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Hedgehog signaling regulates transcription through cubitus interruptus, a sequence-specific DNA binding protein

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ABSTRACT **Hedgehog (Hh) is a member of a family of secreted proteins that direct patterning at multiple stages in both *Drosophila* and vertebrate development. During *Drosophila* embryogenesis, Hh protein is secreted by the cells of the posterior compartment of each segment. *hh* activates transcription of *wingless* (*wg*), *gooseberry* (*gsb*), and *patched* (*ptc*) in the cells immediately adjacent to Hh-secreting cells. Hh signaling is thought to involve the segment polarity gene *cubitus interruptus* (*ci*). *ci* encodes a zinc finger protein of the Gli family of sequence-specific DNA binding proteins. *ci* mRNA is expressed in all non-Hh expressing cells. Here we demonstrate *ci* activity is both necessary and sufficient to drive expression of Hh-responsive genes in the *Drosophila* embryos. We show that Ci is a sequence-specific DNA binding protein that drives transcription from the *wg* promoter in transiently transfected cells. We demonstrate that Ci binding sites in the *wg* promoter are necessary for this transcriptional activation. These data taken together provide strong evidence that Ci is a transcriptional effector of Hh signaling.**

In *Drosophila* embryogenesis Hedgehog (Hh) plays an early role in segmentation and a later role in generating cell type diversity within each segment (reviewed in ref. 1). The *Drosophila* body is divided into parasegments, which are the basic units for patterning in the developing organism. As the embryo cellularizes, the parasegment borders form. A parasegment border is delineated by expression of two secreted proteins (reviewed in ref. 1). Wingless (Wg), a Wnt family member, is expressed in cells anterior to the parasegment border (2), and Hh is expressed by cells posterior to the parasegment border (3–5). During the first 2 h after gastrulation, secreted Hh protein activates a signaling pathway that results in transcription of *wingless* (*wg*), *gooseberry* (*gsb*), and *patched* (*ptc*) in cells that are competent to respond (6). Between 3 and 6 h after gastrulation, Hh takes on a new role. It participates in autoregulatory *engrailed* (*en*) expression (7) and it organizes the pattern of dorsal cuticle hairs (8). In its latter role, Hh has been proposed to act as a morphogen (8), though this remains to be demonstrated (9).

hh also specifies critical aspects of adult morphology in *Drosophila*. The cuticular structures of adult appendages derive from internal epithelial sacs within the larvae, the imaginal discs. The posterior compartment of each disc expresses Hh, and immediately adjacent anterior compartment cells respond to this Hh signal by induction of the secondary signals Wg or the transforming growth factor β family member, Decapentaplegic (Dpp). These signals then organize growth and patterning of both compartments (10–14). While the developing eye lacks distinct anterior and posterior compartments, Hh signaling is critical for organizing the highly ordered structure of the compound eye (reviewed in refs. 5 and 15–18).

Several components of the Hh signaling pathway have been identified in *Drosophila*. *ptc* is a negative regulator of Hh signaling (19). Genetic analysis suggests that Hh signaling either inactivates Ptc or overrides the ability of Ptc to repress transcription of Hh-responsive target genes. Because Ptc is a multiple membrane spanning cell surface protein (20, 21), it could be a Hh receptor (19). However, it cannot be the only Hh receptor, since Hh has effects in *ptc* null embryos (22). Two protein kinases are implicated in Hh signal transduction, *fused* as a positive regulator, and cAMP-dependent protein kinase A as a negative regulator (reviewed in ref. 23). How either of these fit into a signal transduction pathway remains to be determined.

In *cubitus interruptus* (*ci*) mutant embryos, transcription of Hh-responsive genes is lost (24, 25). Ci overexpression or misexpression drives transcription of Hh responsive target genes (26, 27). Ci is a member of the Gli family of sequence-specific DNA binding proteins (28, 29), a family characterized by five tandem zinc fingers with highly similar sequence. Based on the presence of these zinc fingers, Orenic *et al.* (28) proposed that Ci acts as a transcription factor. Alexandre *et al.* (27) showed that Ci can activate transcription in yeast, through a putative Ci binding site. These data suggest that Ci is a transcriptional effector of Hh signaling. However, direct Ci–DNA interaction has not been shown. Here we present genetic evidence confirming that Hh acts through Ci to activate *wg* and *gsb* transcription. We demonstrate that Ci has sequence-specific DNA binding activity and that direct interaction between Ci and sequences in *wg* promoter is required for transcriptional activation from this promoter. We conclude that Ci functions as a transcriptional activator in the Hh signal transduction pathway.

MATERIALS AND METHODS

***Drosophila* Stocks, Crosses, and Analysis.** *Df(4)M62f* flies were obtained from Robert Holmgren (Northwestern University), *yw*; *Hs-hh M3/TM3 y*$^+$ *Ser* from Philip Ingham (Imperial Cancer Research Fund), and *hh*^{GS1}, *ru e/TM3* from Jym Mohler (Barnard College).

