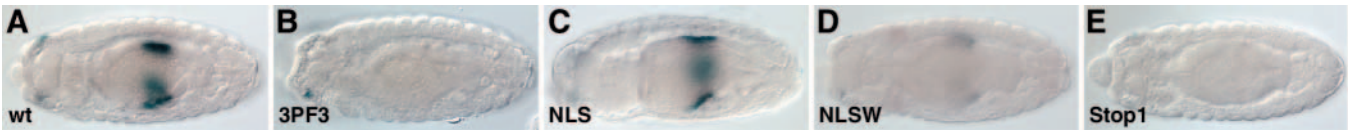

Fig. 6. Repression of the UbxB reporter following the misexpression of modified/mutated forms of Brk in the visceral mesoderm of embryos (X-gal stained), using the 24B Gal4 driver. (A) No *brk* transgene. At this stage, UbxB drives *lacZ* expression in parasegments 6-9 of the midgut mesoderm. (B) Wild-type Brk^{3PF3} completely represses UbxB. (C) Brk^{NLS} has no effect on UbxB expression. (D,E) However, Brk^{NLSW} dramatically reduces, and Brk^{Stop1} completely represses, UbxB expression.

expression is normal in *CtBP* single mutant clones (Fig. 4G), demonstrating that the ectopic expression of *sal* found in *CtBP*/*gro* double mutant clones can be rescued completely by Gro alone. *sal* expression in *gro* single mutant clones, however, is occasionally expanded (Fig. 4H), but not as extensively as in the *CtBP*/*gro* double mutant clones, indicating that CtBP can provide some, but not full, repressor activity to narrow the lateral limit of *sal* expression.

CtBP/Gro-independent repression by Brk is dependent upon a domain, 3R, located just C-terminal to the DBD

The inactivity of Brk^{NLS} in comparison to Brk^{F138} suggested that the region between the DBD and the CiM contains an additional repression domain. This was tested by misexpression of additional modified Brk proteins. Brk^{Stop1} is similar to Brk^{F138}, being truncated immediately before the CiM (Fig. 2), and was tested initially to rule out the possibility that any Brk activity in the brk^{F138} mutant was due to read through of the stop codon. Misexpression of Brk^{Stop1} in the posterior compartment at 25°C resulted in complete repression of *omb* and *sal*, and of the vg-QE (Fig. 5H,L). At 20°C (with concomitant lower expression of the transgene), *omb* was still almost completely repressed, although there was still weak residual expression remaining in the posterior compartment (Fig. 5I). By contrast, at this temperature *sal* was still strongly expressed in the posterior (although the size of its domain was significantly reduced), demonstrating that Brk^{Stop1} is much more effective at repressing *omb* than *sal*, and, thus, that it differs from the wild-type protein.

Although we cannot rule out the possibility that Brk^{Stop1} represses *sal* directly 25°C, it is likely that it does this indirectly: *sal* requires Omb for expression (del Alamo Rodriguez et al., 2004) and *omb* is completely repressed at this temperature (Fig. 5H). It should be pointed out, however, that at 20°C, *omb* expression cannot be detected in some cells that are expressing *sal* (Fig. 5I). Because previous studies (del Alamo Rodriguez et al., 2004), and our own (not shown), indicate that *sal* expression is absolutely dependent upon Omb, the likeliest explanation for this observation is that *omb* is expressed in these cells at levels sufficient for expression of *sal*, but that is just too low to be detected with the *omb-lacZ* line.

Brk^{Stop1} can also repress the reporter UbxB and the vg-QE. It can, in fact, repress UbxB even more effectively than Brk^{NLSW} does (Fig. 6E). It is unclear why Brk^{Stop1} can repress the vg-QE when Brk^{F138} appears to be compromised in this respect (Fig. 4E), and when vg-QE expression is upregulated in *gro* mutant clones (Hasson et al., 2001). It is possible that Gro is required for repression of the vg-QE when Brk is present at physiological levels, but not at the higher levels that can be achieved with the UAS/Gal4 system.

