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dishevelled and *armadillo* act in the Wingless signalling pathway in *Drosophila*

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THE *Wnt* genes encode conserved secreted proteins that play a role in normal development and tumorigenesis[1,2]. Little is known about the signal transduction pathways of *Wnt* gene products. One of the best characterized *Wnt* family members is the *Drosophila* segment polarity gene *wingless*[3–6]. We have investigated whether segment polarity genes with a *wingless*-like phenotype mediate the *wingless* signal. We used a *wingless* transgene controlled by a heat-shock promoter for genetic epistasis experiments. We show that *wingless* acts through *dishevelled* and *armadillo* to affect the expression of the homeobox gene *engrailed* and cuticle differentiation.

During germ-band extension, *wingless* (*wg*) is expressed in stripes flanking a parasegmental border[3]. The Wg protein is secreted[7,8] and taken up by adjacent cells for maintenance of expression of the homeobox gene *engrailed* (*en*)[9–12]. Later, *wg* is necessary for the generation of smooth cuticle[13]. No naked cuticle is formed in the absence of *wg*; instead the ventral cuticle consists of a lawn of denticles.

Genes that mediate the *wg* signal are probably among the segment polarity mutants with a *wg*-like phenotype. Absence of functional products of the genes *dishevelled* (*dsh*), *armadillo* (*arm*), *porcupine* (*porc*) and *hedgehog* (*hh*) lead to cuticle defects similar to that of *wg* mutant embryos and also to a loss of en expression[9,14–16]. It has been difficult to order these mutations in a genetic pathway because their phenotypes are nearly indistinguishable and because expression of *wg* depends on *en* expression. But the order of action of two genes in a common pathway can be found by examining epistasis between a dominant gain-of-function mutant for one gene in combination with a loss-of-function mutant for the other. Dominant *wg* alleles have not been identified, but we previously established a transgenic *Drosophila* strain that expresses *wg* under the control of a heat-shock promoter[17]. Heat-shock during particular stages of development causes expansion of the En expression domain and generation of a completely naked ventral cuticle[17] (Figs 1*b* and 2*b*). We used the HS-*wg* allele to determine which of several segment polarity genes with a *wg*-like phenotype are required for *wg* action.

We first investigated whether the effects of HS-*wg* on expansion of En expression require endogenous *wg* and *en* genes. The en^{CX1} allele produces a cytoplasmic, non-functional protein, in contrast to the nuclear localization of the En protein in wild type. In en^{CX1} embryos, En expression is not maintained[11]. The heat-shocked double mutant en^{CX1};HS-*wg* embryos can be distinguished from the single mutants by their unique pattern of En protein expression, which is ectopically induced at early stage 10 (ref. 18) as in HS-*wg* embryos, but localized in the cytoplasm as in en^{CX1} (Fig. 1*i*, *j*). Normal or ectopic En domains are not maintained: at stage 11 most En protein has disappeared from the ectoderm (data not shown).

In HS-*wg* embryos, an ectopic domain of Wg protein made from the normal *wg* gene is formed just posterior to the expanded En domain[17]. In the heat-shocked double mutant *wg*;HS-*wg* embryos, the ectopic Wg domain does not appear, yet the expanded domain of En expression is induced (compare Fig. 1*a* with *h*) and maintained (data not shown)[20]. Thus neither functional En nor Wg protein made from the endogenous *wg* gene are required to mediate the effects of HS-*wg* on induction of ectopic En.

To examine whether genes with mutant phenotypes similar to *wg* (*dsh*, *arm*, *porc and hh*)[14–16,19] are required for the effects of HS-*wg* on En expression, we made double mutants between HS-*wg* and loss-of-function mutations in these genes. As Dsh, Arm and Porc products are provided both maternally and zygotically, germ-line mosaic females were derived to remove completely the gene product from the developing embryo. In all four single mutants, En expression decays as in *wg* embryos (Fig. 1*c*), but there are some differences in pattern and timing of En decay[9,14]. The En patterns in the double mutant embryos are shown in Fig. 1*d*–*g*. In *dsh*;HS-*wg* embryos (Fig. 1*d*), the En expression pattern is very similar to *dsh* and *wg* embryos, although dorsally the En protein disappears from the ectoderm slightly later in development than in *dsh* embryos. Likewise, *arm*;HS-*wg* embryos (Fig. 1*e*) show a pattern of En distribution similar to *arm* embryos. In contrast, *porc*;HS-*wg* (Fig. 1*f*) and *hh*, HS-*wg* embryos (Fig. 1*g*) show ectopic En expression as seen in HS-*wg* embryos. Thus *dsh* and *arm* are required for induction of ectopic En in HS-*wg*, whereas *hh* and *porc* are not.