Hs-hh M3/+; *Df(4)M62f/Df(4)M62f* embryos were generated by mating *Hs-hh M3/+*; *Df(4)M62f/+* males to *Df(4)M62f/eyD* females. Embryos from this cross were collected and heat-shock treated as in ref. 30.

hh^{GS1}*/hh*^{GS1}*;ci*^{D}*/ci*^{D} embryos were obtained from heterozygous *hh*^{GS1}*/TM3;ci*^{D}*/+* parents. The stage 10/early stage 11 embryos were fixed, processed by *in situ* hybridization, and scored for wild type; *ci*^{D}, *hh*, or *hh; ci*^{D} patterns; and unscorable. Using the χ^2 test, the observed distribution of

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Abbreviations: GST-Ci, glutathione-*S*-transferase/Ci fusion protein; β-gal, β-galactosidase; EMSA, electrophoretic mobility-shift assay.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U84292).

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phenotypes fit the expected 9:3:3:1 ratio with $P = 0.8$ for *ptc*, $P = 0.44$ for *wg*, and $P > 0.9$ for *gsb*.

The *smo* ci^D phenotype was determined by collecting embryos from *smo*^{1} *cn bw sp*/*Cyo*; ci^D/M^{63a} parents overnight at 18°C. The embryos were fixed and assayed for *wg, gsb*, and *ptc* RNA patterns. The stage 10/early stage 11 embryos were scored for wild type; ci^D, *smo*, or *smo;* ci^D patterns; and unscorable. Using the χ^2 test, the observed distribution of phenotypes fit the expected 9:3:3:1 ratio for *wg, gsb*, and *ptc*.

The *fused* (*fu*); ci^D phenotype was determined by collecting embryos from fu^{513}/fu^{513}; ci^D/M^{63a} females crossed to fu^{513}; ci^D/M^{63a} males. Stage 10 and early stage 11 embryos were scored for *fu* or *fu*; ci^D phenotypes. Approximately 25% of the populations had a ci^D like expression pattern for *wg, gsb*, and *ptc*.

In situ hybridizations utilized antisense-strand riboprobes, according to standard procedures (31, 32).

Recombinant DNAs. To build *wg*-Luc, a 5-kb *Eco*RV fragment of *wg* promoter ligated into the *Sma*I site of pXP2Luc (33). To construct Δ*wg*-Luc and Δ*wg**-Luc, a 1-kb *Xho*I fragment from pAlter (Promega) containing a 2-kb *Bam*HI fragment of *wg* promoter was blunt-ended with Klenow and ligated into *tk*-Luc (33) at the *Sma*I site. *wg*-LucΔS was constructed by deletion of a 1-kb *Sal*I fragment from the full-length *wg*-Luc construct. *wg*-LucΔB was constructed by removal of a 2-kb *Bam*HI fragment from *wg*-Luc. *mt*-Ci was constructed by ligating a *Bgl*II/*Not*I fragment containing full-length Ci cDNA from NB40 (34) into pRmHa-1 (35) at the *Bam*HI and *Hin*cII sites. The glutathione-*S*-transferase/Ci fusion protein (GST-Ci) was constructed by ligation of a *Eco*RV/*Hin*cII fragment of Ci cDNA into the *Sma*I site of pGEX-3X (36).

Transient Transfections. *Drosophila* Schneider line 2 cells were transfected using the calcium phosphate precipitation method (37). Each transfection contained 50 ng *wg*-Luc reporter plasmid, variable amounts of expression plasmid *mt*-Ci as indicated, and *pRmHa-1* plasmid in variable amounts to bring the total amount of expression plasmid to 1 μg. A total of 10 μg of DNA was used in each transfection. As an internal control 1 μg of *Copia* long terminal repeat (LTR)-*lacZ* [copia–β-galactosidase (β-gal), obtained from J. Manley, Columbia University] was added to each transfection. β-Gal assays were conducted as described in ref. 38. Luciferase assays were done as in ref. 39.

Transfections with the Δ*wg*-Luc, Δ*wg**-Luc, *wg*-LucΔS, and *wg*-LucΔB reporter constructs contained 200 ng of each reporter. *mt*-Ci concentration was varied as specified. All transfections were performed in duplicate, and each experiment was conducted several times with similar results. Data is presented as the average of the duplicates.

Purification of GST-Ci and DNA Binding Assays. GST-Ci was grown in HB101 *Escherichia coli* to saturation overnight. A 250 ml culture was grown to $OD_{650} = 0.1–0.25$ and induced by addition of 1 mM isopropyl β-D-thiogaloctoside, then grown overnight at 20°C. Growth at lower temperature reduced the formation of inclusion bodies. The cells were then harvested and lysed as described (40), and the protein was purified on glutathione Sepharose (Pharmacia). BSA was added to 1 mg/ml as carrier. Purified protein was concentrated and dialyzed into PBS with a Centricon 30 microconcentrator (Amicon).

