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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-1* 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*^{10638}) and *ptc-lacZ* were obtained from N. Perrimon, G. Struhl, T. Kornberg and B. Noll. *ptc*^{G20} was kindly provided by R. Whittle. *ptc*^{IN} was obtained from C. Nusslein-Volhard. *hh*^{9k94} was obtained from the Tubigen stock center.

DNA constructs

A5C-flip cassette(FC)-ci

The entire *ci* coding region was cloned into pGEM4Z in three fragments: a 400 bp *Dra*I/*Eco*RI cDNA fragment which contains the 5′ *ci* coding sequences and eliminates a large intron, a 1.5 kb *Eco*RI genomic fragment containing sequences from the middle of the coding region and a 6 kb *Eco*RI 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 *Dra*I/*Eco*RI cDNA fragment containing the 5′ *ci* coding sequences and an *Eco*RI cDNA fragment containing the rest sequences (*ci*-pGEM7Z). The pGEM7Z *Xho*I site, on the 3′ side of the cDNA, was converted to a *Bgl*II site, and the cDNA was cloned into the *Bgl*II site of pUAST as a *Bam*HI/*Bgl*II fragment.

UAS-*ci*N[HA]/Zn

ci-pGEM7Z was digested with *Hin*cII and *Bgl*II, and a *Bgl*II linker was added to the *Hin*cII site. The religated 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 *Pst*I 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 *Bam*HI/*Bgl*II fragments.

UAS-*ci*Zn/C

ci-pGEM7Z was digested with *Bam*HI and *Eco*RV, and a *Bam*HI-ATGstart-*Eco*RV adapter was added in frame at the *Eco*RV site (amino acid position 440) (*ci*Zn/C-pGEM7Z). This Zn/C construct was then cloned into pUAST as a *Bam*HI/*Bgl*II fragment.

UAS-*ci*Zn/EnRD

The *en*-repression domain (RD) (amino acids 284-338) was cloned as a PCR fragment with *Hpa*I/*Bgl*II ends into *Hpa*I/*Bgl*II digested *ci*Zn/C-pGEM7Z (*ci*Zn/EnRD-pGEM7Z). The Myc 9E10 epitope was cloned into *Xba*I/*Bgl*II digested *ci*Zn/EnRD-pGEM7Z at the end of the RD. This *ci*Zn/EnRD construct was then cloned into pUAST as a *Bam*HI/*Bgl*II fragment.

UAS-*ci*Zn/GAL4AD

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

UAS-*ci*Zn

*ci*Zn/GAL4AD-pGEM7Z was digested with *Hpa*I and *Bgl*II to cut out the AD region. The *Bgl*II overhang was filled in with a Klenow reaction and the construct was blunt end ligated. This *ci*Zn construct was then cloned into pUAST as a *Bam*HI/*Bgl*II 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 Heuvel et al., 1989), β-galactosidase (Zhang 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*^{9k94}) (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.

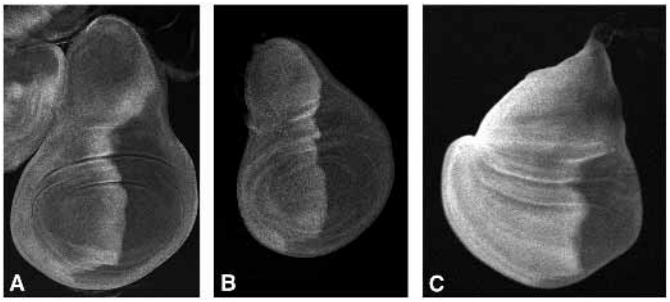

Fig. 1. Hh and Ptc regulate the levels of Ci protein in the wing imaginal disc (dorsal up, anterior to the left). (A) Distribution of Ci protein in a wild-type wing imaginal disc. Unlike the *ci* transcript (Eaton and Kornberg, 1990), the Ci protein is distributed nonuniformly throughout the anterior 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^{9k94}). 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^{G20}/ptc^{IN} larva (Capdevila et al., 1994). Note that the levels of Ci are high throughout the entire anterior compartment.