We then studied the effects of the absence of *dsh*, *arm*, *porc* or *hh* on cuticle pattern formation in HS-*wg*. The cuticle patterns of *dsh*;HS-*wg* (Fig. 2*d*) and *arm*;HS-*wg* (Fig. 2*e*) embryos are indistinguishable from the germ-line clone-derived *dsh* or *arm* embryos and from *wg* embryos (Fig. 2*c*) and develop a continuous lawn of denticles. In contrast, *porc*;HS-*wg* embryos show some restoration of the segmental denticle pattern (Fig. 2*f*). *hh*;HS-*wg* embryos (Fig. 2*g*) have a similar but not identical cuticle pattern to HS-*wg* (Fig. 2*b*)[20]: naked throughout most of the ventral cuticle, with dispersed patches of non-polarized denticles mostly present at the lateral sides. Thus *dsh* and *arm*, but not *porc* and *hh*, are epistatic to and presumably downstream of HS-*wg*, not only in the effect on En expression but also in the generation of naked cuticle. The cuticle of *wg*;HS-*wg* double-mutant embryos is shown in Fig. 2*h*. After a 20-min heat shock during germ-band extension, an embryo of almost wild-type size is formed with partially restored head and tail structures (filzkörper) and a segmental pattern. This unexpected result suggests that differential levels of *wg* are not essential for at least some of its functions[20]. It is possibe that *wg* normally regulates pattern together with other determinants that function in a spatially restricted manner. For example, not all cells may be equally sensitive to *wg* activity, because of differences in concentrations of interpreting molecules.

In conclusion, *dsh* and *arm* are essential components of the *wg* signalling pathway and probably act downstream of *wg*. In contrast to the non-autonomous behaviour of *wg* in mutant cell clones, *dsh* and *arm* act autonomously, suggesting a role in reception of the *wg* signal[16,21]. In addition, *wg* is required for post-transcriptional regulation of *arm*, resulting in an accumulation

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or redistribution of Arm protein in segmentally repeated stripes in the Wg domain[22]. The product of the *arm* gene is homologous to the vertebrate proteins plakoglobin and β-catenin[23], components of cell–cell junctions. Post-transcriptional modification of *arm* by *wg* requires *dsh*, suggesting that *dsh* functions upstream of *arm*[22]. The *dsh* gene encodes a novel protein that is highly conserved in evolution[21].

porc, like *wg*, behaves non-autonomously in mitotic cell clones (J.A.K. and N.P., manuscript in preparation). In *porc* mutant embryos, the distribution of the Wg protein is altered in a way that suggests secretion of the protein is impaired[24]. Possibly, *porc* is required for paracrine functions of *wg*; by expressing *wg* within the *en* cell (under the control of the ubiquitously acting heat-shock promoter), the requirements for *porc* can be bypassed, resulting in induction of ectopic En. But the cuticle phenotype of *porc*;HS-*wg* is not like HS-*wg*, showing a slight restoration of pattern reminiscent of that of *wg*;HS-*wg* embryos, maybe indicating that endogenous *wg* function in these embryos is impaired. We found that Hh protein, a putative transmembrane protein[25], is not necessary for the effects of HS-*wg*. On the basis of genetic evidence, it has been suggested that *hh* is a signal from the *en* cells to maintain Wg expression[26]. *hh* would therefore act upstream of *wg*, which is consistent with our findings.

wg and *dsh* act upstream of *zeste-white* 3 (*zw3*), a serine/threonine kinase and repressor of *en*[27–29], whereas *arm* is epistatic to *zw3* (ref. 30). Taken together with our results, a pathway can now be suggested in which *hh* acts upstream of *wg*, *dsh* downstream of *wg* and upstream of *zw3*, whereas *arm* and *en* act

