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The repressor and activator forms of Cubitus interruptus control Hedgehog target genes through common generic Gli-binding sites

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SUMMARY

The *Drosophila* Gli homolog Cubitus interruptus (Ci) controls the transcription of Hedgehog (Hh) target genes. A repressor form of Ci arises in the absence of Hh signalling by proteolytic cleavage of intact Ci, whereas an activator form of Ci is generated in response to the Hh signal. These different activities of Ci regulate overlapping but distinct subsets of Hh target genes. To investigate the mechanisms by which the two activities of Ci exert their opposite transcriptional effect, we dissect here the imaginal disc enhancer of the *dpp* gene, which responds to both activities of Ci. Within a minimal disc enhancer, we identify the DNA sequences that are necessary and sufficient for the control by Ci, show that the same sequences respond to the activator and repressor forms of Ci, and demonstrate that their activities can be replaced by a single synthetic Gli-binding site. We further show that the enhancer sequences of *patched*, a gene responding only to the activator form of Ci, effectively integrate also the repressor activity of Ci if placed into a *dpp* context. These results provide in vivo evidence against the employment of distinct binding sites for the different forms of Ci and suggest that target genes responding to only one form must have acquired distant *cis*-regulatory elements for their selective behavior.

Key words: Hedgehog target genes, Cubitus interruptus, Gli proteins, *Drosophila*, Transcription, *dpp*, *engrailed*, *patched*

INTRODUCTION

Hedgehog signalling proteins regulate a wide variety of developmental events throughout the animal kingdom. Most, if not all, Hedgehog target genes are controlled through members of the Gli family of transcription factors (reviewed by Altaba, 1999; Ingham, 1998). Major interest is devoted therefore to the mechanisms by which Gli proteins control gene expression. Important advances have recently been made in the system of *Drosophila* limb development, where the function and mode of action of Hedgehog (Hh) and the Gli homolog Cubitus interruptus (Ci) are best understood. Key to our present understanding of Hh signalling was the finding that Ci is regulated post-transcriptionally. In cells that do not receive the Hh signal, a low molecular weight form of Ci, Ci-75, is generated through proteolytic cleavage of the full-length protein Ci-155 (Aza-Blanc et al., 1997). This truncated form of Ci functions as a transcriptional repressor and is here referred to as Ci[rep]. The reception and transduction of the Hh signal prevents the formation of Ci[rep] and at the same time causes the conversion of Ci-155 into an activator form (Ci[act]) (Chen et al., 1999; Méthot and Basler, 1999; Price and Kalderon, 1999; reviewed by Aza-Blanc and Kornberg, 1999). The existence of two forms of Ci with opposite transcriptional activities – the formation of each being subject to Hh control – allows multiple modes of target gene regulation. For example, and as described below, this setup not only provides the means to induce target gene expression in response to Hh but also to ensure tight repression of the same or other genes in cells that do not receive the Hh signal.

Genetic analyses in imaginal discs have recently indicated that the activator and repressor activities of Ci regulate overlapping but distinct subsets of Hh target genes (Méthot and Basler, 1999). Cells of the posterior compartment are programmed by the selector gene *engrailed* (*en*) to secrete Hh (reviewed in Lawrence and Struhl, 1996). During most developmental stages, En is not active in anterior compartment cells and, as a consequence, all these cells express *ci* instead of *hh* (Schwartz et al., 1995), and are thus competent to respond to the Hh signal (Zecca et al., 1995). Only cells in the vicinity of the anteroposterior (AP) compartment boundary, however, receive the Hh signal. The activation of the Hh transduction pathway causes the upregulation of *patched* (*ptc*) expression and the induction of the *decapentaplegic* (*dpp*) gene (Basler and Struhl, 1994; Capdevila et al., 1994; Tabata and Kornberg, 1994). At late stages of wing development, high levels of Hh signalling also induce the expression of the *en* gene (Blair, 1992; Guillen et al., 1995; Strigini and Cohen, 1997). Mutant anterior compartment cells entirely lacking *ci* function fail to upregulate *ptc* and *en* expression, which indicates that these two genes are primarily controlled by the activator form of Ci (Méthot and Basler, 1999). These cells, however, express low levels of *dpp* and *hh*, irrespective of their position (Dominguez et al., 1996; Méthot and Basler, 1999).

This observation led to the interpretation that *dpp* is controlled by both forms of Ci, whereas *hh* is only subject to Ci-mediated repression (Méthot and Basler, 1999). Together these findings indicated that Ci[act] and Ci[rep] can have common as well as distinct targets and raised the question of how different target genes can be differentially sensitive to the two related forms of Ci.

Here we address this question experimentally by identifying regulatory DNA elements for both Ci[act] and Ci[rep] and by exploring the context in which these elements can act. We envisage three scenarios through which differential sensitivity towards Ci[act] or Ci[rep] can be explained. The first possibility is that the two forms of Ci recognize, and act through, different binding sites. Although both forms appear to contain the same zinc-finger DNA-binding domain, it is conceivable that either the C-terminal half of Ci that is lacking in Ci[rep], or some signal-induced covalent modification of Ci[act] results in distinct DNA-binding specificities. The competence of target genes to respond to only one or to both forms of Ci could thus be encoded in the nucleotide sequence of the Ci responsive elements. If these elements are identical in structure, a second possibility would be that they differ in context. Either form of Ci could be specifically associated with DNA-binding cofactors. The function of the resulting complex would depend on the presence of adjacent binding sites for Ci and such cofactors. Finally, in a third scenario, neither sequence nor local context of Ci[act]- and Ci[rep]-binding sites differ. In some target genes, the function of bound Ci may be modulated by distant *cis*-regulatory elements to allow input by one, but not the other form of Ci.

