Development 127, 4095-4103 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV7827

Transcriptional regulation of the Hedgehog effector CI by the zinc-finger gene *combgap*

Gerard L. Campbell[1,2,*] and Andrew Tomlinson[2]

[1]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA
[2]Department of Genetics and Development, Columbia University, New York, NY 10032, USA
*Author for correspondence (e-mail: camp@pitt.edu)

Accepted 25 July; published on WWW 7 September 2000

SUMMARY

Members of the Hedgehog (HH) family of secreted signaling molecules specify cell fate during animal development by controlling the activity of members of the Gli family of zinc-finger transcription factors in responding cells. In *Drosophila* the Gli homolog, cubitus interruptus (CI), is expressed only in the anterior compartment where it represses targets such as the signaling molecule genes *decapentaplegic* (*dpp*) and *wingless* (*wg*). HH is expressed in the posterior and diffuses into the anterior where it antagonizes CI repression resulting in *dpp* and *wg* expression immediately anterior to the compartment border. Reducing CI levels results in misexpression of *wg* and *dpp*, while CI misexpression in the posterior disrupts differentiation. Thus, normal disc patterning requires high levels of CI in the anterior and the absence of CI in the posterior. Here we show that mutations in *combgap* (*cg*) result in deregulation of CI expression, which is now expressed at much lower levels and ubiquitously, i.e., also in the posterior. Consequently, *cg* mutants phenocopy *ci* loss-of-function mutants in the anterior and *ci* gain-of-function mutants in the posterior. *cg* encodes a putative DNA-binding protein that regulates both transcriptional activation and repression of the *ci* gene.

Key words: Hedgehog, Cubitus interruptus, Gli, Zinc finger, Combgap, Imaginal discs, *Drosophila*

INTRODUCTION

Secreted polypeptide signaling molecules belonging to several different families regulate the specification of different cell fates in many animal systems. The Hedgehog (HH) family includes some of the most important regulators of pattern formation (Hammerschmidt et al., 1997; Ingham, 1994). Members control segmental patterning in *Drosophila* embryos (Mohler, 1988), specification of the anteroposterior axis in both vertebrate and insect limbs (Basler and Struhl, 1994; Riddle et al., 1993) and specification of the dorsoventral axis in the vertebrate neural tube (Briscoe and Ericson, 1999). Within these systems, *hh* genes are expressed in discrete domains and secreted HH proteins subsequently regulate patterning by acting as morphogens that induce expression of different target genes above distinct threshold concentrations or by activating expression of additional signaling molecules that in turn act as morphogens (Basler and Struhl, 1994; Briscoe and Ericson, 1999; Strigini and Cohen, 1997).

Activation of the HH signaling pathway in responding cells regulates gene expression by modulating the activity of the Gli family of zinc-finger transcription factors, including cubitus interruptus (CI) in *Drosophila* (Ruiz i Altaba, 1999). The primary function of HH signaling in *Drosophila* appears to be to antagonize the constitutive phosphorylation of CI by protein kinase A (PKA; Chen et al., 1999b; Wang et al., 1999). Full-length CI (CI-155) can act as a transcriptional activator but the fully phosphorylated form is impeded from entering the nucleus and it is targeted for proteolytic cleavage to a truncated form (CI-75), which acts as a transcriptional repressor (reviewed in Aza-Blanc and Kornberg, 1999; Chen et al., 1999a). Thus, HH signaling can activate gene expression directly by raising the level of the activator, CI-155, or more indirectly by reducing the level of the repressor, CI-75, relieving the repression of other genes.

Correct spatial patterning, thus, requires the modulation of CI activity in the appropriate places and this involves the restriction of both *hh* and *ci* expression in developing tissues. The embryonic segments and imaginal discs (the larval tissue giving rise to much of the adult including the appendages) of *Drosophila* are divided into two lineage units, the anterior and posterior compartments (Crick and Lawrence, 1975) which are characterized by the expression of the related homeobox genes *engrailed* (*en*) and *invected* (*inv*) in the posterior and *ci* in the anterior (Blair, 1995; Eaton and Kornberg, 1990; Kornberg et al., 1985; Orenic et al., 1990; Tabata et al., 1995). There is no *ci* expression in the posterior because it is directly repressed by EN (Eaton and Kornberg, 1990; Schwartz et al., 1995), whereas CI plays no role in regulating *en* expression. The posterior compartment is also characterized by the expression of *hh*; it is repressed in the anterior by CI (Dominguez et al., 1996). The segments and discs are thus divided into a CI-positive anterior and a EN/HH-positive posterior.

HH regulates the expression of several genes, including those encoding the secreted signaling molecules *wingless* (*wg*, a WNT, in the embryonic segment and ventral leg disc) and *decapentaplegic* (*dpp*, a TGFβ, in the wing disc and the dorsal leg; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Ingham et al., 1991). These targets are repressed in the posterior by EN (Sanicola et al., 1995; Tabata et al., 1995; Zecca et al., 1995), but HH protein diffusing from the posterior induces their expression in a stripe immediately anterior to the compartment border, the width of the stripe revealing the extent to which HH can diffuse into the anterior. HH signaling in this stripe of cells antagonizes the phosphorylation and degradation of CI, resulting in high levels of the activator form, CI-155 and low levels of the repressor, CI-75 form at the compartment border, whereas outside of this stripe CI-75 is the prevalent form (Aza-Blanc et al., 1997). *dpp* expression in the wing is both activated and repressed by CI away from the compartment border *dpp* is repressed by CI-75 and, in fact, loss of *ci* anywhere in the anterior results in misexpression of *dpp* (Dominguez et al., 1996), which demonstrates that merely reducing the level of CI-75 is sufficient to allow *dpp* expression. HH signaling at the compartment border reduces the level of CI-75 and although this is sufficient for *dpp* expression, it appears that activation of expression by CI-155, stabilized at the compartment border, is required to achieve the normal levels of expression found in wild-type wings (Methot and Basler, 1999). The mechanism of CI regulation of *wg* expression in the embryo and ventral leg is less well understood, because, although it appears that CI can activate *wg* expression (Alexandre et al., 1996; Chen et al., 1999b), it is unclear whether it also is required to repress its expression away from the compartment border.

Gain and loss of HH activity results in characteristic phenotypes in imaginal discs. Ubiquitous expression of HH has no effect in the posterior compartment of leg and wing discs; in the anterior, it results in misexpression of *dpp* in all anterior cells of the wing and dorsal leg discs and of *wg* in all the cells of the ventral leg (Basler and Struhl, 1994). This results in characteristic overgrowth phenotypes in both the wing and the leg that can be directly related to the misexpression of *dpp* and *wg* (Campbell and Tomlinson, 1995; Diaz-Benjumea et al., 1994). Loss of HH activity results in the loss of *dpp* or *wg* expression, and both leg and wing discs fail to grow, again a result that is directly related to the loss of *wg* and *dpp* (Basler and Struhl, 1994). Proteins involved in transducing the HH signal (Murone et al., 1999) can be divided into those required for proteolysis of CI, such as patched (PTC; Ingham, 1998), and those required for antagonizing this and for maintaining the unphosphorylated activator form of CI, such as smoothened (SMO; Alcedo et al., 1996; van den Heuvel and Ingham, 1996); mutations in genes encoding these proteins phenocopy HH gain-of-function and HH loss-of-function, respectively.

