The Drosophila *engrailed* and *invected* Genes: Partners in Regulation, Expression and Function

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Manuscript received September 22, 1995
Accepted for publication December 13, 1995

ABSTRACT

We isolated and characterized numerous *engrailed* and *invected* alleles. Among the deficiencies we isolated, a mutant lacking *invected* sequences was viable and phenotypically normal, a mutant lacking *engrailed* was an embryo lethal and had slight segmentation defects, and a mutant lacking both *engrailed* and *invected* was most severely affected. In seven *engrailed* alleles, mutations caused translation to terminate prematurely in the central or C-terminal portion of the coding sequence, resulting in embryonic lethality and segmentation defects. Both *engrailed* and *invected* expression declined prematurely in these mutant embryos. In wild-type embryos, *engrailed* and *invected* are juxtaposed and are expressed in essentially identical patterns. A breakpoint mutant that separates the *engrailed* and *invected* transcription units parceled different aspects of the expression pattern to *engrailed* or *invected*. We also found that both genes cause similar defects when expressed ectopically and that the protein products of both genes act to repress transcription in cultured cells. We propose that the varied phenotypes of the *engrailed* alleles can be explained by the differential effects these mutants have on the combination of *engrailed* and *invected* activities, that *engrailed* and *invected* share a regulatory region, and that they encode redundant functions.

PAIRS of genes with closely related sequences are not uncommon in eukaryotic chromosomes, but the functional significance of their presence and relatedness is in general unclear. Among the Drosophila segmentation genes, there are several known examples. These are *engrailed (en)* and *invected (inv)* (POOLE *et al.* 1985), *knirps* and *knirps-related (kni, knrl)* (ROTHE *et al.* 1989), *gooseberry-distal (gsb-d)* and *gooseberry-proximal (gsb-p)* (BAUMGARTNER *et al.* 1987), *sloppy paired 1 (slp1)* and *2 (slp2)* (GROSSNIKLAUS *et al.* 1992), and *zerknüllt (zen1)* and *2 (zen2)* (RUSHLOW *et al.* 1987). Each of these gene pairs is characterized by close genetic linkage, by related or nearly identical patterns of expression, and by extensive sequence homology. Because these gene pairs also have regions that are highly divergent, delineating the respective roles and biological importance of each gene is essential to understanding the function of either one.

Several studies have described how the *kni, slp, gsb* and *zen* genes share their respective functions. For instance, *kni* is essential for the abdominal region of the embryo to develop, and *knrl* is unable to substitute, apparently because the presence of a 19-kb intron delays the synthesis of its primary transcript. In contrast, *kni* and *knrl* are functionally redundant for normal development of anterior head structures (GONZALEZ-GAITAN *et al.* 1994). The gene pairs *zen1* and *zen2* and *slp1* and *slp2* appear to encode redundant functions. Mutants that lack *zen2* develop normally, indicating that *zen1* is a sufficient source of *zen+* activity and that *zen2* is not essential (RUSHLOW *et al.* 1987). However, it is unclear if *zen2* can be similarly efficacious in the absence of *zen1,* because mutants lacking only *zen1* have not been isolated. Redundancy among the *slp* genes is suggested by the stronger phenotype of mutants that affect both *slp1* and *slp2* when compared with mutants that affect only *slp1* (CADIGAN *et al.* 1994). Mutants that affect only *slp2* have not been isolated. Ectopic expression of *gsb-d* and *gsb-p* causes similar phenotypes, suggestive of functional redundancy despite the different expression patterns of these two genes (LI and NOLL 1994).

In this report, we address the relationship of *en* and *inv*. These genes are expressed under similar developmental programs—in 14 stripes in the embryo that correspond to the posterior compartments, in the posterior compartment of each imaginal disc and histoblast nest, in one-half of the hindgut, in similar patterns of cells in the central nervous system, and in selected other tissues (POOLE *et al.* 1985; COLEMAN *et al.* 1987; HAMA *et al.* 1990). A conspicuous difference in their expression patterns is the delayed appearance of *inv* transcripts relative to *en,* a difference that is presumably related to the larger size of the *inv* transcription unit (29 kb *vs.* 3.4 kb). The genes encode similarly sized proteins (En: 553 residues; Inv: 576 residues) that contain almost identical homeodomains (53/61 identical residues) that are embedded within a region of high homology

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(86/91 homologous residues) near their respective C termini. Probably reflecting their closely related structures, the proteins function similarly when expressed ectopically. Placed under heat shock control, ubiquitous expression of either gene in blastoderm stage embryos disrupts segmentation and causes denticle belts to fuse (K. G. COLEMAN and T. B. KORNBERG, unpublished data; POOLE and KORNBERG 1988). Despite these many similarities, *en* and *inv* have contrasting genetic properties.

The *en* gene is represented by a viable allele (en^1) and by a large number of recessive, embryonic lethal alleles that are, with one exception, noncomplementing. en^1 embryos are normal, but mutant adults have wings and legs with posterior compartments that are partially transformed toward anterior. Among the lethal alleles, those that have a normal chromosome arrangement ($en^{point\text{-}lethal}$) complement en^1 to produce flies with minor wing vein abnormalities (KORNBERG 1981). In contrast, those that have a visible chromosome rearrangement breakpoint in *en* ($en^{rearrangement\text{-}lethal}$) complement en^1 poorly, producing flies with more extreme abnormalities in wings and other tissues. We suggest two possible explanations for this pattern of complementation—*trans* complementation between mutant *en* genes on paired homologs that is disrupted in the rearrangement mutants (transvection), or alternatively, polar effects that extend the consequences of the chromosome breaks on the mutant chromosomes beyond the *en* gene, possibly to *inv*. We investigate these possibilities in the work described below.

Other puzzling aspects of the *en* phenotype are the effects of *en* mutant cells in genetic mosaics. Clonal patches of en^1 cells faithfully reproduce all aspects of the mutant phenotype that characterizes en^1 flies, including partial anterior transformation of posterior cells and associated slight overgrowth (LAWRENCE and MORATA 1976). In contrast, clonal patches of posterior $en^{point\text{-}lethal}$ cells transform much less effectively (KORNBERG 1981; LAWRENCE and STRUHL 1982). Since the embryonic lethality caused by these alleles suggests more extreme reductions in *en* function than caused by the viable en^1 allele, these marginal phenotypes are surprising.

en mutants have arisen spontaneously (*e.g.*, en^1) and have been isolated in genetic screens for either noncomplementing alleles or for their effects on segmentation. Because the frequencies with which *en* mutants were isolated in these screens are comparable with those of other single loci, single mutations are apparently sufficient to elicit an *en* phenotype (KORNBERG 1981). Although >60 *en* alleles were identified in these screens, *inv* mutants were not. *inv* either has a nonessential role, its phenotype has escaped notice, or it is a poorly mutable target.

Most extant *en* alleles are embryonic lethal, and they perturb segmentation to create a pair-rule phenotype. Although extensive deficiencies have more severe effects and create a lawn phenotype devoid of obvious segmentation, the null phenotype has yet to be clearly defined and the cause for the less extreme pair-rule phenotype characteristic of most *en* alleles is not understood. Possible explanations we have considered are that the existing *en* alleles are not nulls or that *inv* might partially compensate for the loss of *en*.

To address these many unresolved aspects about the roles of *en* and of its relationship to *inv*, we have undertaken a genetic analysis of both genes. We have characterized numerous mutant alleles, including newly isolated *inv* mutants and deletions that remove portions of both genes or of either gene alone. The evidence we obtained suggests that *en* and *inv* are functionally linked, both through their regulation by a shared set of *cis*-regulatory sequences and by their overlapping and redundant roles.

MATERIALS AND METHODS

Fly strains: Most of the *en* and *inv* alleles used were previously described (NÜSSLEIN-VOLHARD and WIESCHAUS 1980; KORNBERG 1981; NÜSSLEIN-VOLHARD *et al.* 1984; KUNER *et al.* 1985; GUSTAVSON 1993; TABATA *et al.* 1995). The deletions $Df(2R)en^C$, $Df(2R)en^E$, and $Df(2R)en^F$ were isolated among progeny of males in which a *P* element that had inserted upstream of the *en* transcription start was mobilized (ryxho25; HAMA *et al.* 1990). $Df(2R)inv^{30}$ was isolated in a similar manner among the progeny of males which contained a *P* element that had inserted at −6 to +2 bp at the *inv* transcription start site (wxba21; HAMA *et al.* 1990). inv^{177}, en^{SF68}, and en^{SF69} were isolated in a F_2 screen for X-ray-induced lethal alleles of en^{SFX31}, and the rearrangement breakpoints were mapped by Southern blot analysis. en^{CX1} was provided by R. HOLMGREN. The position of these and other alleles used in this study are illustrated in Figure 1.

Preparation of heat shocked embryos: Embryos for heat shock treatment were collected for 1–2 hr or overnight. Nuclear cycle 14 embryos were selected, heat shocked as described (POOLE and KORNBERG 1988), fixed and processed for *in situ* hybridization.

