Drosophila Pipe protein activity in the ovary and the embryonic salivary gland does not require heparan sulfate glycosaminoglycans

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Summary

The *Drosophila pipe* gene encodes ten related proteins that exhibit amino acid sequence similarity to vertebrate heparan sulfate 2-O-sulfotransferase. One of the Pipe isoforms, which is expressed in the ventral follicular epithelium, is a key determinant of embryonic dorsoventral polarity, suggesting that Pipe-mediated sulfation of a heparan sulfate proteoglycan provides a spatial cue for dorsoventral axis formation. We used several approaches to investigate this possibility in the work described here. We determined the nucleotide alterations in 11 different *pipe* alleles. Ten of the mutations specifically affect the *pipe* isoform that is expressed in the ovary. Among these ten mutations, two alter an amino acid in the putative binding site for 3′-phosphoadenosine 5′-phosphosulfate, the universal sulfate donor. Using Alcian Blue, a histochemical stain that detects sulfated glycans, we observed a novel, *pipe*-dependent macromolecule in the embryonic salivary glands. Genes known to participate in the formation of heparan sulfate in *Drosophila* are not required for the production of this material. To investigate whether a heparan sulfate proteoglycan is involved in *pipe* function in dorsoventral patterning, we generated females carrying follicle cell clones mutant for heparan sulfate synthesis-related genes. Embryos from follicles with mutant clones did not exhibit a dorsalized phenotype. Taken together, our data provide evidence that Pipe acts as a sulfotransferase, but argue against the hypothesis that the target of Pipe is a heparan sulfate glycosaminoglycan.

Key words: Pipe, Windbeutel, Dorsal group, Dorsoventral polarity, Alcian Blue, Mucopolysaccharide, GAG, Proteoglycan, PAPS

Introduction

The determinant that integrates ovarian and embryonic dorsoventral (DV) polarity in *Drosophila* is encoded by the gene *pipe* (Sen et al., 1998) [for a review of DV patterning, see Roth (Roth, 2003)]. Homozygous *pipe* mutant females produce dorsalized embryos, and mosaic experiments determined that the requirement for *pipe* expression is in the somatic follicle cells (Stein et al., 1991). The *pipe* transcription unit is specifically expressed in the ventral follicle cells of the stage 10 egg chamber. Furthermore, the pattern of *pipe* transcription in the follicle cell layer is dependent on the activity of the Gurken/Epidermal Growth Factor Receptor (EGFR) signal transduction pathway. In the initial molecular characterization of the *pipe* locus (Sen et al., 1998), two alternatively spliced cDNAs were identified that encode independent protein isoforms with significant sequence homology to heparan sulfate 2-O-sulfotransferase (HS2ST) (Kobayashi et al., 1997). HS2ST is a Golgi resident protein that mediates the transfer of sulfate to the '2-O' position of hexuronic (iduronic or glucuronic) acid residues of heparan sulfate (Kobayashi et al., 1996). One of the Pipe isoforms initially identified, designated Pipe-ST2, is expressed in the ventral follicle cells of the ovary. Uniform expression of this isoform in the follicle cell layer leads to the ventralization of progeny embryos, whereas directed expression of this isoform in the dorsal follicle cells of otherwise *pipe* mutant females can invert the polarity of the embryonic DV axis with respect to the intrinsic DV polarity of the egg shell (Sen et al., 1998).

Eight additional protein isoforms are encoded by the *pipe* locus, one of which has also been reported to be expressed in ventral follicle cells (Sergeev et al., 2001). The functional consequences of its expression there are unknown. All isoforms share the same N-terminal 95 amino acids, which are encoded by three common exons, but the C-terminal region of each isoform is distinct because of the existence of 10 alternate sets of exons (Fig. 1A). Like the vertebrate GAG-modifying enzymes, the Pipe isoforms are predicted to exhibit a typical type II transmembrane topology, with a short, N-terminal hydrophilic region followed by a short membrane-spanning hydrophobic region that precedes a catalytic domain residing in the lumen of the Golgi.