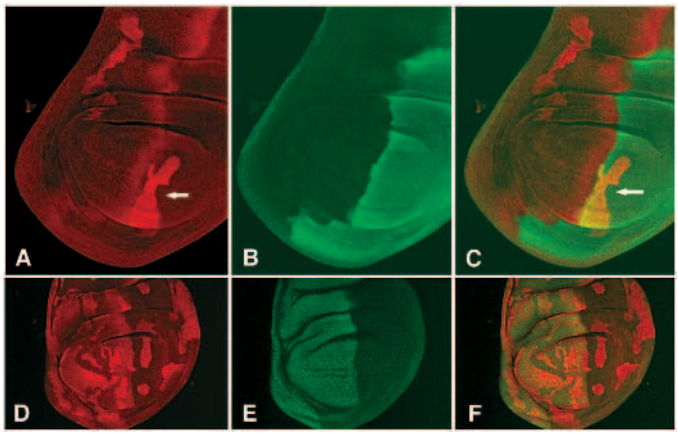

Fig. 2. *ci* gain-of-function clones in the wing do not alter expression of endogenous *ci* or *en* (dorsal up, anterior to the left). (A-C) A wing disc with a Ci-expressing clone in the posterior compartment. The distributions of the Ci protein (A) and β-galactosidase protein from an enhancer trap insertion 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.

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^{G20}/ptc^{IN108}, 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).

Ci activates expression of Hh target genes in wing and leg imaginal discs

To test the role of *ci* in the regulation of Hh target genes, 'flip-out' *ci* gain-of-function clones (Struhl and Basler, 1993; Buenzow and Holmgren, 1995) were generated in both the anterior and posterior compartments of imaginal discs. Clones expressing *ci* were visualized using antibodies against Ci. High levels of ectopic Ci protein did not alter the expression of *lacZ* reporter constructs for the *ci* and *en* genes (Fig. 2). These results show that Ci does not activate its own expression nor does it inhibit the expression of *en* in the posterior compartment.

To follow the expression of the Hh target genes, *dpp*, *wg* and *ptc*, both *lacZ* reporter constructs and antibody stainings were used in each case. Overexpression or misexpression in both wings (Fig. 3D-F) and legs of *ci* caused variable ectopic expression of *dpp* within the clones. Not all cells within the clone expressed *dpp* and ectopic *dpp* expression was not observed within every clone. Clones were generated at either 0-24 hours or 24-48 hours after egg laying (AEL). Ectopic *dpp* expression was most often observed in clones that were generated earlier in development.

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).

Clones overexpressing *ci* in the anterior compartment 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. In the posterior compartment of the wing pouch region, *ptc-lacZ* was uniformly activated to high levels while in the anterior compartment *ptc-lacZ* was activated to lower levels (Fig. 4B). *dpp-lacZ* was variably expressed throughout the wing pouch, although more expression was detected in the posterior compartment (Fig. 4D). Both *ci* constructs were expressed at similar levels in the anterior and posterior compartments and, therefore, the ability of Ci to differentially activate the expression of *ptc* and *dpp* in the posterior compartment may reflect regulation of Ci activity by Hh signaling.

