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Homeo box gene expression in anterior and posterior regions of the *Drosophila* embryo

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ABSTRACT The homeo box is a 180-base-pair coding sequence that has been implicated in the control of *Drosophila* development. A common feature of the nine previously reported homeo box genes is their involvement in the establishment of the segmentation pattern of the embryo. In this report we describe the isolation and properties of two additional homeo box genes, *F90-2* and *S67*. Transcripts encoded by the two genes are detected in embryonic tissues that derive from regions near the anterior and posterior poles of the embryo, which are outside the limits of expression of known homeotic genes. These results suggest that at least some homeo box genes specify positional identity along the anterior–posterior body axis that is independent of the process of segmentation.

A central problem of higher metazoan development is how embryonic cells first acquire and subsequently maintain unique positional identities. The morphogenesis of diverse body segments in *Drosophila* provides a system for analyzing this problem. Two classes of genes are required for establishing the characteristic segmentation pattern of *Drosophila*. Segmentation genes establish the basic metameric repeat along the anterior–posterior axis of the embryo (1–5), and homeotic genes establish the diverse pathways by which each embryonic segment primordium develops a distinct adult phenotype. Mutations in segmentation genes can cause deletions and/or duplications in each segment or in alternating segments. Mutations in homeotic genes cause a partial or complete transformation of one segment into the homologous tissues of another segment (6–8). Many of the segmentation and homeotic genes correspond to "selector" genes that have been postulated to control the developmental fate of a cell by the *trans* regulation of target genes (9, 10).

The molecular characterization of segmentation and homeotic genes has been facilitated by the demonstration that several of these genes share nucleotide homology within a protein-encoding region designated the homeo box (11–13). A total of nine segmentation and homeotic genes have been shown to contain a homeo box (11–16). Six of the homeo box sequences are contained within homeotic genes and three are within segmentation genes (14–16). These nine homeo box genes are located within three gene complexes: The Antennapedia gene complex (ANT-C) (8, 17, 18), the bithorax gene complex (BX-C) (7, 19), and the engrailed gene complex (EN-C) (15, 16).

A common feature of the previously characterized *Drosophila* homeo box genes is that all are involved in specifying different aspects of segment identity during embryogenesis. The homeo box is strongly conserved among higher metazoans (reviewed in ref. 20), and it has been proposed that homeo box genes control pathways of segment morphogenesis in a variety of organisms, including vertebrates (12). To better define the role of the homeo box in development, we isolated and characterized additional homeo box genes in *Drosophila*. In this report we describe the isolation and expression of two novel homeo box genes. *In situ* hybridization studies show that these genes are expressed in anlagen located near the anterior and posterior poles of the embryo. Since these regions give rise to tissues that do not appear to be segmented, we propose that at least some homeo box genes specify positional identity along the anterior–posterior body axis that is independent of the process of segmentation.

MATERIALS AND METHODS

Fly Strains. The cloned genomic DNAs λDmF90-2 and λDmS67 were derived from a Canton S strain (21). Chromosome squashes (Fig. 1 *a* and *b*) were prepared from an Oregon R strain. The tissue sections shown in Fig. 4 were prepared from the following strains: Pc^3/TM1 (*a* and *b*); Scr Ns^{Rc3}/TM3, Sb, Ser (*c–f*).

Homeo Box Screen of Recombinant DNA Library. A total of 4×10^4 recombinants (approximately four genome equivalents) from the Charon 4/*Drosophila* DNA library of Maniatis *et al.* (21) was screened with ^{32}P-labeled (22) homeo box probes derived from the Sex combs reduced (*Scr*) (23) and fushi tarazu (*ftz*) (11) loci of the ANT-C. Nitrocellulose replicas of the Maniatis library were prepared by the method of Benton and Davis (24) and subsequently hybridized with the homeo box probes under reduced-stringency conditions (11). A total of 60 cross-hybridizing recombinant phage was plaque-purified.

