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 dispensable 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 (XPDR: 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 stripped 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 (Fujioika et al., 1996). Two other Prd-binding sites have also been identified in the eve promoter: the e4 site has two half sites in the same order as PH0 and PTE but spaced 1 bp closer; the e4 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 e4, either independently or simultaneously (Treisman et al., 1991; Jun and Desplan, 1995; Fujioika et al., 1996). In contrast, e4 is bound only by Prd protein containing both a functional PD and HD (Treisman et al., 1991). Deletion analysis suggests that e4 may contribute to the strength of late eve expression (Fujioika et al., 1996). The possible in vivo significance of e4 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 e4) 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<sup>am</sup> 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: GGGTG-GAGTTTCCATCAACA; 3’ primer: GTTGGCTTGGGAGAC-GAG). This product was used as a megaprimers in a (simultaneous) second PCR that extended the megaprimer in the 3’ direction to attach prd leader sequence; the second PCR used predfHTD (prd c7340.6 cDNA with a fushi tarazu (fz) homeobox; Frigerio et al., 1986) as template, 5’ primer GTTTCGAGGAGGCT, and 3’ primer GTTGC-GCTTGGGAGAC-GAG. The resulting PCR product was digested with HindIII and EagI and subcloned into the corresponding sites of pGEM3ZF/+prdAPB (Morrissey et al., 1991) to generate a full-length prd gene containing the undulated mutation (prd<sup>am</sup>). 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<sub>2</sub>, 16.6 mM NH<sub>4</sub>SO<sub>4</sub>, 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 fragment, Q9Q10, into the prd cDNA, a 399 bp EagI-PvuII 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<sup>Q9Q10</sup> 3’ primer (ACGCCGCCTGGTTG) 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<sup>am</sup> and prd<sup>Q9Q10</sup> genes were sequentially subcloned (Morrissey et al., 1991) into the heat-shock vector pHSHII (Malicki et al., 1990) and then the P-element vector pW8 (Klemenza et al., 1987). The resulting hs-prd<sup>am</sup> and hs-prd<sup>Q9Q10</sup> constructs were injected with helper plasmid (p25.7w) into Df(1L)w<sup>67Cz</sup> embryos, and white<sup>+</sup> 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 or 5 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 32P-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 transfection 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 pH5B1), CAT reporter genes (PB3-CAT or 3K-TATA-CAT) and D88:GaZ (GaZ driven by a heat-shock promoter). Cells were heat treated (to allow for prd and GaZ expression) and extracted for CAT assays. In all experiments, 1 μg each of prd and GaZ 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 (Morrisey 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 (runt) specify the anterior borders of these stripes, and the even-numbered stripes are specified by fz 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 (prd24.17; 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; Gutjah 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
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 PD-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, prdG15S, 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 prdG15S mutation causes a significant reduction in binding to the XPDRD PCR-optimized PD site (Fig. 5A).

Before testing the full-length PrdG15S protein in embryos, we expressed the hs-prdG15S construct in Schneider cells and verified that its function was compromised when acting through the PD but not the HD. hs-prdG15S was co-transfected with a CAT reporter construct with three tandem copies of the PD-binding site of e5 (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-prdG15S did not activate this promoter (Fig. 6A,B, lane 5). Quantitation of CAT activities (Fig. 6A) indicated that PrdG15S 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-prdG15S (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-prdG15S construct was introduced into embryos by germ-line transformation. Heat-treated hs-prdG15S 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-prdG15S lacks this leader sequence) (not shown). Immunostaining of hs-prdG15S embryos with a polyclonal antibody directed against Prd showed nuclear expression of the PrdG15S protein throughout embryos (Fig. 7D; compare with Fig. 7C). Moreover, hs-prdG15S 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 PrdG15S protein, the
expression of genes downstream of \textit{prd} was unaltered in heat-treated \textit{hs-prd\textsuperscript{un}} embryos. In these embryos, the \textit{hh} and \textit{en} stripes in odd-numbered parasegments were not expanded posteriorly (Fig. 3G,K), whereas in \textit{hs-prd} embryos processed in \textit{hs-prd\textsuperscript{un}} when compared to \textit{prd\textsuperscript{+}} (Fig. 4A) and \textit{prd\textsuperscript{-}}; \textit{hs-prd} embryos (Fig. 4D). It is unlikely that the inactivity of \textit{hs-prd\textsuperscript{un}} in embryos was a consequence of inadequate levels of Prd\textsuperscript{un} protein since similar levels of protein were observed in \textit{hs-prd} and \textit{hs-prd\textsuperscript{un}} embryos. Moreover, no effect was observed in \textit{hs-prd\textsuperscript{un}} embryos treated with two serial 10-minute heat pulses, whereas \textit{hs-prd} embryos show the characteristic expansions in \textit{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 \textit{hs-prd\textsuperscript{un}}, Bertucciolai et al. (1996) have observed that \textit{prd\textsuperscript{un}} under the control of a \textit{prd} promoter is unable to rescue odd-numbered \textit{en} and \textit{gsb} stripes in \textit{prd\textsuperscript{-}} embryos. Hence, even though Prd\textsuperscript{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 \textit{en}, \textit{hh}, \textit{gsb} or L-lacZ (late \textit{eve}) in \textit{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 \textit{eve} expression. Before testing the \textit{prd\textsuperscript{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) 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 \textit{hs-prd\textsuperscript{un}} does not activate L-lacZ in embryos, we found that the full-length Prd\textsuperscript{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 \textit{prd} gene, \textit{prd\textsuperscript{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\textsuperscript{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, \textit{pHD3} and \textit{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\textsuperscript{Q9Q10} bound PTE more weakly than wild-type Prd (>16-fold; Fig. 5C).