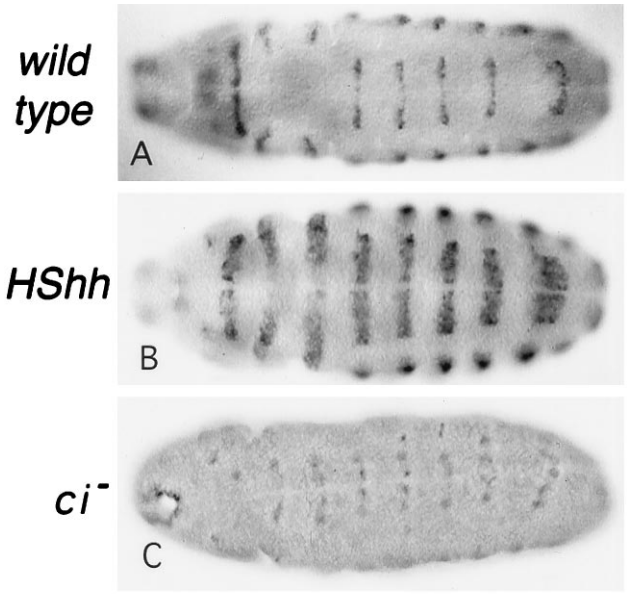
BS-1 and BS-2 plasmids were obtained from David Zarkower (University of Minnesota). The binding site containing the fragment was excised with *Xba*I and labeled with [α-^{32}P]dCTP by Klenow end filling (41). The labeled fragments were then purified on a native acrylamide gel. Electrophoretic mobility-shift assays (EMSAs) were performed in 1× EMSA buffer, 50 mM Hepes (pH 7.5), 10 μM $ZnSO_4$, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 20% glycerol, and resolved on 5% acrylamide native 1× TBE gels. Anti-GST antiserum was provided by Alex Franzusoff (University of Colorado Health Science Center).

Fragments containing mutant and nonmutant *wg* promoter sequences (fragments 1 and 2) were isolated by PCR. PCR fragments were end labeled with [γ-^{32}P]ATP. Quantitation used a scanning densitomiter, Alpha Innotech model IS1000 digital imaging system (Alpha Innotech, San Leandro, CA).

Site-Directed Mutagenesis. Mutagenesis of putative Ci binding sites in *wg* promoter/enhancer DNA used the altered sites *in vitro* mutagenesis kit according to manufacturers instructions (Promega). A 2-kb *Bam*HI fragment of *wg* promoter was inserted into pAlter-1 phagemid vector. Single-stranded DNA was prepared using helper phage M13K07 or R408. Mutagenic primers were synthesized by Life Technologies. Primers used were Alt-1, TCGATGGCTACTCGTCGG; Alt-2, CGCCAGCGTAGACGATGAT; Alt-3, AAAGTGCGTAAACCATGTC; and Alt-4, AAAATGGACTTTCCCAGCGT. Each mutates one of four putative Ci binding sites in the distal 1 kb of *wg* promoter. Successful oligonucleotide mutagenesis was confirmed by sequencing of candidate clones using Sequenase kit (United States Biochemical).

RESULTS

***hh* Acts Through *ci*.** In the *Drosophila* embryo, for 2 h after gastrulation, Hh signaling maintains the expression of *wg, gsb*, and *ptc* (6, 42, 43). To demonstrate *ci* is required for transduction of the Hh signal, we examined expression of *wg* in *ci* null mutant embryos when Hh is ubiquitously expressed under control of a heat-shock promoter (*Hs-hh*). In *Hs-hh* embryos, *wg* is expressed ectopically in anteriorly expanded stripes (Fig. 1*B*) (13, 30). In ci^- embryos *Hs-hh* does not induce ectopic expression of *wg*. Instead *wg* expression decays in the same manner seen in *ci* mutants (Fig. 1*C*). This conclusion is based on statistical analysis of mixed populations. In this experiment 25% of the embryos are *ci* null. If *ci* is required to transduce the Hh signal, then *Hs-hh* will have



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Phenotypes	Expected %	Observed % N	Observed % %
wild type	37.5	100	37.7
Hshh	37.5	100	37.7
ci-	25	65	24.5

FIG. 1. Ci is required for maintenance of Hh-responsive gene expression. (*A*) Wild-type expression of *wg* mRNA, stage 11. (*B*) *wg* mRNA in heat-shocked *Hs-hh* embryo. (*C*) Expression of Wg protein in a *Df(4)M62f* mutant embryo, stage 11. (*D*) Statistical analysis of number (*N*) and relative percents of observed phenotypes, indicates that *ci* is required for Hh signal transduction.

no effect in homozygous ci^- embryos. If *ci* is not required, then only the homozygous ci^- embryos not receiving *Hs-hh* (12.5%) will have the ci^- phenotype. As shown in Fig. 1*D*, 24.5% of the population had the ci^- phenotype. Similar results were obtained for *gsb* expression. The distribution of the three progeny classes for *gsb* expression is as follows: *Hs-hh*, 113 embryos; wild type, 104 embryos and ci^-, 78 embryos—i.e., 26.4% had the ci^- phenotype. Therefore *ci* is required for transduction of the *hh* signal.

ci^D is a gain of function allele of *ci* (28) that produces an abnormally large protein at very low levels (44). In ci^D embryos *wg* expression is initiated normally, but at stage 10 it diverges from the wild-type pattern to form stripes that are more broad and less regular (compare Fig. 2 *G* to *A*; also see ref. 6). In a similar manner, *gsb* expression also diverges from the wild-type pattern to form wider, less regular stripes (compare Fig. 2 *H* to *B*). At stage 11 *ptc* expression remains broad in ci^D embryos, rather than narrowing to two single cell wide stripes at the edges of the anterior compartment (compare Fig. 2 *I* to *C*; also see ref. 6). The ci^D phenotype with respect to *wg* and *gsb* expression is not fully penetrant because some segments show the wild-type expression pattern of these genes. This phenotype is radically different from that of *ci* null embryos and is also different from that of wild-type *ci* (compare Figs. 1 and 2).