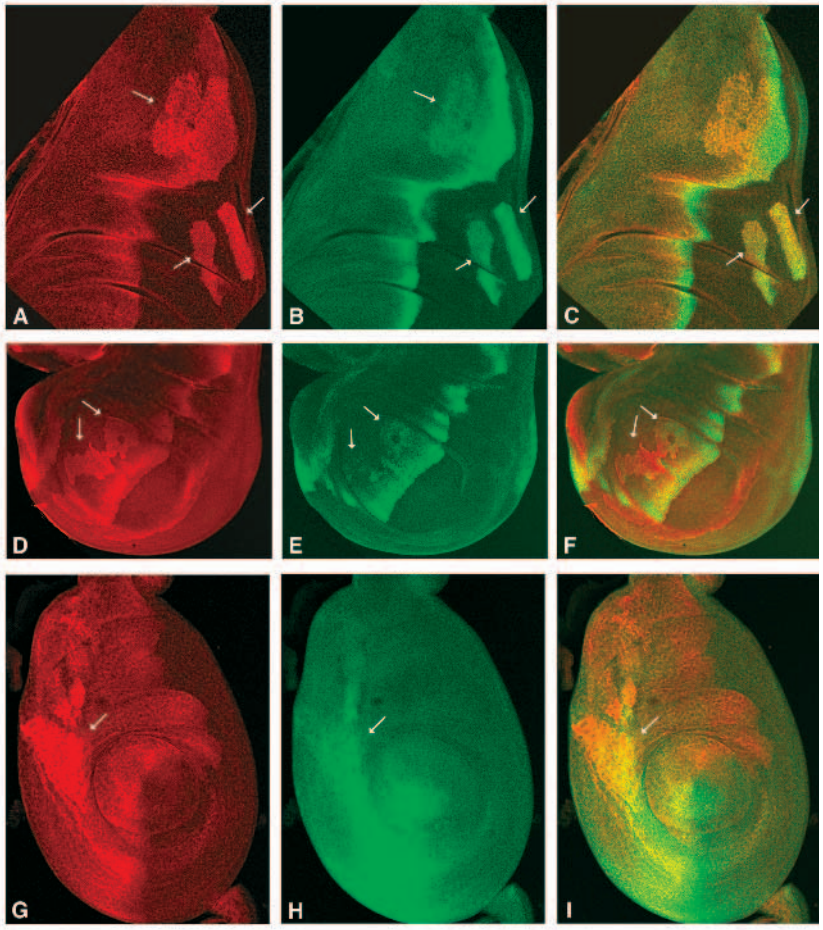

Fig. 3. *ci* gain-of-function clones in the wing and leg imaginal disc can cause alterations in the expression patterns of Hh target genes (dorsal up, anterior to the left). (A-C) A wing imaginal disc with three *ci* gain-of-function clones, one in the anterior compartment and two in the posterior compartment (arrows). This wing imaginal discs has been doubly labeled with antibodies against Ci (A) and Ptc (B). Overlap is shown in C. Misexpression of *ci* in the posterior compartment autonomously activates *ptc* expression within the clone in every case observed. Overexpression of Ci in the anterior compartment can result in overexpression of *ptc* but this effect is variable. The effect of overexpressing or misexpressing Ci on *dpp* expression was assayed by monitoring expression from a *dpp-lacZ* enhancer trap insertion. (D-F) A wing imaginal disc with a clone overexpressing Ci in the anterior compartment of a wing imaginal disc. This disc was doubly labeled with antibodies against Ci (D) and β-galactosidase (E); overlap is shown in F. Overexpression of Ci in regions removed from the A/P boundary stripe of augmentation (D) can cause ectopic expression of *dpp-lacZ*. Ectopic expression is only observed within the clone, although not every cell in the clone misexpresses *dpp-lacZ* (arrows). (G-I) A leg imaginal disc, doubly labeled with antibodies against Ci (G) and Wg (H). Overlap is shown in I. *wg* is expressed in a ventral anterior compartment quadrant adjacent to the A/P boundary of the leg imaginal disc (G). Overexpression of Ci in the anterior compartment (arrow in G) causes ectopic anterior compartment expression of *wg* (arrows in H,I).

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)(CiZn/GAL4AD) or the Engrailed (En) repression domain (Han and Manley, 1993)(CiZn/EnRD) (Fig. 5). The constructs were placed into the pUAST vector (Brand and Perrimon, 1993) and transgenic lines were generated. Expressing the UAS-*ci*Zn/GAL4AD construct with *prd*-GAL4 resulted in complete rescue of the ci^{Ce}/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-*ci*Zn/GAL4AD lines generated was less active and survived to adulthood in combination with *en*-GAL4. Expression of the *ci*Zn/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 ci^{1} and ci^{W} mutants in which the Ci protein is ectopically expressed in the posterior compartment (Locke and Tartoff, 1994; Slusarski et al., 1995).