To discriminate between these possibilities, we set out to identify regulatory elements that mediate Ci[rep] and Ci[act] input. Starting with a 4 kb fragment of the *dpp* gene, we narrowed these elements down to 20 bp by an unbiased, functional assay. We found that both activities of Ci are mediated via the same DNA element, that this element contains a Gli-binding site, and that a synthetic 9 bp Gli consensus binding site can substitute for both activities. In addition, we show that even the Ci responsive element of *ptc*, a gene that normally only responds to Ci[act] input, can confer regulation by Ci[rep] if placed into the *dpp* enhancer context. Finally, we demonstrate that the ability of a Gli consensus binding site to respond both to Ci[act] and Ci[rep] is not limited to the *dpp* enhancer context, but is also observed in combination with an unrelated, naïve enhancer. Taken together, our results indicate that Ci[rep] sites differ neither in DNA sequence nor in local context from Ci[act] sites, which leads us to propose that other *cis*-regulatory properties determine the selective behavior of certain Hh target genes to Ci[act] or Ci[rep] only.

MATERIALS AND METHODS

Clonal analysis

ci^{-} mutant clones were generated for imaginal disc analysis as described by Méthot and Basler (1999), using the enhancer constructs 10ΔH, 10ΔG and 10ΔG+Gli as reporters. Genotypes of the larvae were as follows:

y w hsp70-flp; FRT42 P[ci^{+}] hsp70-GFP/FRT42; P[10ΔH]/+; ci^{94}/ci^{94}

y w hsp70-flp; FRT42 P[ci^{+}] hsp70-GFP/FRT42; P[10ΔG]/+; ci^{94}/ci^{94}

y w hsp70-flp; FRT42 P[ci^{+}] hsp70-GFP/FRT42; P[10ΔG+Gli]/+; ci^{94}/ci^{94}

Transgenes

The 4 kb *Bam*HI fragment was kindly provided by F. M. Hoffmann. Its sequence is now available as part of a genomic sequence in GenBank, accession number U63852. All enhancer fragments were cloned into the reporter vector pX27 (Segalat et al., 1994) that contains a minimal *hsp70* promoter with a canonical TATA box (derived from HZ50, Hiromi et al., 1985). Inserts of constructs 1-10 were obtained by restriction digests using enzymes depicted above construct 1 in Fig. 1. All other inserts were generated by PCR. These constructs were all sequenced to confirm that no unwanted mutations were introduced. The sequence of fragment G is shown on top of Fig. 4.

Fragment E: 5′-AAAGAAAGCGCAGGCAGGAGAATATACCT-TAATTACGGTTAATGGAGCGTTCGAAAAAACAAAACCGATG-GCTTTATATGTGGCCCAGTGTGT-3′

Fragment F (the putative En-binding site is underlined): 5′-GTATCATATGTTGGATCTTCGGCCGAGTGCCACGGCGAAATA-ACTTAATCACATTTCGAGAAGAGACGACCGCAAAAATCTGC-GAGCCATGTTCGTAATTTTGTATATAAATG-3′.

Internal deletions in constructs 10ΔA-10ΔH and 10Δa-10Δe were generated by replacing the appropriate sequences with a *Bam*HI restriction site. In constructs 10ΔG+a-10ΔG+e, a *Bam*HI restriction site was introduced distal (left side in Fig. 4) of fragments a to e.

A 270 bp PCR fragment corresponding to the *ptc* promoter region –811 to –542 was used for construct 10ΔG+*ptc*, (corresponding to the FE minus the GE constructs as published in Alexandre et al., 1996).

Immunochemistry and histochemistry

Imaginal discs from third instar larvae were fixed and stained by standard techniques. Antibodies were rabbit polyclonal anti-β-gal (Cappel) and anti-rabbit Alexa 594 fluorescent secondary antibodies (Molecular Probes). To detect β-galactosidase activity, third instar larval discs were fixed and subjected to a standard X-gal color reaction for 30 minutes at 37°C.

RESULTS

Identification of a minimal *dpp* imaginal disc enhancer

The *dpp* locus comprises more than 25 kb of regulatory sequences 3′ of the transcription unit (position 89 to 114 on the molecular map of *dpp*, Blackman et al., 1987; St Johnston et al., 1990). At least 30 *dpp disc* alleles are known that reduce *dpp* expression in imaginal discs and lack part of these regulatory sequences (St Johnston et al., 1990). Hence, it is likely that these sequences receive and mediate Hh input and thus are targets for Ci. With the aim of identifying a minimal *dpp* enhancer fragment that responds to Ci[act] and Ci[rep], we focussed on region 106 to 110 (see Fig. 1, top) where a 4 kb fragment driving a *dpp* transgene has been shown to complement *dpp disc* alleles (Masucci et al., 1990). This fragment directs *lacZ* expression in most *dpp*-expressing cells of the wing disc and hence served as a starting point for our analysis (fragment 1, Fig. 1). An initial subdivision of fragment 1 into three overlapping subfragments indicated the presence of important regulatory sequences in the proximal region (see fragments 2 to 4). Terminal deletions on either side of fragment 4 led to the identification of fragment 10, an 800 bp enhancer that is still able to direct *lacZ* expression along the AP

compartment boundary in most of the prospective wing and notum tissue (Fig. 1).

The 100 bp fragment G of the *dpp* disc enhancer is required for regulation by both Ci[act] and Ci[rep]

To identify sequences within our *dpp* enhancer that are required for its transcriptional regulation in the wing pouch, we systematically scanned fragment 10 by introducing eight 100 bp deletions (ΔA to ΔH, Fig. 2). Analysis of reporter gene expression revealed three sequence stretches, each necessary for a distinct aspect of the *dpp* enhancer activity. As described in more detail below, fragment E is necessary for general activity, fragment F for repression in posterior compartment cells and fragment G for mediating Ci input.

