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Both the paired domain and homeodomain are required for in vivo function of *Drosophila* Paired

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SUMMARY

***Drosophila paired*, a homolog of mammalian *Pax-3*, is key to the coordinated regulation of segment-polarity genes during embryogenesis. The *paired* gene and its homologs are unusual in encoding proteins with two DNA-binding domains, a paired domain and a homeodomain. We are using an in vivo assay to dissect the functions of the domains of this type of molecule. In particular, we are interested in determining whether one or both DNA-binding activities are required for individual in vivo functions of Paired. We constructed point mutants in each domain designed to disrupt DNA binding and tested the mutants with ectopic expression assays in *Drosophila* embryos. Mutations in either domain abolished the normal regulation of the target genes *engrailed, hedgehog, gooseberry* and *even-skipped*, suggesting that these in vivo functions of Paired require DNA binding through both domains rather than either domain alone. However, when the two mutant proteins were placed in the same embryo, Paired function was restored, indicating that the two DNA-binding activities need not be present in the same molecule. Quantitation of this effect shows that the paired domain mutant has a dominant-negative effect consistent with the observations that Paired protein can bind DNA as a dimer.**

Key words: *Drosophila*, *paired*, homeodomain, paired domain, segmentation, *Pax-3*

INTRODUCTION

The precise spatial and temporal regulation of *Drosophila* segmentation genes is critical for the specification of cell fates in the developing embryo (Nusslein-Volhard and Wieschaus, 1980). The segmentation genes, many of which encode transcription factors, are organized in a regulatory cascade (coordinate → gap → pair rule → segment polarity) in which combinations of genes expressed in coarse patterns regulate the transcription of genes expressed in progressively more refined patterns. In this way, global asymmetries laid down during oogenesis are progressively refined into precise patterns of gene expression that define the developmental fates of individual cells (Akam, 1987; Ingham, 1988). The key to this refinement of spatial information is the observation that the regulators at a given step in the cascade operate combinatorially to define the more refined expression of downstream genes (Gergen et al., 1986). For example, the segment-polarity gene, *engrailed* (*en*), whose RNA and protein is expressed in a zebra-stripe pattern with one-segment periodicity, is regulated by a combination of pair-rule transcriptional regulators each expressed in stripes with two-segment periodicity (DiNardo and O'Farrell, 1987; Ingham et al., 1988; Weir et al., 1988; Manoukian and Krause, 1992, 1993; Cadigan et al., 1994). We have focussed our studies on one of these pair-rule regulators, the *paired* (*prd*) gene, as a paradigm of a gene that acts combinatorially with other pair-rule genes to regulate the patterned expression of several well-defined segment-polarity genes.

prd, a member of the Pax family of genes, encodes a protein with a paired domain (PD) and a homeodomain (HD), both of which have DNA-binding activities (Bopp et al., 1986; Frigerio et al., 1986; Treisman et al., 1989). Point mutations in either the PD or the HD of *PAX-3*, the human homolog of *prd*, are associated with the autosomal dominant disorder Waardenburg Syndrome (Baldwin et al., 1992; Burri et al., 1989; Tassabehji et al., 1992; Lalwani et al., 1995). Both the PD and the HD contain helix-turn-helix structures involved in DNA binding (Treisman et al., 1989, 1991; Xu et al., 1995). Indeed, the PD is bipartite in its structure, containing two subregions each with a helix-turn-helix motif (Czerny et al., 1993; Xu et al., 1995). The C-terminal region containing the second helix-turn-helix is apparently dispensible for in vivo function of Prd (Cai et al., 1994; Bertuccioli et al., 1996), although this region of Prd and other Pax proteins can contribute to DNA-binding

activity (Czerny et al., 1993; Jun and Desplan, 1996). In this study, we investigated whether the remaining two DNA-binding activities of Prd, those of the HD and the N-terminal half of the PD, are required for the individual functions of Prd.

Observations of in vitro DNA binding by the Prd protein support the possibilities that the HD and PD might function either together or independently. Several classes of Prd-binding sites have been defined in vitro. PCR selection experiments have identified sites for the PD alone (XPRD; Jun and Desplan, 1996), and for the HD alone (Wilson et al., 1993), the latter being composed of two HD sites facing each other and separated by 2 bp (P2 site) or 3 bp (P3). A region of the *gsb* promoter sufficient for striped expression (GEE element) contains putative HD sites and is bound by Prd in vitro (Li and Noll, 1994). PCR selection using a peptide containing both the PD and HD gave a composite site (PH0) with adjacent half sites for the PD and HD, respectively (Jun and Desplan, 1996). An almost identical composite site (PTE) has been identified in the late-expression element in the *even-skipped* (*eve*) promoter, and both half sites of this domain have been shown to be critical for Prd regulation of late *eve* expression (Fujioka et al., 1996). Two other Prd-binding sites have also been identified in the *eve* promoter: the e_5 site has two half sites in the same order as PH0 and PTE but spaced 1 bp closer; the e_4 site has half sites in the opposite order (Hoey and Levine, 1988; Treisman et al., 1991). The PD and HD of Prd are able to bind to the respective half sites of PH0, PTE or e_5, either independently or simultaneously (Treisman et al., 1991; Jun and Desplan, 1995; Fujioka et al., 1996). In contrast, e_4 is bound only by Prd protein containing both a functional PD and HD (Treisman et al., 1991). Deletion analysis suggests that e_5 may contribute to the strength of late *eve* expression (Fujioka et al., 1996). The possible in vivo significance of e_4 is unknown. The observation of HD sites in the GEE *gsb* stripe element raises the possibility that the HD alone may be sufficient for regulation of some *prd* target genes. However, the observation of functional composite sites in the *eve* promoter (PTE and e_5) suggests that both the PD- and HD-binding activities may be necessary for regulation of other target genes, although the question remains whether both binding activities need be present in the same Prd molecule. To investigate these possibilities, we made point mutations in the PD and HD of Prd and tested these mutant proteins in vivo.

MATERIALS AND METHODS

Generation of transformant *Drosophila* lines

Construction of the *hs-prd* lines was described previously (Morrissey et al., 1991). The *hs-prd^{un}* construct was made using two simultaneous PCR reactions (Horton et al., 1989; Yon and Fried, 1989; Sarkar and Sommer, 1990). We incorporated the *undulated* mutation from GS15 (Treisman et al., 1991) into a PCR product extending from the mutation site to the C terminus of the Prd HD (5′ primer: GGTGGAGTTTTCATCAACA; 3′ primer: GTGCTGCTTGCGGAGACGAG). This product was used as a megaprimer in a (simultaneous) second PCR that extended the megaprimer in the 3′ direction to attach *prd* leader sequence; the second PCR used *prd/ftzHD* (*prd* c7340.6 cDNA with a *fushi tarazu* (*ftz*) homeobox; Frigerio et al., 1986) as template, 5′ primer GTTTCTGGAGGAGCT, and 3′ primer GTGCTGCTTGCGGAGACGAG. The resulting PCR product was digested with *Hin*dIII and *Eag*I and subcloned into the corresponding sites of *pGEM3Zf+/prdΔPB* (Morrissey et al., 1991) to generate a full-length *prd* gene containing the *undulated* mutation (*prd^{un}*). DNA sequencing was used to confirm that the *undulated* mutation was indeed incorporated into the PCR product, and to ensure the fidelity of the *Taq* polymerase. PCR conditions were according to Sheffield et al. (1989), with 20 μl reactions containing 1-3 ng of template DNA, 10 pM of each primer, 1.25 mM of each dNTP (Pharmacia), 1 unit of *Taq* polymerase (Perkin-Elmer Cetus) and buffered with 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM NH_4SO_3, and 10 mM β-ME. The 5′ primer in the first PCR was used at limiting concentration (0.1 pM). 25 cycles were performed with 1 minute of denaturing at 93°C, annealing at 55°C for 1 minute, and extension at 70°C for 1 minute.

