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Analysis of *cubitus interruptus* regulation in *Drosophila* embryos and imaginal disks

Carol Schwartz[1], John Locke[2], Craig Nishida[1] and Thomas B. Kornberg[1]

[1]Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143, USA
[2]Department of Biological Sciences, University of Alberta, Alberta, Canada

SUMMARY

The *cubitus interruptus* (*ci*) gene of *Drosophila* is expressed in all anterior compartment cells in both embryos and imaginal disks where it encodes a putative zinc-finger protein related to the vertebrate Gli and *C. elegans* Tra-1 proteins. Using *ci/lacZ* fusions, we located regulatory sequences responsible for the normal pattern of *ci* expression, and obtained evidence that separate elements regulate its expression in embryos and imaginal disks. Mutants that delete a portion of this regulatory region express *ci* ectopically in the posterior compartments of their wing imaginal disks and have wings with malformed posterior compartments. Similar deletions of *ci/lacZ* fusion constructs also result in ectopic posterior compartment expression. Evidence that the engrailed protein normally represses *ci* in posterior compartments includes the expansion of *ci* expression into posterior compartment cells that lack engrailed function, diminution of *ci* expression upon overexpression of engrailed protein in anterior compartment cells, and the ability of engrailed protein to bind to the *ci* regulatory region in vivo and in vitro. We suggest that engrailed protein directly represses *ci* expression in posterior compartment cells.

Key words: *Drosophila,* developmental regulation, compartments, *engrailed, cubitus interruptus,* imaginal disk, segment polarity gene

INTRODUCTION

Many of the functions that regulate Drosophila embryonic development are provided by transcription factors (reviewed in Kornberg and Tabata, 1993; Nüsslein-Volhard, 1991). Among known examples are the proteins that establish the anterior-posterior polarity of the embryo, and the proteins that help to subdivide and organise the embryo into metameres. These proteins function, in part, to precisely place the domains of expression of other members of this regulatory network, and since their capacity to do so is dependent upon their relative location in the embryo, the precise pattern in which each one is made is critically important.

To investigate the basis for such interactive relationships, we initiated a study of the mechanism by which transcription of the *cubitus interruptus* (*ci*) gene is regulated. *Ci* is a segment polarity gene that is expressed in anterior compartment cells of embryos and imaginal disks (Eaton and Kornberg, 1990; Orenic et al., 1990). Mutants carrying lethal *ci* alleles die as embryos after severely defective segmentation, and based upon their complementation and phenotype, can be subdivided into two classes (Orenic et al., 1987). Embryos homozygous for ci^D have part of each denticle belt replaced with a mirror image duplication, while embryos homozygous for *Cell* have lost much of the clear cuticle between denticle belts, causing fusion of the belts into a lawn. Although ci^D and *Cell* do not complement each other, they complement other *ci* alleles differentially. In addition to these lethal alleles, many viable *ci* mutants have been isolated; these cause defects predominantly in the wings (Locke and Tartof, 1994; Slusarski et al., 1995). Athough the complex genetic interactions between these different types of alleles led to the suggestion that *ci*, ci^D, and *Cell* mutations represent abnormal forms of three different genes (Orenic et al., 1987), recent observations locate these various mutations in a region neighbouring a single transcription unit (this manuscript; Locke and Tartof, 1994; Slusarski et al., 1995). This suggests, instead, that all are alleles of a single gene, and we refer to this locus as *cubitus interruptus (ci)* henceforth.

Ci encodes a putative zinc-finger protein that is related by sequence to the Gli family of regulatory factors from vertebrates and to the sex determination factor Tra-1 from *C. elegans* (Orenic et al., 1990; Walterhouse et al., 1993; Zarkower and Hodgkin, 1992). Genetic studies implicate *ci* in the regulation of other genes that are essential for anterior compartment development. For instance, the expression of *patched* and *wingless*, two genes that are essential in anterior compartment cells (Baker, 1988; Hooper and Scott, 1989), is reduced in *ci* mutant embryos (Forbes et al., 1993). Curiously, despite its sequence and kinship which suggest that Ci is likely to act as a transcription factor, immunohistochemical analysis indicates that the Ci protein is predominantly, or exclusively, located in the cytoplasm of anterior cells (Schwartz and Kornberg, unpublished). The mechanism through which it acts therefore remains in doubt.

Another gene that is involved in the regulatory networks that

distinguish anterior and posterior compartment cells is *engrailed*. *Engrailed*, like *ci*, is a segment polarity gene that supplies an essential function to segmentation in embryos and to subsequent imaginal development. *Engrailed* is expressed exclusively in posterior compartment cells in both embryos and disks, where it encodes a homeodomain-containing transcription factor (Fjose et al., 1985; Poole et al., 1985). Posterior compartment cells that lack *engrailed* function appear to assume an anterior compartment identity, and it has been proposed that the posterior compartment developmental pathway is orchestrated by En (Garcia-Bellido and Santamaria, 1972). Recent studies suggest that it might do so through its activation of genes such as *hedgehog*, which is expressed specifically by posterior compartment cells, and by its repression of anterior-specific genes such as *ci*, *patched*, and *decapentaplegic* (Eaton and Kornberg, 1990; Martinez Arias et al., 1988; Raftery et al., 1991; Tabata et al., 1992).

The subject of the work described here is the mechanism that limits *ci* expression to anterior compartment cells. To address this issue, we identified sequences in the *ci* regulatory region that mediate the normal patterns of *ci* expression. We also documented the role of the engrailed protein (En) in regulating *ci* expression, monitoring the binding of En to these sequences in vivo and in vitro, and monitoring *ci* RNA and protein in embryos and imaginal disks in which the normal distribution of En had been changed. The association that we observed between En and the critical *ci* regulatory sequences, and the complementary pattern of *ci* and *engrailed* expression that characterised both normal and mutant animals, suggest that *ci* repression by En is direct. These findings therefore support the model that *engrailed* regulates the posterior developmental pathway by controlling the expression of its target genes, and provides the first evidence that links directly differential gene expression in the anterior and posterior compartments.

MATERIALS AND METHODS

Fly culture

Df(1)w^{67c23}*, y* embryos were used for germ line transformation with derivatives of the P-element vector pW8, and, with the exception of 7.1-ci, at least 2 independent lines were analysed for each transgene. *en*^{10} mutant embryos were identified by their fused segments. For FLP/FRT recombination (Xu and Rubin, 1993), FL122; FRT^{43D}, Df(2R) en^{E}/+ males were crossed to either FRT^{43D}, pπMyc$^{46F, 47F}$ or FRT^{43D}, pπMyc$^{46F, 47F}$/+; 2.2dis+1.2prx-ci/+ females. Clones were generated in second instar larvae with a 1 hour heat shock at 37°C.

DNA manipulation

ci/lacZ fusions

The wild-type *ci* region was obtained from λ/wt4-3 (Orenic et al., 1990), and the *ci*^{57g} region from λ/ci57g-3 (Locke and Tartof, 1994). *ci/lacZ* fusions contained either the *ci* promoter or a HSP70 minimal promoter (Amin et al., 1985). For fusions containing the *ci* promoter, the ends of a 1.7 kb *Mlu*I fragment containing the *ci* promoter were filled and the fragment was inserted into a similarly filled *Bam*HI site in the *lacZ* gene. The resulting plasmid had an in frame fusion of the first 5 codons of *ci* to codon 8 of *lacZ*. For the longest fusion (7.1-ci), an additional 4.8 kb was added to the promoter fragment. For fusions with a minimal HSP70 promoter, the wild-type (2.2dis-ci) or mutated (1.6dis-ci^{57g}) fragments were inserted into pWHZ10 (Hiromi and Gehring, 1987).