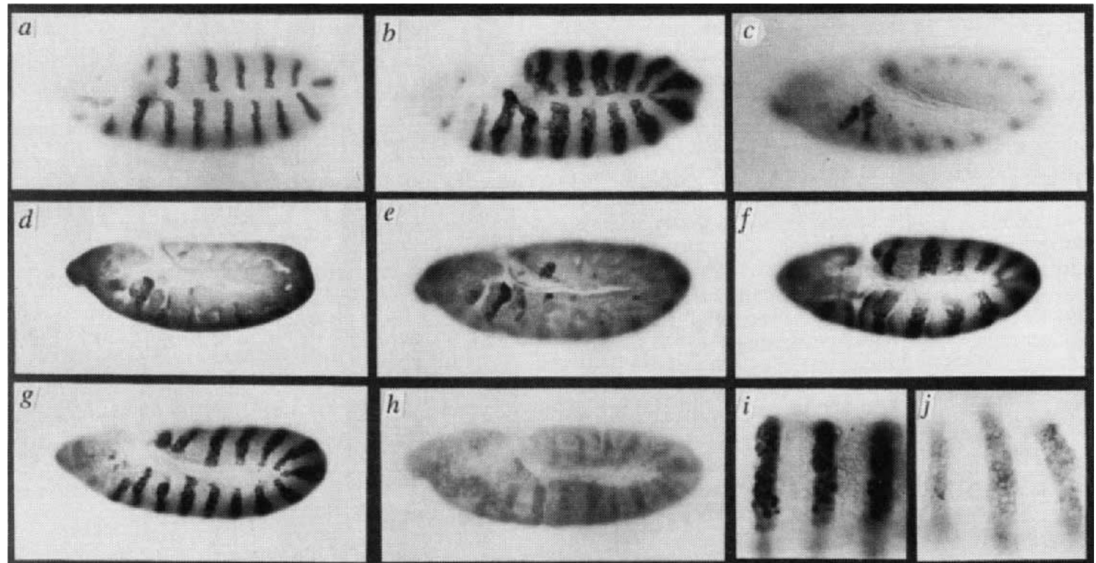

FIG. 1 Effects of the lack of *dsh arm, porc, hh,* endogenous *wg* or functional *en* on expansion of En in stage 10 (ref. 18) HS-*wg* embryos. Anterior is to the left, dorsal is up. *a–h*, Surface views of whole-mount embryos; *i, j*, higher magnification surface views of segments T_1 to T_3. All embryos were heat-shocked as described[17]. *a–c*, En expression pattern in wild-type (*a*), HS-*wg* (*b*) and wg^{CX4} embryos (*c*). *d–f*, Progeny of crosses between germ-line mosaic females of dsh^{477}, arm^{25B} or $porc^{PB16}$ with HS-*wg* males carrying an *FM7, ftz LacZ* balancer were stained for both En and β-galactosidase. In all cases lack of β-galactosidase staining was used as a marker to identify the segment-polarity mutant embryos. *d*, Embryo from the *dsh* × *FM7, ftz LacZ/Y*;HS-*wg*/+ cross; all germ-line clone embryos without *dsh* function lose En expression. The same result is obtained in the cross *arm* × *FM7 ftz LacZ*;HS-*wg*/+ (*e*). In the *porc* × *FM7 ftz LacZ*;HS-*wg* cross, 50% of the germ-line clone embryos without *porc* product show induction of ectopic En (*f*). *g*, A *hh*, HS-*wg* embryo, double labelled for anti En and anti β-galactosidase in which En is induced ectopically. In this case the lack of β-galactosidase was used as a marker for the double-mutant embryos. *h*, A wg^{CX4};HS-*wg*(/+) embryo, labelled for both En and Wg proteins. The absence of Wg protein expression indicates that this embryo is homozygous for wg^{CX4}, but it does show En expansion as observed in HS-*wg*. *i*, Subcellular localization of En protein in HS-*wg* (*i*) and en^{CX1};HS-*wg*(/+) embryos (*j*). In HS-*wg* En expression is localized to the nucleus and in a broader domain than in wild type (*a*). In en^{CX1};HS-*wg*/+ the En domain is broadened but the protein is localized to the cytoplasm as seen in en^{CX1} embryos[11].

