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polyhomeotic appears to be a target of Engrailed regulation in *Drosophila*

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SUMMARY

In *Drosophila*, Engrailed is a nuclear regulatory protein with essential roles in embryonic segmentation and in normal development of posterior compartments. One of its regulatory targets appears to be *polyhomeotic (ph)*, a *Polycomb* group gene. We observed, by immunostaining, that Engrailed protein binds to the site of the *polyhomeotic* locus in region 2D of polytene chromosomes. The same analysis carried out on a transgenic line containing one copy of a *P*(*ph-lacZ*) construct shows an additional Engrailed-binding site at the location of the insert. In vivo, *polyhomeotic* depends on *engrailed* function in germ-band-elongated embryos, when *engrailed* and *polyhomeotic* genes are expressed in similar patterns. By in vitro immunoprecipitations and gel shift assays, we identified two classes of high affinity Engrailed-binding sites upstream of each of the two *polyhomeotic* transcription units. DNA fragments containing these sites were also immunoprecipitated from embryonic UV crosslinked chromatin in presence of anti-Engrailed antibody. These results suggest that *polyhomeotic* activation in germ-band-elongated embryos could be mediated by Engrailed-binding to these sites.

Key words: *Drosophila*, *engrailed*, *polyhomeotic*, target genes, regulation

INTRODUCTION

Proteins that direct embryonic development in *Drosophila melanogaster* are part of a network of transcription regulators. Each of these proteins is expressed in a unique spatial-temporal pattern that is controlled, in part, by other members of the network. Proteins expressed early form broad, partially overlapping domains. Through their combinatorial interactions, smaller domains of expression are specified for the next wave of regulatory proteins (reviewed by Ingham, 1988). This process continues through several nuclear cycles, leading to the cellular blastoderm in which genes like *engrailed* (*en*) are expressed precisely in single segment intervals (Karr et al., 1989).

Engrailed (En) is a 58×10^3 M_r nuclear protein that binds DNA with high affinity in vitro, through its homeodomain (Desplan et al., 1988; Kissinger et al., 1990). These characteristics and its ability to repress transcription after transfection into cultured cells (Jaynes and O'Farrell, 1991) suggest that En could regulate genes directly at the transcriptional level.

In cellular blastoderm-stage embryos, *en* is expressed in single-cell-wide stripes at the anterior edge of each parasegment. These stripes remain throughout embryogenesis, first expanding in width and then contracting (Vincent and O'Farrell, 1992). *en* function is needed in posterior compartment cells to maintain segment and compartment borders and to direct the posterior compartment developmental pathway (Lawrence and Morata, 1976; Kornberg, 1981).

There are several candidates for genes that regulate *en*. For instance, the Fushi tarazu (Ftz), Even-skipped (Eve) and Paired (Prd) proteins appear to activate *en* in the cellular blastoderm (Ingham, 1988). Maintenance of the *en* expression pattern involves mechanisms that are distinct from those that guide pattern establishment. Genes implicated in maintenance of expression include *engrailed* itself and the segment-polarity gene *wingless* (*wg*) (Heemskerk et al., 1991), but also members of the *Polycomb* group (*Pc-G*). Indeed, *en* expression, normally absent from anterior compartment cells, is ectopically activated in several *Pc-G* mutants (Moazed and O'Farrell, 1992). The *Pc* group includes eleven genes that are required for normal segment specification. They are needed generally to repress homeotic gene expression (McKeon and Brock, 1991; Simon et al., 1992). *polyhomeotic (ph)* is one of the *Pc-G* genes. *ph* null mutants cause derepression of *en* and of homeotic genes in the epidermal cells of germ-band-retracted embryos (Dura and Ingham, 1988; Smouse et al., 1988). Polyhomeotic protein (Ph) binds to approximately 100 sites on polytene chromosomes, all of which appear to be shared with the Polycomb (Pc) protein (Franke et al., 1992). One of these common sites is 48A, the site of the *en* gene. *Posterior Sex*

Combs (*Psc*), another *Pc-G* gene, also binds to 48A (Martin and Adler, 1993). Thus *ph* could cooperate with *Pc* and *Psc* to maintain *en* expression after the germ band elongation stage.

Despite evidence for the regulatory capacities of the Engrailed product, there has been little progress in identifying its target genes. The identification of these genes is essential for understanding how combinations of gene products are used to specify pathways of development. Genetic experiments show that *cubitus interruptus* (*ci*, Eaton and Kornberg, 1990) is negatively regulated by *en*, whereas *hedgehog* (*hh*, Lee et al., 1992; Tabata et al., 1992) is positively regulated.

Here we present evidence that *ph* is a direct target of *en* regulation, requiring *en* function for its activation in embryos prior to germ band shortening, while subsequent maintenance of *en* expression during embryogenesis is mediated by Ph and other Pc-G proteins (Moazed and O'Farrell, 1992). Using immunohistochemistry, we identified the cytological location of *ph* as a site of En binding on polytene chromosomes. Transposition of a portion of the *ph* region added a new site of En binding to polytene chromosomes. In vitro, En-binding sites were identified upstream of each *ph* transcription unit. These sites are also specifically immunopurified from intact embryonic nuclei where DNA-protein interactions were stabilized by UV crosslinking.

MATERIALS AND METHODS

Fly strains

The transgenic line *hsen-3* (Poole and Kornberg, 1988) bears a *P*-element containing the *engrailed* coding sequence driven by a *hsp70* promoter and localized in 34B. For heat shock, larvae are put at 37°C for 30 minutes and then returned for 15 minutes to room temperature for recovery. *ttk1* (Xiong and Montell, 1993) is a *P*-element-induced mutation in the *tramtrack* locus. $Df(2R)en^{SFX31}$ is a deficiency of the 48AB region that deletes the *engrailed* and *invected* genes as well as several neighbouring transcription units (Kuner et al., 1985). ph^{lac} was induced by *P(lacW)*-element mutagenesis and contains an insertion in the *polyhomeotic* locus (M. O. Fauvarque and J. M. Dura, unpublished).

Antibodies

Anti-Engrailed antibodies are: (i) a goat polyclonal antibody raised against the N-terminal, non-homeodomain-containing half of Engrailed protein (K. Coleman and T. Kornberg, unpublished); (ii) a rabbit polyclonal antibody raised against a T7-En bacterial protein (F. Payre and A. Vincent, unpublished); (iii) a monoclonal antibody 4F11 which recognizes an epitope in the C-terminal third of Engrailed protein and (iv) a monoclonal antibody 4D9 which recognizes an epitope in the homeodomain (Patel et al., 1989). The anti-Polyhomeotic antibody was raised in rabbits against a Ph-β-galactosidase fusion protein (DeCamillis et al., 1992). Polyclonal anti-β-galactosidase antibody was obtained from Cappel.

Immunostaining of polytene chromosomes

Squashes were made according to Zink and Paro (1989). Late third instar larvae salivary glands (from heat-shocked *hsen-3* strain or from *ttk1* strain) were dissected in PBT (PBS, 0.1% Triton). Glands were fixed in PBS, 1% Triton, 3.7% paraformaldehyde and squashes were performed in 3.7% paraformaldehyde, 50% acetic acid. Squashes were blocked in PBS, 5% powder milk, 5% BSA, 0.2% NP40, 0.2% Tween. For all subsequent incubation steps, slides were kept in a humid atmosphere with a coverslip placed over the chromosomes. Squashes were incubated overnight at 4°C in blocking buffer containing either goat polyclonal anti-Engrailed antibody (dilution 1:200) or rabbit polyclonal anti-Engrailed antibody (dilution 1:75) previously preabsorbed to 0-16 hours embryos. After two 15 minutes rinses in PBS, 300 mM NaCl, 0.2% NP40, 0.2% Tween, squashes were incubated 1 hour in blocking buffer containing secondary antibodies. For immunofluorescence, we used Texas red-conjugated anti-goat antibody (1:1000 dilution), preabsorbed to heat-shocked salivary glands from a wild-type strain and stained with DAPI (4′,6-diamidino-2-phenylindole 1 μg/ml), after rinsing. Chromosomes were embedded in Fluoromount G (Southern Biotechnologies) and examined immediately. Peroxidase was detected by peroxidase-conjugated secondary antibody (1:1000 dilution) (Vectastain; Vector), preabsorbed to 0-16 hours embryos. Chromosomes were then stained with 1% Giemsa in PBS and mounted in 100% glycerol.

Construction of *P*(*ph-lacZ*) transgenic lines

The *P*(*ph-lacZ*) transposon was assembled using the *ph* D fragment (Fig. 6), which corresponds to a 1.5 kb *Sal*I fragment, localized between the two *ph* transcription units. *P*(*ph-lacZ*) corresponds to a *P*-element containing (SCS – *ph* D fragment – minimal *hsp70* promoter – *lacZ* coding sequence – *hsp70* polyA – SCS′ – mini *white* gene) and was constructed using vectors obtained from Kellum and Schedl (1991) and H. Benes (unpublished). *P*(*ph-lacZ*) was injected into early embryos at a concentration of 250 μg/ml together with 50 μg/ml of the helper pUChsπΔ2-3 plasmid. Transformants were selected on eye color. The inserts were localized by in situ hybridization to polytene chromosomes with biotinylated DNA probes, using the nonradioactive gene detection system (BRL Bluegene 8279SA). As a probe, we used the *ph* D fragment in order to map the endogenous *ph* locus as well as the insert.