In situ hybridisation to whole-mount embryos (Eaton and Kornberg, 1990) and to polytene chromosomes (Ashburner, 1989) was essentially as described. *lacZ* probes were used to identify sites of *ci*/*lacZ* integration.

Histochemistry

β-galactosidase activity was detected with X-gal essentially as described by Eaton and Kornberg (1990) after incubation from 1 to 36 hours at 37°C. For immunohistochemistry, embryos were incubated with primary antisera (anti-En: 4D9, Patel et al., 1989; anti-β-galactosidase: Cappell; anti-Ci: Holley and Kornberg, unpublished) overnight at 4°C, and with secondary antibodies (donkey anti-mouse or donkey anti-rabbit) from 1 hour to overnight. HRP staining was carried out with vectastain ABC and DAB staining kits (Vector). Fluorescent images were recorded with a Biorad MRC600 Confocal microscope and analysed with comos software (Biorad).

En bound to polytene chromosomes: Third instar larvae carrying at least one copy of an *engrailed* cDNA transgene under the HSP70 promoter (Gustavson, unpublished data) were subjected to 30 minutes at 37°C. Salivary glands were incubated in Cohen Buffer (25 mM disodium glycerophosphate, 10 mM KH_2PO_4, 30 mM KCl, 10 mM $MgCl_2$, 3 mM $CaCl_2$, 160 mM sucrose, 0.5% Nonidet P-40; Cohen and Gotchel, 1971) for 8-10 minutes, fixed, squashed and stained essentially as described by Weeks et al. (1993) except that 3.7% formaldehyde was used at both stages of fixation. En was detected with a polyclonal rabbit antibody (A. Vincent, unpublished data). Chromosomes were counterstained with DAPI.

Preparation of engrailed protein and footprinting

Full length En was purified from bacterial extracts and used in DNase I footprinting assays essentially as described by Hoey and Levine (1988). For in situ 1, 10- phenanthroline footprinting, En-DNA complexes were separated from free DNA by gel retardation then treated as described by Kuwabara and Sigman (1987).

RESULTS

ci gene structure

To identify sequence elements that regulate the expression of *ci*, gene fusions that couple the *ci* promoter to the *lacZ* gene were constructed. These efforts uncovered numerous differences with the published map of *ci* (Orenic et al., 1990). Most significant is the presence of a single approximately 3 kb intron in the first 4 kb of the transcribed region (Fig. 1A). A similar revision of the gene structure has been reported (Slusarski et al., 1995). Sequence determination of the promoter fragment (proximal fragment; prx, Fig. 2) showed that it overlapped with 430 bp of the cDNA sequence reported by Orenic et al., 1990 (GenBank accession number X54360), but, as indicated in Fig. 2, differed at 17 positions. Primer extension analysis (not shown) indicated that the start of transcription is very close to the 5' end of the published cDNA (position 743), and is coincident with two potential cap sites (positions 742-747 and 747-752; Fig. 2). No consensus TATA sequences are present in the prx fragment upstream of the start site, although several are present at least 100 bp downstream.

Identification of regulatory elements for embryonic and imaginal expression

Among the fusions containing upstream sequences that were tested in transgenic flies, the longest construct (7.1-ci) contained 7.1 kb of upstream and transcribed sequences. This 7.1 kb was colinear with the genomic sequence with the

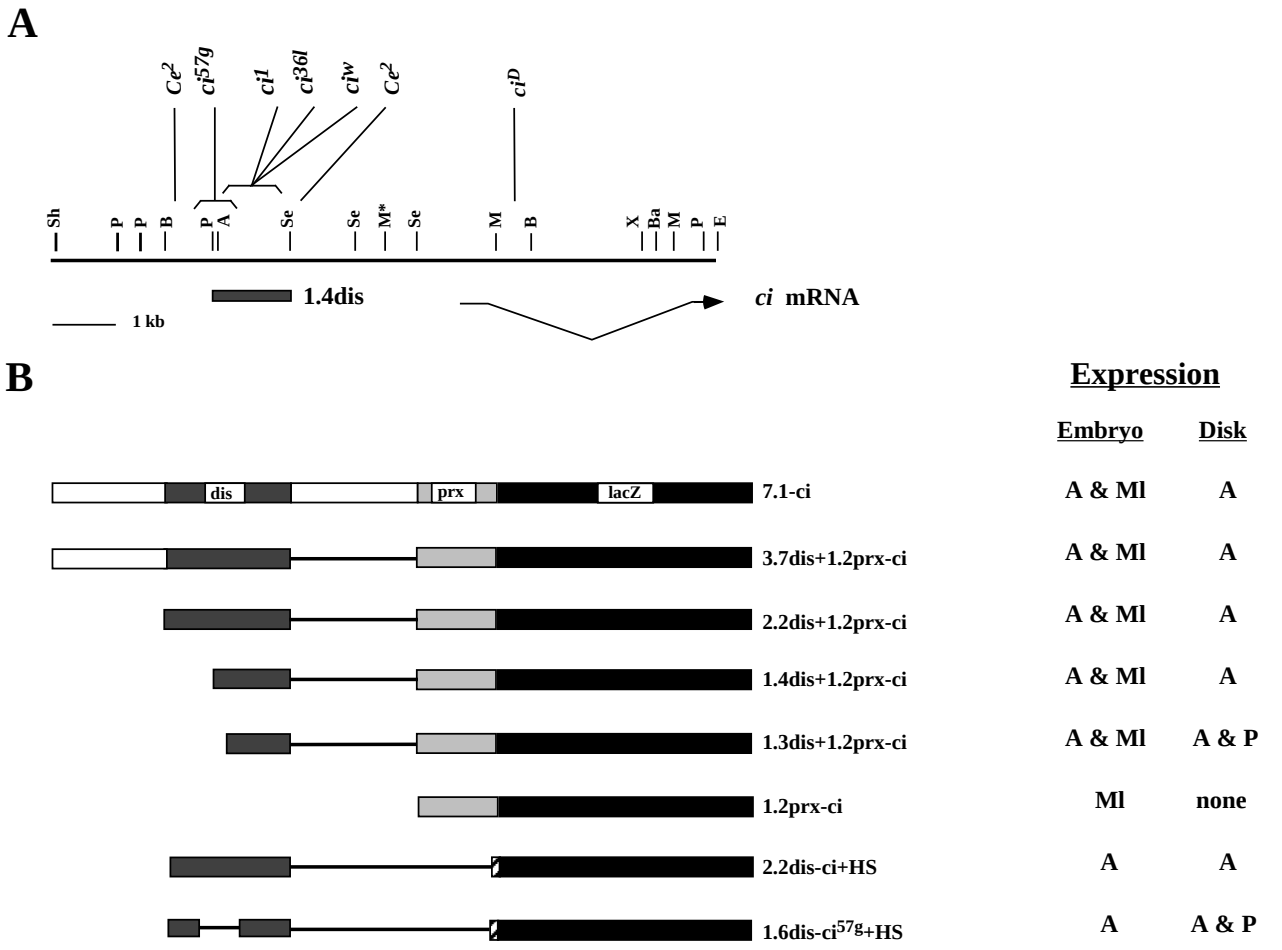

Fig. 1. The *ci* genomic region. (A) Restriction map, with the wild-type genome (thick line), the 5′ end of the *ci* transcript with the structure of the first 3.5 kb of the open reading frame (thin line), a 135 bp deletion between two *Mlu*I sites that occurs in all constructs (*), and positions of mapped *ci* mutations indicated (this manuscript; Locke and Tartof, 1994). ci^D and *Cell* have an additional rearrangement outside of the region included in this map. 1.4dis (stippled box) is the smallest element shown to generate anterior compartment-specific expression in embryos and disks. (B) *ci/lacZ* fusions integrated in transgenic lines, showing sequences included (boxes) and internal deletions (straight lines). Shading of boxes denotes: open – not essential for patterning; dark stippling – distal fragment (dis); light stippling – proximal fragment (prx); black – *lacZ*; and diagonal stripes – crippled HS promoter. Abbreviations: (Sh) *Sph*I, (P) *Pst*I, (B) *Bgl*II, (A) *Afl*II, (Se) *Spe*I, (M) *Mlu*I, (X) *Xba*I, (Ba) *Bam*HI, (E) *Eco*RI, (A) anterior compartments, (P) posterior compartments, and (Ml) ventral midline.

