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Isolation of two tissue-specific *Drosophila* paired box genes, *Pox meso* and *Pox neuro*

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Two new paired domain genes of *Drosophila*, *Pox meso* and *Pox neuro*, are described. In contrast to the previously isolated paired domain genes, *paired* and *gooseberry*, which contain both a paired and a homeodomain (*PHox* genes), *Pox meso* and *Pox neuro* possess no homeodomain. Evidence suggesting that the new genes encode tissue-specific transcriptional factors and belong to the same regulatory cascade as the other paired domain genes includes (i) tissue-specific expression of *Pox meso* in the somatic mesoderm and of *Pox neuro* in the central and peripheral nervous system, (ii) nuclear localization of their proteins, (iii) dependence on *prd* activity and (iv) presence of the paired domain in genes of known regulatory activity. While no mutant phenotypes of *Pox meso* and *Pox neuro* have yet been discovered, a murine gene with a paired domain closely homologous to that of *Pox meso* has recently been identified with the *undulated* mutant. Both *Pox meso* and *undulated* are expressed in tissues derived from the somatic mesoderm. The five known *Drosophila* paired domains fall into three classes: (i) the *prd,gsb*-class, (ii) the *Pox meso*, *undulated*-class and (iii) the *Pox neuro*-class which probably includes the paired domain of the murine gene *Pax 2*.

Key words: Drosophila paired box genes/evolution/gene network concept/mesodermal and neural specific genes

Introduction

Based on evolutionary considerations, we have postulated the gene network concept (Bopp *et al.*, 1986; Frigerio *et al.*, 1986). It assumes that genes consist of 'domains', encoding protein or RNA as well as *cis*-regulatory elements, which assort independently and are used preferentially within the same functional gene network during evolution. Consequently, genes of the same network share a relatively small number of domains that are specific for the particular network, a property that suggests a simple approach to isolate and identify these genes.

To test our concept, we set out to isolate genes that share homologous domains with the *paired* (*prd*) gene of *Drosophila*, a gene belonging to the network of genes that control the progressive subdivision of the early embryo along its antero-posterior axis (Nüsslein-Volhard and Wieschaus, 1980). Of the 15 genes so isolated (Bopp *et al.*, 1986; Frigerio *et al.*, 1986), three were known from genetic studies to belong to the same network as *prd*. The first of these was isolated by hybridization to a *prd* domain consisting essentially of a His-Pro repeat (PRD repeat). Transcripts of this gene accumulate at the anterior pole of developing oocytes and are redistributed during early embryogenesis to form a gradient in the anterior half of the embryo at syncytial blastoderm (Frigerio *et al.*, 1986). This gene was identified with *bicoid* (*bcd*) (Berleth *et al.*, 1988), a maternal gene providing the initial positional cues in the anterior half of the embryo (Frohnhöfer and Nüsslein-Volhard, 1986) and hence belonging to the class of maternal co-ordinate genes (Nüsslein-Volhard and Wieschaus, 1980). Sequence comparison of *prd* and *bcd* revealed an additional domain shared by the two genes, namely two considerably diverged homeodomains, each representing a different class (Frigerio *et al.*, 1986).

The other genes of known phenotype isolated by this approach were shown to belong to the *gooseberry* locus. Surprisingly, this locus was represented by two transcriptional units of opposite polarity (Bopp *et al.*, 1986). Both of these transcriptional units had been isolated as a result of our systematic search by hybridization to a *prd* DNA fragment later found to contain the new type of *prd* homeodomain (Bopp *et al.*, 1986). As *gsb* belongs to the segment-polarity class of segmentation genes (Nüsslein-Volhard and Wieschaus, 1980), these two genes also belong to the same network as *prd*. In addition to the *prd*-type homeodomain, the two *gsb* genes share a considerably larger domain of 129 amino acids with *prd*, termed 'paired domain' (Bopp *et al.*, 1986; Baumgartner *et al.*, 1987).

If domains recombine independently during evolution, one would expect that genes exist in which both the paired domain as well as the homeodomain of the *prd*-type are not associated with each other as in *gsb* and *prd*. Indeed the present study describes two paired box genes of *Drosophila*, *Pox meso* and *Pox neuro*, lacking a paired domain, while the demonstration of genes containing a *prd* homeodomain but no paired domain will be the subject of a future study (K.Schneitz *et al.*, in preparation). *Pox meso* and *Pox neuro* are expressed in a tissue-specific, segmentally repeated pattern, beginning at germ band extension. The experiments described in this paper suggest that both of these genes belong to the same network as *prd* and probably encode tissue-specific transcriptional factors. The two new paired domains of *Pox meso* and *Pox neuro* deviate at positions characteristically conserved in the *prd,gsb*-paired domains and hence form separate types of paired domains, thus con-

firming our previous conclusions derived from comparison of their sequences to those of three human paired domains (Burri *et al.*, 1989).

Unfortunately, no mutant phenotypes have yet been found for *Pox meso* and *Pox neuro*. However, the murine gene *Pax 1* has recently been isolated by hybridization to a *Drosophila* paired box probe (Deutsch *et al.*, 1988) and identified with the phenotype of the *undulated* (*un*) mutant (Balling *et al.*, 1988). It thus represents the rare case in which the phenotype of a vertebrate gene is known before its *Drosophila* homologue. Analogous to *Pox meso* described here, *Pax 1* is expressed in a segmentally repeated pattern in mesodermally derived tissues. The similarity in expression patterns of the two genes has its counterpart in the high degree (90%) of sequence homology of their predicted paired domains. Together these observations suggest that *Pox meso* and *Pax 1* share conserved developmental roles in evolution, in agreement with our gene network concept (Bopp *et al.*, 1986; Frigerio *et al.*, 1986; Burri *et al.*, 1989).

Results

Isolation of two paired domain genes lacking a homeodomain

Paired box probes of the *paired* gene, P_{prd}, and of the two *gsb* genes, P_{BSH9} and P_{BSH4} (Bopp *et al.*, 1986), were used to screen a *Drosophila* genomic library at reduced stringency of hybridization in order to search for genes with paired domains not associated with a homeodomain. Several clones were isolated, which were negative after hybridization with probes flanking the *prd* and *gsb* genes. All inserts of the cloned phage DNAs were derived from two different chromosomal regions which we name the *Pox meso* and *Pox neuro* loci. Figure 1 shows representative sets of overlappping clones from these two loci: P29 and P20 from the *Pox meso* (P29) locus; P4, P35 and P421 from the *Pox neuro* (P4) locus. An additional phage, P29B1.4, was isolated by chromosomal walking to extend the upstream region of the *Pox meso* locus (Figure 1a). The transcriptional organization of the *Pox meso* and *Pox neuro* genes is depicted in Figure 1 and was derived from the analysis of isolated cDNAs and the corresponding genomic DNA sequences as well as from that of Northern blots hybridized to strand-specific RNA probes.

DNA sequence analysis of genomic and cDNA clones allowed us to assess whether the homologies of *Pox meso* and *Pox neuro* with the paired boxes of *prd* and *gsb* were relevant. The longest open reading frames, determined from nearly full-length cDNAs of *Pox meso* and *Pox neuro*, confirmed that both genes encoded proteins with a paired domain located close to the amino-terminal end as in the *prd* and two *gsb* proteins (Figure 2). In addition, translation of the entire open reading frame of *Pox meso* and *Pox neuro* revealed no homeodomain and thus proved that paired domains are not always combined with homeodomains as in *prd* and *gsb* (Bopp *et al.*, 1986).

