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

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Members of the Hedgehog (Hh) family of secreted proteins have been identified recently as key signaling molecules that regulate a variety of inductive interactions central to the development of both *Drosophila* and vertebrates. Despite their widespread importance, the way in which Hh signals are transduced inside the cell remains poorly understood. The best candidate for a transcription factor that mediates Hh signaling in *Drosophila* is the product of the *cubitus interruptus* (*ci*) gene, a zinc finger protein that exhibits significant homology to protein products of the vertebrate GLI gene family. Here, we show that elevated levels of Ci are sufficient to activate *patched* (*ptc*) and other *hh* target genes, even in the absence of *hh* activity. We also show that Ci can function as a transcriptional activator in yeast and demonstrate that the zinc finger domain of the protein is sufficient for its target specificity. Finally, we identify sequences in the promoter region of the *ptc* gene, a primary target of Hh signaling, that are identical to the consensus-binding sequence of the GLI protein and are required for reporter gene expression in response to Hh activity. Taken together, our results strongly support the role for Ci as the transcriptional activator that mediates *hh* signaling.

[Key Words: *hedgehog* signaling; *patched* promoter; GLI family; *cubitus interruptus*; *decapentaplegic*; *wingless*]

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Molecular mechanisms that control pattern formation in many developmental systems have been shown to involve key signaling molecules such as members of the Hedgehog family of secreted proteins. Of particular importance in vertebrates is the Sonic hedgehog (Shh) protein, the activity of which has been implicated in the patterning of the developing limb (Riddle et al. 1993), the neural tube and somites (Echelard et al. 1993; Krauss et al. 1993; Johnson et al. 1994; Roelink et al. 1994, 1995; Fan et al. 1995), as well as in gut morphogenesis (Roberts et al. 1995) and left-right asymmetry (Levin et al. 1995). In addition, other members of the family play important roles in chondrocyte differentiation (Vortkamp et al. 1996), spermatogenesis (Bitgood et al. 1996), and muscle cell specification (Currie and Ingham 1996).

In *Drosophila*, *hedgehog* (*hh*) was first identified as a member of the segment polarity class of genes (Nüsslein-Volhard and Wieschaus 1980), which function collectively to organize the patterning of both the embryonic segments and imaginal discs. Various lines of evidence suggest that the role of *hh* in this process is primarily to regulate the expression of other signal encoding genes. In the embryo, the principal target of *hh* activity is another segment polarity gene *wingless* (*wg*), which like *hh* encodes a secreted protein (Rijsewijk et al. 1987; van den Heuvel et al. 1989). Transcription of *wg* is maintained just anterior to each parasegment boundary by the activity of *hh* in neighboring cells (Hidalgo and Ingham 1990; Ingham 1993), thus ensuring a discrete source of the Wg organizing signal in each segmental field. In the imaginal discs, *hh* performs a similar role, establishing sources of the organizing activities encoded by *wg* (Couso et al. 1993; Struhl and Basler 1993) and *decapentaplegic* (*dpp*) (Nellen et al. 1996) by promoting the transcription of both genes along the anteroposterior compartment boundary (Basler and Struhl 1994; Capdev-

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ila and Guerrero 1994; Tabata and Kornberg 1994; Ingham and Fietz 1995).

Although the patterning activities of Hh molecules are well known, their signal transduction pathways remain unclear. Most of what is known derives from genetic analysis in *Drosophila*, where a number of genes belonging to the segment polarity class have been implicated in *hh* signal transduction (Ingham et al. 1991; Forbes et al. 1993; Ingham 1993; Motzny and Holmgren 1995). Among these genes is *cubitus interruptus* (*ci*), loss-of-function mutations of which are embryonic lethal and have phenotypes similar to those of *hh* (Orenic et al. 1987). Transcription of *wg* disappears from early stage 10 onward in *ci* mutant embryos (Hidalgo 1991; Forbes et al. 1993; Hooper 1994; Slusarski et al. 1995), even in the presence of high levels of ubiquitous *hh* expression (M. Fietz and P. Ingham, unpubl.), strongly suggesting that *ci* is required for the *hh*-dependent regulation of *wg* transcription. Mutations of *ci* also have effects on the patterning of imaginal structures, the gene name deriving from the interrupted fifth or cubitus vein of the wing caused by certain dominant gain-of-function alleles of the locus (Stern and Kodani 1955). Although the basis of this phenotype is unclear, phenotypes associated with a reduction of *ci* activity are similar to those caused by mutations of *fused* (Fausto-Sterling 1978; Slusarski et al. 1995), another segment polarity gene implicated in *hh* signaling (Forbes et al. 1993; Ingham 1993; Thérand et al. 1996).

