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Dual role of the Pax gene *paired* in accessory gland development of *Drosophila*

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SUMMARY

The *Drosophila* Pax gene *paired* encodes a transcription factor that is required for the activation of segment-polarity genes and proper segmentation of the larval cuticle, postembryonic viability and male fertility. We show that *paired* executes a dual role in the development of male accessory glands, the organ homologous to the human prostate. An early function is necessary to promote cell proliferation, whereas a late function, which regulates the expression of accessory gland products such as the sex peptide and Acp26Aa protein, is essential for maturation and differentiation of accessory glands. The late function exhibits in main and secondary secretory cells of accessory glands dynamic patterns of Paired expression that depend in both cell types on the mating activity of adult males, possibly because Paired expression is regulated by negative feedback. The early Paired function depends on domains or motifs in its C-terminal moiety and the late function on the DNA-binding specificity of its N-terminal paired-domain and/or homeodomain. Both Paired functions are absolutely required for male fertility, and both depend on an enhancer located within 0.8 kb of the downstream region of *paired*.

Key words: Accessory gland, Cell differentiation, Cell proliferation, *Drosophila*, Pax genes, *paired*

INTRODUCTION

The *Drosophila* accessory gland is a secretory organ of the male reproductive system and a functional homolog of the human prostate. It secretes a complex mixture of proteins, lipids and carbohydrates that are transferred, together with sperm produced by the testes, to females during copulation (Chen, 1984). Accessory gland secretions (or seminal fluid) induce a number of physiological and behavioral responses in mated females, including increased oviposition, reduced sexual receptivity, diminished attractiveness to males and shortened life expectancy (Chen, 1984; Chen, 1996; Kubli, 1996; Wolfner, 1997). In addition, components of the seminal fluid are absolutely required for sperm fertility (Xue and Noll, 2000) and essential for the storage of sperm in the female genital tract (Tram and Wolfner, 1999).

The accessory glands are a pair of dead-end tubes that branch off the male genital tract at the anterior end of the ejaculatory duct. They arise from a special set of cells in the male primordium of the genital disc (Nöthiger et al., 1977) whose developmental fate is determined by the male sex determination pathway during the third larval instar (Chapman and Wolfner, 1988). Each accessory gland is composed of a single layer of secretory cells surrounded by a sheath of muscle cells that squeeze the gland and force the accumulated secretions into the ejaculatory duct during mating. The secretory cells consist of two morphologically distinct types of cells, the predominant 'main cells', which comprise about 1000 cells per lobe, and the 40-50 'secondary cells' (Bairati, 1968; Bertram et al., 1992). The main cells are flat, hexagonal, binucleate cells that surround the lumen of the glands. Interspersed between the main cells at the distal end of each lobe are the secondary cells, which are large, spherical, binucleate cells with large vacuoles. Each cell type produces and secretes a characteristic set of products, and thus may contribute to a subset of the responses elicited in mated females. Despite extensive studies on the functions of the accessory gland fluid, little is known about the regulation of its components and the molecular mechanisms that specify accessory gland development.

The *Drosophila* Pax gene *paired* (*prd*), initially characterized as a pair-rule segmentation gene required for the establishment of positional information along the anteroposterior axis in the *Drosophila* embryo (Nüsslein-Volhard and Wieschaus, 1980; Kilchherr et al., 1986), encodes a transcription factor whose N-terminal moiety contains two DNA-binding domains, a paired-domain and a *prd*-type homeodomain (Bopp et al., 1986; Treisman et al., 1991; Noll, 1993). In addition to its role in promoting proper segmentation of the larval cuticle (Nüsslein-Volhard and Wieschaus, 1980), *prd* is necessary for postembryonic viability and male fertility (Bertuccioli et al., 1996; Xue and Noll, 1996; Xue and Noll, 2000). Investigation of this male fertility function has revealed that *prd* is required for accessory gland formation (Bertuccioli