Three paired domain classes

The DNA and amino acid sequences of five known paired domains of *Drosophila* are shown in Figure 2. As is evident from Table I, the paired domains of *prd* and the two *gsb* genes exhibit a higher degree of homology with each other (~85%) than with those of *Pox meso* and *Pox neuro* (~70–75%). Hence we might consider the paired domains

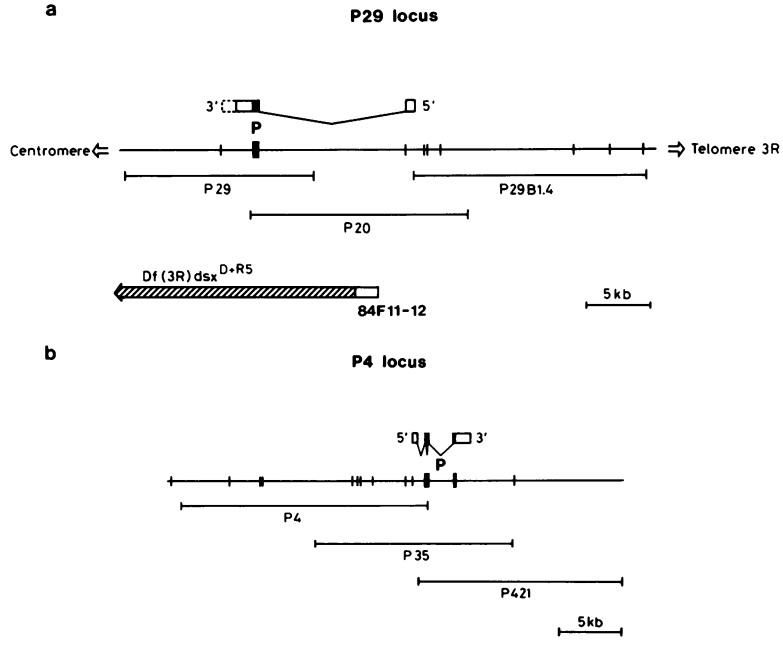

Fig. 1. Maps of the paired box genes *Pox meso* and *Pox neuro*. (**a**) Isolated genomic clones and transcriptional organization of the *Pox meso* (P29) locus at chromosomal bands 84F11-12. The inserts (in EMBL4) of three isolated genomic clones (P29, P20, P29B1.4) are shown below the genomic *Eco*RI restriction map. The direction, extent and exon structure of the *Pox meso* transcript is illustrated above. Two different extents of the 3′ exon are indicated by the solid and dashed line and correspond to two cDNAs differing only in the lengths of their trailer sequences. Their longest open reading frames (370 amino acids) are identical and encode a paired domain P close to the amino-terminal end. The distal breakpoint of the deficiency $Df(3R)dsx^{D+R5}$ (Duncan and Kaufman, 1975) has been mapped to within the region of chromosomal bands 84F11-12 delimited by the open bar at the end of the hatched arrow. (**b**) Isolated genomic clones and transcriptional organization of the *Pox neuro* (P4) locus at chromosomal bands 52C9-D3. Above the *Eco*RI restriction map of the cloned genomic region [represented by three overlapping inserts (P4, P35, P421) in EMBL4], the direction, extent and exon structure of the *Pox neuro* transcript is shown. Its longest open reading frame encodes 425 amino acids comprising the paired domain close to its amino-terminal. The position of the paired box, which is interrupted by two introns, is indicated by filled bars labeled P.

of *prd* and *gsb* to belong to a distinct class of paired domains. In support of this notion is also the observation that the paired domains of *Pox meso* and *Pox neuro* deviate at a number of positions from *prd* and *gsb* by non-conservative amino acid changes (Figure 2b). Since at many of these locations *Pox meso* and *Pox neuro* paired domains differ from each other, they again might represent each a separate class.

Class-specific amino acids appear at a number of positions (boxed in Figure 2b). Some are strictly specific for a single type of paired domain (at positions 4, 31, 55, 58, 67, 79, 85) while others are common to two types and differ only in one type if we disregard conservative amino acid changes (*prd,gsb*-type differs at positions 29, 38, 81, 96, 126; *Pox meso* at 1, 2, 22, 64, 83; *Pox neuro* at 20, 44, 73, 86, 92, 102, 104, 107, 121). At a few positions a deviation of one gene from the two other genes of the *prd,gsb*-class is found also in *Pox meso* (at positions 76, 82, 89, 94, 127) or *Pox neuro* (at amino acids 24, 90, 91, 94, 127) or is specific for *prd* (amino acids 87 and 119) or one of the two *gsb* genes (25, 93, 101, 106, 108, 109, 117). Amino acids appear to be freely variable only at very few positions (amino acids at position 32 and perhaps at 21 and 105).

Paired domains have also been found in mouse (Balling *et al.*, 1988; Deutsch *et al.*, 1988) and man (Burri *et al.*, 1989) and apparently fall into the same three classes. Two human paired domains belong to the *prd,gsb*-type, while a third human gene, *HuP48*, and the *Pax 1* gene of the mouse, encode paired domains that belong to the *Pox meso*-type (Burri *et al.*, 1989). Moreover, the first 29 known amino acids of the paired domains of the mouse *Pax 2* and *Pax 3* genes (Balling *et al.*, 1988) indicate that *Pax 2* has a *Pox neuro*-type (Gly_4, Asp_{20}, Val_{22}, Leu_{29}) and *Pax 3* a *prd, gsb*-type paired domain (Arg_4, Asn_{20}, Ile_{22}, Met_{29}).

A program that increases the accuracy of secondary structure predictions of the Garnier–Osguthorpe–Robson method by averaging the predicted values for five *Drosophila* and three human paired domains (Crawford *et al.*, 1987; T.Niermann, personal communication), strongly suggests that paired domains contain at least three α-helices (Burri *et al.*, 1989). The first α-helix extends from amino acids 23–31 while the second and third α-helices are predicted within a helix–turn–helix motif between positions 80 and 105 (Figure 2b). The first two α-helices are highly amphipathic. It is striking that a cluster of class- or domain-specific amino acids is located in the first α-helix of the helix–turn–helix region. In contrast, the second α-helix (Trp-Glu-Ile-Arg-Asp or WEIRD-helix; Burri *et al.*, 1989) of the helix–turn–helix motif is conserved. Since the homeodomain requires a helix–turn–helix region containing a variable and a conserved α-helix for DNA recognition (for a recent review, see Scott *et al.*, 1988), it is attractive to speculate that this region of the paired domain serves a similar function. Accordingly, the variable first helix in the helix–turn–helix region of the paired domain might indicate differences in its specificity for DNA recognition and hence correspond to the second helix of the helix–turn–helix region in the homeodomain. It might be significant that the first α-helix of the paired domain (amino acids 23–31) is homologous to the first α-helix of the helix–turn–helix region (amino acids 28–38) in the homeodomain (up to seven conserved amino acids).

All five known *Drosophila* paired boxes are separated by an intron from the preceding exon encoding only a short stretch of amino acids (<22) at the amino-terminal end of the corresponding protein (Figure 2b; Frigerio *et al.*, 1986; Baumgartner *et al.*, 1987). The *gsb-BSH4* and the *Pox neuro* gene have an intron also within their paired domain (Figure 2b). In *BSH4* this intron is located immediately after the region encoding the helix–turn–helix motif while in *Pox neuro* it precedes the motif. A separation of the helix–turn–helix structure from the remaining paired domain by two bordering introns is also evident in the two human genes, *HuP1* and *HuP2* (Burri *et al.*, 1989). Such introns may plausibly be understood as remnants of the evolutionary process that gave rise to the paired domain. The observation that the 74 N-terminal amino acids exhibit a considerably higher degree of conservation than the 55 C-terminal amino acids of the paired domain (80–82% versus 51–69% homology between different classes if conservative amino acid changes are ignored, cf. Table I) might also suggest an independent function and separate origin of these two regions in the distant past. Consistent with this view are a deletion of amino acids 111–113 in the *gsb-BSH4* paired domain and an insertion of a Gln after the first 74 amino acids of the *Pox neuro* paired domain (Figure 2b). Apparently, each of these changes has been tolerated by evolution because neither change interrupts the helix–turn–helix motif nor the more highly conserved portion comprising the first 74 amino acids of the paired domain.

Table I. Matrix of amino acid homologies between five *Drosophila* paired domains.

		prd	BSH9	BSH4	P29
	prd				
prd, gsb class	BSH9	0.81 . 0.92 (0.87) . (0.95)			
	BSH4	0.78 . 0.95 (0.85) . (0.97)	0.79 . 0.93 (0.85) . (0.96)		
	P29	0.65 . 0.78 (0.76) . (0.81)	0.70 . 0.77 (0.75) . (0.80)	0.64 . 0.76 (0.72) . (0.81)	
	P4	0.60 . 0.73 (0.71) . (0.82)	0.64 . 0.76 (0.71) . (0.82)	0.60 . 0.74 (0.69) . (0.82)	0.66 . 0.76 (0.71) . (0.80)

The first value of the upper line indicates the fraction of identical amino acids between two paired domains (amino acids 1–129), the second value shows the corresponding fraction for the more highly conserved first 74 amino acids of the paired domain. The values in parentheses underneath represent corresponding fractions of amino acid homologies if conservative changes are neglected (cf. legend to Figure 2). The values comparing paired domains of the *prd,gsb*-class among each other (enclosed by a triangle) are consistently higher than those of paired domains belonging to different classes. P29 is *Pox meso*, P4 is *Pox neuro*.