To incorporate the *prd* homeobox change, Q9Q10, into the *prd* cDNA, a 399 bp *Eag*I-*Pvu*II fragment containing the Q9Q10 mutation was subcloned into the corresponding sites of the *prd* c7340.6 cDNA in pGEM2. The desired subclone was identified by a PCR screen using a *prd^{Q9Q10}* 3′ primer (ACGCCGCTGTTG) and an outside M13 sequencing primer, where only a template containing the Q9Q10 mutation would produce a PCR product. The insertion of the Q9Q10 mutation was then confirmed by DNA sequencing.

The *prd^{un}* and *prd^{Q9Q10}* genes were sequentially subcloned (Morrissey et al., 1991) into the heat-shock vector pHSBJ (Malicki et al., 1990) and then the P-element vector pW8 (Klemenz et al., 1987). The resulting *hs-prd^{un}* and *hs-prd^{Q9Q10}* constructs were injected with helper plasmid (pπ25.7wc) into *Df(1)w^{67c23}y* embryos, and *white^{+}* transformants selected. Analysis was performed on two independent chromosomal insertions of each of the two mutations.

Heat treatment of embryos

Embryos were collected on agar plates (seeded with baker's yeast and acetic acid) for 30-45 minutes and incubated at 25°C until 20-30 minutes before heat treatment, at which time they were rinsed from the plates with water and dechorionated in 50% Chlorox for 2 minutes. The embryos were then rinsed with 0.7% NaCl, 0.04% Triton X-100, and placed in the same solution at 25°C. Heat treatment was performed by submerging the embryos in NaCl-Triton solution at 37°C for two 5 or 10 minute pulses, the first at 140-170 AED and the second 30 minutes later. After heat treatment, the embryos were immediately returned to the NaCl-Triton solution at 25°C and fixed 40-50 minutes after the end of heat treatment except where noted. Heat-treated embryos were processed for either in situ hybridization, or protein staining (see below).

In situ hybridization and immunostaining of embryos

In situ hybridization of heat-treated embryos was as described in Morrissey et al. (1991). DNA probes for in situ analysis were as follows: *prd* c7340.6 cDNA (Frigerio et al., 1986), *en* 1.4 cDNA (Poole et al., 1985), *gsb* BSH9c2 cDNA (Baumgartner et al., 1987), *hh* cDNA (Mohler and Vani, 1992), *eve* cDNA (Harding et al., 1986; Macdonald et al., 1986). The *L-lacZ* reporter construct shows the late *eve* expression pattern and has *eve* promoter sequences (–6415 to –4799 and –275 to +170) contiguous to the *lacZ* reporter. Our use of *hunchback/lacZ* marked balancer chromosomes to identify mutant embryos is described in Morrissey et al. (1991).

Protein staining was carried out essentially as described previously (Karr et al., 1989). Following heat treatment, embryos were fixed and devitellinized. They were then rinsed in methanol, followed by PBS, 0.1% Triton X-100 (PBS/Triton) and were blocked in PBS, 0.1% Triton X-100, 10% normal goat serum (PN) for 30 minutes prior to overnight incubation with a polyclonal Prd antibody (1:200 in PN) (Gutjahr et al., 1993). Embryos were treated sequentially with biotinylated secondary antibody (overnight) and strepavidin-HRP (1 hour) (Vector Research) with extensive washing (PBS/Triton) and blocking (PN) between steps. Following color reactions with diaminobenzidine, embryos were rinsed in PBS/Triton and mounted on slides with Aqua-Poly/Mount (Polysciences).

Electrophoretic mobility shift analysis

Electrophoretic mobility shift assays (EMSA) were performed as described in Fujioka et al. (1996). Briefly, histidine-tagged full-length Prd proteins or truncated proteins containing the PD and HD (amino acids 27-276) were incubated with 0.5-5 ng ^{32}P-labelled probes in 20 μl binding buffer (15 mM Tris-HCl pH 7.5, 60 mM KCl, 0.5 mM DTT, 0.25 mg/ml BSA, 0.05% NP-40 and 7.5% glycerol) which included 100 ng poly(dIdC) to minimize non-specific binding.

Transient transfection assays

Transient tranfection of Schneider-3 cells was used to measure transcriptional activation by *prd* constructs as described previously (Ananthan et al., 1993). Briefly, cells were transfected with *prd* constructs (subcloned in pHSBJ), CAT reporter genes (PB3-CAT or 3K′-TATA-CAT) and D88:lacZ (*lacZ* driven by a heat-shock promoter). Cells were heat treated (to allow for *prd* and *lacZ* expression) and extracted for CAT assays. In all experiments, 1 μg each of *prd* and *lacZ* producer constructs were used.

β-galactosidase activities varied between samples by less than 20%, indicating similar transfection efficiencies. Pilot experiments showed that the activity using 1 μg of wild-type *prd* was within the linear range of CAT activity.

RESULTS

Ectopic Prd can substitute for endogenous Prd function

We have previously described an ectopic expression assay that provides an in vivo assay for the function of an introduced *prd* gene under the control of a heat-shock promoter (Morrissey et al., 1991). Ectopic Prd causes posterior expansion of odd-parasegment *en* stripes, a result consistent with the model that *prd* specifies the posterior borders of these stripes. *sloppy paired* (*slp)* and *runt* (*run*) specify the anterior borders of these stripes, and the even-numbered stripes are specified by *ftz* and *odd-skipped* (*odd*), as illustrated in the model in Fig. 1 (DiNardo and O'Farrell, 1987; Ingham et al., 1988; Weir et al., 1988; Manoukian and Krause, 1992, 1993; Benedyk et al., 1994; Cadigan et al., 1994). A limitation of this ectopic expression assay is that the function of the introduced *prd* gene is measured in cells that normally do not express *prd*. Hence, we transferred this assay to a *prd*$^-$ background (*prd*$^{2.45.17}$; Frigerio et al., 1986) in order to ask whether expression of the introduced *hs-prd* gene can substitute for the endogenous *prd*. In *prd*$^-$ embryos, the odd-numbered *en* stripes are absent (Fig. 2C). However, heat treatment of *prd*$^-$;*hs-prd* embryos results in rescue of these stripes (Fig. 2D), indicating that the introduced *hs-prd* transgene can provide *prd* function in the cells that normally express endogenous *prd*. The rescued stripes are expanded posteriorly (Fig. 2D) as in their *prd*$^+$ siblings (Fig. 2B). The homozygous *prd*$^-$ embryos were unambiguously identified using a lacZ-marked balancer chromosome (see Fig. 2 legend).