There are at least three possible explanations for the unusual activity of ci^D. ci^D might increase the sensitivity of the cells to the Hh signal, ci^D might alter distribution of the Hh signal, or ci^D might substitute for Hh activity. To differentiate between these possibilities, we examined expression patterns of *wg, gsb*, and *ptc* in populations of embryos from $hh^{GS1}/TM3;\ ci^D/M^{63a}$ flies. hh^{GS1} is a strong allele of *hh* that can be considered a null (3). In hh^{GS1}; ci^D homozygous embryos *ptc* expression resembles that of ci^D embryos. *wg* and *gsb* expression in the double mutant embryos shows the *hh* phenotype in some segments and the ci^D phenotype in others. The partial restoration of *wg* and *gsb* expression is consistent with the poor penetrance of ci^D. Apparently ci^D can maintain expression of *hh*-responsive genes in the ectoderm in the absence of active Hh protein. This is consistent with the observation of Alexandre *et al.* (27) that overexpression of wild-type Ci can rescue expression of Hh-responsive genes in a *hh* mutant. We find that *ci* is necessary for Hh signaling and ci^D can substitute for Hh protein in driving expression of Hh-responsive genes. Taken together, these two results confirm that *hh* acts through *ci* to activate transcription of its target genes.

Ci Acts Downstream of *smo* and *fu*. If Ci is the transcriptional effector of Hh signaling then it should act downstream of all other known components of the pathway. Ci has already been shown to act downstream of *ptc* (24, 25, 45). We have tested epistasis of *ci* with two other members of the signaling pathway. The *smoothened* (*smo*) gene encodes a putative receptor for the Hh signal (46, 47). The *fu* gene encodes a Ser/Thr kinase that is necessary for Hh signal transduction (24, 43, 48). *wg, gsb*, and *ptc* expression patterns in $smo;ci^D$ and $fu;ci^D$ double mutants demonstrate that ci^D is epistatic to both *smo* and *fu*. The phenotypes of these double mutants are similar to $hh^{GS1};ci^D$ double mutants (data not shown).

Ci Is a Sequence-Specific DNA Binding Protein. *ci* encodes a zinc finger protein that is a member of the Gli family (29). The zinc finger domains of three of these, Gli, Gli3, and Tra-1, all have sequence-specific DNA binding activity. Furthermore, all three recognize the same 9 bp core consensus sequence: -TGGGTGGTC- (49–51). Ci has a high degree of sequence similarity to these proteins within the zinc finger domains and is identical to Gli in all amino acids that make base contacts, as determined by crystal structure (52). Therefore, we predicted that Ci and Gli would bind DNA with the same sequence specificity.

To demonstrate sequence specificity, we constructed a fusion protein in which the zinc finger domain of Ci is fused to GST (36). Purified GST-Ci fusion protein was used in EMSAs