Because DV signaling involves an extracellular serine protease cascade, it is notable that serine proteolytic activity during blood coagulation is controlled by a complex formed between heparin and antithrombin (Furie and Furie, 1988).

Heparan sulfate and heparin are polymers of repeating disaccharides made up of glucuronic acid and/or iduronic acid residues in β1,4 linkage to N-acetyl glucosamine. Heparin is structurally similar to heparan sulfate, with the distinction that heparin is much more highly sulfated. Of particular interest is the finding that the high affinity antithrombin/heparin interaction occurs via a specific pentasaccharide sequence, the distinguishing feature of which is the 3-0-sulfate group on the internal glucosamine unit (Petitou et al., 2003).

The crucial dependence of DV patterning upon the existence of an extracellular serine proteolytic cascade, and the similarity of Pipe to vertebrate HS2ST, has led to the hypothesis that Pipe-ST2 modifies a glycoprotein that is secreted from the ventral follicle cells and localized ventrally within the perivitelline space (Sen et al., 1998). It is proposed that Pipe-dependent modification mediates an interaction between the glycoprotein and components of the serine protease cascade that lead to ventral processing of the Spätzle ligand. The hypothesis that embryonic DV patterning requires sulfotransferase activity in the follicle cell layer is supported by the finding that females carrying follicle cell clones homozygous for loss-of-function mutations in the gene *slalom* produce dorsalized progeny embryos (Lüders et al., 2003). *slalom* encodes the transporter that mediates uptake of 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the universal sulfate donor, into the Golgi (Kamiyama et al., 2003; Lüders et al., 2003).

In the studies reported here, we have used molecular and genetic methods to investigate whether heparan sulfate is the substrate of Pipe enzymatic activity. First, we characterized the molecular lesions associated with eleven *pipe* mutant alleles. Two of these mutations map to the putative binding site for PAPS (Kakuta et al., 1998), the high energy donor molecule in sulfation reactions. We also demonstrate that in addition to its function in the adult ovary, *pipe* expression in the embryonic salivary glands is correlated with the presence of a material that binds Alcian Blue, a histochemical stain that interacts with highly sulfated molecules (Scott et al., 1964; Scott and Dorling, 1965).

To investigate whether the stained material might represent a heparan sulfate-containing molecule, we took advantage of the existence of mutations in several of the genes involved in heparan sulfate biosynthesis and modification in *Drosophila*. When we examined the salivary glands of embryos that were maternally and zygotically mutant for these genes, the Alcian Blue-stained material was still present. To investigate whether Pipe activity in the ovary is also independent of heparan sulfate synthesis, we generated females carrying follicle cell clones mutant for the heparan sulfate-related genes. In no case did these mutant females produce dorsalized embryos. Together, these observations indicate that proteins encoded by the *pipe* locus play a crucial role in the generation of sulfated macromolecules in the embryonic salivary gland, and by extension during egg formation. Our results, however, strongly argue against the suggestion that a heparan sulfate GAG is the target of Pipe activity.