exception of an approximately 135 bp deletion, 1.2 kb upstream from the start of transcription (Fig. 1A). 7.1-ci generated a striped pattern of expression in germband extended embryos (Fig. 3D) that was indistinguishable from the normal pattern of *ci* (Fig. 3C; Eaton and Kornberg, 1990; Orenic et al., 1990). Striped expression with segmental periodicity was first apparent at the onset of gastrulation and it persisted in germband retracting embryos (not shown). While *ci* expression usually fades during germband retraction, β-galactosidase in these stripes persisted, presumably due to perdurance of the β-galactosidase protein. In addition to the segmentally reiterated stripes of expression, *ci* is normally expressed in a continuous stripe along the ventral midline; 7.1-ci also reproduced this element of the *ci* pattern (not shown). Unlike *ci*, 7.1-ci generated only two broad stripes in cellular blastoderm embryos, not three as in the normal pattern (Fig. 3A,B; Eaton and Kornberg, 1990). Expression of 7.1-ci in imaginal disks was anterior compartment-specific, and was indistinguishable from that of the native gene (not shown).

Distinct elements drive expression in anterior compartments and along the ventral midline

Fusions containing smaller fragments from the regulatory region revealed that the *ci* pattern is a composite made from the contributions of several different pattern elements. A 2.2 kb fragment (2.2dis) 2.5 kb upstream from the start of transcription was sufficient to generate a wild-type pattern of stripes in embryos (Fig. 4A,C) and anterior compartment-specific expression in imaginal disks (Fig. 5B) when coupled with either the *ci* promoter sequence (1.2prx) or a crippled, non-heat-inducible fragment of the HSP70 promoter (HS). To confirm that the β-galactosidase expression in embryos was confined to anterior compartments, embryos were stained with antibodies against both β-galactosidase and En. As *engrailed* expression is confined to posterior compartment cells, these

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ACTAGTATAC CCAATATACC CATAAGATAG GGGTAAAAAT TCGATGCATT   50
AAGTACACTG AGTGCAAAAA TACTGTGTCG CAAATATTAC ACCAAACCAG  100
TCTTGATAGG ATAGAAAACA GTTATGTATG TTTTCATCAA GAATGCTATT  150
CGCAAGGGCA AGGCTATAAT AGATAAATAC GACTACAAAT ACATGTTTGT  200
ATTTTAATAT ACTGGATATA CTGGGTATAA TGGGTACAAA TATCAACGAG  250
ATAGCCAAAA TCAAGATTGA ATCGGGTGTA CAAGCTACTG TAAGATTAAT  300
CTATATATAT ATATATATGT ATGTGTGTAT ATGTATATAG AGATAGGCGT  350
TGCCAATAAC TTTTGCGTTT TTTGCTTAAA AATAATATTG TATCGCCGAG  400
GACAAAAATT ACCCACTGAG AGACACAAAC TACTAGCGTA AAAATATCTA  450
TATTAACAGC ACTGGCATCG AAAAGTATCT TAACTCACGT ACGAGTATAT  500
GTATTGTGTG GCCATCTTCG ATCTGCCCGC CTTCGCCGAC TGTTCGTGAT  550
CCGTAGGTAC ACACAGACAT TTAAATCACC AATGCACTTG TTACTGTATT  600
GTATTTATGT TTGTGAACCT ACACGTATCT ATGTAGAATC TGTACAAGTA  650
TCTGTGTTTG GACAATGGCA TGTGTGAGAG GAGATCCGAA CTGCTCCATC  700
TAAACTAACG AAGCATTGCA ACAGCTAGTG TTAATGTCGC TCAGTCCACT  750
CGTAAGAAAC ATAGGAATAA GACGCAGCAT TCAAAAAAAT ATTGACTTGT  800
CTTACAAAAC TGATTTTCAT TGTTCGCTAC TTAATATTTA GTGATAAATA  850
TATTACTTCG GGACTGATAA TCTTATGAAT ATACGTGTGA GATTTGGCGA  900
TACCCTTCAT TACTTAGGAA GCCGAGCGCA TAAGCTCACA CCATTATAAT  950
ATTTAAGTGA TATGAATATT AAATATTAAA ATTTAAAACC AACTTTTTAA 1000
TCAAAGAGTT TCCTTAAAAT TGTTTAAGTT GCCGATCAAA AGTGGAAATT 1050
TATGCGTGCC GTTATTTGAA ATACCAATCT TTTGAAAATG TTTACGCCGA 1100
CGGTTAATAT TATTTAAAAG GATAATTGAA TTTATAAAAC GACGTCATTC 1150
TTGTTGTGGA CTAACTTTAA TGAAATGGAC GCCTACGCG             1189
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Fig. 2. Sequence of the *ci* promoter. Both strands of the 1189 bp prx fragment were sequenced. The predicted transcription start (bent arrow), cap sites (bold), and translation start (box) are indicated, as are 17 additional bp (underline) not reported in the GenBank (X54360) cDNA sequence.

two expression patterns should not overlap. As expected, the patterns were reciprocal (Fig. 4A).

The *ci* promoter fragment alone (proximal fragment; 1.2prx-ci) was also tested for its ability to mediate expression in either embryos or imaginal disks. Sequences within this fragment mediated expression along the ventral midline and also generated stripes that partially encircled the embryo (Fig. 4B). However these stripes overlapped with En stripes (see arrowheads, Fig. 4B). While the expression along the ventral midline is part of the normal pattern of *ci* expression, these stripes are not. It is interesting to note that when 1.2prx-ci is combined in a fusion with 2.2dis, these ectopic stripes are suppressed (Fig. 4C, see arrowheads). This suggests that there are elements in 2.2dis that repress expression in the posterior compartments of embryos, in addition to activating expression in the anterior compartments. The 1.2prx-ci fragment did not mediate expression in imaginal disks.

Sequences required for repression in the posterior compartments of imaginal disks and embryos are different

While the 2.2dis fragment mediates anterior compartment expression in both embryos and imaginal disks, it is not clear whether this expression is mediated by separate elements within the fragment that control activation and repression, or even if the same elements are used at both developmental stages. To address this question, we analyzed a *ci* mutant, ci^{57g}, that causes an adult phenotype but does not affect embryonic development. ci^{57g} is one of several recessive *ci* alleles that cause vein defects in the posterior compartment of the wing. Southern analysis of an approximately 20 kb region had shown ci^{57g} to have a small deletion in the upstream region of *ci* (Locke and Tartof, 1994). Upon more detailed analysis, the mutation was found to be both a deletion of 646 bp and an insertion of 29 bp at the same site in the 2.2dis fragment (1.6dis-ci^{57g}; Figs 1A, 7B).

