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Genetic evidence that heparin-like glycosaminoglycans are involved in *wingless* signaling

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SUMMARY

We have identified the *Drosophila* UDP-glucose dehydrogenase gene as being involved in *wingless* signaling. Mutations in this gene, called *kiwi*, generate a phenotype identical to that of *wingless*. UDP-glucose dehydrogenase is required for the biosynthesis of UDP-glucuronate, which in turn is utilized in the biosynthesis of glycosaminoglycans. By rescuing the *kiwi* phenotype with both UDP-glucuronate and the glycosaminoglycan heparan sulfate, we show that *kiwi* function in the embryo is crucial for the production of heparan sulfate in the extracellular matrix. Further, injection of heparin degrading enzyme, heparinase (and not chondroitin, dermatan or hyaluronic acid degrading enzyme) into wild-type embryos leads to the degradation of heparin-like glycosaminoglycans and a '*wingless*-like' cuticular phenotype. Our study thus provides the first genetic evidence for the involvement of heparin-like glycosaminoglycans in signal transduction.

Key words: *wingless*, glycosaminoglycans, heparan sulfate, extracellular matrix, *Drosophila*

INTRODUCTION

The *Wnt* genes encode secreted glycoproteins which are required for intercellular communication in a variety of diverse organisms (Nusse and Varmus, 1992; McMahon, 1992). Although the *Wnt* proteins are too large to be classified as growth factors, their various roles do qualify their classification as such (discussed by Klingensmith and Nusse, 1994). Two well-studied members of the *Wnt* gene family are the *Drosophila* segment polarity gene *wingless* (*wg*) and the murine *Wnt-1* gene. Studies on these genes have focused on elucidating the mechanism of signal transduction elicited by *Wnts* (reviewed by Klingensmith and Nusse, 1994). Biochemical studies on *Wnt-1* have demonstrated the paracrine nature of *Wnt* protein function and have shown a close association of *Wnt-1* with the extracellular matrix (ECM) (Papkoff and Schryver, 1990; Bradley and Brown, 1990; Jue et al., 1992). These studies have also implicated a close association of *Wnts* with glycosaminoglycans (GAGs) found in the ECM (Bradley and Brown, 1990; Jue et al., 1992; Burrus and McMahon, 1995). Genetic studies on *Wnt-1* in mice have also shown the importance of this gene during development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Recently, biochemical studies on *wg* protein (Wg) have led to the identification of a receptor molecule for Wg (Bhanot et al., 1996). However, most information regarding the mechanism of *Wnt* signaling has come from genetic studies on *wg* in *Drosophila*.

It has been possible to identify additional genes in *Drosophila* which are involved in *wg* function by focusing on mutations that have phenotypes similar to that of *wg*. This category includes the *dishevelled* (*dsh*), *porcupine* (*porc*) and *armadillo* (*arm*) genes (Perrimon et al., 1989). The phenotype of the *zeste-white 3* (*zw3*) gene appears to be opposite to that of *wg*, leading to its inclusion as a component of *wg* signaling (Siegfried et al., 1992). Genetic epistatis experiments have led to a model whereby *porc* acts upstream of *wg* and *dsh* acts downstream of *wg* (Noordermeer et al., 1994), while *dsh* acts upstream of *zw3* and *arm* acts downstream of *zw3* (Siegfried et al., 1994). These genes have been cloned in *Drosophila* and all have been shown to be well conserved throughout evolution. For example, *zw3* is a homolog of the Glycogen synthase kinase-3 (GSK-3) family, while *arm* is a homolog of vertebrate β-catenin (McCrea et al., 1991). Therefore it is apparent that the Wnt signaling mechanism is well conserved in evolution (reviewed in Klingensmith and Nusse, 1994).

One biochemical aspect of Wnt function has been its close association with the ECM. It has been documented that Wnt-1 is tightly bound to the cell surface and/or the ECM but can be released readily via the exogenous addition of the GAG, heparin (Bradley and Brown, 1990; Papkoff and Schryver, 1990). The ECM consists of a variety of complex carbohydrates such as GAGs, which are frequently linked covalently to a distinct class of glycoproteins called proteoglycans (Kjellan and Lindahl, 1991). Proteoglycans can bind to a variety of growth factors and may even function as co-receptors during signaling (Klagsburn and Baird, 1991). Despite these

results, genetic studies linking GAGs and/or proteoglycans to Wnt or other growth factor function have been lacking. In fact, the importance of proteoglycans for Wnt signaling has come into question recently (Schryver et al., 1996) and it has been demonstrated that not all Wnts can bind GAGs (Burrus and McMahon, 1995).

In this study, we provide the first genetic demonstration of a functional role of GAGs in signal transduction. We report the identification and molecular analysis of a novel gene, *kiwi*, whose activity is critical for *wg* signaling. We show that *kiwi* encodes the *Drosophila* homolog of UDP-glucose dehydrogenase (UDP-GlcDH), an enzyme responsible for the production of UDP-glucuronate (Feingold and Franzen, 1981). UDP-glucuronate is an uronic acid which is a precursor in GAG biosynthesis (Lennarz, 1980). We also show that *kiwi* activity is required for efficient *wg* function and that the injection of either UDP-glucuronate or heparan sulfate (HS) into *kiwi* mutant embryos can rescue their phenotype. We further show that the injection of purified bacterial heparinase (an enzyme degrading heparin-like GAGs) into wild-type embryos can phenocopy the *kiwi* and *wg* mutant phenotype. Our results provide the first genetic evidence for the involvement of heparan sulfate in *wg/Wnt* function.