Additional truncations identified a minimal protein, Brk^{A}, truncated at residue 206 (Fig. 2D), with similar activity to Brk^{Stop1}. A series of additional constructs, which either had repressor activity, Brk^{EC} and $Brk^{Stop1\Delta17}$, or did not, $Brk^{Stop1NA}$ and Brk^{A2}, identified the region 151-206 as being the minimum region sufficient to confer repressive activity. This region has been termed 3R, for the third repression domain, in addition to the CiM and GiM (Fig. 2A). 3R consists of a histidine-rich

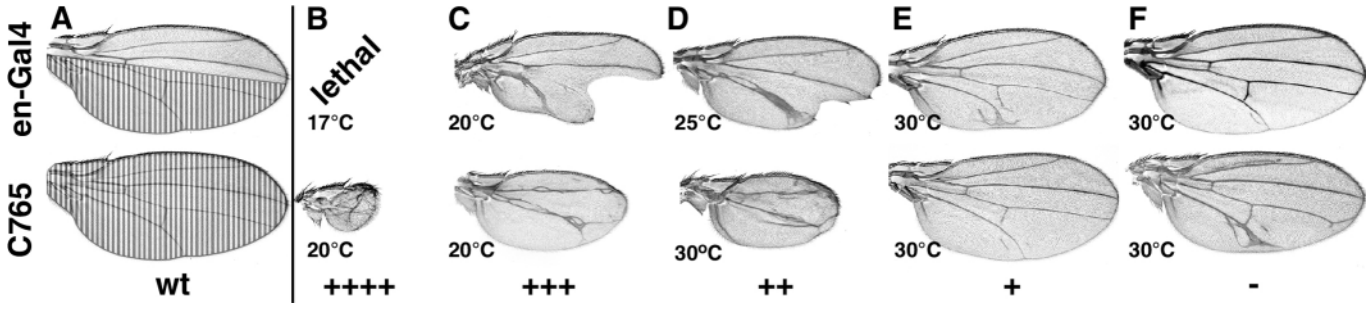

Fig. 7. Phenotype of adult wings following the misexpression of modified/mutated forms of *brk*. UAS-brk transgenes (see Fig. 2D) were driven by en-Gal4 in the posterior compartment and C765 ubiquitously (C765 is weaker than en-Gal4). (A) Wild-type wings shaded to indicate the expression domains of the Gal4 drivers. (B-F) The activity level of different Brk proteins was classified into five categories from '++++' (full, wild-type level) to '–' (no repressor activity; see Materials and methods); examples of each category are shown, for comparison, the temperature must be taken into account (Gal4 is cold sensitive). (B) Full activity of 3PF3 (wild type). With en-Gal4, no animals survived to adult; with C765 the wing size is drastically reduced at 20°C. (C-F) Mutated/modified Brk proteins have weaker activity than wild type. (C) Stop1, 20°C (top); CM, 20°C (bottom). (D) 3M, 25°C (top); 3M, 30°C (bottom). (E) A2, 30°C. (F) NLS, 30°C (top); F124, 30°C (bottom). At high temperatures, ectopic veins are produced with F124, which we attribute to gain of function, as it has no effect on Brk target gene expression.

region, a stretch of poly-alanine and some unique sequence at the C terminus. Further studies are required to narrow down essential sequences in this region; for example, the fact that $Brk^{Stop1\Delta A17}$ still has considerable activity suggests that the stretch of poly-alanine is probably not essential.

Brk protein possessing only the CiM has significant activity

Experiments described above show that a Brk protein possessing only a GiM, or 3R, has significant activity (Fig. 2). We further demonstrated that a protein, $Brk^{Stop1NAC}$, possessing only a CiM (deleting the 3R domain and terminating immediately after the CiM; Fig. 2D) repressed *omb* and *sal*, and significantly reduced the size of adult wings (Fig. 2D, Fig. 5G). It behaved similarly to wild-type Brk, and was more effective at repressing *sal* than *omb*.

Inactivation of a single repression domain/motif reduces the activity of Brk

To test whether the loss of an individual repression domain/motif resulted in reduced Brk activity, we tested the activity of Brk proteins in which either the CiM (Brk^{CM}), GiM (Brk^{GM}) or 3R (Brk^{NA}) was mutated or deleted. In contrast to wild-type Brk^{3PF3} (Fig. 7B), adult flies did survive following the misexpression of each of these single mutant transgenes with en-Gal4 at 20°C (Fig. 7C) indicating that they were less active than the wild-type protein. In terms of gene expression, each could repress *sal*, *omb* and the vg-QE, but were essentially too similar in their activity level to each other, and to the wild-type Brk^{3PF3} protein, to make any clear conclusions.