We also extended the ectopic expression assay by examining the regulation of several other target genes of *prd*. The *hedgehog* (*hh*) gene is expressed in the same cell rows as *en* in the gastrulating and germ-band-elongating embryo, and both genes are likely to be regulated by similar controls (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992). Consistent with this, ectopic Prd causes posterior expansion of odd-numbered *hh* stripes (Fig. 3E,F), just as seen with *en* (Fig. 3I,J). The *gooseberry* (*gsb*) expression stripes are twice the width of those of *en*, and span the cell row in which *en* is expressed as well as the cell row immediately anterior of this (see Fig. 1; Kilchherr et al., 1986; Baumgartner et al., 1987; Gutjahr et al., 1993). In line with the regulation of *en* and *hh*, ectopic Prd expression causes posterior expansion of the odd-numbered *gsb* stripes, as reported by Li and Noll (1994). However, we also observe anterior expansion of the even-numbered *gsb* stripes, causing every other interband to be partially or completely filled in (Fig. 3A,B). As recently reported, *prd* also regulates late *eve* RNA expression as well as *L-lacZ*, a reporter driven by the late *eve* control element (Fujioka et al., 1995, 1996). In *prd*$^-$ embryos, late *eve* RNA stripes fade prematurely during germ-band elongation, and L-lacZ stripes are not activated, whereas in heat-treated *prd*$^-$; *hs-prd* embryos, maintenance of the *eve* RNA and activation of L-lacZ stripes is rescued (see Fujioka et al., 1996, and Fig. 4A,D). The late *eve* expression stripes are in the same cell rows as the odd-numbered *en* stripes and are similarly expanded posteriorly.

DNA binding through the paired domain

Previous ectopic expression studies have shown that deletion

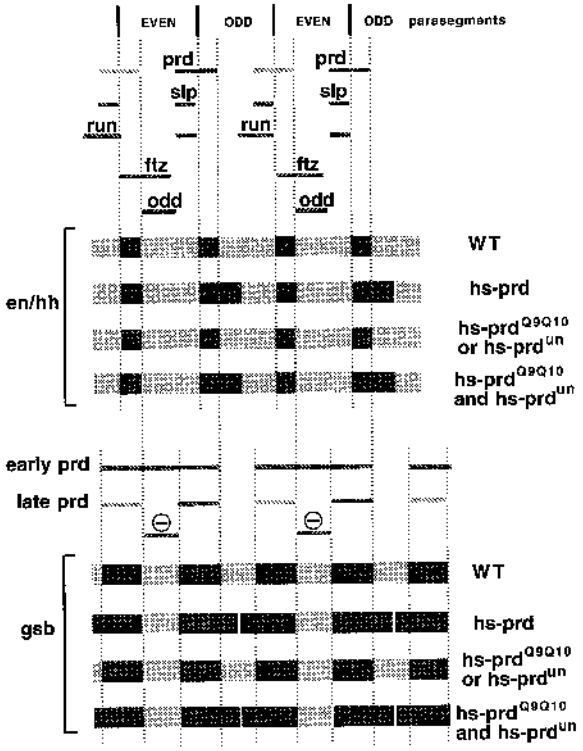

Fig. 1. Schematic summary of ectopic expression experiments. Diagrams (oriented with anterior to the left) summarize the RNA expression patterns of *en*, *hh* and *gsb* in wild-type, *hs-prd*, *hs-prd^{Q9Q10}* and *hs-prd^{un}* embryos. Cycle 14 expression patterns of the postulated pair-rule regulators of *en* are illustrated (see Morrissey et al., 1991). In this combinatorial model, the anterior borders of odd-numbered *en* stripes are defined by *slp* and *run*, and the posterior borders by *prd*; the even-numbered *en* stripes are specified by *ftz* and *odd*. *hh* is thought to be regulated similarly to *en*, based on mutant and ectopic expression studies presented here. *gsb* is regulated by the positive regulator *prd* which defines the anterior and posterior borders of even and odd-numbered *gsb* stripes; consistent with this, *gsb* stripes coincide with those of *prd* at gastrulation (late *prd*). The expression domain of a postulated repressor of *gsb* is illustrated.

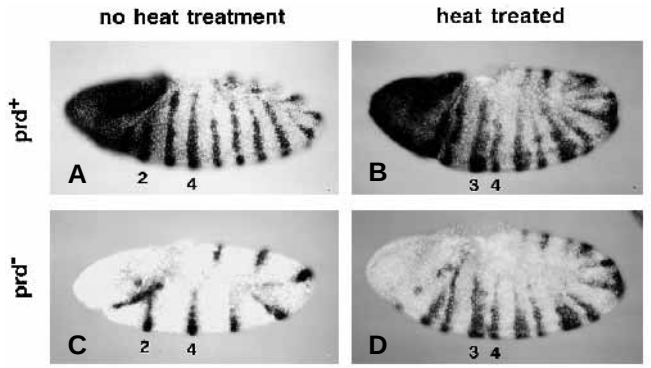

Fig. 2. Rescue of Prd function by ectopic Prd. Illustrated are prd^{+} (A,B) and prd^{-} (C,D) embryos containing a *hs-prd* gene and stained for expression of both *en* and *lacZ* RNA. The homozygous prd^{-} embryos were unambiguously identifiable because they lacked a *hunchback/lacZ* pattern (anterior expression; Driever et al., 1989) exhibited by their prd^{+} siblings which had a *lacZ*-inserted *CyO* balancer chromosome (compare A,B, with C,D). The prd^{-} embryos ($prd^{2.45.17}$) lack *prd* function as a result of a 1.1kb insertion in the paired box of the *prd* gene (Frigerio et al., 1986). In the absence of ectopic *prd* expression (no heat treatment) prd^{-} embryos lack odd-numbered *en* stripes (C). However, ectopic *prd* (heat treated) causes rescue of odd-numbered stripes (D). These stripes are expanded posteriorly as observed in prd^{+} *hs-prd* embryos (B).

of the entire paired domain or its N-terminal half results in loss of in vivo function (Morrissey et al., 1991; Cai et al., 1994). To test whether this loss of function was due to loss of DNA binding by the PD, we made a point mutation designed to disrupt only DNA-binding activity. We made a substitution (G→S) at position 15 of the PD, a residue known to make a DNA base contact (Xu et al., 1995). This substitution, prd^{un}, which is the same as in the *undulated* mutation of mouse *Pax-1* (Balling et al., 1988; Chalepakis et al., 1991), was shown previously by footprinting analysis to disrupt DNA binding by the PD (Treisman et al., 1991). Using a histidine-tagged fragment of the Prd protein, including the PD and HD (amino acids 27-276), we verified by EMSA that the prd^{un} mutation causes a significant reduction in binding to the XPRD PCR-optimized PD site (Fig. 5A).