The *ci* gene encodes a protein with zinc fingers, highly homologous to protein products of the vertebrate GLI gene family and the *Caenorhabditis elegans* sex determination gene *tra-1* (Orenic et al. 1990; Zarkower and Hodgkin 1992). The GLI and *tra-1* proteins bind DNA in a sequence-specific manner (Kinzler and Vogelstein 1990; Zarkower and Hodgkin 1993) and the crystal structure of the GLI-DNA-binding complex has been solved (Pavletich and Pabo 1993). Thus, the Ci protein seems to be a good candidate for the transcription factor that mediates the activation of gene expression in response to *hh* signaling. The distribution of Ci protein in both embryos and imaginal discs is consistent with such a role, being expressed in a pattern complementary to that of *hh* transcription (Johnson et al. 1995; Motzny and Holmgren 1995). Thus, Ci is present in cells that respond to Hh by transcribing *wg* or *dpp*, as well as in cells that normally express neither gene but are capable of responding to ectopic *hh* activity (Ingham 1993; Basler and Struhl 1994). This distribution suggests that the protein is present in most cells in an inactive form, its activation occurring in direct response to cells receiving the Hh signal. In line with this, there is a significant increase in levels of Ci protein, but not *ci* transcript, in cells responding to Hh (Motzny and Holmgren 1995) or in cells in which the Hh pathway is activated constitutively through the removal of protein kinase A activity (Johnson et al. 1995), indicating that *hh* regulates *ci* expression post-transcriptionally. It is, however, notable that the vast majority of Ci protein is localized in the cytoplasm irrespective of whether cells are responding to

Hh (Motzny and Holmgren 1995). Thus, Hh does not seem to direct nuclear uptake of Ci but instead seems to act by increasing the overall levels of Ci within the cell.

In this study we have investigated the relationship between Ci and Hh signaling by using the GAL4 UAS (upstream activating sequences) system (Brand and Perrimon 1993) to direct high-level ectopic expression of the protein both in the embryo and in imaginal discs. We find that elevated levels of Ci are sufficient to activate *hh* target genes, even in the absence of *hh* activity. We also show that Ci activates transcription in yeast by a GLI consensus-binding site and demonstrate that the zinc finger domain is sufficient for its target specificity. Finally, we identify several consensus-binding sites of the GLI protein in the *patched* (*ptc*) promoter region, a primary target of Hh signaling, and demonstrate that these sequences are required for activation of *ptc* transcription in response to Hh activity. Our results strongly support a role for Ci as the transcriptional activator that mediates *hh* signaling and provide the first direct evidence that *ci* performs this function in the imaginal discs as well as in the embryo.

Results

Misexpression of Ci activates wg and ptc transcription in the embryo

During normal embryogenesis the activity of *hh* in the *engrailed* (*en*) expressing cells at the anterior boundary of each parasegment maintains the transcription of the *wg* gene in cells immediately anterior and adjacent to those expressing *en* (Fig. 1A,B; Fig. 2A,B) and of *ptc* in two stripes of cells flanking each *en* domain (Fig. 1G,H). Expression of both genes similarly depends on *ci* activity, suggesting that *ci* mediates *hh* activity (Forbes et al. 1993). To determine whether *ci* activity is sufficient to activate transcription of *wg* and *ptc*, we used the GAL4 system to misexpress *ci* in cells where the gene is not normally transcribed. Flies carrying the *ci* coding region cloned downstream of yeast upstream activating sequences (UAS*ci*) were crossed to *en*GAL4 flies (see Fietz et al. 1995). Embryos derived from this cross die at the end of embryogenesis and express high levels of Ci protein within the *en* domains (data not shown). The expression domains of both *wg* and *ptc* in such embryos are significantly broader than in their wild-type sibs, suggesting that both genes are activated by the ectopically expressed Ci protein (Fig. 1C,D and I,J). To confirm this, the broadened expression domains were related to the *en* domains using double labeling techniques. In both cases, the broadening can be seen to be attributable to the ectopic activation of either gene in the *en*-expressing cells (Fig. 2C–F). Thus, expression of *ci* is sufficient to activate *wg* and *ptc* transcription.

We then asked whether activation of *wg* and *ptc* transcription depends on the levels of Ci protein within a cell. To do this we used the *ptc*GAL4 line (Speicher et al. 1994) to express Ci at high levels throughout the normal *ci* expression domain. Such high-level expression results in the ectopic activation of both *wg* and *ptc* (Fig. 1E,F and

Figure 1. Ectopic transcription of *wg* and *ptc* induced by misexpression of *Ci* during embryogenesis. (Right) lateral view; (left) ventral view. Distribution of *wg* transcripts in wild type (A,B), *enGAL4/+; UASci/+* (C,D) and *ptcGAL4/+; UASci/+* (E,F) embryos at stage 11. Distribution of *ptc* transcripts in wild type (G,H), *enGAL4/+; UASci/+* (I,J) and *ptcGAL4/+; UASci/+* (K,L) embryos at stage 11. In each case, ectopic *Ci* activity results in the broadening of both *wg* and *ptc* expression domains (C–F) and (I–L); note, however, the restricted expansion of *wg* compared to that of *ptc*.