Segmental repeat and tissue specificity of Pox meso and Pox neuro expression

In situ hybridization to salivary gland chromosomes revealed that *Pox meso* and *Pox neuro* were located at chromosomal bands 84F-95A and 52C-D respectively. These regions were narrowed down to 84F11-12 and 52C9-D3 by hybridizations to polytene chromosomes carrying deficiencies flanking or comprising these regions. Southern blot analysis of the $Df(3R)dsx^{D+R5}$ (Duncan and Kaufman, 1975) chromosome showed that the distal breakpoint of its deficiency is located in the intron of the *Pox meso* gene as illustrated in Figure 1a. The *Pox neuro* gene is uncovered by the deficiency *Df(2R)WMG* deleting the region 52C4-E3 (W.Gelbart, personal communication) and was mapped more precisely

to a region between the distal breakpoint of *Df(2R)XTE-18* at 52C9-D1 and the proximal breakpoint of *Df(2R)KL-9* at 52D3, an interval which also contains the non-lethal gene encoding the mitochondrial enzyme α-glycerophosphate oxidase (Davis and MacIntyre, 1988). These two breakpoints map outside the region shown in Figure 1b.

If *Pox meso* and *Pox neuro* belong to the same gene network as *prd* and *gsb*, their mutant phenotypes might reveal a possible involvement of these genes in the process of segmentation. While this is clearly the case for the murine paired box gene *undulated* (Balling *et al.*, 1988), no such mutant phenotypes are known within the chromosomal regions to which *Pox meso* and *Pox neuro* have been mapped. Alternatively, functional integration of *Pox meso* and *Pox neuro* into the network of segmentation genes might manifest itself in a differential distribution of their transcripts or proteins along the antero-posterior axis.

As is evident from Figure 3(a)−(d), *Pox meso* as well as *Pox neuro* transcripts appear in a segmentally repeated pattern during the late stage of germ band elongation (stage 10; Campos-Ortega and Hartenstein, 1985). Transcripts of *Pox meso* are observed posterior to the parasegmental grooves (Martinez-Arias and Lawrence, 1985) in the posterior half of each segment and are restricted to the mesodermal germ layer (Figure 3a and b). Moreover, immunostaining of *Pox meso* protein in whole-mount embryos at the elongated germ band stage demonstrates that this protein is expressed in the somatopleura, giving rise to the somatic musculature, but that it appears to be absent from the splanchnopleura and mesectodermal cells (Figure 4a and E.Jamet, unpublished results). At later stages, *Pox meso* is clearly not expressed in the visceral mesoderm (not shown). Apart from the repetitive pattern of cells that express *Pox meso* in parasegments 3−14, groups of cells in the clypeolabrum (1), the cephalic mesoderm (2), and in the telson and proctodeal primordia (15 and 16 in Figure 3a and b; cf. also Figure 4a) also express *Pox meso*.

Transcripts of *Pox neuro* first appear in a few neuroblasts per segment and their progeny (Figure 3c and d). Evidently, the *Pox neuro* protein is expressed in a segmentally repeated pattern in neural precursors of the peripheral as well as central nervous system (Figure 4b). Expression of *Pox neuro* in the peripheral nervous system (PNS) has been confirmed by the absence of the gene product in homozygous *daughterless*$^-$ embryos in which development of the PNS is blocked (Caudy *et al.*, 1988). Clearly, *Pox neuro* expression appears in the developing CNS as well as PNS as early as ~5 h after fertilization. This finding is consistent with the recently reported temporal overlap of CNS and PNS development (Ghysen and O'Kane, 1989). The pattern of *Pox neuro* expression becomes more complicated as more neurons are generated. It appears, however, that cells expressing *Pox neuro* are clonally related. As expected from our deletion mapping, no *Pox neuro* expression is detectable in homozygous *Df(3R)WMG* embryos. A detailed analysis of *Pox neuro* and *Pox meso* expression patterns during development will be the subject of a future study.

We have shown earlier that *gsb-BSH9* transcripts appear in the posterior half of each primordial segment during germ band elongation and are in register along the antero-posterior axis with *prd* and *gsb-BSH4* transcripts (Baumgartner *et al.*, 1987). It thus appears that *Pox meso* is expressed in a subset

a

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          -30                                                  1                                                  31
prd       GGA TAT TCT ACG ATG CAA GAC ATG AAC AGC GGC CAG GGG CGC GTC AAT CAA CTA GGT GGA GTT
BSH9      ACA CCC TAC TTT GGC GGA TAT CCC TTT CAA GGA CAA GGT CGT GTC AAC CAG TTG GGT GGC GTC
BSH4      CGG CCC CTT TTC GCA GGG TAT CCC TTT CAA GGA CAA GGC CGG GTA AAT CAG CTT GGG GGC GTC
P29       GCC GAA ATG GAC CCA GAG TCG CAG TGT CCG CAG TAT GGC GAG GTG AAC CAG CTG GGC GGC GTC
P4        GTT GAT CAC ATT TCA GCC ATG CCG CAC ACA GGT CAA GCT GGA GTC AAC CAA TTG GGC GGA GTT

                                                      61                                                  91
prd       TTC ATC AAC GGT CGT CCT TTG CCC AAC AAT ATT CGT CTT AAA ATC GTC GAG ATG GCC GCC GAT
BSH9      TTC ATC AAC GGC CGT CCG TTG CCC AAT CAC ATC CGT CGC CAA ATC GTG GAG ATG GCA GCA GCT
BSH4      TTT ATC AAT GGA CGT CCG TTG CCC AAT CAC ATT CGA CTG AAG ATC GTG GAA ATG GCG GCC AGT
P29       TTT GTC AAC GGC CGT CCG CTG CCC AAT GCG ACC AGG ATG CGG ATC GTG GAG CTG GCC CGC CTG
P4        TTT GTG AAT GGC CGT CCT TTG CCG GAC TGC GTT CGT CGG AGG ATC GTC GAC TTG GCT TTG TGC

                                                     121                                                 151
prd       GGC ATT CGG CCC TGT GTG ATC TCC AGA CAG CTA CGT GTA TCC CAT GGC TGC GTA TCG AAG ATC
BSH9      GGA GTC CGT CCC TGT GTC ATC TCC CGC CAG CTG CGC GTC TCT CAT GGC TGC GTC TCA AAG ATT
BSH4      GGA GTG CGG CCT TGT GTA ATA TCG CGC CAG CTC CGC GTG TCT CAC GGC TGC GTA TCG AAG ATT
P29       GGC ATC CGA CCC TGC GAC ATA TCG CGC CAG CTG CGA GTG AGT CAC GGC TGC GTG TCC AAG ATC
P4        GGA GTG AGG CCC TGT GAT ATA TCC CGC CAG CTT CTG GTT TCC CAT GGC TGC GTT TCC AAA ATT

                                                     181                                                 211
prd       CTG AAT CGC TAC CAG GAG ACT GGC TCC ATT AGA CCA GGT GTG ATC GGT GGC TCC AAG CCG AGG
BSH9      CTA AAC CGC TTC CAG GAG ACT GGC TCC ATT CGG CCC GGA GTA ATC GGT GGC AGC AAG CCC CGT
BSH4      CTG AAC CGA TAC CAG GAG ACG GGT TCT ATT AGA CCG GGC GTA ATA GGT GGA TCT AAG CCC AAG
P29       CTG GCC AGG TAC CAC GAA ACG GGC TCC ATA CTA CCC GGC GCA ATT GGG GGA TCC AAG CCG CGC
P4        CTA ACT CGC TTC TAT GAG ACG GGC TCC ATT CGA CCG GGA TCC ATT GGC GGC AGC AAG ACC AAG

                                                     241                                                 271
prd           ATA GCC ACG CCC GAA ATC GAA AAC CGA ATT GAG GAG TAC AAG CGC AGT AGC CCG GGC ATG
BSH9          GTA GCC ACG CCA GAC ATT GAG TCC AGA ATC GAG GAA CTT AAA CAG TCG CAG CCC GGT ATT
BSH4          GTG ACC TCT CCC GAA ATT GAA ACG CGG ATC GAT GAG CTG CGA AAG GAA AAC CCC AGC ATA
P29           GTG ACC ACA CCC AAG GTG GTC AAC TAC ATC AGG GAA CTG AAG CAG CGA GAT CCC GGC ATC
P4        CAA GTG GCC ACG CCC ACC GTG GTG AAG AAG ATC ATC CGG CTA AAG GAG GAG AAC AGC GGC ATG