MATERIALS AND METHODS

Fly stocks and genetic analysis

l(3)08310 or *P1731* (P{ry[+t7.2]=PZ} l(3)08310 ry^{506} / *TM3, ry [RK]*) is from the Spradling collection of P-element lethals and maps cytologically to 65D4-5 (Spradling et al., 1995). *P1731* P-element excisions were generated by selecting single dysgenic males of genotype *P1731/ry e Sb P[Δ2,3]* from the cross between *P1731/TM3* females and males of genotype *w/Y*; *ry e Sb P[Δ2,3]/TM6, Ubx e*. Stocks were then established and scored for viability and loss of ry^+. *Df(3R)W5.4*, *A31*, *N71*, *XAJ36* and *EX34* were generated in a previously reported screen (Anderson et al., 1995). *A31* and *N71* are EMS-induced, while *XAJ36* and *EX34* are X-ray-induced alleles of *l(3)08310*, since they fail to complement the lethality of *l(3)08310*. Complementation was confirmed by crossing GLC females of $kiwi^{P1731}$ to *Df(3R)W5.4*, *A31*, *N71*, *XAJ36* and *EX34* in order to generate the *kiwi* GLC phenotype. In order to perform GLC analysis, FRT^{2A} was recombined on each mutant chromosome (*P1731*, *A31* and *N71*). We were unable to make such a recombinant with *XAJ36* whereas GLC analysis with *EX34* produced only sterile females. GLC analysis with FRT^{2A} was performed as described by Chou and Perrimon (1996). Initial experiments were performed with marked embryos and cuticles to determine the extent of zygotic rescue of the *kiwi* maternal phenotype. Embryos were marked by mating *kiwi* GLC females to males carrying mutant alleles over the *TM3* (*evelacZ*) balancer chromosome. Cuticles were generated in a *yellow* (*y*) mutant background, and the balancer chromosome was *TM3*, y^+. *prdGAL4* insertion on the X chromosome was generated by mobilizing *RG1* (Yoffe et al., 1995) using the *w*; *Sb P[Δ2,3, ry+]* (99B) *e* chromosome (Robertson et al., 1988). For epistasis experiments with *kiwi*, *prdGAL4*/+; *kiwi* GLC females were mated to *UASwg*; *kiwi/TM3* males. These epistasis experiments were performed using *P1731*, *A31* and *N71* with all experiments yielding identical results. To generate wg^{IG22} (*prd-wg*) embryos, *prdGAL4*; wg^{IG22}/*CyO* females were mated to *UASwg*, wg^{IG22}/*CyO* males. wg^{IG22} is a null allele of *wg* and has been used before in similar experiments (Manoukian et al., 1995). For injection experiments, *y kiwi* GLC embryos were generated from the following cross: *y*; $kiwi^{A31}$ GLC females × *y*; $kiwi^{A31}$/*TM3*, y^+ males.

Generation of somatic clones

For the generation of adult somatic clones in the wing, the following cross was used: *yw HsFLPase*; $kiwi^{P1731}$ FRT^{2A}/*TM3* × *yw*; *P[y⁺] FRT*^{2A}/TM3. Larvae from this cross were heat-shocked for 2 hours at 37°C at early crawling stage (72 hours after hatching). Adult wings were dehydrated and then mounted in Euparol™ for observation.

Cuticle preparations

After collection on apple juice-agar plates, embryos were plated onto a fresh plate and aged at 25°C. Unhatched embryos were dechorionated and then cleared in Hoyer's/15% lactic acid medium for 24 hours (Wieschaus and Nüsslein-Volhard, 1986). Preparations were then flattened before analysis and photography. To process injected cuticles, preparations were briefly rinsed in *n*-heptane before mounting in Hoyer's/15% lactic acid.

In situ hybridization

In situ hybridization with digoxigenin (DIG)-labeled *en* and *wg* probes was performed as previously described (Manoukian and Krause, 1992). DIG-labeled *kiwi* probe was generated from clone 51b using the same method.

Genomic DNA analysis

Overlapping phage were isolated from a *Drosophila* genomic library made in bacteriophage EMBL3 (Blackman et al., 1987). Plaque hybridizations, DNA purification and cloning, and Southern blot analysis were done as described by Sambrook et al. (1989). Probe DNAs were labelled with ^{32}P by the random priming method (Feinberg and Vogelstein, 1983), and unincorporated nucleotides were removed using a G-50 Sephadex column. The probe used as an entry into the region was a 120 bp PCR fragment amplified from wild-type genomic DNA using the following primers:

5′CGCTCGGAGAAATCTGACT3′
5′CAGCCTTGTTGTTGTTTT3′.

The sequence of these primers was deduced from that of the STS (sequence tagged site) associated with the P-element insert in the *kiwi* allele *P1731*, which was provided by the Berkeley *Drosophila* Genome Project. The PCR conditions for amplification were 35 cycles of: 94°C, 45 seconds; 60°C, 45 seconds; 72°C, 2 minutes.

cDNA analysis

Putative *kiwi* cDNAs were isolated from a 0- to 4-hour *Drosophila* embryonic cDNA library (Brown and Kafatos, 1988) using the 3.5 kb genomic *Hin*dIII fragment as probe (Fig. 4). Five positives were isolated from an initial screening of approximately 500,000 colonies. The longest clone (51b) was chosen and mapped back to genomic DNA from the walk phage to ensure that it derived from the correct region of the genome. This clone was then subjected to DNA sequence analysis (Amgen ETS program).

Preparation of *kiwi* sense RNA

Clone 51b was linearized with *Not*I, and this template was then used to generate a capped sense transcript with the SP6 mMessage mMachine Kit (Ambion) as per the manufacturer's instructions. Briefly, approximately 1 μg of linearized 51b was transcribed in vitro with SP6 polymerase at 37°C. After termination of the reaction and ethanol precipitation, the total resulting RNA was resuspended in 50 μl of injection buffer (5 mM KCl, 0.1 mM $NaPO_4$, pH 6.8) and stored at −70°C until use.

Microinjection into embryos

For injection experiments, precellular blastoderm embryos (0- to 1.5-hour old embryos) were dechorionated and lined up on double-sided tape and covered with halocarbon oil. At least 200 embryos were injected for each experiment. For 51b mRNA injection, the RNA was injected without further dilution. For protein or glucuronate injections,

the volume of injection was held constant while the concentration of the material being injected was varied. In these manipulations, a compromise was sought where most injected embryos developed to cuticle deposition. These conditions were arrived at for each experiment. Following injections, embryos were allowed to develop at 25°C and were then lifted off the tape and onto apple juice-agar plates. At this point, non-developed embryos were discarded and all other embryos were first rinsed with *n*-heptane (to remove excess halocarbon oil) and then mounted into Hoyer's/15% lactic acid medium for analysis and observation. Each experiment was repeated at least three times and the results were reproduced every time. The results of single experiments are depicted below.