Before testing the full-length Prd^{un} protein in embryos, we expressed the *hs-*prd^{un} construct in Schneider cells and verified that its function was compromised when acting through the PD but not the HD. *hs-*prd^{un} was co-transfected with a CAT reporter construct with three tandem copies of the PD-binding site of e_5 (PB3-CAT; Treisman et al., 1991). Unlike wild-type *prd* (*hs-prd*), which activated PB3-CAT at moderate levels (Fig. 6A,B, lane 1), *hs-*prd^{un} did not activate this promoter (Fig. 6A,B, lane 5). Quantitation of CAT activities (Fig. 6A) indicated that Prd^{un} had background activities similar to those of PrdΔPB, which has a deletion of the entire PD. We note that the level of activation by wild-type Prd was relatively low (approximately 4× background). Possible reasons for this low level will be discussed below. As indicated in Fig. 6A,C, both *hs-*prd^{un} (lane 5) and *hs-prd* (lane 1) activated transcription at similar levels when tested with a reporter construct with HD-binding sites (3K′-TATA-CAT; Han et al., 1989).

The *hs-*prd^{un} construct was introduced into embryos by germ-line transformation. Heat-treated *hs-*prd^{un} embryos showed uniform RNA when tested with a full-length *prd* probe (Fig. 7B; compare with Fig. 7A), but only endogenous striped *prd* expression was observed when a 144 bp leader sequence probe was used (since *hs-*prd^{un} lacks this leader sequence) (not shown). Immunostaining of *hs-*prd^{un} embryos with a polyclonal antibody directed against Prd showed nuclear expression of the Prd^{un} protein throughout embryos (Fig. 7D; compare with Fig. 7C). Moreover, *hs-*prd^{un} was able to activate throughout embryos a Prd3-lacZ reporter gene with Prd HD-binding sites (Treisman et al., 1989; data not shown).

Despite the ectopic expression of the Prd^{un} protein, the

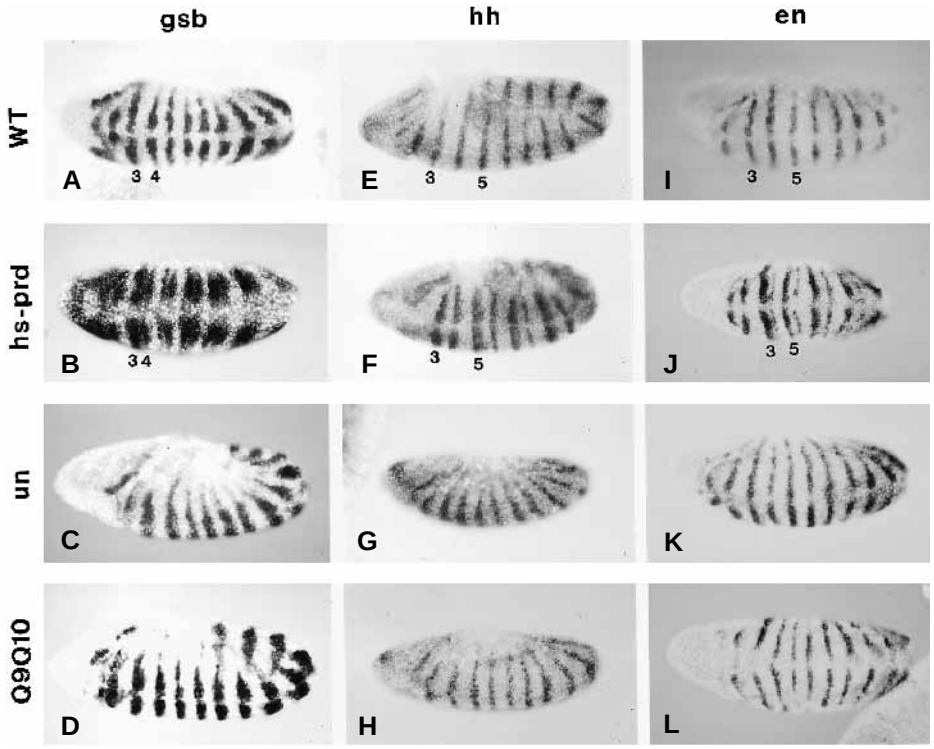

Fig. 3. The Prd^{un} and Prd^{Q9Q10} mutations inactivate Prd function. Illustrated are RNA expression patterns of the segment-polarity genes, *gsb* (A-D), *hh* (E-H), and *en* (I-L) in heat-treated wild-type (A,E,I), *hs-prd* (B,F,J), *hs-*prd^{un} (C,G,K), and *hs-*prd^{Q9Q10} (D,H,L) embryos. Compared to wild-type expression patterns (A), *hs-prd* embryos (B) have posterior expansion of odd-numbered *gsb* stripes, and anterior expansion of even-numbered stripes. Similarly, unlike wild-type embryos (E,I) odd-numbered *hh* (F) and *en* (J) stripes are expanded posteriorly in *hs-prd* embryos. However, these altered expression patterns of *gsb*, *hh*, and *en* are not observed in *hs-*prd^{un} (C,G,K) or *hs-*prd^{Q9Q10} (D,H,L) embryos. Lateral or ventral views of embryos are oriented with anterior to the left.

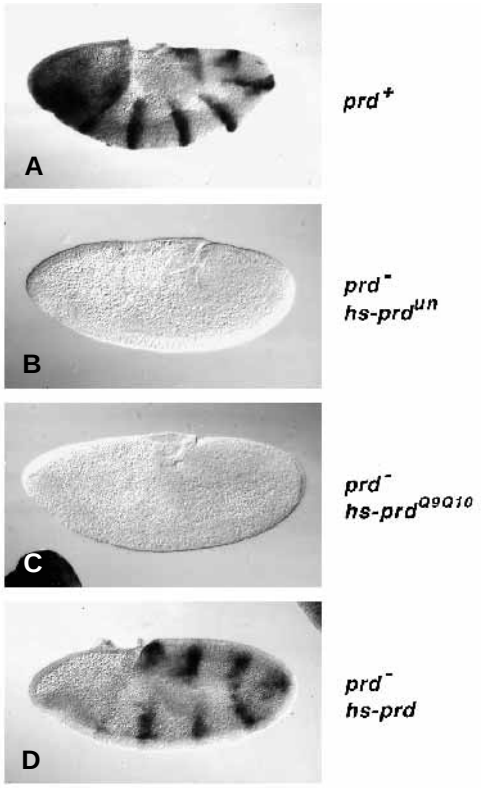

Fig. 4. Prd regulation of late *even-skipped*. Heat-treated embryos (A-D) are stained for lac-Z expression of the late *eve* L-lacZ reporter gene (Fujioka et al., 1995, 1996), and viewed with Nomarski optics. prd^{+} embryos show striped expression of L-lacZ (A). Loss of L-lacZ expression in a prd^{-} background is not rescued by one copy of $hs\text{-}prd^{un}$ (B) nor $hs\text{-}prd^{Q9Q10}$ (C). However, rescue of posteriorly-expanded L-lacZ stripes is observed in prd^{-} *hs-prd* embryos (D).