Loss of CI also phenocopies HH gain-of-function in the wing because, as discussed above, CI is required to repress *dpp* expression in most of the anterior compartment. Misexpression of CI to very high levels in the anterior can also phenocopy HH gain-of-function (Hepker et al., 1997), presumably because this results in high levels of the full-length form and sufficient amounts can gain access to the nucleus (Chen et al., 1999a). Misexpression of CI in the posterior can disrupt normal differentiation in the wing and is associated with defects in wing-vein patterning (Hepker et al., 1997). In fact, many of the original *ci* mutants are gain-of-function alleles in which *ci* is expressed in the posterior as well as the anterior; these mutants have characteristic gaps in wing-vein IV (Slusarski et al., 1995). Thus, normal disc patterning requires *ci* expression to be restricted to the anterior compartment and for CI protein activity to be modulated at the compartment boundary.

Here, we describe a molecular and genetic characterization of the *combgap* (*cg*) gene and show that mutations in this gene result in a hedgehog gain-of-function phenotype in the anterior of the leg. However, unlike other mutants with this phenotype, such as *ptc*, *cg* mutants also disrupt patterning in the posterior of the wing in a similar fashion to ectopic *ci* expression. We show that both anterior and posterior compartment phenotypes in *cg* mutants are due to deregulation of *ci* expression: instead of just being expressed at high levels in the anterior compartment, *ci* is expressed at uniform low levels throughout the disc, i.e., also in the posterior. The hedgehog gain-of-function phenotype appears to be the direct result of the lower levels of CI not being sufficient to repress *dpp* and *wg* expression. The patterning defect in the posterior is also identical to that produced by direct misexpression of *ci*. *cg* encodes a putative transcription factor that, thus, appears to be required for normal regulation of transcription of the *ci* gene.

MATERIALS AND METHODS

P-element screen, fly stocks, mutant analysis and clonal analysis

A collection of P-element insertion lines generated by the Berkeley *Drosophila* Genome Project (BDGP) and maintained by the Bloomington stock center was screened for interesting *lacZ* expression patterns in the leg and wing imaginal discs; line *l(2)07659* was selected for further study based on its homozygous phenotype rather than its *lacZ* expression pattern. This line was maintained over the SM6a-TM6B balancer and homozygous larvae were selected by the absence of the Tb marker. Fly stocks *dpp-lacZ* (dpp^{10638}), *wg-lacZ* (wg^{02657}), hh-lac (hh^{P30}), hh^{ts} (hh^{4}), cg^{1}, en^{59}, ci^{W}, ci^{D}, ci^{94}, ap-Gal4 (ap^{md544}), UAS-ci ($ci^{Scer\backslash UAS.cHa}$) and FRT 42 are described in detail at Flybase (http://flybase.bio.indiana.edu). The ci^{n} mutant is unpublished and was identified fortuitously in another mutant stock. It is homozygous viable with wing duplications in a small percentage of flies and enlarged antennae in most flies. It is pupal lethal over ci^{94}, ci^{D} or a deficiency, with larvae having leg and wing discs showing a hedgehog gain-of-function phenotype. Molecular analyses reveal a 6 kb insertion upstream of the transcription start site (not shown). *cg* clonal analysis was performed using larvae of the genotype hsflp; FRT 42 arm-lacZ/ FRT 42 cg^{07659}, as described previously (Campbell and Tomlinson, 1998). The *cg* hh^{ts} analysis was performed with a cg^{07659}; hh^{ts}/SM6a-TM6B stock; the larvae were raised until early second instars at 17°C and then shifted to 31°C until late thirds, when their discs were dissected and fixed. The *ci* hh^{ts} analysis was performed in a similar fashion by crossing hh^{ts}/TM6B; ci^{n}/+ flies to *dpp-lacZ*/+; hh^{ts}/TM6B; ci^{94}/+.

Gene expression studies

X-gal stains, immunohistochemistry, immunofluorescence and in situ analyses were performed as previously described (Campbell and Tomlinson, 1998). Antibodies used are to AL (rat, diluted 1:1000; Campbell and Tomlinson, 1998), β-gal (Capell, rabbit, diluted 1:2000), CI (AbN; rabbit, diluted 1:250; this recognizes both the full-

length and degraded form; Aza-Blanc et al., 1997), CI (2A1; rat monoclonal, supernatant diluted 1:1; this recognizes only the full-length form; Slusarski et al., 1995). A riboprobe was generated from the X27 cDNA for use in the in situ analysis.

Generation of new *cg* mutants

Initially small deletions were made by excision of the P-element using the Δ2-3 transposase source, yielding several lines with phenotypes equivalent to or less severe than cg^{07659}. Some deletions, such as that in Df(2R)cg315 extended 3′ to the insertion and removed at least one transcription initiation start site and also the adjacent transcription unit. Others, such as the deletion in cg^{262}, extended 5′ to the insertion (in this case extending almost to the start of the X31 cDNA but not into the coding region). Df(2R)cg315 is embryonic lethal and is not rescued by CG247, indicating that it removes more than the *cg* gene, but it has an identical phenotype over both cg^{07659} and cg^{262} as cg^{07659} homozygotes. cg^{262} is rescued by CG247 and homozygotes also have an identical phenotype to cg^{07659}. However, molecular analyses of several hundred deletions revealed that none extended into the protein-coding region of the *cg* gene so they could not be classified as nulls. Because the cg^{07659} line shows *lacZ* expression, the analysis of *dpp* and *wg* expression was carried out using cg^{262}/Df(2)cg315 larvae.

EMS mutants were generated using standard techniques. The initial mutant screen was based on the observation that *en* +/+ *cg* transheterozygotes have a gap in vein IV in a high percentage of flies. Wild-type flies were mutagenized with EMS and crossed to en^{59} and approximately 10,000 en^{59} heterozygotes were screened for gaps in vein IV. This screen yielded a single new *cg* allele, cg^{Y4}, based on its inability to complement cg^{07659} and a substitution of a conserved amino acid in the third zinc finger. An additional screen was performed to revert the dominant negative activity of cg^{Y4}. cg^{Y4} heterozygotes were mutagenized and crossed to en^{59} heterozygotes, and about 10 000 en^{59} +/+ cg^{Y4} transheterozygotes were screened for flies with a reduced gap or no gap in vein IV. A single putative null *cg* mutant, cg^{A22}, was identified in this screen. To determine the molecular basis of these two mutants, genomic DNA was isolated from homozygous larvae and specific primers were used to amplify the *cg* coding region, which was then sequenced.

Rescue of *ci* and *cg* mutants by UAS-*ci*

For rescue of *ci* mutants by UAS-*ci*, *ap*-Gal4/+;ci^{94}/+, flies were crossed to *dpp-lacZ*/+;UAS-*ci*/+;ci^{n}/+ and the discs from the resulting larvae were fixed and stained with X-gal and anti-Ci. *ap*-Gal4/*dpp-lacZ*;UAS-*ci*/+; ci^{n}/ci^{94} discs were identified by overgrowth and by high levels of CI in the dorsal compartment of the wing. For rescue of *cg* mutants by *ci*, *dpp-lacZ* cg^{262}; UAS-*ci*/SM6a-TM6B flies were crossed to Df(2R)cg315; C765/SM6a-TM6B. Leg discs from the non-Tb larvae were stained with X-gal. Gal4 is expressed ubiquitously in C765.