Construction of genomic libraries: A *Hin*dIII restriction site polymorphism present in the *en* gene of en^{LA4}, en^{LA7}, en^{LA9}, en^{LA10} and en^{LA11} mutant chromosomes, but absent in the CyO balancer chromosome, was used to distinguish clones with mutant DNA. A polymorphic *Bgl*II site was used to distinguish en^{IK57}, en^{IM199}, en^{IO34} and en^{B86} DNA, and a polymorphic *Eco*RI site was used to identify en^C DNA. Genomic DNA was digested with *Hin*dIII, *Eco*RI or *Xba*I and size-selected electrophoretically in a 0.7% agarose gel before cloning into commercial plasmid, phage or phagemid vectors.

en^{LA4}, en^{LA7} and en^{LA10} *Hin*dIII fragments were ligated to *Hin*dIII-cut and calf intestinal alkaline phosphatase-treated pEMBL9 (DENTE *et al.* 1983) vector DNA. en^{LA9} and en^{LA11} *Hin*dIII fragments were ligated to λZap and en^C *Eco*RI fragments were ligated to λgt11. en^{IK57}, en^{IM199}, en^{IO34} and en^{B86} *Xba*I fragments were ligated to *Xba*I-cut arms of λ2001 (Stratagene). Phage were packaged using Giga Pak Gold (Stratagene) and plated on P2392 or BB4 cells. Plasmid or phage libraries were screened by colony or plaque hybridization and purified using standard procedures. A 4.7-kb genomic *Eco*RI fragment containing the first exon of the *en* gene as probe for all experiments except with en^C embryos, for which a 0.9-kb *Eco*RI fragment containing the C-terminal two exons was used. Clones with mutant *en* genes were sequenced on one

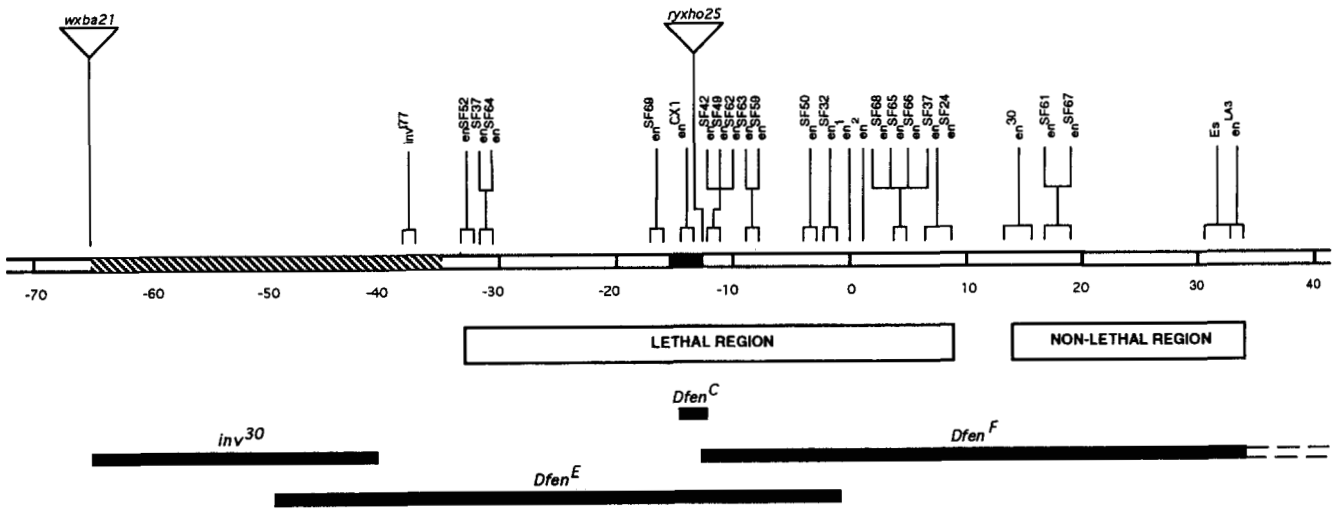

FIGURE 1.—Diagram of the *en-inv* region. The open bar represents ~100 kb of the 2R that includes *en* and *inv*. Distances in kilobases are indicated below the bar. The *en* and *inv* transcription units are shown as a filled bar and a bar with diagonal shading, respectively. *inv* is transcribed from left to right and *en* is transcribed right to left on the map. Positions of *en* rearrangement mutations are indicated above the bar. The positions of the *ryxho25* and *wxba21* transposon insertions are indicated. The extent of *Df (2R)en*^{C}, *Df (2R)en*^{E} and *Df (2R)en*^{F} deletions are shown below the bar. The distal endpoint of *Df en*^{F} is beyond the region shown and is not known.

strand, except in the region of the mutation that was subcloned and sequenced on both.

Immunohistochemistry and *in situ* hybridization: Mutant flies were balanced with a CyO chromosome containing a *P*-element construct in which lacZ is controlled by the *fushi tarazu (ftz)* promoter. Staining for β-galactosidase allowed identification of mutant embryos during germband elongation and subsequent stages. Preparation of fixed embryos, immunohistochemistry and microscopy were essentially as described (KARR *et al.* 1989). Primary antibodies were: anti-En, 1:5000 dilution of a Guinea Pig polyclonal antibody, (K. G. COLEMAN and T. B. KORNBERG, unpublished data); anti-En/Inv, 1:1 dilution of 4D9, (PATEL *et al.* 1989); and anti-β-galactosidase, 1:2500 dilution, Cappel. *In situ* hybridization was as described (EATON and KORNBERG 1990) using specific probes for *en* (the AflII-SalI fragment 123-677 of the *en* cDNA) or *inv* (the *Eco*RI-*Bst*XI fragment 1-996 of the *inv* cDNA or a 550-bp genomic fragment from base 1976 to an *Eco*RI site downstream of the transcription unit) transcripts. The *ci* and *hh* probes were as described in SCHWARTZ *et al.* (1995) and TABATA *et al.* (1992).

Construction of transgenic flies: Plasmids were constructed in pCaSpeR-hs, a modified version of pCaSpeR into which the promoter and 3′ flanking sequences of the hsp70 gene had been inserted. The *en* and *inv* cDNAs were cloned directly into the *Eco*RI site. Injections were into homozygous Df(1)w^{67c23}, *y*.

Transfection assays: Drosophila Schneider S2 cells were grown in 60-mm tissue culture dishes containing 5 ml Schneider medium, 10% fetal calf serum at room temperature. Cells were plated at 50% confluency and cotransfected by calcium phosphate treatment. The reporter plasmid (1 μm) (pT3N6D-33CAT, which contains three glutocorticoid response elements upstream of six homeodomain binding sites, JAYNES and O'FARRELL 1991) was cotransfected with 0.2 μg pPAc-GR (a plasmid encoding the glucocorticoid receptor), 10 ng of pHsp82-lacZ (a plasmid encoding β-galactosidase), and either 300, 30, or 3 ng of either pPAc- En or pPAc- Inv per plate. pPAc (KRASNOW *et al.* 1989) was added to each DNA mixture to bring the total DNA added to each culture to 10 μg. Dexamethasone was added 24 hr after transfection to a final concentration of 100 nM, and cells were grown for an additional 24 hr. Cells were harvested and CAT activity was determined and standardized against β-galactosidase activity.

RESULTS

Only closely linked chromosome rearrangements disrupt inter-allelic complementation among *en* mutants: To determine if complementation between *en*^{1} and the *en*$^{point\text{-}lethals}$ is dependent upon chromosome pairing, flies carrying the *en*^{LA10} allele were subjected to X-rays, and their F_2 progeny were screened for enhanced phenotype when heterozygous with *en*^{1}. If synapsis is required for complementation, rearrangements that disrupt pairing should be readily identifiable by increased disruption of posterior venation and by transformation of posterior row bristles (Figure 2). By analogy with *bithorax* and *decapentaplegic*, such rearrangements should be expected to lie between *en* and the centromere in the proximal region of the right arm of chromosome 2, and they should be frequent products of X-ray mutagenesis. Among 5800 F_2 matings that were scored, only six potential mutants with enhanced phenotype were identified. Five behaved genetically as *en* alleles and had visible chromosome aberrations in the 48A region. Four of these had altered (*en*^{SF64}, *en*^{SF65}, and *en*^{SF66}) or missing [*E(en)eg-13*] restriction fragments in the region of the *en* gene that could be detected by Southern analysis with genomic probes (not shown); the fifth was lost before Southern analysis could be completed. The remaining mutant, *E(en)eg-1 en*^{LA10}, has a pericentric inversion [In(2LR)23C;41], but it is cytologically normal in the 48A polytene interval and has no altered restriction fragments in the *en* region that were detected by Southern analysis. Further

