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Engrailed and *polyhomeotic* maintain posterior cell identity through *cubitus-interruptus* regulation☆

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Abstract

In *Drosophila*, the subdivision into compartments requires the expression of *engrailed* (*en*) and *hedgehog* (*hh*) in the posterior cells and of *cubitus-interruptus* (*ci*) in the anterior cells. Whereas posterior cells express *hh*, only anterior cells are competent to respond to the *hh* signal, because of the presence of *ci* expression in these cells. We show here that *engrailed* and *polyhomeotic* (*ph*), a member of the Polycomb Group (*PcG*) genes, act concomitantly to maintain the repression of *ci* in posterior compartments during development. Using chromatin immunoprecipitation (ChIP), we identified a 1 kb genomic fragment located 4 kb upstream of the *ci* coding region that is responsible for the regulation of *ci*. This genomic fragment is bound in vivo by both Polyhomeotic and Engrailed. In particular, we show that Engrailed is responsible for the establishment of *ci* repression early during embryonic development and is also required, along with Polyhomeotic, to maintain the repression of *ci* throughout development.

Keywords: *Drosophila*; *Polyhomeotic*; *Engrailed*; *Cubitus-interruptus*; Antero-posterior identities

Introduction

During the development of multicellular organisms, cells not only have to proliferate and differentiate, but must, sometimes, segregate from each other. Indeed, certain insect and vertebrate tissues are subdivided into non-intermingling sets of cells called compartments (Blair, 1995; Dahmann and Basler, 1999; Lawrence and Struhl, 1996; Vincent, 1998). Cells from one compartment are kept segregated from cells of a neighboring compartment, leading to a clear boundary between the two sets of cells. This compartmentalization of the cells plays important roles in further pattern formation. The segregation into distinct posterior (P) and anterior (A) compartments is indeed crucial throughout development.

It is therefore important to identify the different factors that are involved in the continuous segregation of A and P cells during development. Among the factors known to be involved in this process is the transcription factor Engrailed (En) that is only expressed in P cells.

En and the products of the segmentation genes are involved in a regulatory cascade that sets the initial transcriptional state of developmental genes, such as the homeotic genes. Later, transcription patterns of homeotic genes are maintained by genes of the *Polycomb* (*PcG*) and the *trithorax* (*trxG*) groups. *PcG* genes were first identified based on their required role in the maintenance of the repressed state of homeotic genes (Soto et al., 1995; Struhl and Akam, 1985). PcG proteins form multimeric complexes that bind to specific chromosomal elements termed Polycomb Group response elements (PREs), and these complexes are thought to maintain gene silencing by acting on the chromatin structure of their target genes. PREs have been identified in transgenic lines by their ability to render the expression of reporter genes responsive to *PcG* genes. Other features of PREs have been described. In particular, transgenes containing a PRE as well as the mini-*white* reporter gene led to the identification of a phenomenon called Pairing-sensitive silencing, in which homozygous lines express lower levels of mini-*white* than the hetero-

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zygous ones, and often show a variegated pattern of expression of this eye-reporter gene (Kassis, 2002).

Several PREs have also been shown to be able to maintain the memory of early regulatory decisions throughout development. In transgenic constructs, these elements drive neighboring reporter genes into silent states in the absence of transcriptional activation. On the other hand, if the reporter is strongly activated by an embryonic pulse of activator, the active state is maintained by the PRE even after the disappearance of the activator. For this reason, these elements were renamed cellular memory modules (CMM) (Cavalli and Paro, 1998; Maurange and Paro, 2002; Orlando, 2003; Poux et al., 2002; Rank et al., 2002).

In addition to the regulation of homeotic genes, PcG proteins have also been shown to play an important role in the regulation of limb patterning genes (Beuchle et al., 2001; Randsholt et al., 2000). Indeed, Polyhomeotic (Ph), which is a member of the PcG, was found to regulate not only *en* (Dura and Ingham, 1988; Smouse et al., 1988), but also other genes of the *en/hh* signaling pathway (Maurange and Paro, 2002; Randsholt et al., 2000).

During wing morphogenesis, the posterior cells (P) express the selector gene *en*, which directs these cells to secrete the short-range signaling molecule Hedgehog (Hh) (Kornberg et al., 1985; Tabata and Kornberg, 1994). Whereas P cells that express *hh* are refractory to respond to the Hh signal, anterior cells (A), which do not express *en*, can respond to Hh (Lawrence and Struhl, 1996; Tabata et al., 1992, 1995; Zecca et al., 1995). This response requires the presence of the transcription factor Cubitus-Interruptus (Ci) in the A cells (Alexandre et al., 1996; Aza-Blanc and Kornberg, 1999; Dominguez et al., 1996).

ph null clones induced in the A compartment show ectopic expression of *en* and *hh*, whereas *ph* null clones induced in the P compartment show an activation of *ci* (Randsholt et al., 2000). These results suggested that the *PcG* genes might be involved in the maintenance of A cell identity by repressing *en* and *hh* (Dura and Ingham, 1988; Maurange and Paro, 2002), and raised the question of whether they might also be involved in the maintenance of P cell identity by repressing *ci*.

It has been clearly established that *en* regulates the posterior developmental pathway by controlling the expression of *ci* both during embryogenesis and during wing development (Eaton and Kornberg, 1990; Schwartz et al., 1995). Moreover, *ph* is directly activated by *en* at different times during development (Maschat et al., 1998; Serrano and Maschat, 1998; Serrano et al., 1995). During wing morphogenesis, *en* and *ph* interactions were found to be important in the maintenance of the position of the A/P boundary and in the incompetence of the P cells to respond to the Hh signal (Maschat et al., 1998). However, the role of *ph* activation by *en* during embryogenesis is not yet understood (Serrano et al., 1995). One hypothesis is that *ph* activation in embryonic P compartments might be involved in the maintenance of *ci* repression and thus of P cell identity. En and Ph could both be involved in the repression of *ci*, En being involved in the establishment of repression, and Ph (with the PcG proteins) in its maintenance. The activation of *ph* by En during embryogenesis and later on during wing morphogenesis would thus ensure the maintenance of *ci* repression during development, which is necessary to maintain the P cell identity.

In this report, we present evidence showing that *ci* is directly repressed by *ph* during embryogenesis. Upstream of the *ci* transcription unit, we identified a 1 kb genomic region that binds both Ph and En in vivo. Whereas En is necessary for both establishment and maintenance of *ci* repression, Ph is only necessary to maintain *ci* repression at later stages. The results suggest that *ci* repression might involve En and PcG separately to ensure the antero-posterior segregation of the cells. *ph* might play a central role in the elaboration of this regulation, since *ph* is directly activated by En in the P compartments, and may recruit other PcG proteins.

Taken together, these results show that *ph* is an important target of *en* regulation, since it is involved in the maintenance of *ci* repression and of the P cell identity, functions that were previously attributed only to *en*.

Materials and methods

Fly strains

The Oregon-R strain was used as wild type. *ci-7.1-LacZ* is a transgenic line containing 7.1 kb of *ci* upstream region in a transgene localized in 63C on chromosome 3; this line was provided by T. Kornberg (Schwartz et al., 1995). ph^{505} (referred as ph^{0}) is an amorphic, lethal allele (Dura et al., 1987). ph^{410} is an hypomorphic allele, corresponding to an inversion that inactivates the proximal unit of the *ph* locus (Dura et al., 1987). Heterozygous *tramtrack* mutation (ttk^{804}) has been used to express *engrailed* ectopically in salivary glands (Fauvarque and Dura, 1993; Solano et al., 2003). The following transgenic stocks were used: UAS-Engrailed (referred as En) (Tabata et al., 1995); UAS-VP16-HA-Engrailed (referred as VP16-En) (Alexandre and Vincent, 2003), (hs-Gal4), allowing ubiquitous Gal4 expression after heat shock and (*MS1096*-Gal4), allowing expression of Gal4 in the entire wing pouch during larval development (Milan and Cohen, 2000). Transgenic lines were obtained by cloning Ci-A region into WH.GFP vector, where fragment has been inserted upstream of the *hsp70* minimal promoter and the GFP reporter gene (Solano et al., 2003).