(K,L) in a manner similar to that caused by ubiquitous expression of *hh* (Ingham 1993; Tabata and Kornberg 1994). Thus, *ptc* is expressed at uniformly high levels between each *en* domain, whereas *wg* transcription expands anteriorly to occupy most or all of the cells competent to express *wg* (Ingham et al. 1991), as defined by the expression of *sloppy paired* (*slp*) (Grossniklaus et al. 1992; Cadigan et al. 1994).

High-level expression of Ci is sufficient to activate wg transcription in the absence of hh activity

Absence of *hh* activity results in the loss of *wg* transcription and concomitantly in the loss of anteroposterior polarity in the larval segments, the posterior naked cuticle being eliminated from every segment. To determine

whether high-level expression of *ci* is sufficient to activate *wg* transcription even in the absence of *hh* activity, we used a *hairyGAL4* line (Brand and Perrimon 1993) to misexpress *ci* in *hh* mutant embryos (see Materials and methods) (Fig. 3). Because *hairy* (*h*) is expressed only in alternating parasegments (Ingham et al. 1985), expression of *ci* under *h* control should rescue only alternate stripes of *wg* expression. Consistent with this, the cuticles of *UASci; hGAL4 hh^{II}* embryos exhibit a pair-rule phenotype, where every other segment now shows naked cuticle, normally indicating *Wg* activity (Fig. 3B). In situ hybridization reveals that the even-numbered stripes of *wg* expression are indeed present in such embryos; in addition, the odd-numbered stripes 9, 11, and 13 are also usually rescued and the more posterior even-numbered stripes are expanded (Fig. 3D). We interpret these latter effects as being attributable to the temporal difference in the resolution of the *h* pattern along the anteroposterior axis, the more posterior stripes extending over a width greater than one segment and hence activating *wg* in adjacent parasegments.

Figure 2. Transcription patterns of *en* (dark brown), *wg* (red) (A–D), and *ptc* (red) (E,F) in embryos misexpressing *Ci*. (Right) ventral view; (left) lateral view. (A,B) In wild-type embryos, *wg*-expressing cells are immediately anterior and adjacent to the *en* domain. In embryos misexpressing *Ci* in the *en* domain, transcription of *wg* (C,D) and *ptc* (E,F) is strongly activated by *Ci* in *en*-expressing cells.

Figure 3. Rescue of *hh* mutant embryos by misexpression of *ci*. (A) Ventral cuticle pattern of a pharate larva homozygous for *hh*; the absence of naked posterior cuticle is presaged by the loss of *wg* expression in the stage 10 embryo (C). (B) Ventral cuticle pattern of a *hh* homozygote expressing Ci under the control of the *hairy* enhancer elements. Note the rescue of posterior naked cuticle in alternate segments and the restoration of *wg* expression in alternate parasegments (D).

High-level misexpression of *ci* activates *hh* targets in imaginal discs

As in the embryo, *ci* is expressed in imaginal discs in a pattern reciprocal to that of *hh*. Thus, in the wing disc, Ci protein is restricted to the anterior compartment with elevated levels accumulating just anterior to the compartment boundary (Johnson et al. 1995; Motzny and Holmgren 1995), a region where transcription of *ptc* and *dpp* is activated in response to *hh* signaling (Basler and Struhl 1994; Capdevila and Guerrero 1994; Ingham and Fietz 1995). To investigate whether high levels of Ci can activate transcription of these *hh* target genes in the disc, we used the GAL4 line 30A (Brand and Perrimon 1993) to drive expression of Ci in a ring around the presumptive wing blade (Fig. 4). Such 30A;UAS*ci* animals die during late larval or pupal stages; pharate adults exhibit outgrowths from the proximal wing region similar to those caused by ectopic *hh* or *dpp* expression driven by the same GAL4 line (data not shown) (Capdevila and Guerrero 1994; Ingham and Fietz 1995). The effects of ectopic Ci (Fig. 4A,D) on *dpp* and *ptc* transcription were assayed using the *dpp-lacZ* reporter gene construct (Blackman et al. 1991) and the *ptc-lacZ* enhancer trap line H84. In the third larval instar disc, expression of *dpp-lacZ* is activated ectopically in all cells expressing high levels of Ci in the anterior compartment, but is not activated in the posterior compartment (Fig. 4B,C). Expression of the *ptc-lacZ* enhancer trap, in contrast, is activated ectopically throughout the 30A expression domain in both compartments (Fig. 4E,F). Thus, as in the embryo, high levels of Ci protein are sufficient to activate transcription of *ptc*, even in the presence of En; however, ectopic Ci activity apparently cannot overcome the repression of *dpp* transcription by En (Sanicola et al. 1995).

Functional domains of Ci protein

The Ci protein contains at least three distinct domains: (1) an amino-terminal region rich in alanine, a character-

istic of repression domains in some *Drosophila* transcriptional repressors, [e.g., Engrailed (En) (Han and Manley 1993) and Krüppel (Kr) (Licht et al. 1990)]; (2) a zinc finger domain that shows a high degree of homology with the DNA-binding zinc finger domain of the vertebrate GLI proteins (84–93%) (Kinzler and Vogelstein 1990; Ruppert et al. 1990); and (3) a carboxy-terminal domain that is highly acidic, a property typical of activation domains in transcription factors (Mitchell and Tjian 1989).