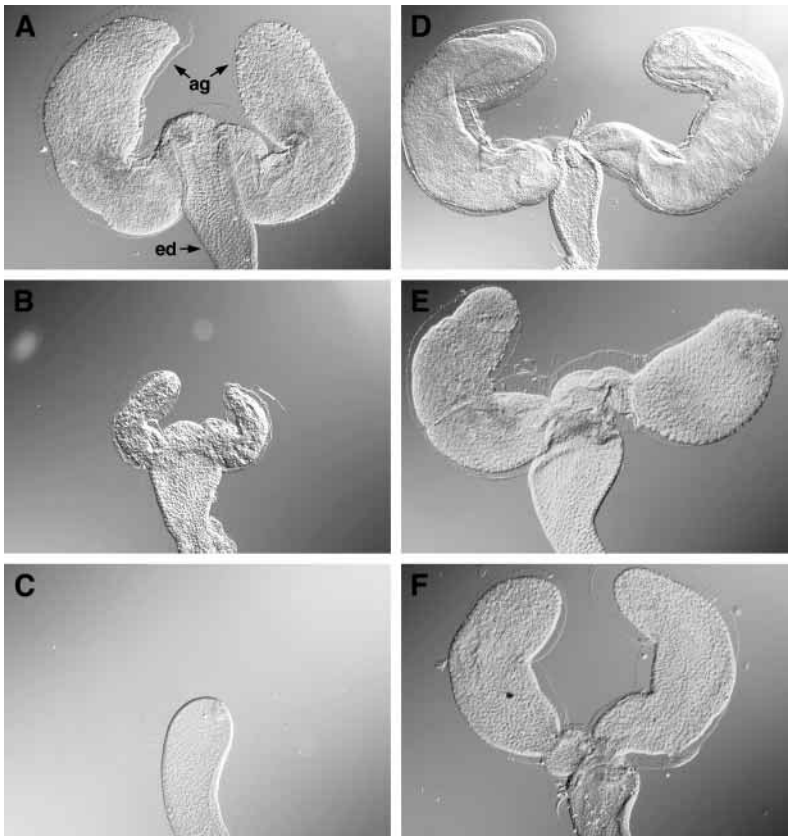

Fig. 1. *prd* is essential for accessory gland formation. (A,D) Accessory glands (ag) dissected from wild-type (A) and *Df(2L)Prl/prd*$^{2.45}$ male rescued by one copy of the *prd*-SN20 transgene (D). (B,C) Highly underdeveloped (B) or absence (C) of accessory glands in *Df(2L)Prl/prd*$^{2.45}$ males rescued to adulthood by two copies of *prd*-Gsb (B) or *prd*Res (C) transgenes. (E,F) Complete rescue of accessory glands by one copy of *prd*-mf5 transgene in *Df(2L)Prl prd*-mf5*/prd*$^{2.45}$ males rescued to adulthood by two copies of *prd*-Gsb (E) or *prd*Res (F) transgenes. Testes and seminal vesicles connecting them to the anterior end of the ejaculatory duct (ed) have been removed here and in Fig. 3, Fig. 4, Fig. 5.

et al., 1996; Xue and Noll, 2000). We demonstrate that this requirement consists of a dual role of *prd* in accessory gland development, an early function required for cell proliferation and a late differentiation function, regulating the expression of accessory gland products. While the early function depends on a domain or motif present in the C-terminal moiety of Prd, the late function crucially depends on the DNA-binding specificity of the N-terminal region of Prd. Both functions are essential for male fertility and require an enhancer located in the downstream *cis*-regulatory region of *prd*.

MATERIALS AND METHODS

Plasmid constructions and generation of transgenic flies

All *prd*-mf transgenes were derived from the *prd*-mf0 vector, which was generated in two steps. First, the 3.9 kb *Pvu*II-*Hin*dIII fragment from *prd*-SN20, encoding 233 bp upstream, 2.8 kb transcribed and 0.90 kb downstream sequences of *prd*, was subcloned by combining its 1.5 kb *Pvu*II-*Pst*I and 2.4 kb *Pst*I-*Hin*dIII subfragments between the *Eco*RV and *Hin*dIII sites of pSK$^-$ to produce the pSK+prdBasal plasmid. The *prd*-mf0 vector was then obtained by inserting, in the appropriate orientation, the 3.6 kb *Spe*I-*Xba*I fragment from pSK+prdBasal into the *Xba*I site of the pDA188.E1 vector, which is a P-element vector containing the Gal4-coding region placed under control of the *hsp70* minimal promoter and including the *tubulinα1* trailer (prepared and kindly provided by D. Nellen and K. Basler).

To generate *prd*-mf1 to *prd*-mf8, the 5.2 kb *Xba*I-*Sal*I *prd* downstream fragment, produced by *Sal*I- and partial *Xba*I-mediated digestion of *prd*-SN20, was first subcloned in the pSK$^-$ plasmid, and the *Xba*I site close to the *Sal*I site was destroyed by partial digestion with *Xba*I, blunt-ending and religation. Subsequently, the *prd* downstream fragments between *Xba*I and *Sal*I, corresponding to *prd*-mf1 to *prd*-mf8, were removed from this plasmid and blunt-end ligated into the *Sma*I site of the pKSpL5 plasmid (Xue and Noll, 1996), from which they were again recovered as *Xba*I fragments and inserted, in the proper orientation, into the *Xba*I site of *prd*-mf0.

To obtain *prd*-mf9 and *prd*-mf10, the 1.6 kb *Eco*RI-*Sal*I *prd* downstream fragment was cloned into the *Eco*RI site of the pKSpL2 plasmid (Gutjahr et al., 1994). From this plasmid, fragments were amplified by PCR by the use of the primers T7 (5′-AAT ACG ACT CAC TAT AG-3′) and pmf9down (5′-CAT TGT GTG TGC GGC CGC GAC TCT AG-3′; underlined bases do not pair with the *prd* downstream sequence to generate a *Not*I site), or pmf10up (5′-CAC TAG TCG CGG GTC CAC ACA CAA T-3′; underlined bases do not pair with the *prd* downstream sequence to generate a *Spe*I site) and T3 (5′-ATT AAC CCT CAC TAA AG-3′), digested with *Spe*I and *Not*I, and inserted between the *Spe*I and *Not*I sites of *prd*-mf0.

All the rescue constructs were injected together with pUChsΔ2-3 P-element helper plasmid (prepared by D. Rio and provided by E. Hafen) into *w*^{1118} embryos, and *w*$^+$ transformants were selected (Rubin and Sprading, 1982).

Dissection, immunostaining and X-Gal staining of accessory glands

Accessory glands were dissected (Xue and Noll, 2000) and stained with antisera directed against Prd (Gutjahr et al., 1993a), Acp26Aa (Monsma et al., 1990) and Gsb (Gutjahr et al., 1993b) as described elsewhere (Monsma et al., 1990). X-Gal staining was performed according to Bertram et al. (Bertram et al., 1992).