Fixation and staining of embryos

Embryos were fixed as described by Sullivan et al. (1993). Fixed embryos were stained with different antibodies diluted to the desired concentration. Primary antibodies were detected either by immunofluorescence (with rhodamine-conjugated or FITC-conjugated secondary antibodies), or by peroxidase-conjugated secondary antibody (Vectastain; Vector), as indicated in the text. Embryos were mounted in Citifluor AF1 for immunofluorescence and in 80% glycerol for peroxidase staining. Double staining with primary antisera from rabbit and mouse was carried out for both antisera simultaneously.

Preparation of Engrailed protein

Purified full-length T7-En protein was kindly provided by H. Bourbon. After transcription from the T7 promoter induced by addition of IPTG, further purifications were performed on phosphocellulose column, followed by FPLC Mono S column (H. Bourbon and T. Kornberg, unpublished).

Schneider 2 cell line (termed HS-EN) transformed with a gene fusion composed of the *Drosophila hsp70* promoter, the coding portion of the *engrailed* cDNA and the *engrailed* polyadenylation site (Gay et al., 1988), was grown in spinner culture at 25°C. To induce Engrailed protein, one liter of cells was heated to 37°C for 45 minutes followed by 2 hours at 24°C. Soluble nuclear extract (referred as HS-EN) was prepared as described (Gay et al., 1988). A typical extract contained 2.5 mg/ml protein, of which 2% is Engrailed (estimated from Coomassie blue staining and Western analysis).

Immunoprecipitation

DNA fragments tested in this experiment correspond to inserts isolated from plasmids covering the *ph* region (Deatrick et al., 1991). The inserts were independently purified from plasmids, end-labeled, analysed on gels and mixed in 3 samples as shown on Fig. 6A. Binding assays were performed in conditions established by M. T. Saenz-Robles (unpublished) with some modifications. Protein A-Sepharose beads (CL 4B Pharmacia) were incubated overnight at 4°C

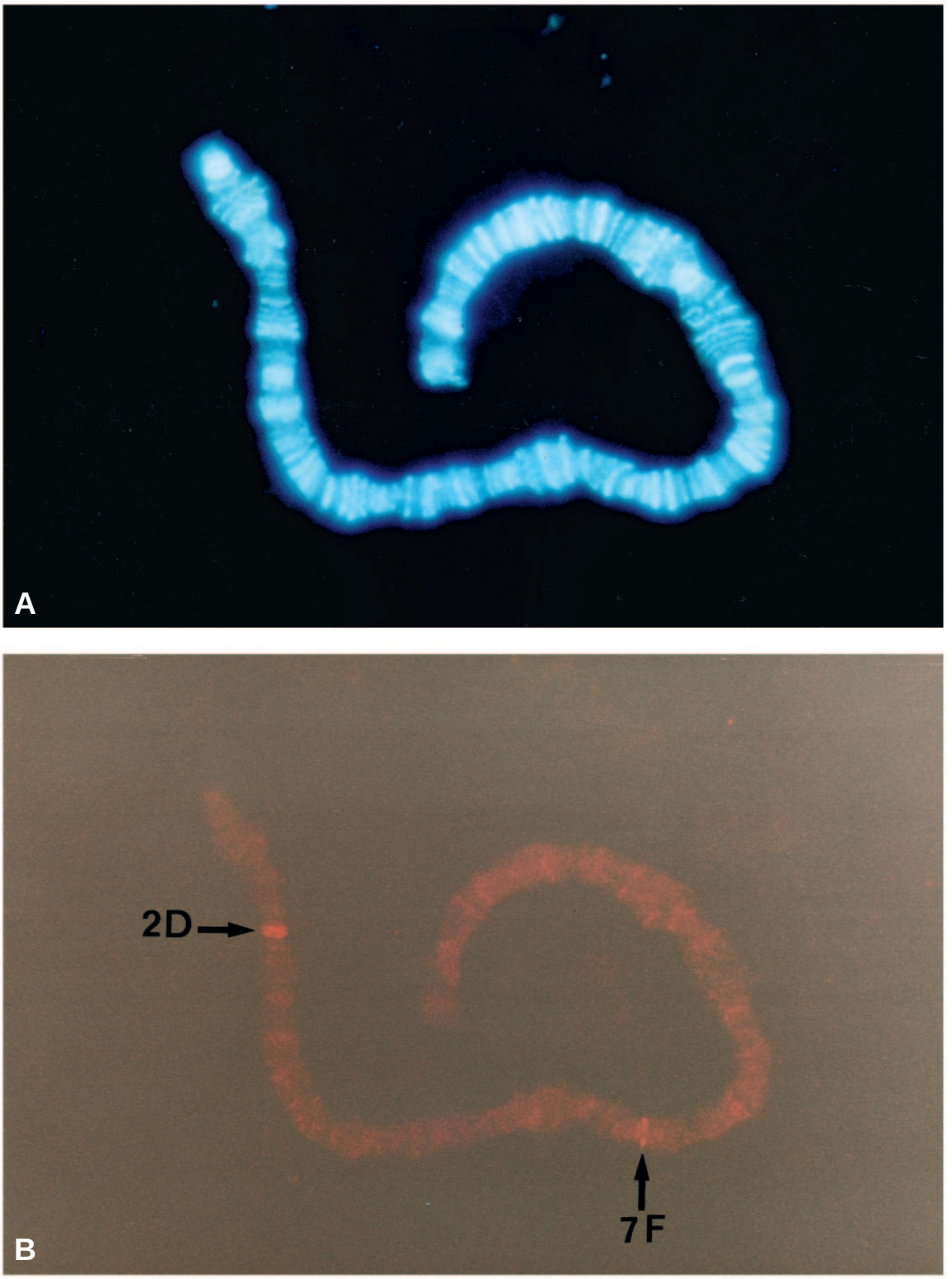


Fig. 1. Localization of Engrailed protein on polytene chromosomes of heat-shocked *hsen-3* larvae. The En protein is detected by goat polyclonal anti-En antibody. (A) X chromosome stained with DAPI to show the polytene banding pattern. (B) The same X chromosome showing bound anti-Engrailed antibody. Strong fluorescent signals are present at 2D and 7F, as indicated by arrows. Weaker, but reproducible sites are also detectable.

in presence of mAb 4F11 supplemented with 1% BSA. The resin was recovered by low speed centrifugation, washed twice in immunoprecipitation buffer (IB: 25 mM Hepes pH 8.0; 100 mM KCl; 0.1 mM EDTA pH 8.0; 0.1% NP40; 12.5 mM $MgCl_2$; 10% glycerol) and incubated for 3 hours at 4°C in presence of approximately 2 mg of HS-EN crude nuclear extract (containing approximately 40 μg of

Engrailed protein) for 0.2 ml of beads. The resin was recovered as above, washed in IB containing 0.1% BSA, resuspended in 0.2 ml of the same buffer and kept at 4°C. Immunoprecipitation was carried out as follows: 50 μl of resin (±HS-EN) in presence of 100 μl of IB supplemented with 0.1% PVA, 0.1% NP40, 0.1% BSA, 100 μg sheared herring sperm DNA and end-labeled DNA fragments (5 to 10 ng) are incubated 30 minutes at 4°C. Pellets were washed 3× with IB containing 0.1% BSA. Immunoprecipitated fragments were eluted from the resin by incubating with 200 μl of TE (10 mM Tris-HCl pH 8.0; 1 mM EDTA) supplemented with 1 M KCl for 30 minutes at 4°C. Prior to electrophoresis, the supernatant was extracted with phenol/chloroform, and the DNA ethanol precipitated in presence of tRNA carrier.

Gel shift assays

Binding assays contained approximately 10^{-10} M DNA. T7-En or HS-EN (diluted in 25 mM Hepes pH 7.6, 10% glycerol, 100 mM KCl) were incubated with end-labeled DNA (0.5 ng) for 30 minutes at 4°C in 10 μl of 25 mM Hepes pH 7.6, 10% glycerol, 100 mM KCl, 1 mM DTT, 1% PVA, 1% NP40, 0.1% BSA, and 200 ng of poly(dI:dC). DNA-protein complexes were resolved on 6% native polyacrylamide minigels in 0.5× TBE (pH 8.3) buffer. Gels were prerun at 4°C for 1 hour at 100 volts and run at 4°C for 2 hours at 120 volts.