expression of genes downstream of *prd* was unaltered in heat-treated $hs\text{-}prd^{un}$ embryos. In these embryos, the *hh* and *en* stripes in odd-numbered parasegments were not expanded posteriorly (Fig. 3G,K), whereas in *hs-prd* embryos processed in parallel, characteristic expansion of these stripes was observed. Similarly, ectopic Prd^{un} did not elicit posterior expansion of odd-numbered, or anterior expansion of even-numbered *gsb* stripes (Fig. 3C). Moreover, we saw no evidence for $hs\text{-}prd^{un}$ function in prd^{-} embryos when *en*, *gsb* and *L-lacZ* (late *eve* element) expression were examined. Fig. 4B illustrates the absence of *L-lacZ* stripes in prd^{-}; $hs\text{-}prd^{un}$ when compared to prd^{+} (Fig. 4A) and prd^{-}; *hs-prd* embryos (Fig. 4D). It is unlikely that the inactivity of $hs\text{-}prd^{un}$ in embryos was a consequence of inadequate levels of Prd^{un} protein since similar levels of protein were observed in *hs-prd* and $hs\text{-}prd^{un}$ embryos. Moreover, no effect was observed in $hs\text{-}prd^{un}$ embryos treated with two serial 10-minute heat pulses, whereas *hs-prd* embryos show the characteristic expansions in *en* expression with the same (2× 10 minutes) or lower levels of heat treatment (2× 5 minutes, or 1× 5 minutes; Morrissey et al., 1991, and data not shown). Consistent with our analysis of $hs\text{-}prd^{un}$, Bertuccioli et al. (1996) have observed that prd^{un} under the control of a *prd* promoter is unable to rescue odd-numbered *en* and *gsb* stripes in prd^{-} embryos. Hence, even though Prd^{un} could still function through its HD to activate artificial promoters in tissue culture cells (3K′-TATA-CAT), or embryos (Prd3-lacZ), it exhibited no activation of *en*, *hh*, *gsb* or *L-lacZ* (late *eve*) in *Drosophila* embryos, suggesting that DNA-binding activity by the paired domain is required for the in vivo regulation of these genes.

This conclusion is supported by our in vitro analysis of DNA binding to the known target of Prd, the PTE sequence, which mediates Prd activation of late *eve* expression. Before testing the prd^{un} mutation, we examined binding to PTE by full-length (WT(F); Fig. 5B) and truncated wild-type protein (WT(T); amino acids 27-276, which includes both the PD and HD). The truncated protein shows a single shift (lane 1, Fig. 5B, and Fujioka et al., 1996), whereas the full-length protein bound with a higher affinity (>8-fold) and showed a more complex pattern (lanes 2 and 3). Compared to the truncated protein, the full-length protein shows a slightly slower migrating doublet, which we interpret as monomer bands (M), as well as a much slower migrating doublet, which we interpret as dimer bands (D). The weak band immediately above the pronounced monomer band (lane 3, Fig. 5B; lane 1, Fig. 5C) may have an altered protein conformation or second DNA molecule bound. The weak band immediately below the pronounced dimer band may be similarly explained. At lower protein concentrations (lane 3, Fig. 5B; lanes 1-4, Fig. 5C), the upper band of the dimer doublet predominates, suggesting that the dimer configuration is more stable than that of the monomer. We see a similar dimer doublet with the truncated protein, but only at high protein concentrations (not shown), suggesting that the C-terminal 337 or the N-terminal 26 amino acids contribute to dimerization. The dimer doublet of the truncated protein is relatively closer to the monomer doublet than is the case for the full-length protein, consistent with the interpretation that the upper doublet represents a dimer. Consistent with our observation that $hs\text{-}prd^{un}$ does not activate L-lacZ in embryos, we found that the full-length Prd^{un} binds as a dimer to PTE with >4-fold lower affinity than wild-type protein (Fig. 5C).

DNA binding through the homeodomain

We undertook a similar approach to investigate the importance of the HD DNA-binding activity for in vivo function. We constructed a mutant *prd* gene, prd^{Q9Q10}, encoding an N→Q substitution at position 10 and an S→Q substitution at position 9 of the HD recognition helix (the latter substitution is the same as that found in Ftz). Mutation of the 10th residue was predicted to disrupt DNA binding by the HD because this residue is absolutely conserved in all homeodomain proteins and makes a DNA base contact (Wilson et al., 1995) (also see Hanes and Brent, 1991). Our in vitro analysis confirmed that Prd^{Q9Q10} protein bound to the P2 HD site with significantly lower affinity than wild-type protein (Fig. 5D). Similarly, DNase-protection analysis with two other HD sites, pHD3 and Prd3 (Treisman et al., 1989, 1991), showed a loss of DNA-binding activity through the HD (E. Harris and C. Desplan, personal communication). Moreover, Prd^{Q9Q10} bound PTE more weakly than wild-type Prd (>16-fold; Fig. 5C).

We tested the transcriptional activity of Prd^{Q9Q10} in S3 cells. Unlike *hs-prd*, the $hs\text{-}prd^{Q9Q10}$ mutant could not activate a reporter (3K′-TATA-CAT) with HD-binding sites (Fig. 6A,C, lane 3) indicating that, as expected, the Prd^{Q9Q10} mutation was ineffective at functioning through its HD. Quantitation of CAT activities from multiple experiments indicated that Prd^{Q9Q10}

