**Drosophila cubitus interruptus** forms a negative feedback loop with patched and regulates expression of Hedgehog target genes

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**SUMMARY**

The *Drosophila* segment polarity gene *cubitus interruptus* (*ci*) encodes a zinc finger protein that is required for the proper patterning of segments and imaginal discs. Epistasis analysis indicates that *ci* functions in the Hedgehog (Hh) signal transduction pathway and is required to maintain *wingless* expression in the embryo. In this paper, the role of the Ci protein in the Hh signaling pathway is examined in more detail. Our results show that ectopic expression of *ci* in imaginal discs and the embryo activates the expression of Hh target genes. One of these target genes, *patched*, forms a negative feedback loop with *ci* that is regulated by Hh signal transduction. Activation is also achieved using the Ci zinc finger domain fused to a heterologous transactivation domain. Conversely, repression of Hh target genes occurs in animals expressing the Ci zinc finger domain fused to a repression domain. To examine Ci function in more detail, regions of the Ci protein that are responsible for its ability to transactivate and its subcellular distribution have been identified.

Key words: *cubitus interruptus*, signal transduction, *hedgehog*, *Drosophila*, feedback loop

**INTRODUCTION**

The embryonic segments of *Drosophila* are patterned in response to signals between cells on either side of the anterior/posterior (A/P) compartment (parasegmental) boundary (reviewed in Peifer and Bejsovec, 1992; Perrimon, 1994). In the posterior compartment, expression of the *engrailed* (*en*) gene is required to maintain the expression of *hedgehog* (*hh*) which encodes a secreted protein (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992). Hh signaling in turn is necessary for continued expression of *wingless* (*wg*) in anterior cells along the A/P compartment boundary (Hidalgo and Ingham, 1990; Forbes et al., 1993; Ingham, 1993; van den Heuvel et al., 1993). The Wg protein is a member of the Wnt family of secreted proteins and is required for the patterning of structures throughout the segment (Baker, 1987; Rijsewijk et al., 1987; Peifer and Bejsovec, 1992).

Hh signaling regulates *wg* expression by modulating the activity of the patched (*ptc*) gene and consequently the levels of the Cubitus interruptus (*Ci*) protein (Ingham et al., 1991; Ingham and Hidalgo, 1993; Motzny and Holmgren, 1995). The *ptc* and *ci* genes are initially transcribed throughout the segment, but En activity restricts their expression to the anterior compartment (Hooper and Scott, 1989; Nakano et al., 1989; Orenic et al., 1990; Eaton and Kornberg, 1990). *ptc* encodes an integral membrane protein (Hooper and Scott, 1989; Nakano et al., 1989), while *ci* encodes a zinc finger protein related to the *gli* genes of vertebrates and the *tra-I* gene of nematode (Kinzler and Vogelstein, 1990; Orenic et al., 1990; Zarkower and Hodgkin, 1992). Genetic analysis suggests that Hh signaling antagonizes negative regulation of Ci by the Ptc protein (Ingham, 1991; Ingham and Hidalgo, 1993). This leads to post-transcriptional elevation of Ci protein levels at the borders of the anterior compartment and activation of *wg* expression anterior to the parasegmental boundary (Motzny and Holmgren, 1995).