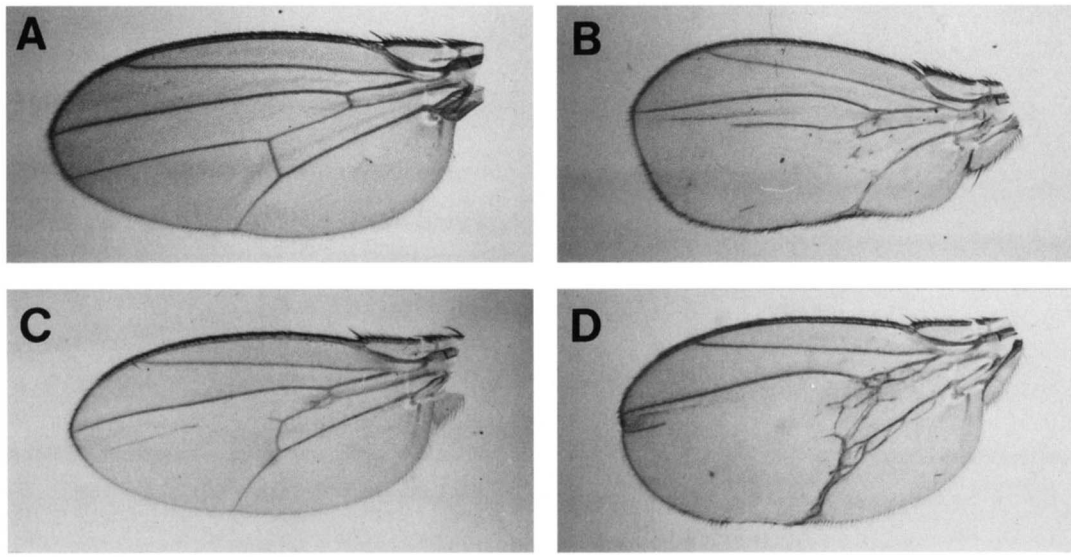

FIGURE 2.—Comparison of wing blades. (A) wild type (B) en^{1}/en^{1} (C) $en^{1}/en^{LA10}/$, (D) $en^{LA10}/E(en)eg\text{-}1$.

characterization suggests that *E(en)eg-1* is a general enhancer of *en*, and that the phenotype of *E(en)eg-1* results from inactivation of a gene that interacts with *en*, not from asynapsis *per se* (E. GUSTAVSON and T. B. KORNBERG, unpublished observations).

In these studies, mutations allelic to *en* were isolated at a frequency similar to that previously observed in screens for noncomplementing alleles (KORNBERG 1981). With one exception, we did not isolate chromosome rearrangements in regions unrelated to *en* function that affect complementation at *en* and so did not obtain evidence for synapsis-dependent complementation. Although it is possible that a closely linked pairing site restricts mutants that disrupt synapsis to the immediate vicinity of the *en* locus (for instance, see HOPMANN *et al.* 1995), we pursued alternative explanations for the observed complementation between en^{1} and the $en^{point\text{-}lethal}$ alleles, and in particular, investigated the possibility that *inv* activity might ameliorate the $en^{1}/en^{point\text{-}lethal}$ phenotype and its absence might contribute to the more extreme phenotype of $en^{1}/en^{rearrangement\text{-}lethal}$ flies.

The nature of $en^{point\text{-}lethal}$ mutations: To first understand the basis for the phenotypes of $en^{point\text{-}lethal}$ alleles, the molecular nature of the responsible mutations was studied. Nine $en^{point\text{-}lethal}$ alleles were chosen for study; seven (en^{LA4}, en^{LA7}, en^{LA10}, en^{IM199}, en^{B86}, en^{IK57} and en^{IO34}) have severe embryonic lethal phenotypes and two (en^{LA9} and en^{LA11}) have much less severe phenotypes. DNA from these nine mutants was isolated and the *en* coding region of each was sequenced. Four, en^{LA4}, en^{LA7}, en^{LA10}, and en^{IM199}, have single base changes that create termination codons in the C-terminal two-thirds of the coding region. en^{B86} and en^{IO34} delete portions of the coding region that shift the reading frame to create sequences that could encode truncated peptides. en^{IK57} has a single base change that changes the splice acceptor junction of intron I and is expected to have aberrant splicing (Figure 3).

en^{LA9} and en^{LA11}, which have the weakest phenotypes, also have single base changes that create termination codons (Figure 3). Unexpectedly, these stop codons are closest to the En amino terminus. These two alleles partially complement other $en^{point\text{-}lethal}$ and $en^{rearrangement\text{-}lethal}$ alleles. en^{LA9} is a larval/pupal lethal. Some en^{LA9} larvae have slight segment fusions, and occasional escapers develop with wing phenotypes comparable to en^{1}. en^{LA11} is viable and can be maintained as a homozygous stock. We were surprised by the nature of these mutants, and so investigated their effects further as described below.

Patterns of *en* and *inv* expression in *en* mutants: We wanted to understand how the various mutants affected *en* and *inv* expression. We first compared the patterns of expression of *en* and *inv* in wild-type embryos, using *in situ* hybridization (Figure 4). In agreement with previous analyses (COLEMAN *et al.* 1987), *en* and *inv* were observed to be expressed in essentially identical patterns throughout most of embryogenesis, and several differences were noted. Whereas stripe 2 of *en* was visible during nuclear cycle 14, *inv* was not detectable until the onset of gastrulation. In addition, *inv* did not appear to have the ordered appearance of stripes that is characteristic of *en*. Finally, *inv* transcripts in the embryonic hindgut were detected earlier (not shown) and more abundantly than *en* transcripts (Figure 4, G and H).

To establish how the patterns of expression were affected in several $en^{point\text{-}lethal}$ mutants, two antibody preparations were used to monitor the synthesis and distribution of En and Inv proteins: 4D9, a monoclonal antibody that recognizes the homeodomains of both En and Inv (anti-En/Inv; PATEL *et al.* 1989), and a polyclonal serum that specifically recognizes En (anti-En). En patterns were examined in en^{LA4}, en^{LA7}, en^{LA10}, en^{B86},

mutation	molecular lesion	position	result	phenotype
en^{LA11}	CAA→TAA	codon 27	stop	weak
en^{LA9}	CAG→TAG	codon 52	stop	weak
en^{IO34}	29bp deletion	codon 72	frameshift	strong
en^{B86}	53bp deletion	codon 75	frameshift	strong
en^{LA10}	CAG→TAG	codon 305	stop	strong
en^{LA7}	TGG→TAG	codon 422	stop	strong
en^{LA4}	TGG→TGA	codon 422	stop	strong
en^{IK57}	G→A	last nt of first intron	splicing errors	strong
en^{IM199}	CAG→TAG	codon 497	stop	strong

FIGURE 3.—Summary of molecular lesions in nine mutant alleles in the *en* gene and expected consequences. Seven out of nine point mutations were due to single base changes. en^{IO34} is a deletion of 29 nucleotides that alters the reading frame after the deletion to add 41 out of frame amino acids before a termination codon at position 113. en^{B86} is a deletion of 54 nucleotides and has an insertion of one nucleotide resulting in a net deletion of 53 nucleotides. There is also a reversal of GC in codon 70. The altered coding sequence encodes a 104 residue polypeptide.

