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Hedgehog signaling regulates transcription through Gli/Ci binding sites in the *wingless* enhancer

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Abstract

The segment polarity gene *cubitus interruptus* (*ci*) encodes a transcriptional effector of Hedgehog (Hh) signaling in *Drosophila*. The Ci gene product is a zinc finger protein belonging to the Gli family of sequence-specific DNA binding proteins. After gastrulation, segmental expression of the segment polarity gene *wingless* (*wg*) is maintained by Hh signaling in a pathway requiring Ci activity. In the absence of Hh or Ci activity, *wg* expression is initiated normally and then fades in the ectoderm after stage 10. We have previously identified a *wingless* enhancer region whose Ci binding sites mediate Ci-dependent transcriptional activation in transiently transfected cells. Here we demonstrate that Hh and Patched (Ptc) act through those Ci binding sites to modulate the level of Ci-dependent transcriptional activation in S2 cells. We demonstrate that this same *wg* enhancer region is Hh responsive in vivo and that its Ci binding sites are necessary for its activity. This provides strong evidence that Hh affects *wg* transcription through post-translational activation of Ci. © 1997 Elsevier Science Ireland Ltd.

Keywords: Cubitus interruptus; *wingless* promoter; Hedgehog signaling

1. Introduction

Formation of the segmentally repeated pattern of denticles and naked cuticle on the ventral surface of the *Drosophila* larvae involves progressive subdivision of the embryos along the anterior/posterior (A/P) axis. Part of this involves spatial restriction of the secreted proteins *wg* and *hh* to cells flanking the parasegment border. Refinement of *wg* expression to a narrow row of cells within each segment is important for generating the segmentally repeated pattern. Loss of *wg* activity results in loss of naked cuticle and a lawn of denticles on the ventral surface of the larvae (Nüsslein-Volhard and Weischaus, 1980), while ubiquitous expression of *wg* results in loss of denticle belts (Noordermeer et al., 1992). Therefore, the activity of Wg appears to inhibit denticle formation and promote naked cuticle, leading to alternating bands of each along the A/P axis of the larvae.

Temporal and spatial regulation of *wg* expression is a dynamic process. During embryogenesis, *wg* transcription is initiated in stripes at the blastoderm stage. At the onset of gastrulation the transcripts become modulated into 14 segmentally repeated stripes one to two cells wide (Baker, 1987). Activation of *wg* in segmental stripes is controlled by pair rule genes, which exert both positive and negative effects. For instance, in the absence of *paired* (*prd*) activity *wg* expression is not activated in even numbered parasegments, indicating that *prd* is a positive regulator of *wg* expression (Ingham and Hidalgo, 1993). As development progresses, cells at the posterior margin of each parasegment continue to express *wg*. However, maintenance of *wg* requires the activity of the segment polarity genes *hh* and *wg* (Hidalgo and Ingham, 1990; Hooper, 1994) as well as the pair-rule gene *sloppy-paired* (*slp*). The *slp* locus contains two transcription units both encoding forkhead domain transcription factors (Grossniklaus et al., 1992). *slp* is an activator of *wg* transcription and its distribution defines the *wg* competent domain (Cadigan et al., 1994). Appropriate *wg* expression at this stage is necessary for proper maintenance of the posterior compartment, for parasegment border for-

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mation and for neuroblast specification (Martinez-Arias et al., 1988; Bejsovec and Martinez-Arias, 1991; Chu-LaGraff and Doe, 1993). Slightly later, *wg* expression becomes independent of *hh* but remains dependent upon *wg* and the segment polarity gene *gooseberry* (*gsb*) (Li and Noll, 1993; Li et al., 1993). Ultimately, restricted *wg* expression is necessary for correct patterning of the ventral denticles of the larval epidermis (Lawrence et al., 1996).