Library of genomic targets for En protein

The library was constructed in conditions described by Graba et al. (1992) and Gould et al. (1990) and modified by N. Serrano, W. Dettman and F. Maschat (unpublished). The different steps are summarized on Fig. 9A. The major modifications concern the steps following immunoselection of the En protein-DNA adducts. Isolation of in vivo targets of DNA-binding En protein was performed by batch incubating *Sau*3A-digested samples with protein A-Sepharose beads coupled to the 4F11 monoclonal antibody. An aliquot of the DNA samples was radiolabeled in order to follow the immunopurification. After proteinase K treatment, recovered DNA fragments (around 1% of the input) were ligated in the *Bam*HI restriction site of a Bluescript vector. To follow the DNA during the subsequent in vitro immunopurification steps, ^{32}P-kinased T3 and T7 primers were used to amplify the selected DNA fragments. The same amount of the input DNA was recovered during the two last steps of immunoprecipitation. After a last PCR amplification, the DNA was digested by *Sau*3A and cloned into the *Bam*HI site of a Bluescript vector.

RESULTS

Engrailed protein binds to specific sites on salivary gland polytene chromosomes

Although Engrailed protein can bind DNA in vitro, its binding specificity is insufficient to unequivocally identify target sequences. We therefore explored the possibility that En could bind more selectively to polytene chromosomes in vivo. Since En is not normally produced in salivary glands, we characterized its distribution in a transgenic strain carrying the *engrailed* gene controlled by a heat shock promoter (*hsen-3*). After heat shock of *hsen-3* larvae, En protein was found in all salivary gland nuclei (data not shown). Despite the fact that salivary gland nuclei do not normally contain En, En protein binds only to a restricted number of specific sites on *hsen-3* squashed polytene chromosomes. Some of these sites showed stronger signals than others (Fig. 1). One of the strong sites appeared to be 2D (Fig. 1). Subsequent genetic and molecular analysis

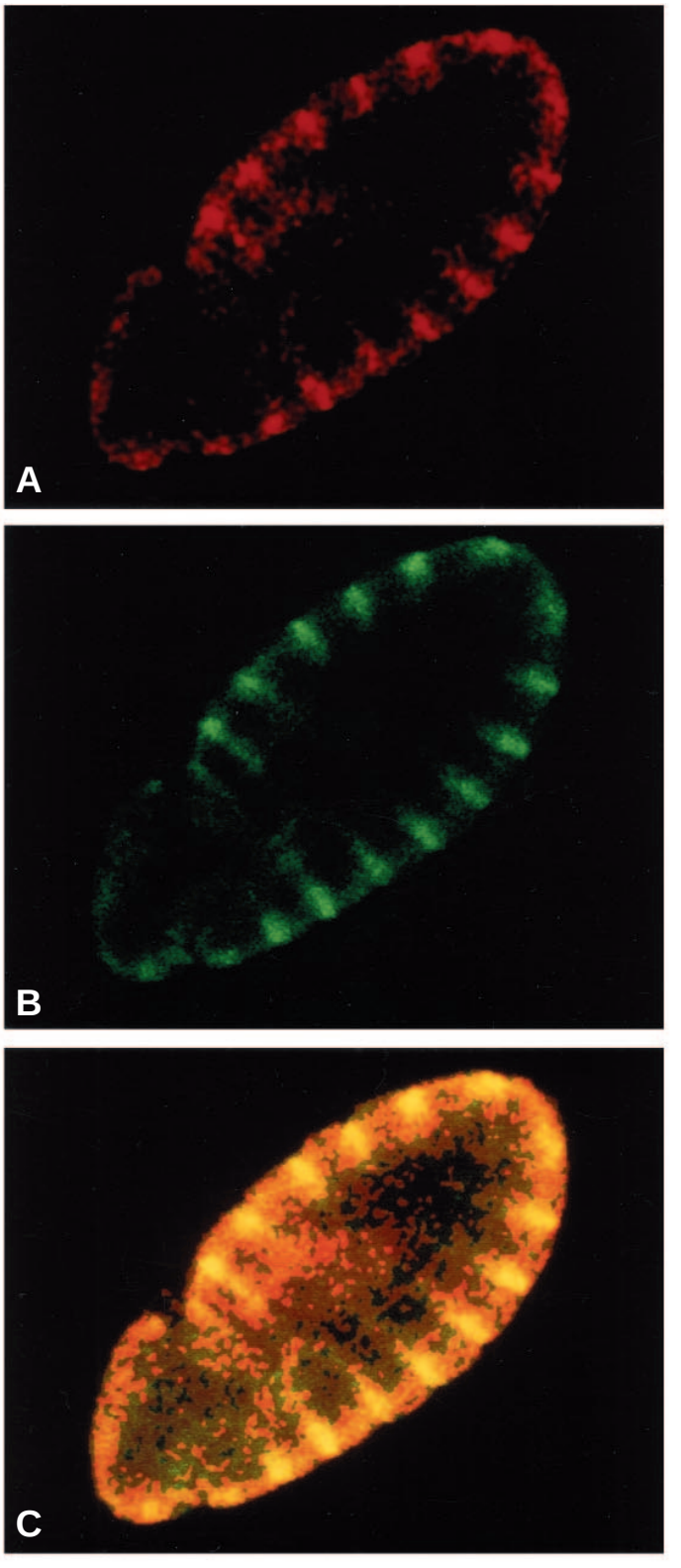


Fig. 2. Comparison of *polyhomeotic* and *engrailed* expression patterns in germ-band extended embryos. Confocal microscopy is used to visualize a stage 10 ph^{lac} embryo stained both with anti-En antibody (4D9) / rhodamine-labeled secondary antibody (A), and with polyclonal anti-β-galactosidase antibody / fluorescein-labeled secondary antibody (B). A composite image of the rhodamine and fluorescein fluorescence reveals yellow cells that express both *polyhomeotic* and *engrailed* (C). Embryos are oriented dorsal up, anterior left.

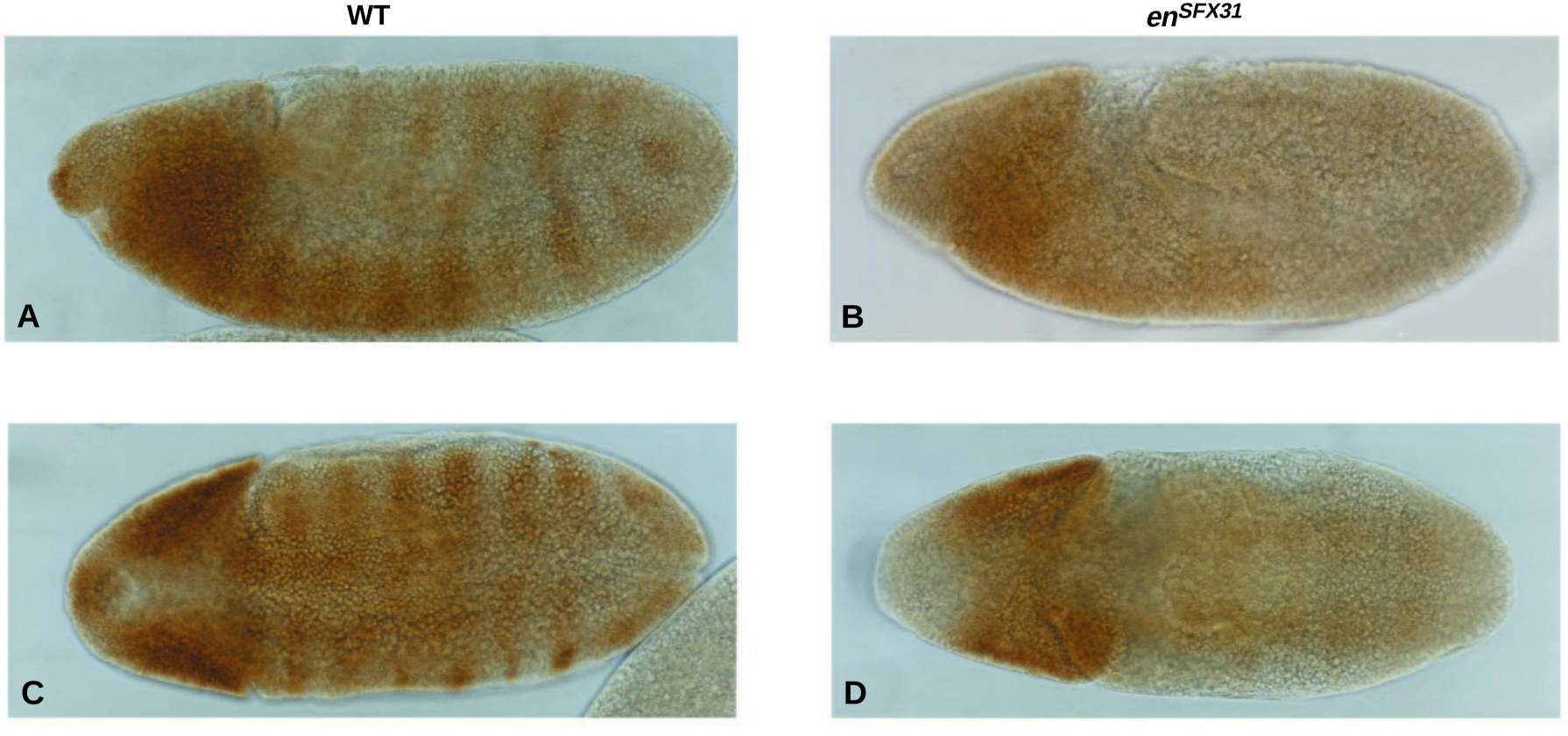


Fig. 3. Dependence of *polyhomeotic* expression on *engrailed*. Stage 10 germ-band-extended ph^{lac} embryos, are stained with anti-β-Galactosidase antibody and peroxidase-conjugated secondary antibody. Embryos either contain two functional *engrailed* genes (A,C) or are homozygous for $Df(2R)en^{SFX31}$ and lack *engrailed* expression (B,D). (A,B) lateral view, dorsal up. (C,D) dorsal view. Embryos are all oriented anterior left. The striped pattern seen in wild-type embryos is absent in *en* mutant embryos.

showed that the *Polycomb* group gene, *polyhomeotic (ph)*, located in 2D is one of the direct targets of *engrailed* regulation.