Deletion of fragment E significantly reduces the levels of reporter gene expression in the wing imaginal disc (10ΔE, Fig. 2), as well as in the eye, leg and antennal discs (not shown). Hence, a general transcriptional activator appears to interact with fragment E, which we have not characterized further. Construct 10ΔF shows ectopic expression in the posterior compartment. Inspection of the nucleotide sequence of fragment F revealed the presence of a putative binding site for Engrailed (En) (Desplan et al., 1988; Kissinger et al., 1990), suggesting that 10ΔF might direct posterior compartment expression because it is not efficiently repressed by En. To test this assumption, this site (TAATCA) was mutated into a *Spe*I recognition site (ACTAGT), which resulted in a similar, even stronger derepression of fragment 10 activity in the posterior compartment (construct 10 En mut, Fig. 2). This result is consistent with previous work by Sanicola et al. (1995) who reported a direct repression of *dpp* expression by En in posterior leg disc cells. Because the deletion of fragment F causes only a partial derepression of *dpp* enhancer activity in posterior compartment cells, we surmise the existence of functional En-binding sites outside region F.

Construct 10ΔG directs *lacZ* expression in the whole anterior wing pouch. This anterior expansion of *dpp* enhancer activity by the ΔG deletion could be explained by a loss of responsiveness to Ci[rep], which normally arises and represses *dpp* expression in anterior wing cells that are not exposed to the Hh signal (Aza-Blanc et al., 1997; Méthot and Basler, 1999). To test whether 10ΔG is no longer subject to Ci[rep] control, its activity was monitored in clones of wing disc cells mutant for *ci94*, a null allele for *ci* (referred to as ci^{-} below). In contrast to the enhancer trap reporter gene $dppZ^{P10638}$ (Méthot and Basler, 1999) or to our minimal *dpp* enhancer (e.g. 10ΔH, Fig. 3A), which both show a derepression of their activities in anteriorly located ci^{-}clones, 10ΔG shows no obvious activity difference in wild-type versus ci^{-}cells (arrow in Fig. 3B). This indicates that Ci[rep] normally represses the activity of the minimal *dpp* enhancer, but not that of 10ΔG.

To test whether 10ΔG also lost Ci[act] input, we compared reporter gene activity in ci^{-} cells to that in wild-type cells exposed to the Hh signal. This is best achieved in the vicinity

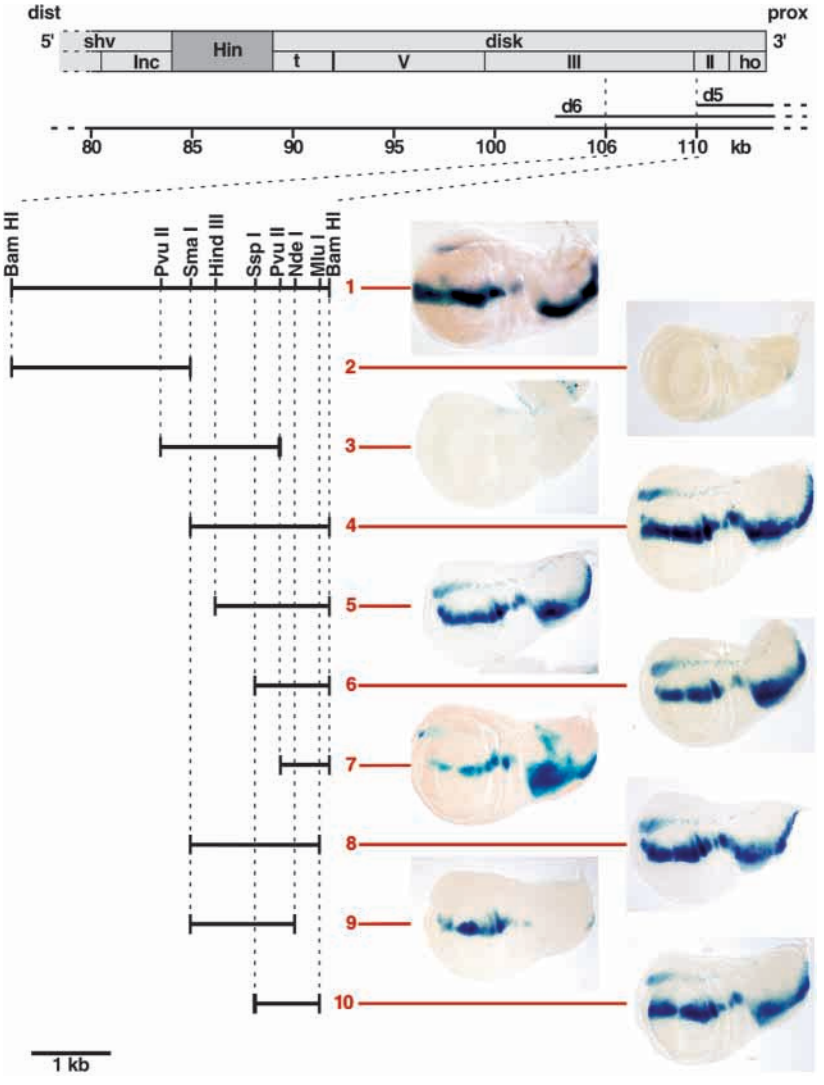

Fig. 1. Identification of an 800 bp minimal *dpp* disc enhancer. Schematic representation of the *dpp* gene and its genetic properties (top of figure, nomenclature and numbering according to St. Johnston et al., 1990); 'Hin' represents the transcribed region of the *dpp* gene. The 4 kb *Bam*HI fragment that served for construct 1 extends from position 106-110 kb on this map and drives expression of a *lacZ* reporter gene in a *dpp*-like pattern. Several rounds of successive terminal deletions (constructs 2 to 10) identified a minimal enhancer element (construct 10) that still exhibits a transcriptional activity similar to that of the *dpp* gene. Further truncations resulted either in a reduction of expression in the ventral wing pouch (compare constructs 6 and 7) or in a loss of expression in the notum (compare constructs 8 and 9). Restriction enzymes used to subclone constructs 1 to 10 are depicted above fragment 1. Representative wing imaginal disc preparations are shown on the right. A minimum of four independent transgenic lines was scored for each reporter construct presented in this study. All preparations were treated identically with the X-gal color reaction proceeding for 30 minutes. In this and all following figures (with exception of Fig. 3) discs are oriented with their anterior side up and dorsal to the right.

of the AP compartment boundary where anterior cells receive and transduce Hh and thus generate Ci[act]. Compared to *dppZ*^{P10638} (Méthot and Basler, 1999) or 10ΔH (arrowhead in Fig. 3A), which show strongly reduced activity in *ci*$^{-}$ cells, 10ΔG is not significantly affected by the lack of Ci. Hence we conclude that 10ΔG is insensitive to both Ci[rep] and Ci[act] and that fragment G must serve as an important and common mediator for these two activities.