Much of the Hh signal transduction cascade appears to be conserved between the embryo and the imaginal discs. Hh targets in the anterior compartments of imaginal discs include *decapentaplegic* (*dpp*), which encodes a secreted factor of the TGF-beta family (Padgett et al., 1987), *ptc* and *wg* (Basler and Struhl, 1994; Capdevila et al., 1994; Tabata and Kornberg, 1994; Felsenfeld and Kennison, 1995; Sanicola et al., 1995). In the wing disc, *dpp* is expressed in an anterior compartment stripe of cells adjacent to the A/P boundary (Masucci et al., 1990; Posakony et al., 1990; Raftery et al., 1995). The *ptc* gene is expressed in all anterior compartment cells, but its levels are augmented in a thin stripe adjacent to the A/P boundary (Phillips et al., 1990; Capdevila et al., 1994). Expression of *dpp* and augmented *ptc* in cells near the A/P boundary requires that the cells receive the Hh signal from the posterior compartment (Basler and Struhl, 1994; Capdevila et al., 1994; Tabata and Kornberg, 1994; Felsenfeld and Kennison, 1995; Sanicola et al., 1995). In a similar fashion, the Hh signal is required in the leg disc for expression of *wg*, in a ventral anterior wedge and *dpp*, in a stripe along the A/P boundary that is most intense in
the dorsal region (Basler and Struhl, 1994; Campbell and Tomlinson, 1995). As in the embryo, post-transcriptional regulation of Ci in imaginal discs leads to elevated levels of Ci protein in anterior cells adjacent to the compartment boundary (Motzny and Holmgren, 1995; Slusarski et al., 1995). The domain of Ci augmentation overlaps the regions expressing wg in the leg disc and dpp in the wing and leg discs. Therefore, Ci is likely to mediate Hh signal transduction in imaginal discs just as it does in the embryo. Consistent with this hypothesis are molecular genetic studies showing that Hh signaling also antagonizes Ptc function in imaginal discs. Ptc represses dpp and its own expression in the region of low level ptc expression (Capdevila et al., 1994; Tabata and Kornberg, 1994). Hh is thought to interfere with Ptc activity in anterior compartment cells near the A/P boundary, which allows expression of dpp and high level ptc in this region.

Here, we demonstrate that, in imaginal discs, Ci plays a central role in the Hh signal transduction pathway. Ci protein levels are regulated by Ptc and Hh function and Ci can activate the expression of ptc, dpp and wg. Further, we demonstrate that Ci forms an interesting negative feedback loop with Ptc. Hh signaling resets this feedback loop and allows the expression of the appropriate Hh target genes. To better understand the basis of Ci function, a series of chimeric and truncated versions of Ci were generated. Results from these experiments suggest that Ci functions as a transcription factor and that sequences C terminal to the zinc finger are required for transactivation and the regulation of Ci subcellular distribution.

MATERIALS AND METHODS

Fly strains
en-GAL4, ptc-GAL4 and 71B-GAL4 were kindly provided by A. Brand and B. Wilder. wg-lacZ, hh-lacZ, en-lacZ, dpp-lacZ (dpp^R635^) and ptc-lacZ were obtained from N. Perrimon, G. Struhl, T. Kornberg and B. Noll. ptc^{G20} was kindly provided by R. Whittle. ptc^{RN} was obtained from C. Nusslein-Volhard. hh^{t3x3} was obtained from the Tubingen stock center.

DNA constructs

**ASC-flip cassette** (FC)-ci

The entire Ci coding region was cloned into pGEM4Z in three fragments: a 400 bp DraI/EcoRI cDNA fragment which contains the 5’ Ci coding sequences and eliminates a large intron, a 1.5 kb EcoRI genomic fragment containing sequences from the middle of the coding region and a 6 kb EcoRI genomic fragment containing 3’ coding sequences. The entire coding region was cloned into the Carnegie 20/actin 5C/FRT cassette vector (Buenzow and Holmgren, 1995).

**UAS-ci-genomic**

The ci coding region described above was cloned into the pUAST vector.

**UAS-ci-cDNA**

The coding region from a near full-length ci cDNA (#2) was cloned into pGEM7Z in two fragments: a 400 bp DraI/EcoRI cDNA fragment containing the 5’ ci coding sequences and an EcoRI cDNA fragment containing the rest sequences (ci-pGEM7Z). The pGEM7Z XhoI site, on the 3′ side of the cDNA, was converted to a BglII site, and the cDNA was cloned into the BglII site of pUAST as a BamHI/BglII fragment.

UAS-ci[N(HA)]/Zn

ci-pGEM7Z was digested with HincII and BglII, and a BglII linker was added to the HincII site. The digested construct contained the amino terminal and Zn finger encoding region of ci and extends through amino acid 684. A triple 12CA5 HA tag was cloned in frame into the PstI site (at amino acid position 29) of ci/N[Zn-pGEM7Z] (ci[N(HA)]/Zn-pGEM7Z). ci/N[Zn] and ci[N-HA]/Zn were cloned into pUAST as a BamHI/BglII fragment.