To determine which regions of the Ci protein are necessary for the regulation of *hh* target gene transcription by Ci, we generated a number of constructs in which these different domains are deleted or replaced by heterologous domains (Fig. 5). The modified constructs were cloned downstream of UAS, transformed into flies, and assayed for their ability to activate *ptc* and *dpp* transcription in imaginal discs. Deletion of the amino-terminal alanine-rich domain has no effect on the ability of the protein to activate either *ptc* or *dpp* transcription (Fig. 6B,E). In contrast, deletion of the carboxy-terminal acidic domain abolishes *ptc* and *dpp* transcriptional activation (data not shown). We then generated a construct in which most sequences both amino-terminal and carboxy-terminal to the zinc finger domain are deleted and the remaining region fused to the herpes simplex virus (HSV)VP16 transcriptional activation domain (see Fig. 5) (Triezenberg et al. 1988). This construct, ZFCiVP16, retains the ability to activate both *ptc* and *dpp* transcrip-

Figure 4. Ectopic induction of *dpp*- and *ptc*- reporter genes by misexpression of Ci in wing imaginal discs. Anterior is to the left in all cases. (A,D) Ectopic expression of Ci (green) in a ring around the presumptive wing blade driven by the GAL4 line 30A. The levels of ectopic protein are much higher than those generated in the anterior compartment by the endogenous gene. Ectopic expression of *dpp* (red) is only present in the anterior compartment (B); whereas the ectopic expression of *ptc* (red) overlaps the misexpressed Ci protein in both compartments (E). (C,F) Superimposition of the individual stainings showing a precise coincidence between Ci and the reporter genes.

Figure 5. Schematic representation of Ci deletion and fusion constructs containing the transcriptional repression domain from En (amino acids 228–282) or the transcriptional activation domain from (HSV)VP16 (amino acids 418–490). The putative Ci repressor domain (RD) is localized 5' to the zinc finger region, whereas the putative activation domain (AD) is localized at the carboxyl terminus of the Ci molecule.

tion in the wing disc, although the induction is not as robust as that achieved with the full-length protein or the amino-terminal deletion (Fig. 6C,F); this may indicate that regions outside the zinc finger domain contribute to the stability or activity of the protein.

Finally, we generated a construct in which the putative activation domain is replaced with the repression domain of the En protein (see Fig. 5) (Han and Manley 1993; Badiani et al. 1994). Expression of this construct in the presumptive wing pouch fails to activate *dpp* transcription; instead it leads to the repression of the normal

Figure 6. Functional domains of the Ci protein. Normal expression of *ptc-lacZ* (A) and *dpp-lacZ* (D) in the wing imaginal disc. Ectopic expression of *ptc-lacZ* (B) and *dpp-lacZ* (E) where a truncated form of Ci (CiΔ5') (see Fig. 5) is expressed ectopically using the 30A GAL4 line. The zinc finger of Ci fused to the activation domain of VP16 (ZFCiVP16) gives similar effects (C,F).

transcription of *dpp* (Fig. 7D) and *ptc* (data not shown) along the anteroposterior compartment boundary, as would be expected if the domain substitution has converted the protein from a transcriptional activator to a repressor. Interestingly, the wings differentiated by such animals are characterized by a fusion between vein L3 and L4 (Fig. 7C); similar effects are induced by the overexpression of Ptc, which suppresses the *hh* signaling pathway, using the same GAL4 driver line (Fig. 7E,F) (Johnson et al. 1995).

Ci can activate transcription in yeast

The functional dissection of the Ci protein described above strongly suggests but does not prove that Ci acts as a direct regulator of transcription. To demonstrate this unequivocally we generated a yeast strain containing a *HIS3* reporter gene under the control of one GLI consensus-binding site (Kinzler and Vogelstein 1990) and tested the ability of Ci to activate transcription from this construct in a heterologous system, *Saccharomyces cerevisiae*. Yeast cells were cotransformed with plasmids containing the *HIS3* reporter gene and the *ci* coding region cloned downstream of the galactose-inducible promoter. Control and cotransformed cells were tested for their ability to grow on yeast medium lacking histidine in the presence or absence of galactose; only cells carrying both plasmids grew on the selective medium and then only in

Figure 7. Repression of *dpp* transcription by a repressor form of Ci (CiΔEn). Wild-type *Drosophila* wing showing the normal distance between L3 and L4 veins (A) and *dpp-lacZ* expression in the wing imaginal disc (B). Wing patterning defects (C,E) and repression of *dpp* transcription in the wing imaginal disc (D,F) caused by the overexpression of CiΔEn and by wild-type Ptc protein, respectively, using the 71B GAL4 enhancer line. Note the fusion of vein L3 and L4 and the reduction in wing blade size.

the presence of galactose (Fig. 8), indicating that the Ci protein can induce transcription from the synthetic promoter containing the GLI-binding site. Moreover, this activation is specific to the GLI-binding site as Ci is unable to activate transcription from the same *HIS3* reporter gene under the control of the yeast transcription factor MCM1-binding site (*STE3* UAS; Bender and Sprague 1987).