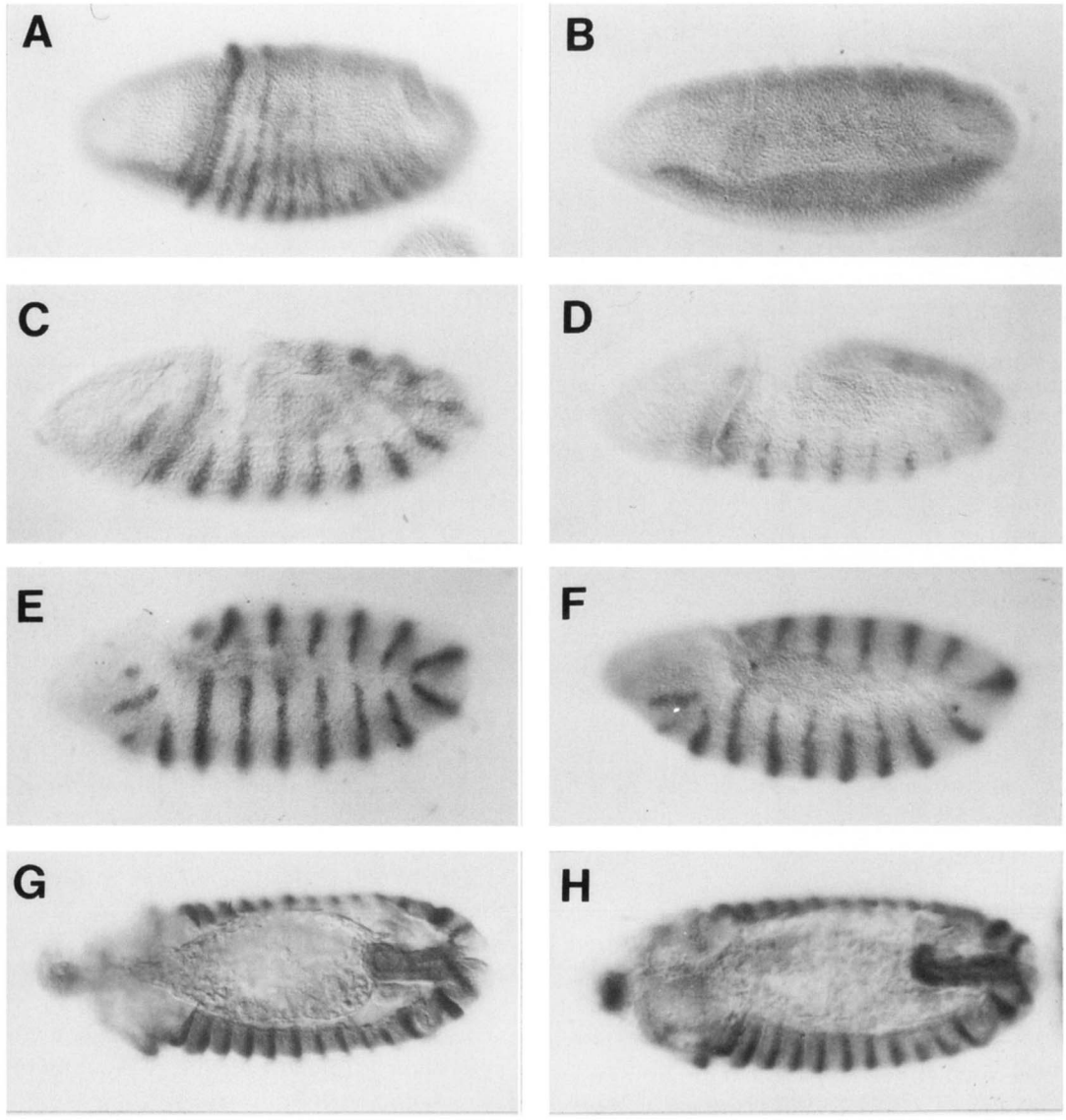

FIGURE 4.—Comparison between patterns of *en* and *inv* expression in embryos. Whole mounts of embryos after *in situ* hybridization with either a probe for *en* RNA (A, C, E, G) or *inv* RNA (B, D, F, H). (A and B) Stage 6; (C and D) Stage 8; (E and F) Stage 9; (G and H) Stage 14. Embryos are oriented anterior to the left and dorsal up, except G and H which are viewed from the dorsal side.

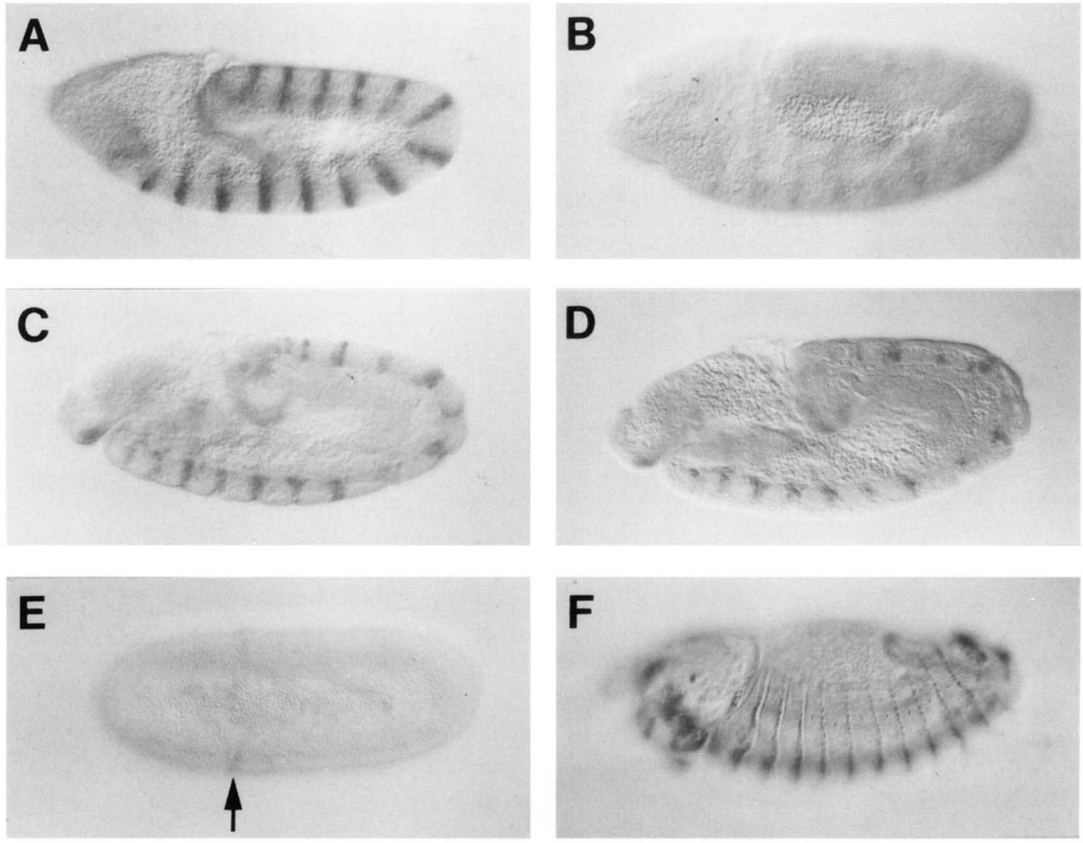

FIGURE 5.—Strong and weak *en* alleles have different effects on the distribution of *en* and *inv* protein in $en^{point\text{-}lethal}$ mutant embryos. (A and C) Distribution of En protein and (B and D) Inv protein as revealed by immunohistochemical detection in (A–D) en^{LA7} embryos stained with (A and C) anti-En antibody or (B and D) anti-En/Inv antibody. Note that the anti-En/Inv antibody detects only Inv in en^{LA7} mutants (see text). (E and F) en^{LA11} embryos stained with the anti-En/Inv antibody. Arrow in (E) points to stripe 2, the first stripe of *en* expression to appear in stage 5 embryos. Embryos are: A and B, stage 9: C and D, stage 11; and F stage 14. Embryos are oriented anterior to the left and dorsal up.

en^{IO34}, en^{IK57}, and en^{IM199} mutant embryos, and Inv patterns were examined in en^{LA4}, en^{LA7}, en^{LA10}. Similar results were obtained for each of the mutants. Patterns in early embryos were similar to wild type, and in stage 9 embryos, both antibody preparations detected similar patterns of antigen (Figure 5, A and B). We conclude that the initial distribution of En and Inv is independent of *en* function, and that the epitope recognized by the polyclonal sera is N terminal to the termination points in these mutants. In older embryos, both En and Inv disappeared from the ectoderm prematurely, although both proteins remained in the CNS, hindgut, and clypeolabrum (Figure 5, C and D). In each of the mutants, *en* and *inv* expression was altered in an identical manner. It has been shown previously that En is required to maintain expression of its own gene in the embryonic ectoderm (WEIR and KORNBERG 1985; HEEMSKERK *et al.* 1991); these new results indicate that *en* function is also required to maintain expression of *inv* in the ectoderm.

The decay in *en* and *inv* expression in $en^{point\text{-}lethal}$ mutants implicates En and/or Inv activities as critical components of the segmentation process in embryos. In contrast, the mild phenotypes of en^{LA9} and en^{LA11}, with their N-terminal stop mutations, raises the possibility that *en* is nonessential or that the phenotype of $en^{point\text{-}lethal}$ mutants has a more complex basis. To characterize en^{LA9} and en^{LA11} further, we stained en^{LA11} embryos with the anti-En and anti-En/Inv antibodies, and found several significant differences between these mutants and the $en^{point\text{-}lethals}$ (Figure 5). First, a low but detectable level of staining was present in cellular blastoderm embryos treated with anti-En/Inv antibody, a stage before the appearance of Inv (Figure 5E). We interpret this signal as evidence for En protein in these mutant embryos. In germ band extended embryos, the staining pattern with the anti-En/Inv serum was indistinguishable from wild type (not shown), and was present in a normal pattern at stage 14 (Figure 5F). To confirm that the antibody staining was detecting En in en^{LA11} embryos, a transgenic line carrying a $hsp70\text{-}en^{LA11}$ fusion was subjected to heat shock at 0–16 hr AEL and the anti-En/Inv antibody was used to stain both embryos and Western blots. Low levels of ubiquitous staining were observed in embryos with the antibody, and the staining was clearly nuclear in the amnioserosa where *en* is not normally expressed (Figure 6A). Western analysis also revealed the presence of En in heat-shocked embryos, whereas the level of En in embryos that had

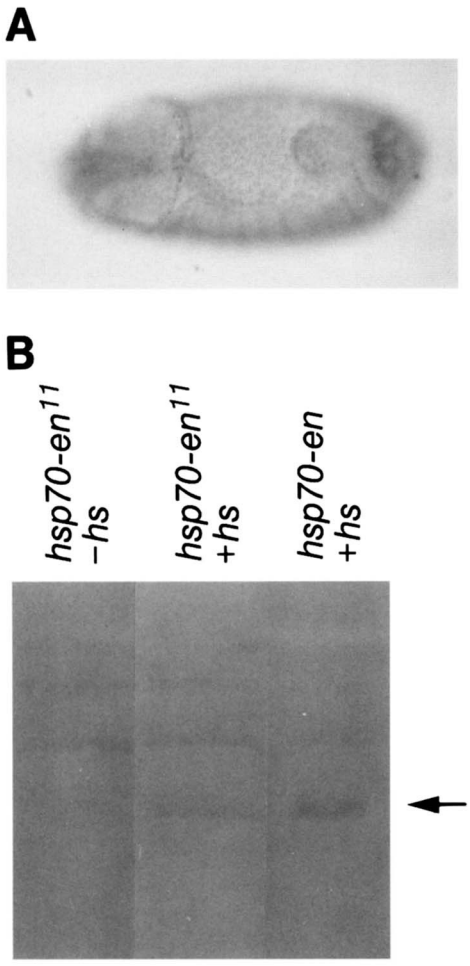

FIGURE 6.—En protein present in en^{LA11} embryos contains the homeodomain and is indistinguishable from full-length En. (A) En was detected in hsp70-en^{LA11} embryos using anti-En/Inv antibody. Note staining in the amnioserosa cells. This embryo is viewed from the dorsal side; anterior is to the left. (B) En was detected in immunoblots of extracts from hsp70-*en* and hsp70-en^{LA11} embryos with the anti-En/Inv antibody and an alkaline phosphatase conjugated secondary antibody. No protein is seen in hsp70-en^{LA11} embryos that were not heat shocked. In treated hsp70-en^{LA11} embryos, protein of a similar or slightly greater electrophoretic mobility than that of wild-type En protein produced in hsp70-*en* embryos is detected. The position of En protein is indicated by an arrowhead.

not been heat-shocked was not detectable (Figure 6B). Comparison of En protein produced by embryos carrying either the *hsp70-*en^{LA11} fusion or a *hsp70-en* fusion did not reveal clear differences in size or quantity. We conclude that the weak phenotype of these alleles does not indicate that *en* function is nonessential, but rather is a consequence of the synthesis of functional En in these mutants. We discuss below (see DISCUSSION) possible mechanisms that may account for the presence of En protein in en^{LA9} and en^{LA11} mutants.