Cloning of *cg* and molecular biology

Genomic DNA 5′ to the P-element in cg^{07659} was cloned by plasmid rescue and used to screen a lambda genomic library; approximately 50 kb of genomic DNA was isolated. This was used to screen cDNA libraries identifying two transcripts close to the insertion site. Further analysis revealed the P-element to be inserted in an intron in the 5′ UTR of one of these transcripts, indicating this was most likely to correspond to *cg*. Over 80 cDNAs were pulled out and the two longest, X27 and X31, were sequenced. This sequence and partial characterization of the other cDNAs by restriction analysis revealed several alternative transcripts, but that almost all of the variation existed outside of the coding region apart from an alternative C terminus (Fig. 5). An 11.5 kb *Xba*I genomic fragment including all of this transcript was cloned into pW8 and used to transform flies (CG247). This fragment minus a central 3.4 *Stu*I fragment was also cloned into pW8 and used to transform flies (CG248).

RESULTS

P-element mutant line, *l(2)07659*, phenocopies HH gain-of-function

Ubiquitous expression of *hh* in the leg imaginal discs of *Drosophila* results in a transformation of the normal circular disc into an anteriorly elongated oval (Campbell and Tomlinson, 1995). This is associated with misexpression of *dpp* in the dorsal anterior and of *wg* in the ventral anterior. The WG/DPP target, *aristaless* (*al*), is expressed as a line at the interface between these two domains. In wild-type discs, there is only a spot of *al* expression in the center (Campbell and Tomlinson, 1995). Screening through a collection of P-element-induced mutations generated by the BDGP identified a line, *l(2)07659*, in which homozygous larvae possessed leg discs morphologically identical to those generated by ubiquitous *hh* expression (Fig. 1B). This phenotype was not associated with ectopic expression of *hh*, and loss of HH activity using a temperature-sensitive mutant had no effect on the phenotype of *l(2)07659* discs (Fig. 1C,D). However, both *dpp* and *wg* were misexpressed in the anterior of these discs, accounting for the overgrowth and ectopic *al* expression (Fig. 1F,J). In the posterior, *wg* and *dpp* were repressed as in wild-type discs. Curiously, although *dpp* was misexpressed, the level of expression, even at the compartment border, was lower than in wild-type discs (Fig. 1G,H). Ubiquitous expression of *hh* also induces ectopic *dpp* expression and overgrowth in the wing (Basler and Struhl, 1994), but there was no overgrowth in *l(2)07659* mutant wing discs and there was only very weak ectopic *dpp* expression (Fig. 1L).

l(2)07659 is an allele of *cg* and interacts with *en* and *ci*

l(2)07659 was mapped to region 50E1-2 by the BDGP (Spradling et al., 1999). Mobilization of the P-element resulted in a high frequency of excisions (>50%) that were completely wild type, indicating that the P-element is responsible for the lethality of this line. Complementation tests with genes in the same region identified it as an allele of *cg* and it has been renamed cg^{07659}. A single viable allele, cg^{1}, had been identified previously with two main phenotypes: first, the number of sex comb teeth on the male first leg is increased (an anterior compartment phenotype associated with ectopic *wg* expression, Fig. 2E); and second, there is a gap in vein IV in the wing (a posterior compartment phenotype; Fig. 2B) (Waddington, 1953). $cg^{1/07659}$ heterozygotes are also viable with a slightly more-severe phenotype than cg^{1} homozygotes (Fig. 2C,F). As described below, it appears that cg^{07659} is a very strong allele identical in severity to a putative null, cg^{A22}.

Although cg^{07659} homozygotes phenocopy mutants such as *ptc*, *Pka-C1* and *costa* (*cos*), which show hedgehog gain-of-function phenotypes, the posterior compartment in the latter mutants is unaffected, unlike *cg* adult wings, indicating that CG function may not be restricted to HH signal transduction. Previous studies showed that cg^{1} interacts strongly with *en* and some *ci* mutants (House, 1953, 1961; Waddington, 1953). The *ci* mutants used in these studies have subsequently been shown to be gain-of-function mutants in which *ci* is misexpressed in the posterior (Slusarski et al., 1995) resulting defects in posterior wing vein patterning, most notably loss

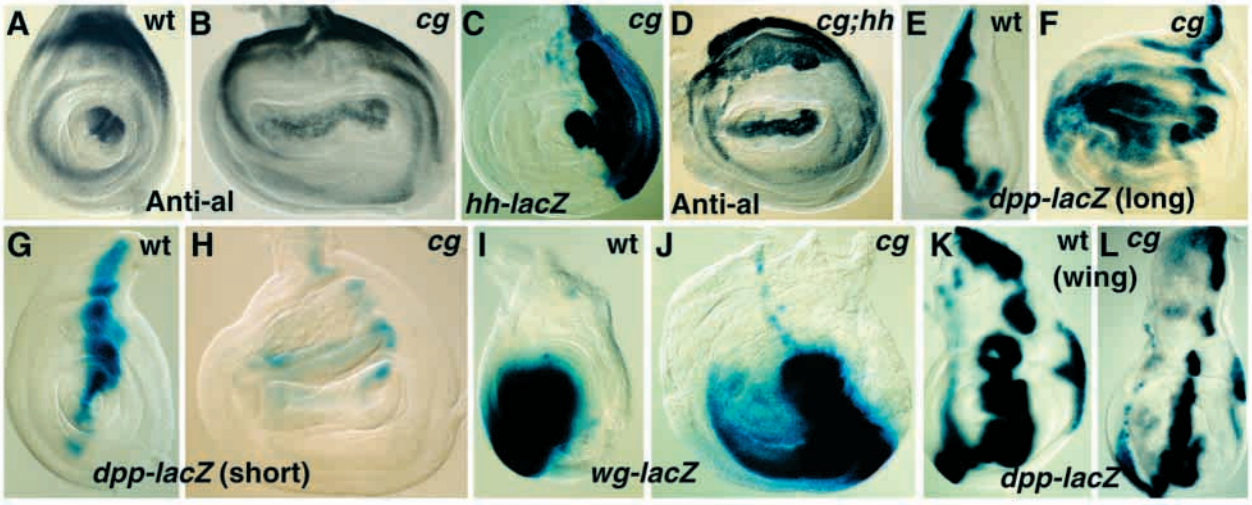

Fig. 1. Phenotype of *cg* mutant leg and wing discs. Late third instar leg discs (A-J) and wing discs (K, L). *cg* mutant discs are cg^{07659} homozygotes for antibody stains and cg^{262}/Df(2R)cg315 for *lacZ*/X-gal stains. (A) Wild-type (wt) leg discs are circular and have a central spot of *al* expression. (B) *cg* leg discs are oval with overgrowth in the anterior compartment where *al* is expressed as a central to anterior line. (C) In *cg* leg discs, *hh* expression (*lacZ*) is restricted to the posterior as in wt discs (not shown). (D) Leg discs from *cg*; hh^{ts} mutant larvae raised at the restrictive temperature during 2nd and 3rd instars and stained for *al* expression. Loss of HH activity has no effect on the phenotype of *cg* discs. (E) *dpp-lacZ* expression in a wild-type leg disc, long X-gal stain. (F) *dpp-lacZ* expression in a *cg* leg disc, long X-gal stain, showing ectopic expression in the anterior. (G) *dpp-lacZ* expression in a wild-type leg disc, short X-gal stain. (H) *dpp-lacZ* expression in a *cg* leg disc, short X-gal stain (same time as in g), showing lowered *dpp* expression at the compartment border in *cg* discs. (I) *wg-lacZ* expression in a wild-type leg disc. (J) *wg-lacZ* expression in a *cg* mutant leg disc showing ectopic expression in the anterior, this is lower than the normal level of *wg* at the compartment border. (K) *dpp-lacZ* expression in a wild-type wing disc. (L) *dpp-lacZ* expression in a *cg* wing disc. There is no overgrowth and although there is some misexpression of *dpp* in the anterior this is at a very low level compared with the leg.