Engrailed is required for *polyhomeotic* expression in embryos

In germ-band-extended embryos, *ph* is expressed by most, if not all, cells of the presumptive neuroectoderm and epidermis. Heightened expression in a portion of these cells generates a transient zebra-stripe pattern that has a periodicity of one segment (DeCamillis and Brock, 1994). In a ph^{lac}strain, an enhancer trap insertion in the *ph* locus that is homozygous viable and has mild homeotic transformations, a similar striped pattern is detected by β-galactosidase staining. This striped pattern is a particularly prominent feature of ph^{lac}strain, because of the perdurance of the β-galactosidase.

β-galactosidase expression in ph^{lac} closely follows the endogenous *ph* expression (M. O. Fauvarque and J. M. Dura, unpublished). ph^{lac} expression was first detected in cellular blastoderm embryos as a broad anterior band extending from approximately 50-80% egg length and a posterior band expanding from approximately 20-30% egg length. This pattern recalls the patterns of the gap genes *hunchback* and *giant* (Deatrick, 1992; M. O. Fauvarque and J. M. Dura, unpublished) and of *engrailed* expression (Karr et al., 1989). During gastrulation, *ph* expression evolves rapidly to form a striped pattern similar to that of *en* in germ-band-elongated embryos (Figs 2B, 3A,C). Indeed, direct comparison of ph^{lac} germ-band-extended embryos stained for both Engrailed protein and β-galactosidase reveals that *en* (Fig. 2A) and *ph* (Fig. 2B) patterns of expression can be precisely superimposed (Fig. 2C).

ph^{0} embryos, which lack *ph* expression, have unaltered patterns of *en* stripes prior to germ-band retraction (Dura and Ingham, 1988; Smouse et al., 1988). This observation suggests that at this stage of development, *en* is not regulated by *ph*. To address the possibility that *ph* might be a downstream target gene of *en*, β-galactosidase expression was examined in an *en* mutant carrying the ph^{lac} enhancer trap. In germ-band-extended *en* mutant embryos, whereas the ph^{lac} expression in the head is not affected, the epidermal stripes of ph^{lac} expression fail to form (Fig. 3B,D). Even though we cannot exclude that prior to germ-band extension, maternal *ph* expression could initiate *en* expression, we conclude that in germ-band-extended embryos, *en* positively regulates *ph*.

polyhomeotic and *engrailed* expression patterns in germ-band-retracted embryos

In germ-band-retracted embryos, patterns of *en* and *ph* expression diverge. At stage 13, *ph* expression fades in the

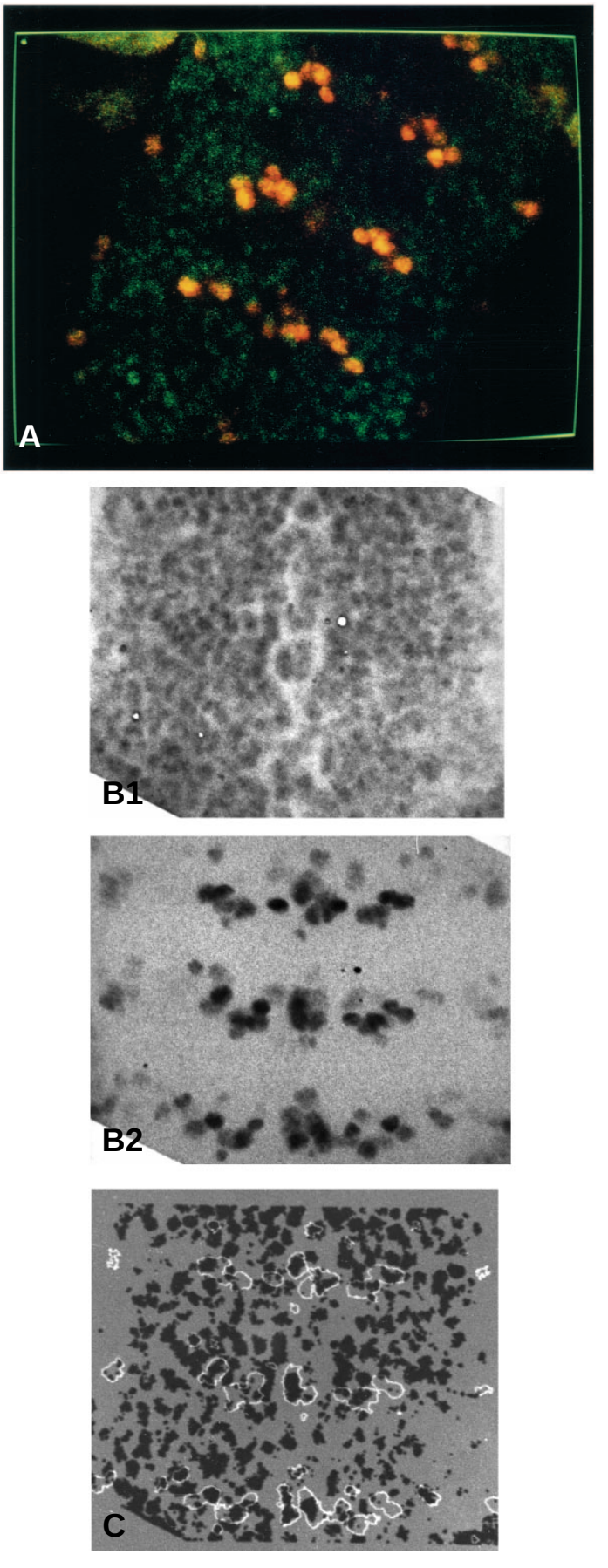


Fig. 4. Comparison of *polyhomeotic* and *engrailed* expression patterns in the CNS of stage 13 germ-band-shortened embryos. Optical sections obtained by confocal microscopy revealed: (A) Polyhomeotic protein detected by rabbit anti-Ph antibody/fluorescein-labeled secondary antibody (green) and Engrailed protein detected by anti-En antibody (4D9)/rhodamine-labeled secondary antibody (red). Cells expressing both Ph and En proteins appear yellow. (B) Images of (B1) fluorescein fluorescence alone (Ph) and (B2) rhodamine fluorescence alone (En) from the doubly-stained embryo in A. (C) Using quantitative analysis, cells expressing high levels of Ph are shown in black, and cells expressing high levels of En are surrounded by white lines. The level of Ph is independent of En.

epidermis and is limited to the central nervous system (CNS), where Ph protein appears somewhat uniform in all cells (Fig. 4). In contrast, *en* is expressed in a segmentally reiterated pattern, in the epidermis and in the CNS. No significant difference in the amount of *ph* expression was noted whether cells do or do not express *en* (Fig. 4C).

The absence of apparent relationship between *en* and *ph* expressions in the CNS is consistent with the pattern of ph^{lac} expression in germ-band-retracted *en* mutant embryos. In such embryos, ph^{lac} expression is still present in the CNS, although the level of expression can vary (compare Fig. 5B and C). Such variations could depend upon indirect effects of severe abnormalities these mutant embryos suffer.

Previous data showed that *en* is not expressed in the CNS of ph^{0} mutant embryos, but is present ubiquitously in the epidermal cell nuclei (Dura and Ingham, 1988; Smouse et al., 1988). We therefore suggest that after germ-band retraction, the regulatory relationships between *ph* and *en* change, since the early pattern of *ph* zygotic expression depends on *en* function, while the later expression does not.

Engrailed-binding sites at the *polyhomeotic* locus

Since both polytene chromosomes localization of En and genetic interactions point to *ph* as a target of En regulation, we looked for En-binding sites within the *ph* genomic region. The *polyhomeotic* genomic region has been cloned (Dura et al., 1987) and the *ph* transcription units have been sequenced (Deatrick et al., 1991; DeCamillis et al., 1992). To identify En-binding sites, monoclonal antibody 4F11 was used to immunoprecipitate genomic fragments from the *ph* region in presence of En protein. Among end-labeled restriction fragments covering 49 kb of the *ph* region, only a 1.5 kb *Bam*HI fragment (P) from the region 5′ to the proximal transcription unit, and a 1.5 kb *Sal*I fragment (D) from the region between the two transcription units were recovered (Fig. 6A,B).