All material to be injected was dissolved and/or diluted in injection buffer (5 mM KCl, 0.1 mM $NaPO_4$, pH 6.8). *kiwi* mutant embryos were marked with *y* and rescue was deemed successful when significant naked cuticle was added back into *y kiwi* mutant embryos – partial rescue was scored as negative. The injection of 51b effectively rescued the *kiwi* mutant embryos at a frequency of 19/38. UDP-glucuronate injection was effective at 1 mg/ml concentration resulting in a rescue frequency of 12/33. Heparan sulfate rescue was also effective at 1 mg/ml concentration, but we did notice that most of the cuticles were becoming converted to the naked phenotype (see below). As we focused on the *y* embryos, we had to remove a significant population of embryos which had the fully penetrant naked phenotype and thus could not be scored. Therefore this rescue frequency was only 8/17.

Expression and purification of heparinase I and III was as has been previously published (Sasisekharan et al., 1993; Ernst et al., 1996). Chondroitinase ABC from *Proteus vulgaris* was from Seikagaku Corporation (Ijamsville, MD). This enzyme can degrade chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate and hyaluronic acid (manufacturer's specifications). Heparinase I, III, chondroitinase ABC and heparan sulfate were injected into w^{1118} embryos. Heparinase I was at a concentration of 0.5 mg/ml. With injection of heparinase, 23/44 embryos showed significant fusion of denticles and 11 of the 23 showed the severe *wg* phenotype. The injection of heparinase III was effective at 0.55 mg/ml and resulted in a *wg*-like phenotype at a frequency of 27/49. Injection of chondroitinase ABC was varied all the way up to a concentration of 1 mg/ml. Injection of heparan sulfate into w^{1118} embryos was at 1 mg/ml (as in above) and denticles were converted to naked cuticle at a frequency of 11/36, with 7 embryos showing severe and fully penetrant effects. The data from these injection experiments are tabulated in Table 1 and depicted in Figs 7 and 8.

RESULTS

Although the *wg* phenotype is zygotic, other *wg* signaling pathway genes are also expressed maternally and thus their phenotypes have to be generated via the production of germ line clone (GLC) females (Perrimon et al., 1989). We have identified *P1731* or *l(3)08310* (Spradling et al., 1995) as having a GLC cuticle phenotype which resembles that of *wg* (Fig. 1B) and we have named this locus *kiwi*. This GLC phenotype is zygotically (paternally) rescuable, but not to wild-type. Zygotically rescued $kiwi^{P1731}$ embryos have weak segmental fusions in the ventral portion of the embryo and are often missing dorsal cuticle pattern elements (Fig. 7B). Excision experiments of *P1731* were used to lose the $rosy^+$ phenotype and generated viability at a frequency of 10/40. The results of this experiment convinced us that the lethal mutation in *l(3)08310* was a result of P-element insertion. We have identified four additional *kiwi* alleles (Anderson et al., 1995), two of which (A31 and N71) we have also used in GLC experiments. In all experiments $kiwi^{P1731}$ behaved as the strongest allele, although the severity of the phenotype compared to A31 and N71 was rather comparable (see Materials and Methods). The GLC phenotype of $kiwi^{P1731}$ is identical to the *wg* phenotype (Fig. 1B) and was not intensified over a deficiency of the region (*Df (3R) W5.4*) (not shown); we therefore believe that $kiwi^{P1731}$ is a null allele.

kiwi is required for efficient *wg* function

The expression of the *engrailed* (*en*) gene has been shown to

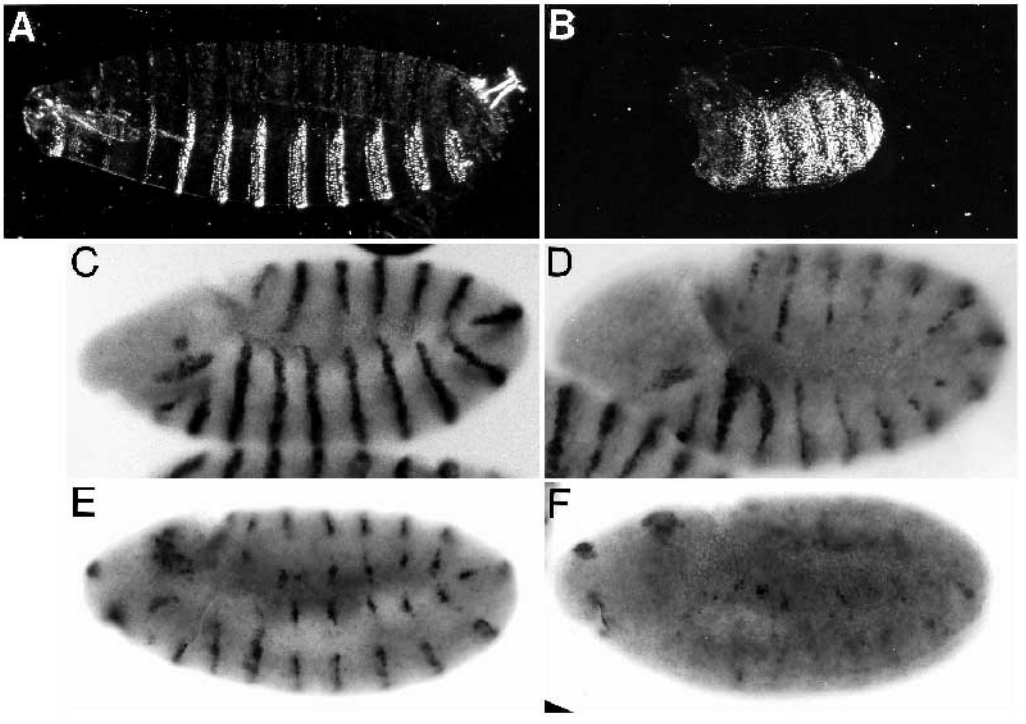

Fig. 1. The *kiwi* germline clone phenotype. Cuticle preparations of wild-type (A), and $kiwi^{P1731}$ GLC (B) embryos. Early stage 10 wild-type (C) and $kiwi^{P1731}$ GLC (D) embryos stained for *en* transcripts. In *kiwi* mutant embryos, *en* transcripts have nearly faded by this stage. Early stage 10 wild-type (E) and $kiwi^{P1731}$ GLC (F) embryos stained for *wg* transcripts. By this stage, almost all *wg* transcription has faded in *kiwi* embryos.