Brk must possess an additional repression domain(s)

If Brk requires a DBD plus a repression domain to function, and 3R, CiM and GiM are the only repression domains/motifs, then mutation or deletion of all three should render the Brk protein inactive. However, in Brk^{3M}, 3R is deleted, and the CiM and GiM are mutated, but this protein still had significant activity (Fig. 2D, Fig. 7D), indicating that there must be at least one more repression domain/motif, which is probably located between the CiM and the C terminus. Preliminary studies indicate a fourth repression domain may lie between the CiM and GiM, but further analysis is required to confirm this finding (not shown).

Discussion

A key step in Dpp acting as a morphogen in the *Drosophila* wing is its generation of lateral-to-medial gradients of Brk expression, a pattern that mirrors the distribution of Dpp itself. Brk represses 'Dpp targets', such as *sal* and *omb*, and their lateral limits of expression are defined by their sensitivity to Brk (Campbell and Tomlinson, 1999; Entchev et al., 2000; Jazwinska et al., 1999; Minami et al., 1999; Muller et al., 2003; Teleman and Cohen, 2000). Consequently, the less sensitive target *omb* is expressed in a wider domain than the more sensitive target *sal*. The question we wished to address was: why are some genes, such as *sal*, repressed by lower levels of Brk than others, such as *omb*? Here, we have investigated the mechanisms that Brk uses to repress gene expression, to determine whether these are the same for different genes. If so, any difference in sensitivity between genes would be simply quantitative: Brk would operate identically, but just more effectively on more sensitive targets. However, if Brk does not always use the same mechanism, then any perceived difference in sensitivity may result from relative differences in the ability of Brk to use alternative mechanisms. We show that Brk can use different mechanisms to repress *sal* or *omb*.

Brk does not appear to repress by simple competition

The simplest method of transcriptional repression involves competition with an activator, and can operate at the level of DNA if the activator and the repressor have the same, or overlapping, binding sites in an enhancer. In theory, assuming a transcription factor is nuclear, it should only require a DNA-binding domain to act in this fashion. Brk has been shown to possess an N-terminal sequence-specific DNA-binding domain (DBD; Fig. 1) (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller et al., 2002; Sivasankaran et al., 2000; Zhang et al., 2001), and here we have identified several mutations in this domain that either completely inactivate or reduce the activity of the protein (Figs 2, 3, Fig. 4A), indicating that this region is essential for Brk activity.

Previous studies suggested that Brk could function by competition, more specifically, by competing with Mad for overlapping binding sites in vitro (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001). However, a nuclear localized Brk protein consisting primarily of the DBD, Brk^{NLS}, cannot repress any Brk target in vivo (Fig. 2D, Fig. 5C, Fig. 6C), including the embryonic UbxB reporter, which has been shown to possess overlapping Brk and Mad binding sites that Brk and Mad can compete for in vitro (Kirkpatrick et al., 2001; Saller and Bienz, 2001). It is possible that Brk^{NLS} cannot bind to DNA in vivo. However, a modified protein, Brk^{NLSW}, which is identical to Brk^{NLS} apart from the addition of the four amino acids WRPW (Fig. 2D) that recruit the co-repressor Gro (Aronson et al., 1997; Fisher et al., 1996), can repress targets (Fig. 5F, Fig. 6D), indicating that Brk^{NLS} should also be capable of binding to these targets in vivo.

Competition has been proposed as a mechanism for many transcriptional repressors. However, direct in vivo support for or against such proposals is rare, at least of the sort presented here, i.e. testing, in vivo, the ability of a protein consisting largely of a functional DBD, which has access to the nucleus, to repress a target for which there is in vitro evidence for overlapping binding sites with an activator. There is some in vivo evidence that the *Drosophila* embryonic repressor Kruppel can repress a synthetic enhancer containing overlapping binding sites with the activators Dorsal and Bicoid (Nibu et al., 2003). However, although this repression was CtBP-independent, and further studies are required to rule out additional domains outside of the DBD being required (Licht et al., 1990) in a similar fashion to the 3R domain in Brk. The paucity of good examples of binding-site competition in vivo in eukaryotes is in stark contrast to in prokaryotes (Ptashne and Gann, 2001), and raises the question of how common this phenomenon really is in eukaryotes.