hh encodes a secreted protein that activates a signal transduction pathway essential for maintenance of *wg* expression (Lee et al., 1992). In *hh* mutant embryos *wg* expression is initiated normally but is not maintained after stage 10 (Hidalgo and Ingham, 1990). Maintenance of *wg* expression by *hh* signaling depends on the activity of several gene products, *patched* (*ptc*), a transmembrane receptor protein, *smoothened* (*smo*), a transmembrane protein, *fused* (*fu*), a serine/threonine kinase, *costal* (*cos*) and a zinc finger containing protein, *cubitus interruptus* (*ci*) (Orenic et al., 1990; Preat et al., 1990; Forbes et al., 1993; Ingham and Hidalgo, 1993; Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Ptc and Smo associate to form a probable Hh receptor in which Ptc has ligand binding activity and Smo initiates an intracellular signaling cascade (Alcedo et al., 1996; Stone et al., 1996). Although the details of their function are not clear, *fu* and *cos* appear to be part of the intracellular signaling cascade. Genetically, *ci* appears to be the final step in *hh* signaling prior to transcriptional activation of target genes such as *wg* (Von Ohlen et al., 1997).

Ci is necessary for Hh-responsive gene expression. Although by immunofluorescence Ci appears to be predominantly cytoplasmic (Motzny and Homgren, 1995), it acts as a transcriptional regulator (Alexandre et al., 1996; Akimaru et al., 1997; Von Ohlen et al., 1997). Ci protein and mRNA are present in all cells of the anterior compartment (Eaton and Kornberg, 1990; Orenic et al., 1990; Motzny and Homgren, 1995) and transcriptional activation by Ci is restricted to cells immediately adjacent to Hh expressing cells. This suggests that activation of Ci by Hh must be post-transcriptional. Alexandre et al. (1996) showed that a fragment of *ptc* promoter containing Gli/Ci binding sites could respond to Sonic hedgehog in imaginal discs and that deletion of a large region including those sites abolished responsiveness. We significantly advance their work by using site-directed mutagenesis to show that it is specifically the Ci binding sites within an enhancer fragment that bestow Hh responsiveness in vivo. Previously, we have demonstrated that a 1 kb element of the *wg* enhancer is sufficient for activation of transcription by Ci in *Drosophila* Schneider line 2 (S2) cells. This element contains four Ci binding sites and mutagenesis of these binding sites abolishes transcriptional activation by Ci (Von Ohlen et al., 1997). Here we demonstrate that both *hh* and *ptc* can regulate transcription from this *wg* enhancer element in S2 cells by modulating Ci activity. We also define this element of *wg* enhancer as Ci-dependent and *hh*-responsive in vivo.

2. Results

hh and *ptc* have opposite effects on *wg* expression in vivo during segmentation. In *ptc* mutant embryos *wg* expression expands to fill the *wg*-competent domain (Ingham et al., 1991). In *hh* mutants *wg* expression disappears at late stage 10 (Martinez-Arias et al., 1988). Thus, *hh* is defined as an activator and *ptc* as a repressor of *wg* expression. Previously, we have used a combination of transient transfection, DNA binding and site-directed mutagenesis to demonstrate that Ci is a transcriptional activator whose interaction with specific target sequences within a fragment of *wg* promoter is essential for transcriptional activation in S2 cells (Von Ohlen et al., 1997). Here we use the same Ci-dependent transcription assay in S2 cells to address whether *hh* and *ptc* regulate target gene expression by regulating Ci activity (Fig. 1A). As we have previously described, a 1 kb fragment of *wg* promoter driving a luciferase reporter gene (Δ*wg*-Luc) is transcriptionally activated by co-transfected Ci cDNA (*mt*-Ci). The response is dose-dependent over a