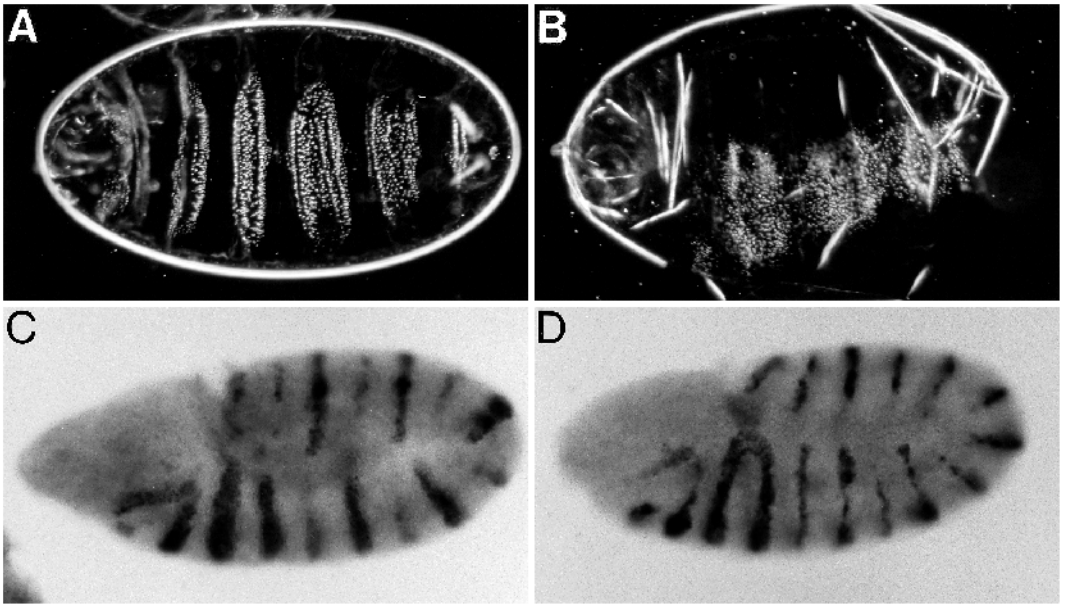

Fig. 2. Effects of ectopic expression of *wg* in *kiwi* mutants. Ectopic expression of *wg* using *prdGAL4/UASwg* (*prd-wg*) in wg^{IG22} (A,C) and $kiwi^{P1731}$ (B,D) mutants. Cuticle preparations of *wg* (*prd-wg*) (A) and *kiwi* GLC (*prd-wg*) (B) larvae. *prd-wg* results in rescue of naked cuticle in alternate segments of *wg* mutants (A). In *kiwi* mutants (B), the effects of *prd-wg* are not as robust and very little naked cuticle is restored. *en* transcription in *wg* (*prd-wg*) (C) and *kiwi* (*prd-wg*) (D) late stage 9 embryos. Wide *en* stripes are restored in *wg* (*prd-wg*) embryos (C) as alternate stripes fade. In *kiwi* (*prd-wg*) (D) embryos, alternate *en* stripes are fading and *prd-wg* rescued stripes are narrow and discontinuous compared to *wg* (*prd-wg*) embryos.

be under the control of *wg* activity in the embryo (DiNardo et al., 1988; Martinez-Arias et al., 1988). In *wg* mutant embryos, *en* transcripts fade during stages 9-10 (Manoukian et al., 1995). In *kiwi* GLC embryos, *en* expression is lost during stages 10-11 (Fig. 1D). It appears that, although *wg* and *kiwi* GLC embryos have identical cuticle phenotypes, *en* expression persists longer in *kiwi* GLC embryos. Therefore, *en* expression is more resistant to the loss of *kiwi* activity than *wg* activity. In *wg* mutants, *wg* transcription is lost during stages 9-10 before the loss of *en* expression in the same embryos (Manoukian et al., 1995). In *kiwi* GLC embryos, *wg* expression is also lost before *en* during stage 10 (Fig. 1F). However, as in the case of *en* transcription, *wg* transcripts are lost earlier in *wg* mutants compared to *kiwi* mutants.

As *wg* expression is gone relatively early (stage 10) in *kiwi* mutants, we decided to test the activity of ectopic *wg* in these mutants by using the *prdGAL4* transgene to activate *UASwg* in alternate segments (Brand and Perrimon, 1993; Yoffe et al., 1995; Manoukian et al., 1995). In *wg* mutant embryos, *prdGAL4/UASwg* (*prd-wg*) can rescue *wg* activity (naked cuticle and *en* expression) in alternate segments (Fig. 2A,C). In *kiwi* mutants, *prd-wg* results in a minor rescue of the *kiwi* cuticular phenotype but cannot specify naked cuticle (Fig. 2B). *en* expression is also rescued by *prd-wg* in *kiwi* mutants (Fig. 2D), although this effect is weak. These experiments suggest that *kiwi* is not upstream of *wg*, but do suggest that *wg* can have some activity in the absence of *kiwi*. Since *hedgehog* (*hh*) mutants have a phenotype similar to that of *kiwi* and *wg*, this opens up the possibility that *kiwi* functions in *hh* signaling and thus affects *wg* indirectly (Manoukian et al., 1995). As ectopic *wg* is fully functional in the absence of *hh* and *smoothened* activity (van den Heuvel and Ingham, 1996), our epistasis results with *prd-wg* suggest that *kiwi* is involved in *wg* signaling directly as opposed to being an exclusive component of *hh* signaling.

Further evidence to support a direct role for *kiwi* in *wg* signaling comes from the fact that *kiwi* somatic clones result in loss of anterior margin bristles of the adult wing (Fig. 3B), which have been shown to be specified by *wg* (Couso et al., 1994). This wing margin phenotype also gives us the opportunity to test the autonomy of *kiwi* function. In the *wg* pathway, *wg* and its upstream component *porc* function non cell-autonomously, whereas the downstream components (*dsh*, *zw3* and *arm*) are cell-autonomous in mosaics (Klingensmith and Nusse, 1994). Making *yellow* marked clones of *kiwi* demonstrates that *kiwi* functions non cell-autonomously (Fig. 3C), suggesting that *kiwi* is at least genetically upstream of *dsh*.