Antibodies

Anti-En antibodies are rabbit polyclonal antibody against the entire protein (anti-En) used for immunostaining of embryos (1:200) (Randsholt et al., 2000); rabbit polyclonal antibody against a truncated form of En lacking the homeodomain (anti-En-ΔHD) for immunostaining of polytene chromosomes (1:40) and for chromatin immunoprecipitation

(ChIP) (1:15). Monoclonal Ci antibody is used for immunostaining of embryos (1:100) (Randsholt et al., 2000). Anti-Ph antibody is a rabbit polyclonal used either for ChIP (1:50) or for immunostaining (1:200).

Chromatin immunoprecipitation and Southern blot analysis

Chromatin immunoprecipitation (ChIP) was performed from staged, 0–4, 4–8, or 8–21 h wild-type embryos using a standard procedure (Cavalli et al., 1999) that was modified as follows. Approximately 1 g of embryos were cross-linked in 5 ml buffer A1 (60 mM KCl, 15 mM NaCl, 15 mM Hepes (pH 7.6), 4 mM $MgCl_2$, 0.5% Triton X-100, 0.5 mM DTT, and complete EDTA-free protease inhibitor cocktail [ROCHE]), in the presence of 1.8% formaldehyde, by homogenization in a Potter and then in a Dounce homogenizer with type A pestle (three strokes each), followed by incubation for 15 min at room temperature. Cross-linking was stopped by adding glycin to 225 mM for 5 min. The homogenate was transferred to a 15-ml tube and centrifuged for 5 min, 4000 *g* at 4 °C. The supernatant was discarded and the crude nuclei pellet was washed three times in 3 ml A1 buffer and once in 3 ml A2 buffer (140 mM NaCl, 15 mM Hepes (pH 7.6), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, and protease inhibitors) at 4 °C. After the washes, nuclei were resuspended in A2 buffer in the presence of 0.1% SDS and 0.5% N-lauroylsarcosine (0.5 ml per 0.15 g of cross-linked embryos), and incubated for 10 min in a rotating wheel at 4 °C. After sonication (four pulses of 30 s with 2-min intervals, using a Branson Sonifier 250 equipped with a 5-mm probe, and regulated at power 2, duty cycle 100%,) and 10 min high speed centrifugation, fragmented chromatin (DNA fragment size ranging from 200 bp to 5 kb) was recovered in the supernatant. Cross-linked chromatin was loaded on CENTRICON YM-100 columns (AMICON cut-off 100 kDa) and washed with 3 volumes of A2 buffer in presence of 0.05% SDS (referred as A3 buffer) at 4 °C to eliminate uncross-linked proteins and small DNA fragments. For 1 g of embryos, approximately 500 μg of chromatin were recovered.

For each immunoprecipitation, 50 μg of chromatin in A3 buffer was pre-incubated in the presence of 100 μl of Protein A-Sepharose suspension (Amersham Pharmacia, 50% w/v, called PAS) for 1 h at 4 °C. PAS was removed, antibodies were added (a control in the absence of antibody named Mock IP was included), and samples were incubated overnight at 4 °C in a rotating wheel. Then, 50 μl of PAS was added and incubation was continued for 4 h at 4 °C. Samples were centrifuged at maximum speed for 1 min and the supernatant was discarded. Samples were washed four times in A3 buffer and twice in 1 mM EDTA, 10 mM Tris (pH 8) (TE) buffer (each wash 5 min at 4 °C). Chromatin was eluted from PAS in two steps; first in 100 μl of 10 mM EDTA, 1% SDS, 50 mM Tris (pH 8) at 65 °C for 10 min, followed by centrifugation and recovery of the supernatant. PAS material was re-extracted in 150 μl of TE, 0.67% SDS. The combined eluate (250 μl) was incubated 6 h at 65 °C to reverse cross-links and treated by Proteinase K for 3 h at 50 °C and by RNaseI. Samples were phenol–chloroform extracted and ethanol precipitated in the presence of 20 μg glycogen. DNA was resuspended in 25 μl of water and treated with 10 Units of Polynucleotide Kinase (New England Biolabs) for 30 min at 37 °C. Staggered DNA ends were repaired by incubation with 5 Units of Klenow fragment polymerase (PROMEGA) for 30 min at 37 °C in the presence of 2 mM of dNTPs. Linkers were ligated to the DNA, and Linker-mediated PCR was performed as previously described (Cavalli et al., 1999). After removal of nucleotides and oligos, the amplified DNA amounts were accurately determined by loading 1 μl of DNA on a Slot Blot membrane (PerkinElmer life sciences) containing a standard curve of genomic DNA, and hybridizing the slot blot membrane with a radiolabeled genomic DNA probe (prepared by using the Rediprime kit labeling system of Amersham Pharmacia, following manufacturer's instructions). Signals were quantified by PhosphorImager and Imagequant v1.2 software. Seventy-five nanograms of this PCR-amplified DNA purified after ChIP either in the presence or in the absence of antibody (Mock IP) was radioactively labeled by the random priming Rediprime kit. These labeled probes were used to hybridize Southern blots containing 6 kb from the *ci* genomic region to be analyzed. *ci* upstream region (covering region −7024 to −1093) were obtained by *Spe*I/*Pst*I/*Eco*RI digestions of the Pcs100-Ci vector that contains 11 kb of *ci* genomic sequences (Schwartz et al., 1995). To obtain the Southern membranes, restriction digested DNA was run on 1% agarose gels at 40 V and blotted on a nylon membrane (PerkinElmer life sciences). The remainder downstream 5 kb *ci* region, as well as the vector, is cut from the gel before blotting. Signals were quantified by PhosphorImager and the ratio of signals from α-Ph IP and α-En IP samples versus signals from the Mock IP gave rise to the Fold Enrichment values plotted on the *Y* axis of Fig. 2.

Immunostaining and in situ hybridization

Embryos were fixed as described in Sullivan et al. (1993) and stained with different antibodies diluted to the desired concentration as indicated. Dissected L3 larvae were fixed in conditions described in Solano et al. (2003) and either immunostained or hybridized by DIG-labeled antisense RNA probe prepared from pGEM-*ci* cDNA (Orenic et al., 1990) in conditions described in Alexandre et al. (1996).

Constructions of transgenic lines

Ci-A genomic fragment was obtained by PCR performed on the Pcs100-Ci vector and was cloned in WH.GFP, upstream of the *hsp*70 promoter and the GFP reporter gene. The construct was injected in w^{1118} embryos. Ten independent transgenic lines were obtained, which express the GFP according to a same pattern similar to the *ci* expression. GFP expression was analyzed in detail in three lines, and is

shown in one of them in Fig. 3. Otherwise, immuno-FISH experiments have been performed on a Ci-A-GFP line inserted on chromosome 2.

Heat shock procedure

Heat shock (Hs) was performed on early and mid-L3 larvae during 1 h at 37°C to induce Gal4 in (*hs*-Gal4/Ci-A-GFP; UAS-En/+). Larvae were dissected at late L3, 24 h or 48 h after *hs*, as indicated.