Fly stocks

Fly strains used in this work are: *Df(2L)Prl*, *prd*$^{2.45}$ and *prd*-Gsb (Xue and Noll, 1996), *prd*Res (Bertuccioli et al., 1996), *UAS*-Prd (Jiao et al., 2001), *UAS*-Myc (Johnston et al., 1999), *UAS*-CycE (Neufeld et al., 1998), *UAS*-P35 (Hay et al., 1994), *UAS*-Dp110 (Leevers et al., 1996), *Df(3L)th102*, *h kni*$^{ri-1}$ *e*^{s}*/TM6C*, *Sb* (Meier et al., 2000), *Df(3L)H99*, *kni*$^{ri-1}$ *p*^{p}*/TM3*, *Sb* (White et al., 1994) and *sp-lacZ* (D. Styger-Schmucki, PhD Thesis, University of Zürich, 1992).

RESULTS AND DISCUSSION

prd is required for accessory gland formation

As a member of the pair-rule gene family, the *prd* gene regulates the expression of segment-polarity genes in a double-segment periodicity and thus specifies the segmental pattern of the larval cuticle. All known *prd* mutant alleles are deficient for this function of *prd* and hence embryonic lethal (Tearle and

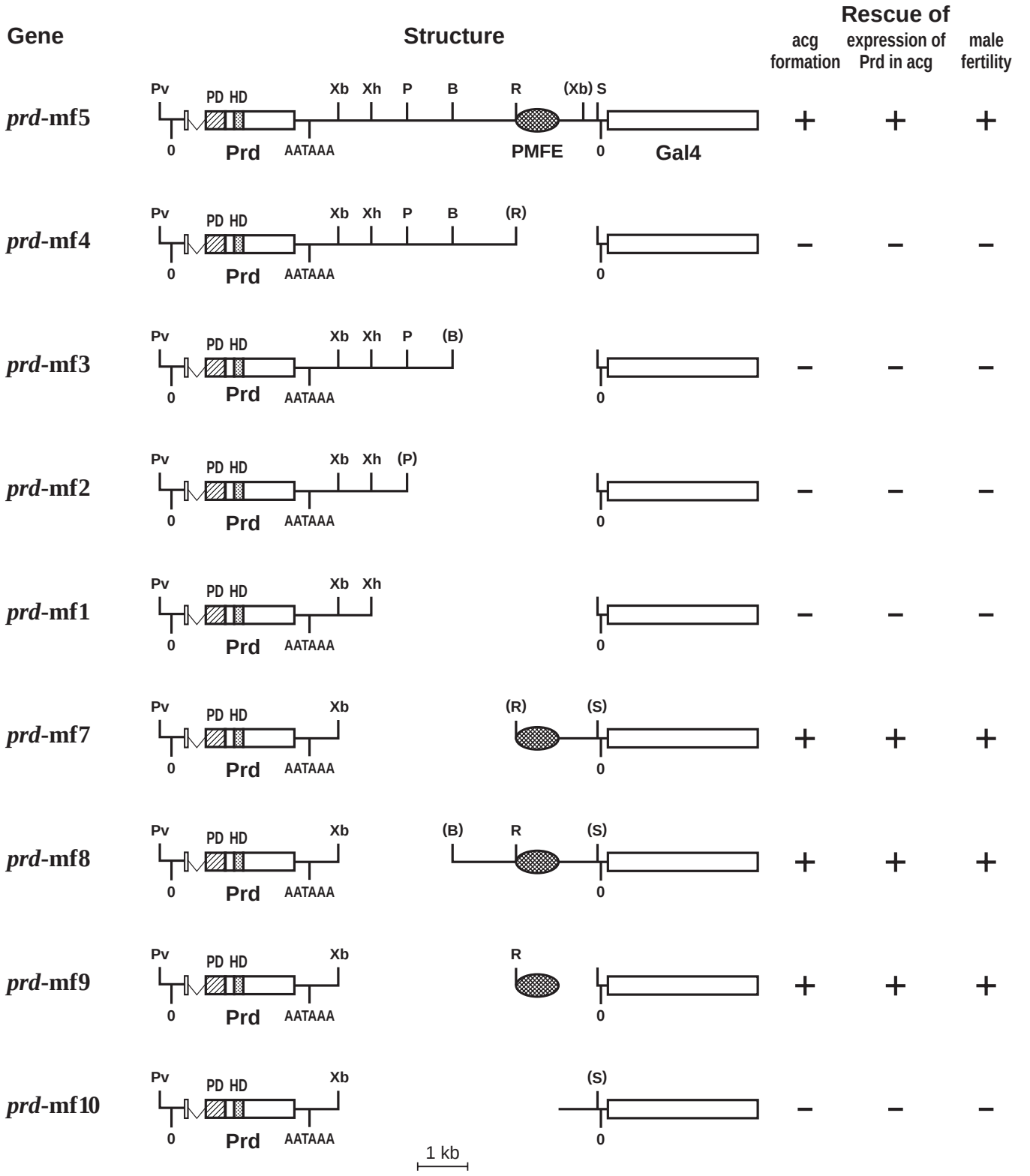

Fig. 2. Rescue by *prd* transgenes of *prd* functions in accessory gland development. Maps of *prd* transgenes listed in the left column are shown, while their abilities to rescue accessory gland (acg) formation, Prd expression in adult accessory glands, and male fertility in *prd* mutant males rescued by *prd*-Gsb or *prd*Res are indicated on the right. The Prd-coding region is indicated as open boxes, interrupted by the v-shaped *prd* intron, with the paired-domain PD hatched and the *prd*-type homeodomain HD stippled, and upstream and downstream sequences as straight line. Positions of transcriptional starts (0) and poly(A) addition signal (AATAAA) are marked, and the Gal4-coding region is shown as open box. The region including the enhancer responsible for the development of functional accessory glands, PMFE (Prd Male Fertility Enhancer), has been mapped to a 0.8 kb fragment downstream of the *Eco*RI site in the *prd* downstream region. Restriction sites in parentheses have been destroyed. B, *Bam*HI; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; Xb, *Xba*I; Xh, *Xho*I.