*Sau*3A digests of the P and D fragments were analyzed by immunoprecipitation with or without En protein and by gel shift assays (not shown). En-binding sites were only detected in a 441 bp *Bam*HI-*Sau*3A fragment in the P region (termed P1; Fig. 7B1) and within two 300 bp *Sal*I-*Sau*3A and 334 bp *Sal*I-*Dde*I fragments in the D region (termed D1 and D2; Fig. 7B2). Studies of these fragments by gel shift assays and DNaseI footprinting analysis revealed the presence and precise location of several sites that bind En with high affinity (Fig. 7). Many homeodomains can bind with high affinity to DNA containing matches to either the consensus TCAATTAAAT or (TAA)n (Desplan et al., 1988). Whereas the P1 and D1 fragments contain 6 to 7 sequences closely related to the TCAATTAAAT consensus, the D2 fragment contains long stretches of TAA motifs (Fig. 7B1, B2). Engrailed affinities for these different fragments were estimated by mobility shift

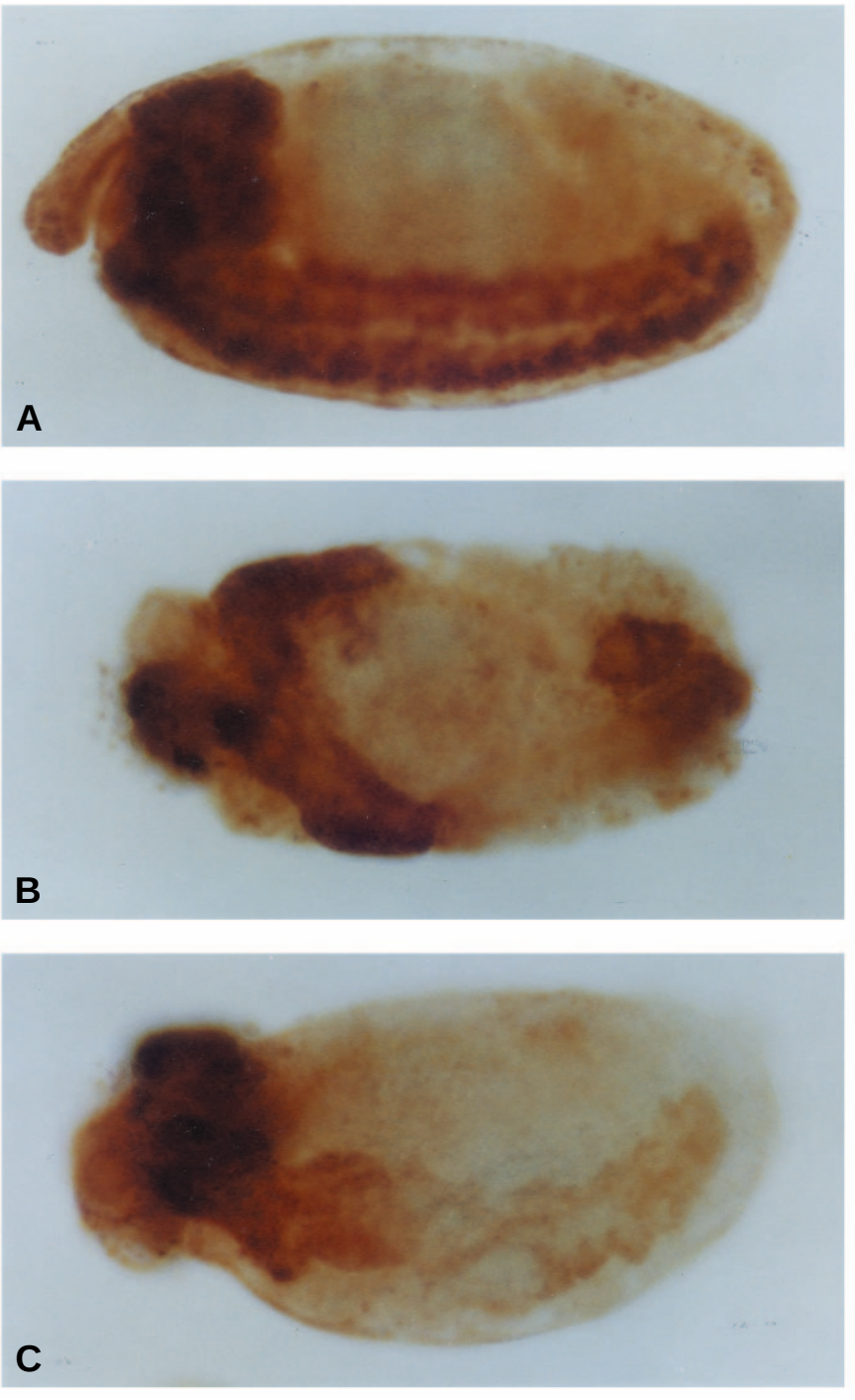
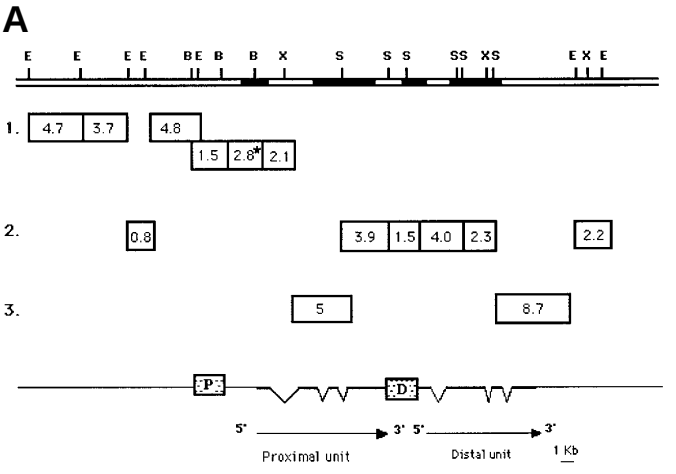


Fig. 5. Influence of *engrailed* on *polyhomeotic* expression in germ-band-retracted embryos. Stage 13 ph^{lac} embryos are stained with anti-β-Galactosidase antibody and peroxidase-conjugated secondary antibody. (A) Embryos contain two functional *en* copies, lateral/ventral view. B,C are homozygous for $Df(2R)en^{SFX31}$ and lack *en* expression. The embryo in B is twisted and shows normal level of *ph* expression in the ventral CNS. The embryo in C shows weak *ph* expression in the CNS. Embryos are oriented dorsal up, anterior left.



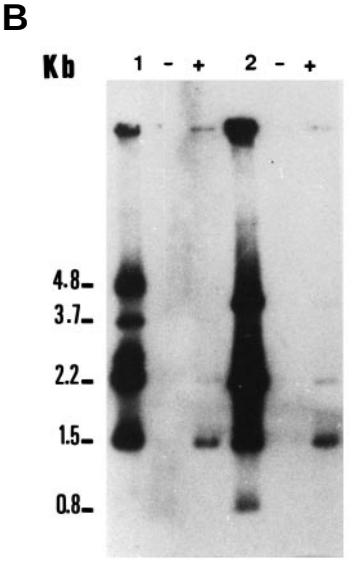


Fig. 6. Localization of Engrailed protein-binding sites in the *polyhomeotic* region. (A) Diagram and restriction map of 49 kb of the *polyhomeotic* genomic region (Dura et al., 1987). Black boxes correspond to repeated sequences in the two transcription units. The intron/exon structure of the proximal and distal transcripts is shown below. The 1.5 kb *Bam*HI and the 1.5 kb *Sal*I fragments (named respectively P and D) that were immunoprecipitated by Engrailed protein are indicated by stippled boxes. Purified restriction fragments were pooled in three groups for immunoprecipitation. Groups 1 and 2 indicate fragments tested in the immunoprecipitation experiments shown in B. Fragments in group 3 did not immunoprecipitate in presence of the Engrailed protein (not shown). (B) Autoradiograph of an agarose gel resolving restriction fragments spanning the *polyhomeotic* genomic region that were monitored for Engrailed-binding sites by immunoprecipitation assay. Lane 1, mixture of end-labeled genomic DNA fragments (21 kb) covering the upstream region and the first exon of the proximal unit. Immunoprecipitation of these fragments was carried out in absence (–) or in presence (+) of HS-EN protein. A 1.5 kb *Bam*HI fragment (P fragment) is specifically immunoprecipitated in presence of En protein. The 2.8 kb *Bam*HI fragment that has been tested in vivo (refered to as ph N fragment on Fig. 9). Lane 2, end-labeled genomic DNA fragments (15 kb) covering the two transcription units of *polyhomeotic* as well as a small part of the downstream region (2.2 kb *Eco*RI fragment). Note that a 0.8 kb fragment of the upstream region is loaded in this lane. Immunoprecipitation of these fragments was carried out in absence (–) or in presence (+) of HS-EN protein. A 1.5 kb *Sal*I fragment (D fragment) is specifically immunoprecipitated in presence of En protein. Fragments in the mixtures are not always well separated on the gel, but they were previously analysed separately on gels before immunoprecipitation (not shown). E, *Eco*RI; B, *Bam*HI; S, *Sal*I; X, *Xho*I.