METHODS. The HS-*wg*/*TM3, Sb* stock has been described previously[17]. Several segment polarity mutations used here have previously been characterized as strong alleles: en^{CX1} (ref. 11), wg^{CX4} (ref. 3), hh^{G51} (ref. 19). From the mutant alleles arm^{25B}FRT[101], arm^{XM19}FRT[101] (ref. 23), dsh^{477}FRT[101], dsh^{75} FRT[101] (N.P., unpublished) and dsh^{V26}FRT[101] (ref. 15), mosaic germ lines were made by the FLP-DFS technique[31], whereas from $porc^{PB16}$ germ-line clone embryos were made by X-ray irradiation using the DFS technique[32]. To study the HS-*wg* phenotype in a segment polarity mutant background, we generated embryos that contain HS-*wg* and lack one of the following segment polarity genes: *hh*, *en*, *wg*, *dsh*, *arm* and *porc*. Because HS-*wg* flies are very weak, it was in most cases not possible to establish stocks that contain the HS-*wg* P-element over a *TM3* balancer chromosome and a balanced segment polarity mutation. A recombinant stock was obtained for *hh* and HS-*wg* on the third chromosome and balanced over a *TM3* chromosome, marked with a P-element carrying the β-galactosidase gene under the control of the *hunchback* promoter (G. Struhl, unpublished results). Using this marker we could identify embryos that are homozygous for the HS-*wg* P-element and hh^{G51}. In the case of the second chromosome mutations en^{CX1} and wg^{CX4}, crosses were made between HS-*wg*/*TM3* males and en^{CX1}/Gla or wg^{CX4}*b pr*/*CyO* females. F_1 males and females, carrying the particular segment-polarity mutation and one copy of the HS-*wg* P-element, were selected and F_2 progeny collected and heat-shocked. In a double-antibody staining of WG and EN proteins in wg^{CX4};HS-*wg* embryos and a single En staining in en^{CX1};HS-*wg*, embryos were identified that showed a novel staining pattern not present in the mutant or the HS-*wg* embryos alone. In this way we were able to identify the double mutant embryos unambiguously. Females carrying homozygous germ-line clones of *dsh*, *arm* and *porc* were crossed with the *FM7, ftz LacZ/Y*;HS-wg/+ males. The *FM7, ftzLacZ* balancer chromosome marked with the β-galactosidase gene under the control of the *fushi-tarazu* promoter[33] allows marking of the embryos from about stage 6 to 13. From this cross, stage 6–13 embryos that do not stain with anti-β-galactosidase antibody lack the particular segment polarity gene on the first chromosome. Half of these embryos carry the HS-*wg* P-element. By double labelling with anti-β-galactosidase and anti-*wg* or anti-*en* antibodies the pattern of Wg and En proteins in the double mutant embryos could be determined. The maximal broadening of En in stage 10 to 12 (ref. 18) HS-*wg* embryos was only seen after multiple heat-shocks[17]. As a consequence of the heat-shock procedure, generalized HS-*wg* protein is present in these embryos starting at around 2 h until 6.5 h AEL (stages 4 to 10). Fixation of embryos and double labelling for En and Wg or for En and β-galactosidase (Promega, Cappel) were as described[17]. For antibody stainings and cuticle preparations (Fig. 2) the appropriate heat-shocked segment polarity mutant embryos were used as a control for the general effects of heat shock.