of vein IV. These genetic interactions were confirmed with cg^{07659}, as follows. ci^{W} is a weak dominant mutation with heterozygotes showing a variable loss of vein IV tissue (Fig. 2G). The cg^{07659}/+; ci^{W}/+ transheterozygotes have a much stronger phenotype with vein IV being completely absent in all flies (Fig. 2H). Many viable *en* loss-of-function mutants have gaps in vein IV and some, for example en^{59}, also show this phenotype very occasionally (<1%) as heterozygotes, but en^{59} +/+ cg^{07659} transheterozygotes showed this at a much higher frequency (25%; Fig. 2I). This suggested *ci* and possibly *en* activities may be disrupted in *cg* mutants.

CI expression is abnormal in *cg* mutant discs but posterior EN expression is unaffected

Analysis of *cg* mutant discs revealed CI expression was abnormal. Instead of high levels in the anterior and no expression in the posterior, CI was expressed at low levels throughout (Fig. 3A-D). An antibody that recognizes all CI proteins (AbN; Aza-Blanc et al., 1997) revealed fairly uniform levels of expression in both anterior and posterior compartments of the leg and wing discs, but at much lower levels than are found in the anterior of wild-type discs. A CI antibody (2A1) that recognizes only the full-length form (stabilized by HH signaling; Slusarski et al., 1995) revealed

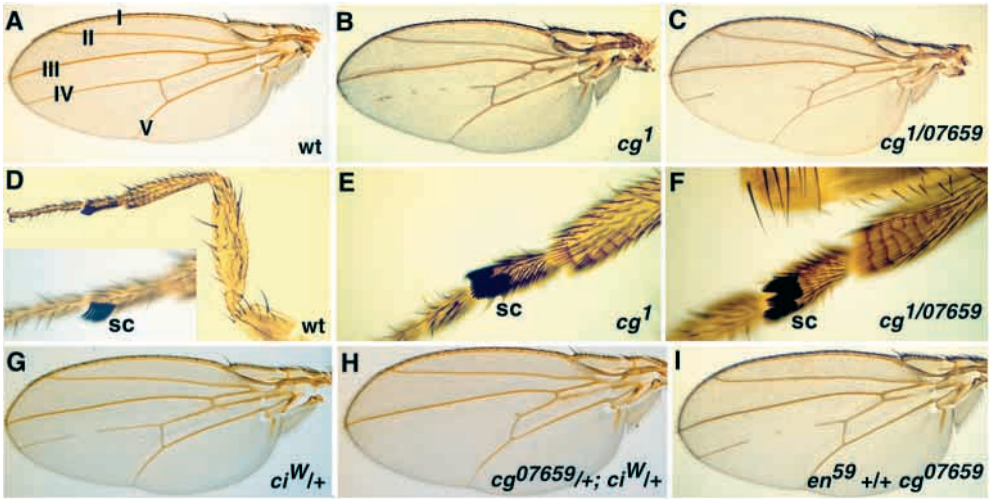

Fig. 2. Phenotype of *cg* adults and genetic interaction with *ci* and *en*. (A) Wild-type (wt) wing with five wing veins (I-V). (B) Wing from cg^{1} homozygote showing gaps in vein IV. (C) cg^{1}/cg^{07659} wing also showing gap in vein IV. (D) Wild-type male prothoracic leg, inset shows magnification of the region carrying the sex comb (sc). (E) Leg from cg^{1} homozygote showing extra sex-comb teeth. (F) cg^{1}/cg^{07659} leg also showing extra sex-comb teeth. (G) ci^{W}/+ wing showing minor gap in vein IV. (H) cg^{07658}/+; ci^{W}/+ wing, vein IV is completely lost distal to the posterior crossvein. (I) en^{59} +/+ cg^{07659} wing with gap in vein IV.

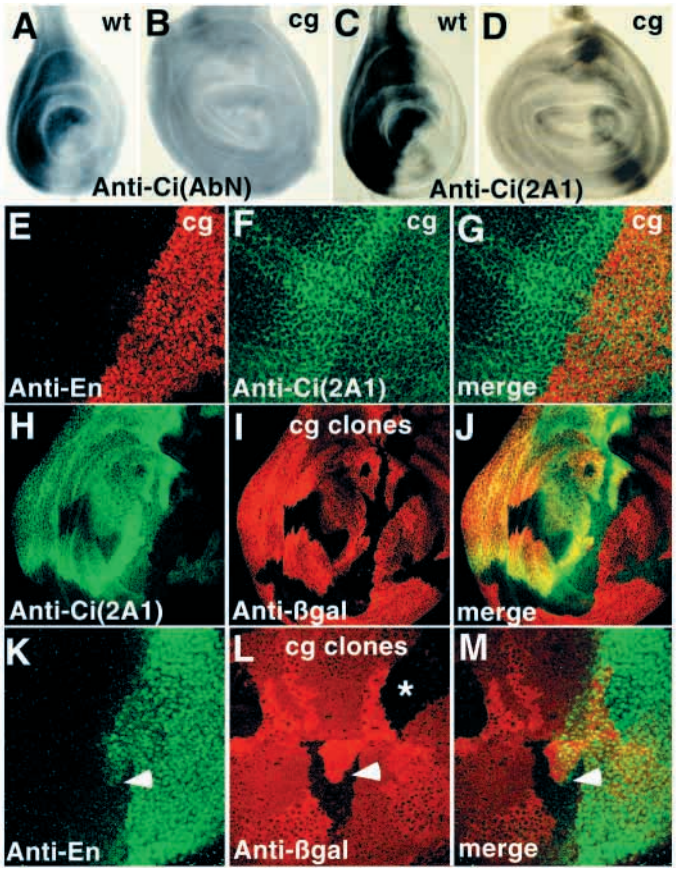

Fig. 3. CI and EN expression in *cg* mutant discs. (A) and (C) In wild-type (wt) leg discs, CI is expressed only in the anterior compartment. In A the Ci (AbN) antibody detects all CI protein; in C the CI (2A1) antibody detects only the full-length form. (B) and (D) In cg^{07659} leg discs, CI protein is ubiquitously expressed, i.e., also in the posterior, but at much lower levels than in wt discs. The ectopic expression in the posterior is clearly revealed by the CI (A2A1) antibody. (E-G) The wing pouch in a cg^{07659} mutant disc showing a clear boundary between the EN-positive posterior (red) and EN-negative anterior. At the compartment boundary there is a slight upregulation of full-length CI protein (green) in the anterior compared with the level of expression in posterior. (H-J) cg^{07659} clones in a wing disc (identified by loss of the ubiquitously expressed marker β-gal, red) show autonomous reduction of CI expression (green) in the anterior and autonomous ectopic expression in the posterior. (K-M) cg^{07659} wing clones in the posterior have identical levels of EN (green) as surrounding wt cells. Clones in the anterior adjacent to the compartment border do not show EN expression (arrowhead). EN is normally expressed in this position in late third instar discs.

higher levels in the posterior than in most of the anterior. Clonal analysis was also used to demonstrate that the effect of *cg* on CI expression is cell autonomous. *cg* clones in the anterior showed autonomous reduction in *ci* levels whilst clones in the posterior showed autonomous gain of *ci* expression (Fig. 3H-J).