Materials and methods

Stocks

All stocks were maintained and crosses carried out employing standard conditions and procedures. Larval cuticles were prepared according to Van der Meer (Van der Meer, 1977). The wild-type stock used was Oregon R. Mutant alleles have been described as follows: $pipe^{1}$ and $pipe^{2}$ (formerly $pipe^{386}$ and $pipe^{664}$) (Anderson et al., 1985); $pipe^{4}$ (Chasan et al., 1992); $pipe^{ZH1}$ (Sergeev et al., 2001); Df(3L)$pipe^{A13}$ (Sen et al., 1998); transgenic lines carrying pUAST-*pipe*-ST2 insertion (Sen et al., 1998); Df(3L)*kto2* (Kennison and Tamkun, 1988). $wind^{RP}$, $wind^{T6}$, $wind^{M88}$, ndl^{4} (formerly ndl^{133}), gd^{7}, ea^{1}, snk^{2} (formerly snk^{229}), spz^{4} (formerly spz^{rm7}), sll^{7E18}, sgl^{08310}, sfl^{03844} and frc^{00073} are described in FlyBase (http://flybase.bio.indiana.edu/). $pipe^{3}$, $pipe^{5}$, $pipe^{6}$, $pipe^{7}$, $pipe^{8}$, $pipe^{9}$, $pipe^{11}$ and $pipe^{12}$ were gifts from Dr Kathryn Anderson. $pipe^{C14}$ was identified in a P-element screen for new alleles of the locus (D.S., unpublished). The following mutations were obtained from Dr Jim Kennison, who isolated them in a screen for lethal mutations that fail to complement the chromosomal deficiency *Df(3L)kto*^{2}: $l(3)76BDd^{4}$, $l(3)76BDi^{2}$, $l(3)76BDj^{1}$, $l(3)76BDk^{1}$, $l(3)76BDl^{5}$, $l(3)76BDm^{6}$, $l(3)76BDn^{1}$, $l(3)76BDn^{2}$, $l(3)76BDn^{3}$, $l(3)76BDn^{4}$, $l(3)76BDo^{1}$ [now named $Su(z)12^{3}$ (Birve et al., 2001)], $l(3)76BDq^{1}$ [now named $asf1^{1}$ (Moshkin et al., 2002)], $l(3)76BDr^{1}$, $l(3)76BDs^{1}$ [now named $Su(Tpl)^{s1}$ (Eissenberg et al., 2002)], $l(3)76BDt^{1}$, $l(3)76BDu^{1}$, trc^{2} and kto^{1}. The mutations $l(3)76BDn^{1}$, $l(3)76BDn^{2}$, $l(3)76BDn^{3}$ and $l(3)76BDn^{4}$ have been renamed $papss^{1}$, $papss^{2}$, $papss^{3}$ and $papss^{4}$, respectively. The stock carrying third chromosomal insertions of P{w[+mC]=ovoD1-18}3L and P{w[+mW.hs]=FRT(w[hs])}2A was obtained from the *Drosophila* stock center in Bloomington, as were stocks carrying GFP-expressing balancer chromosomes. The stocks *y w* dec^{VA28}/FM7; p[dec^{+}2L-21] p{FRT(w[hs])}2A and *y w* dec^{VA28} P[hsFLP, ry^{+}]/FM6; *ru Pr ca*/TM3, *Ser* were kind gifts from Dr Trudi Schüpbach.

Sequencing of *pipe* alleles

Genomic DNA was prepared from transheterozygous adult flies carrying each of the mutant *pipe* alleles in trans to Df(3L)*kto2*, which uncovers the *pipe* locus. Oligonucleotide primers were generated that permitted PCR amplification of each of the six exons that constitute the Pipe-ST2 ovary-specific isoform. For each of the 11 alleles, exon-specific amplification products were purified and subjected to direct sequence analysis.

Staining of embryos with Alcian Blue

Stocks were constructed in which chromosomes with mutations of interest were carried in trans to balancers carrying insertions of *Krüppel*-Gal4 and UAS-GFP. Overnight collections of embryos were dechorionated in 50% bleach, then transferred to a glass plate and covered in hydrocarbon 27 oil (Sigma). Stage 12-16 embryos were collected and separated into groups containing fluorescent wild-type or non-fluorescent mutant embryos using a Leica MZFLIII dissecting microscope equipped for detection of GFP. Sorted embryos were transferred to a solution of 4% formaldehyde in PEMS buffer (0.1 M PIPES, 2 mM $MgSO_4$, 1 mM EGTA, pH 6.9):heptane (4.5 ml:5 ml) and fixed for 20 minutes with shaking. Following fixation, the lower phase containing fixative was aspirated. Methanol (5 ml) was then added and the embryos shaken vigorously for 1 minute to remove vitelline membranes from the embryos. The devitellinized embryos were then rinsed several times with methanol and stored in methanol at –20°C.