Two Gli-binding sites are necessary for the repressor activity of Ci

Ci has been shown to directly bind to the Hh target genes *ptc*, *wg* and *dpp* (Alexandre et al., 1996; Hepker et al., 1999; Von Ohlen and Hooper, 1997). In all cases analyzed, however, the binding sites for Ci conferred transcriptional activation. Fragment G represents a unique situation therefore to investigate the mechanism by which the repressor activity of Ci is integrated into the transcriptional control of Hh target genes. As outlined in the Introduction, there are in principle three possibilities: (i) Ci[rep], by virtue of its different structure, could bind to target sequences that are distinct from those of Ci[act], (ii) Ci[rep] and Ci[act] could bind the same core sequence, but local, context-dependent binding sites for co-factors could confer selective binding or selective transcriptional activities to the two proteins, or (iii) Ci[rep] and Ci[act] both could bind to all and the same binding sites and a selective responsiveness to only one of the two forms would be accomplished by the employment of specific, other *cis*-regulatory elements.

To distinguish between these possibilities, we wanted to narrow down the sequence requirements in fragment G for Ci[rep] input. Five non-overlapping 20 bp deletions, covering the extent of fragment G, were introduced into fragment 10 (Fig. 4, 10Δa to 10Δe; note that lower case letters are used for this finer subdivision). In parallel, five corresponding overlapping 30 bp fragments, also covering the extent of fragment G, were added back into the 10ΔG deletion (Fig. 4, 10ΔG+a to 10ΔG+e).

From the deletion series, only Δa and Δd showed an effect, a partial anterior expansion of *dpp* enhancer activity (Fig. 4, 10Δa and 10Δd), which suggested that, in both cases, input of Ci[rep] is impaired. Moreover, fragments a and d were also partly sufficient to repress ectopic anterior enhancer activity of 10ΔG (Fig. 4, 10ΔG+a and 10ΔG+d). From this, we conclude that the two small fragments a and d contain important regulatory sites through which Ci[rep] can control *dpp* enhancer activity. Thorough sequence examination of fragment 10 then revealed three putative Gli-binding sites, all located within fragment G. Each site differs by two unrelated mismatches to the Gli consensus site TGGG(T/A)GGTC (Kinzler and Vogelstein, 1990). Two of the putative Gli-binding sites map to a and d, a third one to e. No regulatory activity could be attributed to fragment e. However, fragments a and d are necessary and partly sufficient for anterior repression of *dpp* enhancer activity, which suggests that their activity might depend on the presence of a Gli-binding site. To test this presumption, single nucleotide mutations were introduced into the putative Gli-binding sites of fragments a and d (highlighted in red in Fig. 4). Based on the three-dimensional structure of a Gli-DNA complex, these nucleotides have been reported to be essential determinants for Gli/Ci binding (Pavletich and Pabo, 1993). The activity of fragment a as well as fragment d was abolished by this single base pair substitution (Fig. 4, 10ΔG+a* and 10ΔG+d*). Together these results are taken as evidence that two Gli-binding sites in the minimal *dpp* disc enhancer are required for Ci[rep] input.

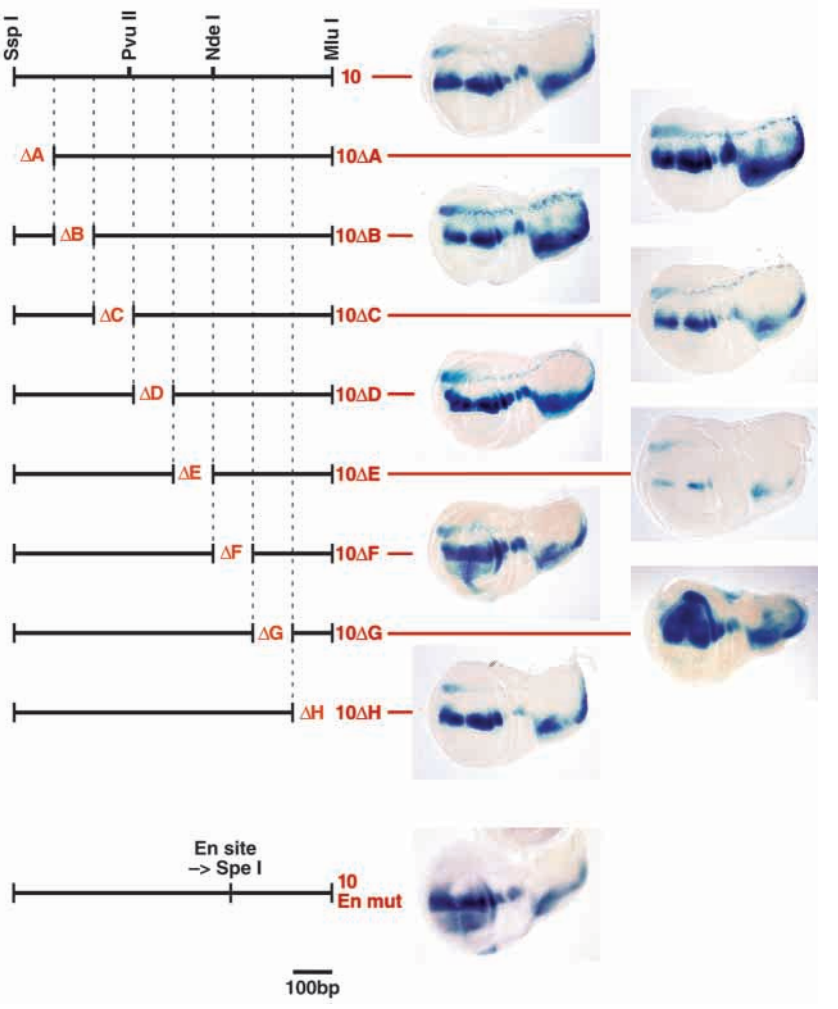

Fig. 2. Dissection of the *dpp* disc enhancer by serial internal deletions: identification of regulatory elements. Eight non-overlapping 100 bp deletions (ΔA to ΔH) were introduced into the 800 bp fragment 10. Five deletions do not result in an alteration of enhancer activity (ΔA to ΔD and ΔH). Three deletions have an effect: ΔE results in a general reduction of expression, ΔF in a partial derepression of expression in the P compartment, and ΔG results in a expanded expression that extends throughout the anterior wing pouch. Replacement of a presumptive En-binding site within fragment F causes a similar, even stronger derepression of expression in posterior compartment cells (construct 10 En mut).