Expression of *ci*Zn/EnRD or *ci*Zn 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 ci^{Ce} mutation appears to be a moderate dominant negative (Motzny and Holmgren, 1995; Slusarski et al., 1995) and ci^{Ce}/+ animals

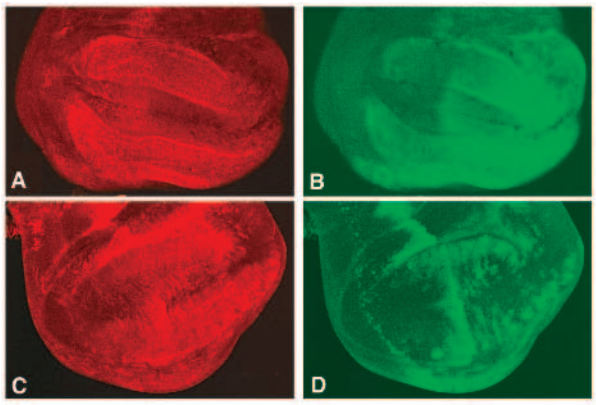

Fig. 4. *ci* activation of Hh target genes is stronger in the posterior compartment (dorsal up, anterior to the left). (A) Ci protein distribution in a wing disc from larvae carrying UAS-*ci*-cDNA and 71B-GAL4 insertions. The 71B-GAL4 insertion directs high level expression in the wing pouch. (B) *ptc-lacZ* expression in the disc shown in A. *ptc-lacZ* expression is activated in both the anterior and posterior compartments but the levels are higher in the posterior compartment. High level Ci expression in the wing pouch (C) also activates *dpp-lacZ* expression better in the posterior compartment (D).

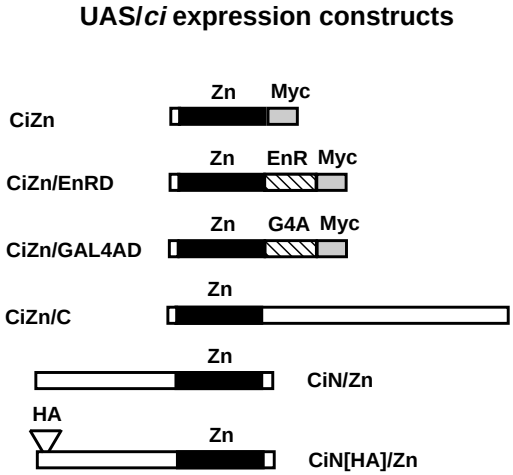

Fig. 5. UAS-Ci expression constructs. Diagram of constructs used to express chimeric and truncated versions of Ci. CiZn has an initiator ATG followed by the regions encoding amino acids 440-684 of Ci (the zinc finger domain includes amino acids 453-603). CiZn/EnRD has the same region of Ci followed by 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. CiN/Zn has the coding sequence through amino acid 684. CiN[HA]/Zn is the same as CiN/Zn except that a triple 12CA5 HA epitope tag has been placed after amino acid position 29.

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 (*ci*Zn/C) or C terminal (*ci*N/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 *ci*Zn/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