Sequences upstream of *ptc* that include GLI-binding sites drive transcription in response to Hh activity

Previous studies have shown that sequences from –2000 to +500 of the *ptc* transcription unit are sufficient to drive reporter gene expression in cells adjacent to the *hh* domain in both embryos and imaginal discs, indicating that they contain elements responsive to the *hh* signal (Forbes 1992; Forbes et al. 1993). Sequence analysis of this fragment reveals the existence of a cluster of three GLI consensus-binding sites centered at position –684 relative to the transcription start site (Fig. 9). Deletion of sequences distal to these binding sites (FE construct; Fig. 9) leaves the compartment boundary-specific expression of reporter gene intact (Fig. 10A). Moreover, expression of this reporter construct can be induced ectopically by *shh* activity driven by the GAL4 line 30A (Fig. 10C), indicating that FE still retains the *hh* responsive elements. Deletion of a further 156 bp, however, which includes all three GLI consensus sites (GE construct; Fig. 9), abolishes both the robust compartment boundary-specific expression and the *shh* inducibility (Fig. 10B,D). Therefore, we conclude that sequences

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GAAAGCTCCA TTGGGCAGCG CTGCTGACGC TGAAGGCCGA CATCGGCAGA -764
GCTGAACGTT TGGGTAGGG ACCACCCACA TCGCTTGCGG GTTTCAGTTT -714
AATGAAGGCA GAAACAAATT TATTTTGGG TGGTCACAC TGGAGCGAAA -664
ATAAATACATA GTGGCAACAA CAAACCAGCA GCCAAGGCAC TTGGGTGGT -614
CTATGCAGAAA AAAAAACAAA TTACGGCATG CGAATAACAA TAGAAATTAG -564
CGCTCTCGTG GCGGAGCTAT TTGGGTATAT TAGAGCTACA TATTTTATTT -514
GTTTATAAAA AGTATAAATG TAAACAATGA GTTCCAAGCA TTAAGTCCGT -464
ATGCTCAACA ATTACATTAT CATTATTATT ATCACTTAAA TATTTACAAA -414
GGATATTTAA ACAGTAATAG ATATATATTT TATTTCTTAA TTTCTGTTAA -364
CATATGTATT TACATTGGTA GTTATTCTTT ATGTTGCAAC AAGCATTCAT -314
AAATTTTATA TAACAAACTT GGTATTTTCT CGGAAAACT CCTGAATCAC -264
CCCTCGGTAT TTTGTGCGTT GAGCTATCGT TAAAGCAGCC CTCGCAGAGA -214
GCGTTCTCAA ACCAAAATGG CCGCACACGA AACAAGAGAG CGAGTGAGAG -164
TAGGGAGAGC GTCTGTGTTG TGTGTTGAGT GTCGCCACG CACACAGGCG -114
CAAAACAGTG CACACAGACG CCCGCTGGGC AAGAGAGAGT GAGAGAGAGA -64
AACAGCGGCG CGCGCTCGCC TAATGAAGTT GTTGGCTTGG CTGGCGTGCC -14
GCATCCACGA GATACAGATA CATCTCTCAG ACTGCGTGCG ATCCTCGAAC +37
GAAACGGTTG TAAGTGCAGA GCGCGACGAC TTGTTATTTCG TATTTCCGAC +87
TACTGGCACT CTCTGTGTGT GGTATACTAA CAAGATAGAT ATCAGACAAC +137
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Figure 9. Sequence of the *ptc* promoter. The predicted transcription start site is indicated (angled arrow). The three GLI consensus-binding sites are indicated by boxes. The two different fragments cloned upstream of the *lacZ* reporter construct are indicated in solid arrowheads (FE construct) or solid circles (GE construct).

within this deleted 156-bp fragment (including the three GLI consensus-binding sites) are necessary for *hh*-dependent transcriptional activation.

Discussion

Signaling by *hh* plays a central role in segmental patterning both in the embryo and in the imaginal discs of *Drosophila*, acting to regulate the transcription of other signal encoding genes, *wg* or *dpp*, or both, at the antero-posterior compartment boundary. Genetic epistasis analyses have implicated a number of other segment polarity genes in the *hh* signaling pathway (for review, see Ingham 1995; Perrimon 1995), but among these only one, *ci*, stands out as a possible candidate for the factor that activates directly transcription of target genes in response to *hh* signaling. The spatial regulation of *ci* expression is consistent with such a role, both transcript and protein being distributed in a pattern reciprocal to that of Hh in the embryonic segment and the imaginal discs (Guillen et al. 1995; Johnson et al. 1995; Schwartz et al. 1995). Thus, Ci protein accumulates throughout the anterior compartment such that it is present both in cells that transcribe actively *hh* target genes as well as in those that do not express these genes but have the potential to do so in response to ectopic *hh* activity (Ingham 1993; Basler and Struhl 1994). These observations suggest that Ci is inactive in the majority of cells in which the protein is expressed, its activity being induced only in response to reception of the Hh signal. One way in which