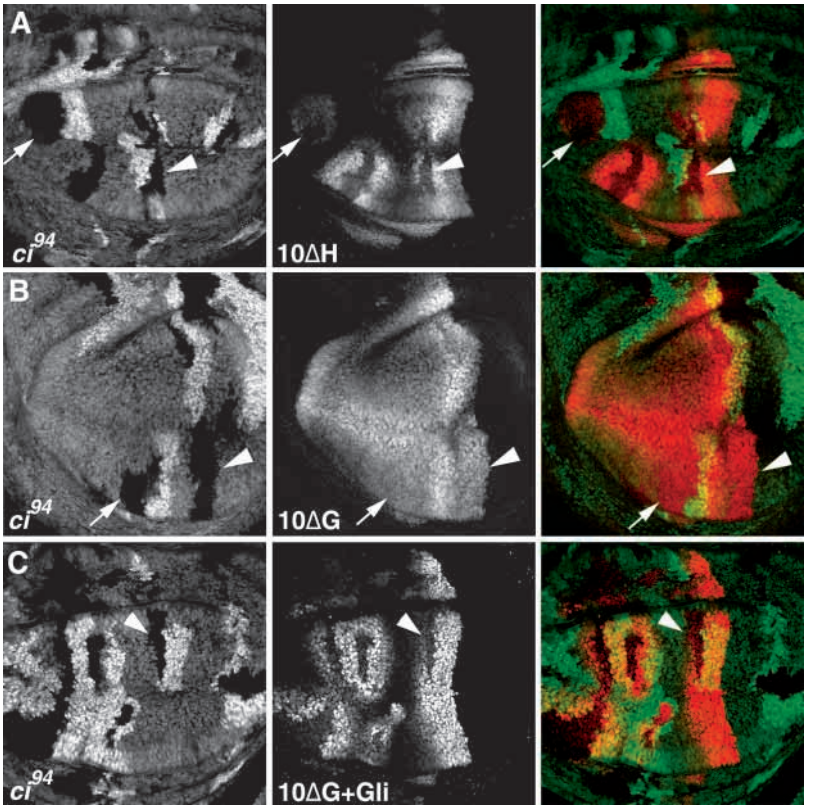

Fig. 3. Responsiveness of wild-type and mutant *dpp* enhancer constructs to Ci[act] and Ci[rep]. Confocal micrographs showing the wing primordium of three different genotypes. In each case, clones have been introduced that lack Ci product entirely, revealing in anteriorly located regions the contribution of Ci[rep] (arrows) and in cells near the AP boundary the contribution of Ci[act] (arrowheads). Discs are oriented anterior to the left and dorsal up. The left-hand panels show the expression of the GFP marker gene (loss of expression indicates ci^- genotype), panels in the center show the same discs stained for β-galactosidase (β-gal), and the merge of both is shown to the right (β-gal in red). (A) The minimal *dpp* enhancer is sensitive to both Ci[act] and Ci[rep]. Construct 10ΔH (representative for fragment 10 here) drives expression at low levels in ci^- clones, independent of their location. These levels are lower (arrowhead) than those seen in the Hh receiving cells along the boundary, indicating that Ci[act] normally activates expression of the 10ΔH-*lacZ* transgene. The levels in ci^- cells however are higher (arrow) than those of wild-type cells in the anterior compartment away from the AP boundary, indicating that Ci[rep] normally represses the activity of 10ΔH. (B) Construct 10ΔG has a severely impaired sensitivity to both Ci[act] and Ci[rep]. Unlike 10ΔH, the activity of 10ΔG is not upregulated in anteriorly located ci^- clones (arrow), indicating that this enhancer has lost its responsiveness to Ci[rep]. Clones that are located in the vicinity of the AP boundary where cells normally receive the Hh signal and produce Ci[act] show only a slight reduction of expression (arrowhead), indicating that this enhancer has also lost most of its sensitivity to Ci[act]. (C) The ability to respond to Ci[rep] and Ci[act] is restored in the 10ΔG construct carrying a single Gli-binding site (10ΔG+Gli). The effect of ci^- clones on the expression of construct 10ΔG+Gli is qualitatively the same as for construct 10ΔH (panel 3A): downregulation of expression in clones at the boundary (arrowhead) and upregulation in clones further away from the boundary. The β-gal staining in cells surrounding the ci^- clones is caused by ectopic *hh* expression in some of these *ci*- clones (see also in A, cf. Méthot and Basler, 1999).

A synthetic Gli-binding site can provide the activator and repressor functions of fragment G

While the results described above indicate that Gli-binding sites are required for the integration of Ci[rep] activity, they do not address the question of whether these sites specifically differ from those conferring Ci[act] activity, either in sequence or in context. To address this issue, we inserted a single, synthetic Gli consensus binding site (TGGGTGGTC) into 10ΔG and asked whether this site can substitute for fragment G in mediating both Ci[act] and Ci[rep] input. As shown in Fig. 5A, the Gli consensus site effectively represses the ectopic anterior enhancer activity of 10ΔG (construct 10ΔG+Gli). Thus this Gli site is able to confer input from Ci[rep]. Moreover, we found strongly reduced levels of 10ΔG+Gli activity in cells mutant for *ci* (Fig. 3C, arrowhead), indicating that 10ΔG+Gli also responds to Ci[act]. Thus a single Gli site can at the same time confer responsiveness to Ci[rep] and Ci[act] in the *dpp* enhancer. From this, we conclude that Ci[act] and Ci[rep] require neither different core sequences nor specific flanking co-factor sites for their binding to the *dpp* enhancer or for their opposite transcriptional activities.