Immuno-FISH on polytene chromosomes

This protocol of immuno-FISH was performed as previously described (Dejardin and Cavalli, 2004; Lavrov et al., 2004). Briefly, after a standard immunostaining protocol (Franke et al., 1992) with first primary antibody and then secondary anti-rabbit antibody coupled to Cy3, chromosomes were washed for 15 min in PBS supplemented with 300 mM NaCl, 0.1% Tween 20, and PBS-400 mM NaCl, 0.1% Tween 20. Slides were post-fixed 15–20 min in PBS-3.7% formaldehyde. Then, slides were washed in PBS several times and dehydrated by passing through 70% (2 × 5 min) and 95% (2 × 5 min) ethanol. Slides were air-dried. Next, slides were incubated in 2× SSC 45 min at 70°C and dehydrated as described above. DNA denaturation on the slides was performed by immersion in 0.1 N NaOH during 10 min. Slides were washed several times in 2× SSC and dehydrated. Biotinylated DNA probes were produced using Bionick labeling system (Invitrogen) according to manufacturer's instructions. The probes were hybridized overnight at 37°C. After hybridization, slides were washed 3 × 5 min in 2× SSC at 42°C and once at room temperature. Detection was performed using FITC anti-biotin antibody (Vector, 1:100) diluted in PBS supplemented with BSA 1% for 1 h. Then, slides were washed 2 × 5 min in 2× SSC and once in PBS and stained with DAPI and analyzed with a Leica DMRA2 microscope equipped with a CoolSnap HQ high resolution CCD camera (Roper Scientifics). Images were acquired using Metamorph software (Universal Imaging Corporation) and processed with Adobe Photoshop software. DNA probes used for in situ hybridization were Pcs100-ci for the endogenous locus or *ci-LacZ* transgene localization, and WH.GFP for Ci-A transgene. Immuno-FISH labeling of the endogenous *ci* locus was performed on a w^{1118} strain, while labeling of transgenes was performed on corresponding *ci-7.1-LacZ* and *ci-A-GFP* transgenic lines.

Results

Polyhomeotic is involved in the maintenance of posterior and anterior cell identities during embryogenesis

To determine whether *ph* is involved in the maintenance of A and P cell identities during embryonic development, we analyzed *en* and *ci* expression in ph^0 embryos, in comparison to wild-type embryos. At the beginning of gastrulation, in stage 8 wild-type embryos, when the 14 stripes of *en* expression are just formed (Fig. 1A), *ci* is ubiquitously expressed (Fig. 1A). Later on, in germ-band extended stage 11 embryos, the expression domains of *en* and *ci* become mutually exclusive and remain that way throughout development (Fig. 1A). Embryonic *ci* repression has been described to depend on *en* regulation (Eaton and Kornberg, 1990). Interestingly, *ph* activation by En in P compartments is transient and occurs in germ-band elongated embryos, when segmentation is completing (Serrano et al., 1995). In ph^0 germ-band retracted embryos, segments form normally, but their identities are affected, since *en* is no longer restricted to its normal domain of expression (Fig. 1B). Deregulation of *en* and *ci* in ph^0 embryos is detectable only from stage 13 embryos. Interestingly, most cells express both *en* and *ci* (Fig. 1C), showing that ph^0 mutant cells express *ci* even in the presence of En.

These results suggested a role for *ph* during embryogenesis in determining the A and the P cell identity. Indeed, this confirmed that *ph* is involved in the maintenance of *en* repression in the A compartments (Dura and Ingham, 1988; Smouse et al., 1988), but also suggested that *ph* is involved in the maintenance of *ci* repression in P compartments.

In vivo En and Ph binding on the ci locus during embryogenesis

To test whether En and Ph act directly on the *ci* locus, we used a ChIP approach. In this experiment, chromatin was isolated from wild-type staged embryos (0–4 h for early gastrulation [stage 1–8], 4–8 h for germ-band extension [stage 8–11], and 8–21 h for late embryogenesis [stage 11–17]). At 4 h of development, the *en* expression stripes are already formed and the germ-band is elongating. This is the time at which *ci* begins to be repressed in the P compartments (Fig. 1A). *ph* activation by En only occurs later, during germ-band elongation (Serrano et al., 1995).

To analyze the binding profiles of En and Ph on the *ci* regulatory sequences, chromatin was immunoprecipitated, either in the presence of anti-En antibody (α-En IP) or in the presence of anti-Ph antibody (α-Ph IP). The results were compared to a control immunoprecipitation without antibody, corresponding to the background of the experiments (Mock IP in Fig. 2C). Chromatin preparations were further used as radiolabeled probes to hybridize Southern blots covering 6 kb of the *ci* upstream region (positions −7024 to −1093), that contain regulatory elements sufficient to drive the normal expression and regulation of *ci* during both embryogenesis and imaginal disc development (Schwartz et al., 1995).

As shown in Fig. 2, during early development (0–4 h), specific En binding can be detected (Figs. 2C, D), whereas no Ph binding is detectable (Figs. 2C, E). Later on, during germ-band extension (4–8 h development), at a time when

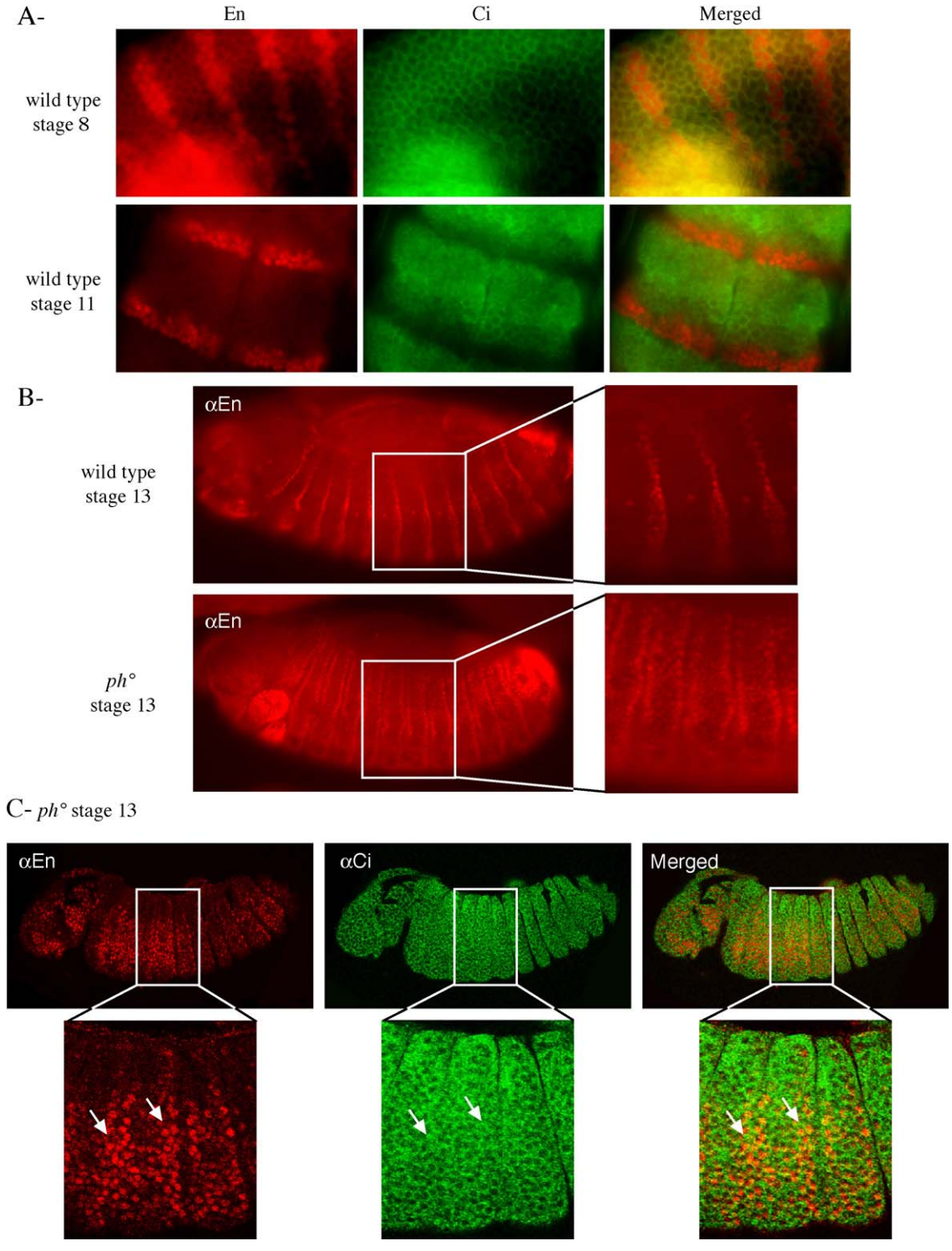

Fig. 1. Analysis of Engrailed and Cubitus-interruptus expression in wild-type and ph^0 backgrounds during embryogenesis. (A) En and Ci expressions in stages 8 and 11 wild-type embryos, as indicated. (B) En expression in wild-type and ph^0 stage 13 embryos. An enlargement of each panel is shown on the right. (C) En and Ci expression in stage 13 ph^0 embryos. Enlargements are shown below. A and C are confocal microscopy. Images in B were acquired by wide field microscopy. En expression is detected with a polyclonal anti-En primary antibody, followed by a secondary anti-rabbit antibody conjugated with Cy3 (A–C). Ci expression is detected using a monoclonal anti-Ci antibody, followed by a secondary anti-mouse antibody conjugated with FITC (A and C). Arrows indicate cells expressing both En and Ci.