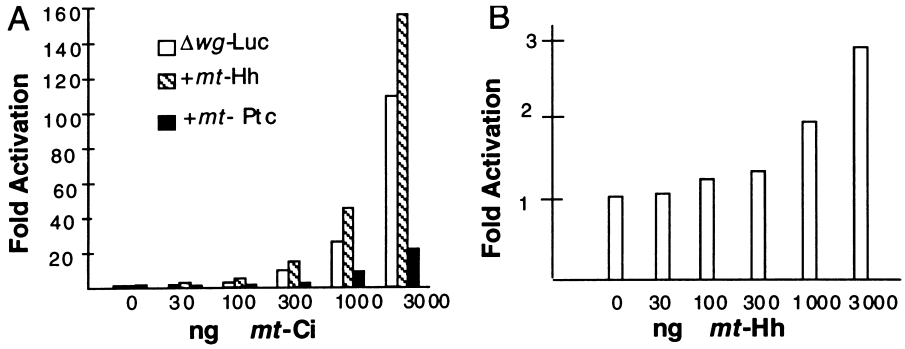

Fig. 1. Hh and Ptc modulate Ci activity in S2 cells. (A) Transfection of increasing amounts of the Ci expression plasmid (*mt*-Ci) in the presence of 500 ng of the Hh expression plasmid (*mt*-Hh) (striped bars), or 500 ng of the Ptc expression plasmid (*mt*-Ptc) (solid bars). Compare to open bars in absence of either. All transfections contained 200 ng of Luciferase reporter plasmid (Δ*wg*-Luc). Fold increases are shown relative to zero Ci. All transfections were done in parallel and variability between duplicates was less than 14%. (B) Transfection of increasing amounts of *mt*-Hh in the presence of 200 ng *mt*-Ptc and 300 ng *mt*-Ci. Fold increase is shown relative to activity in the absence of Hh.

wide range of Ci cDNA concentrations with a greatest activation observed of 110-fold (Fig. 1A, open bars) and the response requires intact Ci binding sites in the Δ*wg*-Luc promoter (see Von Ohlen et al., 1997). S2 cells can process and secrete active Hh (Thérond et al., 1996). To test for effects of Ptc and Hh, we performed parallel titrations of Ci cDNA either alone, in the presence of constant Hh cDNA (500 ng *mt*-Hh, striped bars), or in the presence of constant Ptc cDNA (500 ng *mt*-Ptc, solid bars). We found that the reporter luciferase activity was approximately 3-fold inhibited in the presence of co-transfected Ptc. This was true across the entire range of Ci levels tested. Ptc has no effect on the basal level of Δ*wg*-Luc activity seen in the absence of Ci. Previously, we used site-directed mutagenesis to introduce four single base changes into Δ*wg*-Luc that virtually abolish binding of Ci to this enhancer fragment (Von Ohlen et al., 1997). This mutated construct (Δ*wg**-Luc) which is no longer transcriptionally activated by Ci is also unresponsive to the addition of Ptc (data not shown). Thus, Ptc exerts its effects on transcription of Δ*wg*-Luc in S2 cells through modulating Ci.

The addition of Hh resulted in a 1.5-fold increase in luciferase activity over that observed for Ci alone. As with Ptc, the effect of Hh is dependent upon the presence of co-transfected Ci. Hh also had no effect on the mutated construct, Δ*wg**-Luc. Therefore, like Ptc, Hh also exerts it effects by modulating Ci activity. Although significant (see Section 4), the 1.5-fold increase observed was less than expected given the potent effect of Hh in vivo. One explanation for this might be that S2 cells do not express appropriate levels of the proteins necessary for transduction or reception of the Hh signal.

Biochemical and genetic studies suggest that Smo and Ptc together form a receptor complex for Hh and that the balance between Hh, Ptc and Smo determines the activity of the *hh* signaling pathway (Alcedo et al., 1996; Stone et al., 1996). In the absence of *hh*, *ptc* renders *smo* inactive while in the absence of *ptc*, *smo* is constitutively active and is no longer regulated by *hh*. We examined the levels of endogenous *hh*, *ci*, *ptc* and *smo* mRNAs in the S2 cells by Northern blot analysis (Fig. 2). We found *smo* mRNA expression similar to levels found in embryos. *ptc* expression is detectable on long exposures but present at much lower levels than in embryos, while *hh* (data not shown) and *ci* expression are undetectable. The high levels of *smo* expression relative to *ptc* expression suggests that the downstream signal transduction pathway should be relatively active in the absence of Hh and relatively insensitive to the presence of Hh. If this were true then increasing levels of *ptc* should reconstitute Hh responsiveness of S2 cells. To test this, we titrated Hh in the presence of moderate levels of Ptc and Ci (Fig. 1B). Under these conditions, Hh increased luciferase activity in a dose-dependent manner up to threefold over the range of Hh titrated (Fig. 1B). The presence of additional *ptc* reduces Ci activity. Further addition of *hh* restores Ci activity to levels similar to that observed in the absence of either *hh* or *ptc*. Thus, addition of Ptc improves the Hh responsiveness of S2 cells, perhaps by reconstituting a functional Hh receptor. As a result, Ci-dependent transcriptional activation is modulated by Hh, much as transcription of Hh-responsive genes is Ci-dependent and Hh-modulated in vivo.