Selective responsiveness to Ci[act] or Ci[rep] is not determined by the local context of their binding sites

We have previously reported that different subsets of Hh target genes are controlled by different activities of Ci (Méthot and Basler, 1999). For example, *ptc* expression is controlled solely by Ci[act], and only *dpp* was found to be under the control of both activities of Ci. To address the mechanisms underlying these differences in responsiveness, we grafted the Ci-binding domain of the *ptc* gene into the context of the *dpp* enhancer and asked if it retains its selective responsiveness to Ci[act]. A 270 bp enhancer fragment of the *ptc* gene, which contains three Ci-binding sites and is necessary for transcriptional activation in response to the Hh signal (Alexandre et al., 1996), was inserted into 10ΔG. This *ptc* fragment efficiently represses ectopic *dpp* enhancer activity (Fig. 5A). Thus, in contrast to its normal role, the *ptc* fragment in its new environment mediates responsiveness to Ci[rep]. This result provides further evidence against the existence of local co-factor sites conferring selectivity to Ci-binding sites.

Neither Ci[act] nor Ci[rep] require a dedicated enhancer composition for their transcriptional activities

It is possible that the *dpp* gene represents a rare case with an enhancer that is especially equipped for the interpretation of both activities of Ci, whereas other genes, such as *ptc*, *en* or *hh* would be competent to respond to only one activity of Ci, and most genes to none. Alternatively, responsiveness to both forms of Ci could represent the default state, and uni-

responsive genes such as *ptc* and *hh* feature designated mechanisms to prevent responsiveness to Ci[rep] or Ci[act], respectively. To test the effect of Ci-binding sites on a naïve enhancer, we fused the *dpp* fragment G or the synthetic Gli consensus binding site to a ubiquitously expressed minimal enhancer of the *brinker* (*brk*) gene (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Minami et al., 1999). This enhancer is normally expressed throughout the wing pouch of wild-type discs with slightly reduced activity in the center of the disc (Fig. 5B, B. M. and K. B., unpublished results). Fragment G as well as the single Gli site resulted in a modulation of the *brk* enhancer activity, with upregulation in Hh-receiving cells and downregulation in anterior cells not exposed to Hh signal. We interpret this result as evidence that neither Ci[act] nor Ci[rep] require a special dedicated enhancer context for their transcriptional activities.

DISCUSSION

Cells lacking Ci protein do not behave like cells lacking Hh input. Ci mutant cells show a gain, rather than a lack of organizer activity. This seminal discovery led to the idea that Ci plays at least two roles: apart from its function in the Hh signal transduction pathway, it also plays a critical role in repressing growth-promoting genes, such as *dpp* and *hh*. The general question arose, therefore, as to how Ci can carry out these overtly opposite roles, with the specific challenge to explain how it can repress target genes that should not be activated by Hh (such as *hh*) and how it can activate target genes that should not be repressed (such as *ptc*, see below).

There are preceding cases of other signalling pathways where the nuclear mediator can have two opposite transcriptional activities. In the Spätzle/Toll signalling pathway, for example, the nuclear mediator Dorsal functions as a bona fide activator of transcription of target genes (Jiang et al., 1991). Dorsal can also transcriptionally repress certain targets through its association with neighboring DNA-binding proteins, which induce the potential of Dorsal to recruit the co-repressor protein Groucho (Dubnicoff et al., 1997; Jiang et al., 1992; Kirov et al., 1993). As a key difference to Dorsal, however, Ci was found to exist in two molecularly distinct forms (Ci-75 and Ci-155) which correlate with its two genetically ascribed functions, Ci[rep] and Ci[act], respectively. Both functions of Ci are subject to Hh control, which constitutes another difference to Dorsal whose activities can only be regulated concomitantly. As a consequence, activated and repressed targets of Dorsal constitute two non-overlapping sets of genes, whereas Ci targets can be regulated by both activities synergistically. This latter situation is exemplified by the *dpp* gene which is Ci-repressed in cells lacking Hh input, but derepressed and at the same time Ci-activated in cells receiving Hh signal (Méthot and Basler, 1999). The identification of Ci[rep] and Ci[act] as two molecularly distinct entities raised