Figure 8. Ci activates transcription specifically through a GLI consensus-binding site in an heterologous system (*S. cerevisiae*). (A) Panel showing the three different cotransformations: The full-length Ci and the zinc finger region fused to VP16 activation domain are under the control of the galactose-inducible promoter. Note the activation of the *HIS3* reporter gene by these two different Ci constructs when they are cotransformed with the reporter gene under the control of one GLI consensus-binding site and streaked on His[–], 2% galactose plate (C), but no growth is detected in the presence of 2% glucose (B). A cotransformation of the *HIS3* reporter gene under the control of the MCM1-binding site and the Ci expression vector does not permit growth on His[–], 2% galactose plate (C).

Figure 10. A /58-bp minimal *ptc* promoter region responds to Shh activity. *Ptc-lacZ* expression of FE construct containing the three GLI consensus-binding sites (A) and GE deleted from these three sites (B). Note the absence of the compartment boundary when the GLI-binding sites are deleted. (C) Ectopic expression of *ptc-lacZ* (FE construct) following the overexpression of Shh using the 30A GAL4 enhancer line. (D) In the absence of the GLI-binding sites, the ectopic expression of *ptc-lacZ* is abolished.

this control might be achieved is by the modulation of protein translation or stability, a possibility suggested by the finding that the levels of Ci protein are significantly elevated in cells that are actively responding to Hh (Motzny and Holmgren 1995). Moreover, similarly elevated levels of Ci are also induced in clones of cells that lack protein kinase A (PKA) activity (Johnson et al. 1995), which itself acts to antagonize the Hh signal response (Jiang and Struhl 1995; Lepage et al. 1995; Li et al. 1995; Pan and Rubin 1995). A possible implication of this latter observation is that PKA acts by modifying the Ci protein (either directly or indirectly), causing its inactivation and concomitantly targeting it for degradation. According to this interpretation the increased levels of the protein in Hh responding cells might be a secondary consequence of the reversal of this modification, leading to increased protein stability that would not in itself be sufficient for the activity of Ci. Although our experiments do not rule out this possibility, they tend to argue against it. Using the GAL4 UAS system we were able to increase artificially the levels of Ci protein in cells that are not actively responding to Hh activity. In these experiments, the activity of PKA is unaffected, therefore the exogenous Ci protein should be subject to any PKA-mediated modification experienced by its endogenous counterpart. Nevertheless, such elevated levels of Ci were sufficient to activate all of the targets of Hh signaling tested, suggesting that it is the level of Ci

protein that is the principal determinant of its activity. We cannot exclude the possibility, however, that the unusually high levels of Ci induced in these experiments titrate out the activity of PKA or other negative regulators of Ci activity.

An interesting and unexpected aspect of our findings is the differential response of the different Hh target genes to this ectopic Ci activity. Using the GAL4 line 30A to drive expression around the entire wing blade primordium, we find that Ci activity is sufficient to activate *ptc* transcription in both anterior and posterior compartments, whereas activation of *dpp* is restricted precisely to the anterior compartment. This suggests a fundamental difference in the way in which these two genes are normally regulated; previous studies have shown that *dpp* is under direct negative regulation by En (Sanicola et al. 1995), and our results indicate that Ci activity is unable to overcome this repression. In contrast, the repression of *ptc* transcription by *en* activity, appears to be an indirect consequence of the repression of *ci* transcription by *en* (Eaton and Kornberg 1990). Normally this results in the absence of Ci protein from the posterior compartment, but it is clear from our experiments that provision of Ci protein is sufficient to activate *ptc* transcription in these cells irrespective of the presence of En protein. It follows that the repression of *ci* transcription by *en* could provide the basis for limiting the competence of cells to respond to *hh* signaling to the anterior compartment. A similar situation is revealed in the embryo using the *en*-GAL4 line to drive ectopic *ci* expression in the posterior "compartment" of each segment. Here, such expression results in the activation of *ptc* transcription within *en*-expressing cells. Significantly, such expression also results in the activation of *wg* transcription; thus, unlike *dpp*, its signaling counterpart in the wing disc *wg* appears not to be under the direct control of *en*. Paradoxically, although *wg* transcription can be induced in the posterior compartment by ectopic Ci activity, its induction in the anterior compartment by the same protein is restricted to the so-called *wg* competent cells. These cells are defined by the expression of the Sloppy paired proteins, transcription factors whose activity is essential for normal *wg* expression (Cadigan et al. 1994). Neither Slp protein is expressed in the posterior compartment, however, yet Ci activity appears to be sufficient to activate *wg* transcription in these cells. This apparent difference in the requirement for *slp* activity remains to be explained.