UAS-ciZn/C

ci-pGEM7Z was digested with BamHI and EcoRV, and a BamHI-ATGstart-EcoRV adapter was added in frame at the EcoRV site (amino acid position 440) (ciZn/C-pGEM7Z). This Zn/C construct was then cloned into pUAST as a BamHI/BglII fragment.

UAS-ciZn/EnRD

The en-repression domain (RD) (amino acids 284-338) was cloned as a PCR fragment with HpaI/BglII ends into HpaI/BglII digested ciZn/C-pGEM7Z (ciZn/EnRD-pGEM7Z). The Myc 9E10 epitope was cloned into XbaI/BglII digested ciZn/EnRD-pGEM7Z at the end of the RD. This ciZn/EnRD construct was then cloned into pUAST as a BamHI/BglII fragment.

UAS-ciZn/GAL4AD

This construct was generated using the same methods to generate UAS-ciZn/EnRD except that the GAL4 activation domain (AD) (amino acids 768-881) was used instead of the EnRD.

UAS-ciZn

ciZn/GAL4AD-pGEM7Z was digested with Hpal and BglII to cut out the AD region. The BglII overhang was filled in with a Klenow reaction and the construct was blunt end ligated. This ciZn construct was then cloned into pUAST as a BamHI/BglII fragment.

Immunohistochemistry

Imaginal discs were prepared as in Carroll and Whyte (1989). Embryos were prepared as in Buenzow and Holmgren (1995). Stainings were visualized on a BioRad MRC 600 Lasersharp Confocal system. Imaginal discs and embryos were singly or doubly labelled with antibodies against Ci (Motzny and Holmgren, 1995), Ptc (Capdevila et al., 1994), Dpp (Panganiban et al., 1990), Wg (van den Boogaard et al., 1994), Myc (provided by N. Brown and S. Carroll), and HA (Wilson et al., 1984).

RESULTS

Hh and Ptc regulate the levels of Ci protein in imaginal discs

In wing and leg discs, the region of augmented Ci protein is reminiscent of the expression of the Hh target genes dpp, ptc and wg. To examine whether Hh might influence Ci protein levels in the anterior compartment, the distribution of Ci protein was followed in imaginal discs homozygous for a temperature-sensitive allele of hh (hh^{t3x3}) (Mohler, 1988). Mutant larvae were transferred to the nonpermissive temperature in the second larval instar and the pattern of Ci protein distribution assayed in late third instar larvae. In both wing (Fig. 1B) and leg imaginal discs, there were low levels of Ci protein throughout the anterior compartment, but the augmented expression along the A/P boundary was greatly reduced. This suggests that, as occurs in the embryo, Hh function is required for the accumulation of high levels of Ci protein along the A/P boundary.
Ptc is known to repress the expression of Hh target genes and Hh signaling is thought to interfere with Ptc function in cells near the A/P boundary. To determine whether Ptc also regulates the levels of Ci protein, the distribution of the Ci protein was examined in imaginal discs mutant for ptc. In wing (Fig. 1C) and leg imaginal discs transheterozygous for ptc<sup>G20</sup>/ptc<sup>en108</sup>, the levels of Ci protein were elevated and uniform throughout the anterior compartment. This suggests that Ptc functions to reduce the levels of Ci protein in cells that do not receive the Hh signal. Similar results have been reported by Sanchez-Herrero et al. (1996).

**Fig. 1.** Hh and Ptc regulate the levels of Ci protein in the wing imaginal disc (dorsal up, anterior to the left). (A) Distribution of Ci protein in a wild-type wing imaginal disc. Unlike the ci transcript (Eaton and Kornberg, 1990), the Ci protein is distributed nonuniformly throughout the anterior compartment of the wing imaginal disc. The Ci protein levels are elevated in a stripe 7-8 cells wide adjacent to the A/P boundary. (B) Ci protein distribution in a wing imaginal disc homozygous for a temperature-sensitive allele of hh (hh<sup>9k28</sup>). In these discs, the elevated levels of Ci protein along the A/P boundary are reduced. (C) Ci protein distribution in wing imaginal disc from a ptc<sup>G20</sup>/ptc<sup>en108</sup> larva (Capdevila et al., 1994). Note that the levels of Ci are high throughout the entire anterior compartment.