***In Situ* Hybridization to Polytene Chromosomes.** Total DNA was extracted from each recombinant phage and biotinylated by nick-translation (25) with an analog of thymidine triphosphate, bio-16 dUTP (purchased from Enzo Biochemicals, New York). Biotinylated DNA was hybridized to salivary gland chromosome spreads as described (25). Hybridization was done at 58°C for 12–16 hr. Nonspecifically bound probe was removed by washing three times in 0.3 M NaCl/0.03 M sodium citrate at 53°C for 10 min each. Immunohistochemical detection of specifically bound probe was done with a strepavidin/biotin-treated peroxidase polymer and diaminobenzidine exactly as described by the supplier (Enzo Biochemicals).

Nucleotide Sequence Analysis. The 550-base-pair (bp) *Pst* I fragment from pBam-2.7 that contains the homeo box was inserted (26) in both orientations into *Pst* I-cut mp18 vector (27). The 450-bp *Bgl* II/*Eco*RI fragment from the S67 cDNA was inserted into *Eco*RI/*Bam*HI-cut mp18 vector. The 400-bp *Sac* I/*Pst* I fragment from pS67 was ligated into *Sac* I/*Pst* I-cut mp19; and the 350-bp *Bgl* II/*Pst* I fragment was inserted into *Bam*HI/*Pst* I cut mp19. DNA was sequenced by the Sanger dideoxy method (28).

***In Situ* Hybridization to Tissue Sections.** *In situ* hybridizations to embryo tissue sections were done as described (29). Homeo-box-containing DNA fragments were used as probes

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Abbreviations: CNS, central nervous system; PMG, posterior midgut; ANT-C, antennapedia gene complex; BX-C, bithorax gene complex; EN-C, engrailed gene complex; *Abd-B*, abdominal B locus; kb, kilobase(s); bp, base pair(s).

for localizing transcripts within tissue sections: for λDmF90-2, the 2.7-kb *Bam*HI fragment (see Fig. 1*c*); for λDmS67, the 3.7-kb *Eco*RI/*Bam*HI fragment (see Fig. 1*d*); and for Abd-B, a cDNA sequence was used (C.W. and M.L., unpublished results; refs. 14 and 23). The probes were radiolabeled with tritiated deoxynucleotides by nick-translation to a specific activity of approximately 5×10^7 to 1×10^8 cpm/μg of DNA. Embryos were not fixed prior to sectioning. Frozen tissue sections were prepared as described (29). Autoradiographic exposures were for 2–6 wk.

RESULTS

In a search for homeo box sequences not previously characterized, we screened a *Drosophila* DNA library for clones that cross-hybridize with the fushi tarazu (*ftz*) and sex combs reduced (*Scr*) homeo box probes. From a screen of approximately four genome equivalents, a total of 60 cross-hybridizing clones were plaque-purified. On the basis of high-stringency hybridizations with the previously isolated homeo box genes, a total of six clones were found to contain homeo box sequences not previously identified (ref. 30; H. Radomska, C.W., H.J.D., and M.L., unpublished results). In this report we characterize two of the clones, called λDmF90-2 and λDmS67.

The cytogenetic map locations of λDmF90-2 and λDmS67 were determined by *in situ* hybridization to salivary gland chromosome spreads. Fig. 1 shows photomicrographs of polytene chromosomes after hybridization with λDmF90-2 (Fig. 1*a*) and λDmS67 (Fig. 1*b*). λDmF90-2 maps to the 84A1,2 region on the right arm of chromosome 3, and λDmS67 maps to the 38E/F region on the left arm of chromosome 2.

Evidence that *F90-2* and *S67* contain copies of the homeo box was obtained by nucleotide sequence analysis. The nucleotide sequence of the *F90-2* homeo box is shown in Fig. 2. On the basis of the locations of stop codons and the occurrence of highly conserved donor and acceptor splice sequences, it appears that the *F90-2* homeo box is interrupted by an intervening sequence between codons 44 and 45. Removal of the putative intron, as indicated by the large arrows in Fig. 2, would result in a processed homeo box that contains 64% nucleotide and 67% amino acid homology with the *Antp* homeo box sequence. Several homeo box cross-hybridizing restriction fragments from the pS67 cDNA were also sequenced (data not shown). Comparison of the *S67* and *Antp* homeo boxes revealed that these share 61% nucleotide and 54% amino acid homology.