Deficiencies of *en* and *inv*: To define the null phenotype for *en* and *inv*, several deletions were isolated using two screens for imprecise excisions of *P* elements integrated in the *en/inv* region. We first used the *ryxho25* strain, which carries a *P* element 257 nucleotides upstream of the *en* transcription start (Figure 1), and the Δ2-3 *P* element as a source of P transposase. *ry* flies (500) were identified, and among the 20 that failed to complement the lethality of $en^{point\text{-}lethal}$ mutants, Southern analysis identified several, including *Df(2R)*en^{C}, *Df(2R)*en^{E} and *Df(2R)*en^{F}, that had deleted all or part of the *P* element as well as neighboring genomic DNA. en^{C} and en^{E} were analyzed extensively, en^{C} because of its surprisingly mild phenotype and en^{E} because its deletion removes most of the *en* and *inv* transcription units. en^{F} deletes sequences distal to the *en* transcription unit; it has only mild effects on embryogenesis, but its interactions with en^{1} and other mutants yielded the strongest adult phenotype among the extant *en* alleles.

The second screen identified 33 F_1 *w* flies among the offspring of a strain, *wxba21,* which carries a *w+ P* element upstream of the *inv* transcription start. Southern analysis identified one of the revertants, *Df(2R)*inv^{30}, as having a large deletion that removes most of the *inv* transcription unit, and it was characterized further.

The *en* deficiencies have phenotypes that vary in severity from extreme to mild. They failed to complement the adult phenotype of en^{1}, with en^{F} generating the most extreme abnormalities (not shown). en^{E} also has extreme embryonic segmentation defects: its denticle belts fused into an almost continuous lawn, and no mutants survived embryogenesis (Figure 7D). In contrast, en^{C} embryos frequently hatched, and segment fusions varied from pair-wise fusions to milder fusions in which one or two pairs of denticle belts partially fused (Figure 7B). Because this phenotype is weaker than that of the $en^{point\text{-}lethal}$ alleles (compare Figure 7, B and C), en^{C} DNA was cloned and sequenced. The en^{C} deletion removes the entire *P* element insertion as well as 36 upstream base pairs and extends through the first exon and to within 3 bp of the splice acceptor of the first intron. Although it is possible that the remaining protein fragment, which includes the homeodomain, could be expressed, a monoclonal antibody that specifically recognizes the En homeodomain (E. MARTIN-BLANCO and T. B. KORNBERG, unpublished data) failed to detect any immunoreactive material in en^{C} mutant embryos (Figure 8B). These results indicate that En protein is not produced in detectable amounts in en^{C} mutants and that this does not result in an extreme segmentation phenotype. Instead, the en^{C} phenotype indicates that a deletion of the *en* transcription unit that leaves most of the surrounding regulatory region intact has milder effects.

We also monitored *inv* expression in en^{C} mutant embryos. Using the 4D9 anti-En/Inv antibody that recognizes the homeodomain of both En and Inv, we detected normal striped patterns of antigen in gastrulating mutant embryos (Figure 8C). Because the anti-En monoclonal detected no antigen, this signal must represent Inv protein. These stripes of Inv protein

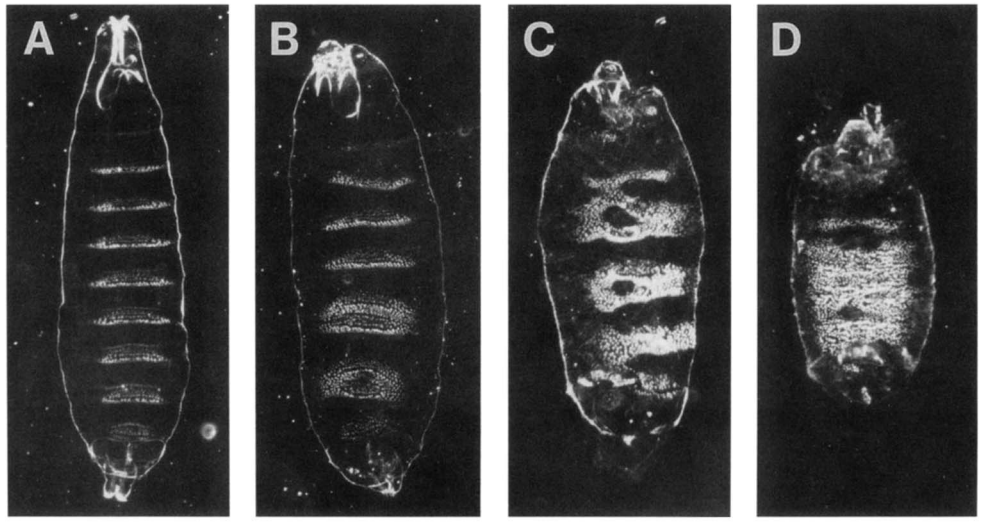

FIGURE 7.—Comparison of embryo cuticles. (A) Wild type; (B) *Df(2R)en^{C}*; (C) *en^{LA10}*; and (D) *Df(2R)en^{E}* embryos.

decayed during germ band extension. In germ band retracted embryos, antigen was detected in the clypeolabrum, hindgut, and ventral nerve cord, but ectodermal staining had disappeared (Figure 8D). As described above, Inv and En synthesis in *en$^{point\text{-}lethal}$* embryos also appeared with normal patterns initially, but decayed during germ band extension. This suggests that *en* has an autoregulatory function and that its activity is necessary for the maintenance but not the activation of *en* and *inv* expression. The decay of Inv synthesis we observed in *en^{C}* embryos therefore reinforces the conclusion that *en^{C}* is deficient for *en*.

***en* and *inv* are regulated by shared sequences:** If *inv* and *en* expression are both dependent upon *en* function and En is involved in direct autoregulation, then we can consider three possible mechanisms for *inv* regulation. The En-responding autoregulatory element in the *en* gene might also regulate *inv*. *inv* might be directly regulated by a second, independent En-responding element. Or, the *en* and *inv* genes, which are juxtaposed in the genome, may be regulated by the same set of regulatory elements that are indirectly dependent upon En. To investigate the mechanism of *inv* regulation, we analyzed *inv* expression in *en$^{rearrangement\text{-}lethal}$* alleles, which by virtue of their rearrangement breakpoints within the *en* gene, remove regulatory elements that would normally be downstream of *inv*. We analyzed *inv* expression in most of the *en$^{rearrangement\text{-}lethal}$* mutants and report here on results with the *en^{CX1}* mutant that illustrate the general findings from these studies. *en^{CX1}* mutants have a strong *en* phenotype. Mutant embryos developed extreme denticle belt fusions and had a severe segmentation phenotype (not shown). They were more severely affected than any of the *en$^{point\text{-}lethal}$* mutants. In fact, they

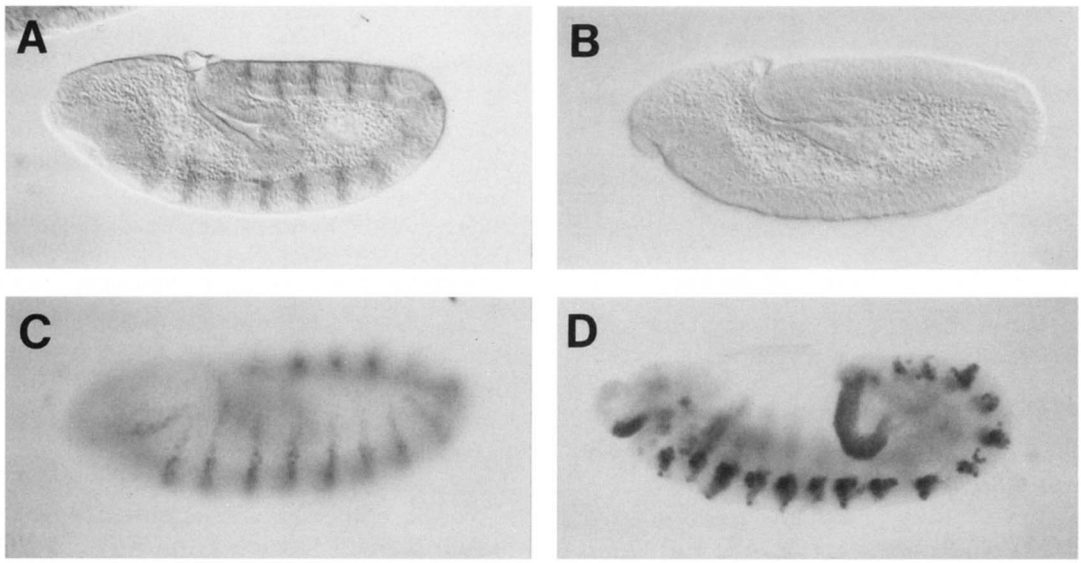

FIGURE 8.—Distribution of En and Inv proteins in *Df(2R)en^{C}* embryos. (A) Wild type and (B) *Df(2R)en^{C}* embryos stained with an antibody directed against the En homeodomain. Note that En is not detectable in *Df(2R)en^{C}* embryos. (C and D) *Df(2R)en^{C}* embryos stained with the anti-En/Inv antibody.

