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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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 Pablo 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-translationally. 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 [UASci] 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 *ptcGAL4* 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
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/ 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.

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

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,F} 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.

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.
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Figure 3. Rescue of hh mutant embryos by misexpression of ci. [A] Ventral cuticle pattern of a pharate larva homozygous for hh\(^{10}\), the absence of normal cuticle expression in stage 10 embryo (C). [B] Ventral cuticle pattern of a hh homzygote expressing Ci under the control of the hairy enhancer elements. Note the rescue of posterior cuticle expression 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;UASci 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 characteristic 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 transcription.
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 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 (CiA5') (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 ($C,F$).

**Figure 7.** Repression of $dpp$ transcription by a repressor form of Ci (CiAEn). 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 ($C,F$) and repression of $dpp$ transcription in the wing imaginal disc ($D,F$) caused by the overexpression of CiAEn 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 promoter containing the GLI-binding site. Moreover, this activation is specific to the GLI-binding site as 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

**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).

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 boundary-specific expression and the shh inducibility (Fig. 10B, D). Therefore, we conclude that sequences within this deleted 156-bp fragment (including the three GLI consensus-binding sites) are necessary for hh-dependent transcriptional activation.
Transcriptional activation of patched by ci

**Figure 10.** A 158-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, the 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 [Orecic 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; Hammerschmidt 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 [Harvard Medical School, Cambridge, MA]. The dpp-lacZ (BS3.0; Blackman et al. 1991), ptc-lacZ (H84), and hh" 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 30Adpp-lacZ and 30Aptc-lacZ recombinants were generated in the laboratory. The hh mutant embryos used in the hh rescue experiment were obtained by crossing UASCi; hh" females to hGal4 hh" males.

Generation of transgenic fly lines

The different pUAS.T and ptc-lacZ constructs described in the plasmid section were microinjected into Drosophila embryos from a yw" czas 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 KScic5'. The remaining ci sequence was removed from Ci1 [kindly provided by R. Holmgren] and cloned as an EcoRI fragment into KScic5' to generate KScicl. The full-length ci cDNA was then removed from this vector and cloned into pUAS.T as an EagI fragment to generate UASCi.

UASCiA3' KScfl was digested with SpeI and SmaI. The SpeI site was filled in with Klenow and the vector was ligated to itself to generate KScicA3'. This vector contains the ci cDNA with a deletion from amino acids 970-1235 that corresponds to a putative transcriptional activation domain. This ciA3' was then cloned in pUAS.T as an Eag1–Kpn1 fragment to generate UAScicA3'.

UAS.TciAEn 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 KScicA3' to generate KScicEn. This vector was then cut with Eag1 and Kpn1 and the ciAEn insert was cloned into pUAS.T to generate UAS.TciAEn.

UAS.TciA5' KScfl was digested with NcoI in position 21846 and Eagl in amino acids 3' of the coding sequence. The 3233-bp ci fragment was then cloned into KSZFlcI digested with the same restriction enzymes to generate KScicA5'. This vector was then cut with EcoRI and NotI and cloned into pUAS.T to generate UAS.TciA5'.

UAS.TZFCiVP16 A fragment encoding amino acids 314–609 that includes the entire zinc finger region was generated by PCR, digested by BglII in 5' and BamHI in amino acids 3' and then cloned into KSFlcI to generate KSZFlcI. A BamHI–XhoI PCR fragment encoding the carboxy-terminal transcriptional activation domain of HSV VP16 was cloned into KSZFlcI to generate KSZFlcI. This construct was then cut with EcoRI and NotI and inserted into pUAS.T to generate UAS.TZFCiVP16.
\textbf{p601Cibs} A double-stranded oligonucleotide corresponding to the GLI consensus-binding site \(5'\-\text{GACCCACCA-3'}\) was cloned in the yeast vector p601 \cite{Alexandre1993} at the EcoRI site to generate p601Cibs.

\textbf{YCGTci} The ci-coding sequence (amino acids 18–1262) was cloned in the yeast vector YCGT \cite{Alexandre1993} to generate YCGTci. The same vector was used to generate YCGTZeClFP16.

\textbf{ptc-lacZ construct} A 888-bp PCR fragment corresponding to the ptc promoter region \(-758\) to \(+130\) was generated and cloned into the \textit{wac-Z} vector \cite{Yakamoto1989} as a BamHI-NcoI 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 \(+130\).

\textbf{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-\(\beta\)-galactosidase (Promega) at 1:500; rabbit polyclonal anti-Ci at 1:1000 \cite{M.Fietz1991}; rabbit polyclonal anti-Hh (raised against the amino-terminal portion of the protein) \cite{A.M.Taylor1991} 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.

\textbf{Detection of \(\beta\)-galactosidase activity} To detect \(\beta\)-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 \cite{Ashburner1989}. Stained discs were dissected from the carcass in PBS and mounted in 70% glycerol for microscopic analysis.

\textbf{In situ hybridization} Whole embryos were fixed and hybridized with digoxigenin-labeled single-stranded \textit{wg} or \textit{ptc} RNA probes, as described \cite{Ingham1991}. For double in situ hybridization, the \textit{wg} or \textit{ptc} probes were used with a fluorescein-labeled single-stranded \textit{en} RNA probe. After the first reaction (using NBT/BCIP) for \textit{wg} or \textit{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 \textit{en} staining was then revealed with fastred/naphthol in 100 mM Tris (pH 8.2).

\textbf{Wing mounting} Wings from adult flies were dissected in 70% ethanol, dehydrated, and mounted in Euparal for examination with the compound microscope.

\textbf{Yeast transformation} The yeast \textit{S. cerevisiae} strain W303-1A [\textit{mata}, his3-11, 15, \textit{trpl-1}, \textit{ade2-1}, \textit{leu2-3, 112}, \textit{ura3}, \textit{ho}, \textit{can1-100}] [kindly provided by Nic Jones, Imperial Cancer Research Fund, London, UK] was transformed with the various plasmids using the rapid transformation protocol \cite{Alexandre1993}.

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