The tissue distribution of RNAs homologous to F90-2 was determined by *in situ* hybridization using the 2.7-kb *Bam*HI fragment as a hybridization probe (see Fig. 1*c*). This probe detects a single 2.8-kb transcript in advanced-stage embryos (Fig. 3).

A tissue autoradiogram of a 12- to 14-hr embryo after hybridization with the *F90-2* probe shows that one of the sites of labeling corresponds to a region within the central nervous system (CNS) that is just posterior to the brain (Fig. 4 *a* and *b*). In addition to the CNS, F90-2 transcripts were detected in epithelial cells of the midgut. Intense labeling was seen throughout the middle midgut, whereas substantially weaker hybridization signals are present within portions of the

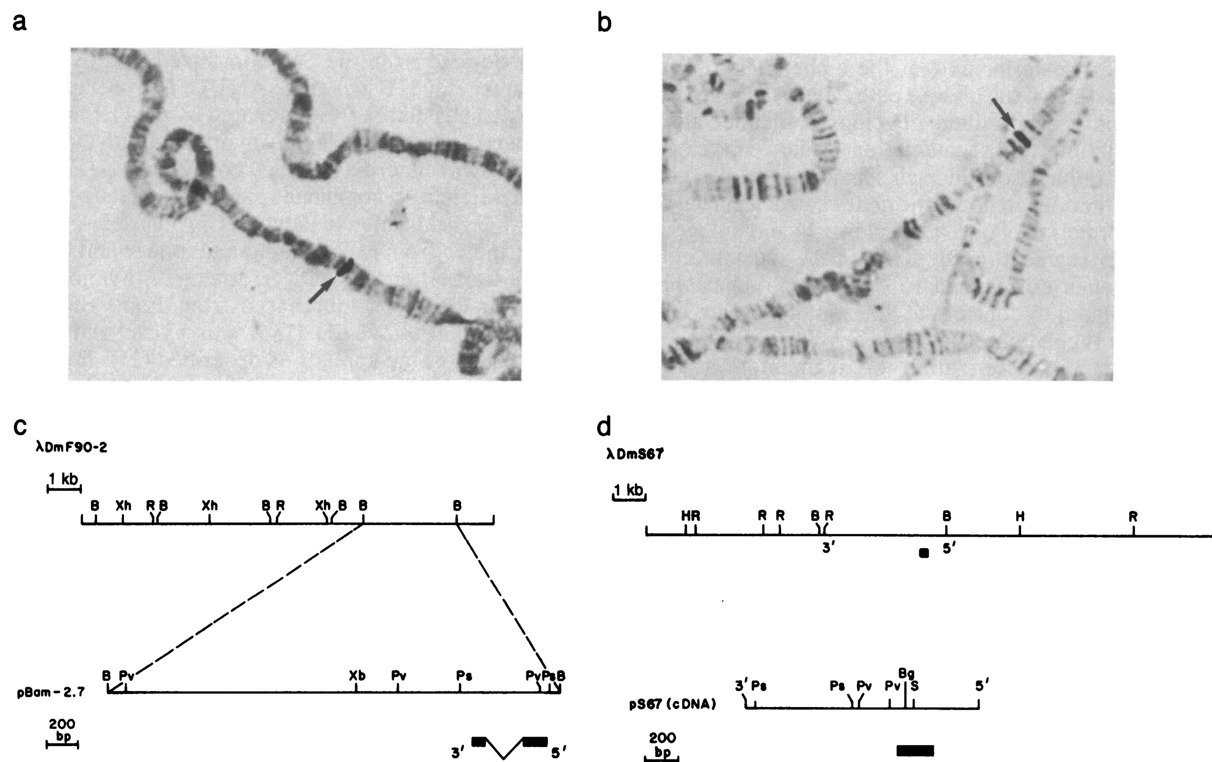

FIG. 1. Cytogenetic locations and restriction maps of λDmF90-2 and λDmS67. (*a* and *b*) Photomicrographs of salivary gland chromosomes after hybridization with biotinylated λDmF90-2 and λDmS67 DNA probes, respectively. The arrows indicate the cytogenetic sites of hybridization: λDmF90-2 maps to location 84A1,2 on the right arm of chromosome 3 and λDmS67 maps to 38E/F on the left arm of chromosome 2. (*c* and *d*) Restriction maps of λDmF90-2 and λDmS67, respectively. The *Drosophila* genomic DNA insert of λDmF90-2 is ≈12 kb in length, and λDmS67 is ≈16.5 kb. A more detailed restriction map of a 2.7-kb *Bam*HI fragment from λDmF90-2 is also shown in *c*. This fragment was used as an *in situ* probe for transcript distribution studies in tissue sections (see Fig. 4). A restriction map of a λDmS67 cDNA, pS67, is shown in *d*. pS67 was isolated from an unfertilized egg cDNA library that was provided by B. Yedvobnick. The positions of homeo box cross-homology are indicated by