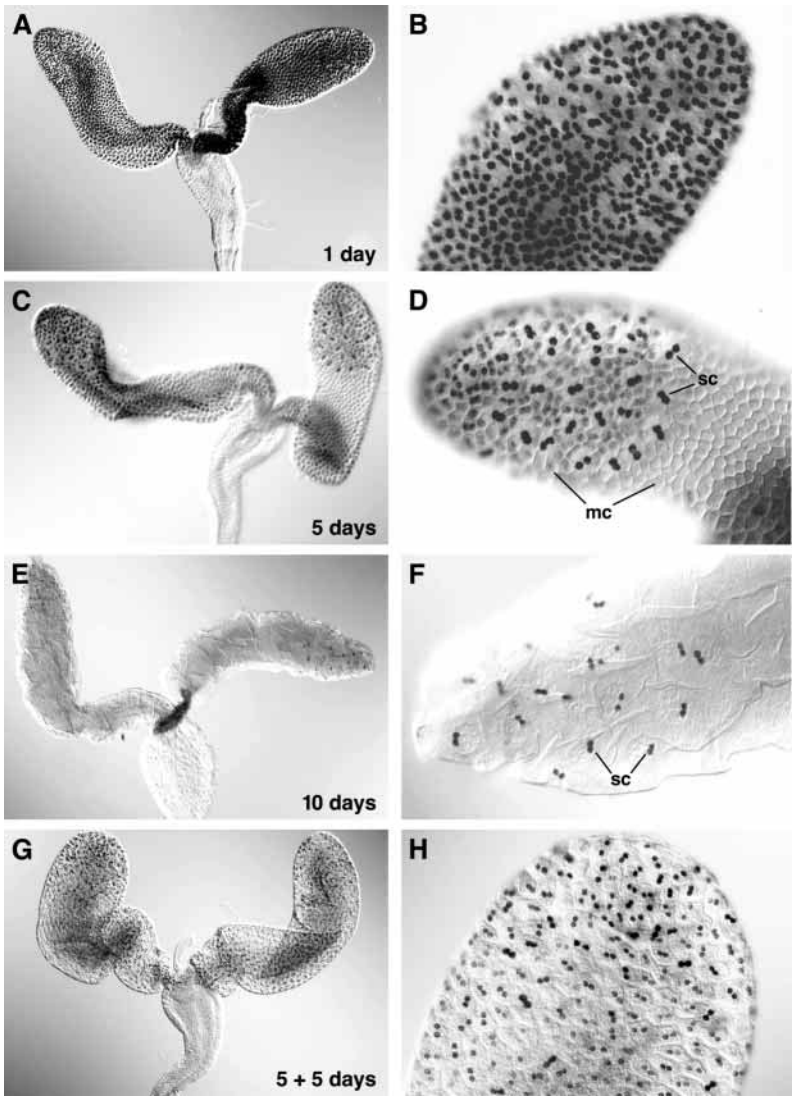

Fig. 3. Dynamic expression of Prd in adult accessory glands. Expression patterns of Prd protein in accessory glands from a 1-day- (A,B), 5-day- (C,D), or 10-day-old (E,F) virgin male, or from a 5-day old-virgin male mated with females for the next 5 days (G,H) are shown at low (A,C,E,G) and high (B,D,F,H) magnification. Accessory glands were stained with rabbit anti-Prd antiserum. mc, main cells; sc, secondary cells.

Nüsslein-Volhard, 1987). We have previously shown that the mouse homolog of the Prd protein, Pax3, when expressed under the control of the complete *cis*-regulatory region of *prd*, is able to rescue this 'cuticular' function of *prd*, yet not its embryonic lethality (Xue and Noll, 1996). Therefore, Prd has a 'viability' function that is separable from its cuticular function (Xue et al., 2001). The *prd* transgene *prd*-SN20, a genomic fragment extending from 9.8 kb upstream to 5.7 kb downstream of the transcribed region of *prd*, rescues *prd* null mutants to fertile wild-type adults (Gutjahr et al., 1994) and hence includes the enhancers of all *prd* functions. Two additional *prd* transgenes are also able to rescue *prd* mutants to viable adults: *prd*Res, which lacks the distal 5.2 kb of the downstream region of *prd*-SN20 (Bertuccioli et al., 1996), and *prd*-Gsb, in which the coding region of *prd*-SN20 has been replaced by that of *gsb* (Xue and Noll, 1996). However, in both these cases all rescued males are sterile, while rescued females are fully fertile (Bertuccioli et al., 1996; Xue and Noll, 2000; Xue et al., 2001). It follows that the wild-type *prd* gene includes, in addition to its cuticular and viability functions, functions required for male fertility. The sterile *prd* males rescued by *prd*-Gsb or *prd*Res possess severely reduced (Fig. 1B) or no accessory glands (Fig. 1C), which is the primary cause of the sterility (Xue and Noll, 2000). As *prd* males rescued by *prd*-SN20 have accessory glands of normal size (Fig. 1D) (Xue and Noll, 2000) and are fertile (Gutjahr et al., 1994), the 5.2 kb downstream sequences of *prd*-SN20, which are missing in *prd*Res, might include the enhancers that are essential for accessory gland formation and the male fertility function of *prd*. To test this conjecture, we constructed *prd*-mf5, a *prd-Gal4* transgene consisting of the *prd* promoter, *prd* transcribed region, and 5.7 kb adjacent downstream sequences placed upstream of the *hsp70* basal promoter and the yeast *Gal4*-coding region (Fig. 2). This transgene is expected to function both as Prd rescue construct for functions mediated by downstream enhancers of *prd* and as Gal4 reporter construct, because it drives the expression of Prd as well as Gal4 proteins under the control of the same *cis*-regulatory region. Indeed, *prd*-mf5 rescues both the accessory gland phenotype (Fig. 1E,F) and the male fertility (Fig. 2) of *prd* mutant males rescued to adulthood by either *prd*-Gsb or *prd*Res. It follows that the enhancer(s) required for *prd* functions in accessory gland formation and male fertility are located within the 5.7 kb downstream region of *prd*.