Brk possesses at least three independent repression domains/motifs

If Brk cannot repress by competition it must possess repression

domains/motifs, and previous studies identified interaction motifs for the co-repressors CtBP and Gro (CiM and GiM) (Hasson et al., 2001; Saller and Bienz, 2001; Zhang et al., 2001). However, repression of at least one Brk target, *omb*, was previously shown not to require CtBP or Gro (Hasson et al., 2001). This is consistent with our demonstration that the protein produced by the endogenous mutant brk^{F138}, which is truncated before the CiM and GiM, can still repress *omb* (Fig. 4D). Truncated proteins that lack the CiM and GiM, Brk^{Stop1}, Brk^{EC} and Brk^{A} (Fig. 2D), can also repress *omb* (Fig. 5H), but only if they contain a specific region between the DBD and CiM that has been classified as a third repression domain, 3R (Fig. 2A). Further studies are required to determine if 3R is a true autonomous repression domain, i.e. if it can function outside of Brk, or if it is more specific (for example, antagonizing activators such as Mad), and to determine what its specific properties are (for example, how close do Brk sites have to be to activator sites for 3R to be effective?).

Differential activity of the repression domains

The three repression domains/motifs of Brk are not equivalent (Fig. 2D). Wild-type Brk and proteins containing only a GiM, Brk^{NLSW}, or only a CiM, $Brk^{Stop1NAC}$, can repress both *sal* and *omb*, and they are more effective at repressing *sal* than *omb* (Fig. 5A,B,F,G). Analysis of *gro* and *CtBP* single and double mutant clones revealed that Gro is required for normal repression of *sal* in wing discs, and that CtBP can provide some, but not always complete, activity for the repression of *sal* in the absence of Gro (Fig. 4F-H). By contrast, Gro and CtBP are not required for repression of *omb* (Hasson et al., 2001).

The 3R domain is sufficient for Brk to repress *omb* (Fig. 4D, Fig. 5H) and the UbxB enhancer in embryos (Fig. 6E), but is deficient for the repression of *sal* (Fig. 4D). Furthermore, misexpression of proteins possessing only the 3R domain (plus the DBD) are much more effective at repressing *omb* than *sal*, i.e. the converse of wild-type Brk or Brk possessing only a GiM or a CiM (Fig. 5I). Although some results suggested that 3R may confer a limited ability to repress *sal* (Fig. 5H), this is probably indirect, because a previous study (del Alamo Rodriguez et al., 2004), and our own (not shown), demonstrated that *sal* requires Omb to be expressed, and if *omb* is repressed directly, *sal* will be lost also. However, we cannot completely rule out the possibility that high levels of proteins possessing only the 3R domain can repress *sal* directly.

Contradictory results were obtained regarding the ability of 3R to repress the vg-QE. Expression of the vg-QE did show expansion in some brk^{F138} clones (Fig. 4E), indicating that the truncated protein produced in this mutant (which only has the 3R repression domain) cannot efficiently repress this enhancer. However, similar in vitro truncated proteins, such as Brk^{Stop1}, could efficiently repress vg-QE expression when misexpressed using the UAS/Gal4 system (Fig. 5L). Such a difference could simply be a reflection of the high levels of expression achieved with the Gal4/UAS system, and that, at physiological levels, the 3R domain is not sufficient for complete repression of the vg-QE.

Whether a single repression domain is sufficient for Brk to repress a particular target may depend upon the positioning of Brk sites in relation to activator sites (or possibly the promoter) at that target. The UbxB reporter has overlapping Brk and activator (Mad) sites (Kirkpatrick et al., 2001; Saller and Bienz, 2001). Analysis of an *omb* enhancer revealed that an important Brk site may also overlap with an activator (Sivasankaran et al., 2000). Conversely, analysis of the cis-regulatory elements of the *sal* gene indicate that activator and Brk sites are separated (Barrio and de Celis, 2004). Proteins possessing only 3R can repress UbxB and *omb*, but not *sal*, suggesting that 3R may only be sufficient for the repression of genes in which the Brk sites are situated very close to activator sites.