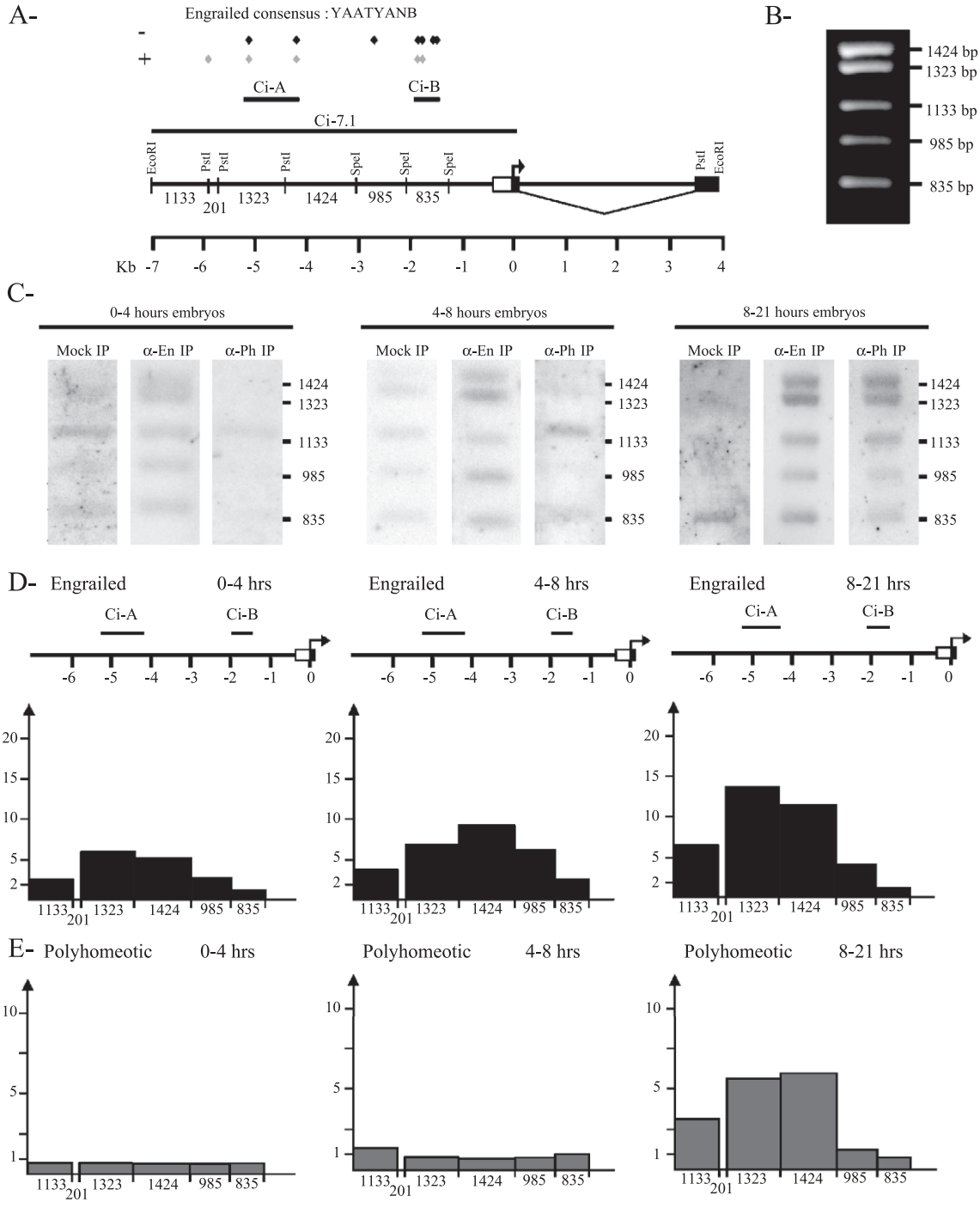

Fig. 2. Analysis of the binding of En and Ph to the *ci* genomic region. (A) Map of the 11 kb *ci* genomic region, with ATG called 0 (according to transcript CG2125 from Flybase), the open box corresponds to 5′ -UTR, and black boxes correspond to exons. ♦ correspond to En consensus on the minus strand and ◈ on the plus strand present in the Ci-7.1 kb region (Solano et al., 2003). Fragments enriched in En consensus are Ci-A covering region −5214 to −4184 and Ci-B covering region −1942 to −1456. Ci-7.1 kb is covering region from −7024 to +8. (B) shows an ethidium bromide-stained agarose gel with the triple restriction digests of the 6 kb *ci* upstream region by *Eco*RI/*Spe*I/*Pst*I. (C) Southern blot of the 6 kb *ci* upstream region, covering region −7024 to −1093 hybridized with chromatin extracts from 0 to 4, 4 to 8, or 8 to 21 h wild-type embryos. Chromatin from formaldehyde-fixed embryos was immunoprecipitated with either anti-En (α-En IP) or anti-Ph (α-Ph IP) antibodies or with no antibody (called Mock IP). D–E correspond to the quantification of the Southern blots shown in C. Enrichment was quantified relative to the Mock IP and displayed on the *Y* axis. (D) Enrichment of En binding in 0–4, 4–8, or 8–21 h embryos, respectively, from left to right, (E) enrichment of Ph binding in 0–4, 4–8, or 8–21 h embryos. The region from −1093 to +8 was tested separately and does not present significant enrichments (data not shown).

ph is activated by En in the P compartments, En binding gets stronger on the 6 kb region upstream of *ci*, whereas Ph binding is still not detected (Figs. 2C–E). Finally, once the germ-band is elongated (8–21 h development), both En and Ph are detected (Figs. 2C–E). Interestingly, the highest enrichment is detected for both proteins within the same 1323 and 1424 bp genomic fragments.

Altogether, these experiments show that both En and Ph directly bind in vivo to *ci* regulatory regions, and that En binding precedes Ph binding. We further observed that En is not displaced from the *ci* upstream region, upon Ph recruitment.

The Ci-A region is able to drive repression by En

The *ci-7.1-LacZ* line has been shown to generate a pattern that was indistinguishable from the normal pattern of *ci* and also depends on *en* regulation (Schwartz et al., 1995).

We recently identified a consensus motif for En binding (YAATYANB) among 203 genomic sequences that bind to En in vivo (Solano et al., 2003). Within the 7.1 kb genomic region covering the *ci* regulatory sequences, we identified two fragments that are enriched in En consensus binding motifs, named Ci-A and Ci-B (Fig. 2A).