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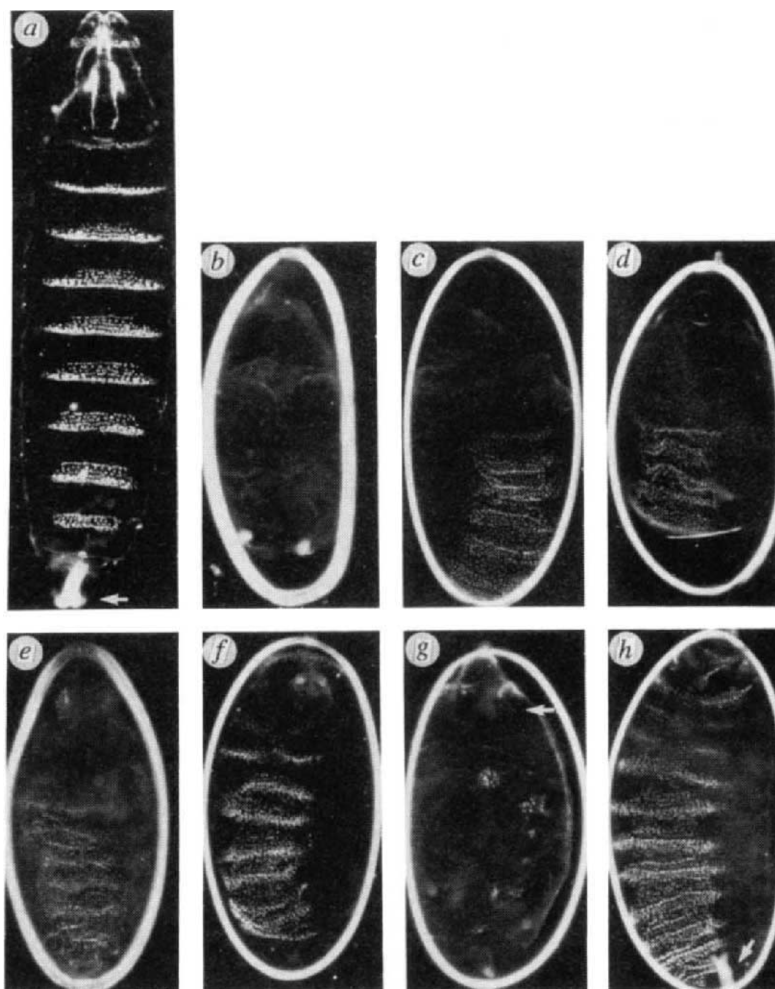

FIG. 2 Effects of lack of *dsh*, *arm*, *porc*, *hh* or endogenous *wg* on cuticle phenotype of HS-*wg* embryos. Anterior is up. All embryos used for cuticle preparations were heat-shocked as described[17]. Cuticles were mounted in Hoyers' and photographed under dark-field optics at the same magnification. *a*, Ventral view of wild-type larvae. The posterior spiracle and associated Filzkörper material is indicated by the arrow. *b*, Ventral cuticle pattern of an HS-*wg* embryo. No ventral denticles are present, except for the beard[17]. *c*, A wg^{CX4} embryo, showing a lawn of disoriented denticles. Head structures are abnormal and filzkörper are missing. *d*, A dsh^{477};HS-*wg*/+ embryo. These embryos are indistinguishable from *dsh* and *wg* with the exception that in some larvae some rudimentary filzkörper material is present. *e*, An arm^{25B};HS-*wg*/+ embryo. These embryos are indistinguishable from *arm* and *wg* embryos. *f*, A $porc^{PB16}$;HS-*wg*/+ embryo. These embryos show a slight restoration of pattern and naked cuticle. In addition, the posterior spiracles with their filzkörper materials are present (Not shown). *g*, Ventral view of a hh^{G51}, HS-*wg* embryo. Some patches of denticles are left, but most of cuticle is naked except for the beard (indicated by the arrow). *h*, Cuticle pattern of a wg^{CX4};HS-*wg* embryo. A 20-min heat shock between 4 to 9 h AEL substantially rescues segmental pattern, head structures and filzkörper materials (arrow).

METHODS. The most extreme cuticle phenotype in HS-*wg* was generated with a single heat shock between 4 to 9 h of development (stages 8 to 11 (refs 17, 18)). Therefore, to analyse the cuticle phenotypes of the double-mutant larvae, stage 8 to 11 embryos were individually selected and heat-shocked once for 20 min. Because we did not have any markers to distinguish between the single- and double-mutant embryos, we counted the numbers in different progeny classes. We were thus able to determine the phenotype of the double mutant larvae. Larvae were mounted[18] in Hoyers' mountant.