Multiple repression domains

Why does Brk possess at least three, probably four, independent repression domains/motifs? There are two obvious answers: qualitative, different repression domains/motifs are required for the repression of different targets; and quantitative, more domains/motifs provide greater repressor activity. Other transcription factors have multiple repression domains and there is evidence that they have these for either qualitative or quantitative reasons, and, in some cases, both. For example, in the *Drosophila* embryo, the pair-rule protein Runt requires Gro for the repression of one stripe of the pair-rule genes, *even skipped* (*eve*) and *hairy*, but not for the repression of *engrailed* (Aronson et al., 1997). The gap protein Knirps represses different stripes of *eve*; for stripes 4 and 6 it requires CtBP, but for stripes 3 and 7, it does not. However, this appears to be a quantitative difference, because increasing the levels of Knirps allows it to repress stripes 4 and 6 even in the absence of CtBP (Struffi et al., 2004). Similarly, Gro appears to increase the repressor activity of the Eve protein (Kobayashi et al., 2001).

As discussed above, there is some difference in the ability of the three domains/motifs in Brk to repress different targets. For example, the 3R domain is sufficient for the normal repression of *omb* but not *sal*. However, either the CiM or GiM appear to be sufficient for the repression of both *sal* and *omb* (Fig. 2D, Fig. 5F,G), so why does Brk need the 3R domain? In the absence of Gro and CtBP, the Brk protein appears fully active in its ability to repress *omb*, and recruiting Gro and CtBP does not seem to increase its activity towards *omb*; otherwise, the width of the *omb* domain would be expected to shift in brk^{F138} mutant cells, which have no CiM or GiM, or in *CtBP gro* double mutant cells, but it does not (Hasson et al., 2001). It is possible that, in regard to *omb*, the 3R domain is more efficient than either of the other two and provides Brk with sufficient activity to establish the *omb* domain in the correct position.

Brk needs to recruit either CtBP or Gro for the repression of some targets, including *sal* (Fig. 3E-G) and *brk* itself (Hasson et al., 2001), or just Gro for some others, including the vg-QE (Hasson et al., 2001). Consequently, why does Brk need to recruit CtBP? Mutation of the CiM alone, in common with mutation or deletion of just the GiM and 3R, does reduce activity of Brk, as judged by its effect when misexpressed (Fig. 2D). However, there is no evidence that CtBP is required specifically for the repression of any Brk target in the wing, because CtBP mutant clones have no effect on the expression of any known Brk target in the wing (Fig. 4G) (Hasson et al., 2001). The CtBP and Gro motifs in Brk have been conserved over millions of years (Fig. 1), and thus, recruiting CtBP is presumably important for Brk activity. It is possible that CtBP is required outside of the wing – for example in the embryo

(Hasson et al., 2001) – or for some other, as yet, uncharacterized targets in the wing.

Recruiting both CtBP and Gro does appear to be a little illogical from what is known about their basic properties, CtBP acting only over a short range, while Gro acts over much longer ranges. It might be assumed that different transcription factors would use either Gro or CtBP (Zhang and Levine, 1999), because the primary advantage of recruiting CtBP is that it would allow a transcription factor to repress one enhancer without disrupting the activity of one nearby, which would be repressed if Gro was recruited, although this simple model does not always hold (Nibu et al., 2001). Consequently, most transcription factors do recruit only one of these co-repressors. However, there are two other exceptions, Hairy and Hairless. In Hairy it appears that CtBP may actually be functioning to antagonize Gro activity and not in its standard role as a co-repressor (Phippen et al., 2000; Zhang and Levine, 1999). There is no evidence that it does this in Brk, where it can provide repressor activity. For Hairless, there is genetic evidence that both CtBP and Gro provide repressor activity to the protein (Barolo and Posakony, 2002), although it is not clear if CtBP is required to increase the general activity of Hairless, or for repression of specific targets that cannot be repressed adequately by Gro.

With the exception of the brk^{F138} mutant, our analysis has been limited to analyzing the effects of misexpressing modified Brk proteins in positions where the endogenous protein is not found. Consequently, further insights into the precise roles of individual repression domains will require replacing the endogenous gene with one in which only one or two repressions domain have been mutated or deleted.

Thresholds

To conclude, it is often assumed that the sensitivity of one enhancer to a transcription factor compared with that of another enhancer is based largely upon the number or the affinity of the binding sites for that transcription factor in each enhancer. However, other factors are also important; for example, the ability of the Giant transcription factor to repress a promoter is related to how closely it binds (Hewitt et al., 1999). Here, we have shown that the two best characterized outputs of the Dpp morphogen gradient, *sal* and *omb*, appear to be regulated differently by Brk. Consequently, simply counting binding sites and measuring their affinity will not reveal why one is more sensitive to Brk than the other, and we need to factor in what specific repressive mechanisms are being used, and the relative efficiencies of each.