To determine how the ci^{57g} deletion affects *ci* expression, an antibody raised against a portion of the ci protein (Holley and Kornberg, unpublished data) was used to analyse mutant wing imaginal disks. Unlike wild-type disks, Ci was present in both the anterior and posterior compartments (Fig. 5E), although the level in the posterior compartment was lower. Wild-type disks had no detectable Ci in posterior compartment cells (see Fig. 5A). Analysis of other members of this class of *ci* mutants has also revealed misexpression of Ci in the posterior compartment of wing imaginal disks (Slusarski et al., 1995). To determine if the ectopic *ci* expression in ci^{57g} mutants is caused by the observed rearrangement, a transgenic fly was generated carrying a fusion construct with the dis fragment from ci^{57g} (1.6dis-ci^{57g}+HS; Fig. 1B). This construct is identical to 2.2dis-ci+HS, except for the substitution of a mutant ci^{57g} distal restriction fragment. β-galactosidase expression in embryos harbouring 1.6dis-ci^{57g}+HS was unchanged (not shown), but expression in imaginal disks differed significantly (Fig. 5F). There was appreciable expression in both the anterior and posterior compartments. β-galactosidase activity was present ectopically in all areas of the posterior compartment, but was strongest in the notum region. We conclude that the ci^{57g} lesion eliminates sequences that are necessary for repression in the posterior compartment of imaginal disks, and we suggest that such ectopic expression accounts for the phenotype of the mutant in its wing posterior compartments. We note that the observation that ci^{57g} and other *ci* alleles (ci^{1} and ci^{W}, not shown) do not affect embryonic expression is at variance with the results of Slusarski et al. (1995).

Sequences deleted in ci^{57g} are necessary for repression in imaginal disks

Using the limits of the ci^{57g} deletion as a guide, we sought to identify smaller constructs that contain sufficient sequences to generate proper imaginal disk expression. A series of constructs was made in which successively greater portions of the distal region of the 2.2dis fragment were deleted. Testing in transgenic animals revealed that none of the sequences within the 2.2dis fragment that are distal to the ci^{57g} deletion are necessary for proper expression of fusion constructs in either embryos or imaginal disks. In fact, normal patterns of expression in embryos (not shown) and imaginal disks (Fig. 5C) were observed when all sequences distal to the ci^{57g} break, as well as an additional 296 bp from within the deleted region (1.4dis+1.2prx-ci), had been removed. Thus, the regulatory elements for both developmental stages appear to be at a more proximal location within the fragment.

Removing an additional 148 bp creates a construct (1.3dis+1.2prx-ci) that retains only the most proximal 207 bp of the sequences deleted in ci^{57g}. Although embryonic

expression was unaffected in 1.3dis+1.2prx-ci, imaginal disk expression expanded into the posterior compartment and was reduced in the anterior compartment (Fig. 5G). This suggests that the 148 bp that had been deleted in 1.3dis include sequences that are necessary for proper expression in imaginal tissues; these sequences are not, apparently, involved in embryonic expression. As activation of expression in imaginal disks was largely intact with 1.3dis and since the 1.6dis fragment from ci^{57g} activated expression normally, it is likely that the sequences required for activation are outside of the deleted 148 bp. Similarly, since de-repression in the posterior compartment is only partial, there are likely to be additional sequences necessary for repression either within the remaining 207 bp from the ci^{57g} deletion or proximal to this region.

Repression is *engrailed*-dependent

Having identified some of the sequence elements required for proper *ci* patterning, we also wished to identify *trans*-acting factors that regulate *ci* expression. To identify such factors, the expression of either the native *ci* gene or of the fusion construct 2.2dis+1.2prx-ci was tested in a variety of mutant backgrounds.

engrailed had been identified in a previous study as a potential repressor of *ci*, since *ci* expression expands into both embryo and disk posterior compartments of *engrailed* mutants (Eaton and Kornberg, 1990). Expression mediated by the 2.2dis+1.2prx-ci transgene expanded into the posterior compartments of embryos that are homozygous for the lethal en^{10} allele (Fig. 4D). This indicates that at least some of the sequences that respond to *engrailed* are present in this fusion construct. To extend these observations to imaginal tissue, the 2.2dis+1.2prx-ci construct was crossed into an en^{1} mutant background. en^{1} is a viable allele that reduces *engrailed* expression in the wing pouch region (Condie and Brower, 1989), and it has been shown previously that *ci* is de-repressed in the posterior compartment of the wing pouch in en^{1} mutants (Eaton and Kornberg, 1990). We found the 2.2dis+1.2prx-ci construct to be similarly de-repressed in the wing pouch region of the posterior compartment in en^{1} wing imaginal disks (Fig. 5D).

As a second means to test how the endogenous *ci* regulatory region and the 2.2dis+1.2prx-ci construct in imaginal disks respond to changes in *engrailed* function, the FLP/FRT recombination system was used to generate clones of en^{-} cells in otherwise normal imaginal disks (Broach et al., 1982; Xu and Rubin, 1993). Flies homozygous for FRT insertions at 43D and heterozygous for a deficiency that removes both the *engrailed* and *invected* genes were briefly exposed to FLP recombinase during the second larval instar. Clones that were generated were detected in the imaginal disks of third instar larvae by scoring the distribution of En, Ci, or β-galactosidase proteins. Several disks were identified with areas of their posterior compartment that lacked En, indicating the position of somatic clones. Both Ci (see Fig. 5H) and β-galactosidase (not shown) were found to be expressed in these clones. These results suggest that *engrailed* response elements are present in the *ci* regulatory region and are located within the 2.2dis+1.2prx-ci construct.

As a further test of the role of *engrailed* in *ci* regulation, *ci* expression was monitored by in situ hybridisation after *engrailed* had been expressed ubiquitously with a *HS-En* transgene. The *HS-En* construct was used in two different types of experiments. The first was a time course in which *ci* expression was monitored at various times following a pulse of heatshock to embryos at a variety of developmental stages: if *ci* is a direct target of En, its RNA would be expected to disappear quickly following the heat pulse (15-20 minutes), whereas an indirect target might be expected to respond only after sufficient time for some intermediate factor to be affected (45 minutes to 1 hour; Manoukian and Krause, 1992). The second was to monitor *ci* expression 2-3 hours following a heat pulse that had been delivered at the onset of gastrulation; *engrailed* stripes widen after recovery from such a heat pulse (Heemskerk et al., 1991), so the absence of *ci* expression in the cells that newly express *engrailed* would suggest that the presence of En is incompatible with the expression of *ci*.

In the first experiment, a pulse of heat shock led to ubiquitous synthesis of En that reached a maximum approximately 20 minutes later and faded away after 60 minutes. 20 minutes after heat shock, these embryos had greatly reduced levels of *ci* RNA. 60 minutes after heat shock, the stripes of *ci* RNA had returned (not shown). This rapid response to *HS-En* suggests a direct interaction between En and the *ci* regulatory region. Similarly, after recovery from heatshock at the onset of gastrulation, stripes of *ci* RNA narrowed (Fig. 4H). We conclude