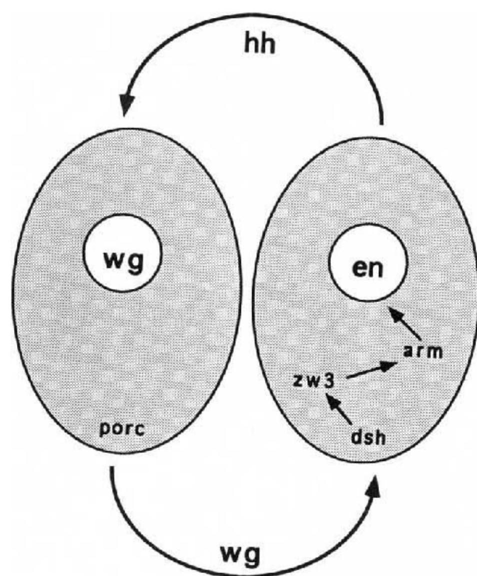

FIG. 3 A model for intercellular signalling between Wg- and En-expressing cells in the embryonic epidermis. Wg protein is secreted[7,8] and required in the neighbouring cells to maintain the nuclear protein En[9-12]. Secretion of Wg appears to be mediated by the product of the *porc* gene[24]. *dsh* and *arm* are downstream of *wg* in their effect on *en* expression and act cell-autonomously, indicating that they play a role in reception rather than secretion of Wg. By genetic analysis it has been shown that *zw3* is downstream of *dsh* and upstream of *arm* (ref. 30). *hh* encodes a putatively secreted protein and is transcribed in the En cells[25]. Its function is upstream of *wg* in our analysis. It has been postulated that the Hh protein antagonizes repression of *wg* expression[26] and might be the signal emanating from the En cells to maintain Wg. The arrows indicate genetic relationships; little is known about the biochemistry of these interactions.

downstream of *zw3* (Fig. 3). Because all the cloned genes in the *wg* pathway have homologues in mammals, the mechanism of action of *Wnt* genes during development of highly diverse species might be conserved. □

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Evolution of distinct developmental functions of three *Drosophila* genes by acquisition of different *cis*-regulatory regions

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It is generally accepted that the specific function of a gene depends on its coding sequence. The three paired-box and homeobox genes *paired* (*prd*), *gooseberry* (*gsb*) and *gooseberry neuro* (*gsbn*) have distinct developmental functions in *Drosophila* embryogenesis[1-5]. During the syncytial blastoderm stage, the pair-rule gene *prd*[4,6] activates segment-polarity genes, such as *gsb*[7], *wingless* (*wg*), and *engrailed* (*en*), in segmentally repeated stripes[8]. After germ-band extension, *gsb* maintains the expression of *wg*, which in turn specifies the denticle pattern by repressing a default state of ubiquitous denticle formation in the ventral epidermis[9]. In addition, *gsb* activates *gsbn*[5], which is expressed mainly in the central nervous system[2,3], suggesting that *gsbn* is involved in neural development. Here we show that, despite the functional difference and the considerably diverged coding sequence of these genes, their proteins have conserved the same function. The finding that the essential difference between genes may reside in their *cis*-regulatory regions exemplifies an important evolutionary mechanism of how function diversifies after gene duplication.

The most conspicuous feature of the segmental organization of a *Drosophila* larva is its ventral denticle pattern (Fig. 1*a*). Recently, we have shown that *gsb* regulates this pattern through a *wg–gsb* autoregulatory loop that maintains the expression of *wg*, which represses denticle formation[9]. Thus, when *Hsgsb* embryos carrying a transgenic *gsb* gene under the control of the heat-inducible *hsp70* promoter were heat-shocked between 3 h 10 min and 6 h 20 min of development at 25 °C (early time interval), ubiquitous expression of *gsb* generated a naked larval cuticle (Fig. 1*e*). An earlier heat shock between 2 h 10 min and 3 h 10 min of development at 25 °C (early time interval), however, induced a pair-rule phenotype (Fig. 1*b*). This result is unexpected because it differs dramatically from the normal *gsb* gain-of-function phenotype (Fig. 1*e*). In wild-type embryos, *gsb* begins to be expressed only by the end of this early time interval, which coincides with the time of pair-rule gene rather than segment-polarity gene function. In fact, ubiquitous expression of pair-rule genes is known to result in pair-rule phenotypes that are nearly reciprocal to their loss-of-function phenotypes[10-14]. Therefore, we suspected that activation of *Hsgsb* during the early time interval mimics the function of a ubiquitously expressed pair-rule protein. The most likely candidate was the Prd protein, as its N-terminal half consists of a paired-domain and a *prd*-type homeodomain and thus is highly homologous to the N-terminal half of Gsb[2]. Indeed, early ubiquitous expression of *prd* in *Hsprd* embryos produced a phenotype[12] (Fig. 1*c*) indistinguishable from the *Hsgsb* pair-rule phenotype (Fig. 1*b*).