Alcian Blue is a cationic histochemical stain that has been used extensively for the in situ detection of sulfated molecules (Scott et al., 1964; Scott and Dorling, 1965; Goso and Hotta, 1994; Schumacher and Adam, 1994). For Alcian Blue staining, fixed embryos were incubated for 30 minutes each in 70% methanol:30% PBT (PBS containing 0.1% Tween-20), 50% methanol:50% PBT, 30% methanol:70% PBT, and finally PBT. The PBT was then aspirated and the embryos were resuspended in a solution of 0.00125% Alcian Blue-tetrakis (Methyl-Pyradinium) chloride in 0.3 $MgCl_2$, 0.1 M sodium acetate (CH_3COONa) (pH 5.8). Following staining overnight, the

staining solution was aspirated and embryos were destained for several hours in a solution of 0.7 M $MgCl_2$, 0.1 M sodium acetate (pH 5.8).

Generation of P-element transformants expressing PAPS synthetase

A plasmid carrying a full-length cDNA encoding *Drosophila PAPS synthetase* (Jullien et al., 1997) cloned in pBS (SK^-) was obtained from Genome Systems (St Louis, MO 63134). The *PAPS synthetase* (*papss*) cDNA was excised and subcloned into phs-CaSPer (Bang and Posakony, 1992) at the unique *Xba*I site downstream of the *hsp70* promoter. Transgenic lines carrying phs-CaSPeR-*papss* were generated by conventional microinjection (Rubin and Spradling, 1982) with a P-element transposase-expressing helper plasmid.

Immunostaining and in situ hybridizations

A peptide of the sequence AFKYRRIPYPKRSVE, corresponding to amino acid residues 9-23, which are common to all Pipe isoforms, was synthesized by SynPep Corporation and purified by HPLC. Peptide (5.0 mg) was conjugated to Keyhole limpet hemocyanin using glutaraldehyde as a crosslinking reagent. Antibodies directed against the immunogen were generated in a rabbit at Covance Research Products. Staining of embryos was carried out according to Macdonald and Struhl (Macdonald and Struhl, 1986) using antibody preabsorbed against wild-type embryos at a dilution of 1:1000. The rabbit polyclonal antibody directed against the Windbeutel protein (Ma et al., 2003) was used at a dilution of 1:2000. Primary antibodies were used in conjunction with a biotinylated goat anti-rabbit secondary antibody (1:500 diluted, pre-absorbed against wild-type embryos) and visualized with avidin/HRP complex (Vector Laboratories).

To examine *pipe-ST2* RNA expression in $pipe^{C14}/pipe^{C14}$ embryos, we balanced the $pipe^{C14}$ mutation over TM3, Sb, *Krüppel*-Gal4, UAS-GFP. Progeny embryos from this stock were sorted into fluorescent (wild-type) and non-fluorescent ($pipe^{C14}/pipe^{C14}$) groups. Sorted embryos were then subjected to whole-mount in situ hybridization (Tautz and Pfeifle, 1989) using a digoxigenin-labeled DNA probe synthesized using a DNA fragment corresponding to the full-length, mature *pipe*-ST2 cDNA, excised as an *Eco*RI/*Xho*I fragment from the plasmid pBluescriptSK-*pipe*-ST2. In situ hybridization to ovaries (Hong and Hashimoto, 1995) was carried out using the same DNA fragment.

Generation of follicle cell and germ-line clones

To test whether follicle cell expression of genes previously implicated in the synthesis or modification of GAGs is required maternally for embryonic DV patterning, we generated follicle cell clones that were homozygous for mutations in genes of interest by FLP/FRT-mediated site specific recombination (Golic and Lindquist, 1989). Clones were generated in females carrying a mutation-bearing FRT chromosome in trans to an FRT-bearing, but otherwise wild-type chromosome. To identify embryos derived from follicles containing mutant clones, we used the marking system of Nilson and Schüpbach (Nilson and Schüpbach, 1998).

Embryos lacking both maternal and zygotic expression of *sgl*, *sfl*, *frc* and *papss* were generated using the dominant female-sterile technique of Chou and Perrimon (Chou and Perrimon, 1996).