**Fig. 2.** ci gain-of-function clones in the wing do not alter expression of endogenous ci or en (dorsal up, anterior to the left). (A-C) A wing disc with a Ci-expressing clone in the posterior compartment. The distributions of the Ci protein (A) and β-galactosidase protein from an enhancer trap insertion in the en gene (B) were visualized by antibody labeling. (C) Merge of the two images. The arrow points to a clone expressing Ci in the posterior compartment; expression of the β-galactosidase reporter is unaffected. (D-F) A wing disc with multiple Ci-expressing clones. The distribution of Ci protein (D) and β-galactosidase from an enhancer trap insertion in the ci gene (E) were visualized by antibody labeling. (F) Merge of the two images. Multiple clones expressing Ci protein are present throughout the anterior and posterior compartments, but they do not affect the expression of β-galactosidase from the ci enhancer trap.

**Role of ci in hh signaling**

**Fig. 3.** Overexpression of ci in the wing pouch region. Two UAS ci constructs were used. The first construct was primarily derived from genomic sequences, while the second was a cDNA. Both gave similar results, though the cDNA appeared to have somewhat higher activity. Overexpression was observed between anterior and posterior compartment cells may be due to the presence of high levels of Hh in the posterior compartment.

**Fig. 4.** Overexpression of ci in the wing pouch region caused overexpression of ptc (Fig. 3A-C), but, as observed with dpp, there was variability in the response; some clones expressed ptc and others did not. In contrast, posterior compartment clones ectopically expressing ci caused autonomous high level misexpression of ptc (Fig. 3A-C). The differential ptc response observed between anterior and posterior compartment cells may be due to the presence of high levels of Hh in the posterior compartment.

To better compare the effects of high level Ci expression in the anterior and posterior compartments, the GAL4 system of Brand and Perrimon (1993) was used to express ci throughout the wing pouch region. Two UAS ci constructs were used. The first construct was primarily derived from genomic sequences, while the second was a cDNA. Both gave similar results, though the cDNA appeared to have somewhat higher activity. Overexpression was observed between anterior and posterior compartment cells may be due to the presence of high levels of Hh in the posterior compartment.

When ‘flip-out’ ci clones were generated in either the anterior or posterior compartment of leg imaginal discs, ectopic expression of wg was observed (Fig. 3G-I). In both the wing and leg, Dpp and Wg functions are required for formation of the proximal/distal axis, and ectopic expression of either can cause duplication of wing and leg structures. Similar limb duplications are observed with ci gain-of-function clones that have been induced early in development (data not shown).

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The Ci zinc finger domain can function with heterologous activation and repression domains to regulate Hh target genes

To examine the function of the Ci zinc finger domain, it was assayed alone as well as in combination with either the GAL4 activation domain (Fischer et al., 1988) or the Engrailed (En) repression domain (Han and Manley, 1993) (Fig. 5). The constructs were placed into the pUAST vector (Brand and Perrimon, 1993) and transgenic lines were generated. Expressing the UAS-ciZn/GAL4AD construct with prd-GAL4 resulted in complete rescue of the ciCe/ci Ce cuticle defect within the segments expressing prd-GAL4 (Fig. 6B). As expected, wg expression was restored in every other segment (data not shown). The level of rescue was equivalent to that obtained using UAS-ci-cDNA.

One of the UAS-ciZn/GAL4AD lines generated was less active and survived to adulthood in combination with en-GAL4. Expression of the ciZn/GAL4AD in the posterior compartment caused activation of Ptc protein (Fig. 6C) and ptc-lacZ expression (Fig. 6D). Disruption of posterior wing veins (Fig. 6E) was observed in adults, and this phenotype was similar to ciTei and ciTeiB mutants in which the Ci protein is ectopically expressed in the posterior compartment (Locke and Tartoff, 1994; Slusarski et al., 1995).