black boxes. The direction of transcription is based on the orientation of the homeo box sequence within the cloned DNA. The position of a putative intron within the *F90-2* homeo box is indicated below the pBam-2.7 map (see Fig. 2). Restriction endonucleases used: R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Xh, *Xho* I; Ps, *Pst* I; Pv, *Pvu* II; Xb, *Xba* I; Bg, *Bgl* II.

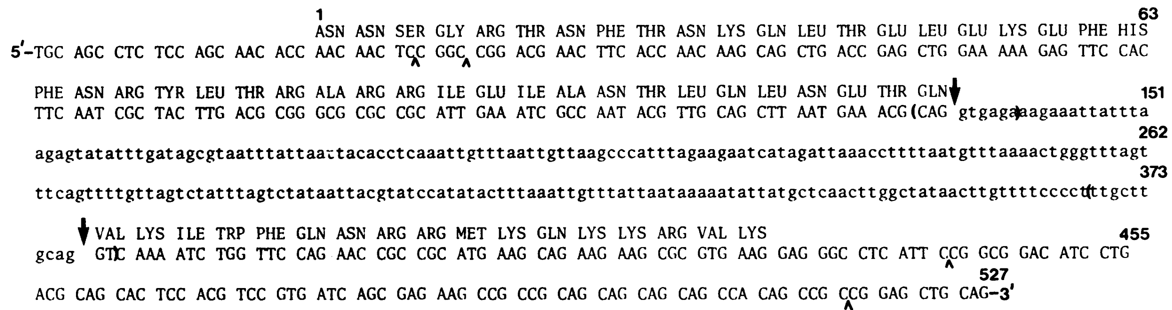

FIG. 2. Nucleotide and amino acid sequences of the F90-2 homeo box. The entire nucleotide sequence of the 550-bp *Pst* I fragment that contains the homeo box is shown (see Fig. 1*c*). The putative intron/exon boundaries are indicated by large arrows. The consensus sequences (31) for splice donor ($^{C}_{A}$-A-G↓G-T-$^{A}_{G}$-A-G-T) and splice acceptor (Y-Y-Y-Y-Y-Y-N-$^{C}_{T}$-A-G↓G-G, in which Y is an unspecified pyrimidine nucleoside and N is an unspecified nucleoside) are highly conserved (indicated by brackets). Sequences that correspond to the putative exons are shown in upper case letters; the 245-bp intron is indicated by lower case letters. There are stop codons in all three reading frames of the putative intron. The first nucleotide of the *F90-2* homeo box is labeled "1." The arrowheads at positions 8/9, 12/13, 441/442, and 516/517 are *Hpa* II restriction sites.

posterior midgut. Both the middle midgut and PMG arise from the PMG rudiment of gastrulating embryos (32, 33). The PMG is established by the invagination of blastoderm cells that reside near the posterior pole. In contrast, the region of the CNS where *F90-2* is expressed originates from blastoderm cells near the anterior pole of the embryo. Thus, *F90-2* is expressed in tissues that arise from primordia near opposite ends of the embryo (see Fig. 5).