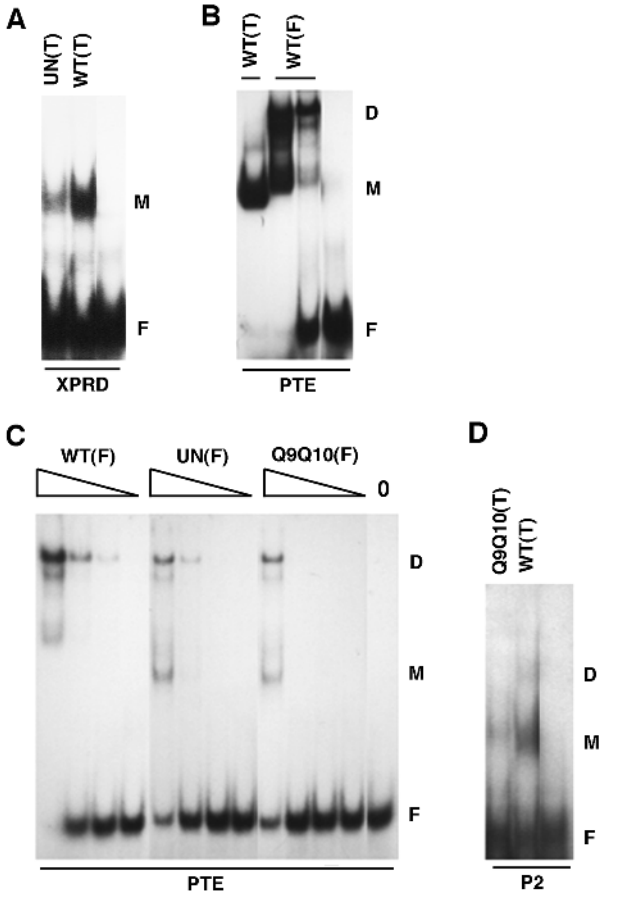

Fig. 5. Gel shift analysis of Paired. Gel shifts were performed using histidine-tagged full-length (613 amino acids) or truncated (amino acids 27-273) proteins containing both PD and HD. (A) Truncated Prd^{un} binds the PD site, XPRD (27 bp probe; GATCAGTGTCA-ACCGTGACGACTGATC, site underlined), with significantly lower affinity (lane 1) than wild-type protein (lane 2). Both proteins, 1.6×10^{-6} M. F, free probe. (B) Truncated wild-type Prd (lane 1; 1.6×10^{-6} M) binds as a monomer (M) to the PD/HD composite site, PTE (26 bp; GATCCACTCACCGTGGCTAATTGTAC). Full-length wild-type protein (lanes 2 and 3; 2.4×10^{-7} M and 6×10^{-8} M) shows a slightly slower migrating monomer doublet, as well as a much slower migrating doublet which we interpret as dimer bands (D). (C) PTE was incubated with 4-fold decreasing concentrations of wild-type Prd, Prd^{un} or Prd^{Q9Q10} starting at 6×10^{-8} M. Wild-type Prd bound PTE with higher affinity than Prd^{un} (>4-fold) or Prd^{Q9Q10} (>16-fold). The monomer bands for UN(F) and Q9Q10(F) are shifted less than that of WT(F), perhaps due to differences in protein conformation or the number of DNA molecules bound. (D) Truncated Prd^{Q9Q10} (lane 1) binds P2 (24 bp; GATCTGATAATTGATTATCAGATC), which contains two HD sites, with lower affinity than wild-type protein (lane 2). Both proteins, 1.6×10^{-6} M.

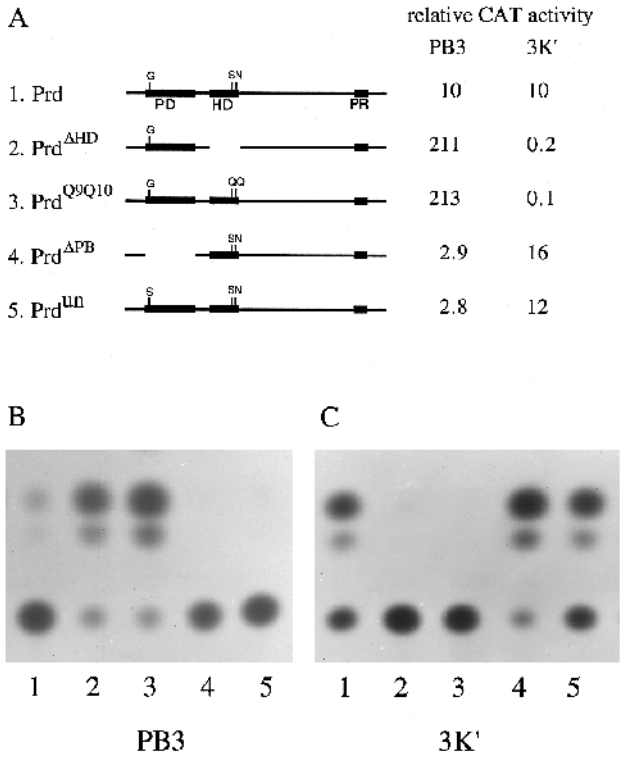

Fig. 6. Transcriptional activation in Schneider cells. Transcriptional activation by *prd* constructs was tested using transient transfection assays. The PB3-CAT reporter gene has 3 binding sites for the PD derived from e_5 (underlined: CTGAGCACCGTTCCGCTCA-GCTGAGCACCGTTCCGCTCAGATAGCACCGTTCCGCTCATA; Treisman et al., 1991); the 3K′-TATA-CAT reporter has multiple consensus HD-binding sites (TCAATTAAAT) embedded within 3 repeats of a 97-nucleotide segment from the *engrailed* promoter (Han et al., 1989). Tested constructs are illustrated in A. Quantitation of relative CAT activities in A is based on four or more independent experiments. (B,C) Examples of experiments with PB3-CAT and 3K′-TATA-CAT. Lanes 1-5 in B and C correspond to constructs 1-5 in A. Prd^{un} (lane 5) and Prd^{Q9Q10} (lane 3) do not activate through PB3-CAT and 3K′-TATA-CAT, respectively; however, Prd^{Q9Q10} activates through PB3-CAT at levels much higher than wild-type Prd. The relative CAT activities in A are standardized to those of wild-type Prd. Comparisons of the absolute CAT activities for PB3-CAT and 3K′-TATA-CAT indicate that both the background levels (e.g. PrdΔHD with 3K′-TATA-CAT) and the activities of single-domain mutants functioning through the unmutated domain (e.g. PrdΔHD with PB3-CAT) were about 5-fold higher with PB3-CAT than 3K′-TATA-CAT. In contrast, for wild-type Prd, which has a functional PD and HD, the absolute activation levels with 3K′-TATA-CAT were approximately 3-fold higher than with PB3-CAT.

had activities similar to those of PrdΔHD (which has a deletion of the HD) which were two orders of magnitude lower than those of wild-type Prd. However, when tested with PB3-CAT, which has PD-binding sites, *hs-prd*^{Q9Q10} retained its ability to activate transcription through its unaltered PD (Fig. 6B, lane 3), indicating that a biologically active protein could be made in S3 cells. Surprisingly, the levels of activation of PB3-CAT by *hs-prd*^{Q9Q10} were considerably higher (over 20-fold) than those of wild-type *hs-prd* (Fig. 6A). Consistent with this being an effect of the HD mutation, similar high activation levels were observed with PrdΔHD (Fig. 6A,B, lane 2). The low activation of PB3-CAT by wild-type *hs-prd* could be a result of titration by other DNA-binding events through the unmutated HD.

To test its in vivo functions, *hs-prd*^{Q9Q10} was introduced into embryos by germ-line transformation. Heat-treated *hs-prd*^{Q9Q10} embryos revealed protein localized to nuclei throughout the embryo (not shown), just as observed with *hs-prd*^{un} (see above).

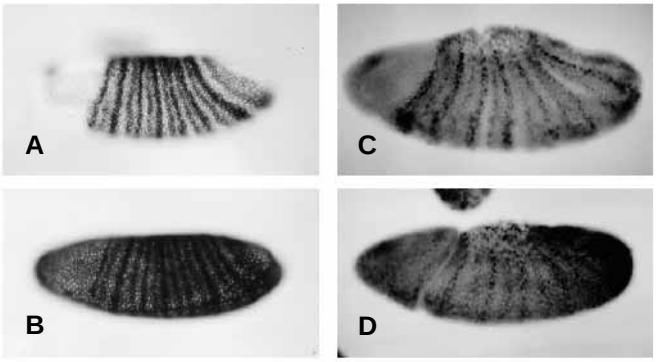

Fig. 7. Prd RNA and protein distribution. Expression of *prd* RNA (A,B) and protein (C,D) is illustrated in wild-type (A,C) and *hs-prd*^{un} (B,D) embryos. Embryos were fixed for in situ hybridization and immunostaining at 10 and 30 minutes respectively after the end of heat treatment. The wild-type embryos (A,C) show striped *prd* expression characteristic of late cycle 14 (see Fig. 1). Superimposed on this striped expression, *hs-prd*^{un} embryos have uniform RNA (B) and protein (D) throughout the embryo. (A,B) Photographed with simultaneous bright-field and DAPI optics to show the distribution of nuclei. The bright-field-only images in C and D illustrate that the Prd protein is localized to the nuclei.