                                                     301                                                 331
prd       TTC TCG TGG GAG ATC AGG GAG AAG CTG ATC CGC GAG GGT GTC TGC GAC AGG AGC ACA GCA CCA
BSH9      TTT AGT TGG GAA ATC CGC GCC AAG CTA ATC GAA GCG GGA GTC TGC GAC AAG CAA AAT GCT CCG
BSH4      TTC AGC TGG GAA ATA CGC GAA AAG CTG ATA AAG GAG GGC TTT GCG GAT             CCA CCA
P29       TTT GCG TGG GAA ATC CGG GAC CGT TTG CTC AGC GAG GGC ATA TGC GAC AAA ACG AAT GTA CCC
P4        TTC GCG TGG GAA ATT CGC GAG CAG CTG CAG CAG CAG CGC GTC TGC GAT CCC AGT TCG GTG CCC

                                                     361
prd       TCT GTG TCC GCC ATA TCG CGC CTG GTG CGC GGC CGA GAT GCT CCA
BSH9      TCG GTG AGC TCT ATT TCG CGT CTT CTG CGA GGA TCC TCC GGA TCA
BSH4      TCA ACA TCG TCG ATC AGT CGC TTA TTG CGG GGA AGC GAT CGC GGC
P29       AGT GTG AGC TCC ATA TCG AGA ATC CTG CGC AAC AAG TTG GGC AGC
P4        TCG ATC AGC TCC ATC AAC CGG ATT CTG CGC AAC AGC GGT CTG TGG
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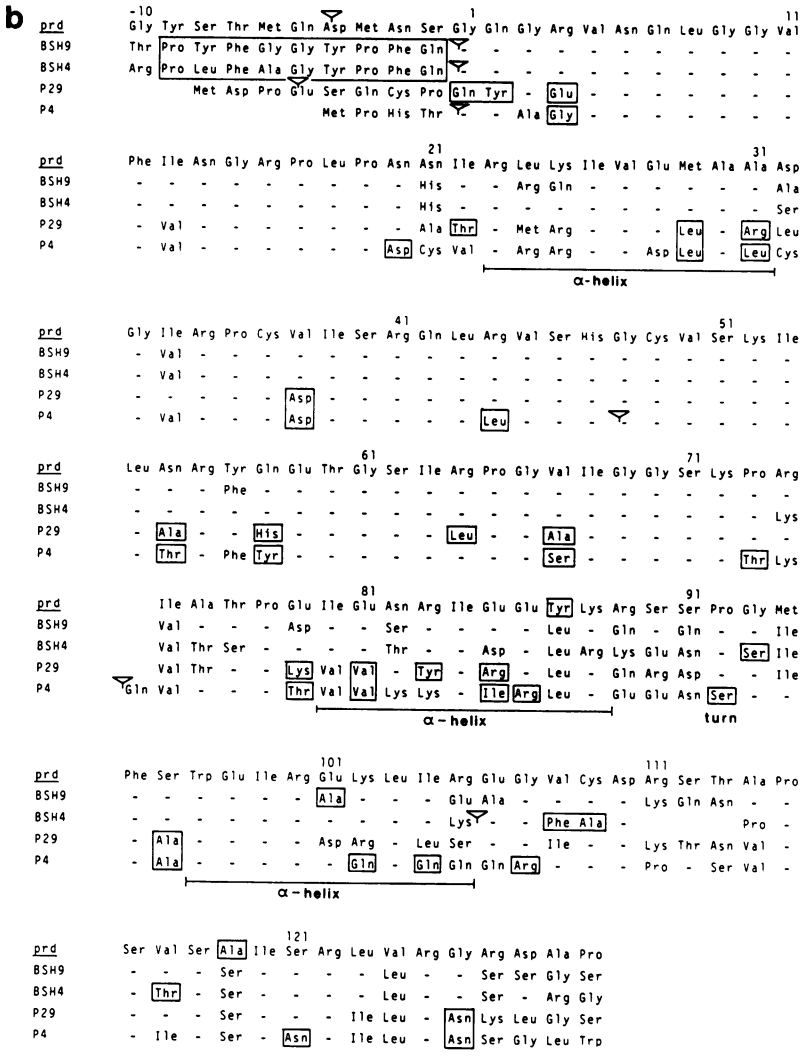

Fig. 2. Paired box sequences of the five *Drosophila* genes, *prd*, *gsb-BSH9*, *gsb-BSH4*, *Pox meso* and *Pox neuro*. The DNA (**a**) and corresponding amino acid sequences (**b**) of the paired box regions of the *prd* gene and the two *gsb* genes, *BSH9* and *BSH4* (Bopp *et al.*, 1986), as well as of the newly isolated *Drosophila* genes, *Pox meso* (P29) and *Pox neuro* (P4) have been aligned. Amino acids identical to those at corresponding positions of the *prd* sequence are represented by a dash. To illustrate class specificities of paired domains, amino acids of the *Pox meso* and *Pox neuro* paired domains that deviate from amino acids conserved at corresponding positions in *prd* and *gsb* as well as amino acids that are specific for only one of the five paired domains have been boxed [conservative amino acid alterations (Lys-Arg; Val-Ile-Leu; Thr-Ser; Phe-Tyr; Ala-Gly) are neglected]. Also boxed is the extended homology between the two *gsb* genes preceding their paired domains. Three predicted α-helices, and a region of high flexibility and very poor helical or β-pleated sheet structures indicative of a turn, between the second and third α-helix, are indicated (Burri *et al.*, 1989). Amino acids 111–113 are absent from the paired domain of *gsb-BSH4* (Bopp *et al.*, 1986) while the *Pox neuro* paired domain contains an additional Gln after the first more highly conserved 74 amino acids. The positions of introns are marked by triangles.

of the cells that transcribe *prd* and *gsb-BSH9*. To test this supposition, labeled probes of *BSH9* and *Pox meso* (P29) were hybridized separately (Figure 3e and g) and together (Figure 3f and h) to adjacent serial sections of embryos at the elongated germ band stage. As shown in Figure 3(e)–(h), *Pox meso* transcripts appear in the same cells of the mesoderm that express *gsb-BSH9*. Hence *prd*,*gsb* and *Pox meso* are all expressed in register along the antero-posterior axis, in the posterior half of each segment.

Expression patterns of gsb, Pox meso and Pox neuro in prd⁻ embryos

The expression patterns of *Pox meso* and *Pox neuro* suggest that these genes refine in the somatic mesoderm and neurectoderm the positional information passed on to them by the segmentation genes proper (Nüsslein-Volhard and Wieschaus, 1980). If this is the case, one would expect that their expression patterns depend on the activity of at least some of the segmentation genes. However, regardless of the specific function of *Pox meso* and *Pox neuro*, altered expression patterns in segmentation mutants would suggest that the two paired box genes are integrated into the network of segmentation genes.

Since *prd*, the two *gsb* genes and *Pox meso* are expressed in overlapping cell populations, a direct regulatory interaction between them would be possible. To test this hypothesis, we first examined transcript patterns of *gsb-BSH9* and *gsb-BSH4* in *prd⁻* embryos. Clearly, the transcript patterns of both genes are altered in a similar and rather simple manner. In the absence of functional *prd* protein, both *gsb* genes are activated only in every other segment (Figure 5a–d) as compared to the single-segmental repeat of *gsb* transcripts observed in wild-type embryos (Figure 3e; Bopp *et al.*, 1986; Baumgartner *et al.*, 1987; Côté *et al.*, 1987).