A tissue autoradiogram of an advanced-stage (16–18 hr) embryo after hybridization with the S67 cDNA probe is shown in Fig. 4 *e* and *f*. The primary site of hybridization corresponds to the malpighian tubules. Weaker signals are detected over portions of the posterior midgut and hindgut. The malpighian tubules derive from a region of the blastoderm that is just posterior to the hindgut rudiment and anterior to the blastomeres that become invaginated during formation of the PMG (32, 33). Thus, the primary site of *S67* expression corresponds to tissues that derive from a region of the blastoderm that is just anterior to the domain of *F90-2* expression in the PMG.

F90-2 and S67 RNAs accumulate in tissues that derive from regions near the posterior pole of the embryo. Of the known homeotic loci that contain a homeo box, the abdominal B locus (*Abd-B*) of the BX-C acts on the posterior-most regions of the embryo (14, 19, 23, 34). In order to determine the

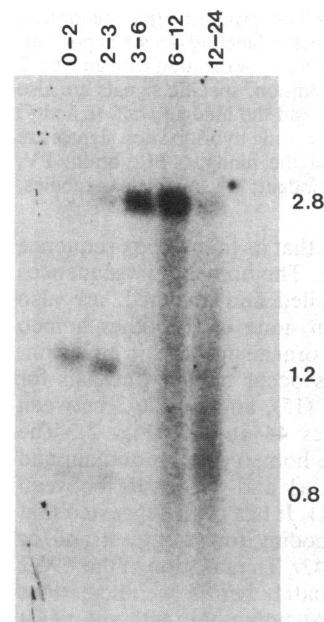

FIG. 3. RNA blot analysis of F90-2 transcripts. Transcripts 2.8, 1.2, and ≈0.8 kb long hybridize with the 2.7-kb *Bam*HI fragment from the F90-2 clone. The hybridization probe was ^{32}P-labeled by nick-translation. Aliquots containing 2 μg of poly(A)$^{+}$ RNA from each of the indicated embryonic stages (in hours after fertilization) were electrophoresed in an agarose/formaldehyde gel and transferred to nitrocellulose.

spatial limits of *F90-2* and *S67* expression relative to *Abd-B*, tissue sections of advanced-stage embryos were hybridized with an Abd-B cDNA probe (Fig. 4 *c* and *d*). As previously shown, Abd-B transcripts are detected in posterior regions of the ventral nerve cord, including the fourth (A4) through ninth (A9) abdominal ganglia (23, 35). In addition, Abd-B RNAs are also found in the visceral musculature surrounding the hindgut as well as in some of the cells that comprise the hindgut wall. The hindgut and associated visceral musculature derive from regions of the blastoderm that are just anterior to the anlage for the malpighian tubules (33), which is the primary site of *S67* expression.

The distribution of F90-2 transcripts in the anterior and posterior regions of advanced-stage embryos (see Fig. 4 *a* and *b*) was also detected in younger embryos (data not shown). It is surprising that *F90-2* is expressed in tissues that derive from opposite ends of the embryo. To determine whether one site of labeling resulted from hybridization to a linked transcript encoded by the 2.7-kb *Bam*HI probe (see Fig. 1*c*), tissue hybridizations were done with a smaller 0.43-kb *Hpa* II probe containing most of the homeo box sequence (see Fig. 2). The 0.43-kb *Hpa* II probe that was used is completely contained within the sequenced 550-bp *Pst* I fragment. This smaller probe also showed both anterior and posterior sites of hybridization. In addition, a cDNA homologous to F90-2 showed the same *in situ* hybridization pattern as the genomic probes (data not shown). We propose that the transcripts detected at both sites derive from the same transcription unit. It is not known whether the transcripts encode the same product or if the products have the same role in the development of the two poles. One implication of this result is that the poles of the developing embryo might contain similar positional information or common developmental cues.