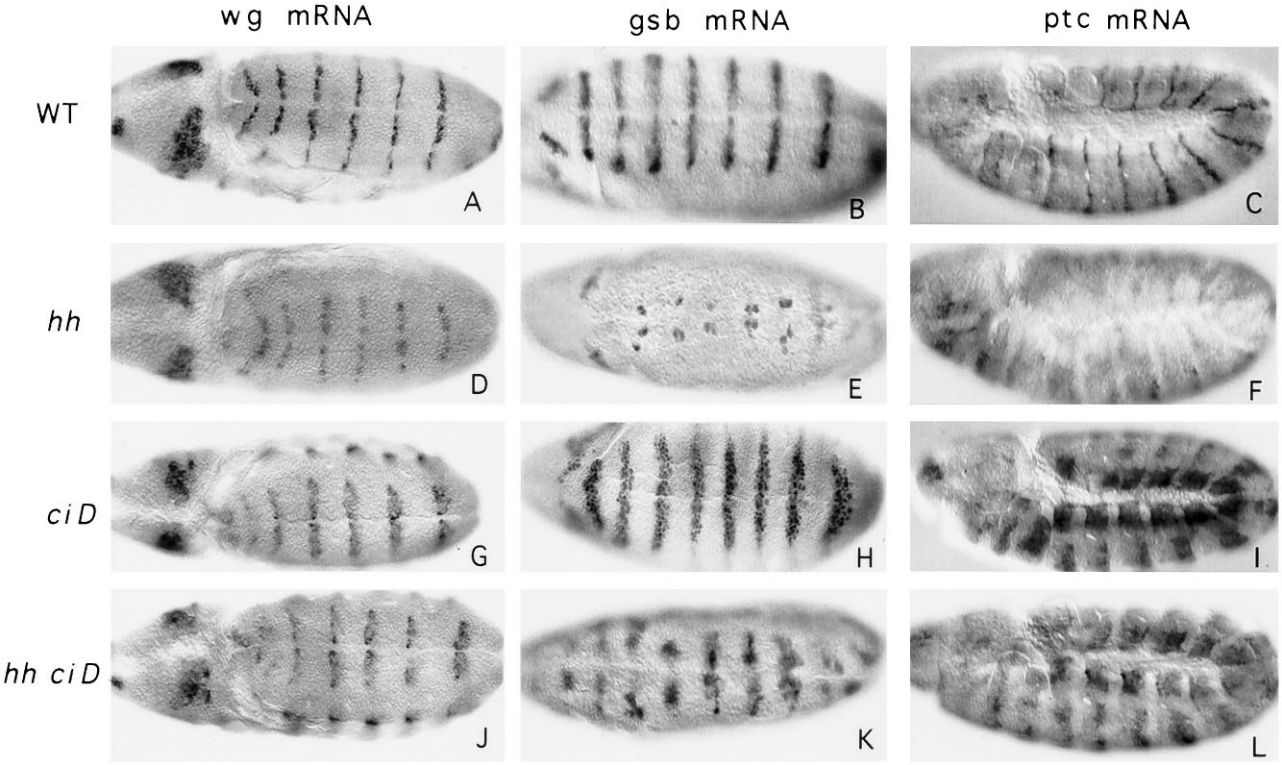

FIG. 2. Ci acts epistatically to Hh. (*A*, *D*, *G*, and *J*) *wg* mRNA expression patterns, in late stage 10 embryos: (*A*) wild type, (*D*) hh^{GS1} mutant, (*G*) ci^D mutant, and (*J*) hh^{GS1} ci^D double mutant. (*B*, *E*, *H*, and *K*) *gsb* mRNA expression patterns late stage 10 embryos: (*B*) wild type, (*E*) hh^{GS1} mutant, (*H*) ci^D mutant, and (*K*) hh^{GS1} ci^D double mutant. (*C*, *F*, *I*, and *L*) *ptc* mRNA expression patterns, stage 11 embryos: (*C*) wild type, (*F*) hh^{GS1} mutant, (*I*) ci^D mutant, and (*L*) hh^{GS1} ci^D double mutant.

(Fig. 3). We found that purified GST-Ci bound to a 36-bp nucleotide that contained the 9-bp core consensus binding site (BS-1), but not to a control DNA (BS-2) containing a single G-A mutation that changes the core consensus binding site (data not shown). Competition with unlabeled BS-1 and unlabeled BS-2 showed markedly different effects. BS-1 competed efficiently for for binding to GST-Ci whereas BS-2 did not. Furthermore, an antibody that recognizes GST is able to supershift the complex, demonstrating that the complex is formed with GST-Ci/BS-1. Thus, the zinc finger domain of Ci binds DNA in a sequence-specific manner.

Ci Activates Transcription from the *wg* Promoter in Cultured Cells. As shown above, Ci is a sequence-specific DNA binding protein that is necessary for *wg* expression. Therefore we predicted that Ci would be capable of activating transcription from *wg* promoter in transiently transfected Schneider line 2 (S2) cells. The reporter construct, *wg*-Luc, drives luciferase expression with a 5-kb fragment of *wg* promoter (Fig. 4*A*). *In vivo* this *wg* promoter fragment drives LacZ expression in a pattern that accurately reproduces most aspects of endogenous *wg* expression (D.L. and R.N., unpublished data). *tk*-Luc used as a control for promoter specificity, drives Luciferase expression off the thymidine kinase minimal promoter (33). All transfections included the *copia–β*-gal plasmid as an internal control for transfection efficiency.

wg-Luc expression but not *tk*-Luc expression is stimulated by coexpression of Ci (Fig. 4*B*). The effect of Ci on *wg*-Luc is bimodal. Low amounts of Ci stimulated *wg*-Luc to a maximum of 3.6-fold with 20 ng of *mt*-Ci. Higher concentrations of Ci decreased luciferase activity. This complex response to Ci dosage probably reflects interaction of distal activating elements and proximal inhibitory elements within the *wg* promoter; the inhibitory phase of the Ci response is lost when only a distal fragment of *wg* promoter is used (see below). This experiment demonstrates that Ci regulates gene expression from the *wg* promoter and that this effect is promoter-specific.

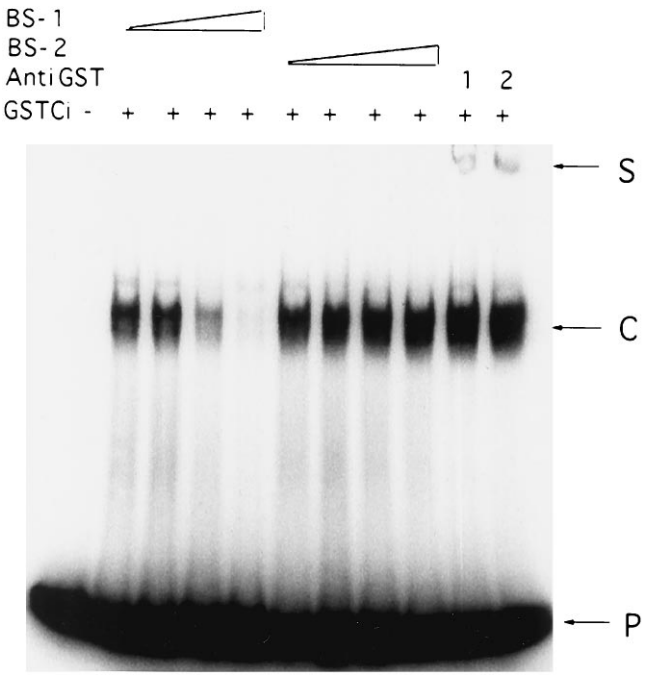

FIG. 3. Sequence-specific DNA binding of GST-Ci. P, free BS-1 DNA; C, GST-Ci/BS-1 complex; S, supershifted complex. Lane 1, BS-1 (TGGGTGGTC) alone. Lanes 2–5, GST-Ci/BS-1 complex with 0, 10, 100, and 500 ng competing unlabeled BS-1. Lanes 6–9, GST-Ci/BS-1 complex with 0, 10, 100, and 500 ng competing unlabeled BS-2 (TGGGTAGTC). Lanes 10 and 11, GST-Ci/BS-1 complex supershifted with 1 and 2 μl GST antiserum.