studies in which the concentration of En was varied relative to a constant amount of DNA. Binding sites in P1, D1 and D2 were titrated with purified T7-En protein or HS-EN crude extracts. The estimated K_D for P1 (Fig. 7A1) and D1 (not shown) were similar to the estimated affinity for both T7-En and HS-EN proteins for the TCAATTAAAT consensus (not shown) and were estimated to 10^{-9} M with the HS-EN protein. For D2, affinity was fivefold higher than for P1 and D1 and was estimated to 2×10^{-10} M with the HS-EN protein (Fig. 7A2). The affinities for the HS-EN protein were 10-fold higher than the affinities for the T7-En protein for the same binding site (Fig. 7A1, A2). This is probably due to post-translational modifications like phosphorylation that occur in *Drosophila* cells (Gay et al., 1988). The identification of DNA-protein complexes in presence of T7-En protein confirms that there are specific bindings between the P1, D1 and D2 fragments and En. The specificity of the binding with HS-EN was also verified by supershift with the 4F11 antibody (data not shown).

We noticed that the footprinted sequences in D2 are larger than the ones in P1 and D1 (sequences underlined on Fig. 7B1, B2). The footprinted region in D2 comprises two blocks of 92 and 30 bp covering the TAA stretches, whereas 6 to 7 shorter regions covering TCAATTAAAT related sequences are protected against the DNaseI in the D1 and P1 regions (Fig. 7B1, B2).

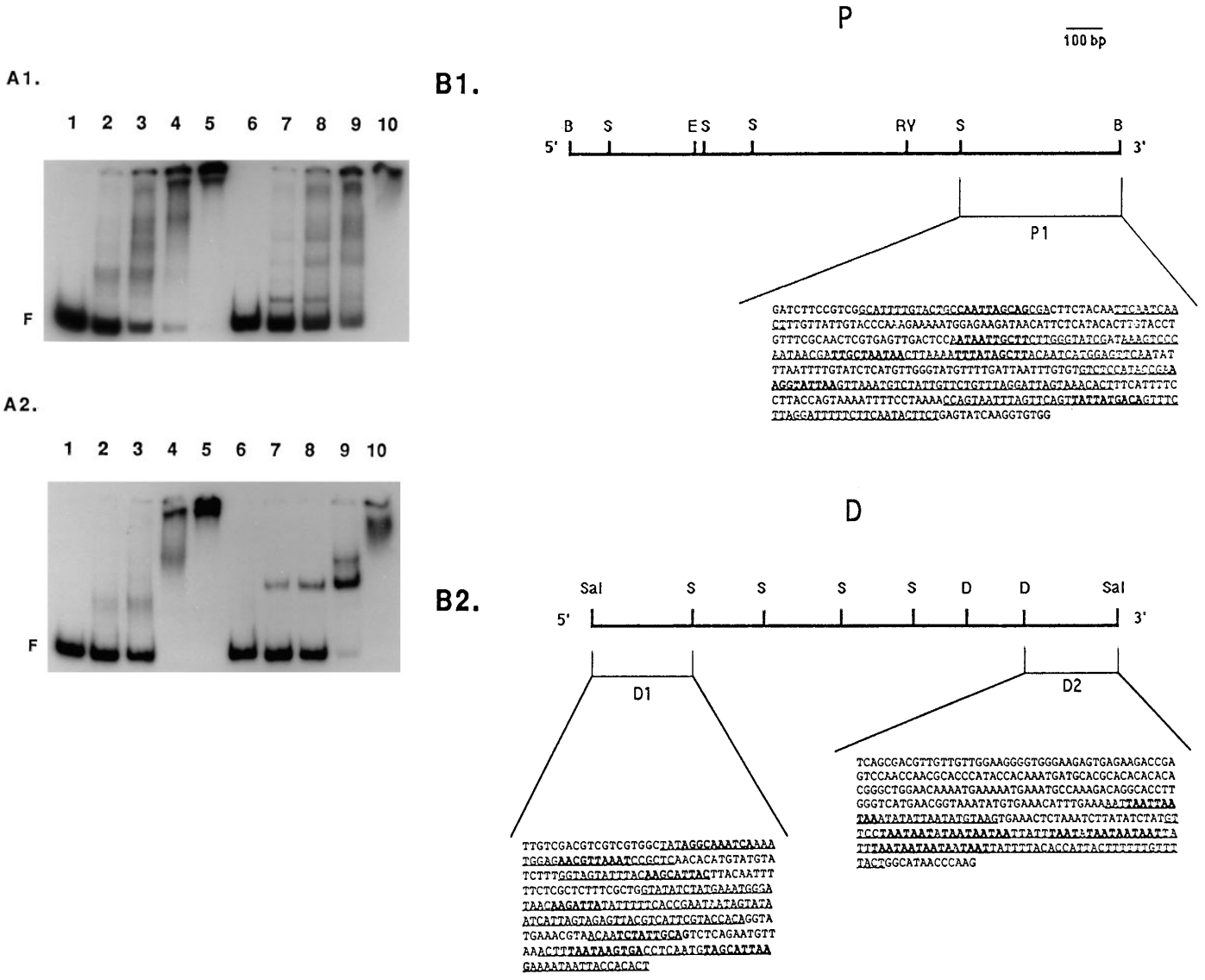
Together, these observations suggest that P1 (or D1) and D2 represent two different classes of En-binding sites, which differ in their sequences, the length of the footprinted regions and their affinity for En (Fig. 7C).

Polytene chromosomes immunostaining of *ph* transgenic lines

In order to verify that the signal detected at 2D on polytene chromosomes results from En binding on the *ph* locus, we constructed a transgenic line where the *ph* D fragment (Fig. 6A) was placed upstream to a *hsp70-lacZ* reporter gene. After microinjection of this *P*(*ph-lacZ*)-element in embryos, several transgenic lines were isolated. This 1.5 kb *Sal*I D fragment confers a low level of *lacZ* expression in germ-band-extended embryos without detectable spatial restriction, whereas β-galactosidase expression can be detected in the salivary glands, a site of normal *ph* expression (not shown). In situ hybridization to polytene chromosomes was first carried out in order to localize the inserts. One insert was located at the tip of the X chromosome in 1A (Fig. 8A).

We examined En antibody polytene binding sites in the transformed strain containing the 1.5 kb *Sal*I fragment inserted in 1A. Because En is ectopically expressed in salivary glands of a *tramtrack* mutant (*ttk1*) (Xiong and Montell, 1993), the experiments were carried out in a *ttk1* background. Fig. 8B shows that there is a new site of En antibody binding in region 1A in the *P*(*ph-lacZ*) transgenic line that is not present in the *ttk1* control (Fig. 8C). The other En-binding sites are identical in both cases (Fig. 8B,C). We noticed that the rabbit anti-En antibody gave stronger signals than the goat antibody (Fig. 1). Around 50 strong En-binding sites were obtained with the rabbit antibody (unpublished), the 2D site being one of them. The use of heat-shocked *hsen-3* strain or *ttk1* strain gave the same pattern of En-binding sites, except that the morphology of the chromosomes was in general better in the case of the *ttk1* strain and so gave more reproducible results.

This experiment showed that the En signal detected in 2D can be attributed, at least in part, to En binding on the *ph* gene and that En is able to recognize and bind the D fragment in vivo.



C.