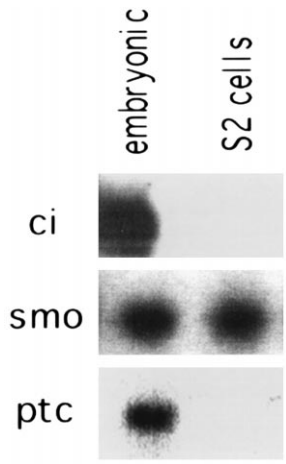

Fig. 2. Comparison of RNA levels in embryos and S2 cells. Northern blots of total RNA were probed for *ci* mRNA (top), *smo* mRNA (middle) and *ptc* mRNA (bottom). Equal loading for all lanes was confirmed by ethidium bromide detection of ribosomal RNA.

2.1. Δwg also regulates Hh-dependent gene expression in vivo

We have identified a 1 kb fragment of *wg* enhancer that is sufficient to support Ci-dependent Hh-modulated transcriptional activation in S2 cells. Mutagenesis of the Ci binding sites in this fragment provided compelling evidence that binding to specific enhancer sequences is essential both for Ci to activate transcription (Von Ohlen et al., 1997) and for Hh to regulate transcription (data not shown). To test whether the same is true in vivo, we constructed transgenic flies with either the wild-type (Δ*wg-lacZ*) or mutagenized (Δ*wg*-lacZ*) *wg* promoter fragments driving *lacZ* expression.

Expression of *lacZ* in Δ*wg-lacZ* transgenic flies accurately reproduces most aspects of Hh-dependent *wg* expression in embryos from stages 11 to 13 (Fig. 3D–F). Like endogenous *wg*, Δ*wg-lacZ* is expressed in single cell wide stripes in the ventral ectoderm. Also present are the dorsal spots in each segment and the wide stripe in the mandibular segment. This fragment of *wg* enhancer does not direct many *hh*-independent aspects of expression. For instance, embryos younger than stage 11 do not express *lacZ* at detectable levels, while endogenous *wg* is expressed at earlier stages but in a *hh*-independent manner. It is at stage 11 that *wg* expression is lost in *hh* mutant embryos, identifying this as the stage when *wg* expression becomes Hh-dependent. During gut development *wg* is expressed in the mid-, fore- and hind-gut in a *hh*-enhanced but not *hh*-dependent manner (Fig. 4A,B) (Hoch and Pankratz, 1996). *lacZ* mes-