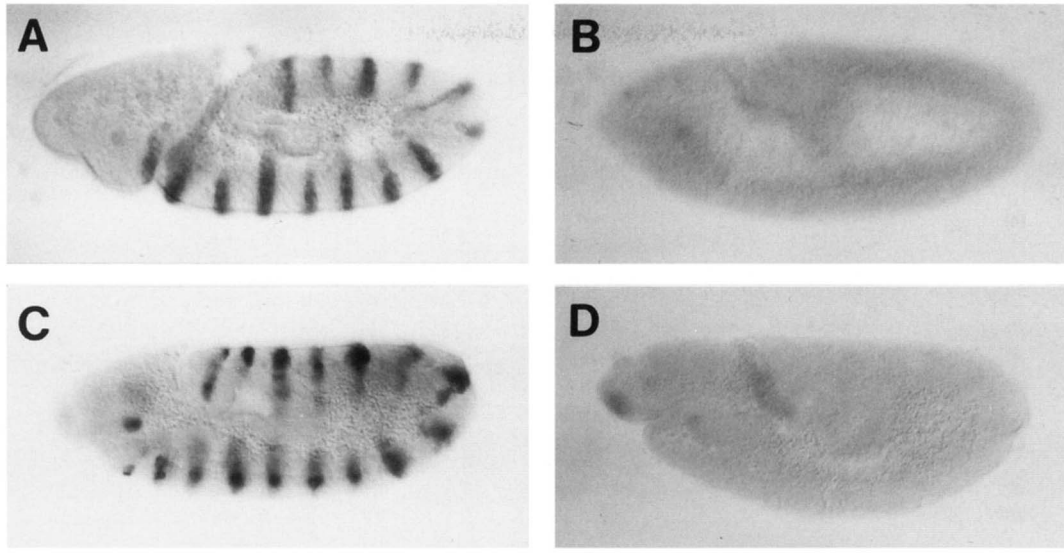

FIGURE 9.—Distribution of En and Inv in $en^{rearrangement\text{-}lethal}$ embryos. en^{CX1} embryos stained with (A and C) anti-En or (B and D) anti-En/Inv antibody. Note that the anti-En/Inv antibody detects only Inv in this mutant (see text). Embryos are stage 9 (A and B) and stage 11 (C and D), and are oriented anterior to the left and dorsal up.

were comparable with embryos that are deleted for both *en* and *inv*, such as en^{E}. en^{CX1}/en^{1} adults had severely disrupted wings, legs with fused tarsal segments, and a low frequency of eclosion.

The en^{CX1} mutation is a rearrangement that severs the *en* transcription unit either in the first exon or first intron. During gastrulation, mutant embryos produced a truncated form of En that was recognized by the anti-En serum but not by the anti-En/Inv antibody, which binds to the homeodomain. The mutant protein was not localized to the nucleus and was not synthesized in embryos after germ band extension. The pattern was similar to the $en^{point\text{-}lethal}$ mutants with one notable exception: it was absent from the clypeolabrum and hindgut (Figure 9, A and C). We suggest that the regulatory sequences that control expression in the hindgut and clypeolabrum have been separated from those required for expression in the ectoderm and nervous system by the en^{CX1} breakpoint.

Staining en^{CX1} embryos with the anti-En/Inv antibody detects Inv protein only, because the truncated En produced by the mutant lacks a homeodomain. With the anti-En/Inv antibody, Inv could not be detected through the first 10 stages of embryogenesis (Figure 9B). At stage 11, staining appeared in the hindgut and clypeolabrum (Figure 9D) and was clearly visible by stage 14 (not shown). In late embryos that have formed cuticle, staining was also detected in the ventral nerve cord, indicating that these cells can activate *inv* expression independently at this stage (not shown). With the exception of this late expression, *inv* expression was abolished in all tissues except the hindgut and clypeolabrum and was complementary to the pattern of En. These complementary patterns suggest that the en^{CX1} breakpoint separates proximal regulatory sequences that direct expression in the hindgut and clypeolabrum from distal sequences that direct expression in the ectoderm and nervous system. The regulatory sequences responsible for *en* and *inv* expression in these various locations are apparently either tightly linked or are shared.

***en* and *inv* are functionally redundant:** Among the collection of *en* mutants, those with the strongest phenotype inactivate both *en* and *inv*. Examples are en^{E}, which deletes both *en* and *inv*, and en^{CX1}, which separates *inv* from the regulatory sequences that activate ectodermal expression and that truncates the *en* transcription unit. The slightly less extreme phenotype of the $en^{point\text{-}lethal}$ alleles may be a consequence of early *inv* expression, which although it is not maintained, may nevertheless ameliorate the effects of loss of *en* function. This possibility suggests that *inv* might substitute for *en* to some extent.

We have obtained two mutant *inv* alleles, inv^{l77} and inv^{30}. inv^{l77} has a translocation breakpoint in the *Eco*RI genomic restriction fragment that contains the third *inv* exon (Figure 1); it survived without phenotypic abnormality as a heterozygote with $Df(2R)en^{SFX31}$, a deletion that removes the entire *en-inv* region. inv^{30} was also viable over a deficiency and was phenotypically normal with the exception of slight effects on the anterior crossvein. It deletes the first exon, leaving the portion of the gene that encodes the homeodomain intact. To exclude the possibility that inv^{30} produces a C-terminal protein fragment with partial function, a probe specific for the 3′ end of the gene was used for *in situ* hybridization analysis of inv^{30} embryos. This probe detected transcripts in wild-type embryos, but not in inv^{30} mutant embryos (not shown). We conclude that this allele is a null and that *inv* is not required for viability.

To compare *inv* and *en* function, two types of assays were carried out. In the first, *inv* was ectopically expressed as a *hsp70-inv* transgene, and the consequences of its overexpression were monitored and compared with similar studies with a *hsp70-en* transgene. Previous studies showed that heat-shocked *hsp70-en* embryos and heat-shocked *hsp70-inv* embryos develop segment fusions comparable to those of *en* mutants (K. G. COLEMAN and T. B. KORNBERG, unpublished data; POOLE and KORNBERG 1988). Heat shock also induces stable expression of the endogenous *en* gene in *hsp70-en* embryos, causing stripes 5–15 to be abnormally broad in ventral and lateral regions (HEEMSKERK *et al.* 1991). To ask if *inv* elicits the same response after ectopic expression, new transgenic lines that express *en* and *inv* under heat shock control were established and embryos were subjected to heat shock. Segment fusions similar to those previously described were observed in both the *hsp70-en* and *hsp70-inv* lines (not shown). Probes for *en* RNA and for *inv* RNA were used for *in situ* hybridization and revealed that the patterns of both *en* and *inv* transcripts broadened in similar ways (Figure 10 A–F). In addition, expression of both genes disappeared from dorsal regions. We conclude that ectopically expressed *inv* can induce stable expression of the endogenous *en* and *inv* genes and that the consequences are similar to those previously described for ectopically expressed *en* (HEEMSKERK *et al.* 1991).

We also investigated the distribution of the *hedgehog (hh)* and *cubitus interruptus (ci)* transcripts. These two genes are likely to be regulated by *en,* because *ci* expression is derepressed in *en* mutants (EATON and KORNBERG 1990), and stripes of *ci* RNA narrow and *hh* is expressed ectopically after heat shock of *hsp70-en* (TABATA *et al.* 1992; SCHWARTZ *et al.* 1995). We observed that after heat shock of either *hsp70-en* or *hsp70-inv* embryos, stripes 5–15 of *hh* RNA broadened ventrally and laterally and decayed dorsally (Figure 10, G–I) in a manner similar to the *en* and *inv* stripes. All the stripes of *ci* RNA narrowed in response to ectopic *en* or *inv* expression (Figure 10, J–L) and they did not decay dorsally. These data demonstrate that *inv* and *en* have similar activities in these assays.

A second approach asked whether Inv can repress transcription in a transfected cell culture assay as has been previously demonstrated for En. Figure 11 shows that synthesis of either En or Inv reduced expression of a target sequence by more than 15-fold in a concentration dependent manner. We conclude that both proteins can repress the same targets, strengthening the conclusion that they have partially redundant functions.