The 0.5 kb Ci-B fragment is located within a 835 bp genomic fragment that does not correspond to a peak of En binding during embryogenesis, according to ChIP experiments (Figs. 2C, D). On the other hand, the 1 kb Ci-A fragment is located 4 kb upstream of the transcription start site and overlaps in part with the 1323 and 1424 bp genomic fragments that are preferentially bound by En in vivo (Figs. 2C, D). Moreover, this fragment is part of the distal regulatory elements previously identified (Schwartz

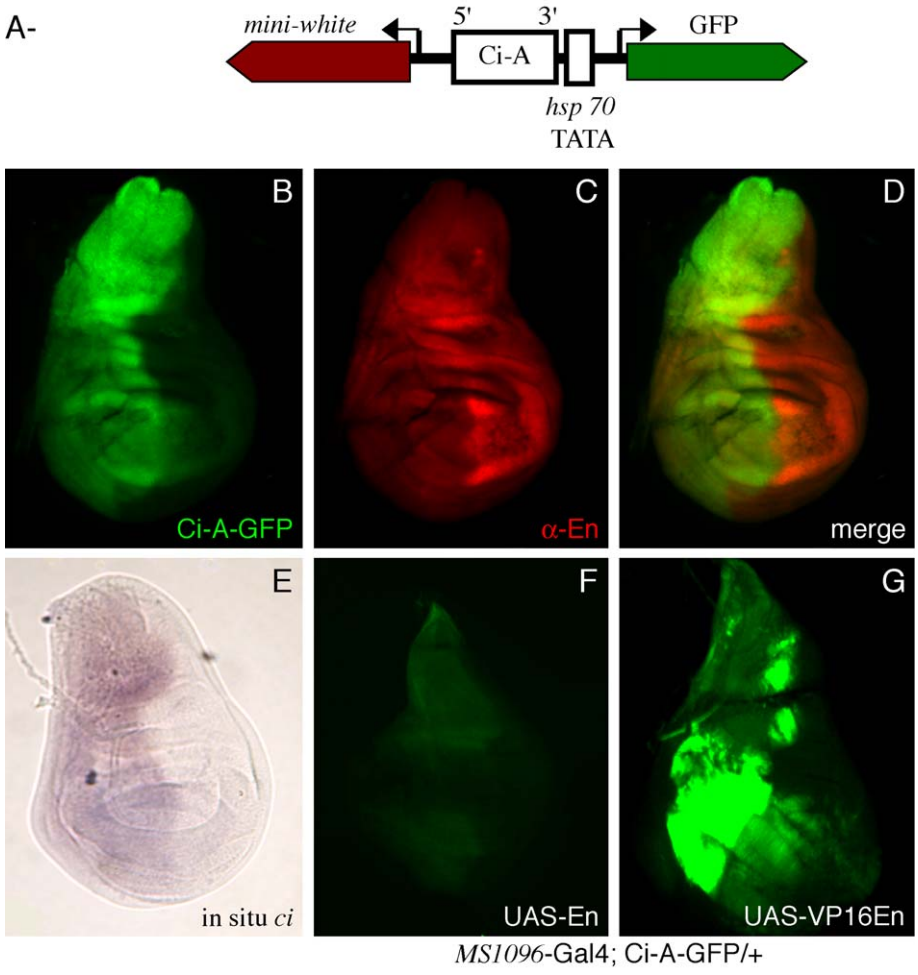

Fig. 3. Ci-A is able to respond to *en* regulation in vivo. (A) Schematic representation of Ci-A cloned upstream of the *hsp70* minimal promoter and the GFP reporter gene. Note that the *mini-white* gene is in the opposite orientation. (B) GFP expression driven by Ci-A in wing imaginal discs of third instar larvae. (C) The same disc is immunostained with a polyclonal anti-En antibody, followed by a secondary anti-rabbit antibody conjugated with Cy3. (D) corresponds to B and C merged images. (E) *ci* endogenous expression detected by in situ hybridization using an anti-sense RNA probe. (F) GFP expression of (*MS1096*-Gal4; Ci-A-GFP/+; UAS-En/+) in L3 imaginal discs. (G) GFP expression of (*MS1096*-Gal4; Ci-A-GFP/+; UAS-VP16-En/+) in L3 imaginal discs. Discs are oriented anterior to the left and dorsal up.

et al., 1995). Therefore, the Ci-A fragment was tested for its ability to respond to En in vivo. For this purpose, we constructed transgenic lines, where Ci-A was placed upstream of a GFP reporter gene (Fig. 3A).

We found that the transgenic lines containing the Ci-A fragment are able to mediate regulation by En. Indeed, Ci-A is able to drive GFP expression, both in embryos and imaginal discs, with a pattern similar to the endogenous *ci* expression pattern (Figs. 3B, E and data not shown) and mutually exclusive to the pattern of *en* expression (Figs. 3C, D). In addition, in L3 wing imaginal discs from *ci-A-GFP* transgenic flies, continuous over-expression of En protein represses GFP expression (Fig. 3F), whereas continuous over-expression of a chimeric activator form of En (VP16-En) (Alexandre and Vincent, 2003; Solano et al., 2003) activates GFP expression, in both A and P compartments (Fig. 3G).

In sum, a combination of in silico analysis of En consensus sites and ChIP assays allowed us to identify a 1 kb regulatory region within the *ci* locus that can drive reporter gene expression in the *ci* pattern and which can mediate repression by En.

The Ci-A element is sufficient to recruit Polyhomeotic in vivo

Genomic regions that bind En or Ph in vivo were shown to overlap (Fig. 2). In particular, a strong binding of En and Ph occurred on the two 1323 and 1424 bp restriction fragments

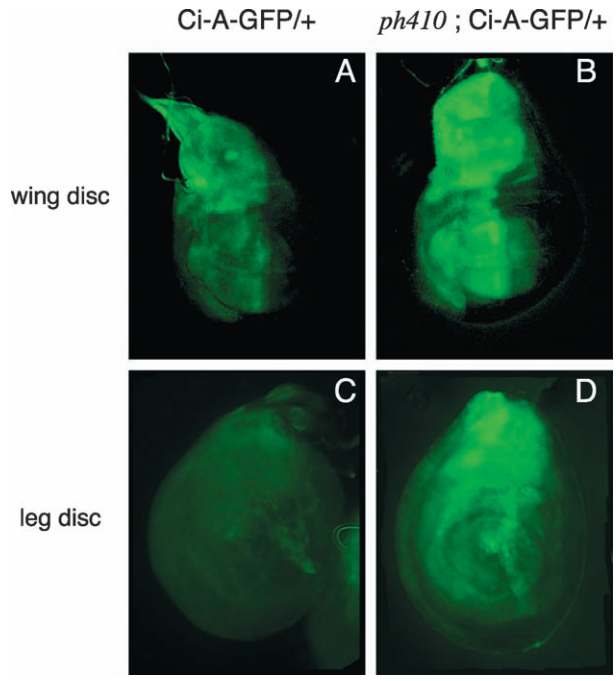

Fig. 4. Ci-A-GFP is able to respond to *ph* regulation in vivo (A) GFP expression in L3 imaginal wing discs of *ci-A-GFP* line (B) GFP expression in (ph^{410}, *ci-A-GFP*/+) mutant wing discs. (C) GFP expression in leg discs of *ci-A-GFP* line. (D) GFP expression in (ph^{410}, *ci-A-GFP*/+) mutant leg discs. Discs are oriented anterior to the left and dorsal up.

that contain the Ci-A region. Therefore, it appeared important to test whether the Ci-A region responds to *ph* regulation. For this purpose, we analyzed the GFP expression of the *ci-A-GFP* transgenic line, in a genetic background where *ph* expression is reduced. A higher level of GFP expression is detectable in ph^{410} mutant wing (Fig. 4B) or leg (Fig. 4D) imaginal discs, when compared to wild type (Figs. 4A, C). We observed an ectopic expression of GFP in the P compartment, consistent with the results obtained by Randsholt et al. (2000) showing that *ci* is activated in ph^{0} clones induced in the P cells. We also noticed a much higher level of GFP expression in the A compartment (Figs. 4B, D). These results show that the 1 kb Ci-A region is sensitive to *ph* regulation, and that it might be involved in the maintenance of both the repression of *ci* in P cells and the control of the level of *ci* expression in A cells.

We further analyzed the binding of PcG proteins on *ci* cis-regulatory sequences in vivo, using an approach that combines immunostaining and fluorescent in situ hybridization on polytene chromosomes (Immuno-FISH, Dejardin and Cavalli, 2004; Lavrov et al., 2004). The *ci* endogenous locus is detected on chromosome 4 by using a DNA probe that covers the *ci* genomic region (Fig. 5A). On the same chromosome preparation, an anti-Ph antibody allowed us to identify a Ph binding site at the *ci* locus (Figs. 5B, C).

Direct Ph binding to *ci* was also confirmed when applying this immuno-FISH approach to the *ci-7.1-lacZ* transgenic line. In this case, an additional Ph binding site is detected at the site of insertion of the transgene, at cytological position 63C (Figs. 5D–F). An additional site was also detected for Polycomb (Pc), another member of the PcG, in the *ci-7.1-lacZ* transgenic line (data not shown). These results are consistent with the presence of a PRE within the 7.1 kb regulatory region of *ci*.