Expression of ciZn/EnRD or ciZn along the A/P boundary of the anterior compartment caused a fusion between wing veins 3 and 4 (Fig. 7H and data not shown). This is the expected phenotype of a dominant negative mutation. The ciCe mutation appears to be a moderate dominant negative (Motzny and Holmgren, 1995; Slusarski et al., 1995) and ciCe/+ animals
have partial fusion between wing veins 3 and 4. Similarly the fused (fu) gene appears to be required for Ci function (Limbourg-Bouchon et al., 1991; Forbes et al., 1993) and fu mutants also have fusions between wing veins 3 and 4. The CiZn/EnRD protein was nuclear and was expressed in a broad stripe that coincided with that of expanded high level endogenous Ci expression (Fig. 7A,B). High level Ptc at the A/P boundary was eliminated as would be expected with a dominant negative molecule competing for Ci target sites (Fig. 7E). The normal stripe of high level Ptc was replaced by lower Ptc levels that were found throughout the broad stripe of Ci expression (Fig. 7E,F). In a similar fashion, Dpp levels were also lower and spread throughout the broad stripe of Ci expression (Fig. 7C,D).

Sequences C terminal to the zinc finger domain are required for transactivation and regulate the subcellular distribution of Ci

To define the regions of the Ci protein that are responsible for its function, we generated two UAS constructs in which sequences either N terminal (cizn/C) or C terminal (cizn/Zn) to the zinc finger domain were removed (Fig. 5). These constructs were expressed in either the anterior or posterior compartment by using the ptc-GAL4 or the en-GAL4 lines, respectively. Effects were assayed in developing embryos because very few animals survived to adulthood. Expression of cizn/C in the posterior compartment caused high level expression of ptc in the posterior compartment (Fig. 8B). The Cizn/C protein appeared to have the same subcellular distribution as the full-length Ci protein and was primarily cytoplasmic (Fig. 8A). Cuticles prepared from this line were relatively normal, though the denticle belts seemed to have fewer denticles (data not shown). Expression of Cizn/K in the anterior compartment gave rise to animals with a near normal cuticle pattern (data not shown). However, expression of cizn/Zn or an epitope tagged version of this construct (cizn[HA]/Zn) in the anterior compartment caused a deletion of naked cuticle and a partial duplication of the denticle belts (Fig. 9A). This phenotype is similar to that caused by loss of wg function in the later stages (10-12) of

![UAS/ci expression constructs](image)

Fig. 5. UAS-Ci expression constructs. Diagram of constructs used to express chimeric and truncated versions of Ci. Cizn has an initiator ATG followed by the regions encoding amino acids 440-684 of Ci (the zinc finger domain includes amino acids 453-603). Cizn/EnRD has the same region of Ci followed by the repression domain of En (amino acids 284-338) and the 9E10 Myc epitope. Cizn/GAL4AD has the same region of Ci followed by the activation domain of GAL4 (amino acids 768-881) and the 9E10 Myc epitope. Cizn/C has an initiator ATG followed by the Ci sequences starting at amino acid 440 and continuing to the end of the coding sequence. Cizn/Zn has the coding sequence through amino acid 684. Cizn[HA]/Zn is the same as Cizn/Zn except that a triple 12CA5 HA epitope tag has been placed after amino acid position 29.
embryonic development (Bejsovec and Martinez-Arias, 1991). In embryos expressing ciN/Zn in the anterior compartment, wg expression was lost by stage 11 (Fig. 9E). The subcellular distribution of CiN[HA]/Zn was examined in ptc-GAL4 embryos by using the 12CA5 anti-HA antibody. In contrast to wild-type Ci expression, which is primarily cytoplasmic, the CiN[HA]/Zn protein was present at uniformly high levels throughout the nucleus and the cytoplasm (Fig. 9B,C).