EN is required to repress *ci* in wild-type discs (Eaton and Kornberg, 1990), but posterior EN expression in *cg* mutant discs appeared normal (Fig. 3E-J). This was clearly demonstrated by clonal analysis as EN expression in *cg* mutant clones in the posterior was indistinguishable from that in surrounding wild-type cells (Fig. 3K-M). Thus, it appears that En is unable to repress *ci* expression normally in *cg* discs. Loss of *cg* had a minor effect on EN expression, but this was in the anterior compartment of the wing. Although EN is usually restricted to the posterior, in late third instars, *en* expression is activated in the anterior of the wing at the compartment boundary and this is HH-dependent (Blair, 1992; Strigini and Cohen, 1997). *cg* mutant cells immediately anterior to the compartment border (defined by clonal analysis) do not express EN in late third instar wing discs (Fig. 3K-M).

Loss of *ci* in the leg phenocopies HH gain-of-function

Loss of *ci* activity in the wing results in HH-independent *dpp* expression in the anterior compartment (Dominguez et al., 1996; Methot and Basler, 1999) so it appeared possible that the ectopic *dpp* and *wg* in the anterior of *cg* leg discs may result directly from the lowered CI levels found in these discs. Initially we investigated whether loss of *ci* in leg discs also resulted in a hedgehog gain-of-function phenotype as it does in the wing. This was achieved by analysis of hypomorphic *ci* loss-of-function allelic combinations ci^{n}/ci^{94} or ci^{n}/ci^{D}. These are pupal lethal with larvae having wing and leg discs that phenocopy ubiquitous *hh* expression (Fig. 4A,C,D). CI levels were dramatically reduced in these discs and the mutant phenotype was HH independent (Fig. 4B,F). *dpp* was misexpressed in the anterior wing pouch and in the dorsal anterior of the leg discs (Fig. 4C,D) and *wg*

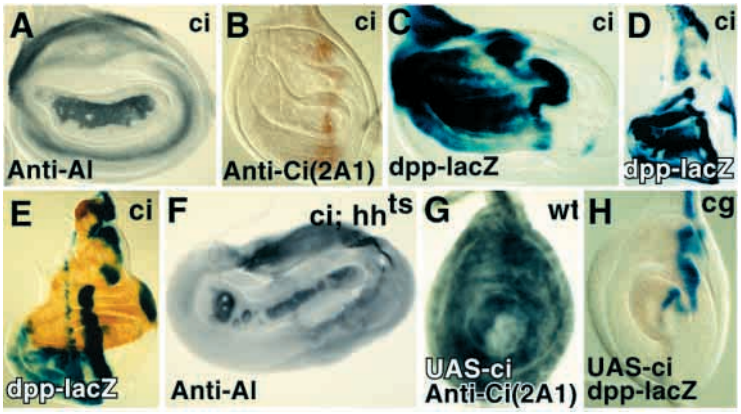

Fig. 4. Phenotype of CI hypomorphs and rescue of cubitus interruptus anterior phenotype by misexpression of CI. (A) ci^{n}/ci^{94} leg disc stained for *a1* expression showing a hedgehog gain-of-function phenotype identical to *cg* mutant leg discs. (B) CI protein levels are clearly reduced in these *ci* mutant discs. (C) *dpp* is misexpressed in the dorsal anterior of *ci* mutant leg discs and (D) in the wing pouch of *ci* mutant wing discs, which also show overgrowth. (E) This *dpp* misexpression is rescued by directly raising the *ci* levels, here with UAS-*ci* driven by *ap*-Gal4 (CI is brown) in the dorsal compartment of the wing. (F) *ci*; hh^{ts} mutant discs are indistinguishable from *ci* mutant discs, indicating the phenotype is HH independent. (G) Wild-type leg disc stained for CI expression in which *ci* is misexpressed using UAS-*ci* and the ubiquitously expressed C765 Gal4 line. (H) *cg* mutant leg discs in which *ci* levels have been raised with UAS-*ci*/C765: there is no overgrowth and no ectopic *dpp* expression.

was misexpressed in the ventral anterior of the legs (not shown). The ectopic *dpp* expression was repressed by raising *ci* levels with a wild-type UAS-*ci* transgene driven by a Gal4 line (Fig. 4E). Thus, normal *dpp* and *wg* expression requires high levels of CI in the anterior because this functions to repress their expression away from the compartment border.

Raising CI levels in *cg* mutant leg discs suppresses the hedgehog gain-of-function phenotype

To test whether the ectopic *dpp* and *wg* in *cg* leg discs is a direct result of the lowered *ci* levels in these discs, *ci* levels were raised in *cg* mutants with a ubiquitous Gal4 driver (Fig. 4G). The higher *ci* levels rescued the overgrowth and *dpp* misexpression phenotypes (Fig. 4H), indicating that these anterior compartment combgap phenotypes are the direct result of lowered *ci* levels and consequently that one of the functions of *cg* is to maintain high levels of *ci* expression in the anterior.

cg encodes a ubiquitously expressed, putative transcription factor

cg was cloned by plasmid rescue of the P-element in cg^{07659} and subsequent screening of genomic and cDNA libraries. This P-element is inserted in an intron in the 5′ UTR of a transcription unit that encodes for a protein consisting largely of ten C2H2 zinc fingers (and a defective eleventh finger, Fig. 5A-C). A genomic fragment, CG247, containing this unit can fully rescue the cg^{07659} mutant while the same fragment containing a small deletion removing part of this zinc-finger gene can not (Fig. 5A), indicating that it corresponds to *cg*. *cg* is expressed ubiquitously at uniform levels in the imaginal discs (Fig. 5E,F), consistent with it being required in both anterior and posterior compartments.

Generation of a putative null *cg* allele

The molecular analysis cannot confirm that cg^{07659} is a null allele so additional mutants were generated. Mobilization of the P-element in line cg^{07659} generated several deletions (see Materials and Methods for more details) that had identical phenotypes to cg^{07659} homozygotes. However, none of these deletions extended into the protein-coding region of the *cg* gene, so it was impossible to determine if they were complete loss-of-function alleles.