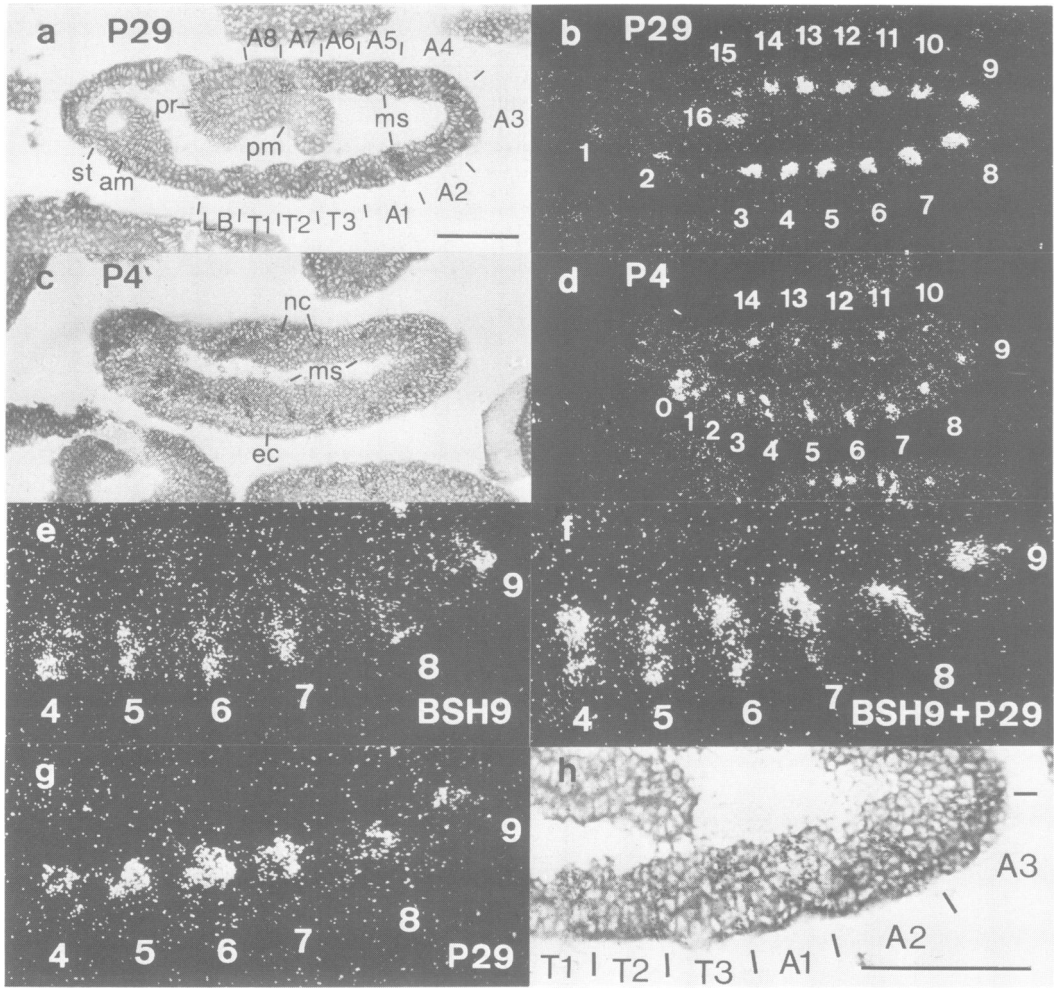

Fig. 3. Segmentally repeated distribution of *Pox meso* and *Pox neuro* transcripts and localization of *Pox meso* relative to *gsb-BSH9* transcripts in *Drosophila* embryos at the extended germ band stage. **Panels a–d** show photomicrographs of tissue sections of two stage 10 embryos (Campos-Ortega and Hartenstein, 1985) taken under phase-contrast (**left**) or dark-field illumination (**right**). The embryos are oriented with their dorsal side up and their anterior end to the left. Parasagittal sections were hybridized *in situ* with a ^{3}H-labeled cDNA probe of *Pox meso* (P29) (**a**,**b**) or *Pox neuro* (P4) (**c**,**d**) as described in Materials and methods. **Panels e–g** are dark-field photomicrographs of three consecutive sections of an embryo hybridized *in situ* with ^{3}H-labeled DNA probes of *gsb-BSH9* (**e**), *Pox meso* (P29) (**g**) or a combination of *gsb-BSH9* and *Pox meso* (BSH9 + P29) (**f**). **Panel h** shows the same section as *f* under phase-contrast illumination. The portion of the embryo shown corresponds to the three thoracic (T1–T3) and the first three abdominal segment primordia of an embryo at the extended germ band stage (stage 10). Note that *gsb-BSH9* transcripts are in register with those of *Pox meso*. The numbering refers to band numbers of *gsb-BSH9* or *en* transcripts whose posterior boundaries conicide (Baumgartner *et al.*, 1987). Horizontal bars in (**a**) and (**h**) indicate a length of 0.1 mm in (**a**)–(**d**) and (**e**)–(**h**) respectively. Abbreviations: am and pm, primordium of the anterior and posterior midgut; ec, ectoderm; ms, mesoderm; nc, neural precursor cells; pr and st, proctodeal and stomodeal primordium; LB, labial, T1–T3 thoracic; A1–A8, abdominal segment primordia.

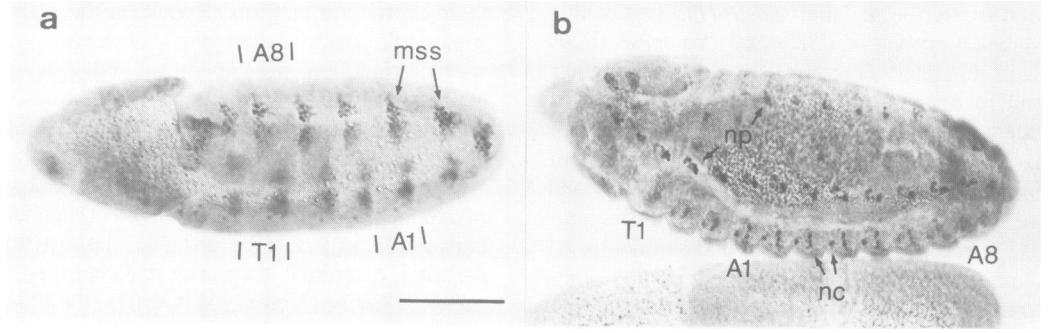

Fig. 4. Tissue-specific expression of *Pox meso* and *Pox neuro*. Embryos at the extended germ band stage (stage 11; Campos-Ortega and Hartenstein, 1985) in (**a**) or after germ band retraction (stage 15) in (**b**) have been stained immunocytochemically with purified anti-*Pox meso* (**a**) or anti-*Pox neuro* antibodies (**b**) as described in Materials and methods. The embryos are oriented with their dorsal side up and their anterior end to the left. The observed *Pox meso* and *Pox neuro* protein patterns appear to precisely parallel those of their transcripts with a short temporal delay. Arrows point to cells of the somatic mesoderm (mss) and of the central (nc) and peripheral nervous system (np). Other abbreviations are as in the legend to Figure 3. The length of the horizontal bar in (a) represents 0.1 mm.