**DISCUSSION**

Ptc and Hh regulate Ci protein levels

Previous work by Johnson et al. (1995) has shown that high levels of Ptc block dpp expression and the elevation of Ci protein levels along the compartment boundary of the wing imaginal disc. In a reciprocal experiment, Capdevila et al. (1993) showed that loss of ptc function leads to ectopic...
expression of dpp and ptc itself. Results by Dominguez et al. (1996) and Sanchez-Herrero et al. (1996) showed that loss of Ptc function leads to elevation of Ci protein levels. These results support a strong correlation between Ptc negative regulation of Ci protein levels and its ability to block the expression of Hh target genes. The augmentation of Ci protein levels along the A/P compartment boundary of imaginal discs is dependent on hh function as it is in the embryo. Thus, as was initially suggested in the embryo, Hh signaling in imaginal discs appears to relieve negative regulation by the Ptc protein (Ingham, 1991; Ingham and Hidalgo, 1993).

**Ectopic ci activates the expression of Hh target genes**

Elevation of Ci protein levels and its activity is likely to be responsible for the activation of Hh target genes in imaginal discs. To test this hypothesis, clones expressing high levels of the Ci protein were generated using the ‘flip-out’ technique and the GAL4 system was used to direct ci expression in the wing pouch. Our results show that ectopic expression of Ci has the ability to activate the expression of wg, dpp and ptc and are in general agreement with the results of Dominguez et al. (1996) and Alexandre et al. (1996). These results were obtained with both reporter constructs and antibody stainings to the protein products, suggesting that the effects are transcriptional. One discrepancy in these studies is that we and Dominguez et al. (1996) find that Ci expression in the posterior compartment activates dpp expression while Alexandre et al. (1996) did not observe posterior compartment activation of dpp. All three groups used different ci cDNAs, so it is possible that alternative forms of Ci with varying activities are produced.

Previous work has shown that the inability of posterior compartment cells to express Hh target genes is probably dependent on en expression (Zecca et al., 1995). Therefore, we wanted to examine whether ectopic expression of ci blocked the expression of en. Expression of en was unaffected by ectopic ci expression, demonstrating that the activation of dpp, wg and ptc by ectopic Ci was not an indirect effect due to loss of en. Instead these results suggest that en regulation of these genes is probably indirect and reflects silencing of ci by the En protein (Eaton and Kornberg, 1990).

**Ci and Ptc form a negative feedback loop that is regulated by Hh signaling**

By having Ci control the expression of ptc, its own negative regulator, it should be possible to maintain Ci protein levels and activity within a fairly narrow range. If Ci levels or activity increase, it will lead to higher expression of ptc, which will...
tend to return Ci to lower levels. Lower Ci levels will cause decreased expression of ptc and an increase in Ci levels. Such a negative feedback loop should contribute to homeostasis and ensure that cells respond appropriately to developmental signals. Hh signaling interrupts this feedback loop, allowing high levels of Ci to accumulate and the expression of the Hh target genes. A diagram of this regulatory pathway is shown in Fig. 10.

Chimeric proteins suggest that Ci functions as a transcription factor and confirm the role of Ci in regulating Hh target genes

Taking advantage of many previous studies that have shown the modular organization of transcription factors, the zinc finger domain of Ci was combined with either the transcription activation domain of GAL4 (Fischer et al., 1988) or the repression domain of En (Han and Manley, 1993). If Ci functions as a transcriptional activator, we would expect CiZn/GAL4AD to activate the expression of Hh target genes and CiZn/EnRD to repress Hh target genes. By deleting most of the Ci coding sequences, potential negative regulatory sites might be eliminated and the small size of the chimeric protein should allow access to the nucleus even in the absence of a nuclear localization signal (NLS) (Dingwall and Laskey, 1991). As expected, the CiZn/GAL4AD construct activated the expression of ptc and even has the ability to rescue the cuticle phenotype of ci mutants. This suggests that the primary function of Ci is to bind DNA through its zinc finger and activate the expression of target genes. The CiZn/EnRD gave a reciprocal phenotype and repressed the narrow stripe of high level ptc expression along the compartment boundary of imaginal discs.