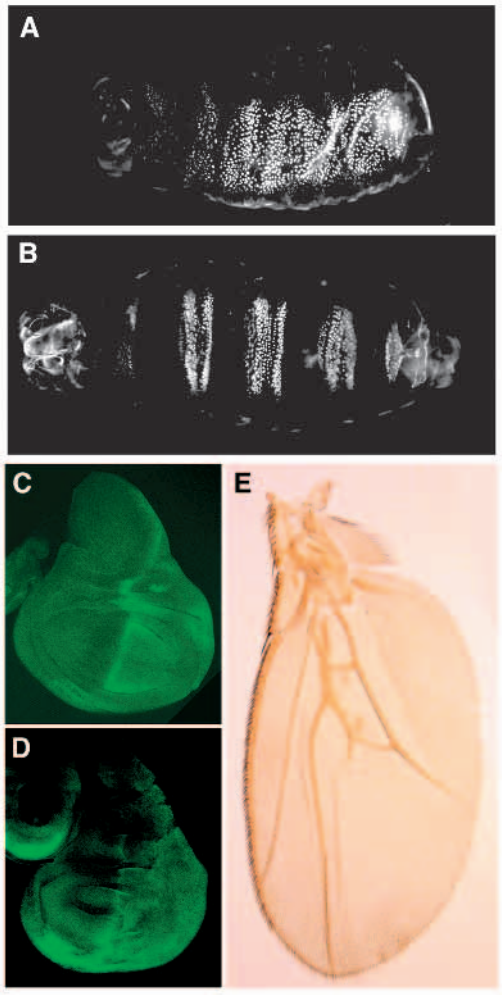

Fig. 6. The CiZn/GAL4AD protein rescues ci^{Ce} mutants and activates expression of the Hh target gene, *ptc*. In all panels, anterior is to the left. Animals homozygous for ci^{Ce} fail to form ventral naked cuticle and are covered with a lawn of denticles (A). Expression of UAS-*ci*Zn/GAL4AD via *prd*-GAL4 rescues naked cuticle in every other segment and leads to the generation of phenotypically normal denticle belts (B). To test the ability of this construct to activate *ptc* expression, UAS-*ci*Zn/GAL4AD was expressed in the posterior compartment of wing imaginal discs using *en*-GAL4. Ectopic Ptc protein is shown in green (C). Activation of *ptc* is at the level of transcription, as a *ptc-lacZ* reporter construct is also activated by posterior compartment expression of UAS-*ci*Zn/GAL4AD. Antibody staining to β-galactosidase is shown in green (D). Ectopic Ptc in the posterior compartment leads to posterior wing vein defects in adults (E; wild-type wing shown in Fig. 7G).

prepared from this line were relatively normal, though the denticle belts seemed to have fewer denticles (data not shown). Expression of Ci Zn/C in the anterior compartment gave rise to animals with a near normal cuticle pattern (data not shown).

Expression of *ci*N/Zn in the posterior compartment did not lead to activation of *ptc* expression (data not shown). However, expression of *ci*N/Zn or an epitope tagged version of this construct (*ci*N[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