Cloning of the *kiwi* locus

To clone the *kiwi* locus, a chromosomal walk in the 65D region was initiated using as an entry point the STS (sequence tagged site) generated from the *kiwi* P-element-induced allele *P1731* by the Berkeley *Drosophila* Genome Project. Overlapping phage clones were isolated that span approx. 30 kb of wild-type genomic DNA. Genomic fragments that hybridized to the STS were sequenced and used as probes to screen a 0- to 4-hour embryonic cDNA library. The longest clone (designated 51b) was chosen for further analysis. Southern blot analysis indicates that the cDNA extends over approx. 5 kb of genomic DNA (Fig. 4). Sequence analysis of genomic DNA and comparison to that of the cDNA indicates that the *P* element associated with *P1731* has inserted into the 5′ untranslated region of the transcription unit. A developmental northern analysis using 51b as probe detects a single transcript of approx. 2.4 kb present at most stages of development, including 0- to 2 hours (data not shown). In situ hybridization to wild-type whole-mount embryos with 51b as probe shows that *kiwi* transcripts are present uniformly throughout the embryo at blastoderm stage (Fig. 5A), and subsequently become localized to specific cephalic regions and to the presumptive amnioserosa beginning at stage 9 (Fig. 5B). This expression in the amnioserosa persists well into stage 14 of embryogenesis (not shown). To demon-

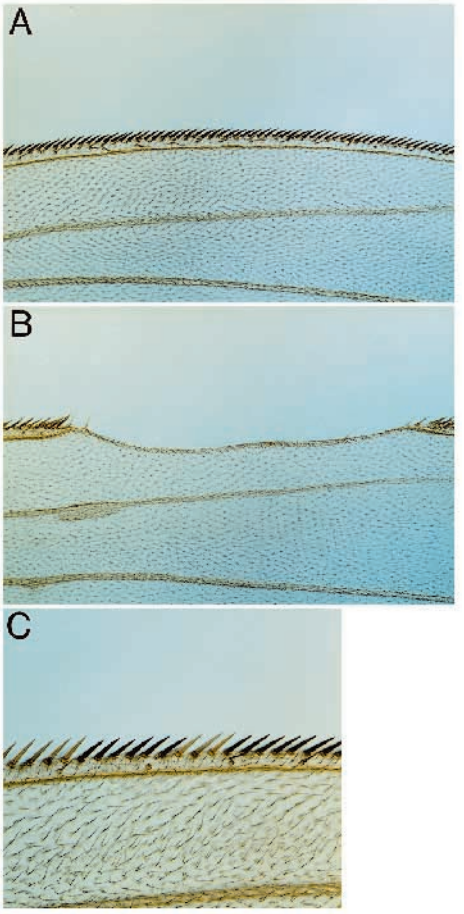

Fig. 3. Clonal analysis of the *kiwi* mutation. (A) Close-up of a wing margin of wild-type fly. (B) A large *kiwi*$^{-}$/*kiwi*$^{-}$somatic clone at the wing margin showing loss of anterior margin bristles and wing tissue. (C) Close up of *y kiwi* clones demonstrating the non cell-autonomy of the *kiwi* mutation. The existence of *y* bristles demonstrates the rescue of the *kiwi* mutant clone by the surrounding wild-type cells.

strate that 51b encodes *kiwi* function, an SP6-generated transcript corresponding to the sense strand of 51b was injected into *kiwi* germline clone-derived embryos, where it led to significant rescue of the *kiwi* phenotype (Table 1; Fig. 7C).

kiwi encodes the *Drosophila* homolog of UDP-glucose dehydrogenase

The nucleotide sequence of 51b (2343 bp) encodes a conceptualized open reading frame beginning with an ATG at position 315 and extending 1428 nucleotides to position 1742, thus encoding a putative protein of 476 amino acids. Stop codons in all three reading frames follow the open reading frame. The nucleotides immediately upstream of the putative initiation codon (CGAA) are a reasonably good match to the consensus sequence for *Drosophila* translation start sites [(C/A)AA(C/A)] (Cavener, 1987). The open reading frame is preceded by an untranslated region of 215 nucleotides and followed by an untranslated region of 601 nucleotides containing the consensus polyadenylation recognition sequence AATAAA (Birnstiel et al., 1985) 16 bases from the 3′ end of the cDNA.

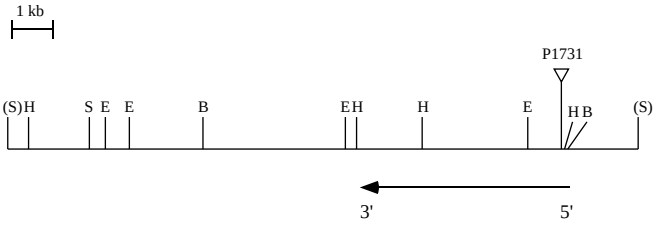

Fig. 4. Molecular map of the *kiwi* locus. Shown is a restriction map of the genomic DNA encompassing the *kiwi* locus. The enzymes indicated above the horizontal line are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), and *Sal* I(S). The parentheses enclose restriction sites not present in genomic DNA but present at the junction of phage and genomic DNA in the phage used to generate this map. Below the restriction map is a schematic of the *kiwi* transcript, indicating the direction of transcription and the extent of genomic DNA detected by the *kiwi* cDNA by Southern blotting. The intron-exon structure of the genomic DNA corresponding to the cDNA has not been determined. The site of P-element insertion in the *kiwi* allele *P1731* is also indicated.

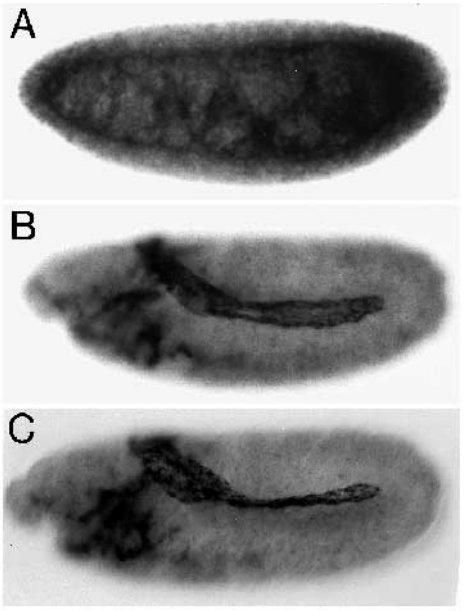

Fig. 5. Distribution of *kiwi* transcripts in embryos. Distribution of *kiwi* transcripts in stage 4 (A), late stage 10 (B), and stage 11 (C) wild-type embryos. Although *kiwi* expression is uniform at stage 4 before blastoderm, it becomes restricted to regions surrounding the cephalic furrow and to the presumptive amnioserosa during gastrulation.