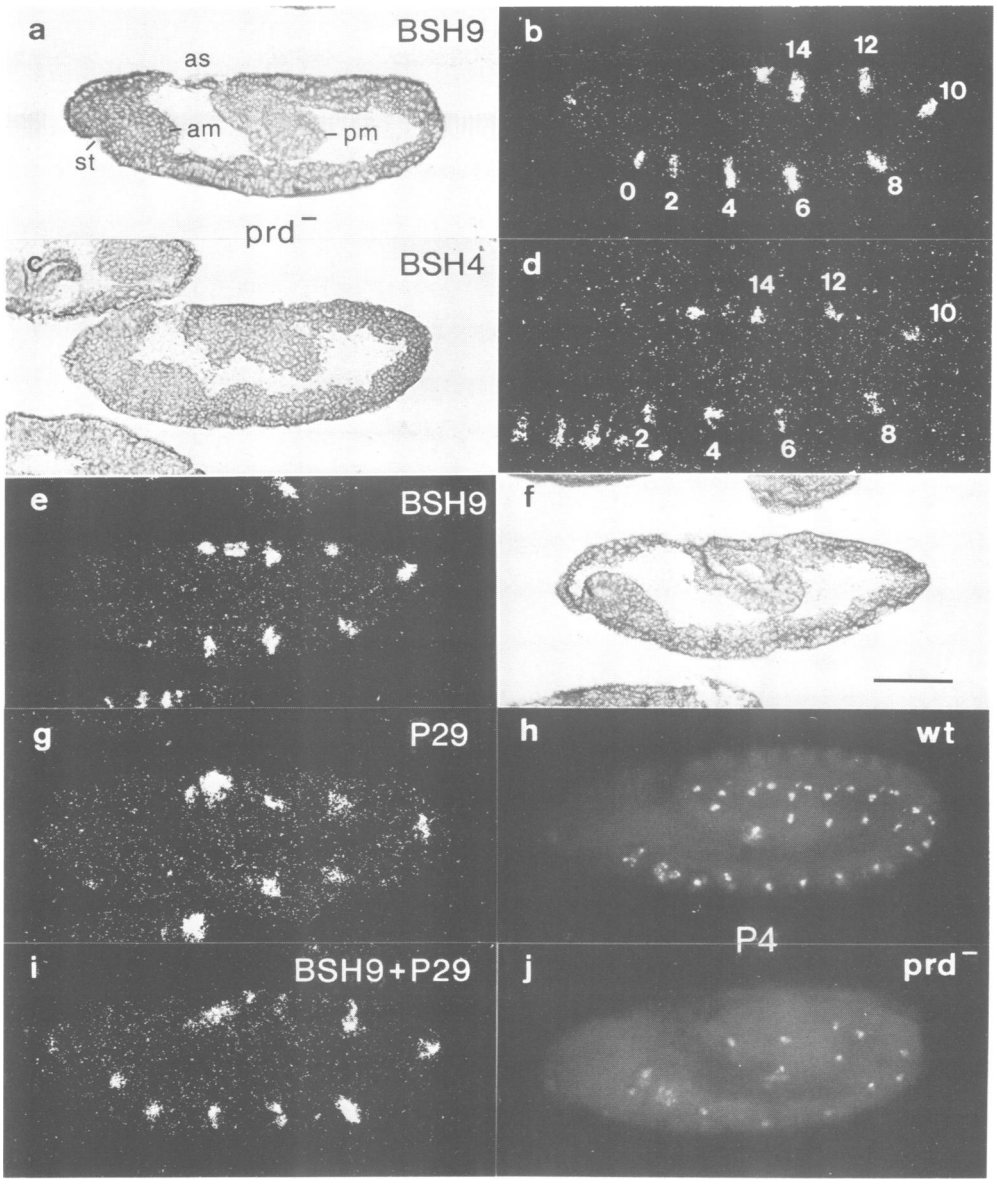

Fig. 5. Expression pattern of *gsb-BSH9* and *gsb-BSH4*, *Pox meso* and *Pox neuro* in *prd*$^-$ embryos. In (**a**)−(**d**) parasagittal sections of two homozygous *prd*$^-$ (*prd*$^{2.45.17}$/*prd*$^{2.45.17}$) embryos at the extended germ band stage (stage 10; Campos-Ortega and Hartenstein, 1985) were hybridized *in situ* with ^{3}H-labeled DNA probes of *gsb-BSH9* (**a**,**b**) or *gsb-BSH4* (**c**,**d**) and are shown under phase-contrast (**left**) or dark-field illumination (**right**). Panels **e**, **g** and **i** are dark-field photomicrographs of three consecutive sections of a stage 10 *prd*$^-$ embryo hybridized *in situ* with ^{3}H-labeled DNA probes of *gsb-BSH9* (**e**), *Pox meso* (P29) (**g**) or a combination of *gsb-BSH9* and *Pox meso* (BSH9 + P29) (**i**). **Panel f** is a phase-contrast photomicrograph of the section shown in (**e**). **Panels h** and **j** show immunofluorescent stainings (see Materials and methods) with purified anti-*Pox neuro* antibodies of a wild-type (**h**) and a *prd*$^-$ (**j**) embryo at the extended germ band stage. All embryos are oriented with their dorsal side up and their anterior end to the left. The length of 0.1 mm is indicated by the horizontal bar in (**f**). Abbreviation: as, amnioserosa. For other abbreviations and numbers see legend to Figure 3.

The same alteration in *prd*$^-$ embryos has been found for the expression of the *engrailed* (*en*) gene (DiNardo and O'Farrell, 1987). Hence, for the activation of both *gsb* and *en*, the *prd* protein is required only in the posterior half or compartment of alternating segments, i.e. in the anterior portions of the odd-numbered parasegments. This double-segmental repeat resulting from the elimination of the *gsb* and *en* bands in *prd*$^-$ embryos might provide an explanation for the similar cuticular pattern of the *prd*$^-$ phenotype (Nüsslein-Volhard and Wieschaus, 1980). The alternative explanation that cell death rather than the absence of *prd* activity is responsible for the missing *gsb* bands in *prd*$^-$ embryos is improbable because in another pair-rule mutant, *ftz*, cell death begins only at the completion of germ band extension (Magrassi and Lawrence, 1988).

In analogous experiments, the transcript pattern of *Pox meso* and its relation to that of *gsb-BSH9* transcripts was analyzed in consecutive serial sections of *prd*$^-$ embryos (Figure 5e−g and i). Again, the same cells that fail to express *BSH9* in *prd*$^-$ embryos fail to transcribe *Pox meso* (Figure 5e and g). This is particularly clear from an embryonic section hybridized to both *BSH9* and *Pox meso* probes (Figure 5i). Thus, expression of *Pox meso* depends on *prd* activity in every other segment as it does in the case of the two *gsb* genes. A simple explanation for these results might be offered by a regulatory scheme in which *prd*

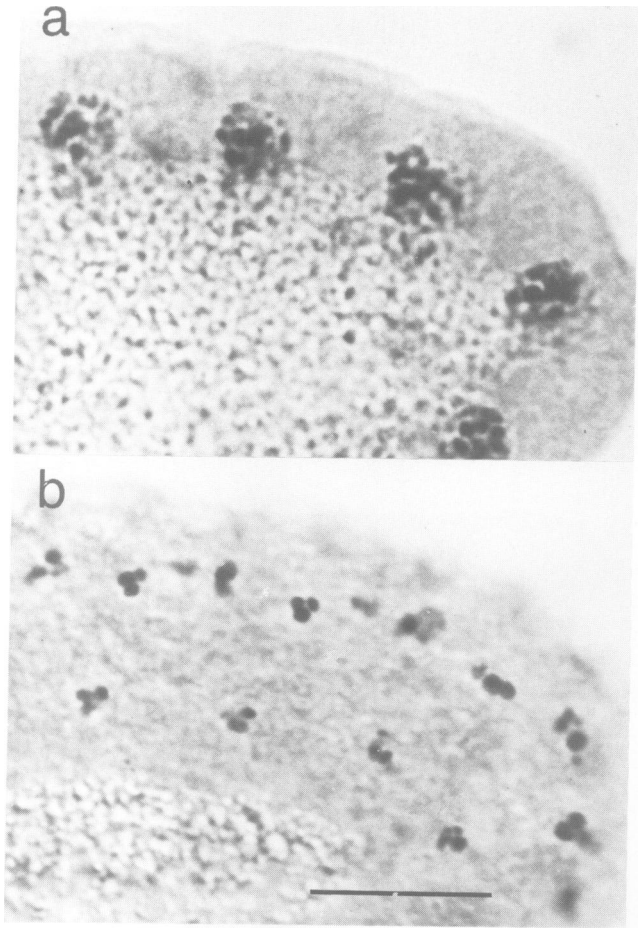

Fig. 6. Nuclear localization of *Pox meso* and *Pox neuro* proteins. Embryos at the extended germ band stage were stained with anti-*Pox meso* (**a**) and anti-*Pox neuro* (**b**) antibodies as described in the legend to Figure 4. The portions of the embryos shown correspond to parasegments 8–12. The horizontal bar in (b) indicates a length of 0.1 mm.

activates *Pox meso* directly or via *gsb-BSH9*. From the observation that the activation of the two *gsb* genes and *Pox meso* depends on *prd* only in odd-numbered parasegments, it follows that *gsb* and *Pox meso* activation do not require the *prd* product in even-numbered parasegments. Such a dependence of the activation on the *prd* protein could be explained if the *prd* protein acted on *gsb* and *Pox meso* in combination with an additional factor which is absent in the anterior portion of even-numbered parasegments. Hence activation of *gsb* and *Pox meso* in the even-numbered parasegments could not occur by the same combination of factors but would have to depend on different proteins (S.Baumgartner and M.Noll, in preparation).

We have also compared the expression patterns of *Pox neuro* in wild-type (Figure 5h) and prd^- embryos (Figure 5j). Although in this case *Pox neuro* expression also depends on *prd* activity, it is unclear at present whether its regulatory interactions with *prd* might be as simple as for *gsb* and *Pox meso*.