It does not address whether Ci acts directly. In addition, *wg*-Luc transcripts retain 436 nt of *wg* 5′ untranslated region, leaving open the possibility that the effects of Ci on *wg*-Luc are posttranscriptional.

Ci-Responsive Elements Map to the Distal End of the *wg* Promoter. To demonstrate direct transcriptional involvement of Ci, we mapped the Ci-responsive elements in *wg*-Luc. *wg*-LucΔS and *wg*-LucΔB, which respectively delete 1 kb and 2 kb from the distal end of *wg*-Luc (Fig. 4*A*), were tested for transcriptional activation by cotransfection of *mt*-Ci in S2 cells (Fig. 4*C*). *wg*-LucΔS showed a bimodal response to Ci similar to that of the intact *wg* promoter fragment, though the magnitude was significantly reduced. Maximal activation of only 1.7-fold was achieved with 200 ng *mt*-Ci while more *mt*-Ci decreased activation. *wg*-LucΔB showed poor transcriptional activation, only 1.4-fold activation at 300 ng *mt*-Ci. This suggests that the primary elements that are activated by Ci are in the distal regions of the promoter/enhancer.

To test this we used the distal 1 kb of *wg* promoter to drive Luciferase expression from the *tk* minimal promoter (Δ*wg*-Luc; Fig. 4*A*). Δ*wg*-Luc responded over a 100-fold range of increasing *mt*-Ci concentrations with maximal stimulation of 109-fold by 3,000 ng *mt*-Ci (Fig. 4*D*). This unambiguously maps Ci-activated elements of the *wg* promoter to the distal 1-kb fragment. Because Δ*wg*-Luc is not inhibited by high levels of Ci, specific elements in the deleted proximal region probably mediate the Ci inhibition seen with the full-length promoter (Fig. 4*B*). Because no *wg* 5′ untranslated region is retained in Δ*wg*-Luc, this eliminates concerns that the Ci effect is posttranscriptional. The difference in the magnitude of activation of Δ*wg*-Luc versus *wg*-Luc (109-fold versus 3.6-fold) could be related to the much higher basal expression of *wg*-Luc relative to Δ*wg*-Luc. The 4 kb deleted in Δ*wg*-Luc might eliminate many Ci-independent activating elements. In addition, the *tk* minimal promoter might respond less well than the *wg* minimal promoter to enhancer elements retained in Δ*wg*-Luc. While these issues are germane to an integrated view of *wg* promoter function, they are not critical to understanding whether Ci directly regulates transcription from the *wg* promoter.

Ci Binding Sites Mediate Transcriptional Activation of Δ*wg*-Luc by Ci. If Ci directly regulates transcription from *wg* promoter, then binding of Ci to sequences in *wg* promoter will be necessary for transcriptional activation. The *wg* promoter sequence has 10 possible Gli consensus binding sites, with 3 pairs of sites in the distal 1.2 kb (Fig. 4*A*). *wg*-LucΔS eliminated 2 of these pairs and *wg*-LucΔB eliminated all 3. Δ*wg*-Luc included the distal 2 of these pairs of putative Ci binding sites.

To confirm direct interaction of Ci with its putative binding sites, DNA fragments containing each of the distal two pairs of putative Ci binding sites were analyzed by EMSA (Fig. 5). Fragment 1 corresponds to the region of *wg* promoter containing the first two putative Ci binding sites (−4652 to −4422), and fragment 2 contains the second pair of putative Ci binding sites (−4037 to −3787). GST-Ci was able to shift both fragments 1 and 2. This confirmed the presence of Ci binding sites in *wg* promoter fragments that mediate transcriptional activation.

To directly test whether the Ci binding sites in Δ*wg*-Luc mediated its activation by Ci, we mutagenized the Ci binding sites in Δ*wg*-Luc. In Δ*wg**-Luc the four putative Ci binding sites were mutagenized with the same G → A mutation that distinguishes BS-1 from BS-2 and eliminates Ci binding (Fig. 3). These mutations severely compromised activation by Ci in the transient transfection assay (Fig. 4*D*). The mutant Δ*wg**-Luc was activated only 9.1-fold with maximal Ci concentrations, compared with 109-fold for Δ*wg*-Luc. That is, mutation of the Ci binding sites resulted in greater than 90% reduction in Ci-dependent transcriptional activation. An EMSA was used to confirm that the mutations in Δ*wg**-Luc compromised DNA binding (Fig. 5). Mutagenesis of fragment 2 completely eliminated the mobility shift by GST-Ci (Fig. 5*B*). Mutagenesis of fragment 1 reduced its