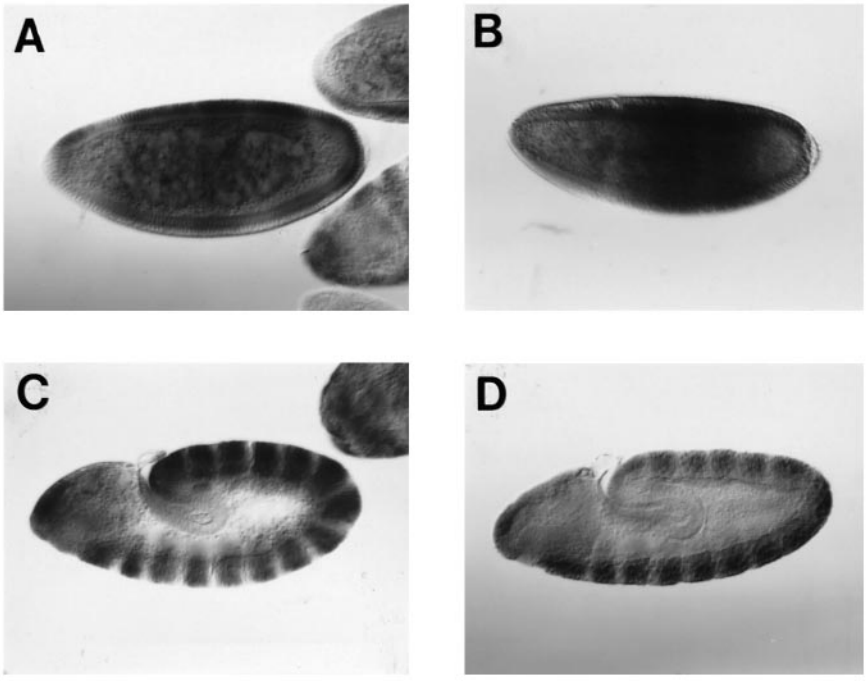

Fig. 3. Comparison of endogenous *ci* and 7.1*ci*/lacZ expression. Anti-β-galactosidase antibodies (D), X-gal staining (B), or in situ hybridisation to *ci* mRNA (A, C) were used to identify cells expressing 7.1-ci and *ci*, respectively. (A,B) cellular blastoderm embryos, (C,D) germband extended embryos.

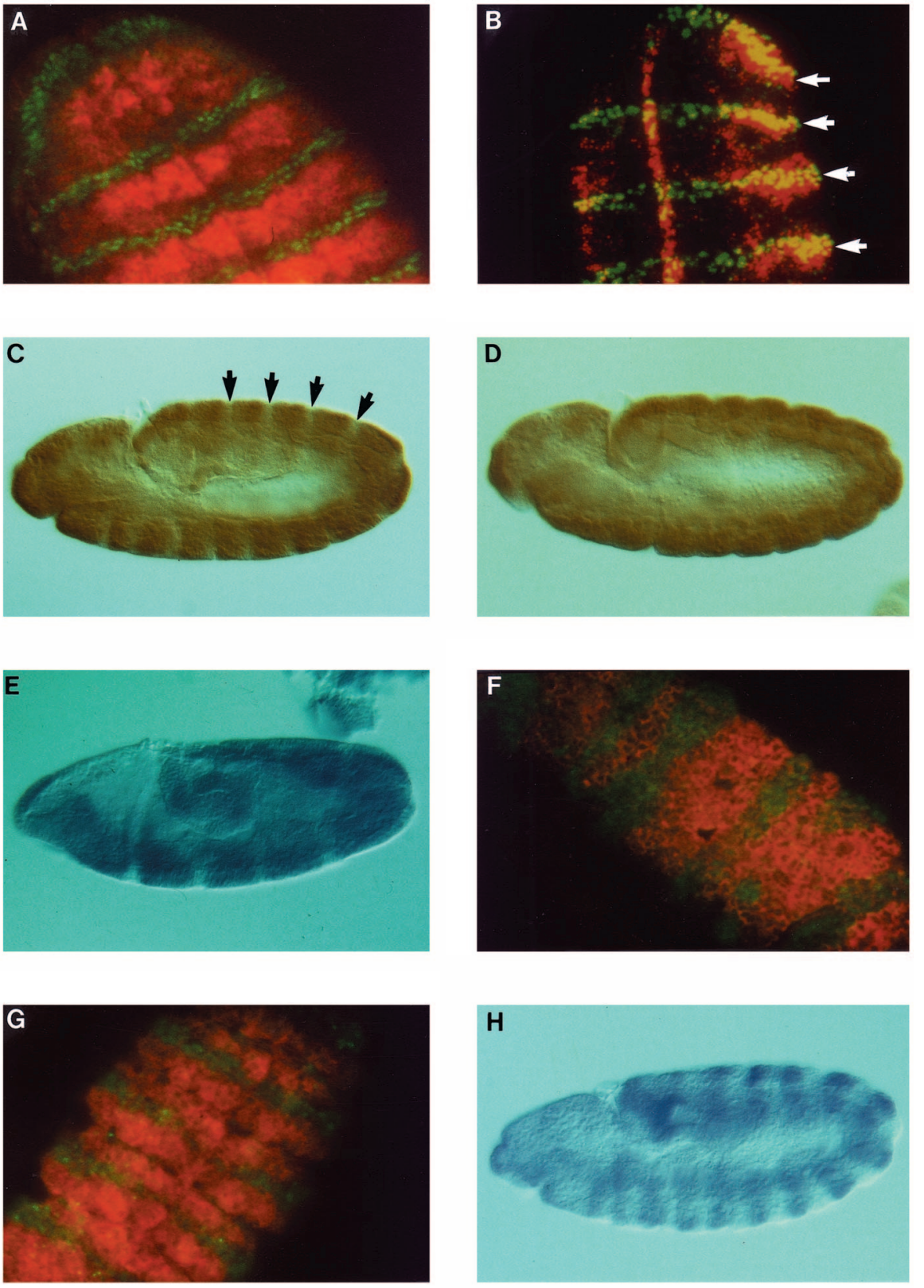

Fig. 4. En and β-galactosidase protein and ci transcripts in embryos. En (green) and β-galactosidase (red: A,B,F,G; brown: C,D) proteins were localised by antibody staining, *ci* transcripts by in situ hybridisation (blue: E,H). (A) 2.2dis-ci+HS, (B) 1.2prx-ci, (C) 2.2dis+1.2prx-ci in a wild-type embryo, (D) 2.2dis+1.2prx-ci in an en^{10} embryo, (E) a ftz^{w26} embryo, (F) 2.2dis+1.2prx-ci in an $eve^{3.77}$ embryo, (G) 2.2dis+1.2prx-ci in a ptc^{IF85} embryo (H) an HS-EN3 embryo that had recovered for 3 hours after a 30 minutes pulse at 37°C at the onset of gastrulation. A,B and G, ventral view; C,D,E,F and H, lateral view. White arrowheads indicate ectopic stripes of expression generated by 1.2prx fragment alone. Black arrowheads point to the same regions in an embryo carrying, in addition, 2.2dis.