Finally, Ph binding was also detected on the Ci-A fragment, as shown by the presence of an ectopic Ph binding site at the locus of insertion of the *ci-A-GFP* transgene at position 32C (Figs. 5G–I). The intensity of Ph staining detected at Ci-A is weaker than the binding seen at the 7.1 kb region (Figs. 5E, H), indicating that PcG binding to *ci* might involve additional regions outside Ci-A. This is consistent with the idea that PREs can be composite silencing elements capable of PcG protein recruitment (Horard et al., 2000). In particular, sequences outside of the Ci-A region, but within the 1323 pb and 1424 pb fragments that are highly enriched using ChIP (Fig. 2), might be involved in PRE functions. Interestingly, several potential binding sites for Pleihomeotic (Pho), a member of the PcG complex that encodes a DNA-binding protein (Brown et al., 1998), are present within the Ci-7.1 kb regulatory region. One is located within the Ci-A fragment, and another can be detected 30 bp downstream, within the 1424 bp fragment (not shown).

Other PRE features were also tested on Ci-A. In contrast to most PREs, the Ci-A 1 kb fragment neither causes variegation of *mini-white* nor a clear pairing-sensitive silencing effect. In contrast, eight out of ten indepen-

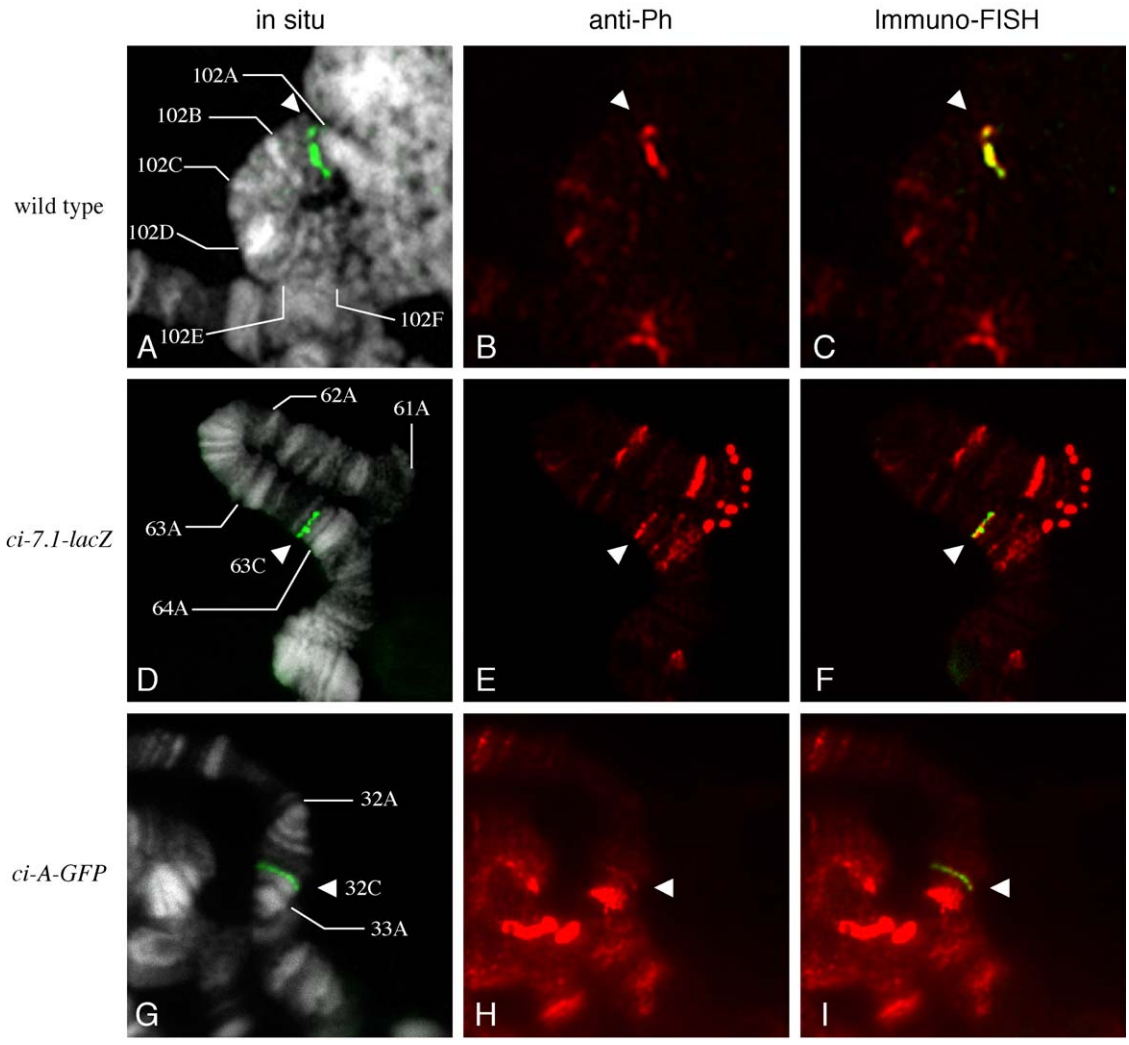

Fig. 5. Ph binds to the *ci* locus. Immuno-FISH experiments are performed on polytene chromosomes of salivary glands. A, D, and G correspond to FISH detection and DAPI staining of the chromosomes. *ci* locus and *ci* transgenic constructions are detected using a biotinylated probe, detected with FITC anti-biotin. B, E, and H correspond to immunostaining with anti-Ph polyclonal antibody, followed by detection with a secondary anti-rabbit antibody conjugated with Cy3. C, F, and I correspond to immuno-FISH merged images. (A–C) Ph detection on the *ci* endogenous locus in w^{1118} strain at 102A. (D–F) Ph detection on the *ci-7.1-LacZ* transgenic line at 63C. (G–I) Ph detection on the *ci-A-GFP* transgenic line at 32C. The arrows indicate the Ph binding sites on *ci* loci. Note that Ph does not bind normally in 63C and 32C loci when tested on w^{1118} larvae (data not shown).

dent transgenic lines bearing Ci-A present an unusually strong red eye color. This suggests the presence of a strong eye-specific enhancer within the Ci-A 1 kb fragment, since insertions of the vector alone result in orange eyes in most fly lines and show red eyes only in two lines out of seven. We also noticed that the GFP reporter in the transgene was strongly expressed in the eye imaginal discs, in the normal territory of endogenous *ci* expression (data not shown). This shows that the Ci-A 1 kb region may contain enhancers involved in establishing normal *ci* expression. No pairing-sensitive silencing of *mini-white* reporter was detected among the lines when the eye color was tested. In contrast, a pairing-sensitive effect is detected on wing disc GFP expressions, since homozygous flies show a lighter GFP expression than heterozygous flies in the P compartment (data not shown). Moreover, GFP expression in the wing discs was found to depend on *ph* (Fig. 4), whereas *Pc* mutation only slightly increases the effect of *ph* mutation, but does not show any effect when tested alone (data not shown).

These results show that *ci* is a direct target of PcG proteins. Moreover, its regulation by these proteins involves the same DNA region, which contains En binding sites. The Ci-A fragment does not, however, show all of the features normally assigned to PREs.