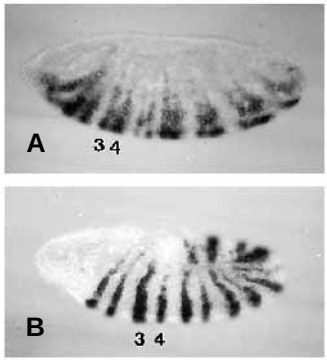

Fig. 8. Co-expression of Prd^{un} and Prd^{Q9Q10} provides Prd function. Illustrated are *gsb* (A) and *en* (B) expression patterns in embryos containing 1 or 2 copies of *hs-prd*^{un} and 2 copies of *hs-prd*^{Q9Q10}. The combination of both mutants reconstitutes ectopic Prd function indicating that the HD and PD DNA-binding activities need not be present in the same Prd molecule for in vivo function.

Moreover, in situ hybridization with a full-length *prd* cDNA probe revealed *prd*^{Q9Q10} RNA throughout embryos and ectopic expression was not observed with the 5′ leader probe specific to the endogenous *prd* RNA (not shown). Furthermore, *hs-prd*^{Q9Q10} was able to activate throughout embryos a PB3-lacZ reporter gene with paired box-binding sites (not shown). However, examination of *gsb*, *hh*, *en* and *L-lacZ* RNA expression in *hs-prd*^{Q9Q10} embryos revealed no evidence for in vivo ectopic function. Unlike *hs-prd* embryos processed in parallel, the staining of *gsb*, *hh* and *en* in *hs-prd*^{Q9Q10} embryos (Fig. 3D,H,L) was indistinguishable from that of heat-treated wild-type embryos with no introduced *prd* gene (Fig. 3A,E,I). Moreover, *hs-prd*^{Q9Q10} was unable to rescue odd-parasegment *en*, *gsb* and *L-lacZ* (late *eve*; Fig. 4C) stripes in *prd*$^{-}$ embryos. Together, the inability of *hs-prd*^{un} and *hs-prd*^{Q9Q10} to regulate *en*, *hh*, *gsb* or *L-lacZ* (late *eve*) suggests that DNA binding by both the HD and PD is required for the in vivo regulation of these genes.

The homeodomain and paired domain DNA-binding activities are not required within the same Prd molecule

Although the above results indicate that DNA-binding activities are required through both the PD and the HD, they do not distinguish whether these activities must be present in the same Prd molecule. In principle, the promoter targets to which Prd molecules bind might require binding through both the PD and HD of the same Prd molecule, as is the case for binding to the e_4 site. Alternatively, the promoter targets may contain a combination of sites that can be bound by either the PD or HD of different Prd molecules. Indeed, the latter possibility is supported by our observation that full-length Prd appears to bind PTE as a dimer. To distinguish between these possibilities, we tested whether the Prd^{un} and Prd^{Q9Q10} proteins could complement each other when co-expressed within the same embryos. Our results indicate that embryos with one or two copies of *hs-prd*^{un} and two copies of *hs-prd*^{Q9Q10} showed the characteristic alterations in *gsb* (Fig. 8A) and *en* (Fig. 8B) expression normally observed in *hs-prd* embryos. These results suggest that the DNA-binding activities of the PD and HD can be provided by different Prd molecules and yet give successful in vivo regulation of target genes.

Quantitation of this *trans*-complementation effect (Table 1) indicated that, surprisingly, the *hs-prd* effect was observed in fewer embryos from parents with two copies of each of *hs-prd*^{un} and *hs-prd*^{Q9Q10} compared to embryos from parents with one copy of *hs-prd*^{un} and two copies of *hs-prd*^{Q9Q10}. About half of the progeny of *hs-prd*^{un}/*SM1*; *hs-prd*^{Q9Q10} parents should have received one copy of *hs-prd*^{un}, and this correlates with the relatively higher levels of stripe expansion seen in these embryos, suggesting that Prd^{un} has a dominant-negative effect on *trans*-complementation. Consistent with this, we have observed cases of *hs-prd*^{un} embryos with deletions of odd-numbered *en* stripes, suggesting poisoning of endogenous Prd function by Prd^{un}. Similar poisoning effects by Prd^{un} have also been seen by Bertuccioli et al. (1996).

DISCUSSION

The results of mutant and ectopic expression studies have provided support for combinatorial models for the function of *prd* and other pair-rule segmentation genes in the regulation of

Table 1. Quantitation of *hs-prd*^{un}/*hs-prd*^{Q9Q10} complementation

parents	*hs-prd*^{un} / *SM1*	*hs-prd*^{Q9Q10} / *hs-prd*^{Q9Q10}	*hs-prd*^{un} / *hs-prd*^{un}	*hs-prd*^{Q9Q10} / *hs-prd*^{Q9Q10}	*hs-prd* / *hs-prd*
en embryos	33% (97)*		12% (51)		90% (96)
gsb	75% (59)†		66% (32)		98% (60)

*Tabulated are the percentages of embryos showing expansion of 2 or more odd-numbered *en* or *gsb* stripes. The total numbers of scored embryos of appropriate age are illustrated in parenthesis. Similar results were obtained in multiple experiments; the combined results from two experiments for each probe are illustrated.

†The levels of stripe expansion were consistently higher for *gsb* than for *en*.

downstream segment-polarity genes. The pair-rule genes are generally expressed in coarse striped patterns with two-segmental periodicity and the regions of overlap between combinations of these regulators define the more refined patterns of segment-polarity genes. Both loss-of-function mutations in pair-rule genes and ectopic expression of these genes redefine the regions of overlap, and thereby modify in predictable ways the expression patterns of the downstream genes. For example, *prd* defines the posterior borders of the odd-numbered *en* stripes and ectopic expression of *prd* shifts these borders posteriorly (Morrissey et al., 1991). In this study, we found that the stripes of *hh* expression, which coincide with those of *en*, are similarly expanded posteriorly in *hs-prd* embryos. This result, and the parallel effects of pair-rule mutants on *hh* and *en* expression (DiNardo and O'Farrell, 1987; Howard and Ingham, 1986; Ingham et al., 1988; Lee et al., 1992), suggest that both genes are regulated by similar mechanisms. Ectopic expression experiments indicate that *prd* also defines the posterior borders of the late *eve* stripes, which are expressed in the same cells as the odd-numbered *en* stripes and are hypothesized to reinforce these stripes by excluding repressors (Fujioka et al., 1995, 1996). The expression of *gsb*, which coincides with the late 14-stripe pattern of *prd*, is also modified in *hs-prd* embryos: the posterior borders of odd-numbered *gsb* stripes are expanded posteriorly, and anterior borders of even-numbered stripes are expanded anteriorly, suggesting that *prd* is a positive regulator of *gsb* and normally defines the respective expression borders of the odd- and even-numbered stripes. As illustrated in Fig. 1, cells in the *gsb* interband unaffected by ectopic *prd* (posterior of even-numbered *gsb* stripes) transiently express *prd* during its earlier seven-stripe pattern. We suggest that this *gsb* interband is probably defined by another pair-rule regulator acting as a repressor in this domain. Although our present study suggests that *prd* is a positive regulator of both even- and odd-numbered *gsb* stripes, previous analysis (Bopp et al., 1989) showed that in prd^{-} embryos, only the odd-numbered stripes are deleted. This retention of even-numbered stripes in prd^{-} mutants may reflect a redundancy in the specification of even-numbered stripes, which may also be regulated by a second activator yet to be identified.