Although the correlation between the levels of Ci protein and the activation of Hh target genes is strong, the precise manner in which Ci might effect such transcriptional activation has remained a contentious issue (Johnson et al. 1995; Motzny and Holmgren 1995). Although Ci shows strong sequence homology to members of the GLI family of vertebrate transcription factors (Orenic et al. 1990), the protein is barely detectable in the nuclei of either embryonic or imaginal disc cells (Motzny and Holmgren 1995). To address this paradox, we have taken a functional approach, seeking to define the minimal regions of both the Ci protein and the *cis*-

acting regulatory regions of the *hh* target gene *ptc*, sufficient to mediate its transcriptional activation. The finding that the zinc finger domain of Ci alone, when fused to the herpes simplex VP16 activation domain, can activate transcription of both *ptc* and *dpp* in imaginal discs, indicates that the specificity of Ci activity is determined by its putative DNA-binding domain. This result, taken with the effects of replacing the putative activation domain of the protein with the En repressor domain, strongly suggests that Ci acts as a transcription factor. Conclusive evidence for this comes from the demonstration that the intact Ci protein can activate transcription in yeast from a synthetic promoter containing one consensus-binding site for the vertebrate GLI proteins. These findings, however, cannot distinguish between Ci regulating the *hh* target genes directly or regulating the expression of some other transcription factor that itself controls transcription of *ptc*, *wg*, and *dpp*. Compelling evidence in favor of the former possibility comes from our analysis of the *ptc* promoter region; by deletion analysis, we have defined a 758-bp *ptc* upstream regulatory element that directs robust expression along the anteroposterior compartment boundary and is induced by ectopic Shh activity. Deletion of 156 bp containing three consensus GLI-binding sites from this fragment completely abolishes this expression.

Taken together, these results represent the first demonstration that Ci acts as a transcription factor and provide compelling evidence that the induction of *ptc* transcription by *hh* is mediated by a direct interaction between Ci and sequences upstream of the *ptc* promoter. Although we have not undertaken an analogous analysis of the *dpp* and *wg* regulatory elements, we think it likely that they too will be similarly under the direct control of Ci.

Recent studies have indicated a striking conservation of some elements of the *hh* signaling pathway between *Drosophila* and vertebrates. Thus, homologs of *ptc* have been identified in mouse (Goodrich et al. 1996), chicken (Marigo et al. 1996), and zebrafish (Concordet et al. 1996), which in each case are regulated in response to *hh* signaling in a manner analogous to their *Drosophila* counterpart. In addition, several lines of evidence have implicated PKA in signaling by vertebrate Hh family proteins (Fan et al. 1995; Concordet et al. 1996; Hammer-schmidt et al. 1996). Although GLI proteins have yet to be implicated directly in such signaling, their patterns of expression are highly suggestive of their involvement (Hui et al. 1994; Vortkamp et al. 1996). It is a strong prediction of our analysis that the induction of direct targets of Shh activity will be mediated similarly by GLI-binding sites in their *cis*-acting regulatory regions.

Materials and methods

Fly stocks

Wild-type flies were obtained from Oregon R strain (Lindsley and Zimm 1992). The different GAL4 lines were kindly provided by U. Hinz (University of Köln, Germany), A. Brand (Wellcome/CRC Institute, Cambridge, UK), and N. Perrimon (Har-

vard Medical School, Cambridge, MA). The *dpp-lacZ* (BS3.0; Blackman et al. 1991), *ptc-lacZ* (H84), and *hh^{fl}* stocks were kindly provided by R. Blackman (University of Illinois, Urbana), C. Goodman (University of California, Berkeley), and C. Nüsslein-Volhard (Max Planck Institut für Entwicklungsbiologie, Tübingen, Germany), respectively. The 30*Adpp-lacZ* and 30*Aptc-lacZ* recombinants were generated in the laboratory. The *hh* mutant embryos used in the *hh* rescue experiment were obtained by crossing *UASci*; *hh^{fl}* females to *hGAL4 hh^{fl}* males.

Generation of transgenic fly lines

The different pUAS.T and *ptc-lacZ* constructs describe in the plasmid section were microinjected into *Drosophila* embryos from a *yw^{67c23}* stock using standard procedures. For each case, several independent lines were obtained and analyzed to ensure that the observed effects are independent of insertion site.

Cuticle preparation

Larvae were collected just before hatching, removed from their vitelline membranes manually and mounted in 1:1 lactic acid/Hoyer's medium.

Plasmids

UASci A double-stranded oligonucleotide corresponding to the first 53 nucleotides of the Ci-coding sequence was cloned into pBSKS as an *EagI*-*EcoRI* fragment to generate *KSci5'*. The remaining *ci* sequence was removed from Ci1 (kindly provided by R. Holmgren) and cloned as an *EcoRI* fragment into *KSci5'* to generate *KSciFL*. The full-length *ci* cDNA was then removed from this vector and cloned into pUAS.T as an *EagI* fragment to generate *UASci*.