SITES	K_D	ENGRAILED BINDING SITE	FOOTPRINT
P1	10^{-9} M	TCAATTAAAT Consensus	SHORT
D1	10^{-9} M	TCAATTAAAT Consensus	SHORT
D2	2.10^{-10} M	"TAAT" stretches	LARGE

Fig. 7. Mobility-shift analysis of En protein-binding sites. (A) Relative affinities of En-binding sites were measured by maintaining a constant concentration of DNA fragment (0.5 ng/lane) and varying the concentration of T7-En or HS-EN protein. Panel A1, 441 bp P1 fragment. Protein concentrations were calculated to be for (i) T7-En: lane 1, 0; lane 2, 10^{-10} M; lane 3, 10^{-9} M; lane 4, 10^{-8} M; lane 5, 5×10^{-8} M. (ii) HS-EN: lane 6, 0; lane 7, 10^{-11} M; lane 8, 10^{-10} M; lane 9, 10^{-9} M; lane 10, 5×10^{-9} M. The estimated K_D is 10^{-9} M for HS-EN. Panel A2, 334 bp D2 fragment. Protein concentrations were calculated to be for (i) T7-En: lane 1, 0; lane 2, 5×10^{-10} M; lane 3, 10^{-9} M; lane 4, 10^{-8} M; lane 5, 5×10^{-8} M. (ii) HS-EN: lane 6, 0; lane 7, 5×10^{-11} M; lane 8, 10^{-10} M; lane 9; 10^{-9} M; lane 10, 5×10^{-9} M. The estimated K_D is 2×10^{-10} M for HS-EN. F indicates free DNA. (B) Localization of Engrailed-binding sites in the P (B1) and D (B2) fragments. Restriction maps of the fragments are shown above. Immunoprecipitation and gel shift experiments (not shown) localized Engrailed-binding sites in the P fragment to a 441 bp *Bam*HI-*Sau*3A fragment, referred to as P1 and in the D fragment to two 300 and 334 bp fragments, referred to, respectively, as D1 and D2. The nucleotide sequences of these fragments (Deatrick et al., 1991) are indicated. Sequences related to the consensus Engrailed-binding site are in bold. DNaseI footprinted sequences are underlined. B, *Bam*HI; E, *Eco*RI; S, *Sau*3A; RV, *Eco*RV; Sal, *Sal*I; D, *Dde*I. (C) Table summarizing the different properties of the En-binding sites localized within the P1, D1 and D2 fragments.

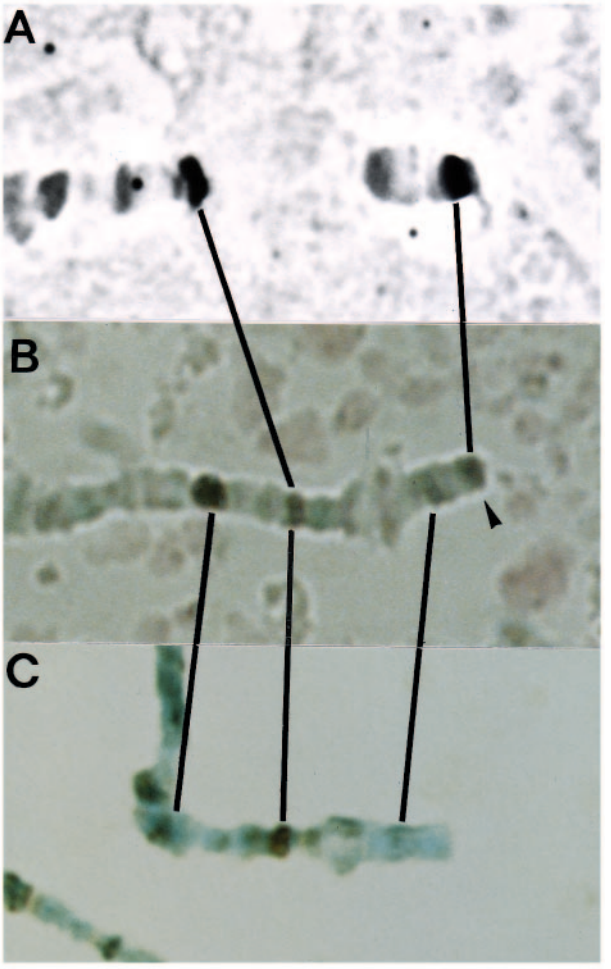


Fig. 8. Engrailed immunodetection in a *P*(*ph-lacZ*) transgenic line. (A) Localization of the *P*(*ph-lacZ*) insert to region 1A by in situ hybridization on polytene chromosomes, using a biotinylated *ph* D fragment as a probe. Note the endogenous *ph* locus in 2D. (B,C) Localization of Engrailed protein on polytene chromosomes. En-binding sites were identified by using the rabbit polyclonal anti-En antibody, detected by a secondary antibody coupled to horseradish peroxidase. (B) Immunostaining of the X chromosome from a *P(ph-lacZ)*; *ttk1* line. (C) Immunostaining of the X chromosome from a *ttk1* line. Common sites are indicated, and the new signal in B is indicated with an arrowhead.

In vivo immunopurification of Engrailed-binding sites

To identify En target sequences in embryos, we developed an immunoprecipitation approach that enriches for short chromatin fragments associated with endogenous En protein. The procedure is outlined in Fig. 9A and follows procedures of Graba et al. (1992) and Gould et al. (1990). Native embryonic chromatin was stabilized by UV light irradiation. After *Sau*3A digestion of the chromatin, En protein-DNA complexes existing in intact embryonic nuclei were affinity purified with a resin containing antibodies to En protein. *Sau*3A DNA fragments from the immunopurified chromatin were ligated in a Bluescript vector at the *Bam*HI site. Prior to transformation into competent bacterial cells, several rounds of PCR using the T3 and T7 primers followed by in vitro immunoprecipitation helped to enrich for En-binding sites.

Specific enrichment of En-binding sites was verified by comparing PCR amplification of a known En-binding site in the *en* gene itself (K fragment; Desplan et al., 1988), or of a *ph* fragment (N fragment; * on Fig. 6A) that cannot bind En in vitro. Both fragments were verified to be equivalently present in chromatin prior to immunoprecipitation (data not shown). Fig. 9B shows that the *en* K fragment was readily detectable by PCR in the ligation mix after in vivo immunopurification and was strongly represented in the final library after three rounds of PCR / in vitro immunoprecitation, whereas the *ph* N fragment was never detected.

The presence of P1, D1 and D2 fragments was confirmed by PCR, using specific sets of primers. Fig. 9C shows that the three fragments were amplified, as much as was the *en* K fragment (Fig. 9B). Moreover, screening of the final library with a *ph* D fragment probe, showed that around 0.5 % of plasmid recombinants were positive (data not shown). This is in agreement with the expected ratio for immunoprecipitated *Sau*3A fragments, if we consider that there are approximately 50 En target genes, as suggested by chromosomes immunostaining.

Thus, immunopurification of chromatin with an anti-En antibody results in an enrichment of the three *ph* sequences shown to bind En in vitro. This indicates that the regulation of *ph* in embryos could directly depend on En binding and that this regulation could be mediated through the P1, D1 and D2 fragments.

DISCUSSION

The En protein is a homeodomain-containing nuclear protein that binds DNA with high affinity in vitro and could thus regulate genes directly at the transcriptional level. If En acts as an on/off switch, it might activate genes that execute posterior compartment-specific functions, or it might repress genes that specify anterior functions. Such target genes should have expression patterns that are either similar to or complementary to *en* expression at relevant stages of the interaction. Candidates that satisfy these criteria are *cubitus interruptus* (*ci*) and *hedgehog* (*hh*). *ci* is expressed in patterns complementary to those of *en*, and in posterior compartment cells its lack of expression is *en*-dependent (Eaton and Kornberg, 1990). Apparently, *ci* is negatively regulated by *en* in both embryonic and imaginal cells. In contrast, *hh* seems to be a positively regulated target of *en* at germ band elongation (Lee et al., 1992; Tabata et al., 1992). In this report, we show that *polyhomeotic* is also directly activated by En.

en and *ph* interact at various steps during development. Both genes are expressed in broad domains in the cellularizing blastoderm and in similar segmentally reiterated zebra-stripe patterns shortly thereafter. At these stages, *ph* expression is dependent upon *en* function. In particular, the zebra-stripe pattern is severely reduced in *en* mutant germ-band-extended embryos (Fig. 3). Later, *ph* expression becomes independent of *en* in germ-band-retracted embryos, and a transition in the regulation occurs, making *en* maintenance dependent of *ph* expression (Dura and Ingham, 1988; Smouse et al., 1988). In larvae, *en* activates *ph* expression during wing morphogenesis, whereas it represses *ph* expression in the hindgut (N. Serrano and F. Maschat, unpublished). Thus En could act as an activator or as a repressor on *ph* expression.