Two DNA-binding activities of Paired are required for in vivo function

In this study of *Drosophila* Prd, we tested the functions of point mutants in the paired domain and homeodomain (Prd^{un} and Prd^{Q9Q10}, respectively), which lack normal DNA-binding activities of the respective mutated domains. Since the mutations involve substitutions of only one (Prd^{un}) or two (Prd^{Q9Q10}) amino acids, it is unlikely that the mutations affect any other properties of the Prd protein such as protein-protein interactions. Our results indicate that when tested alone, neither mutant exhibited any in vivo function as judged by our ectopic expression and rescue assays monitoring four target genes. This result shows for the first time that DNA binding through both the PD and the HD of wild-type Prd protein is required for the in vivo regulation of normal Prd targets. For none of the tested targets is there evidence that DNA binding through one of the two domains is sufficient for in vivo regulation. Why are both of these binding activities required for in vivo function? Previous in vitro studies of homeoproteins have revealed that, in general, homeoproteins have surprisingly ubiquitous DNA-binding specificities, despite the distinct functions that these proteins have in vivo (Scott et al., 1989). It has been suggested that protein interactions with cofactors can enhance the DNA-binding specificities of homeoproteins (Goutte and Johnson, 1988; Grueneberg et al., 1992; Smith and Johnson, 1992; Chan et al., 1994). For example, binding of MCM1 protein to $\alpha 2$ homeoprotein dimers places constraints on the permitted spacing between the adjacent monomer target sequences (Smith and Johnson, 1992). From our current study, we suggest that another strategy for conferring specificity of targeting to a homeoprotein is for the protein (or protein multimer) to have a second DNA-binding activity that functions in combination with the HD, thereby constraining the range of possible target sequences that the protein(s) bind with high efficiency. By this model, the targeting specificities of Prd would be defined at least in part by the combination of specificities of the Prd HD and PD, consistent with our observations that both binding activities are required for in vivo function.

Paired can bind DNA as a dimer

Our gel shift analysis suggests that full-length wild-type Prd binds PTE DNA as a dimer. Prd^{Q9Q10} and Prd^{un} bind PTE more weakly than wild-type Prd, suggesting that DNA-binding activities of both the HD and PD of wild-type protein contribute to dimer formation. Our observations that both mutant proteins can bind PTE as a dimer, and our finding that wild-type Prd can bind the single PD site, XPRD, as a dimer (as well as a monomer; data not shown), together suggest that protein interactions between Prd molecules probably contribute to dimer formation. However, the putative protein interaction is probably dependent upon DNA binding through the PD or HD, given that no protein interaction was observed between full-length Prd/LexA fusions in a yeast two-hybrid test using the LexA DNA-binding domain (data not shown). Moreover, the protein interaction appears to be stabilized by the N-terminal 26 or C-terminal 337 amino acids of the protein, given that a protein truncated shortly after the HD (amino acids 27-276) will only bind as a dimer at high protein concentrations (data not shown). In principle, dimer formation could be mediated by DNA binding through the HD of one molecule and the PD of the other. However, although isolated PD and HD protein fragments bind cooperatively to PH0, a composite site very similar to PTE (Jun and Desplan, 1996), we have not thus far seen evidence for significant cooperativity between full-length Prd^{Q9Q10} and Prd^{un} (data not shown).

Prd^{un} and Prd^{Q9Q10} can *trans*-complement

When Prd^{un} and Prd^{Q9Q10} are co-expressed in the same embryo, the two mutants complement each other and exhibit apparently wild-type Prd function. This result indicates that although the PD- and HD-binding activities are both required, they can be present in separate protein molecules, suggesting that the in vivo binding sites are qualitatively unlike the e_4 site, which requires the two binding activities to be in the same molecule (Treisman et al., 1991). Instead, the critical in vivo sites appear to be equivalent to either single-domain sites for the PD (e.g., XPRD; Jun and Desplan, 1996) or the HD (e.g., P2; Wilson et al., 1993), or PD/HD composite sites that can be bound simultaneously by the HD and PD of separate molecules, as has been shown for example in footprinting analysis of e_5 (Treisman et al., 1991). Quantitation of the *trans*-

complementation result (Table 1) indicates that embryos with two copies of each of *hs-prd*^{un} and *hs-prd*^{Q9Q10} have lower activation of *en* and *gsb* than embryos with one copy of *hs-prd*^{un} and two copies of *hs-prd*^{Q9Q10}, suggesting that higher levels of Prd^{un} protein have a dominant-negative effect on *trans*-complementation. Similarly, Prd^{un} has a dominant-negative effect on wild-type Prd function (this study and Bertuccioli et al., 1996). These results are consistent with the observations that Prd can bind DNA as a dimer (this study and Wilson et al., 1993, 1995), and that protein-protein interactions may contribute to dimerization. It is possible that Prd^{un} competes with Prd^{Q9Q10} or wild-type Prd through protein interactions and causes a dominant-negative effect because its PD DNA-binding activity is mutated. Consistent with this model, *trans*-complementation by *hs-prd*^{un} and *hs-prd*^{Q9Q10} gives rise to significantly less function than wild-type *hs-prd* (Table 1). Similarly, Bertuccioli et al. (1996) have not observed *trans*-complementation between equivalent Prd mutants driven by a *prd* promoter fragment, probably due to insufficient expression levels. Moreover, difficulties in combining sufficient heat-shock transgenes in the same embryo have not allowed testing for *trans*-complementation in a *prd*$^{-}$ background.

Paired is a model for PAX-3 function

The functions of the *Drosophila* Prd protein are of particular interest because a number of vertebrate homologs of Prd containing both a PD and HD have been identified and shown to be developmentally important. The closest mammalian homolog of *Drosophila* Prd is *Pax-3*. Mutations in mouse *Pax-3* are responsible for the spina-bifida-associated *Splotch* phenotype (Epstein et al., 1991) and mutations in Human *PAX-3* (*HuP2*; Burri et al., 1989) are associated with Waardenburg Syndrome, which involves deafness and pigment and facial structure defects caused by dysfunction of embryonic neural crest cells (Baldwin et al., 1992; Tassabehji et al., 1992). Molecular characterization of several human *PAX-3* mutations (Baldwin et al., 1992; Tassabehji et al., 1992; Hoth et al., 1993; Chalepakis et al., 1994) has revealed amino-acid substitutions (clustered close to the *undulated* mutation) or small deletions in the PD, which are expected to disrupt normal DNA binding of the PD. These *PAX-3* mutations behave as autosomal dominants. The observations that Prd^{un} has dominant-negative effects (this study and Bertuccioli et al., 1996) provides a possible model for the dominant effects of these *PAX-3* mutations, which may similarly be explained by protein interactions with functional PAX-3 molecules (see also Chalepakis et al., 1994).

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