We tested the transcriptional activity of Prd\textsuperscript{Q9Q10} in S3 cells. Unlike \textit{hs-prd}, the \textit{hs-prd\textsuperscript{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\textsuperscript{Q9Q10} mutation was ineffective at functioning through its HD. Quantitation of CAT activities from multiple experiments indicated that Prd\textsuperscript{Q9Q10}
had activities similar to those of Prd\(\Delta\)HD (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 Prd\(\Delta\)HD (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\(\Delta\)HD (see above).
Moreover, in situ hybridization with a full-length prd cDNA probe revealed prd<sub>Q9Q10</sub> 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<sub>Q9Q10</sub> 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<sub>Q9Q10</sub> embryos revealed no evidence for in vivo ectopic function. Unlike hs-prd embryos processed in parallel, the staining of gsb, hh, en and L-lacZ RNA expression in hs-prd<sub>Q9Q10</sub> embryos was indistinguishable from that of heat-treated wild-type embryos with no introduced prd gene (Fig. 3A,E,I). Moreover, hs-prd<sub>Q9Q10</sub> was unable to rescue odd-parasegment en, gsb or L-lacZ (late eve; Fig. 4C) stripes in prd<sup>-</sup> embryos. Together, the inability of hs-prd<sub>Q9Q10</sub> 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<sub>i</sub> 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<sup>un</sup> and Prd<sup>Q9Q10</sup> 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<sup>un</sup> and two copies of hs-prd<sup>Q9Q10</sup> 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<sup>un</sup> and hs-prd<sub>Q9Q10</sub> compared to embryos from parents with one copy of hs-prd<sup>un</sup> and two copies of hs-prd<sub>Q9Q10</sub>. About half of the progeny of hs-prd<sup>un</sup>/SM1; hs-prd<sub>Q9Q10</sub> parents should have received one copy of hs-prd<sup>un</sup>, and this correlates with the relatively higher levels of stripe expansion seen in these embryos, suggesting that Prd<sup>un</sup> has a dominant-negative effect on trans-complementation. Consistent with this, we have observed cases of hs-prd<sup>un</sup> embryos with deletions of odd-numbered en stripes, suggesting poisoning of endogenous Prd function by Prd<sup>un</sup>. Similar poisoning effects by Prd<sup>Q9Q10</sup> 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 target gene activity. These results are consistent with the idea that Prd functions as a dimer, providing DNA binding through both the PD and HD.

**Table 1. Quantitation of hs-prd<sup>un</sup>/hs-prd<sub>Q9Q10</sub> complementation**

<table>
<thead>
<tr>
<th>Parents</th>
<th>hs-prd&lt;sup&gt;un&lt;/sup&gt;</th>
<th>hs-prd&lt;sub&gt;Q9Q10&lt;/sub&gt;</th>
<th>hs-prd&lt;sup&gt;un&lt;/sup&gt;</th>
<th>hs-prd&lt;sub&gt;Q9Q10&lt;/sub&gt;</th>
<th>hs-prd</th>
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<td>SM1</td>
<td></td>
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<tr>
<td>en</td>
<td>33% (97)†</td>
<td>12% (51)</td>
<td></td>
<td>90% (96)</td>
<td></td>
</tr>
<tr>
<td>gsb</td>
<td>75% (59)†</td>
<td>66% (32)</td>
<td></td>
<td>98% (60)</td>
<td></td>
</tr>
</tbody>
</table>

†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.

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<sup>un</sup> (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<sup>un</sup> 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<sup>un</sup> and Prd<sub>Q9Q10</sub> provides Prd function. Illustrated are gsb (A) and en (B) expression patterns in embryos containing 1 or 2 copies of hs-prd<sup>un</sup> and 2 copies of hs-prd<sub>Q9Q10</sub>. 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.
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 (Fujioika 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 (Prdum and PrdQ9Q10, respectively), which lack normal DNA-binding activities of the respective mutated domains. Since the mutations involve substitutions of only one (Prdun) or two (PrdQ9Q10) 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 α2 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. PrdQ9Q10 and Prdun 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 PHO, a composite site very similar to PTE (Jun and Desplan, 1996), we have not thus far seen evidence for significant cooperativity between full-length PrdQ9Q10 and Prdun (data not shown).

Prdun and PrdQ9Q10 can trans-complement

When Prdun and PrdQ9Q10 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 e4 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 e5 (Treisman et al., 1991). Quantitation of the trans-
complementation result (Table 1) indicates that embryos with two copies of each of hs-prdΔun and hs-prdQ9Q10 have lower activation of en and gsb than embryos with one copy of hs-prdΔun and two copies of hs-prdQ9Q10, suggesting that higher levels of PrdΔun protein have a dominant-negative effect on trans-complementation. Similarly, PrdΔun 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 PrdΔun competes with PrdQ9Q10 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-prdΔun and hs-prdQ9Q10 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 *Sploch* phenotype (Epstein et al., 1991) and mutations in human *PAX-3* (*HufPa*; 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; Hoth et al., 1993; Chalepakis et al., 1996) 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 PrdΔun 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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