Dynamic expression of Prd in main and secondary cells of adult accessory glands

In addition to its requirement for accessory gland development, Prd is expressed in the differentiated glands of adult males (Bertuccioli et al., 1996). To examine the expression pattern of Prd in adult accessory glands more closely, genital tracts were dissected from virgin males 1 day, 5 days and 10 days after eclosion, and stained for Prd protein by the use of a Prd antiserum (Gutjahr et al., 1993a). Prd is initially expressed at high levels in all secretory accessory gland cells of newly eclosed flies (Fig. 3A,B), but levels are gradually reduced with increasing age of virgin males, rapidly in main cells and slowly in secondary cells (Fig. 3C,D). In 10-day-old virgin males, Prd protein remains detectable only in a few scattered cells in the distal region of the glands (Fig. 3E,F). As these cells are large and round, they are probably secondary cells, a conclusion that was confirmed by double staining for Prd and β-gal in the enhancer trap line 23ZΔ-280.1.4 (data not shown) expressing β-gal specifically in secondary cells (Bertram et al., 1992).

To determine the effect of mating on Prd expression in accessory glands, these were dissected from 10-day old males that had been allowed to mate after 5 days. Such males display enhanced Prd levels in both main and secondary cells throughout the entire glands (Fig. 3G,H). Similar patterns were

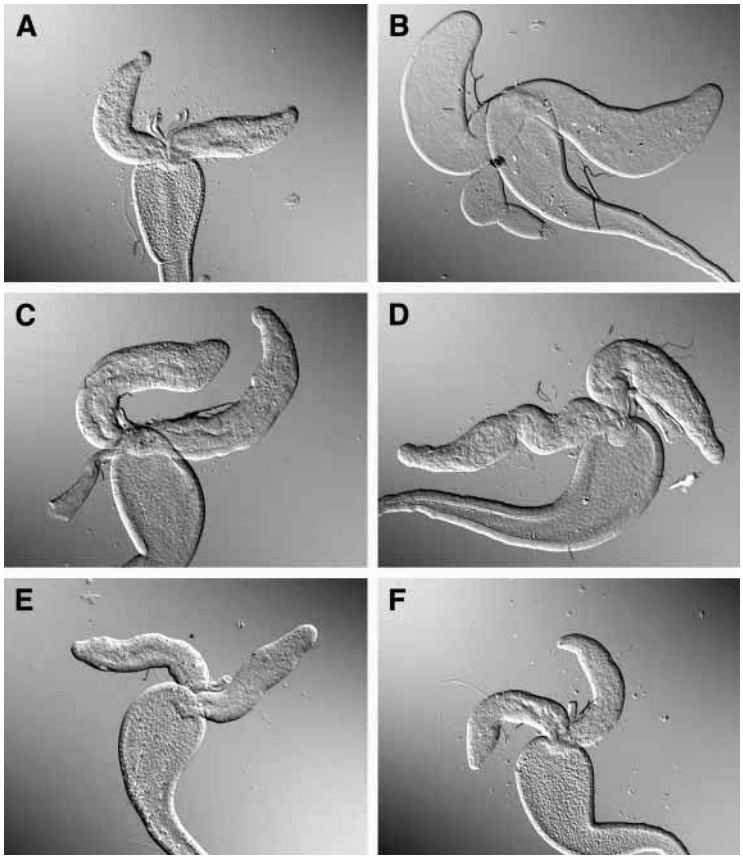

Fig. 4. *prd* is required to promote cell proliferation in accessory gland development. Accessory glands are shown that have been dissected from *Df(2L)Prl prd*-mf9.7/*prd*$^{2.45}$; *prd*Res/+ males (A), carrying an additional copy of a *UAS*-Prd (B), *UAS*-dMyc (C), *UAS*-CycE (D), or *UAS*-P35 (E) transgene, or hemizygous for *Df(3L)H99* (F), uncovering the proapoptotic genes *reaper*, *grim* and *hid*. Note that one copy of *prd*Res, as used here, rescues about 20%, whereas two copies, used in Fig. 1C,F, rescue about 75% of *prd* mutants to adult males that have no accessory glands. The rescue efficiencies are not affected by the presence of a *prd*-mf9.7 transgene, nor does the presence of an additional copy of *prd*Res augment the size of the accessory glands shown in A.

observed in glands of 13-day-old males mated only after 10 days (data not shown). Therefore, the elevated Prd levels resulted from an increase in synthesis rather than a slower decay of the Prd protein after mating. It is possible that mating induces factor(s), for example a hormonal response, that regulate *prd* positively. Alternatively, Prd expression might be regulated by negative feedback that inhibits Prd synthesis in the presence of high concentrations of accessory gland fluid or at least one of its products. Accumulation of these secreted factors in the absence of mating would thus downregulate Prd protein, whereas a reduction in concentration as a result of mating would in turn relieve the inhibition of Prd synthesis.