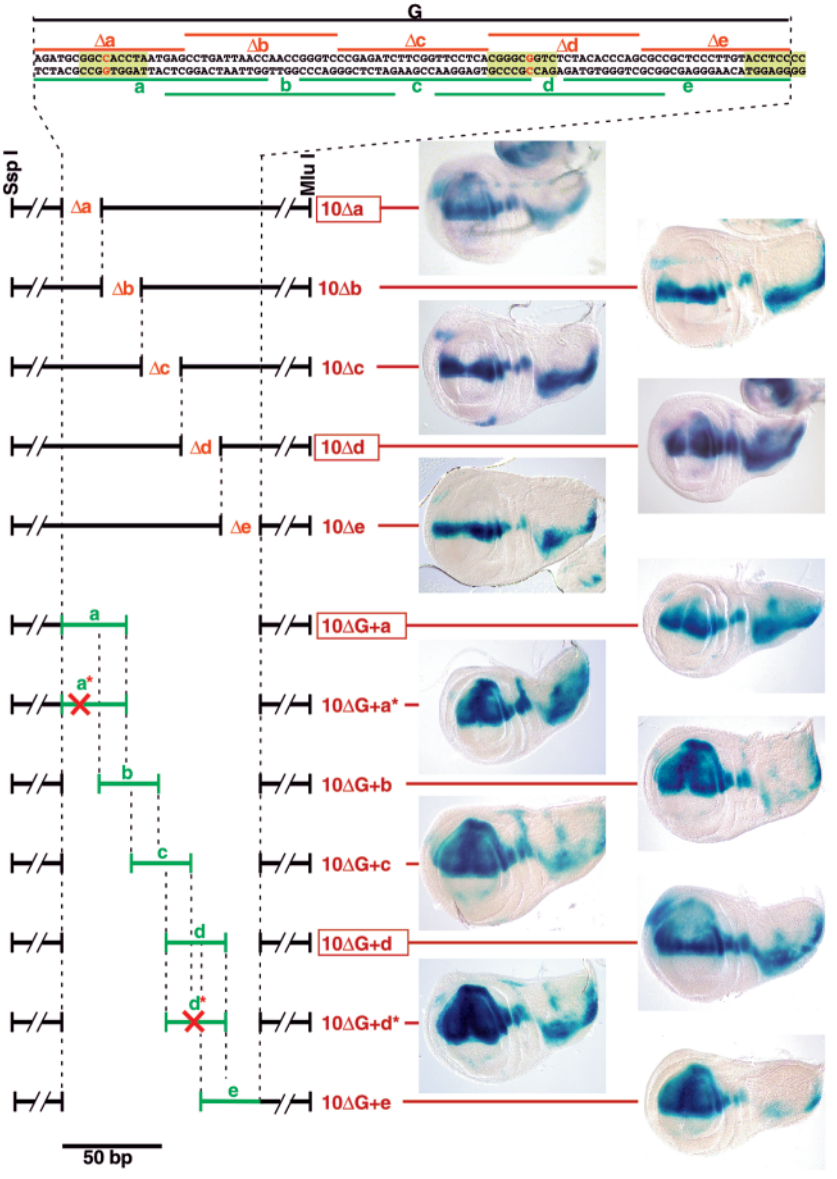

Fig. 4. Identification of two Ci-binding sites in fragment G that are necessary for responsiveness to Ci[rep]. The nucleotide sequence of fragment G is shown at the top. Orange bars denote the exact position and extent of the five deletions Δa to Δe shown below. Green bars indicate the small fragments that were reintroduced into 10ΔG (a to e, see bottom half of the figure). Deletion and substitution constructs which show a transcriptional activity that differs from their parental construct are boxed. 10Δa and 10Δd show a partial derepression of β-gal expression in the anterior region of the wing pouch. Conversely, 10ΔG+a and 10ΔG+d show a partial repression of anterior β-Gal expression compared to 10ΔG. Sequence examination of construct 10 revealed three putative Ci-binding sites, all of which map to subregion G and are highlighted in yellow. Base pairs which are essential for Ci binding and which have been mutated in constructs 10ΔG+a* and 10ΔG+d* are shown in red. Note that the transcriptional repression activity of the small fragments a and d is abolished by these point mutations.

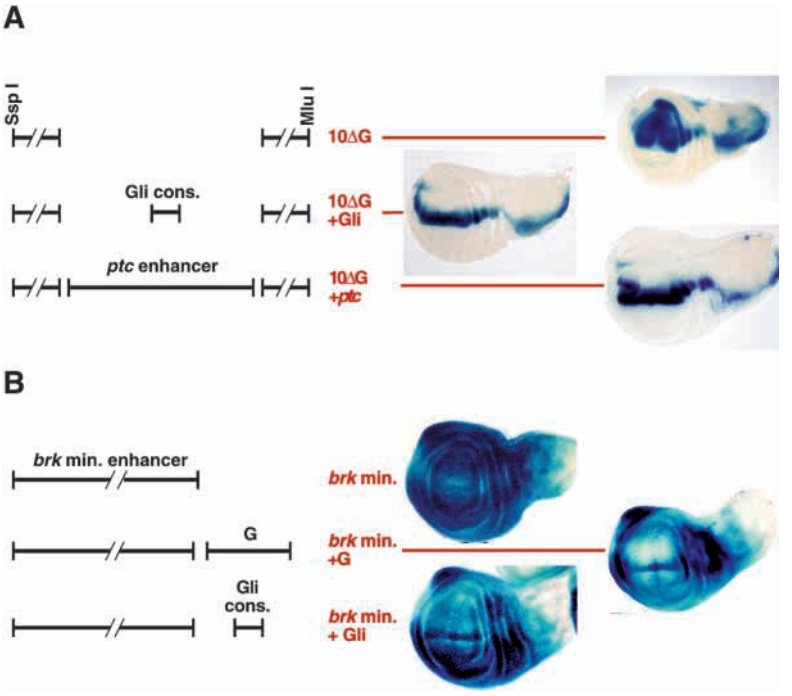

Fig. 5. Hybrid enhancers reveal that a single Gli consensus binding site can mediate both activator and repressor functions of Ci independent of their local context. (A) A single Gli-binding site or a *ptc* enhancer fragment can restore Ci[rep] responsiveness to construct 10ΔG. A single Gli consensus site was introduced into construct 10ΔG. The *dpp*-like β-gal expression is restored perfectly, indicating that 10ΔG+Gli responds to Ci[rep]. In addition, it also responds to Ci[act] (see Fig. 3C). *ptc* normally only responds to Ci[act] (Alexandre et al., 1996; Méthot and Basler, 1999). A 270 bp enhancer fragment required for this response was inserted into 10ΔG. The resulting construct 10ΔG+ptc shows a *dpp*-like expression of β-gal, demonstrating that the *ptc* fragment mediates responsiveness to Ci[rep]. (B) Fragment G as well as a single Gli-binding site can confer anterior activation and repression to an unrelated enhancer fragment. Transcriptional activity of a minimal (2 kb) *brk* enhancer construct in the wing pouch. Its activity is symmetrical relative to the AP boundary with a slight reduction in the center of the disc. Fragment G does modulates the expression of the minimal *brk* enhancer in the anterior compartment. It upregulates expression close to the AP boundary and downregulates expression more anteriorly (the slight repression observed in the posterior compartment is likely due to a weak En-binding site in fragment G). A single Gli-binding site is similarly able to modulate expression of the minimal *brk* enhancer (bottom construct). In this case, however, no posterior repression is observed.

the possibility that the two forms of Ci exert their regulatory activity through distinct binding sites.