A homology search of the available databases using the BLAST algorithm (Altschul et al., 1990) indicates that *kiwi* encodes the *Drosophila* homolog of uridine diphosphate glucose dehydrogenase (UDP-GlcDH) (Dougherty and van de Rijn, 1993; Hempel et al., 1994). The sequence with maximum homology is bovine UDP-GLcDH with an overall identity of 68% (see Fig. 6). UDP-GlcDH catalyzes the reaction UDP-glucose + 2NAD^{+} + H_2O to yield UDP-glucuronic acid + 2NADH + 2H^{+} (Dougherty and van de Rijn, 1993). To show that the *kiwi* GLC phenotype is due to the loss of UDP-GlcDH activity, we attempted to rescue the *kiwi* phenotype by injecting

Fig. 6. Alignment of UDP-glucose dehydrogenase amino acid sequences. Deduced amino acid sequence of *Drosophila melanogaster* (Dm) UDP-glucose dehydrogenase and alignment with bovine (bov) and *C. elegans* (Ce) homologs. All *Drosophila* residues, as well as identical residues in homologs, are indicated by bold type. The gaps in the sequence are represented with '-'.

UDP-glucuronic acid into *kiwi* GLC embryos. Since this leads to the rescue of the *kiwi* phenotype (Table 1; Fig. 7D), we suggest that UDP-glucuronic acid (or UDP-glucuronate) production in the embryo is essential for *wg* signaling.

Heparin-like GAGs are involved in *wg* signaling

UDP-glucuronate is one of the uronic acids incorporated into several complex polysaccharides or glycosaminoglycans (GAGs), including hyaluronic acid, chondroitin sulfate, heparin and heparan sulfate. Reichsman et al. (1996) have recently shown the involvement of GAGs in *wg* signaling in tissue culture cells, consistent with studies on *Wnt-1* which had implicated the involvement of heparin in *Wnt* localization and function (Bradley and Brown, 1990; Jue et al., 1992; Burrus and McMahon, 1995). Given this background information of the potential roles for heparin-like and/or chondroitin sulfate GAGs in *wg* function, we injected separately, purified heparinases I and III (degrading heparin-like GAGs) and chondroitinase ABC (degrading chondroitin sulfates, hyaluronic acid and dermatan sulfate) into wild-type embryos to determine if either or both enzymes had any effect on *wg* signaling. We found that injection of heparinases I (Table 1; not shown) and III (Table 1; Fig. 8A) into wild-type embryos can mimic the *kiwi* and *wg* phenotypes, whereas injecting chondroitinase ABC does not

Table 1. Results of micro-injection experiments depicted in Figs 7 and 8

Experiment number	Genotype of embryos injected	Material injected	Genotype analyzed	Number of embryos analyzed	Phenotype observed	Number of embryos with observed phenotype
1	*y kiwi GLC*	51b mRNA	*y kiwi*	38	Rescue	19
2	*y kiwi GLC*	UDP-glucuronate	*y kiwi*	33	Rescue	12
3	*y kiwi GLC*	Heparan sulfate	*y kiwi*	17	Rescue	8
4	w^{1118}	Heparinase I	All	44	*wg*-like	23
5	w^{1118}	Heparinase III	All	49	*wg*-like	27
6	w^{1118}	Heparan sulfate	All	36	'Naked' cuticle	11

See Materials and Methods for details of experiments.