These results demonstrate that Prd exhibits dynamic expression patterns in main and secondary cells of differentiated accessory glands that depend on age and mating activity in both secretory cell types. Similar age-dependent and mating-stimulated expression patterns in both secondary and main cells have been observed for several accessory gland proteins (DiBenedetto et al., 1990; Monsma et al., 1990) and accessory gland-specific enhancer trap lines (Bertram et al., 1992), which are probably regulated at the transcriptional level (Bertram et al., 1992). The fact that the expression of the Prd transcription factor correlates with that of these accessory gland products suggests that Prd is involved in their transcriptional regulation.

Delimiting the *prd* enhancers regulating accessory gland development and transcription in adult accessory glands

The *prd*-mf5 transgene is not only able to rescue accessory gland development (Fig. 1E,F), but also to express Prd in adult accessory glands with the same profile as endogenous Prd (Fig. 2; data not shown). In addition, it restores fertility (Fig. 2) in *prd* mutant males rescued by either *prd*-Gsb or *prd*Res transgenes. These results indicate that the 5.7 kb *prd* downstream sequences include all enhancers that are necessary for *prd* functions in accessory gland development and any possible later functions of *prd* required for fertility in differentiated glands of adult males. To map these enhancers, we constructed a series of *prd* transgenes derived from *prd*-mf5 by deleting different portions of the downstream sequences (Fig. 2). These transgenes were introduced into *prd* mutant males, rescued by either *prd*-Gsb or *prd*Res, and scored for their abilities to rescue accessory gland formation, drive Prd expression in adult accessory glands and restore fertility (Fig. 2). Expression of these transgenes in accessory glands was further tested and confirmed by examining their ability to express Gal4 and activate β-gal expression from a *UAS-lacZ* transgene (data not shown). The *prd*-mf1, -mf2, -mf3 and -mf4 transgenes all lack the most distal 1.6 kb of the *prd* downstream region and are unable to perform any of these three functions (Fig. 2), which therefore strictly depend on enhancers partly or completely included in this 1.6 kb *Eco*RI-*Sal*I fragment. By contrast, the *prd*-mf7 and *prd*-mf8 transgenes contain this fragment, and are able to execute all three functions (Fig. 2). It follows that the *prd* enhancers endowed with these functions are completely included in this region. To further delimit the enhancer region, *prd*-mf9 and *prd*-mf10 were constructed that subdivide this region into two halves of 0.8 kb (Fig. 2). While *prd*-mf9, which includes the proximal half, is again able to perform all three functions, *prd*-mf10 is unable to support accessory gland development (Fig. 2). Evidently, the 0.8 kb of the *prd* downstream region included in *prd*-mf9 harbor the *prd* male fertility enhancer (PMFE), which is necessary and sufficient for all *prd* functions required for accessory gland development and male fertility. Additional experiments would be required to elucidate whether this region contains a single or two separate enhancers responsible for the *prd* functions in accessory gland formation and its dynamic expression in adult accessory glands.

prd is required for cell proliferation during early accessory gland development

prd mutant males rescued by *prd*-Gsb or *prd*Res exhibit severely reduced (Fig. 1B) or no accessory glands (Fig. 1C), a phenotype that may result from an excess of apoptosis or a