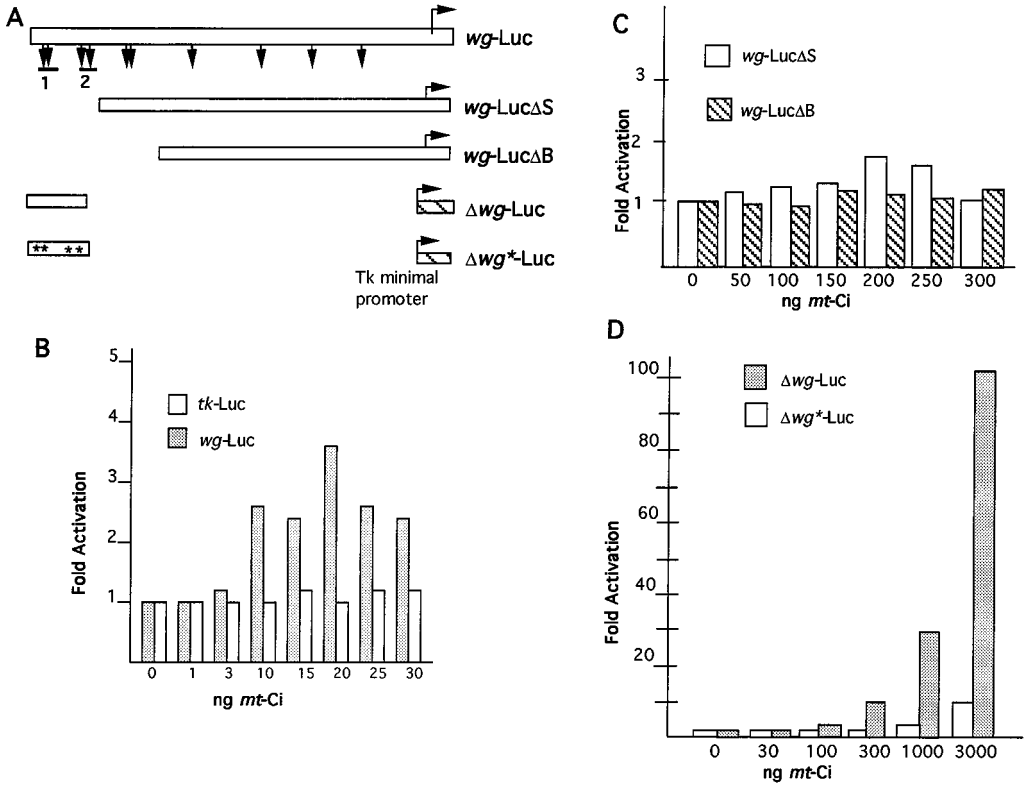

FIG. 4. Binding of Ci to *wg* promoter is required for transcriptional activation. (*A*) There are 10 putative Ci binding sites (defined as at least 5 of 9 match with BS-1) in the *wg* promoter/enhancer region. *wg*-LucΔS deletes the distal 1 kb of enhancer. *wg*-LucΔB deletes the distal 2 kb. Δ*wg*-Luc and Δ*wg**-Luc eliminate all but the distal 1 kb of *wg* promoter and are fused to the *tk* minimal promoter. Δ*wg**-Luc contains mutations in the four putative Ci binding sites. The sequences of Ci binding sites 1–4 in Δ*wg*-Luc are TGGCTGCTC, AGCGTGGAC, TGCGTGAAC, and GACCTCCCA, respectively. Underlined letters indicate mutagenized bases in Δ*wg**-Luc. The complete sequence for the *wg* promoter is available from GenBank. (*B*) Ci activates transcription from the *wg* promoter. Schneider line 2 (S2) cells were cotransfected with the indicated amounts of *mt*-Ci expression vector and 50 ng *wg*-Luc (shaded bars) or *tk*-Luc (open bars). Luciferase activity is normalized relative to β-gal activity as an internal control for transfection efficiency. Fold activation is presented as the increase in luciferase activity relative to the value from transfections lacking *mt*-Ci. Duplicate samples varied by less than 18%. Basal levels of luciferase units for reporter constructs in the absence of *mt*-Ci were 30,000 for *wg*-Luc and 5,000 for *tk*-Luc. (*C*) Deleting distal regions within the *wg* promoter/enhancer results in reduced activation by Ci. Cotransfection of varying concentrations of *mt*-Ci, as specified, with 200 ng *wg*-LucΔS (open bars), or with 200 ng *wg*-LucΔB (striped bars). Basal levels of luciferase units for reporter constructs in the absence of *mt*-Ci were 45,000 for *wg*-LucΔS and 30,000 for *wg*-LucΔB. (*D*) Transcriptional activation of Δ*wg*-Luc is reduced when Ci binding sites are mutagenized. Cotransfection of *mt*-Ci with Δ*wg*-Luc (shaded bars) or Δ*wg**-Luc (open bars) resulted in a 90% reduction in the ability to activate luciferase expression. Basal levels of luciferase units for reporter constructs in the absence of *mt*-Ci were 400 for Δ*wg*-Luc and 500 for Δ*wg**-Luc.

efficiency of binding to GST-Ci by 7-fold (Fig. 5*A*), rather than completely eliminating the binding. The residual binding could be explained by weak Ci binding sites missed by sequence search or by residual binding at either of the two mutagenized sites. Nevertheless, the overall binding of Ci to Δ*wg**-Luc was greatly reduced by mutagenesis of four Ci binding sites. Reduced binding affinity correlates with greater than 10-fold difference in transcriptional activation (Fig. 4*D*).