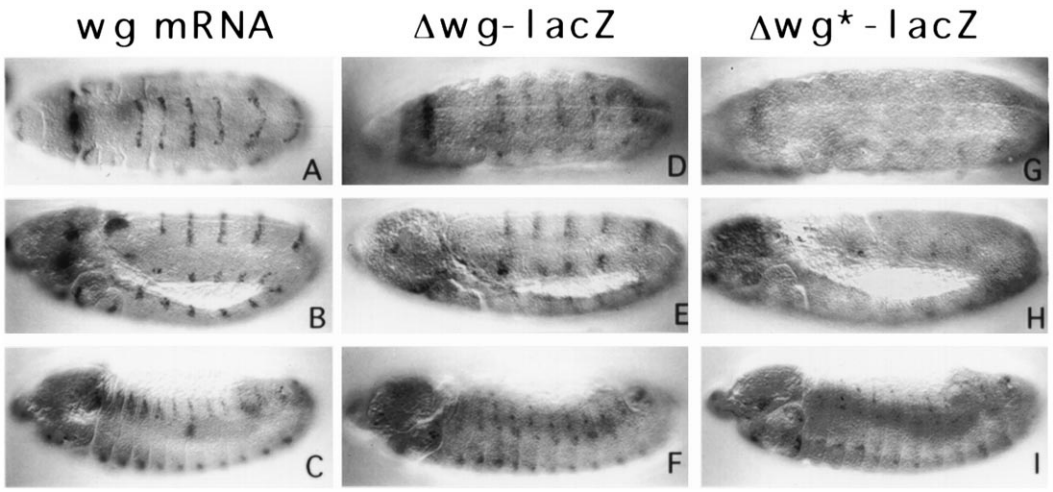

Fig. 3. In vivo expression of Δ*wg*-lacZ and Δ*wg**-lacZ, lacZ mRNA detected in transgenic embryos by in situ hybridization. (A,B) Endogenous *wg* mRNA expression in wild-type stage 11 embryos. (C) *wg* mRNA expression in a stage 13 embryo. (D,E) *lacZ* mRNA expression in Δ*wg*-lacZ stage 11 embryos. (F) *lacZ* expression in a stage 13 embryo. (G,H) *lacZ* mRNA expression in Δ*wg**-lacZ stage 11 embryos. (I) *lacZ* mRNA expression in Δ*wg**-lacZ stage 13 embryos. (A,D,G) Dorsal views. (B,E,H) Ventral views.

sage is not detected in these areas in Δ*wg*-lacZ embryos (Fig. 4C,D). Together these data suggest that the element of *wg* promoter/enhancer we have identified is largely specific for Hh-dependent aspects of *wg* expression.

In order to demonstrate that Δ*wg-lacZ* expression was dependent upon Ci binding sites in the *wg* enhancer fragment, we examined *lacZ* expression from the mutagenized enhancer. Δ*wg**-*lacZ* transgenic embryos are lacking most aspects of *lacZ* seen with Δ*wg-lacZ* (Fig. 3G–I). In particular, the segmental stripes are absent. The dorsal spots remain as does the stripe in the mandibular segment. This is similar to what happens to endogenous *wg* expression in *hh* or *ci* mutant embryos (Hidalgo and Ingham, 1990). This result demonstrates that binding of Ci protein to sequences in *wg* promoter/enhancer is necessary for Hh-dependent expression in transgenic embryos. This is consistent with results seen previously in S2 cells and in vitro binding assays (Von Ohlen et al., 1997). Taken together these results suggest that Ci binding to these sequences is necessary for Hh-dependent activation of *wg* transcription.

2.2. Expression of Δwg-lacZ is modulated in vivo by ci, hh and ptc

ci is required for maintenance of *wg* expression but not for its initiation. In ci^{-} embryos *wg* expression is initiated normally but fades during late stage 10/early stage 11 (Forbes et al., 1993; van den Heuvel et al., 1993; Motzny and Homgren, 1995). This corresponds to the time at which we see *lacZ* expression activated in Δ*wg-lacZ* transgenic embryos suggesting that this fragment of *wg* enhancer is the element directly required for Ci mediated *wg* transcription. To demonstrate that Ci activity is required for *hh* mediated *wg* expression we examined *lacZ* mRNA expression from Δ*wg-lacZ* in the near null *ci* allele ci^{Ce} (Orenic et al., 1987). In ci^{Ce} mutant embryos expression of Δ*wg-lacZ* is absent from stripes in the ectoderm (Fig. 5C). Thus, Ci activity is necessary for transcription from the enhancer fragment in Δ*wg-lacZ* embryos as it is for *wg* transcription in wild-type embryos.