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References

Aronson, B. D., Fisher, A. L., Blechman, K., Caudy, M. and Gergen, J. P. (1997). Groucho-dependent and –independent repression activities of Runt domain proteins. *Mol. Cell. Biol.* **17**, 5581-5587.

Barolo, S. and Posakony, J. W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev.* **16**, 1167-1181.

Barrio, R. and de Celis, J. F. (2004). Regulation of spalt expression in the Drosophila wing blade in response to the Decapentaplegic signaling pathway. *Proc. Natl. Acad. Sci. USA* **101**, 6021-6026.

Blackman, R. K., Sanicola, M., Raftery, L. A., Gillevet, T. and Gelbart, W. M. (1991). An extensive 3′ cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in Drosophila. *Development* **111**, 657-666.

Campbell, G. and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by brinker. *Cell* **96**, 553-562.

Chen, G. and Courey, A. J. (2000). Groucho/TLE family proteins and transcriptional repression. *Gene* **249**, 1-16.

Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol. Cell* **9**, 213-224.

Clyde, D. E., Corado, M. S., Wu, X., Pare, A., Papatsenko, D. and Small, S. (2003). A self-organizing system of repressor gradients establishes segmental complexity in Drosophila. *Nature* **426**, 849-853.

del Alamo Rodriguez, D., Terriente Felix, J. and Diaz-Benjumea, F. J. (2004). The role of the T-box gene optomotor-blind in patterning the Drosophila wing. *Dev. Biol.* **268**, 481-492.

Entchev, E. V., Schwabedissen, A. and Gonzalez-Gaitan, M. (2000). Gradient formation of the TGF-beta homolog Dpp. *Cell* **103**, 981-991.

Fisher, A. L., Ohsako, S. and Caudy, M. (1996). The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol. Cell. Biol.* **16**, 2670-2677.

Grigliatti, T. (1986). Mutagenesis. In Drosophila*: A Practical Approach* (ed. D. B. Roberts), pp. 39-58. Oxford: IRL Press.

Hasson, P., Muller, B., Basler, K. and Paroush, Z. (2001). Brinker requires two corepressors for maximal and versatile repression in Dpp signalling. *EMBO J.* **20**, 5725-5736.

Hewitt, G. F., Strunk, B. S., Margulies, C., Priputin, T., Wang, X. D., Amey, R., Pabst, B. A., Kosman, D., Reinitz, J. and Arnosti, D. N. (1999). Transcriptional repression by the Drosophila giant protein: cis element positioning provides an alternative means of interpreting an effector gradient. *Development* **126**, 1201-1210.

Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S. and Rushlow, C. (1999). The Drosophila gene brinker reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563-573.

Jiang, J., Rushlow, C. A., Zhou, Q., Small, S. and Levine, M. (1992). Individual dorsal morphogen binding sites mediate activation and repression in the Drosophila embryo. *EMBO J.* **11**, 3147-3154.

Kalderon, D., Roberts, B. L., Richardson, W. D. and Smith, A. E. (1984). A short amino acid sequence able to specify nuclear location. *Cell* **39**, 499-509.

Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene. *Nature* **382**, 133-138.

Kim, J., Johnson, K., Chen, H. J., Carroll, S. and Laughon, A. (1997). Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. *Nature* **388**, 304-308.

Kirkpatrick, H., Johnson, K. and Laughon, A. (2001). Repression of dpp targets by binding of brinker to mad sites. *J. Biol. Chem.* **276**, 18216-18222.

Kobayashi, M., Goldstein, R. E., Fujioka, M., Paroush, Z. and Jaynes, J. B. (2001). Groucho augments the repression of multiple Even skipped target genes in establishing parasegment boundaries. *Development* **128**, 1805-1815.

Lammel, U. and Saumweber, H. (2000). X-linked loci of Drosophila melanogaster causing defects in the morphology of the embryonic salivary glands. *Dev. Genes Evol.* **210**, 525-535.

Lammel, U., Meadows, L. and Saumweber, H. (2000). Analysis of Drosophila salivary gland, epidermis and CNS development suggests an additional function of brinker in anterior-posterior cell fate specification. *Mech. Dev.* **92**, 179-191.