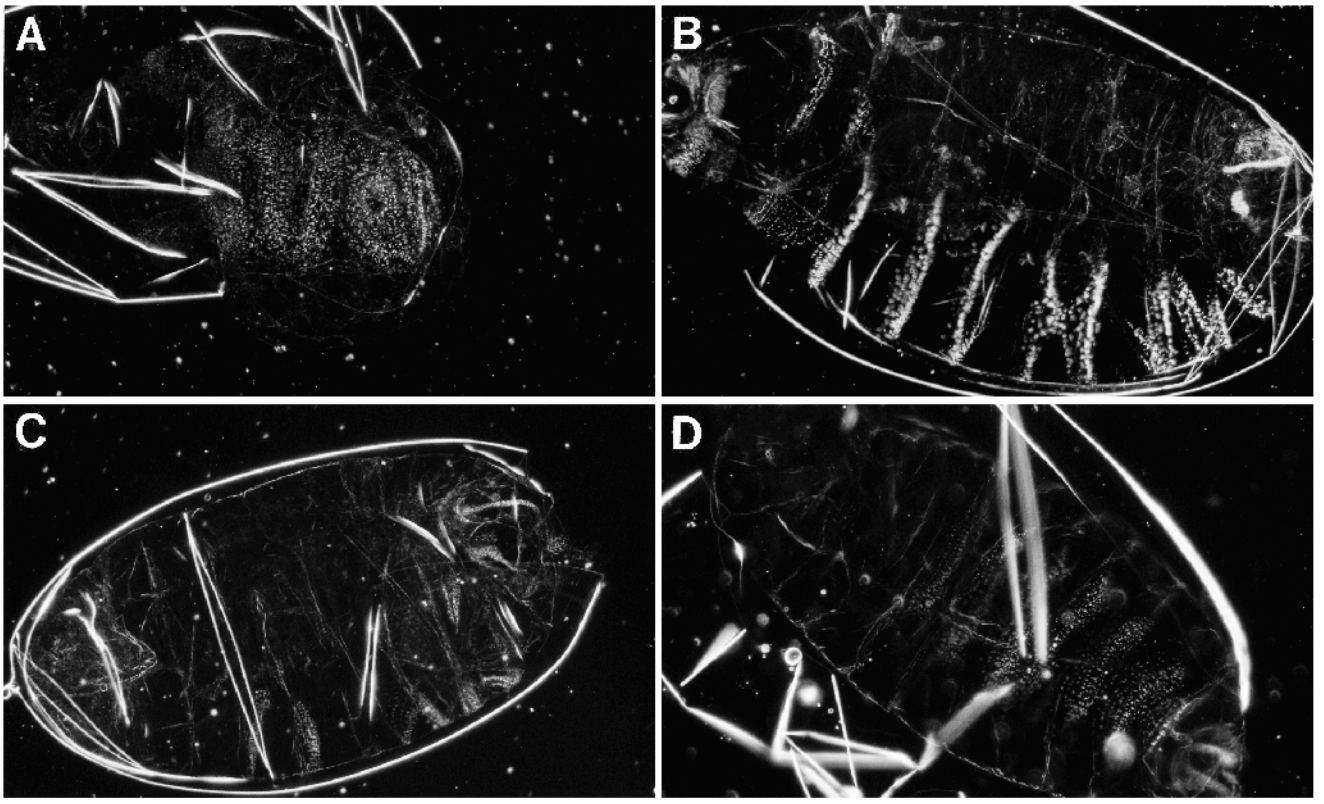

Fig. 7. Rescue of the *kiwi* phenotype. Cuticle preparations of *y kiwi* GLC embryos. Non-paternally rescued *kiwi* embryos are marked with the *y* mutation (see Materials and Methods) which results in a diminution of the brightness of denticles under dark-field illumination (A, C-D). When *y kiwi* GLC embryos are rescued with y^+ $kiwi^+$, denticle brightness and much of segmentation is restored (B). Rescue of segmentation of *kiwi* GLC embryos with in vitro transcribed *Drosophila* UDP-glucose dehydrogenase (51b) mRNA (C), and UDP-glucuronate (D). These cuticles are clearly marked with *y* (thus being $kiwi^-$) and are rescued to varying degrees, although the rescue of filzkorper is complete in each case.

mimic the *wg* phenotype (unpublished observations; see Materials and Methods). This suggests that heparin-like (heparin and heparan sulfate) GAGs are involved in *wg* signaling. As heparinase III is much more specific to heparan sulfate (Ernst et al., 1995), we attempted to rescue the *kiwi* phenotype by injecting exogenous heparan sulfate into *kiwi* GLC embryos. Since heparan sulfate was able to rescue the *kiwi* phenotype (Table 1; Fig. 8B), we suggest that the loss of UDP-GlcDH activity in *kiwi* embryos reflects a requirement of heparin-like GAGs and, perhaps more specifically, heparan sulfate in *wg* function. Furthermore we find that injection of excess heparan sulfate into wild-type embryos results in lethality and a 'naked' cuticle phenotype (Table 1; Fig. 8C), which is identical to that of Wg over-expression in embryos (Noordermeer et al., 1992). Thus it appears that excess heparan sulfate in embryos results in hyperactivity of Wg, consistent with the in vitro results obtained by Reichsman et al. (1996).

DISCUSSION

We have shown that the *kiwi* gene is required for full *wg* function in embryos and at the wing margin. We have also shown that *kiwi* encodes the *Drosophila* homolog of UDP-GlcDH, an enzyme involved in the biosynthesis of UDP-glucuronate. UDP-glucuronate is further utilized in the biosynthesis of complex polysaccharides such as hyaluronic acid, chondroitin sulfate, heparin and heparan sulfate. This fact implicates all of these polysaccharides as having a role in *wg* function. Since chondroitinase ABC can degrade a wide range of GAGs such as hyaluronic acid, chondroitin sulfate and dermatan sulfate (Ernst et al., 1995), our inability to generate a *wg* phenotype by injecting chondroitinase ABC (not shown) suggests that the loss of only heparin-like GAGs results in the *wg*-like phenotype observed in *kiwi* mutants. This idea is fortified by our ability to rescue the *kiwi* phenotype with excess heparan sulfate.

Heparin-like GAGs are required for *wg* signaling

We have demonstrated a crucial role for heparin-like GAGs in *wg* function by four criteria. First, the injection of heparinase III (Fig. 8A) and not chondroitinase ABC (not shown) leads to phenocopy of the *wg* (and also *kiwi*) phenotype. Second, and more specifically, injection of excess heparan sulfate leads to rescue of the *kiwi* phenotype (Fig. 7). Third, the effect of expression of ectopic *wg* is partially suppressed in *kiwi* embryos (Fig. 2). Finally, injection of heparan sulfate into wild-type embryos results in excess Wg signaling and a 'naked' phenotype (Fig. 8C). Our results are therefore consistent with those of Reichsman et al., (1996) which demonstrated a role for GAGs in *wg* signaling in tissue culture cells. In this study, we have been able to provide the first genetic evidence for the requirement of GAGs in *Wnt* function. Furthermore, we have specifically established a role for heparan-like GAGs in