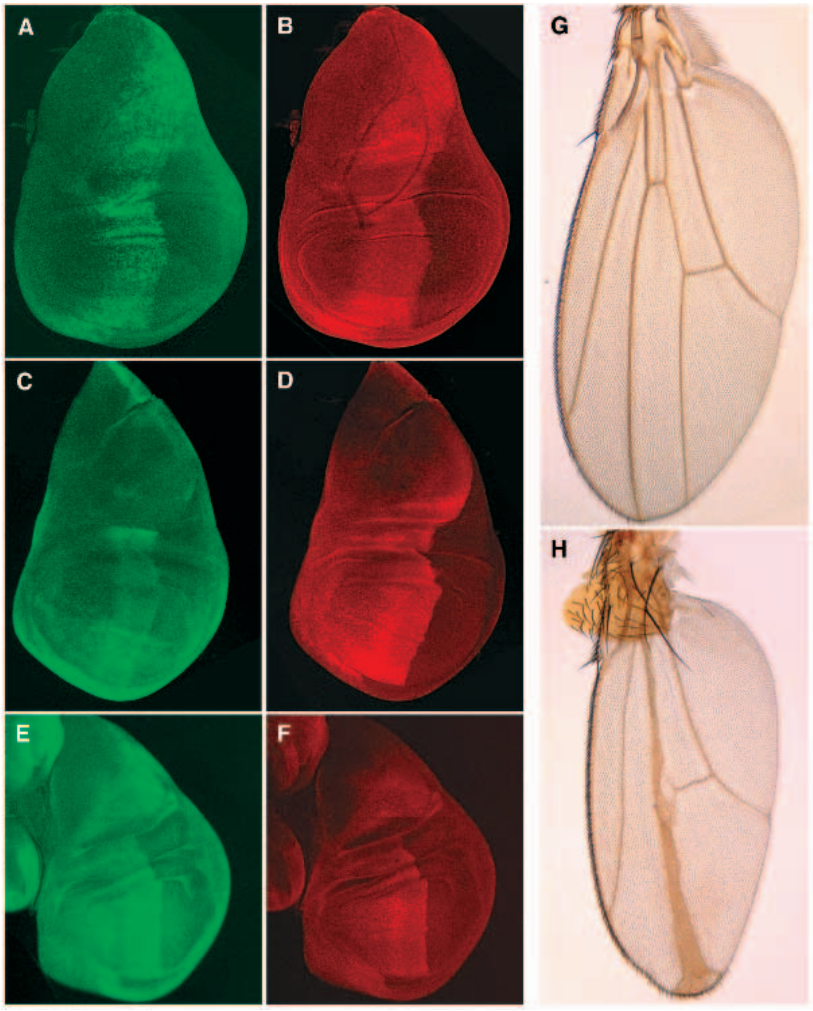

Fig. 7. Expression of *ci*Zn/EnRD via *ptc-GAL4* negatively regulates *ptc* and *dpp* expression. Anterior is to the left in all panels. (A-F) Wing imaginal discs expressing *ci*Zn/EnRD via *ptc-GAL4*. Antibody staining to the Myc epitope shows that *ci*Zn/EnRD is expressed in a broad stripe just anterior to the compartment boundary (A). It should be noted that *ptc*-GAL4 driven UAS-*lacZ* is also expressed in a wider stripe than the endogenous *ptc* gene (Johnson et al. 1995). In the same disc as A, the endogenous Ci protein is also augmented in a broad stripe that coincides with that of CiZn/EnRD (B). While Ci is cytoplasmic, the CiZn/EnRD is nuclear. Antibody staining of the Dpp protein shows that it is also present in a broad stripe, but its levels are lower than in wild type (C). Visualization of Ci expression in the same disc as C shows that the low level Dpp expression overlaps the broad stripe of augmented Ci protein (D). The narrow stripe of high level Ptc protein is eliminated and replaced by a broad stripe of low level Ptc expression (E) that coincides with augmented Ci (F). The antibody staining of Ptc in panel E has been overexposed to allow visualization of the Ptc stripe. Adult wing vein phenotypes are also observed with expression of *ci*Zn/EnRD in the anterior compartment (H). A wild-type wing is shown in G.

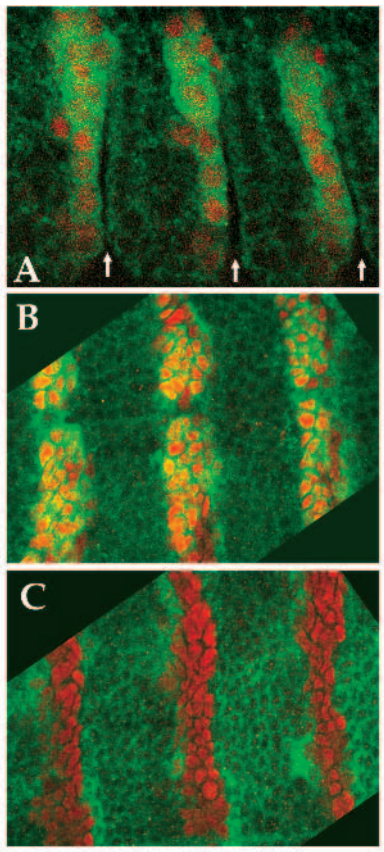

Fig. 8. Expression of *ci*Zn/C activates high level expression of *ptc* in the posterior compartment. Anterior is to the left. (A) *ci*Zn/C is expressed via *en*-GAL4 in the posterior compartment of a late stage 12 embryo. The embryo is doubly stained for En in red to mark the posterior compartment and for Ci in green. The 2A1 monoclonal antibody recognizes both Ci and CiZn/C. Low level endogenous Ci is present in the anterior compartment and higher level CiZn/C is found in the posterior compartment. The CiZn/C protein is primarily cytoplasmic, as is endogenous Ci. The segmental grooves are pointed out by arrows. (B) High level expression of *ptc* is induced in the posterior compartment of a stage 11 embryo by CiZn/C. The embryo carries the enhancer trap element *hh-lacZ*. It is doubly stained for β-galactosidase in red to mark posterior compartment and Ptc in green. Yellow color represents the overlap between *hh-lacZ* and ectopic Ptc. (C) No *ptc* expression (note absence of yellow color) is observed in the posterior compartment in embryos not expressing CiZn/C. Shown is a stage 11 embryo carrying *hh-lacZ* but not UAS-*ci*Zn/C. It is doubly stained for Ptc in green and β-galactosidase in red.

embryonic development (Bejsovec and Martinez-Arias, 1991). In embryos expressing *ci*N/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