It is significant that the zinc finger region alone functions as a dominant negative, while the zinc finger region plus a heterologous activation domain complements loss of endogenous Ci function. This observation and the reciprocal results obtained with the activation and repression domains are consistent with Ci functioning as a transcription factor and are inconsistent with most other potential roles of Ci. For example, if Ci primarily regulates some aspect of RNA metabolism, the GAL4AD and the EnRD would not be expected to have specific functions, and it would be likely that both CiZn/GAL4AD and ciZn/EnRD would have similar phenotypes. Similar results with chimeric molecules have been reported by Alexandre et al. (1996).

There are a number of interesting aspects to the experiments in which ciZn/EnRD was expressed via ptc-GAL4. Before carrying out the studies, it was not possible to predict the levels of CiZn/EnRD that would be obtained. CiZn/EnRD should negatively regulate the expression of the ptc-GAL4 just as it does ptc and limit its own expression. The final phenotype will depend upon the relative levels of endogenous Ci and CiZn/EnRD and their competition for target sites.

The pattern of expression is also curious. The ciZn/EnRD is expressed in a broad stripe along the A/P boundary. Expression of the ciZn/EnRD eliminates expression of high level ptc and leads to broadening the augmented stripe of endogenous Ci. This results in broad low level expression of ptc and dpp.

The phenotype of the animals expressing ciZn/EnRD via ptc-GAL4 is the loss of structures between wing veins 3 and 4 and a fusion between these wing veins. This phenotype is similar to that of fused (fu) mutants and is more extreme than that of ci<sup>fu</sup> animals. The fu gene encodes a serine/threonine kinase (Preat et al., 1990) that appears to be required for activation of Ci function. The ci<sup>fu</sup> allele produces a truncated Ci protein that appears to behave as a dominant negative. In fu mutants and ci<sup>fu</sup> mutants, the region expressing high level Ci expands to fill the entire anterior compartment (Slusarski et al., 1995). The elevation of Ci protein levels in fu and ci<sup>fu</sup> mutants could be explained by the same process that occurs with ciZn/EnRD. Disruption of Ci activity lowers ptc expression and causes a decrease in negative regulation of Ci. As a result, Ci protein levels rise and reach a new homeostatic plateau.

Transactivation by Ci and its cytoplasmic localization depend on sequences C terminal to the zinc finger domain

Deletion of sequences N terminal to the zinc finger domain results in a protein that is still able to activate ptc expression and has a subcellular distribution very similar to that of the wild-type Ci protein. While our initial assays have not identified a role for the N-terminal domain, it almost certainly has a function since it contains a 60 amino acid region that is 68% identical between Gli3 (Ruppert et al., 1990) and Ci.

Deletion of sequences C terminal to the zinc finger domain results in a protein that behaves as a strong dominant negative. It appears to be more potent than the CiZn/EnRD because animals expressing ciN[Zn via ptc-GAL4 die as embryos and lack wg expression while those expressing ciZn/EnRD survive embryogenesis and only show defects in imaginal disc patterning. These results suggest that the C-terminal domain contains a region required for transactivation. Similar results have been reported by Alexandre et al. (1996).

Deletion of C-terminal sequences also results in a shift in the subcellular distribution of the CiN[Zn protein. The wild-type Ci protein is primarily cytoplasmic, while the CiN[HA]/Zn protein is found throughout the cell and is present at high levels in the nucleus. The CiN[HA]/Zn protein is approximately 70x10<sup>4</sup> M<sup>-1</sup> and would require a nuclear localization signal (NLS) or interaction with a protein containing an NLS for translocation into the nucleus. Ci does not contain a canonical NLS though it is possible that some related sequence in Ci serves this role. The presence of CiN[HA]/Zn in the nucleus suggests that the Ci protein itself may have the ability to enter the nucleus, but that nuclear transport is normally blocked by the C-terminal domain. C-terminal sequences could either tether Ci in the cytoplasm or mask a region required for Ci nuclear import. Regulation of nuclear translocation would lead to modulation of Ci activity.

The goal of future work will be to define the regions of Ci responsible for its functions and to identify the molecular events that regulate Ci protein levels and activity.

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