DISCUSSION

We have described the molecular cloning and transcript distribution patterns of two homeo box genes, *S67* and *F90-2*. The tissues in which these genes are expressed derive from regions along the anterior–posterior body axis of the embryo that are outside the limits of known ANT-C and BX-C homeotic loci (23, 29, 36, 40). S67 RNAs accumulate primarily in the malpighian tubules, which arise from embryonic primordia that are just posterior to the domain of *Abd-B* expression (see Fig. 5; refs. 14, 23, and 35). F90-2 transcripts are detected in regions of the midgut that derive from the posterior-most portions of the PMG invagination. Fig. 5 summarizes the RNA accumulation patterns for F90-2 and S67 and relates these to the limits of ANT-C and BX-C gene expression. The posterior regions of the embryo give rise to the midgut, malpighian tubules, and the hindgut/procto-

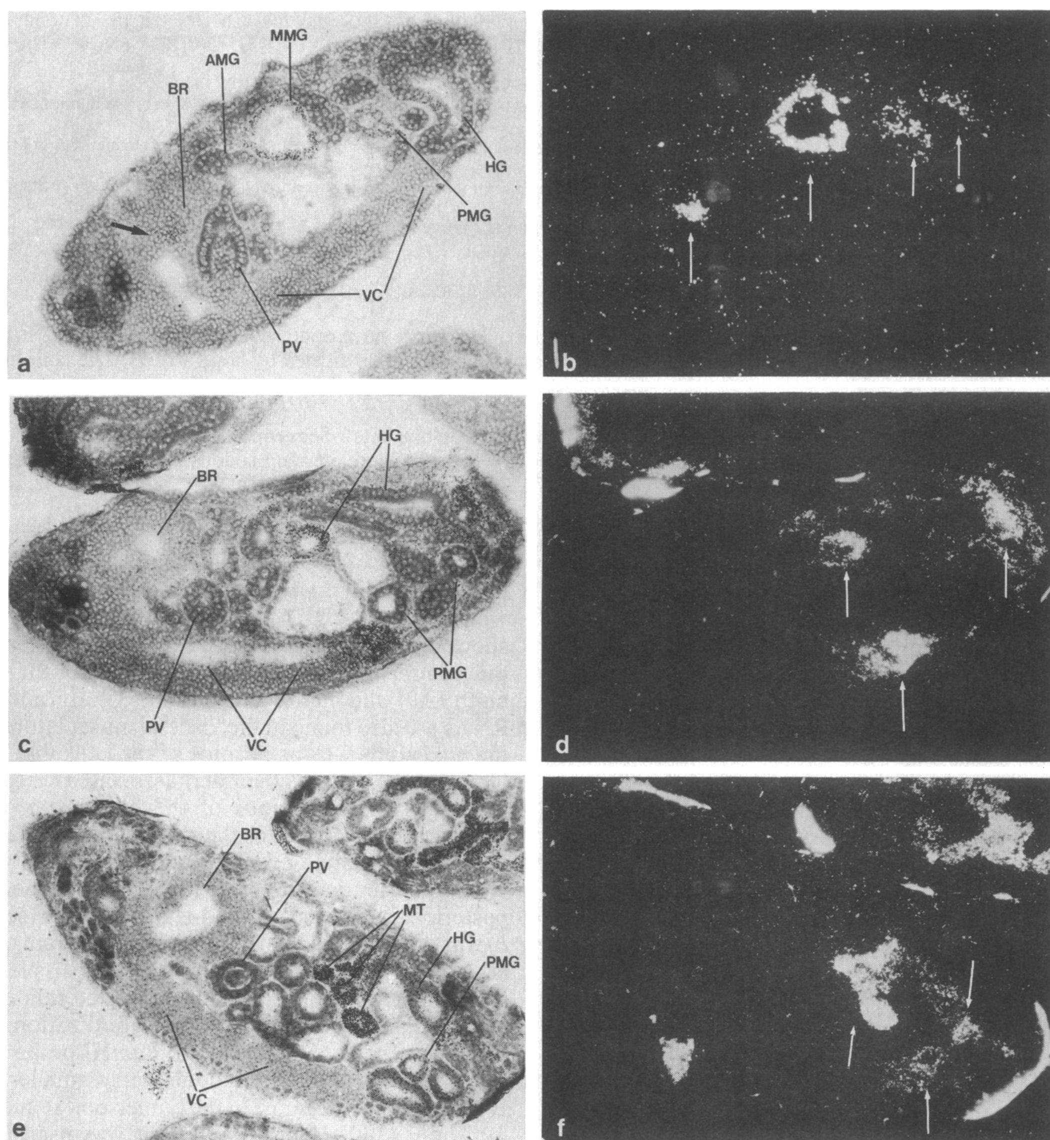

FIG. 4. Localization of F90-2, S67 and Abd-B RNAs within embryo tissue sections. All sections are sagittal and oriented so that anterior is to the left and dorsal is up. (*a* and *b*) Bright- and dark-field photomicrographs of a 12- to 14-hr embryo after hybridization with the *F90-2* probe. The arrow indicates specific hybridization to an anterior region of the CNS. This labeling appears to be located just anterior to the brain. However, during 9–10 hr after fertilization, head involution results in the posterior displacement of the brain so that the subesophageal ganglia become the anterior-most regions of the CNS. The portion of the CNS that is labeled by the *F90-2* probe derives from primordia that are initially located posterior to the brain rudiment (32). Strong labeling is also seen to a region of the middle midgut, and weaker labeling is seen in portions of the posterior midgut and hindgut. (*c* and *d*) Bright- and dark-field photomicrographs of a 14- to 16-hr embryo after hybridization with an Abd-B cDNA probe. Strong hybridization signals are seen in the A4→A8/A9 ganglia of the ventral nerve cord. In addition, specific signals are also detected over regions of the posterior spiracle, the visceral musculature of the hindgut and posterior midgut, and the hindgut