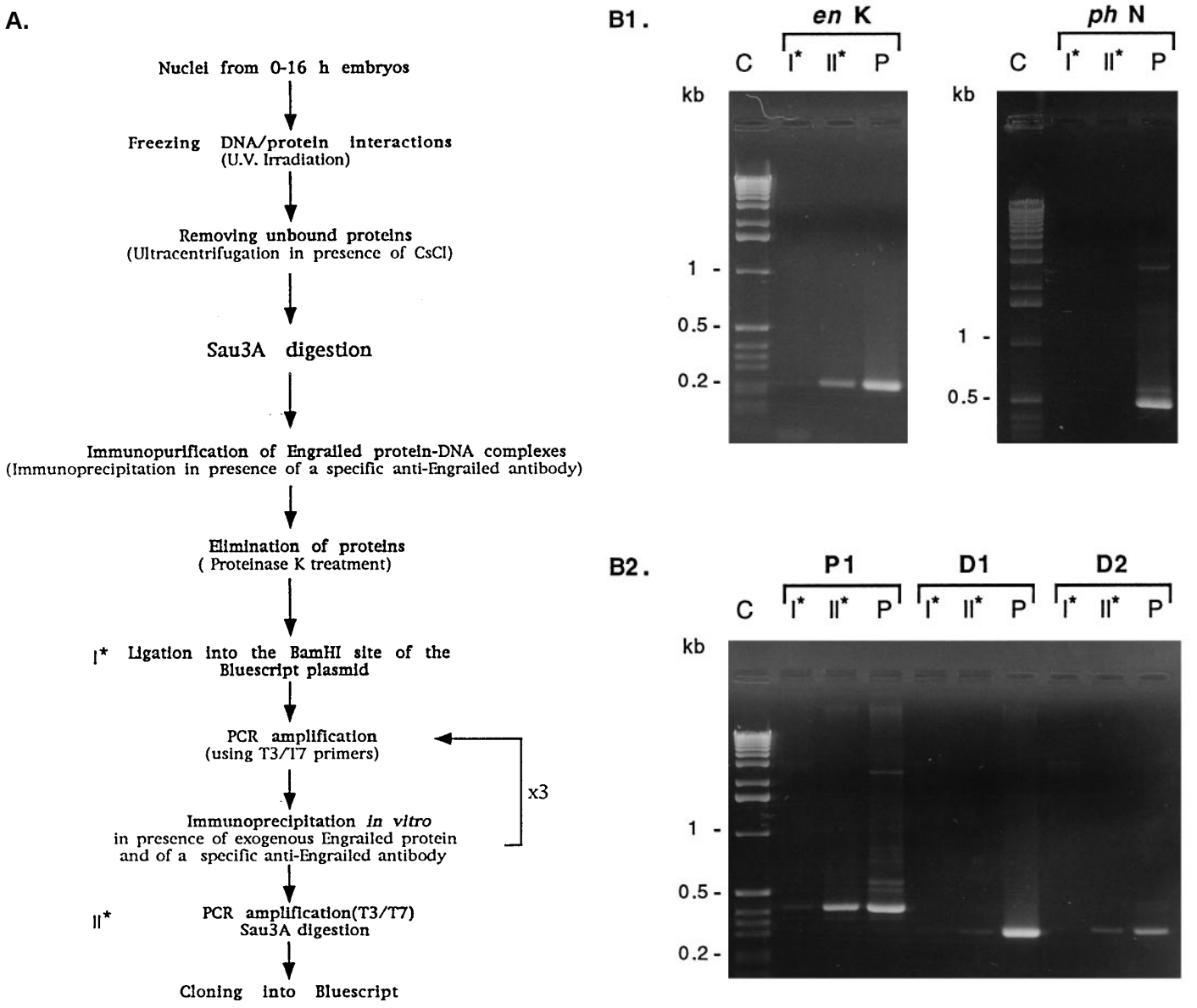


Fig. 9. Immunopurification of sites bound in vivo by En protein. (A) Immunopurification strategy. UV-irradiated chromatin was immunoprecipitated in presence of monoclonal anti-En antibody (4F11). Several steps of PCR followed by in vitro immunoprecipitation were carried out in order to further enrich for En-binding sites. Detailed descriptions of this in vivo immunopurification method and DNA analysis will be published elsewhere (N. Serrano, W. Dettman and F. Maschat, unpublished data). (B) The presence of several sites during this immunopurification has been tested by PCR amplification at two different steps during the procedure, using specific sets of primers. The primers have been chosen to lie within *Sau3A* fragments. 10 ng of total DNA were thus amplified: I* on ligation mix after the in vivo immunoprecipitation; II* in the final library before cloning, as indicated in A; P is a control of PCR amplification. The entire product of PCR is loaded in I*, whereas only a fifth is loaded in II* and P on a 0.8% agarose gel. C is the 1 kb ladder migration control (Gibco, BRL). Panel B1: amplification of the *en* K fragment as a positive control and of the *ph* N fragment as a negative control. Panel B2: amplification of the P1, D1 and D2 En-binding sites identified in vitro on the *ph* locus.

Several observations support the proposal that *ph* is a direct target of *en* regulation in germ-band-extended embryos. En protein accumulation at the cytological location 2D indicates that En might directly regulate genes in this region. Region 2D is known to contain several loci, including *corkscrew*, *polyhomeotic*, *Phosphogluconate dehygrogenase*, and *l(1)C204* (Perrimon et al., 1985). The detection of an additional En-binding site in 1A in the *P*(*ph -lacZ*) transgenic line suggests that *en* might directly regulate *ph* expression in vivo. This result does not exclude that genes in 2D, other than *ph*, might contribute to the accumulation of En protein at this position.

That En directly regulates *ph* expression is also supported by the presence of high affinity En-binding sites in *ph* DNA (Fig. 6). Binding sites are located upstream of the 5′ end of the longest cDNAs available for the proximal transcription unit (termed P fragment; Fig. 7A). Additional binding sites are present in a short region of unique sequence between the 3′ end of the proximal transcription unit and the 5′ end of the distal

transcription unit (termed D fragment; Fig. 7B). These En-binding sites are presumably localized in the promoter regions of each of the *ph* transcription units. They conform to the consensus sequences for En binding (Desplan et al., 1988) and their affinity for En protein is as high or higher than the affinities shown by the En homeodomain for a consensus binding site. The *P*(*ph-lacZ*) transgene localized in 1A contains the *ph* D fragment, which binds En with high affinity in vitro (Fig. 6A). This suggests that *en* regulation in vivo might occur, at least in part, through this fragment.

Since the En-binding sites identified in vitro in the *ph* locus are also recovered by in vivo immunopurification of En targets, we suggest that En regulates *ph* directly in embryos. The mechanism of activation by En is particularly interesting to elucidate. Indeed while repression by En has been well studied (Jaynes and O'Farrell, 1991), little is known about the mechanisms involved in activation by En, even though En acts in vivo as an activator in several circumstances. En activates its own expression (Heemskerk et al., 1991) and also *hh* expression (Lee et al., 1992; Tabata et al., 1992). Only the K fragment of the *en* gene has been clearly identified as a high affinity En-binding site (Desplan et al, 1988). However, this fragment mediates repression of CAT activity by En in transfection assays (Jaynes and O'Farrell, 1991). The K fragment was clearly immunopurified from embryonic native chromatin in our experiments. Likewise, the P1 and D1 fragments were immunoprecipitated from native chromatin, and we have shown, by CAT assays, that these fragments mediate repression by En (N. Serrano and F. Maschat, unpublished). The identification on one hand, of the three P1, D1 and D2 *ph* fragments in the in vivo library and, on the other hand, of two different classes of En-binding sites (Fig. 7C) leads to the possibility that activation of *ph* might depend on the occupancy of all the sites. This supports the idea that repression or activation could depend upon En concentration, if different sites are occupied at different concentrations of En, or upon the presence of cofactors, such as Extradenticle (Exd), which is able to modify En DNA binding (van Dijk and Murre, 1994).

The significance of the transient peak of *ph* activity driven by *en* regulation in the posterior compartment of the segments of germ-band-extended embryos is interesting to consider. Embryos carrying either a mutation in one of the *Pc-G* genes (or a deletion in the *bithorax* complex) develop the normal number of segments, despite the fact that most segments have abnormal segmental identity. This suggests that segmentation genes act upstream of both homeotic and *Pc-G* genes. Later during embryonic development, this relationship changes, and *en* expression becomes dependent upon *ph*. This reversed regulatory relationship can be understood in the context of the different roles that *en* and *ph* assume. Dependence of a *Pc* group gene (*ph*) on a segmentation gene (*en*), might therefore reflect this hierarchical relationship. Subdivision into segments is controlled by segmentation genes, while segmental identity is maintained by *PcG* genes and homeotic genes. One of the functions of *en* appears to down-regulate *Ultrabithorax (Ubx)* and *Deformed* (*Dfd*) in the epidermis of germ-band-extended embryos (Martinez-Arias and White, 1988; Mann, 1994). Once segmentation has created the metameric pattern and the pattern of homeotic gene expression has been established, the global control of the homeotic genes becomes the province of the *Pc-G* genes. Indeed, later in development, *Pc-G* genes are thought to maintain repressed states of gene expression by imprinting *cis*-regulatory elements with local heterochromatinization (Paro, 1990; DeCamillis et al., 1992; Fauvarque and Dura, 1993). *Pc-G* regulation of the *bithorax* and *Antennapedia* gene complexes is essential during embryogenesis (Dura and Ingham, 1988) and binding sites for Ph and Pc proteins are present at the polytene chromosome sites of these complexes (Franke et al., 1992). Also, multimeric *Pc-G* protein complexes could have a direct role in maintaining the regionally specified states of *bithorax* genes expression, set up by the segmentation genes. From our observations, it appears that this regulatory transition between the segmentation and the *Pc-G* genes becomes effective as the expression patterns of *ph* and *en* diverge and their respective prominent roles change, perhaps at the time when imprinting becomes active.

Based upon the genetic and molecular interactions that we observed between *en* and *ph*, we propose that, during early embryonic development, *ph* is a direct target of En. Further mutational and molecular studies are now warranted to elucidate the molecular mechanisms involved.

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