Pox meso and Pox neuro encode nuclear proteins

If *Pox meso* and *Pox neuro* are part of the same gene regulatory network as *prd* and *gsb*, the question arises whether they also encode gene regulatory proteins. Such a

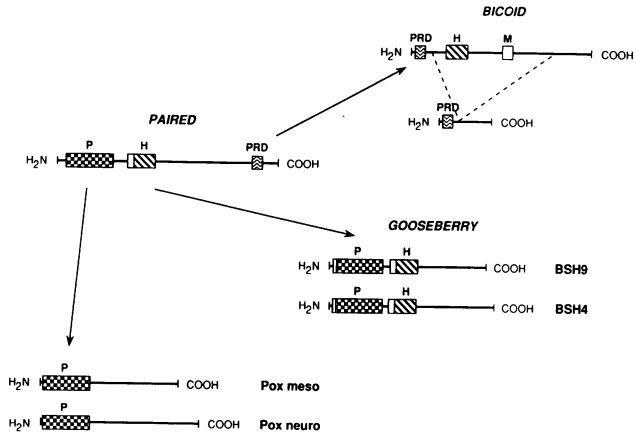

Fig. 7. Network of genes sharing homologous domains with the *paired* gene. The structural relationship between the six products of five genes isolated by searching for homologies to the three domains of the *paired* gene (arrows) is indicated by the following domains: P, paired domain; H, homeodomain; PRD, His-Pro repeat; M, M- or opa-repeat (poly-Gln) (McGinnis *et al.*, 1984; Wharton *et al.*, 1985). Amino-terminal extensions of the homeodomains in *paired*, *gooseberry-HSB9* and *gooseberry-BSH4* and of the paired domains in the two *gooseberry* gene products are shown as attached open boxes (Bopp *et al.*, 1986; Baumgartner *et al.*, 1987). The dashed lines indicate the origin of a minor splice product of the *bicoid* gene (Berleth *et al.*, 1988).

role for *Pox neuro* and *Pox meso* is suggested by the presence of a paired domain in the *Pox meso* and *Pox neuro* proteins. The presence of a conserved paired domain in genes whose regulatory function has been established—e.g. in *prd* and *gsb* by the additional presence of a homeodomain (Bopp *et al.*, 1986)—implies that the paired domain itself has a gene regulatory function as well. To test whether the cellular localization of the *Pox meso* and *Pox neuro* proteins is compatible with such a function, the proteins were immunostained on whole-mount embryos. As shown in Figure 6, both proteins are found predominantly in nuclei. This observation is consistent with a possible role of the *Pox neuro* and *Pox meso* products as gene regulators.

Discussion

The gene network concept states that genes sharing homologous domains are functionally related and members of a network of genes whose products interact, directly or indirectly, to perform an integrated function. The homology between two genes may consist of a homologous protein or RNA 'domain' or a homologous *cis*-regulatory element required, for example, for their co-ordinate activation (Frigerio *et al.*, 1986). To test the concept, we have selected for our analysis the network of segmentation genes, or, more generally, the network of genes specifying position along the antero-posterior axis of the *Drosophila* embryo in ectodermal as well as internal tissues.

We began by scanning the previously isolated *prd* gene (Kilchherr *et al.*, 1986), a representative of the pair-rule class of segmentation genes (Nüsslein-Volhard and Wieschaus, 1980), for homologous domains shared with other *Drosophila* genes (Bopp *et al.*, 1986; Frigerio *et al.*, 1986). With the isolation and analysis of the *Pox meso* and *Pox neuro* genes reported here, we have extended to five the number of genes that share a protein domain with *prd* and that have been shown to belong to the same functional network (Figure 7). The first gene, isolated on the basis of

the PRD or His-Pro repeat of *prd*, was *bicoid*. In addition to the His-Pro repeat, it shares a homeodomain with *prd* which is, however, of a different type than that of *prd* (Frigerio *et al.*, 1986). The *bicoid* gene belongs to the functional network of *prd* because it provides the maternal positional cues for anterior development (Frohnhöfer and Nüsslein-Volhard, 1986) in the form of a RNA gradient established at syncytial blastoderm (Frigerio *et al.*, 1986). The two other genes that have been shown previously to share a *prd*-type homeodomain, H, as well as a paired domain, P, with *prd* and to be part of the same network as *prd*, are the two *gsb* genes, *BSH9* and *BSH4* (Bopp *et al.*, 1986; Baumgartner *et al.*, 1987), which belong to the segment-polarity class of segmentation genes (Nüsslein-Volhard and Wieschaus, 1980). Such genes harboring both a paired and a homeobox we propose to call *PHox* genes (Figure 7).

According to the gene network concept the paired domain is expected to occur independently of the homeodomain in other *Drosophila* genes as well. This has been verified in this study for the two genes *Pox meso* and *Pox neuro* which contain a paired domain but lack a homeodomain (Figure 7). Conversely, we have isolated several genes that possess a *prd*-type homeodomain but no paired domain (R. Rutschmann and K.Schneitz, unpublished results).

The following evidence suggests that *Pox neuro* and *Pox meso* belong to the same gene network as *prd*. (i) Like the two *gsb* genes, *Pox meso* and *Pox neuro* are expressed in a tissue-specific segmentally repeated pattern, *Pox meso* in a subset of cells that express *prd*. (ii) They are regulated by segmentation genes including the *prd* gene. (iii) The *prd* gene controls *Pox meso* and *Pox neuro* expression in the same, or a similar, way as that of *gsb*, two other genes that belong to the same network as *prd* (Nüsslein-Volhard and Wieschaus, 1980; Bopp *et al.*, 1986).

All six genes, *bcd*, *prd*, *gsb-BSH9*, *gsb-BSH4*, *Pox meso* and *Pox neuro* are most likely encoding transcription factors. Although this seems obvious only for *bcd*, *prd* and the two *gsb* genes as they contain a homeodomain, such a role is strongly suggested by the nuclear localization of the *Pox meso* and *Pox neuro* products and by the fact that they harbor a paired domain which probably also has a gene regulatory function as argued above. Interestingly, *Pox neuro* encodes at its C-terminal a highly acidic region (E.Jamet *et al.*, in preparation) that might act as a gene activator in combination with the paired domain (Ma and Ptashne, 1987). Another putative transcription factor containing a PRD repeat and active in early development was recently found to be encoded in the maternal gene, *daughterless*, which is thought to control the *Sex lethal* gene in sex determination (Cronmiller *et al.*, 1988).

An attractive hypothesis for the role of *Pox meso* and *Pox neuro* is that they act further down in the gene regulatory cascade to which *bcd*, *prd* and the two *gsb* genes belong, by specifying positional information in a tissue-specific manner. The maternal gene *bcd* functions at the top of this cascade and probably does not regulate *prd* directly. On the other hand, the activation of the *gsb* genes probably consists of a direct interaction of the *prd* protein with the *gsb* promoter (S.Baumgartner and M.Noll, in preparation). The striking similarity of the expression patterns of *gsb-BSH9*, *gsb-BSH4*, *Pox meso* and *Pox neuro* in prd^- embryos suggests that the activation of *Pox meso* and *Pox neuro* in odd-numbered parasegments is either a direct effect of *prd* or occurs via the *gsb* genes. Preliminary experiments, however, appear to exclude the latter possibility (E.Jamet, unpublished results).

The two genes, *Pox meso* and *Pox neuro*, encode paired domains that are different from the *prd,gsb*-type previously described (Bopp *et al.*, 1986). We have proposed that paired domains contain three α-helices, two of which are part of a helix−turn−helix region in its C-terminal portion (Burri *et al.*, 1989). It is interesting that characteristic differences between different paired domain types accumulate in the first helix of the helix−turn−helix motif. If, analogous to the helix−turn−helix region of the homeodomain, this region is involved in DNA recognition, these variations might reflect the recognition of different DNA sequences. If the paired domain binds to DNA, another interesting consequence would be that the *prd* and *gsb* proteins carry in their paired and homeodomains two independent DNA binding sites. The existence of such paired domains has recently been demonstrated in prokaryotes (Moitoso de Vargas *et al.*, 1988).

Paired domains have been conserved in many organisms (Balling *et al.*, 1988; Deutsch *et al.*, 1988; Dressler *et al.*, 1988; Burri *et al.*, 1989). Of the three sequenced human paired domains two are of the *prd,gsb*-type and one of the *Pox meso*-type (Burri *et al.*, 1989). The paired domain of the mouse *Pax 1* gene (Deutsch *et al.*, 1988) is clearly of the *Pox meso*-type while the first 29 amino acids of the paired domain of *Pax 2* and *Pax 3* suggest that they are of the *Pox neuro*- and *prd,gsb*-type.