In summary, these results demonstrate that Ci is capable of binding to *wg* promoter fragments, that mutagenesis of the putative Ci binding sites in these fragments greatly reduces Ci binding, and that the reduced binding correlates with greater than 90% reduction in transcriptional activation. This demonstrates that Ci binding sites profoundly influence transcriptional activation by Ci. In other words, Ci binding to DNA is necessary for Ci to mediate transcriptional activation. We conclude that Ci activates transcription by direct binding to its recognition sites *wg* promoter/enhancer.

DISCUSSION

The genetic data presented here show that *ci* is the last identified step in the Hh signal transduction pathway. First we demonstrated that *ci* is necessary for maintenance of Hh-responsive gene expression. Our use of a *ci* null allele provides rigorous support for previous studies which used hypomorphic alleles (24, 26, 44, 53), and further show that when *ci* is absent *hh* overexpression is unable to activate transcription of target genes. Second, we demonstrated that *ci* activity is sufficient to activate Hh-responsive gene expression in the absence of *hh*, *smo*, or *fu*. This supports and extends recent work by Alexandre *et al.* (27) who demonstrated *ci* overexpression rescues *wg* expression in *hh* mutants. Combined with previous work showing that *ci* is epistatic to *ptc* (24, 25, 45), this argues strongly that *ci* is the last step in the Hh signaling pathway before transcriptional activation.

This work demonstrates that Ci acts as a sequence-specific DNA binding protein. Orenic *et al.* (28) suggested DNA binding activity for Ci based on its zinc fingers. The sequence-specific DNA binding of other Gli family zinc finger domains supports this notion (49–51). Here we have demonstrated that the Ci zinc finger domain binds DNA with the same sequence specificity as other Gli zinc finger domains. We have identified the *wg* promoter as a target for Ci interaction and have identified Ci binding sites in the *wg* promoter. Most important, we have shown that direct interaction between Ci and sequences in the promoter/enhancer region of the *wg* gene is sufficient for Ci-mediated transcriptional activation. *ptc*, another target for Hh, can respond to Hh via a promoter fragment containing Gli consensus binding sites (6, 27). The genetic and molecular results taken together provide strong evidence that Ci is a transcription factor that mediates transcriptional activation of Hh-responsive genes.

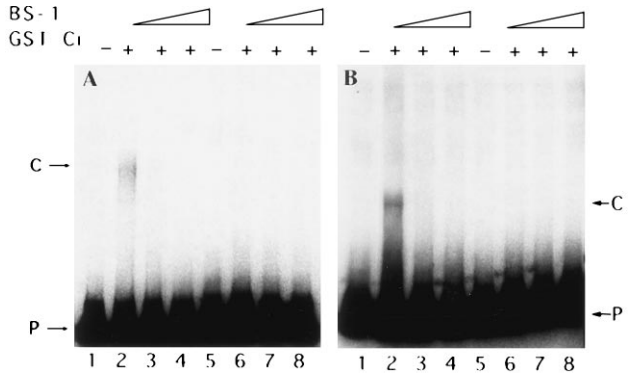

FIG. 5. GST-Ci binds wild-type *wg* promoter fragments more efficiently than mutagenized fragments. P, free DNA; C, GST-Ci/DNA complex. (*A*) Electrophoretic mobility shifts of *wg* promoter DNA fragment 1, containing Ci binding sites 1 and 2. Lanes 1–4, wild-type fragment 1. Lanes 5–8, mutagenized fragment 1. Lane 1, no GST-Ci; lanes 2–4, with GST-Ci and 0, 100, or 500 ng competing cold BS-1, respectively. (*B*) EMSA of *wg* promoter DNA fragment 2, containing Ci binding sites 3 and 4. Lanes 1–4, wild-type fragment 2. Lanes 5–8, mutagenized fragment 2. Lane 1, no GST-Ci; lanes 2–4, with GST-Ci and 0, 100, or 500 ng unlabeled BS-1 competitor, respectively; lane 5, no GST-Ci; lanes 6–8, with GST-Ci and 0, 100, or 500 ng unlabeled BS-1 competitor.

How is Ci activated by Hh? Ci protein is expressed in a complimentary pattern to Hh in both embryos and imaginal discs (25, 54). Ci is activated in cells adjacent to Hh-expressing cells but not in cells at a distance from Hh-expressing cells. Because Ci mRNA levels are not modulated by the Hh signal (28, 55), *ci* must be posttranscriptionally activated by Hh. Ci protein levels are elevated in cells responding to the Hh signal (25, 26, 54) and Ci overexpression can drive expression of Hh-responsive genes in the absence of Hh (27), so Ci protein levels may play a role in response to the Hh signal. However, Ci protein must also have active and inactive states to account for the lack of activity in cells that do not receive the Hh signal but still express the modest levels of Ci protein.

Ci protein is predominantly cytoplasmic in all cells, regardless of whether they receive the Hh signal (25). Because Ci acts as a transcription factor, the small amount of nuclear Ci detected adjacent to Hh-secreting cells (25) must be sufficient to activate transcription of Hh responsive genes. Future studies must resolve the apparent paradox of why this transcription factor is predominantly cytoplasmically localized and how its subcellular localization relates to its activation.

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