wall. (*e* and *f*) Bright- and dark-field photomicrographs of a 16- to 18-hr embryo after hybridization with an S67 cDNA probe. Strong hybridization signals are seen over the malpighian tubules. Weaker signals are detected over regions of the posterior midgut and the hindgut. BR, brain; PV, proventriculus; VC, ventral cord; AMG, anterior midgut; MMG, middle midgut; PMG, posterior midgut; HG, hindgut; MT, malpighian tubules.

deum. Each of these larval tissues shows expression of a different homeo box-containing gene: *F90-2* is expressed in the midgut, *S67* in the malpighian tubules and *Abd-B* in the hindgut/proctodeum.

The pattern of *F90-2* expression is complicated because it includes both anterior and posterior regions of the embryo. F90-2 RNAs are detected in a posterior region of the brain, in addition to derivatives of the PMG. The site of CNS labeling is just anterior to the domain of *Dfd* expression (see Fig. 5). Since *Dfd* is expressed in the neural and hypodermal tissues of the mandibular and maxillary head segments (refs. 23 and 35; W. McGinnis, personal communication), it appears that the anterior domain of *F90-2* expression corresponds to a site anterior to the mandibular ganglion, possibly the clypeolabrum.

An unusual feature of *F90-2* is that its homeo box sequence appears to be split by an intron. The homeo box sequences of the two EN-C genes, engrailed and invected, are also interrupted by introns (15), but none of the other homeo boxes from *Drosophila* or other organisms contain an intron. The introns in the EN-C genes occur between codons for amino acid residues 18 and 19 (15), and in *F90-2* between codons for amino acid residues 44 and 45 (Fig. 2). The greatest homology between the homeo protein domain and the yeast mating-type proteins a1 and α2 occurs between amino acid residues 44 to 57 (41). It has been suggested that introns occur between exons coding for structural and/or functional domains in proteins (42). The location of the F90-2 splice junction, which is immediately before the nucleotides encoding the domain of greatest homology with the yeast

mating type proteins, could correspond to a structural or functional domain within the homeo box.