UASciΔ3' *KSciFL* was digested with *SpeI* and *SmaI*. The *SpeI* site was filled in with Klenow and the vector was ligated to itself to generate *KSciΔ3'*. This vector contains the *ci* cDNA with a deletion from amino acids 970–1235 that corresponds to a putative transcriptional activation domain. This *ciΔ3'* was then cloned in pUAS.T as a *EagI*-*KpnI* fragment to generate *UASciΔ3'*.

UAS.TciΔEn A PCR fragment encoding the transcriptional repression domain of Engrailed (amino acids 228–282) (Han and Manley 1993) was cloned as a *BglII*-*BamHI* fragment into *KSciΔ3'* to generate *KSciΔEn*. This vector was then cut with *EagI* and *KpnI* and the *ciΔEn* insert was cloned into pUAS.T to generate *UAS.TciΔEn*.

UAS.TciΔ5' *KSciFL* was digested with *NcoI* (position 2184) and *EagI* (3' of the coding sequence). The 2325-bp *ci* fragment was then cloned into *KSZFci* digested with the same restriction enzymes to generate *KSciΔ5'*. This vector was then cut with *EcoRI* and *NotI* and cloned into pUAS.T to generate *UAS.TciΔ5'*.

UAS.TZFciVP16 A fragment encoding amino acids 314–609 that includes the entire zinc finger region was generated by PCR, digested by *BglIII* (5') and *BamHI* (3') and then cloned into pKS to generate *KSZFci*. A *BamHI*-*XbaI* PCR fragment encoding the carboxy-terminal transcriptional activation domain of HSV VP16 (amino acids 418–490) was cloned into *KSZFci* to generate *KSZFciVP16*. This construct was then cut with *EcoRI* and *NotI* and the insert was cloned into pUAS.T to generate *UAS.TZFciVP16*.

p601Cibs A double-stranded oligonucleotide corresponding to the GLI consensus-binding site (5'-GACCACCCA-3') was cloned in the yeast vector p601 (Alexandre et al. 1993) at the *EcoRI* site to generate p601Cibs.

YCGTci The *ci*-coding sequence (amino acids 18–1262) was cloned in the yeast vector YCGT (Alexandre et al. 1993) to generate YCGTci. The same vector was used to generate YCGTZFcVP16.

ptc-lacZ construct A 888-bp PCR fragment corresponding to the *ptc* promoter region (–758 to +130) was generated and cloned into the *wlac-Z* vector (Y. Nakano, unpubl.) as a *BamHI*–*NotI* insert to create the FE construct. The same strategy was used to create the GE construct but the 731-bp PCR fragment corresponded to the region –601 to +130.

Immunocytochemistry

Wing imaginal discs were dissected in phosphate-buffered saline (PBS) and fixed for 20 min at 4°C in 4% paraformaldehyde in PBS (pH 7.2). Incubation with primary antibodies was carried out overnight at 4°C using the following dilutions: monoclonal anti- β -galactosidase (Promega) at 1:500; rabbit polyclonal anti-Ci at 1:1000 (M. Fietz, unpubl.); rabbit polyclonal anti-Hh (raised against the amino-terminal portion of the protein) (A.M. Taylor, unpubl.) at 1:1000. Double stainings were done in parallel using mouse and rabbit primary antibodies. Confocal images were collected with a Bio-Rad MRC 1000 system and processed with Bio-Rad COMOS and Adobe Photoshop software.

Detection of β -galactosidase activity

To detect β -galactosidase activity in imaginal discs, mature third instar larvae were cut in half in PBS. The anterior halves were inverted and fixed in 4% glutaraldehyde for 5 min and washed in PBS. The discs were then stained as described (Ashburner 1989). Stained discs were dissected from the carcass in PBS and mounted in 70% glycerol for microscopic analysis.

In situ hybridization

Whole embryos were fixed and hybridized with digoxigenin-labeled single-stranded *wg* or *ptc* RNA probes, as described (Ingham et al. 1991). For double in situ hybridization, the *wg* or *ptc* probes were used with a fluorescein-labeled single-stranded *en* RNA probe. After the first reaction (using NBT/BCIP) for *wg* or *ptc* detection, the embryos were refixed in 4% formaldehyde and the alkaline phosphatase was inactivated by incubating the embryos overnight in 100 mM glycine (pH 2.2). The next day, the embryos were incubated with the anti-fluorescein AP antibodies for 1 hr and the *en* staining was then revealed with fast-red/naphthol in 100 mM Tris (pH 8.2).

Wing mounting

Wings from adult flies were dissected in 70% ethanol, dehydrated, and mounted in Euparal for examination with the compound microscope.

Yeast transformation

The yeast *S. cerevisiae* strain W303-1A (*mata*, *his3-11,5*, *trp1-1*, *ade2-1*, *leu2-3,112*, *ura3*, *ho*, *can1-100*) (kindly provided by Nic Jones, Imperial Cancer Research Fund, London, UK) was trans-

formed with the various plasmids using the rapid transformation protocol (Alexandre et al. 1993).

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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.

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