Polyhomeotic binding occurs in the presence or in the absence of Engrailed

Since Ph and En bind the same 1 kb *ci* regulatory sequences, we asked whether the bindings of En and Ph were mutually exclusive. To answer this question, we analyzed by

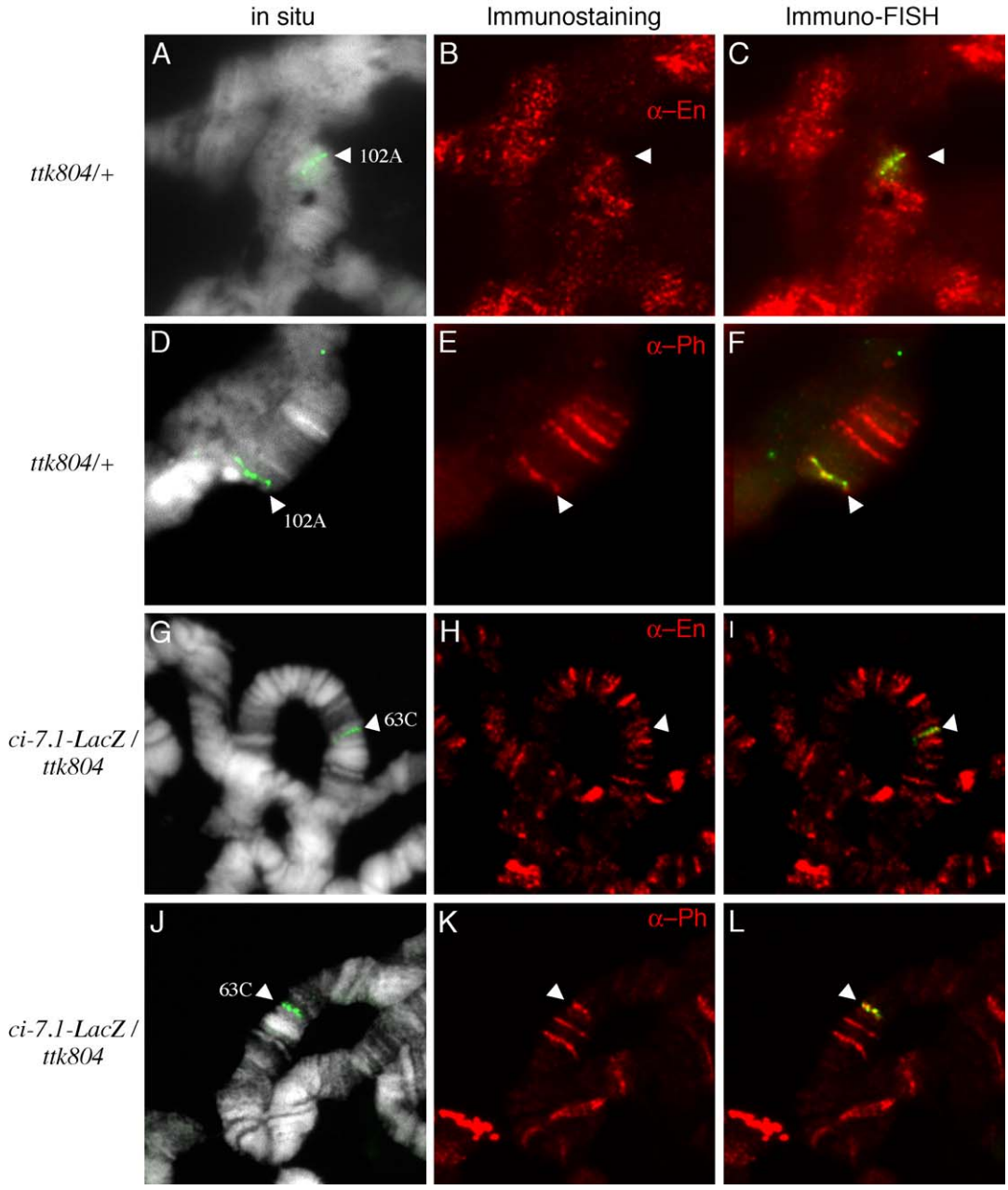

Fig. 6. En binding on polytene chromosome does not interfere with Ph binding. Immuno-FISH experiments are performed in (ttk^{804}/+) mutant salivary glands. A, D, G, and J correspond to FISH detection and DAPI staining of the chromosomes. *ci* loci are detected using a probe, secondary labeled with FITC anti-biotin. B and H correspond to immunostaining with the anti-EN-ΔHD polyclonal antibody, followed by detection with a secondary anti-rabbit antibody conjugated with Cy3. E and K correspond to immunostaining with an anti-Ph polyclonal antibody, followed by detection with a secondary anti-rabbit antibody conjugated with Cy3. C, F, I, and L correspond to immuno-FISH merged images. (A–C) En detection on the *ci* endogenous locus. (D–F) Ph detection on the *ci* endogenous locus. (G–I) En detection on the *ci-7.1-LacZ* transgenic line at 63C. (J–L) Ph detection on the *ci-7.1-LacZ* transgenic line at 63C. The arrows indicate the Ph or En binding sites on *ci* loci.

immuno-FISH, the binding of Ph at the *ci* locus in the presence of En.

As shown in Fig. 5, PcG proteins bind *ci* in salivary glands. This tissue does not normally express *en*. Since *ci* is also not expressed in salivary glands, this suggests that *ci* repression in this tissue does not require En. In a *tramtrack* (ttk^{804}) mutant background, where *en* is derepressed in salivary glands (Solano et al., 2003), En binding can be detected at the *ci* endogenous locus (Figs. 6A–C). Under these conditions, Ph still binds to the *ci* locus, even in the presence of En (Figs. 6D–F). We further found that Ph is able to strongly bind the 7.1 kb *ci* regulatory region in the presence of En, by using the *ci-7.1-LacZ* transgenic line inserted in 63C region in a *ttk* mutant background (Figs. 6G–L).

These results show that Ph binding does not prevent En binding. Similarly, En binding is not able to modify Ph

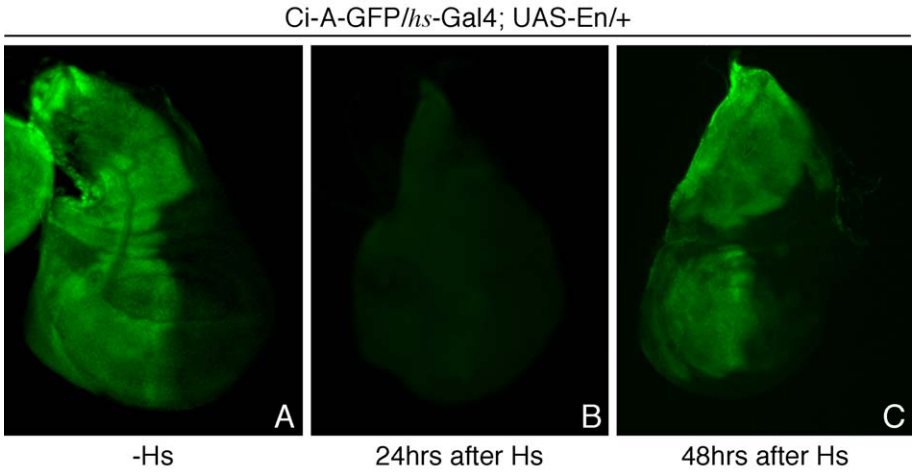

Fig. 7. Ci-A cannot mediate cellular memory of the repressed state. A–C shows GFP expression of (Ci-A-GFP/*hs*-Gal4; UAS-En/+) L3 imaginal discs A, without heat shock (–Hs); B, GFP expression 24 h after heat shock; C, GFP expression 48 h after heat shock. Anterior is to the left and dorsal is up.

binding. Thus, the binding of En and Ph to *ci* is not mutually exclusive.

Transient En binding does not induce cellular memory of gene silencing through the Ci-A element

The binding of PcG proteins at PREs has been associated with transcriptional memory, presumably by setting and maintaining epigenetic marks during DNA replication and mitosis (Cavalli and Paro, 1998; Maurange and Paro, 2002). For instance, in the case of chromosomal elements of the *Drosophila bithorax* complex that contain PREs, the early factors necessary to establish repression are not involved in further maintenance of silencing, once the PcG complex is installed, leading to cellular memory (Poux et al., 1996).

To test whether the Ci-A 1 kb fragment is able to maintain the initial state established by En, we monitored GFP expression in the wing disc of *ci-A-GFP* transgenic flies at different times after induction of repression by En (Fig. 7). If initial binding of En is able to induce a memory of silencing through Ci-A by recruiting PcG proteins, repression should be maintained once the initial repressor disappears. On the other hand, if En is not able to induce stable memory of repression, normal *ci* expression should be recovered following a relatively short delay after the disappearance of the repressor.