It is uncertain whether the products of *F90-2* and *S67* are critically required for the proper morphogenesis of the tissues in which they are found. However, for *F90-2* the cytogenetic mapping and transcript localization data are consistent with a role as an anterior "head-forming" homeotic gene. There is a colinear correspondence between the chromosome order of homeotic loci that contain a homeo box within the ANT-C and BX-C, and the embryonic segments along the body axis of the fly that accumulate transcripts encoded by these loci (7, 8, 23, 34). ANT-C homeotic loci near the centromere (i.e., *Dfd* and *Scr*) act on anterior body segments, whereas telomeric BX-C loci (i.e., *abd-A* and *Abd-B*) act on posterior body segments (see Fig. 5). There is some genetic evidence for additional homeotic functions that act on embryonic segments that are anterior to the primary domain of *Dfd* expression (17, 18). By analogy with the previously characterized homeotic loci, it is possible that such anterior "head" functions reside within regions of the ANT-C that are proximal (closer to the centromere) to the *Dfd* locus. The ANT-C maps within the 84A1,2–84B1,2 region of chromosome 3 (8), and *F90-2*, to 84A1,2. Therefore, *F90-2* appears to reside within a region of the ANT-C that is proximal to *Dfd*. Moreover, F90-2 RNAs are detected in embryonic tissues that derive from a region along the body axis that is just anterior to the domain of *Dfd* expression (see Fig. 5). More definitive evidence that *F90-2* might correspond to an anterior "head-forming" gene will require genetic analyses.

There are at least 15 copies of the homeo box sequence in the *Drosophila* genome (ref. 30; W. McGinnis, personal communication), nine of which have been previously shown to be contained within well-defined genes or gene complexes that are known to control some aspect of segment morphogenesis. On this basis, and because the homeo box is evolutionarily conserved, it has been proposed that the homeo box somehow controls segment morphogenesis in a variety of higher metazoans, including vertebrates (12). In this report we have shown that at least two homeo box genes are expressed in regions of the embryo that do not appear to be segmented. Thus, the homeo box might specify positional identity that is independent of the process of segmentation. A common feature of the 11 *Drosophila* homeo box genes that have been characterized so far is that each shows a unique pattern of expression along the anterior–posterior body axis

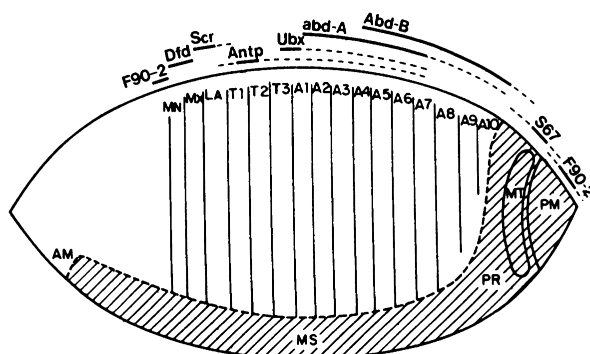

FIG. 5. Sites of *F90-2*, *S67*, ANT-C, and BX-C gene expression in an embryonic fate map. The fate map shows the origins of various embryonic tissues at the blastoderm stage of development. The solid lines above the map show the primary domains of expression for each of the eight homeo box genes (23, 29, 35–40). The dashed lines indicate sites of weaker expression. The map is adapted from Hartenstein *et al.* (33) and is oriented so that dorsal is up and anterior is to the left. MS, mesoderm; MT, malpighian tubules; PM, posterior midgut; PR, proctodeum; MN, mandibular head segment; MX, maxillary segment; LA, labium; T1-T3, pro-, meso-, and metathorax; A1–A10, abdominal segments.

during embryogenesis. An additional implication of these observations is that homeo box genes might be involved in the elaboration of anterior-posterior positional identity in organisms which are not overtly segmented.

Note Added in Proof. On the basis of its cytogenetic map location, restriction map, and nucleotide sequence, S67 corresponds to a previously isolated homeo box gene called caudal (43).

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