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 Pox 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 bico (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, Pprd, and of the two gsb genes, PBSH9 and PBSH4 (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 Pprd 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 overlapping 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 pprd 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 pprd 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 pprd 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 pprd 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

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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 EcoRI 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)Rd3k+85 (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 EcoRI 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.

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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 *Drosophila* and *Pox meso* 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).

Table 1. Matrix of amino acid homologies between five *Drosophila* paired domains.

<table>
<thead>
<tr>
<th>prd</th>
<th>BSH9</th>
<th>BSHH</th>
<th>P79</th>
</tr>
</thead>
<tbody>
<tr>
<td>prd,gsb class</td>
<td>prd,gsb class</td>
<td>prd,gsb class</td>
<td>prd,gsb class</td>
</tr>
<tr>
<td>0.81, 0.92</td>
<td>0.87, 0.95</td>
<td>0.77, 0.83</td>
<td>0.78, 0.85</td>
</tr>
<tr>
<td>0.85, 0.78</td>
<td>0.77, 0.86</td>
<td>0.64, 0.74</td>
<td>0.85, 0.78</td>
</tr>
<tr>
<td>0.48, 0.73</td>
<td>0.64, 0.74</td>
<td>0.48, 0.74</td>
<td>0.48, 0.74</td>
</tr>
</tbody>
</table>

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*.

32–31 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 amphiphatic. 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 1) 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.

**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)dsxB5+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 \( \alpha \)-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 (Ballal 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 3a–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 the embryos mesodermal posterior half of pattern during Pox itself. Alternatively, regions to paired mutant such of segmentation.

While 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 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 prostomial 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...
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 neururectoderm 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 3H-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 3H-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 coincide (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− (prd243/243) embryos at the extended germ band stage (stage 10; Campos-Ortega and Hartenstein, 1985) were hybridized in situ with 3H-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 3H-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, fit, 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
activates \textit{Pox meso} directly or via \textit{gsb-BSH9}. From the observation that the activation of the two \textit{gsb} genes and \textit{Pox meso} depends on \textit{prd} only in odd-numbered parasegments, it follows that \textit{gsb} and \textit{Pox meso} activation do not require the \textit{prd} product in even-numbered parasegments. Such a dependence of the activation on the \textit{prd} protein could be explained if the \textit{prd} protein acted on \textit{gsb} and \textit{Pox meso} in combination with an additional factor which is absent in the anterior portion of even-numbered parasegments. Hence activation of \textit{gsb} and \textit{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 \textit{Pox neuro} in wild-type (Figure 5h) and \textit{prd}− embryos (Figure 5j). Although in this case \textit{Pox neuro} expression also depends on \textit{prd} activity, it is unclear at present whether its regulatory interactions with \textit{prd} might be as simple as for \textit{gsb} and \textit{Pox meso}.

\textbf{Pox meso and Pox neuro encode nuclear proteins}

If \textit{Pox meso} and \textit{Pox neuro} are part of the same gene regulatory network as \textit{prd} and \textit{gsb}, the question arises whether they also encode gene regulatory proteins. Such a role for \textit{Pox neuro} and \textit{Pox meso} is suggested by the presence of a paired domain in the \textit{Pox meso} and \textit{Pox neuro} proteins. The presence of a conserved paired domain in genes whose regulatory function has been established—e.g. in \textit{prd} and \textit{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 \textit{Pox meso} and \textit{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 \textit{Pox neuro} and \textit{Pox meso} products as gene regulators.

\section*{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 \textit{Drosophila} embryo in ectodermal as well as internal tissues.

We began by scanning the previously isolated \textit{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 \textit{Drosophila} genes (Bopp et al., 1986; Frigerio et al., 1986). With the isolation and analysis of the \textit{Pox meso} and \textit{Pox neuro} genes reported here, we have extended to five the number of genes that share a protein domain with \textit{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 (Frohnhofer 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.Schnieit, 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 Pashine, 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 (Cronmüller 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 (Grüneweg, 1954). The Pax 1 gene is first expressed in the sclerotomes of differentiating somites, in the perichondral 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 splanchonpeural 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 melanogaster* were prepared in EMBL4 according to Frischauer et al. (1983) and screened with paired box probes of the *pre* gene (0.7 kb HindIII–PstI of *c7340A*; Frigerio et al., 1986) and of the two gsb genes (0.4 kb EcoRV–BamHI fragment of *BSH9c2* and 0.4 kb NcoI–EcoRI 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 DNA sequences were determined by the dideoxy nucleotide method of Sanger et al. (1977), using the M13 vector mW3296 (Frigerio et al., 1986), its counterpart, mW3226, which contains the M13mp18 polylinker in opposite orientation (Baumgartner et al., 1987), or a derivative of mW3296, mW3297, in which a 132 bp PvuII–MsII fragment of the lacZ gene has been deleted to eliminate the unique restriction sites PvuII, PvuI, FplI, BstI, HaeIII and MsII. All sequencing vectors were derived from M13 vectors described by Barnes et al. (1983).

**In situ hybridization to polytenes 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 Hsu 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 BamHI–EcoRI fragment of *BSH9* and a 2.4 kb terminal EcoRI fragment of *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 BamHI–EcoRI 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 meso* and *Pox neuro* proteins in *Escherichia coli* BL21 (DE3) (Studier and Moffatt, 1986). A 1.01 kb BamHI cDNA fragment spanning most of the *Pox neuro* coding region was cloned into the *BamHI* 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 BamHI–PstI 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 *BamHI* 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 Gault 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 Bamberg, 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 (incorpoare for booster injections) Freund's adjuvant. Antiserum 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 MgSO4. 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 IgG (Dakopatts, Denmark), pre-absorbed 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 (Giboh 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).

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