Modulation of luciferase activity in S2 cells by addition

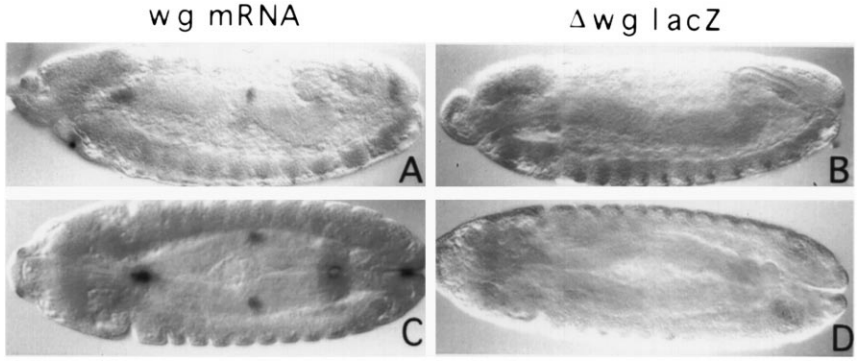

Fig. 4. Δ*wg*-lacZ does not regulate non-Hh-responsive aspects of *wg* expression. (A,C) Endogenous *wg* mRNA expression in gut. (B,D) lacZ mRNA expression in Δ*wg*-lacZ.

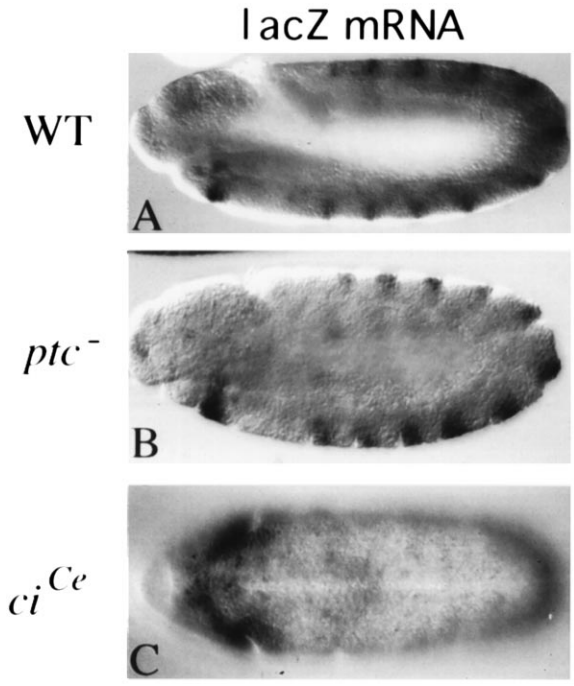

Fig. 5. Δ*wg*-lacZ expression in *ci* and *ptc* mutants. (A) Δ*wg-LacZ* mRNA in a stage 11 wild-type embryo. (B) Δ*wg*-LacZ mRNA expression in stage 11 ptc^{6P43} embryos. (C) Δ*wg*-LacZ mRNA expression in a stage 11 ci^{Ce} embryo.

of *ptc* and/or *hh* suggests that both Hh and Ptc affect transcription by modulating Ci activity. To determine if this is also true in vivo we examined the effects of *ptc* and *hh* on *lacZ* expression in Δ*wg-lacZ* embryos. In *ptc* mutant embryos *wg* expression is expanded anteriorly, filling half of the parasegment (Martinez-Arias et al., 1988). Ubiquitous Hh expression under control of a heat shock promoter (*Hs-hh*) results in similar anteriorly expanded stripes of *wg* expression (Ingham, 1993). We predicted that *lacZ* expression would be expanded in both Δ*wg-lacZ*; ptc^{-} embryos and in *Hs-hh*; Δ*wg-lacZ* embryos. When we examined Δ*wg-lacZ* expression in ptc^{-} embryos, we observed expanded *lacZ* stripes similar to the *wg* stripes described above (Fig. 5B). When we analyzed *lacZ* expression in *Hs-hh* in Δ*wg-lacZ* embryos again we observed expansion of *lacZ* stripes similar to but less regular than that seen in *ptc* mutants (data not shown). These results demonstrate that we have mapped a Hh response element in *wg* enhancer. These data also suggest that because both *hh* and *ptc* act through an enhancer element that requires Ci binding sites, they also affect transcription through modulating Ci activity.