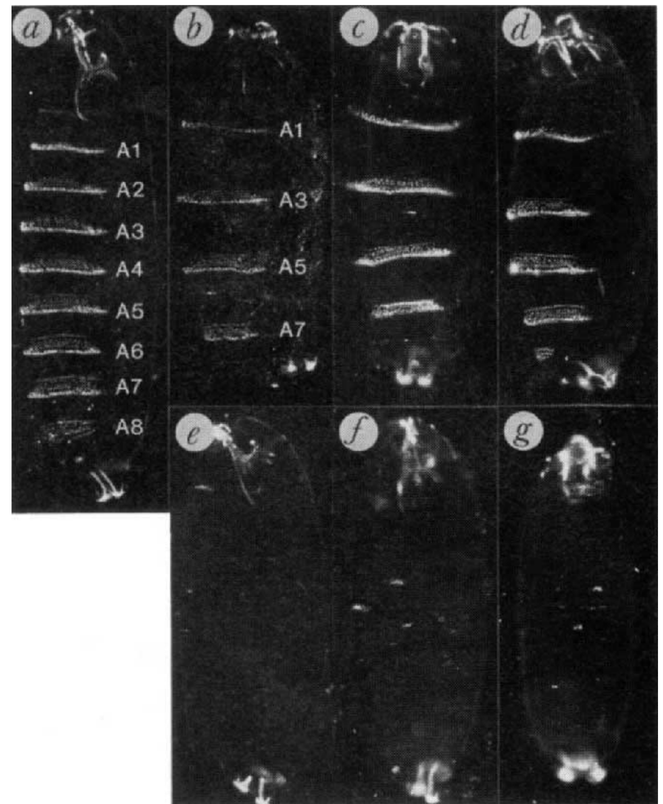

FIG. 1 Identical cuticular phenotypes induced by the ubiquitous expression of *gsb*, *prd* or *gsbn*. Cuticular preparations of wild-type (*a*), *Hsgsb* (*b*, *e*), *Hsprd* (*c*, *f*) and *Hsgsbn* (*d*, *g*) embryos heat-shocked during the early (*a–d*) or late (*e–g*) time interval are shown as ventral views under dark-field illumination. Ubiquitous activation of *Hsgsb*, *Hsprd* or *Hsgsbn* during the early time interval generates a pair-rule cuticular phenotype. In all cases, even-numbered abdominal denticle belts (A2, A4, A6, A8) and their anteriorly adjacent naked regions are lost, with the occasional exception of a few remaining denticles. This phenotype is nearly reciprocal to that of prd^- embryos in which the odd-numbered denticle belts and their anteriorly neighbouring naked regions are deleted. Ubiquitous activation of *Hsgsb*, *Hsprd* and *Hsgsbn* by a heat shock during the late time interval produces a naked cuticular phenotype.
METHODS. Transgenic *Hsgsb*, *Hsprd* or *Hsgsbn* embryos, collected between 2 h 10 min and 3 h 10 min AEL (after egg laying) (early time interval) or between 3 h 10 min and 6 h 20 min AEL (late time interval), were heat-shocked for 15 min at 37 °C. After 24 h of development at 25 °C, cuticles were prepared essentially as described[28]. Transgenic *Hsprd*, *Hsgsb* and *Hsgsbn* fly stocks were produced, as previously described[29], by cloning a *prd*-cDNA, c7340.4 (ref. 19), a *gsb*-cDNA, BSH9c2, or a *gsbn*-cDNA, BSH4c4 (ref. 3), into the P-element vector pKB255 (K. Basler and E. Hafen, unpublished) and subsequent germ-line transformation of w^{1118} embryos according to standard procedures[30].

As *gsb* maintains the expression of *wg*[9], we expect that the pair-rule phenotype of *Hsgsb* and *Hsprd* results from ectopic expression of the endogenous *gsb* and *wg* genes. Indeed, ubiquitous activation of either *gsb* or *prd* during the early time interval generates ectopic Gsb (Fig. 2*b*, *c*) and Wg stripes (Fig. 2*f*, *g*) anterior to the even-numbered wild-type Gsb (Fig. 2*a*) and corresponding Wg stripes (Fig. 2*e*). The observed pair-rule phenotype (Fig. 1*b*, *c*) is thus consistent with the ectopic *wg* expression and the resulting repression of denticle formation (Fig. 1*b*, *c*).

Activation of *Hsprd* has been shown to expand the odd-num-

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