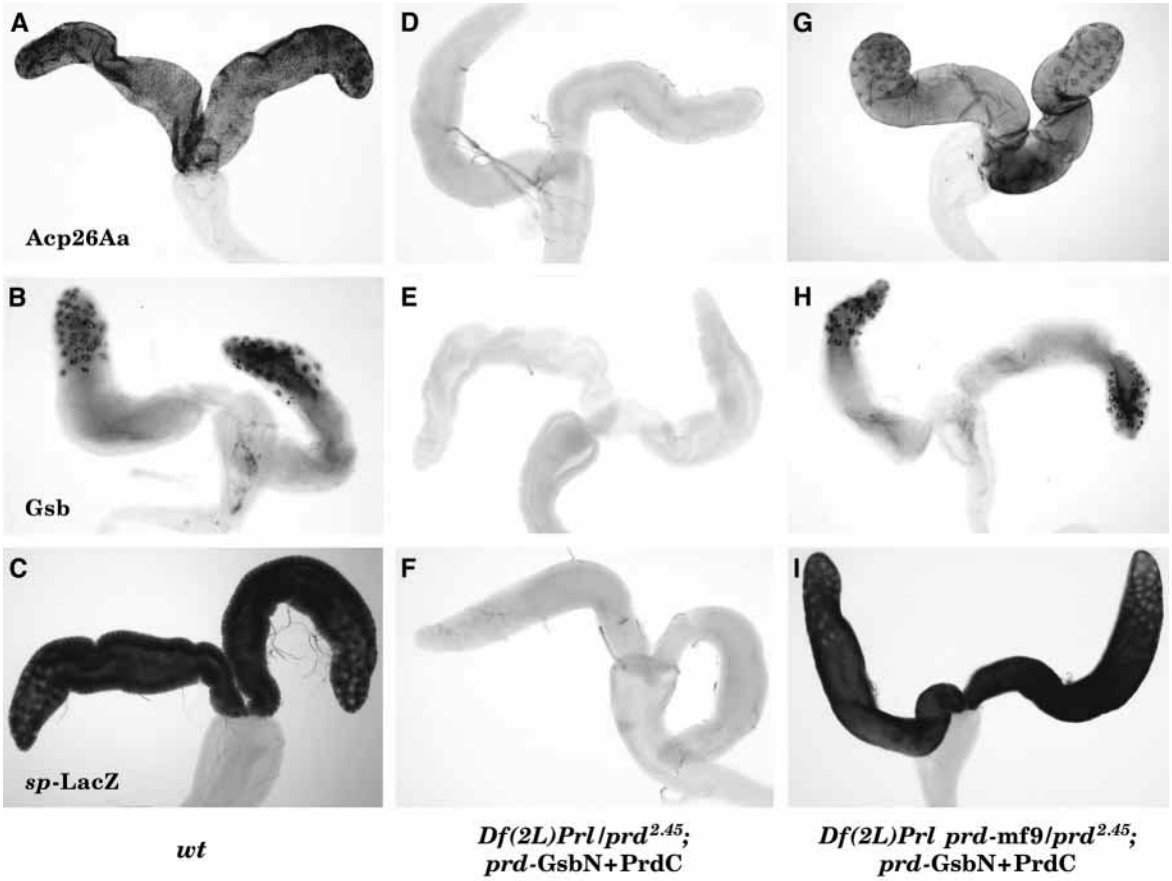

Fig. 5. *prd* is required for accessory gland maturation. Expression of Acp26Aa protein (A,D,G), Gsb (B,E,F) and SP (C,F,I) in accessory glands of wild-type males (A-C), or *Df(2L)Prl/prd*$^{2.45}$; *prd*-GsbN+PrdC/*prd*-GsbN+PrdC males carrying no (D-F) or one copy of a *prd*-mf9 transgene recombined onto the *Df(2L)Prl* chromosome (G-I) is detected by rabbit antisera against Acp26Aa and Gsb, or visualized by X-Gal staining of the product from an *sp-lacZ* reporter gene.

block in cell proliferation during early accessory gland development. To discriminate between these alternatives, we took advantage of a transgenic line, *prd*-mf9.7, that rescues the accessory glands of *prd*Res mutant males (Fig. 1C) completely with two copies of *prd*-mf9 (data not shown), but only partially with one copy (Fig. 4A), while restoration of fertility requires two copies. By contrast, most other *prd*-mf9 lines display a complete rescue with a single copy (data not shown). The weak rescue efficiency of the *prd*-mf9.7 line is presumably the result of a position effect on the *prd*-mf9 transgene causing its low expression (data not shown). The fact that, in addition to the expression of Prd, *prd*-mf9 drives Gal4 expression ubiquitously in developing accessory glands (data not shown) under the control of the same enhancer (Fig. 2) permits us to express any protein in developing accessory glands under the control of this enhancer by the use of the Gal4/UAS system and to subsequently test its ability to rescue the accessory gland phenotype of *prd* mutant males that carry one copy each of the *prd*Res and *prd*-mf9.7 transgenes.

As expected, one copy of *UAS*-Prd rescues the accessory glands to nearly wild-type size (Fig. 4B). In addition, overexpression of Myc or CycE, both of which are required for promoting cell proliferation (Neufeld et al., 1998; Johnston et al., 1999), rescues the accessory glands to a large extent (Fig. 4C,D) and restores male fertility. The rescue by CycE or Myc completely depends on the low level of Prd expression from *prd*-mf9.7 in developing accessory glands. This is evident from the complete absence of accessory glands in *prd* mutant males that are rescued by *prd*Res and carry a *prd3.1*-Gal4 transgene driving *UAS*-CycE or *UAS*-dMyc expression in accessory glands under control of the *prd* downstream region (5.03 kb *Xba*I fragment in Fig. 2; data not shown). In contrast to CycE and Myc, expression of P35, the baculoviral protein that specifically inhibits caspase-mediated apoptosis (Hay et al., 1994), is unable to rescue the accessory gland phenotype (Fig. 4E). Consistent with this result, removing one copy of the *thread* gene (*Df(3L)th102*; data not shown), which encodes the inhibitor of apoptosis Diap1 (Hay et al., 1995), or removing one copy of the three *Drosophila* proapoptotic genes *reaper*, *grim* and *hid* (*Wrinkled* – FlyBase) (Quinn et al., 2000), uncovered by the deficiency *Df(3L)H99* (White et al., 1994), has no effect on this phenotype (Fig. 4F). Similarly, overexpression in developing accessory glands of Dp110, the *Drosophila* PI3-kinase, is unable to rescue their reduced size (data not shown), although this kinase activates the insulin signaling pathway promoting cell growth and proliferation (Leevers et al., 1996).