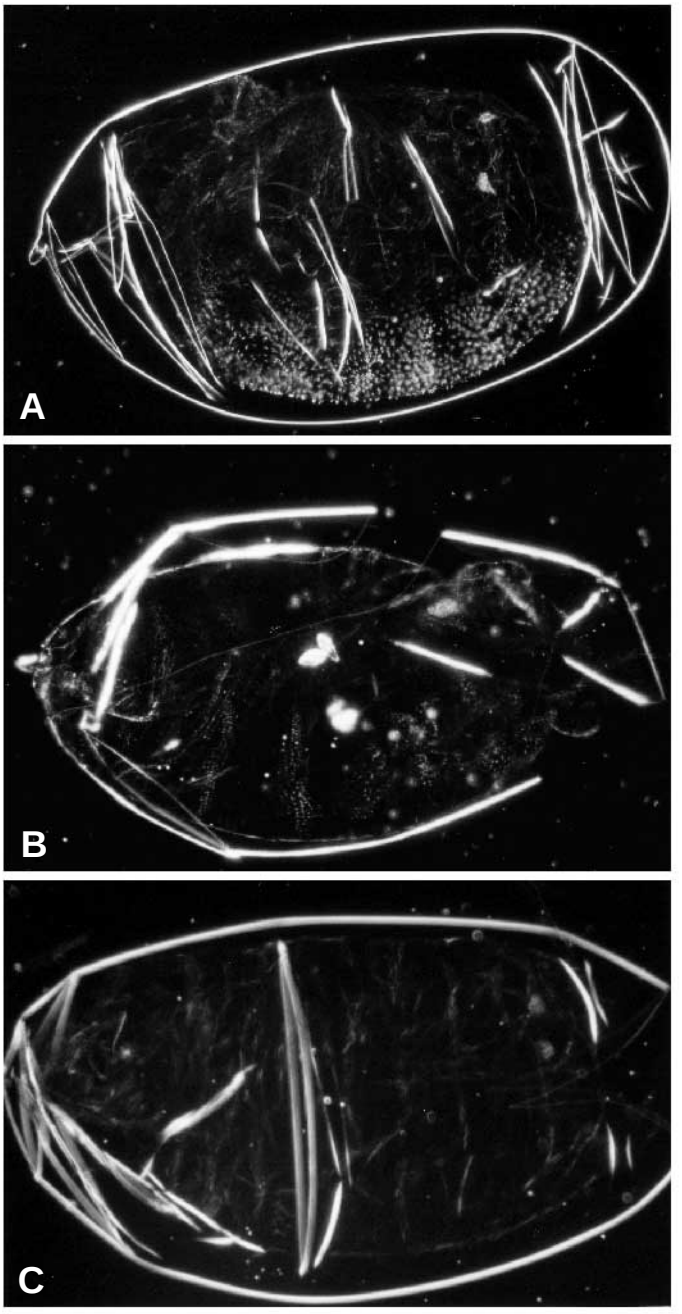

Fig. 8. Injection of heparinase and heparan sulfate. Cuticle preparation of w^{1118} embryos injected with heparinase III protein (A), *y kiwi* GLC embryos injected with heparan sulfate (B), and w^{1118} embryos injected with heparan sulfate (C). With the injection of heparinase III, w^{1118} embryos die and their cuticle phenotype resembles that of *kiwi* GLC embryos (A). Injection of heparan sulfate rescues the *kiwi* GLC phenotype (B). Note the robust rescue of 'naked' cuticle between the first two abdominal segments. With the injection of heparan sulfate, w^{1118} embryos die and have a 'naked' cuticular phenotype (C).

wg signaling. Our ability to rescue *kiwi* with exogenous heparan sulfate suggests that heparan sulfate is the crucial moiety in *wg* function. Heparan sulfate is similar to heparin except for a lower degree of sulfation and it contains a higher percentage of glucuronate residues consistent with the role of UDP-GlcDH (Ernst et al., 1996). Another important fact to note is that heparin is largely concentrated in the intracellular portion of cells, whereas heparan sulfate is extracellular.

Genetic mosaic experiments show that *kiwi* acts non cell-autonomously (Fig. 3), consistent with *kiwi* encoding UDP-GlcDH. As heparan sulfate is localized to the ECM, the UDP-GlcDH function would reflect non cell-autonomy. Since *kiwi* has a GLC phenotype which is zygotically rescuable (Fig. 7B), it is logical to assume that maternal *kiwi* expression (Fig. 5) has a crucial role in *wg* signaling. *kiwi* expression is at first uniform and persists until cellularization of blastoderm, gradually becoming restricted during gastrulation (Fig. 5). We therefore propose that maternal and pre-cellular blastoderm expression of *kiwi* generates enough UDP-glucuronate to be incorporated into the ECM as it is synthesized before cellularization. Once the ECM is formed, *kiwi* expression is not uniform and becomes restricted to specific tissues where it may be required for similar or perhaps different functions. Interestingly, the zygotic phenotype of *kiwi* shows extensive loss of dorsal cuticle structures and perhaps the amnioserosa (not shown), and may reflect the high level of *kiwi* expression in these tissues during gastrulation. The GLC phenotype of *kiwi* is identical to the *wg* phenotype and may only reflect the earliest crucial role for *kiwi* function. UDP-glucuronate may be required for many other processes which are either required later on in development or whose phenotypes are masked by the severe '*wg*-like' phenotype apparent in *kiwi* GLC embryos.

Role of heparin-like GAGs in Wg signaling

Heparin-like GAGs are complex acidic polysaccharides that are important soluble components of the ECM. They provide the necessary hydration to the ECM scaffold and hence solubilize and modulate the function of several of the transient components of the matrix such as growth factors (Kjellen and Lindahl, 1991). The role of heparin-like GAGs in regulating the biological activities of several heparin-binding growth factors has garnered attention for some time. For example, heparin-like GAGs are thought to tether fibroblast growth factor (FGF) in the ECM so as to create a reservoir of FGF (reviewed by Jackson et al., 1991). In one model for FGF-GAG interaction, stable FGF-heparin complexes are released, diffuse and activate FGF receptor at a site distant from the release site (Jackson et al., 1991). A different model (the dual receptor model) suggests that heparin-like GAGs bind FGF and 'present' this ligand to the FGF receptor. Specifically, it is thought that heparin-like GAGs participate in a dual receptor system as low-affinity receptors to facilitate the interaction of ligands with their high-affinity receptors (Klagsbrun and Baird, 1991; Schlessinger et al., 1995). It has also been suggested that heparin binding to FGF causes FGF oligomerization leading to clustering of FGF receptors for signal transduction (Ornitz et al., 1992).

wg protein (Wg) has been shown to bind GAGs in vitro, suggesting that GAG moieties in the ECM sequester Wg (Reichsman et al., 1996). This interaction appears to be crucial for cells that receive Wg and not those that produce it (Reichsman et al., 1996). We propose that the extracellular GAG, heparan sulfate, physically interacts with Wg in cells which receive the Wg signal. The tight binding of Wg to heparin-like GAGs could regulate the stability, diffusion and effective concentration of the Wg reservoir in the ECM, while

generating a gradient by limiting the diffusion of Wg (Lawrence and Struhl, 1996). This aspect is particularly intriguing in light of the finding that different threshold concentrations of Wg elicit distinct responses to organize cellular pattern during morphogenesis (Zecca et al., 1996). Alternatively, reception of the Wg signal could be regulated by heparan sulfate. With the recent identification of a Wg receptor (Bhanot et al., 1996), testing of models for regulation of Wg-receptor interactions by heparan sulfate can now be undertaken.

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Note: The sequence of 51b has been submitted to GenBank; accession number AF 000570.

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