3. Discussion

This paper demonstrates that Hh and Ptc exert their effects on *wg* transcription through Ci and its ability to bind DNA. We had previously used a 1 kb fragment of *wg* promoter in S2 cells to demonstrate that binding of Ci to its recognition sequences is essential for Ci to activate transcription. Here we use the same promoter fragment, both in vivo and in S2 cells, to show that Hh regulates transcription through Ci and its DNA binding sites in target promoters. We also define this element of *wg* enhancer as that which directs Hh-responsive *wg* transcription. We conclude that Hh regulates transcription of its target genes by modulating the activity of Ci, its transcriptional effector.

Ci can function both as a transcriptional activator and as a repressor. The state of *hh* signaling dictates which function Ci adopts. In the absence of Hh signaling Ci is proteolytically processed to the repressor form, whereas in the presence of *hh* signaling Ci functions as an activator (Aza-Blanc et al., 1997; Von Ohlen et al., 1997). Ci is not processed to the repressor form in S2 cells (Aza-Blanc et al., 1997). This is expected given the data presented here showing that *hh* signaling is constitutively active in S2 cells. Thus, monitoring transcription in S2 cells allows us to look at the activator function of Ci in the absence of the confounding effects of processing to the repressor form. Our results strongly suggest that Ci is activated in response to Hh signaling. That is, modulation of Ci by Hh involves both inhibition of processing and some as yet unknown activation step. Unprocessed Ci can be found in a complex with the kinesin related protein, Costal2 (Cos2) and the serine/threonine kinase Fused (Fu) (Robbins et al., 1997; Sisson et al., 1997). Components of the complex are likely to be involved in the activation step.

It is clear that Ci activation is post-transcriptional from at least two independent lines of evidence. First, Ci mRNA is present at significant levels in all anterior compartment cells, whether or not they are receiving and responding to the Hh signal (Eaton and Kornberg, 1990; Orenic et al., 1990; Motzny and Homgren, 1995). Second, Ci transcription in our S2 cell assay is from a heterologous promoter and so cannot be regulated by Hh. Ci protein accumulates to higher levels in cells which have been activated by Hh, but significant levels of Ci protein are present in unstimulated cells as well (Johnson et al., 1995; Motzny and Homgren, 1995). This makes it likely that an important part of Ci regulation is via protein modification, either directly through modification of Ci or indirectly through modification of a Ci-associated protein. Indeed, stoichiometric association with a negative regulatory protein is suggested by the observation that overexpression of wild-type Ci can drive expression of Hh-responsive genes, even in the absence of Hh (Alexandre et al., 1996). The most provoking aspect of Ci is that it is cytoplasmically localized, both in unstimulated and in stimulated cells (Motzny and Homgren, 1995). Since DNA binding is essential for Ci transcriptional activity (Von Ohlen et al., 1997) and this must take place in the nucleus, the active levels of Ci which reach the nucleus must be very low. Consistent with this, 10-fold more *mt*-Ci DNA must be transfected into S2 cells for detection of Ci expression by immunofluorescence than the amount of *mt*-

Ci DNA which gives maximal transcriptional activation (T.V.O. and J.E.H., unpublished observation).

Recently *Drosophila* CBP has been identified as essential for expression of the Hh-responsive gene *ptc*, to enhance Ci-activated transcription in transiently transfected cells and to bind to the carboxy terminal region of Ci (Akimaru et al., 1997). In mammalian systems CBP has been found to associate with CREB and several other transcriptional activators (Arias et al., 1994; Kwok et al., 1994; Bannister and Kouzarides, 1995; Bhattacharya et al., 1996; Dai et al., 1996). CBP is thought to facilitate activation of the basal transcription apparatus. The histone acetyltransferase activity of CBP may function to alter or disrupt chromatin structure (Ogryzko et al., 1996). In mammalian systems CREB is activated by PKA phosphorylation. *Drosophila* PKA is a negative regulator of Hh responsive gene expression (reviewed in Perrimon, 1995). Binding of CBP to Ci occurs in a phosphorylation-independent manner whereas CBP binding to dCREB is phosphorylation-dependent (Akimaru et al., 1997). It is possible that competition for CBP binding between Ci and CREB could be a factor in Ci activation (Akimaru et al., 1997).