We conclude that an inhibition of the cell cycle, presumably in G1 (Neufeld et al., 1998; Johnston et al., 1999), rather than induced apoptosis is the primary cause for the reduction or loss

of accessory glands in *prd* mutant males, and that *prd* is required for promoting cell proliferation during early accessory gland development.

prd is essential for accessory gland maturation

In adult accessory glands, *prd* exhibits a dynamic expression profile that depends on aging and mating activity (Fig. 3). This suggests that *prd* might be required for the regulation of accessory gland products. In support of this hypothesis, *prd* mutant males rescued to adulthood by two copies of a particular *prd* transgene are sterile even though the size of their accessory glands appears normal (Fig. 5D-F). This transgene, *prd*-GsbN+PrdC, expresses a chimeric protein consisting of the N-terminal half of Gsb and the C-terminal region of Prd under the control of the complete *prd* cis-regulatory region (Xue et al., 2001). This finding suggests that development of accessory glands to normal size does not strictly depend on the binding specificities of the paired-domain and homeodomain in the N-terminal moiety of Prd when compared with those in the homologous half of Gsb. Moreover, as the accessory glands of *prd* mutant males rescued by two copies of *prd*-Gsb are severely reduced (Fig. 1B), their development to normal size requires functions in the C-terminal region of Prd having the N-terminal moiety of the protein derived from Gsb. These C-terminal functions reside partially, though not exclusively, in the PRD transactivation domain of Prd (Xue et al., 2001).

The sterility of *prd* mutant males, whose accessory glands have been rescued to normal size by *prd*-GsbN+PrdC, might result from a failure to express certain accessory gland factors required for sperm fertility (Xue and Noll, 2000). To test this supposition, we examined the expression of Acp26Aa, Gsb and sex peptide (SP) in the accessory glands of wild-type and *prd* mutant males rescued by *prd*-GsbN+PrdC. While the Acp26Aa protein is important for enhanced female oviposition during the first day after copulation (Herndon and Wolfner, 1995), SP is a key component of accessory gland secretions responsible for increased oviposition and reduced sexual receptivity in mated females (Chen et al., 1988; Kubli, 1996). The function of Gsb in adult accessory glands is not known. In wild-type accessory glands, Acp26Aa is expressed in all secretory cells (Monsma et al., 1990) (Fig. 5A), while Gsb is expressed only in secondary cells (Fig. 5B) and SP only in main cells as assayed by the expression of a *lacZ* reporter gene under control of the *sp* enhancer (D. Styger-Schmucki, PhD Thesis, University of Zürich, 1992) (Fig. 5C). In *prd* mutant males rescued by *prd*-GsbN+PrdC, the accessory glands fail to express Acp26Aa, Gsb and SP (Fig. 5D-F), which suggests that *prd* is indeed also required in late accessory gland development to regulate the expression of at least these three accessory gland products. This is corroborated by the introduction into these males of the *prd*-mf9 transgene, which expresses Prd in adult accessory glands (Fig. 2) and is able to restore both male fertility (data not shown) and the expression of Acp26Aa, Gsb and SP in accessory glands (Fig. 5G-I).

Although Prd and Gsb share a highly conserved N-terminal moiety, including two DNA-binding domains, a paired-domain and a *prd*-type homeodomain (Bopp et al., 1986; Baumgartner et al., 1987; Treisman et al., 1991), the N-terminal region of Gsb is apparently unable to substitute for this particular function of Prd. It seems therefore probable that the enhancers of *Acp26Aa*, *gsb*, *sp*, and perhaps of other genes specifically expressed in adult accessory glands include DNA-binding sites recognized by one or both DNA-binding domains of Prd, but not by those of Gsb, whose expression depends on Prd. Preliminary experiments suggest that the enhancer of the *sp* gene includes DNA-binding sites recognized by the paired-domain of Prd but not that of Gsb. It is possible, however, that other genes whose expression in accessory glands depends on Prd are regulated more directly by the Gsb transcription factor.

We conclude that *prd* performs a dual role in accessory gland development, an early function promoting cell proliferation that is required for accessory gland formation and a late function promoting cell differentiation that is essential for accessory gland maturation. The early function demands a domain or motifs present in the C-terminal region of Prd, whereas the late function depends on the DNA-binding specificity of at least one of the two N-terminal DNA-binding domains of Prd. Both functions are essential for male fertility.

Interestingly, *Pax3*, which encodes a vertebrate homolog of Prd, also seems to be necessary for both cell proliferation and differentiation. While *splotch* mutations in *Pax3* of mice lead to the absence of limb muscles and a reduction in trunk muscle mass (Franz et al., 1993; Tajbakhsh et al., 1997), overexpression of Pax3 in cultured cells produces foci of transformed cells that are able to develop tumors in nude mice (Maulbecker and Gruss, 1993). Moreover, a *Pax3* gain-of-function mutation produces alveolar rhabdomyosarcoma, a highly proliferative cancer (Shapiro et al., 1993). In addition to its role in regulating cell proliferation, *Pax3* acts upstream of *MyoD* and can induce muscle differentiation (Maroto et al., 1997). The fact that both Prd and its vertebrate homolog Pax3 play pivotal roles in the regulation of cell proliferation and differentiation might reflect an evolutionary mechanism important during the evolution of Pax genes as well as many other genes encoding transcription factors (Noll, 1993).

Because gene networks have been conserved during evolution (Noll 1993), it is reasonable to expect that many of the factors present in seminal fluid whose synthesis depends on the late male fertility function of *prd* are also synthesized in the human prostate and required for sperm fertility, a proposition now testable on the basis of the results reported here.

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