DISCUSSION

The functional relationship between *en* and *inv*: The nearly identical homeodomains and expression patterns of *en* and *inv* suggest that the En and Inv proteins may have overlapping or partially redundant functions, and the analysis that we report here is consistent with this hypothesis. We base this conclusion on the consequences that ensue when either gene is expressed ectopically, on the activity of the En and Inv proteins in a cultured cell assay, and on the manner in which the different mutant alleles interact.

Although the normal role of a gene cannot always be deduced from the phenotypes that develop after its ectopic expression, such phenotypes do reflect the activity of the gene, and the similar consequences of heat shock-induced expression of *en* and *inv* on the endogenous genes we examined is most likely indicative of the similar activities that En and Inv have. Stable ectopic expression of *en* follows heat shock induced expression of *en* (HEEMSKERK *et al.* 1991), and we found that heat shock induced expression of *inv* induced a similar pattern of *en* expression. In addition, we found that heat shock-induced expression of either gene induced a similar pattern of *inv* expression and that the changes in the expression of *hh* and *ci* induced by heat shock induced expression of either gene were the same. Both En and Inv proteins also repress transcription of target sequences in transfected Schneider cells in a similar manner. These observations certainly suggest that the En and Inv proteins have similar activities, but they also raise the question of why the endogenous *inv* gene does not rescue *en* alleles.

Because both *en* and *inv* are expressed normally in gastrulating *en* mutant embryos, the transcription of both genes is apparently activated by proteins encoded by other genes. However, neither *en* nor *inv* expression is sustained in *en* mutant embryos (WEIR and KORNBERG 1985; HEEMSKERK *et al.* 1991), indicating that En is an essential component of an autoregulatory circuit that is either direct or indirect in its action. The ectopic expression of *en* in heat shocked *hsp70-inv* embryos indicates that Inv can activate *en* transcription, but the lethal phenotype of *en* mutant embryos is evidence that Inv cannot substitute for En. In addition, the loss of *inv* expression in mutant embryos indicates that Inv cannot activate the autoregulatory circuit to maintain its own expression. We cannot assess whether Inv is capable of carrying out other aspects of *en* function.

There are many possible explanations for the failure of Inv protein to substitute for En in embryos. A likely one might relate to the fact that *en* is expressed in young embryos, and morphogenetic abnormalities are evident in *en* mutants even during the precellular stages (KARR *et al.* 1985). Because the embryo develops rapidly, functional expression in the zygote requires that RNA transcripts be compact, and the *en* transcription unit is relatively small (3.7 kb; POOLE *et al.* 1985). In contrast, the *inv* transcription unit is much larger (>30 kb; COLEMAN *et al.* 1987), and its large size may not permit *inv* to rescue *en* function in embryos. Indeed, studies of *knrl* indicate that whereas a small form of the *knrl* gene,

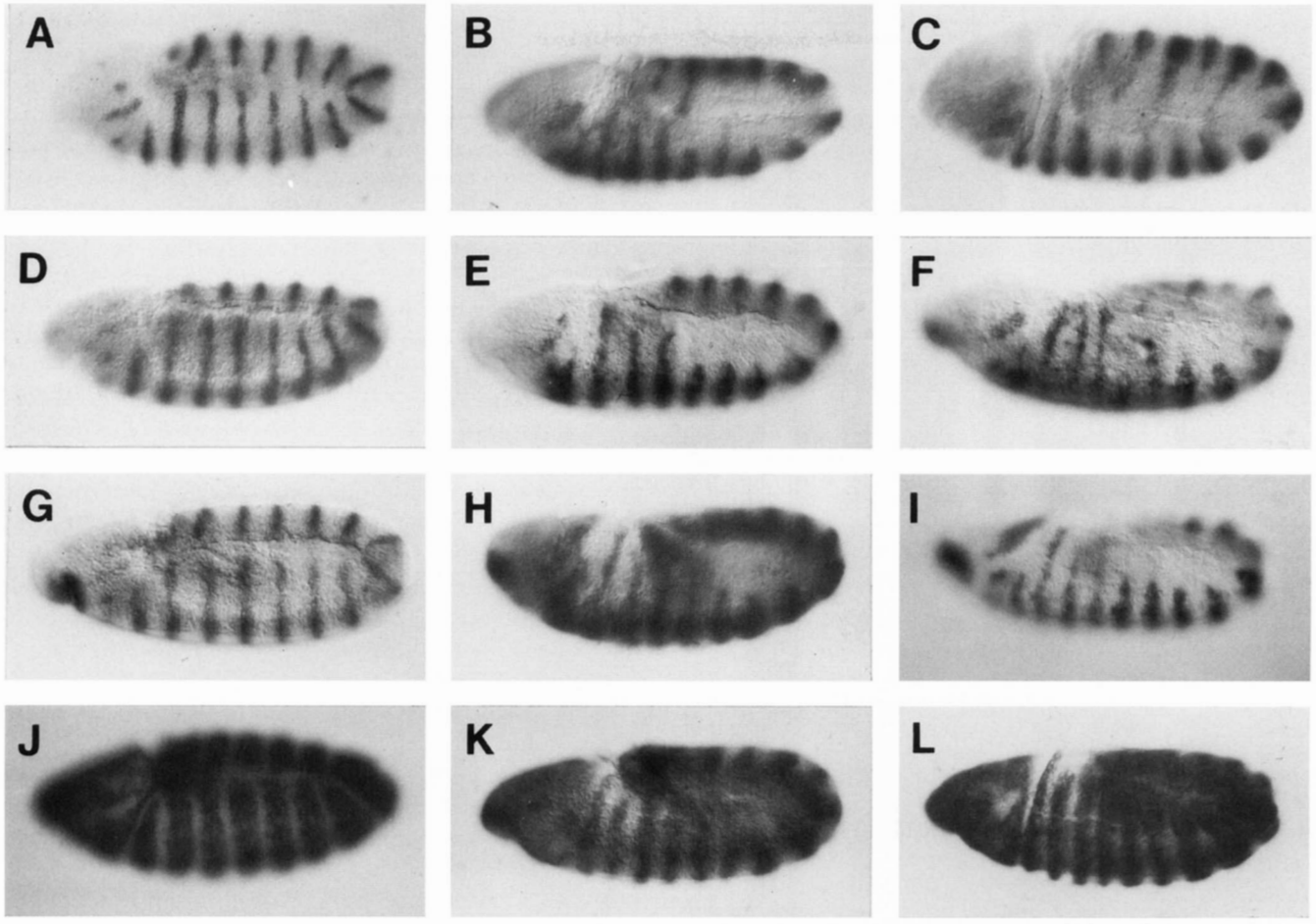

FIGURE 10.—Ectopic expression of *en* and *inv* alters expression of *en*, *inv*, *hh*, and *ci*. *hs-en* and *hs-inv* strains that can express En or Inv proteins, respectively, under the control of the hsp70 promoter, were subjected to a pulse of heat shock at 37° as described in Materials and Methods. Whole mounts of stage 9 (A, D, G, and J) wild type, (B, E, H, and K) *hs-en* or (C, F, I, and L) *hs-inv* embryos after *in situ* hybridization with probes for either (A–C) *en* RNA, (D–F) *inv* RNA, (G–I) *hh* RNA or (J–L) *ci* RNA. Note that after heat shock, *en*, *inv*, and *hh* stripes are wider ventrally and decay dorsally, and *ci* stripes are narrower ventrally compared to the stripes in wild-type embryos. Embryos are oriented anterior to the left and dorsal up.

which lacks a 19-kb intron can rescue *kni* mutants, the endogenous gene cannot (ROTHE *et al.* 1992). The wild-type *kni* gene has only 1 kb of intron sequence. In addition, mutations that lengthen the mitotic cycles in the early embryo suppress *kni* mutants, presumably by providing time for completion of *knrl* transcripts (RUDEN and JÄCKLE 1995).

Although *inv* mutants are essentially without phenotype (see RESULTS), *en inv* double mutants have more extreme embryonic and adult phenotypes than do *en* mutants (TABATA *et al.* 1995). Double mutants also have a more severe neural phenotype (M. LUNDELL and J. HIRSH, personal communication). If this increased severity means that *inv* can partially compensate for lack of *en* function, it follows that the kinds of mutants isolated in screens might represent a selected subset. We classify extant *en* alleles by their cytological/molecular type and by the severity of their phenotype, and, with the exception of en^{1A9} and en^{1A11}, the phenotype of the $en^{point\text{-}lethals}$ is quite extreme. These phenotypes contrast with that of en^{C}, a mutant that is deleted for most of the *en* transcription unit and in which we could not detect any En product. en^{C} has a very mild phenotype, and some mutant embryos have only slight segmentation defects. We must consider the possibility that en^{C} represents the null condition for *en*, and that mutants with more severe phenotypes, such as the $en^{point\text{-}lethals}$ and the $en^{rearrangement\text{-}lethals}$, owe their greater severity to the loss of both *en* and *inv* functions. Alternatively, *inv* may be up-regulated in en^{C} mutants. Increased expression of *inv* could arise by elimination of promoter competition if relevant sequences are deleted in the mutant, and the consequent increase in Inv might account for the mild phenotype of en^{C} mutants relative to the $en^{point\text{-}lethal}$ mutants. Our analysis of $en^{rearrangement\text{-}lethals}$ suggests that the regulatory region that surrounds the *en* transcription unit contains regulatory elements for both *en* and *inv*, so rearrangement breakpoints in this region are likely to affect both genes directly. This conclusion is supported by data demonstrating that $en^{rearrangement\text{-}lethal}$ mutants reduce *inv* expression in wing imaginal discs (GOLDSBOROUGH and KORNBERG 1994). We do not yet