Repression of the GFP induced during larval stages was obtained in the A compartment after a pulse of En expression as expected, but was not maintained 48 h after the pulse (Fig. 7). Since the chromatin state might be more difficult to reprogram at late developmental stages, we also tested the effect of pulses of En at different times during embryonic development. Again, *ci-A-GFP* was normally expressed in L3 wing imaginal discs (data not shown). This suggests that the ectopic repression established by the En protein cannot be maintained through the Ci-A element after disappearance of the primary repressor.

Hence, while En is able to repress *ci* in the A compartments, it is not able to induce a stable switch of the *ci* locus into a silenced state, indicating that continuous recruitment of both En and PcG complex might be required for maintenance of *ci* repression. In molecular terms, this indicates that either En does not recruit Ph and PcG complex, or that if required for recruitment, it is continuously needed to maintain PcG proteins anchored at the PRE of *ci*, these two alternatives being non exclusive.

Discussion

Patterning and organization of tissues are established according to different processes that first involve selector genes like *en*. The expression of En determines the position of the A/P boundaries necessary to segregate adjacent groups of cells that are destined to proliferate separately. An important mechanism to ensure that A and P cells do not intermingle is to segregate Hh-secreting and Hh-receiving cells into distinct but juxtaposed compartments (Dahmann and Basler, 1999). En is involved in this process by directly activating *hh* (Alexandre and Vincent, 2003; our unpublished results) and repressing *ci* in the P compartment (Schwartz et al., 1995). *PcG* genes, and in particular *ph*, also play a role in the maintenance of the segregation of these two populations of cells. Indeed, a clear link between *en* and *ph* has been established during wing morphogenesis, since transheterozygous *ph/en* flies present a wiggly boundary and P cells become competent to respond to the Hh signal in this genetic background (Maschat et al., 1998). Moreover, induction of ph^0 clones during wing morphogenesis confirms that *PcG* genes are involved in the maintenance of the expression patterns of different genes of the *en/hh* signaling pathway (Randsholt et al., 2000). Interactions between *en* and *ph* have been also detected during

embryogenesis (Serrano et al., 1995). The studies reported here suggest that the activation of *ph* by En during embryonic A/P patterning may contribute to the maintenance of silencing of the *ci* gene in posterior compartments throughout development, once *ci* repression has been established by En.

Engrailed is necessary for ci repression throughout development

Early in development, during germ-band elongation, *ci* repression in the P compartments occurs. We have shown, using ChIP, that during germ-band extension, En binds to genomic fragments within the *ci* regulatory region. This binding is maintained at later stages, after *ci* repression is established. Identification of En binding motifs in association with ChIP experiments helped us to further restrict to 1 kb the fragment responsible for the establishment and maintenance of *ci* repression by En. These observations suggest that En is directly necessary not only to establish, but also to maintain *ci* repression throughout development, although some tissues such as salivary glands escape this requirement. This result is in agreement with a previous genetic analysis of *en/inv* null clones in wing discs, showing that cells lacking *en* in P compartments activate *ci* (Tabata et al., 1995).

PcG genes are involved in ci repression

Both genetic and molecular evidence supports the implication of *PcG* genes, and in particular *ph*, in *ci* repression. First, *ci* is mis-expressed in ph^0 embryos. Moreover, during wing morphogenesis, ph^0 clones induced in the P compartment express both *ci* and *en* (Randsholt et al., 2000). This clearly shows that *ci* repression involves *ph* and *en* independently.

Using ChIP experiments and immunostaining of polytene chromosomes, we found that Ph binds directly to the *ci* regulatory region in vivo. Repression of *ci* seems not to be restricted to the action of the Ph protein alone, since we detected a binding to *ci* region of both Ph and Pc (Fig. 5 and data not shown) that form the core of the PcG complex PRC1 (Shao et al., 1999). One of the features of a PRE is indeed to directly recruit PcG proteins (Kassis, 2002). Therefore, according to those criteria, the Ci-7.1 kb fragment might be considered as a PRE. We also show that the *ci* PRE presents different features compared to PREs typically found in homeotic genes. Recently, another PRE from the *ph* upstream region was shown to differ from homeotic PREs (Bloyer et al., 2003). This may suggest that multiple classes of PREs exist in *Drosophila*, tailored to the specific regulatory features of the genes that they regulate.

Ph and En bind within a small region in the ci locus

ChIP experiments showed that En and Ph bind preferentially to the same genomic fragments within the *ci* locus. Indeed, Ph binding was identified within a 1 kb genomic fragment located about 4 kb upstream of the *ci* coding region. This fragment also contains En binding sites. Moreover, this 1 kb fragment, tested in a transgene, is able to drive GFP expression in a pattern similar to endogenous *ci* expression in both embryonic and larval tissues (Fig. 3 and data not shown) and to bind and to respond to En regulation in vivo. The same transgene is bound by Ph, and GFP expression driven by this fragment depends on the level of *ph* expression.

From this study, it appears that the 1 kb Ci-A fragment contains several regulatory elements. In addition to a PRE, there are also enhancers that allow expression in the A compartment, and silencers that respond to En, and allow repression in the P compartment. Further dissection of this 1 kb fragment will help to better characterize the features of the *ci* PRE and of other flanking regulatory regions. In particular, further experiments will be needed to separate the PRE from enhancer sequences and from the En binding sites.

Transition from establishment to maintenance

Since En was found to be necessary, but not sufficient (since it requires PcG proteins), to maintain *ci* repression after germ-band extension, the role of PcG protein recruitment might be to sustain En-dependent *ci* repression. Indeed *en/inv* null clones drive *ci* activation in the P compartment of the wing disc (Tabata et al., 1995), even though we know that Ph is present and is also involved in *ci* repression at that stage (Fig. 4; Randsholt et al., 2000).

An interesting question is to understand how the different members of the *PcG* complex are recruited, after En has induced the repression of *ci*. Direct interactions between PcG and transcription factors have been suggested (Zhang and Bienz, 1992). One example of such an interaction concerns the gap protein Hunchback (Hb) and the dMI2 protein. Hb is able to induce the early repression of *Ubx*, and by directly interacting with dMI2, it might lead to the recruitment of PcG proteins (Kehle et al., 1998). In the case presented in this report, such an interaction might occur between En and some PcG proteins to account for maintenance of *ci* repression.

However, most En binding sites detected on polytene chromosomes do not correspond to Ph binding sites (data not shown), suggesting that Ph recruitment is not a general consequence of En function in vivo. Moreover, preliminary immunoprecipitation experiments using antibodies directed against Pc and Ph did not induce co-immunoprecipitation of En (data not shown). Therefore, En might not directly recruit PcG proteins such as Ph. An alternative possibility is that the regulation of *ci* by *en* and *ph* might represent partially redundant mechanisms to ensure *ci* repression in the P compartments even when either *en* or *ph* are slightly defective. Partially redundant mechanisms might be important to ensure that important functions, such as the A/P segregation of cells, take place correctly. *ph* might play a central role in the elaboration of this regulation, since *ph* is

directly activated by En in the P compartments, and may trigger PcG recruitment.

PcG genes are involved in the maintenance of the expressions of different genes of the *en/hh* signaling pathway (Dura and Ingham, 1988; Maurange and Paro, 2002; Randsholt et al., 2000). Recently, *PcG* genes have been shown to be involved in *hh* repression in the A compartment of the wing disc, independently of *en* (Randsholt et al., 2000). Indeed, PREs have been detected upstream of the *hh* transcription unit (Maurange and Paro, 2002; our unpublished results).

Taken together, these results show that *PcG* genes also play a role in the maintenance both of A cell identity during development by directly repressing *en* (Kassis, 1994; Strutt and Paro, 1997) and *hh* (Maurange and Paro, 2002; Randsholt et al., 2000), and of P cell identity by repressing *ci*. The correct expressions of *ci* and *en* were shown to control most aspects of the distinct sorting properties of A and P cells, leading to the compartmentalization of cells (Dahmann and Basler, 2000). Our results show that *PcG* genes are involved in this process, an early and crucial event in pattern formation.

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