Consequently, an alternative approach was taken to generate point mutants, cg^{Y4} and cg^{A22}. cg^{Y4} was identified because en^{59} +/+ cg^{Y4} adults show a loss of vein IV (Fig. 6A). cg^{Y4} is associated with a substitution of a conserved amino acid in the third zinc finger (Fig. 5D) and appears to be a dominant negative mutant because, even though the vein IV phenotype over en^{59} (Fig. 6A) is more penetrant than cg^{07659} (100% compared with 25%), it has a weaker phenotype as a homozygote, showing no overgrowth in leg discs. In addition, cg^{Y4} homozygotes have a weak dominant phenotype, having

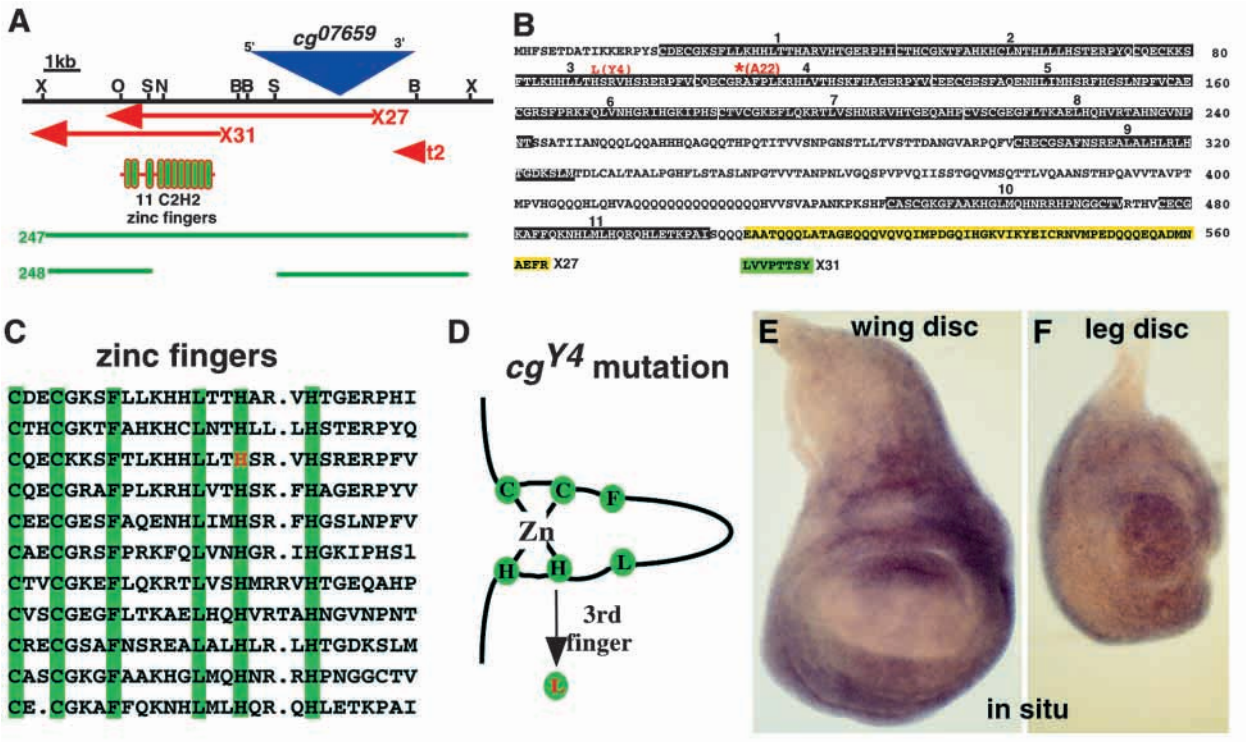

Fig. 5. Molecular biology of *cg*. (A) The *cg* genomic region showing the insertion site of the P-element in cg^{07659}, the location of *cg* cDNAs X27 and X31 (introns are not indicated) and an additional transcript T2. The 11.5kb fragment CG247 fully rescues *cg* mutants whilst the smaller fragment, CG248, lacking the 3.4kb *Stu* fragment, does not rescue. (B) *cg* encodes a protein containing ten C2H2 zinc fingers (and a defective 11th finger). The location of mutations in cg^{Y4} (H90L) and cg^{A22} (a stop after amino acid 106) are marked (L and asterisk, respectively). Although there is differential splicing at the cg locus, all of the cDNAs characterized encode proteins containing all of the zinc fingers. Alternative C-terminal regions found in X27 and X31 are indicated. (C) A line-up of the zinc fingers highlighting the invariant amino acids. The putative 11th finger has only one amino acid between the first two cysteines, suggesting that it would not be functional. (D) The mutation in cg^{Y4} substitutes one of the histidines required for binding the zinc in the third finger. (E) and (F) *cg* expression in leg and wing discs (in situ) is ubiquitous and uniform.

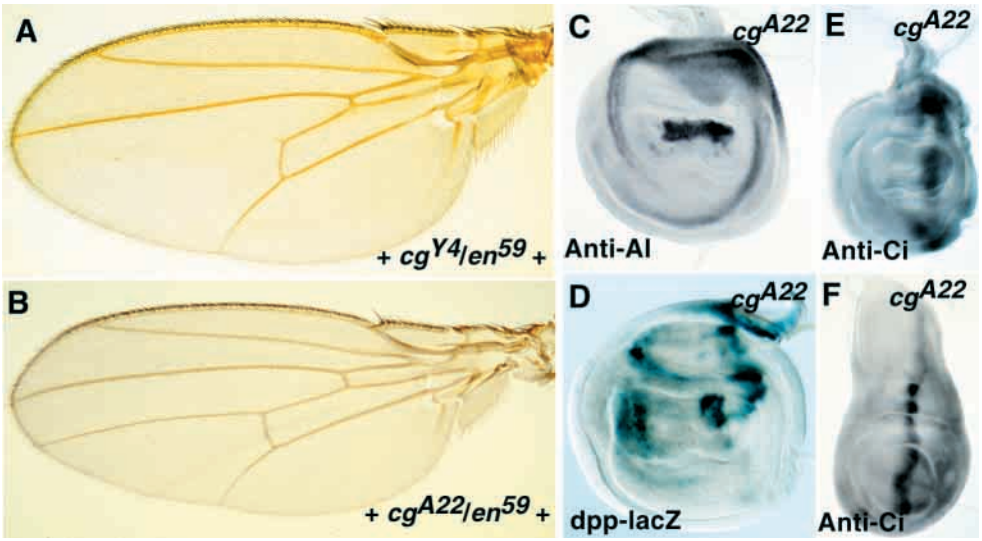

Fig. 6. Phenotype of cg^{A22} and cg^{Y4} mutants. (A,B) Adult wings from transheterozygotes with en^{59}. (A) + cg^{Y4}/en^{59} + flies have a fully penetrant loss of vein IV phenotype. (B) + cg^{A22}/en^{59} + flies only very rarely (>1%) show a gap in vein IV. (C-E) leg discs and (F) wing discs from cg^{A22} mutant larvae stained for *al* , *dpp* and *ci* expression. The phenotype of cg^{A22} is indistinguishable from cg^{07659}.

an increase in the number of sex comb teeth (11.9±0.93) compared with wild type (10.8±0.97), and it is not fully rescued by the CG247 genomic fragment. cg^{A22} was identified as a revertant of the dominant negative activity in cg^{Y4}. en^{59} +/+ cg^{A22} transheterozygotes are indistinguishable from en^{59}/+ heterozygotes with regard to vein IV (Fig. 6B). cg^{A22} is fully rescued by CG247 and has a stop codon between the third and fourth zinc fingers, suggesting that it may be a null (Fig. 5B), although it is impossible to rule out that the first three zinc fingers provide some function. cg^{A22} homozygotes have a phenotype almost identical to that of cg^{07659} (Fig. 6C-F), indicating that the latter is close to being a complete loss-of-function allele.