The tissue culture assay for Hh signaling which we have developed gives dose-dependent responses to Hh in terms of easily assayed luciferase activity. It is regulated by a balance of Hh, Ptc and Smo, just as Hh-responsive gene expression is regulated in vivo. It offers opportunities to manipulate physiological, pharmacological and genetic conditions in a homogeneous cell population. It should be very useful for future studies addressing how Ci is activated by Hh.

4. Experimental procedures

4.1. Fly stocks

yw; *Hs-hh M3/TM3 y*$^{+}$ *Ser* flies were obtained from Phil Ingham (Imperial Cancer Research Fund). *yw*; Δ*wg*-LacZ and *yw*; Δ*wg**-LacZ; transgenic flies were made according to standard procedures (Spradling and Rubin, 1982). Transgenes Δ*wg*-LacZ and Δ*wg**-LacZ are both homozygous viable on chromosome II. One line of each was obtained and tested.

4.2. In situ hybridizations

In situ hybridizations utilized antisense-strand riboprobes according to standard procedures (Tautz and Pfeiffle, 1989; Jiang et al., 1991). LacZ antisense probe was a gift from Dr Grace Panganiban (University of Wisconsin, Madison, WI).

4.3. Northern blots

Total RNA was isolated from unstaged wild-type embryos and from non-transfected S2 cells according to Chomczynski and Sacchi (1987); 10 μg of each was loaded per lane and separated on formaldehyde gels (Maniatis et al., 1982), confirmed by ethidium bromide detection of ribosomal RNA, transferred to zetabind (Cuno) and probed with appropriate ^{32}P-labeled probes according to Maniatis et al. (1982).

4.4. Recombinant DNAs

mt-Hh was constructed by inserting a 1.6 kb *Mse*I fragment of Hh cDNA coding sequence into the *Hinc*II site of the inducible expression vector pRmHa-1 (Bunch et al., 1988). *mt*-Ptc was constructed by insertion of the *ptc* coding region described in Schuske et al. (1994) into the *Hinc*II site of pRmHa-1. *mt*-Ci was also made be insertion into pRmHa-1 as described in Von Ohlen et al. (1997).

Δ*wg*-lacZ and Δ*wg**-lacZ were constructed by excision of the *Bgl*II/*Bam*HI fragments containing the promoter fragments and the thymidine kinase minimal promoter from Δ*wg*-Luc and Δ*wg**-Luc, respectively (Von Ohlen et al., 1997). These were inserted into the *Bam*HI site of CasAUGβgal (Thummel et al., 1988).

4.5. Transient transfections

Cell culture, transfection, induction and analysis is described in Von Ohlen et al. (1997). Briefly, *Drosophila* Schneider line 2 cells were transfected using the calcium phosphate precipitation method (Han et al., 1989). Each transfection contained 200 ng Δ*wg*-Luc reporter plasmid, variable amounts of expression plasmids, *mt*-Ci as indicated and variable amounts of pRmHa-1 to bring the total amount of expression plasmid to 3 μg. As an internal control for transfection efficiency 1 μg of *copia* long terminal repeat-*lacZ* was added to each transfection (gift from J. Manley). β-Galactosidase assays were conducted as described in Miller (1972). Luciferase assays were done as in de Wet et al. (1987). Transfections that included Hh and Ptc expression vectors contained 500 ng of each of these or variable amounts as indicated. All data points are the mean of duplicate samples and are normalized to β-gal internal control for transfection efficiency. Variation between duplicates was never more than 14%. To determine if the difference between fold activation observed ±Hh was significant, we applied a paired *t*-test with independent variances. According to these numbers we can accept with 99.5% certainty that the observed differences are significant.

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