Here we have isolated a minimal *dpp* imaginal disc enhancer, which, like the endogenous *dpp* gene, is regulated by Ci[act] and Ci[rep] activities. In the absence of the internal 100 bp fragment G, sensitivity to both Ci[act] and Ci[rep] is lost. Two Ci-binding sites are necessary for the activity of fragment G and a single synthetic Gli consensus site is sufficient to replace their function, restoring sensitivity to both Ci[act] and Ci[rep]. Furthermore, the ability of a single synthetic Gli-binding site to respond both to Ci[act] and Ci[rep] is preserved in the context of a different, naïve enhancer, indicating that a single Gli/Ci-binding site is intrinsically able to mediate both inputs. In contrast therefore to the aforementioned case of Dorsal, whose repressing activity is dependent on the fixed arrangement of binding sites for Dorsal and its cofactors, Ci can function as a repressor by binding to the same site as the activator form of Ci, irrespective of the local sequence context.

For the minimal *dpp* and *brk* enhancers, we have to postulate a Hh-independent activator input that causes a basal expression level (see Fig. 6). This transcriptional activity can either be synergistically enhanced by Ci[act] or suppressed by Ci[rep], depending on which form of Ci is prevailing and thus predominantly binding to the Gli/Ci-binding site. This scenario represents the simplest case of a Hh target gene, one that responds to both forms of Ci.

To achieve selective responsiveness to only one form of Ci, as in the cases of *hh* and *ptc*, additional *cis*-acting elements

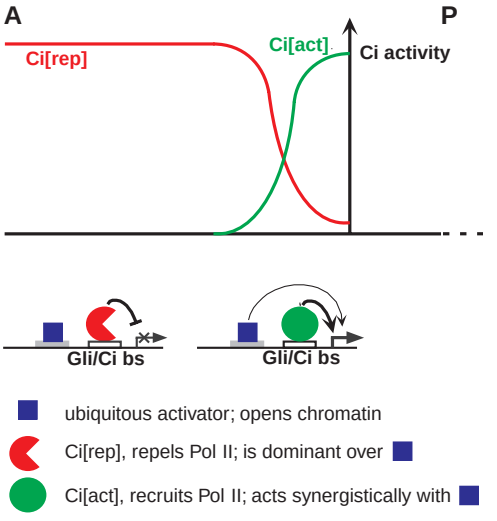

Fig. 6. Model of how Ci acts. The activity of Ci along the anteroposterior axis of imaginal discs is schematically depicted on top of the figure. The opposing transcriptional activities (Ci[rep] in red and Ci[act] in green) are exerted by the distinct molecular forms Ci-75 and activated Ci-155, respectively. The distribution and activity of these different forms of Ci is controlled by Hh signaling. Close to the compartment boundary where Hh signaling activity is high, Ci[act] is prevailing. More anterior, in cells that do not receive Hh, Ci[rep] predominates. The expression of *dpp* and of the synthetic 'min *brk* + Gli' construct responds both to Ci[act] and Ci[rep].This responsiveness to both forms of Ci is mediated by common Gli/Ci-binding sites. In A cells close to the AP boundary, these sites would be occupied by Ci[act] and in more anterior cells by Ci[rep]. Both forms of Ci would alter the activity of a ubiquitously present activator (shown in blue) which on its own might enable low basal transcription by opening the chromatin structure. Ci[act] would recruit the *Pol*II-associated transcriptional machinery and hence synergistically enhance this basal activity, whereas Ci[rep] might repel this same complex and suppress the basal activity.

must have evolved. At present, we can only speculate about the *cis* elements that make some Ci target genes different from others. However, we can make a firm case for the relevance of this yet unknown mechanism. Let's consider first the *hh* gene, which is particularly interesting because it responds effectively and selectively to Ci[rep]. *hh* expression levels can neither be increased in the posterior compartment nor ectopically induced in the anterior compartment by overexpression of constitutively active forms of Ci (data not shown). But even very low levels of Ci[rep] suffice to repress *hh* expression in anterior cells near the AP boundary, despite the presence of high levels of Ci[act]. This was revealed by the observation that anterior *ci* mutant clones located close to the AP boundary ectopically express the *hh* gene (Méthot and Basler, 1999). If the *hh* gene failed to resist to Ci[act], *hh* transcription could no longer be stably maintained in imaginal discs. Instead it would propagate anteriorly until all cells secrete Hh and Hh could no longer locally maintain the Dpp-expressing organizer cells. Thus, if Ci controls *hh* directly, by binding to Gli sites of the *hh* gene, the *hh* promoter must be configured to assemble a transcriptional complex that is unable to effectively interact with Ci[act].

The converse situation is found for *ptc*, which is regulated by Hh exclusively via Ci[act] (Méthot and Basler, 1999) and for which a direct binding of Ci to enhancer elements has been shown (Alexandre et al., 1996). The *ptc* gene is normally 'off' in P compartment cells but can readily be induced by expressing Ci ectopically. Similarly, the low expression levels found in A compartment cells can be augmented by ectopically providing Ci[act]. But this low level expression of *ptc* is not controlled by Ci[rep]. *ci* mutant clones in anterior regions, where Ci[rep] is the predominant form of Ci, show no increase in *ptc* expression (Méthot and Basler, 1999). In addition, overexpression of Ci[rep] in A cells does not reduce the low levels of *ptc* (data not shown). The arrangement of transcription factors on the *ptc* promoter must either facilitate the binding of Ci[act] versus Ci[rep], or they must be largely insensitive to Ci[rep] activity. If this were not the case and the *ptc* gene would be effectively repressed by Ci[rep], insufficient levels of Ptc protein would cause Hh-independent Smo signalling. This in turn would prevent the formation of Ci[rep] which plays a critical role in the repression of genes such as *dpp* and *hh*. Therefore an important question that remains to be answered in the future is how Ci targets, such as *hh* and *ptc*, evolved their selective responsiveness to Ci through distant *cis*-regulatory elements.

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