DISCUSSION

Mutations in the *cg* gene result in an anterior overgrowth phenotype in leg imaginal discs that is similar to that produced by ubiquitous expression of *hedgehog*. The *cg* mutant phenotype is HH independent. Although this phenotype is very similar to other mutants such as *ptc*, which encode for negative regulators of the HH signaling pathway, it differs in one main respect: viable *cg* mutants reveal that CG also functions in the posterior compartment because adult wings have defects in venation here. Loss or gain of HH signaling has no effect in the posterior where the outputs are normally repressed by EN/INV.

CG regulates *ci* expression

An understanding of the *cg* mutant phenotype, why it causes overgrowth in the anterior and defects in differentiation in the posterior, comes not from the HH signaling pathway itself but from analysis of the protein primarily regulated by this pathway: CI. The primary function of HH signaling is to regulate the transcriptional activity of CI: to antagonize its degradation to a repressor and to maintain it as an activator (Aza-Blanc and Kornberg, 1999). A genetic interaction between *cg* and some *ci* mutants was identified many years ago (House, 1953, 1961; Waddington, 1953). The posterior wing venation defect in *cg* hypomorphs is very similar to that found in these *ci* mutants and it was shown that this phenotype is enhanced in *cg*/+; *ci*/+ transheterozygotes. These *ci* mutants, however, are gain-of-function mutants; they show ectopic expression of *ci* in the posterior (Slusarski et al., 1995). In fact, direct misexpression of *ci* in the posterior using the UAS/Gal4 system can also produce the same vein defects as seen in these mutants and in *cg* mutants (Hepker et al., 1997). Analysis of *cg* mutant discs reveals ectopic *ci* expression in the posterior (Fig. 3D), indicating that the *cg* posterior phenotype is almost certainly the direct result of deregulation of *ci* expression in this compartment.

CI expression is also abnormal in the anterior of *cg* mutant discs, being found at much lower levels than in wild-type discs. Loss of *ci* expression in the wing results in hedgehog gain-of-function phenotypes, including overgrowth and misexpression of *dpp*. Reduced CI levels in the leg also result in the characteristic overgrowth phenotype, with ectopic expression of *wg* and *dpp*, found following ubiquitous expression of HH – i.e., the same phenotype as that found in *cg* mutant leg discs. Support for the proposal that the anterior combgap phenotype in the leg is also the direct result of deregulation of *ci* expression, in this case lowered levels of expression, comes from the observation that raising *ci* levels in *cg* mutant leg discs using the UAS/Gal4 system can suppress the overgrowth and ectopic *dpp* expression.

One difference between *ci* and *cg* mutants is that wing discs from the former have a hedgehog gain-of-function phenotype with overgrowth and ectopic *dpp* in the anterior (Dominguez et al., 1996), whilst the latter do not show overgrowth and only very weak ectopic *dpp*. It is possible that the leg and wing are differentially sensitive to CI levels and the CI levels are still high enough in the wing in *cg* mutants to repress most *dpp* expression. Protein levels detected with antibody staining in *ci* hypomorphs and *cg* mutants are too low to detect significant differences with confidence, so the reason for the difference between *ci* and *cg* wings remains to be determined. CI is also required during embryogenesis, but the putative null *cg* mutant survives to the early pupal stage. This suggests either that lower levels of CI are sufficient for embryonic but not larval

development or that *cg* RNA is maternally supplied. The first possibility is supported by the observation that hypomorphic *ci* mutants (Fig. 4) are not embryonic lethal and survive to the early pupal stage. However, in situ analysis reveals that *cg* RNA is maternally supplied so that the question of whether *cg* is required during embryogenesis will require the generation of germline clones.

Full-length CI acts as a transcriptional activator and there is evidence that the lowered levels of CI in *cg* mutants also compromises CI function as an activator. Although, *dpp* is misexpressed in *cg* discs, the level of expression, even at the compartment border, is lower than that found in wild-type discs. A similar phenomenon has been demonstrated for loss of *ci* in the wing and it appears that the high levels of *dpp* in wild-type discs require activation by CI-155, as well as the absence of CI-75 (Methot and Basler, 1999). Thus, the lower levels of *dpp* in *cg* discs are presumably due to lower levels of CI-155. Another gene directly activated by CI is *en* in late third instar wing discs. CI-dependent *en* activation in the anterior compartment (Blair, 1992; Strigini and Cohen, 1997) does not occur in *cg* mutant cells, again presumably because the level of the CI-155 activator form is too low.

The anterior/posterior (A/P) lineage restriction appears to be maintained by adhesive differences between A and P cells; this requires both CI in the anterior and EN in the posterior, and it has been proposed that CI activates and EN represses expression of an adhesion molecule that regulates cell mixing (Dahmann and Basler, 2000). In *cg* mutant discs, although CI levels across the compartment border are almost uniform, the lineage restriction is maintained, presumably because EN repression overcomes any activation of this adhesion molecule by the CI present in the posterior compartment.

Mechanism of CG regulation of *ci*

We have shown that CG is required to activate *ci* expression to its normal levels in the anterior compartment and to repress *ci* expression in the posterior. The CG protein contains multiple zinc fingers and is most probably a DNA-binding protein that would be expected to bind to elements at the ci locus. However, understanding the mechanism by which it regulates *ci* expression requires further studies. It is possible that CG functions as a standard transcription factor and activates *ci* transcription in the anterior and represses it in the posterior. If this is the case, its activity must be modified in either the anterior or posterior compartments. Analysis of the CG protein outside of the zinc fingers does not reveal any classical activator or repressor domains, but as these are often not well defined it is impossible to determine whether the protein has these activities without more-detailed studies.

An argument against such a direct involvement of CG in transcription is the well-documented role of EN in regulating *ci* expression. EN is a transcription factor that represses expression of several genes including *ci*, *dpp* and *wg*, and has been shown to bind to elements at the ci locus (Schwartz et al., 1995). It would appear likely that EN is the primary factor that represses transcription of *ci* in the posterior. If this is the case, the function of CG in regulating transcription may be indirect and may be to assist the binding of other transcription factors to the *ci* gene. If so, the misexpression of CI in the posterior of *cg* mutant discs would be due to a lowered ability of EN to bind in the absence of CG protein, while the lowered Ci levels in the anterior would be due to a lowered ability of an, as yet unidentified, transcriptional activator of *ci* to bind. There are several possible mechanisms by which CG might affect the binding of other factors. For example, there may be direct physical interactions between CG and these other factors. Alternatively, CG action could be more indirect, for example, it could modify chromatin structure at the ci locus producing a more open conformation. Further studies are required to test these possibilities.

Given the similarity between HH signaling and regulation of CI/Gli protein activity in *Drosophila* and vertebrates (Murone et al., 1999; Ruiz i Altaba, 1999), it will be important to determine whether the transcriptional activity of the *Gli* genes is also regulated by a CG homolog. Disruption of HH signaling or activity of Gli proteins can result in different human pathologies that include specific types of cancer or limb abnormalities (Ingham, 1998; Ruiz i Altaba, 1999), indicating that individuals with mutations in a human *cg* homolog may exhibit similar diseases.