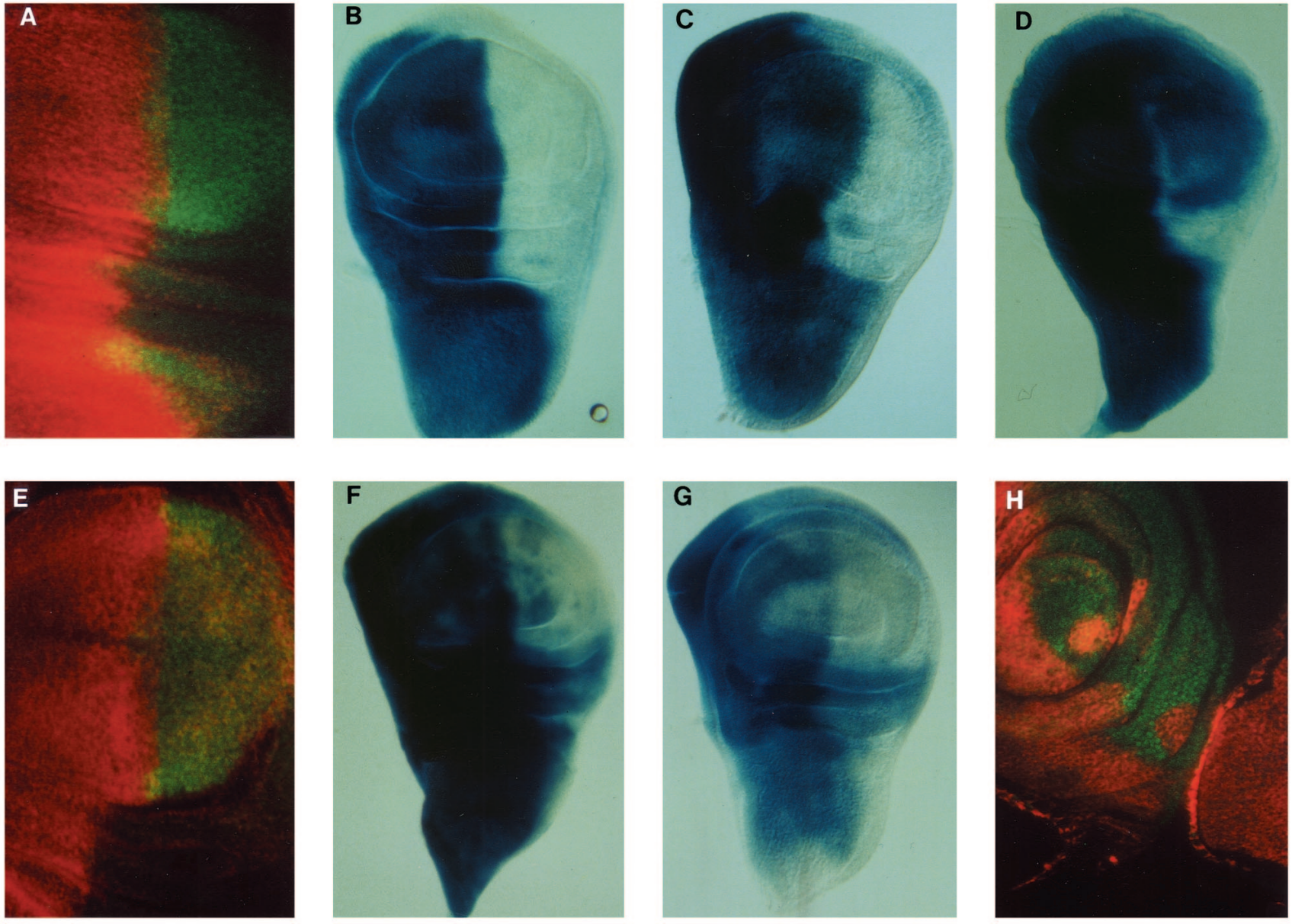

Fig. 5. Expression of En, Ci and β-galactosidase in imaginal disks. En (green) and Ci (red) in imaginal disks were localised by antibody staining (A,E,H), and β-galactosidase (blue) with X-gal (B,C,D,F,G). (A) Wing pouch from a wild-type wing disk. The overlap of En and Ci at the A/P border is due to late expression of En on the anterior side of the border (Blair, 1992). (B) Wing disk carrying 2.2 dis+1.2prx-ci, (C) wing disk carrying 1.4dis+1.2prx-ci, (D) *en*^{1} wing disk carrying 2.2 dis+1.2prx-ci, (E) *ci*^{57g} wing pouch, (F) wing disk carrying 1.6dis-ci^{57g}+HS, (G) wing disk carrying 1.3dis+1.2prx-ci, and (H) leg disk with En^{-} clones in the posterior compartment. Anterior, left.

that the reciprocal patterns of *engrailed* and *ci* expression are functionally linked, and that an important aspect of *engrailed* function is to repress *ci*.

The expression of *ci* and the 2.2dis+1.2prx-ci transgene were also tested in embryos mutant for *fushi tarazu* (ftz^{w26}), *even-skipped* ($eve^{3.77}$), *patched* (ptc^{IF85}), and *wingless* (wg^{IG22}). *ftz* and *eve* are both transcription factors that regulate *engrailed* expression in embryos, but each is also expressed in parts of the anterior compartment where they could regulate *ci* (Lawrence et al., 1987). Similarly, both *patched* and *wingless* are expressed in anterior compartments in embryos and have been implicated in signalling between the compartments (Baker, 1988; Hooper and Scott, 1989; Ingham et al., 1991). In ftz^{w26}, the embryo forms only half as many segments as normal and only half the normal number of En stripes (Howard and Ingham, 1986). Correspondingly, *ci* is expressed in half as many stripes and these stripes are twice as wide as normal (Fig. 4E). $eve^{3.77}$ is a hypomorph which causes the stripes of *eve* expression to fuse partially (Frasch et al., 1988) and the stripes of En to form at irregular intervals (Fig. 4F). The pattern of expression from the 2.2dis+1.2prx-ci transgene is reciprocal to the pattern of *engrailed* expression in $eve^{3.77}$ embryos (Fig. 4F). ptc^{IF85} mutant embryos form twice as many segmental grooves as normal and have twice the number of En stripes (Martinez Arias et al., 1988); in each of these ectopic grooves, *ci* expression disappears (Fig. 4G). Finally, wg^{IG22} mutant embryos do not retain their segment borders after germ band extension and *engrailed* expression is not maintained (Bejsovec and Martinez Arias, 1991); *ci* expression is initially normal in these embryos, but the periodicity of *ci* stripes deteriorates during germ band extension, and *ci* begins to be expressed throughout the embryo (not shown).

In each of the abnormal conditions described above, expression of both *ci* and the transgene changed in a manner that was reciprocal to changes in *engrailed* expression (Fig. 4F,G). Similar results have been noted with *naked* mutants and in a *HS-Wg* background (Noordermeer et al., 1992). Although it is not possible to derive precise regulatory relationships from such studies, these studies are consistent with the suggestion that *ftz, eve, ptc,* and *wg* affect *ci* expression through their effects on *engrailed*.

Engrailed protein binds to *ci* sequences in polytene chromosomes

Since *ci* transcription in embryos appears to respond directly to En, and since the 2.2dis fragment contains *engrailed* reponse elements, it is important to determine if En binds to sequences within the *ci* regulatory region. To determine if En could be detected bound to *ci* in vivo, polytene chromosomes from salivary glands were scanned with anti-En antibody to detect bound protein. Although *engrailed* is not normally expressed in salivary glands, it can be induced in these cells in *HS-En* strains, and previous studies in this laboratory demonstrated that En binds to a limited number of sites on polytene chromosomes in such *HS-En* strains (e.g. 10-12 prominent sites of binding were detected on the X chromosome; F. Maschat, unpublished data). This technique can indicate the chromosomal region but not the actual site of binding. However, if a region containing putative binding sites was moved to a new chromosomal location and a novel band of staining appeared, it would argue strongly that this DNA contained binding sites. The insertion sites of *ci/lacZ* fusions provide a potential relocation of binding sites, and one of the strains with an insertion in the 1E-F region provided an optimal cytological location for examination (Fig. 6A). Although no En binding was detected in region 1E-F in chromosomes lacking this insert (Fig. 6B), a novel band of En was present in chromosomes from the strain carrying an insertion of 3.7dis+1.2prx-ci at 1E-F (Fig. 6C). This observation suggests that En can bind with high selectivity to *ci* regulatory sequences in vivo, and that it could act to regulate *ci* directly.

Engrailed protein binding sites in the *ci* 1.4dis fragment

Since En binds to *ci* sequences in vivo, its ability to bind to 1.4dis in vitro was tested. Footprinting analysis identified two high affinity sites. DNase I footprinting of small fragments representing the entire 1.4dis fragment located a single footprint spanning the sequence ATGTAATTGAGCA between nucleotides 757 and 769 (Figs 7A, 8A). 1,10-phenanthroline copper ion footprinting of En-DNA complexes identified a second footprint spanning the sequence CTAATTAAG between nucleotides 192 and 200. The ci^{57g} deletion removes these residues (Figs 7A, 8B). Although both sites contain the TAATT motif that is common to most homoeodomain binding sites, neither site contains a perfect match to the previously determined consensus for En (Desplan et al., 1988). Curiously, a precise match to that consensus located between nucleotides 540 and 549 was not footprinted in either assay.