By the demonstration that the *undulated* (*un*) phenotype is the result of a point mutation in the paired domain of the *Pax 1* gene, *Pax 1* was identified with the *un* locus of the mouse (Balling *et al.*, 1988). Homozygous *un* mice are viable and exhibit malformed vertebrae along their entire rostro-caudal (antero-posterior) axis because anterior sclerotome cells fail to join the posterior cells of the adjacent sclerotome during vertebrae formation (Grüneberg, 1954). The *Pax 1* gene is first expressed in the sclerotomes of differentiating somites, in the perichordal condensations around the notochord (the intervertebral disk anlagen), in the third and fourth pharyngeal pouches (thymus anlagen), and finally in the intervertebral disks, the sternum and the thymus (Deutsch *et al.*, 1988). The *Drosophila Pox meso* gene, on the other hand, is first expressed at stage 10 of embryogenesis in the somatic mesoderm when the mesoderm separates laterally into splanchnopleural and somatopleural cell layers, defining a series of hollow spaces that could be considered as incipient somites (Campos-Ortega and Hartenstein, 1985).

This striking similarity of the *Pax 1* and *Pox meso* expression patterns parallels their evolutionary relationship. The paired domain of *Pax 1* is clearly of the *Pox meso*-type rather than of the *prd,gsb*- or *Pox neuro*-type. Thus, if *Drosophila* has a gene with an analogous function to that of *Pax 1*, we would expect it to be *Pox meso* rather than the segmentation genes *prd* or *gsb* as previously proposed (Balling *et al.*, 1988). Furthermore, if genes exerting analogous functions in insects and vertebrates share the same type of paired domain, *Pax 3* would be a likely candidate for a segmentation gene in the mouse.

If *Pox meso* plays a similar developmental role to *Pax 1*, we might suspect that both are part of a cascade of regulatory genes and act in this cascade below the proper segmentation genes as defined in *Drosophila* (Nüsslein-Volhard and

Wieschaus, 1980). Similar to the segmentation genes, *Pox meso* and *Pox neuro* could then be understood as genes further subdividing segments and defining position along the antero-posterior axis in mesodermal and neural precursor cells. The mutant phenotypes of such genes, if lethal, are not expected to be expressed in the cuticle and hence would escape screens for cuticular phenotypes. Our approach to screen for genes sharing network-specific domains appears to be one way of discovering these genes.

Materials and methods

Screening of genomic libraries at low stringency

Genomic libraries of *Drosophila melaongaster* were prepared in EMBL4 according to Frischauf *et al.* (1983) and screened with paired box probes of the *prd* gene (0.7 kb *Hin*dIII−*Pst*I of c7340.4; Frigerio *et al.*, 1986) and of the two *gsb* genes (0.4 kb *Eco*RV−*Bam*HI fragment of BSH9c2 and 0.4 kb *Nco*I−*Eco*RI fragment of BSH4c4; Baumgartner *et al.*, 1987) at reduced stringency (McGinnis *et al.*, 1984).

Isolation of cDNA clones

A cDNA library constructed in λgt10 of poly(A)$^+$ RNA from 3−12 h old embryos (Poole *et al.*, 1985), was kindly provided by T.Kornberg and screened for cDNAs of *Pox meso* and *Pox neuro* according to standard procedures (Maniatis *et al.*, 1982).

DNA sequencing

All DNA sequences were analyzed on both strands, reading each sequence at least twice on independent cDNA and genomic clones. The DNAs were sequenced by the dideoxynucleotide method of Sanger *et al.* (1977), using the M13 vector mWB3296 (Frigerio *et al.*, 1986), its counterpart, mWB3226, which contains the M13mp18 polylinker in opposite orientation (Baumgartner *et al.*, 1987), or a derivative of mWB3296, mWB3297, in which a 132 bp *Pvu*II−*Mst*II fragment of the *lacZ* gene has been deleted to eliminate the unique restriction sites *Pvu*II, *Pvu*I, *Fsp*I, *Bgl*I, *Hgi*EII and *Mst*II. All sequencing vectors were derived from M13 vectors described by Barnes *et al.* (1983).

In situ hybridization to polytene chromosomes

In situ hybridizations to salivary gland chromosomes were carried out with biotinylated probes according to the method of Langer-Safer *et al.* (1982) essentially as described (Frei *et al.*, 1985).

In situ hybridization to tissue sections

In situ hybridizations to embryonic tissue sections were carried out essentially according to Hafen *et al.* (1983) as previously described (Baumgartner *et al.*, 1987). The following purified DNA fragments were used as nick-translated probes: a 1.3 kb *Bam*HI−*Eco*RI fragment of *BSH9* and a 2.4 kb terminal *Eco*RI fragment of the *BSH4* clone as described previously (Bopp *et al.*, 1986; Baumgartner *et al.*, 1987), a nearly full-length (2.0 kb) cDNA of *Pox neuro* and a 1.7 kb *Bam*HI−*Eco*RI 3′-terminal fragment of a nearly full length (2.6 kb) *Pox meso* cDNA. Autoradiographic exposure occurred for 28 days.

Preparation of antibodies against Pox meso and Pox neuro antigens

Two recombinant DNA clones were constructed in pAR vectors to express truncated *Pox neuro* and *Pox meso* proteins in *Escherichia coli* BL21 (DE3) (Studier and Moffatt, 1986). A 1.01 kb *Bam*HI cDNA fragment spanning most of the *Pox neuro* coding region was cloned into the *Bam*HI site of the pAR3040 plasmid. A protein with an apparent mol. wt of 47 kd, including the C-terminal half of the paired domain (65 amino acids) and all but the last 17 amino acids of the C-terminal end of the *Pox neuro* protein, was obtained after induction of the transformed BL21 (DE3) bacterial strain with IPTG. For the expression of a truncated *Pox meso* protein, a 1.8 kb *Bam*HI−*Pst*I genomic DNA fragment (whose 5′ end is located 179 nucleotides upstream of the 3′ end of the *Pox meso* paired box) was ligated with blunt ends into the *Bam*HI site of the pAR3039 vector. A corresponding truncated *Pox meso* protein (apparent mol. wt of 36 kd), encompassing 292 amino acids of the carboxy-terminal portion of the *Pox meso* protein (including 59 amino acids of the paired domain), was induced in BL21 (DE3).

The proteins expressed in *E.coli* were purified according to Gaul *et al.* (1987). The last step of the purification procedure consisted of a preparative electrophoresis in a SDS−polyacrylamide gel. Proteins were stained with KCl (Nelles and Bamburg, 1976) and the IPTG-induced band was excised from the gel and homogenized with a Polytron tissuemizer. Rabbits were immunized and boosted every 2 months with this homogenate containing ~100 μg of protein emulsified 50:50 with complete (incomplete for booster injections) Freund's adjuvant. Antisera were taken by bleeding 8 days after each boost and purified by affinity chromatography over the purified antigen coupled to CNBr-activated Sepharose 4B according to the manufacturer's specifications (Pharmacia).

Immunocytochemical and immunofluorescent staining of embryos

Embryos were fixed and prepared for immunostaining by a modified version of the method of Dequin *et al.* (1984) which itself is based on the technique of Mitchison and Sedat (1983). Staged embryos were collected from a cage, dechorionated in 25% javel water (corresponding to 60% Chlorax), extensively rinsed with water, and devitellinized and fixed for 20 min in a 1:1 mixture of heptane and 8% formaldehyde in 0.1 M PIPES, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$. Embryos were then rinsed in methanol, rehydrated in PBS, and permeabilized for 30 min in PBS containing 0.05% each of Triton X-100, NP-40, deoxycholate and Tween 20, and 0.02% BSA. Unspecific binding of the purified antibodies was abolished by pre-incubation of the embryos in buffer A [PBS containing 0.1% Triton X-100 and 0.5% BSA (for immunofluorescence) or 4% dry milk powder (for immunocytochemistry)]. The embryos were incubated with affinity-purified antibodies, pre-adsorbed to 0−16 h old embryos for 1 h at room temperature, at a 1:10 dilution in buffer A for 2 h at room temperature or overnight at 4°C. Subsequently, either rhodamine- or HRP-conjugated swine anti-rabbit IgGs (Dakopatts, Denmark), pre-adsorbed in the same manner, were applied as secondary antibodies at a 1:50 dilution in buffer A again for 2 h at room temperature or overnight at 4°C. For immunofluorescence, embryos were mounted in 90% glycerol containing 2% *n*-propyl gallate (Giloh and Sedat, 1982). For HRP immunocytochemistry, embryos were fixed with 2% glutaraldehyde in PBS and stained, essentially according to Steller *et al.* (1987), by incubation with 0.5 mg/ml of diaminobenzidine and 0.0015% hydrogen peroxide in PBS for 15 min at room temperature. The embryos were washed in PBS and stepwise dehydrated in 70, 90 and 100% methanol for 5 min at each step. Embryos were cleared by replacing residual ethanol with methylsalicylate for 15 min at room temperature and mounted in DPX (BDH, UK).

Acknowledgements

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