For technical assistance we thank Dangu Li, Justin Campbell and Eric Hitchcock. We thank the following people for materials: Gary Struhl, Jin Jiang, Bob Holmgren, John Locke, Kathy Matthews, Kevin Cook and the Bloomington Stock Center, Tom Kornberg, Pat O'Farrell, Nikita Yakubovich, Carol Schwartz, and Teresa Orenic. We also thank Rich Carthew for comments on the manuscript.

REFERENCES

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M. and Hooper, J. E. (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* **86**, 221-232.

Alexandre, C., Jacinto, A. and Ingham, P. W. (1996). Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. *Genes Dev.* **10**, 2003-2013.

Aza-Blanc, P. and Kornberg, T. B. (1999). Ci: a complex transducer of the hedgehog signal. *Trends Genet.* **15**, 458-462.

Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B. (1997). Proteolysis that is inhibited by Hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. *Nature* **368**, 208-214.

Blair, S. S. (1992). Engrailed expression in the anterior lineage compartment of the developing wing blade of Drosophila. *Development* **115**, 21-33.

Blair, S. S. (1995). Compartments and appendage development in Drosophila. *BioEssays* **17**, 299-309.

Briscoe, J. and Ericson, J. (1999). The specification of neuronal identity by graded Sonic Hedgehog signalling. *Semin. Cell Dev. Biol.* **10**, 353-362.

Campbell, G. and Tomlinson, A. (1995). Initiation of the proximodistal axis in insect legs. *Development* **121**, 619-628.

Campbell, G. and Tomlinson, A. (1998). The roles of the homeobox genes aristaless and Distal-less in patterning the legs and wings of Drosophila. *Development* **125**, 4483-4493.

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. *EMBO J.* **13**, 4459-4468.

Chen, C. H., von Kessler, D. P., Park, W., Wang, B., Ma, Y. and Beachy, P. A. (1999a). Nuclear trafficking of Cubitus interruptus in the transcriptional regulation of Hedgehog target gene expression. *Cell* **98**, 305-316.

Chen, Y., Cardinaux, J. R., Goodman, R. H. and Smolik, S. M. (1999b). Mutants of cubitus interruptus that are independent of PKA regulation are independent of hedgehog signaling. *Development* **126**, 3607-3616.

Crick, F. H. C. and Lawrence, P. A. (1975). Compartments and polyclones in insect development. *Science* **189**, 340-347.

Dahmann, C. and Basler, K. (2000). Opposing transcriptional outputs of

Hedgehog signaling and engrailed control compartmental cell sorting at the Drosophila A/P boundary. *Cell* **100**, 411-422.

Diaz-Benjumea, F. J., Cohen, B. and Cohen, S. M. (1994). Cell interaction between compartments establishes the proximal-distal axis of Drosophila legs. *Nature* **372**, 175-179.

Dominguez, M., Brunner, M., Hafen, E. and Basler, K. (1996). Sending and receiving the hedgehog signal: control by the Drosophila Gli protein cubitus interruptus. *Science* **272**, 1621-1625.

Eaton, S. and Kornberg, T. B. (1990). Repression of ci-D in posterior compartments of Drosophila by engrailed. *Genes Dev.* **4**, 1068-1077.

Hammerschmidt, M., Brook, A. and McMahon, A. P. (1997). The world according to hedgehog. *Trends Genet.* **13**, 14-21.

Hepker, J., Wang, Q. T., Motzny, C. K., Holmgren, R. and Orenic, T. V. (1997). Drosophila cubitus interruptus forms a negative feedback loop with patched and regulates expression of Hedgehog target genes. *Development* **124**, 549-558.

House, V. L. (1953). Some observations on the interaction of comb-gap with Hairless, engrailed and alleles at the cubitus interruptus locus in Drosophila melanogaster. *Genetics* **38**, 669-670.

House, V. L. (1961). Mutant effects in multiple heterozygotes of recessive venation mutants in Drosophila melanogaster. *Genetics* **46**, 871.

Ingham, P. W. (1994). Pattern formation. Hedgehog points the way. *Curr. Biol.* **4**, 347-350.

Ingham, P. W. (1998). The patched gene in development and cancer. *Curr. Opin. Genet. Dev.* **8**, 88-94.

Ingham, P. W., Taylor, A. M. and Nakano, Y. (1991). Role of the Drosophila patched gene in positional signalling. *Nature* **353**, 184-187.

Kornberg, T. B., Siden, I., O'Farrell, P. and Simon, M. (1985). The engrailed locus of Drosophila: In situ localization of transcripts reveals compartment-specific expression. *Cell* **40**, 45-53.

Methot, N. and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* **96**, 819-831.

Mohler, J. (1988). Requirements for hedgehog, a segmental polarity gene, in patterning larval and adult cuticle of Drosophila. *Genetics* **120**, 1061-1072.

Murone, M., Rosenthal, A. and de Sauvage, F. J. (1999). Hedgehog signal transduction: from flies to vertebrates. *Exp. Cell Res.* **253**, 25-33.

Orenic, T. V., Slusarski, D. C., Kroll, K. L. and Holmgren, R. A. (1990). Cloning and characterization of the segment polarity gene cubitus interruptus Dominant of Drosophila. *Genes Dev.* **4**, 1053-1067.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

Ruiz i Altaba, A. (1999). Gli proteins and Hedgehog signalling. *Trends Genet.* **15**, 418-425.

Sanicola, M., Sekelsky, J. J., Elson, S. and Gelbart, W. M. (1995). Drawing a stripe in Drosophila imaginal disks: negative regulation of decapentaplegic and patched expression by engrailed. *Genetics* **139**, 745-756.

Schwartz, C., Locke, J., Nishida, C. and Kornberg, T. B. (1995). Analysis of cubitus interruptus regulation in Drosophila embryos and imaginal disks. *Development* **121**, 1625-1635.

Slusarski, D. C., Motzny, C. K. and Holmgren, R. (1995). Mutations that alter the timing and pattern of cubitus interruptus gene expression in Drosophila melanogaster. *Genetics* **139**, 229-240.

Spradling, A. C., Stern, D., Beaton, A., Rhem, E. J., Laverty, T., Mozden, N., Misra, S. and Rubin, G. M. (1999). The Berkeley Drosophila genome project gene disruption project. Single P-element insertions mutating 25% of vital Drosophila genes. *Genetics* **153**, 135-177.

Strigini, M. and Cohen, S. M. (1997). A Hedgehog activity gradient contributes to AP axial patterning of the Drosophila wing. *Development* **124**, 4697-4705.

Tabata, T., Schwartz, C., Gustavson, E., Ali, Z. and Kornberg, T. B. (1995). Creating a Drosophila wing de novo, the role of engrailed, and the compartment border hypothesis. *Development* **121**, 3359-3369.

van den Heuvel, M. and Ingham, P. W. (1996). smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature* **382**, 547-551.

Waddington, C. H. (1953). The interactions of some morphogenetic genes in Drosophila melanogaster. *J. Genet.* **51**, 243-258.

Wang, G., Wang, B. and Jiang, J. (1999). Protein kinase A antagonizes Hedgehog signaling by regulating both the activator and repressor forms of cubitus interruptus. *Genes Dev.* **13**, 2828-2837.

Zecca, M., Basler, K. and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. *Development* **121**, 2265-2278.