DISCUSSION

Two distinct regulatory elements control three different aspects of *ci* transcription

The developmental program of *ci* expression features a variety of patterns. In embryos, the first pattern appears in three broad bands. These bands resolve into anterior compartment-specific, segmentally reiterated stripes in gastrulating embryos. Gastrulating embryos also express *ci* in a continuous stripe of cells

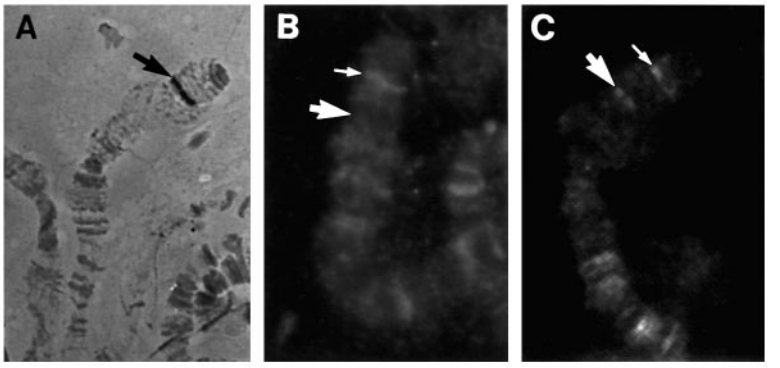

Fig. 6. En binds to *ci/lacZ* sequences in vivo. (A) The black arrowhead indicates site of hybridisation of a *lacZ* probe to 3.7dis+1.2prx-ci integrated at 1 E-F. Anti-En antibody does not stain 1 E-F in wild-type (B), but does when 3.7dis+1.2prx-ci is integrated (C). Small white arrowhead: En staining distal to 1 E-F present on all chromosomes. Large white arrowhead: 1 E-F.

A

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CTGCAGCTTG CTCCGTCTTA ATCGAAAAAG AAACTGGATT CAAGAAGACA   50
TTATACGTTA ACGGCTTGAA TGTATATACA GTATACGTAG ATATATGATA  100
TAAATGTGTG TATACATATA CGTATACACA AGTGTGCGAG CATATCGCTT  150
AAGCCGAACG TTCTCCACCC TTTTCGGCTT CGAACCGGTG ACTAATTAAG  200
CCTTTAAAAT TTGTACTGAA CCAAAGAAGA GAGGATAATA AAGTCATTGG  250
GTACACCGCA TAACTAAAGT GCTGTAATGG CAAGCAAAAA GTAGAAAAAA  300
TATTAAATAT TAAAAATTAT TTTGAACCAT TTTTATATTA CTGCTTTAAC  350
ACTGAATTTA TTATTTAGAC GATGTGATTA TTGATTTGTT AATAAATTAT  400
ATATATACAT TTAAAAATTA TATAAATTGT TATAAATTAT ATAGATAAAT  450
ATATACATAT ATCTACATAT GTATATTTAT GAAGACAACT CAAAGAATTT  500
TTTAAAACCA CATATGTATG TACATGTCTT ATAATACTTA TTTAATTGTG  550
GAAAACATAT GTACATGCTT CAATACACTA AATAATACTA ATAATACTAA  600
ATAGATATGA ATACATATGT TTCATTACGG ACCTACGCAC AATCCGTTAT  650
TTTTTTGCAA ATTATGAAGA TGTGCAAAGT TGTTTGAAAT TATTTACAAT  700
TTACATATAT ACATTTATAT ATATAAGTTT GGATGCGAAA ATGTACGTAC  750
ATATGTATGT AATTGAGCAT ATGTGTATGT ATTTATTTTT ATGATAATAC  800
TTAGAGATAT GTATGTATTT ATGTTCATAT GTATAACAAA ATTTCTGGTT  850
TTGTAGTTTC CAAGAATCGA CAAAACGTTT TTTATAAAAA GGAATGGGAC  900
CACCAAACAT ACATACATAC CTATATATCT ATGTATATTT ATGTATGGGT  950
GCATATACAC GCTTAAAGAA GCGTACAGAC GTACCTGCAA ATACGATTAT 1000
CTTGTGCGGA TATTTATACA CACATATACA TACATTCATA AAGGGTCTGG 1050
AAAATCACAC AAATGTACGT TAAGCAAACA TACAGATTTG TTTGTATGTG 1100
TAAGCTGTAG AAGCACAGCA TGTGTCCGCT TCTTCATAGA GTTTTGAGTA 1150
AGACGTTAAA TTACAAAAGG CCTGGCACAT ACACACACAA GTATGTATGT 1200
ATATAGATGC ATACAATATA CATACAAACG CATGGGTATC AAAAGAATCT 1250
GACGATACCA GATTCAATCC TGCTGTGCAC TTGCAGGAAA AATGTTATTT 1300
TTCTTGTTTT AATGCCGGAC AAGGATACAC CATACACATC TACATATGTA 1350
TGTATGTACA GAGTTGTTTT ATAGTTAGGA AATTCATTGC CAAGACTAGT 1400
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B