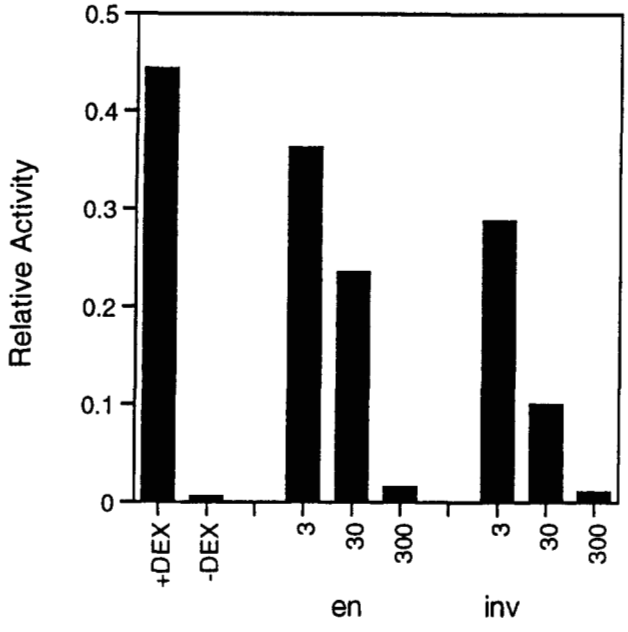

FIGURE 11.—En and Inv proteins repress transcription from a hormone stimulated promoter in tissue culture cells. Plasmids encoding the En and Inv proteins were transfected into Schneider S2 cells along with a reporter plasmid containing three glucocorticoid response elements upstream of six homeodomain binding sites (JAYNES and O'FARRELL 1991), a plasmid encoding the glucocorticoid receptor, and a control reference plasmid encoding β-galactosidase. Promoter activity of the reporter plasmid was measured by CAT activity relative to a cotransfected reference gene. CAT activity was dependent upon hormone (lanes 1 and 2) and was sensitive to the amounts of transfected *en* (lanes 3–5) and *inv* (6–8).

have data to indicate whether the control sequence elements within this shared regulatory region are distinct for each gene, although the simplest arrangement would have all, or a subset, of these regulatory elements shared by the two. It is not clear whether there are any differences between the expression patterns of the two genes and whether any cells uniquely express either one.

For the $en^{point\text{-}lethals}$ to inactivate both genes, these alleles must have a negative, antimorphic activity. Unfortunately, standard tests that might better define the nature of these alleles are not possible, because *en* is so tightly linked to a redundant function. However, ectopic expression of en^{LA10} under either heat shock or GAL4-UAS control does not lead to mutant phenotypes (E. GUSTAVSON and T. B. KORNBERG, unpublished), so we have not been able to obtain direct evidence for any negative function of this point-lethal allele. It is worth noting that the type of mutations that the $en^{point\text{-}lethals}$ represent is a narrowly defined one—all generate protein truncations—and that perhaps the presence of *inv* has limited the ability of screens to identify *en* mutants to those of this type. A way to address the uncertain nature of the $en^{point\text{-}lethal}$ alleles is to ask if novel classes of *en* alleles can be isolated in a genetic background that lacks *inv*. Under these conditions it may be possible to detect mutations that simply reduce the activity of En. We mutagenized inv^{30}, the deletion that our data suggest is null for Inv, and isolated five mutants that fail to complement en^{LA10} (E. GUSTAVSON, Z. ALI and T. B. KORNBERG, unpublished). Preliminary tests indicate that these mutants are of three types: embryonic lethal with severe segmentation defects, embryonic lethal with mild segmentation defects, and a viable allele with a visible en^{1}-like wing phenotype. The viable mutant fails to complement the lethality of a subset of $en^{rearrangement\text{-}lethal}$ alleles. Additional analysis will be required to confirm the genetic and molecular nature of these mutations.

SIMMONDS *et al.* (1995) recently described a mutant, inv^{D}, that causes transformation of anterior wing to posterior wing. inv^{D} ectopically expresses *inv* in the anterior compartment of the wing disc, but surprisingly, changes in *en, hh, ci, dpp,* or *ptc* expression that are expected to correlate with the anterior inv^{D} expression could not be seen in late third instar wing discs. These observations contrast with the changes that ensue after ectopic expression of *inv* in embryos, in which *en* and *hh* expand their domains and *ci* contracts its domain of expression. They would also appear to contrast with our observation that ectopic expression of *inv* after heat shocking *hs-inv* larvae causes pupal lethality (E. GUSTAVSON and T. B. KORNBERG, unpublished). It is certainly possible that the roles of *en* and *inv* change as the animals mature, and that *inv* and *en* take on different functions in imaginal discs. Resolution of these apparent discrepancies must await additional studies that can directly compare the consequences of *inv* and *en* misexpression.

Interallelic complementation requires continuity of the *en-inv* region: Although the $en^{point\text{-}lethal}$ and the $en^{rearrangement\text{-}lethal}$ alleles have similar embryonic phenotypes, their effects on the adult differ. As discussed above, the $en^{point\text{-}lethals}$ complement en^{1}, an allele that affects only adult structures, and the phenotype of $en^{point\text{-}lethals}$ in mosaic clones is surprisingly mild. We investigated the possibility that transvection might promote interallelic complementation between synapsed homologs, but our screens failed to obtain evidence for such interactions. Our results indicate either that the region that promotes pairing at the *en-inv* locus is tightly linked and thereby severely reduces the target size for rearrangements that disrupt pairing or that the interactions between these alleles do not involve trans complementation. We cannot distinguish between these possible explanations, but in view of the redundancy between *en* and *inv*, we also consider another possibility: the $en^{point\text{-}lethal}$ and the $en^{rearrangement\text{-}lethal}$ alleles have different adult phenotypes because of their different effects on *inv*.

The mechanisms that regulate *en* expression differ in embryos and imaginal discs (BUSTURIA and MORATA 1988; SERRANO *et al.* 1995), and we assume that the type of autoregulatory and interdependent relationship that

characterizes *en* and *inv* expression in embryos may change in imaginal tissues as well. We have observed that $en^{rearrangement}$ mutants reduce *inv* expression in wing imaginal discs (GOLDSBOROUGH and KORNBERG 1994), so we conclude that the regulatory elements that control *inv* expression in discs are also tightly associated with the elements that control *en* expression in discs. The inability of these alleles to complement en^{1} might be a consequence of the reduction in both *en* and *inv* function in discs. We can postulate that the $en^{point\text{-}lethal}$ alleles, which do not affect disc functions so severely, do not interfere with *inv* function in discs, enabling Inv to partially compensate for loss of *en* function more effectively at this stage than it could in the different regulatory environment in embryos.

Mechanism of En synthesis in en^{LA9} and en^{LA11}: The curious observation that en^{LA9} and en^{LA11} have weak phenotypes despite the N-terminal location of their termination codons, and the demonstration that embryos can abundantly express a full-length en^{LA11} peptide after heat shock induction (Figure 6), demands explanation. Possible mechanisms we consider are ribosome hopping and internal reinitiation, and differences in their efficiency might explain the phenotypic differences between these two alleles. Either of these two mechanisms might account for the synthesis of functional En protein in en^{LA9} and en^{LA11} mutant embryos.

Ribosome hopping (reviewed by ATKINS *et al.* 1990) could produce mutant protein that is a similar size to the wild-type product. Short hops can occur when the paired codon and the anticodon disengage and re-pair with a similar sequence downstream. We note that the codon upstream and the four codons immediately downstream of the stop codon in en^{LA11} are identical (CAG). The codons preceding and following the en^{LA9} stop codon are both CAA.

Internal reinitiation occurs efficiently when a termination codon near the N terminus of a protein occurs between 79 and 147 nucleotides upstream of another potential translation start (KOZAK 1987). Potential reinitiation sites for en^{LA9} and en^{LA11} are located 33 nucleotides and 108 nucleotides downstream of their respective stop codons. Internal reinitiation is therefore a possible mechanism that produces functional En protein in these mutants. However, if internal reinitiation occurs in these mutants, we must conclude that En can function without its N-terminal 63 residues. We note that although the N-terminal region of En is not conserved in Inv, 11 of the 15 N-terminal residues are conserved between the Drosophila and Bombyx En proteins (HUI *et al.* 1992).

We thank Y. HIROMI for *CyO ftz lacZ*, C. RUSHLOW for pCaSpeR-hs, and R. HOLMGREN for en^{CX1}. We gratefully acknowledge I. REVEILLAUD for initiating the cloning of $en^{point\text{-}lethal}$ alleles, C.-N. CHEN for help with embryo transformations, S. POOLE and J. KASSIS for support and valuable discussions, C.-T. WU for helpful discussions, B. GELBART for help with the cytology of *E(en)eg-1*, and many colleagues in the Kornberg lab over the last decade for their advice and support. We also thank the reviewers and the editor (R. DENELL) for helpful suggestions. This work was supported by grants from the National Institutes of Health to T.B.K.

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Communicating editor: R. E. DENELL