Langeland, J. A., Attai, S. F., Vorwerk, K. and Carroll, S. B. (1994). Positioning adjacent pair-rule stripes in the posterior Drosophila embryo. *Development* **120**, 2945-2955.

Lawrence, P. A. and Johnston, P. (1986). Methods of marking cells. In

Drosophila*: A Practical Approach* (ed. D. B. Roberts), pp. 229-242. Oxford: IRL Press.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature* **381**, 387-393.

Licht, J. D., Grossel, M. J., Figge, J. and Hansen, U. M. (1990). Drosophila Kruppel protein is a transcriptional repressor. *Nature* **346**, 76-79.

Marty, T., Muller, B., Basler, K. and Affolter, M. (2000). Schnurri mediates Dpp-dependent repression of brinker transcription. *Nat. Cell Biol.* **2**, 745-749.

Masucci, J. D., Miltenberger, R. J. and Hoffmann, F. M. (1990). Pattern-specific expression of the Drosophila decapentaplegic gene in imaginal disks is regulated by 3′ cis-regulatory elements. *Genes Dev.* **4**, 2011-2023.

Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H. and Tabata, T. (1999). brinker is a target of Dpp in Drosophila that negatively regulates Dpp-dependent genes. *Nature* **398**, 242-246.

Muller, B., Hartmann, B., Pyrowolakis, G., Affolter, M. and Basler, K. (2003). Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. *Cell* **113**, 221-233.

Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.

Nibu, Y., Zhang, H. and Levine, M. (2001). Local action of long-range repressors in the Drosophila embryo. *EMBO J.* **20**, 2246-2253.

Nibu, Y., Senger, K. and Levine, M. (2003). CtBP-independent repression in the Drosophila embryo. *Mol. Cell. Biol.* **23**, 3990-3999.

Phippen, T. M., Sweigart, A. L., Moniwa, M., Krumm, A., Davie, J. R. and Parkhurst, S. M. (2000). Drosophila C-terminal binding protein functions as a context-dependent transcriptional co-factor and interferes with both mad and groucho transcriptional repression. *J. Biol. Chem.* **275**, 37628-37637.

Ptashne, M. and Gann, A. (2001). *Genes and Signals*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Rivera-Pomar, R. and Jackle, H. (1996). From gradients to stripes in Drosophila embryogenesis: filling in the gaps. *Trends Genet.* **12**, 478-483.

Rushlow, C., Colosimo, P. F., Lin, M. C., Xu, M. and Kirov, N. (2001). Transcriptional regulation of the Drosophila gene zen by competing Smad and Brinker inputs. *Genes Dev.* **15**, 340-351.

Saller, E. and Bienz, M. (2001). Direct competition between Brinker and Drosophila Mad in Dpp target gene transcription. *EMBO Rep.* **2**, 298-305.

Saller, E., Kelley, A. and Bienz, M. (2002). The transcriptional repressor Brinker antagonizes Wingless signaling. *Genes Dev.* **16**, 1828-1838.

Sivasankaran, R., Vigano, M. A., Muller, B., Affolter, M. and Basler, K. (2000). Direct transcriptional control of the Dpp target omb by the DNA binding protein Brinker. *EMBO J.* **19**, 6162-6172.

Stathopoulos, A. and Levine, M. (2002). Dorsal gradient networks in the Drosophila embryo. *Dev. Biol.* **246**, 57-67.

Struffi, P., Corado, M., Kulkarni, M. and Arnosti, D. N. (2004). Quantitative contributions of CtBP-dependent and -independent repression activities of Knirps. *Development* **131**, 2419-2429.

Teleman, A. A. and Cohen, S. M. (2000). Dpp gradient formation in the Drosophila wing imaginal disc. *Cell* **103**, 971-980.

Thuringer, F., Cohen, S. M. and Bienz, M. (1993). Dissection of an indirect autoregulatory response of a homeotic Drosophila gene. *EMBO J.* **12**, 2419-2430.

Zhang, H. and Levine, M. (1999). Groucho and dCtBP mediate separate pathways of transcriptional repression in the Drosophila embryo. *Proc. Natl. Acad. Sci. USA* **96**, 535-540.

Zhang, H., Levine, M. and Ashe, H. L. (2001). Brinker is a sequence-specific transcriptional repressor in the Drosophila embryo. *Genes Dev.* **15**, 261-266.