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                    -290                              356
GTCTATATTA GAAACATTTG AATTTATTTA TTAACTGAAT AAATTATTTA
TTTATTATTT AGACGATGT
```

Fig. 7. DNA sequence of 1.4dis and the junctions of the *ci*^{57g} deletion. (A) 1.4dis was sequenced on both strands; 0, the first 5′ base of the *Pst*I site (see Fig. 1A). The 3′ end of the *ci*^{57g} deletion (arrowhead) is at position 356, with the sequences deleted in 1.3dis+1.2prx*ci* surrounded by a dashed line. The positions of the En footprints are indicated by boxes. (B) Sequence of the junctions of the *ci*^{57g} deletion, including 29 bp of inserted sequence (bold) and 20 bp distal and proximal to the deletion. No other changes between wild type and *ci*^{57g} were identified in over 1 kb of sequence.

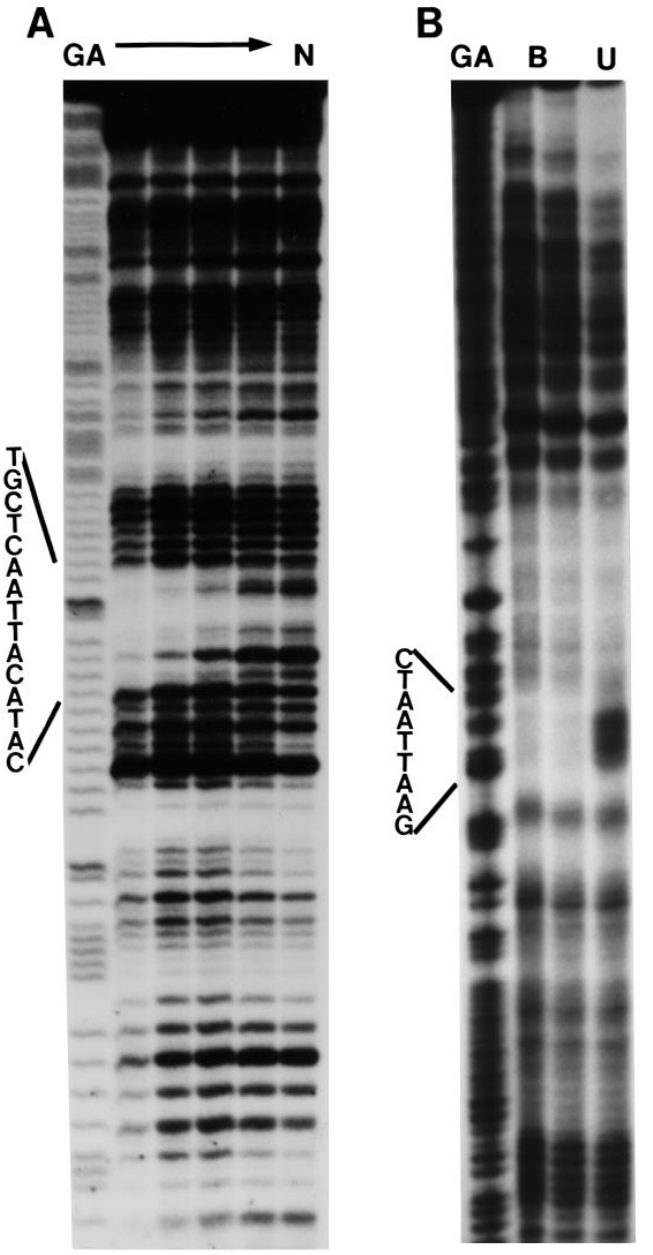

Fig. 8. Footprints of En on sequences in 1.4dis. (A) DNase I footprint of ATGTAATTGAGCA (upper strand sequence) between nucleotides 757 and 769: (GA) sequencing standard, (N) no protein. The arrow indicates decreasing protein concentration. (B) 1, 10-phenanthroline copper ion footprint of CTAATTAAG between nucleotides 192 and 200: (GA) sequencing standard, (B) bound, (U) unbound.

along the ventral midline, in all mesodermal cells, in most of the cells in the head, and in selected cells of the central nervous system. Larval imaginal disks express *ci* exclusively in their anterior compartments. In this study, we analysed the upstream region of the *ci* gene to identify sequences that regulate these various patterns of expression. We discovered two clusters of regulatory elements that recreate several aspects of the *ci* pattern. One activates *ci* along the ventral midline. The other generates anterior compartment-specific expression in the epidermal cells of embryos and imaginal disks. It appears to do so through a two step process that involves ubiquitous activation and posterior compartment-specific repression.

Transcriptional activation along the ventral midline was observed with a 1189 bp DNA fragment (1.2prx-ci) that contains 742 bp of proximal promoter sequence as well as a portion of the 5′ untranslated region. 1.2prx provided robust expression either in the absence of additional regulatory or promoter sequences, or when coupled with other *ci* regulatory elements.

Anterior compartment-specific expression in embryos and imaginal disks was observed with fragments of the *ci* gene located between 2.75 to 5 kb upstream of the transcription start site (1.4dis - 2.2dis). This region of *ci* sequence element was equally active when coupled to either the *ci* proximal promoter

region (1.2prx-ci) or to a crippled HSP-70 promoter. 1.4dis was observed to generate anterior compartment-specific expression as well as to repress expression in embryonic posterior compartments that were produced by the 1.2prx-ci fragment alone. This suggests that the compartment-specificity of its patterns results from generalized activation and compartment-specific repression, a model that is supported by truncations of 1.4dis that retain their ability to activate transcription, but lose their compartment specificity.

The functional importance of 1.4dis is underscored by its proximity to the chromosome rearrangements associated with several *ci* alleles (Fig. 1A). Most notable is ci^{57g}, a deletion of 646 bp that removes the distal portion of 1.4dis. ci^{57g} causes ectopic expression in the wing posterior compartment, as do distal truncations of 1.4dis. Moreover, there is an excellent correlation between the mutant phenotype of ci^{57g} in the posterior wing compartment and the posterior compartment *lacZ* expression of truncated 1.4dis. Other mutations in this region, ci^{W}, ci^{36l}, and ci^{1}, also have adult-specific phenotypes, in contrast to the lethal ci^{D} and *Cell* alleles, which have rearrangements that map elsewhere. We infer that 1.4dis contains the principal sequence element involved in imaginal disk expression. Since 1.4dis also recapitulates the anterior compartment specific program of expression in embryos, and this program is unaffected by distal truncation of 1.4dis or ci^{57g}, the sequences involved in posterior compartment repression in embryos and disks are apparently distinct.

The apparent simplicity of the *ci* regulatory region contrasts with the organization of the *engrailed* gene, whose segmental stripes emerge individually at the cellular blastoderm stage (DiNardo et al., 1985; Weir and Kornberg, 1985). In contrast, the *ci* pattern resolves from broad bands at approximately the time when the *engrailed* pattern matures (Eaton and Kornberg, 1990). The different ways in which these patterns emerge is reminiscent of the differences between primary and secondary pair rule genes. Whereas the primary pair rule genes such as *hairy* and *eve* have separate regulatory elements for the initial expression of each stripe (Goto et al., 1989; Harding et al., 1989; Riddihough and Ish-Horowicz, 1991; Small et al., 1991), the stripes of the secondary pair rule genes such as *ftz* are activated concurrently by single elements (Dearolf et al., 1989; Hiromi and Gehring, 1987). We suggest that from a similar perspective, *ci* might be considered as a 'secondary segment polarity gene', one that depends upon the prior establishment of another segment polarity gene, *engrailed*.

Factors regulating *ci* expression

We have shown that proper *ci* expression is dependent upon *engrailed* function, and that its regulatory region can be bound by En both in vitro and in vivo. Since, in addition, *ci* responds rapidly to En-mediated repression in embryos, we believe that the conclusion that En is a direct repressor of *ci* is justified. We are intrigued by the presence of an En binding site within the ci^{57g} deletion and also by the presence of sequences that are required for posterior compartment repression in imaginal disks that are not bound by En. At this point, it is not clear whether the latter sequences are bound by some other factor, or if their removal simply perturbs binding to the adjacent En binding site. If the sequences are bound by some other protein, it will be interesting to know if it is ubiquitously expressed or if it is restricted to the posterior compartment. At this time, En is the only transcription factor known to be restricted to the posterior compartment.

We know little about the factors that activate *ci* expression. As the initial pattern in embryos has broad stripes, it is possible that *ci* expression is initiated by a combination of gap genes rather than by the pair rule genes. Consistent with this possibility is the observation that *ci* expression expands into abnormally broad stripes in *eve* and *ftz* mutant embryos (Fig. 3F,G). This suggests that neither *eve* nor *ftz* is uniquely responsible for activation or repression in any particular domain.

Maintenance of compartment identity by *engrailed*

While *engrailed* is essential for the maintenance of posterior compartment identity (Garcia-Bellido and Santamaria, 1972), the mechanism by which it carries out this role remains unclear. We propose a dual role for *engrailed* in this process. The first is direct, and involves the repression of genes whose lack of expression in posterior compartment cells is essential to their posterior compartment identity. Such genes include *ci* as well as, perhaps, *patched*, and *decapentaplegic* (Eaton and Kornberg, 1990; Hidalgo, 1991; Raftery et al., 1991). The repression of *ci* by *engrailed* would ensure its absence in the posterior compartment and would also help enforce the absence of potential targets that *ci* might activate. The second role is indirect, and in it *engrailed* signals to cells in the anterior compartment by activating *hedgehog* (Tabata and Kornberg, 1994). In embryos, these two roles of *engrailed* appear to be interdependent - direct regulation by *engrailed* turns off inappropriate signalling pathways in the posterior compartment while the consequence of its signalling to the adjacent anterior cells elicits a response (Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991) that helps to maintain *engrailed* expression in posterior compartment cells.

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Note added in proof

The sequences contained in this paper have been submitted to GenBank and have the following accession numbers: ci prx fragment – U21390, ci dis fragment – U21389.

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