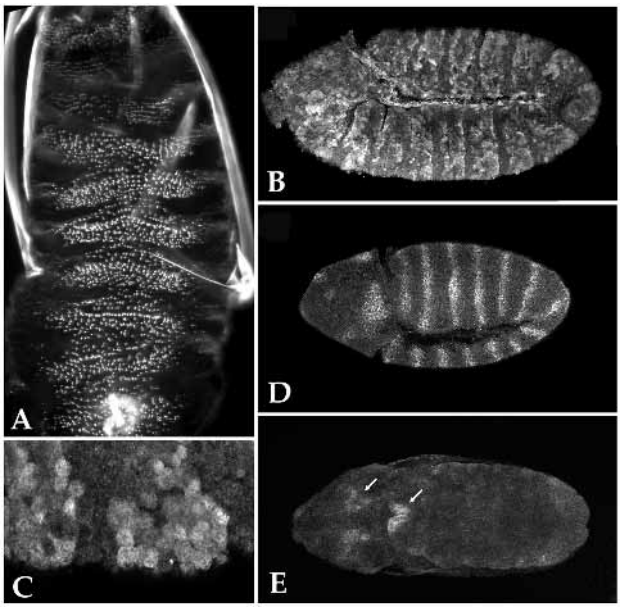

Fig. 9. Expression of *ci*N/Zn in the anterior compartment abolishes the expression of *wg*. (A) Anterior is up; (B-E) anterior is to the left. (A) Anterior compartment expression of the *ci*N/Zn eliminates the naked cuticle and causes a partial duplication of the denticle belts. (B) The CiN[HA]/Zn protein is distributed equally throughout the cell including the nucleus. Also shown is a higher magnification picture (C). (B,C) Embryos are stained for HA. (D) A stage 10 wild-type animal. *wg* is expressed as 14 thin stripes circling the embryo. (E) Expression of *ci*N/Zn or *ci*N[HA]/Zn by *ptc*-GAL4 abolishes *wg* expression during stage 11. Notice that *wg* expression is not disrupted in the regions where the *ptc* promoter is not active (arrows).

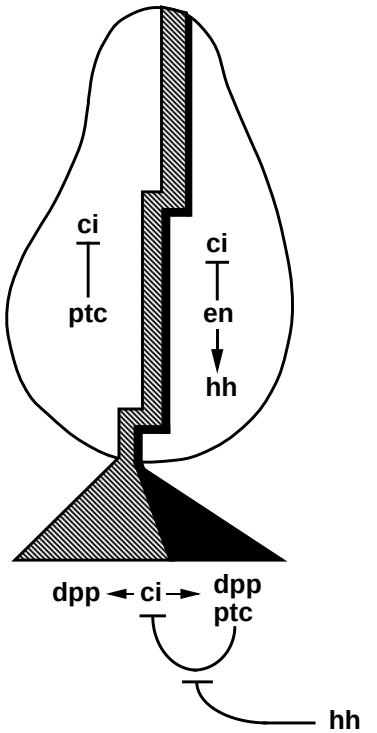

Fig. 10. Model for regulation of Hh target genes along the A/P compartment boundary. Ci protein levels are negatively regulated by Ptc in cells throughout the anterior compartment of imaginal discs. Hh signaling interferes with Ptc-negative regulation leading to elevation of Ci protein levels in a 8- to 9-cell-wide stripe. Within this region Ci activates *dpp* expression. Ci is only able to activate high level expression of *ptc* in the 2-3 cells closest to the A/P compartment boundary. Thus, there appears to be two distinct responses to Hh signaling. Highest levels of Hh lead to Ci activation of *ptc* expression while lower levels are sufficient for augmentation of Ci protein levels and its activation of *dpp*. By having Ci control the expression of *ptc*, its own negative regulator, Ci function can be regulated across the anterior compartment of imaginal discs.

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 transactivation 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 and CiZn 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^{Ce} 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 *ci*Zn/GAL4AD and *ci*Zn/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 *ci*Zn/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 *ci*Zn/EnRD is expressed in a broad stripe along the A/P boundary. Expression of the *ci*Zn/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 *ci*Zn/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^{Ce}/+ 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^{Ce} allele produces a truncated Ci protein that appears to behave as a dominant negative. In *fu* mutants and ci^{Ce}/+ 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^{Ce}/+ mutants could be explained by the same process that occurs with *ci*Zn/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 *ci*N/Zn via *ptc*-GAL4 die as embryos and lack *wg* expression while those expressing *ci*Zn/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 70×10^3 M_r 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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