Results

Analysis of the sequence of *pipe* mutant alleles

Most alleles of *pipe* are homozygous viable with the production of dorsalized embryos by mutant females being the sole phenotype. Two alleles are semi-lethal. In crosses to $Df(3L)pipe^{A13}$, $pipe^3$ and $pipe^{C14}$ produced only 21% and 33%, respectively, of the expected number of transheterozygous *pipe* mutant progeny. In addition, the viable transheterozygous *pipe* mutant flies eclosed an average of 3 days later than wild-type flies and were small (Fig. 2J). Interestingly, this phenotype was also observed for flies transheterozygous for mutations in *windbeutel* (*wind*) (Fig. 2K), which is believed to encode a chaperone that is necessary for correct localization and function of Pipe protein (Sen et al., 2000).

Deviations from the wild-type *pipe* sequence were identified in each of the 11 EMS-derived mutant alleles (Fig. 1B). In 10 of these mutations, the lesions identified were associated with Pipe-ST2 specific exons. The $pipe^3$ allele carries a stop codon in the third exon, which is common to all *pipe* isoforms. The $pipe^{C14}$ allele was identified in a screen for P-element mutations in the *pipe* locus. Although we have not identified the lesion associated with the $pipe^{C14}$ allele, we believe the mutation is in a 5′ regulatory region of the gene that affects all isoforms (see below).

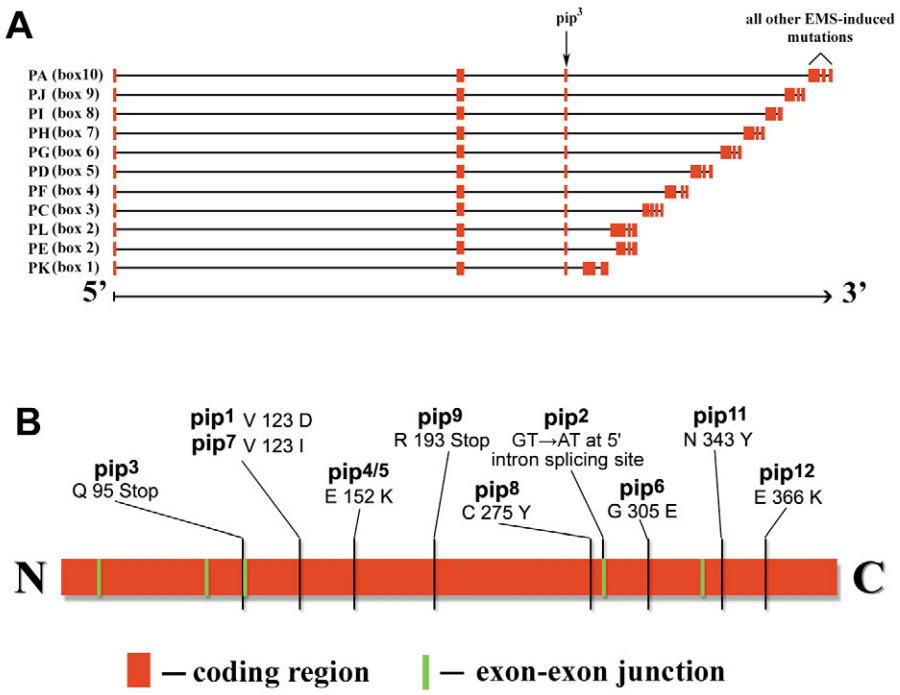

Fig. 1. Structure of the *pipe* locus and location of EMS-generated *pipe* mutations. (A) Intron structures of the various *pipe* isoforms are shown. On the far left are the isoform designations of the BDGP (PA-PK) and of Sergeev et al. (Sergeev et al., 2001). Box 10 is *pipeST2*. All mutations except $pipe^3$ are associated with *pipe*ST2-specific exons. $pipe^3$ is located in the third common exon. (B). Structure of the coding region of PipeST2 showing the location of the eleven EMS-generated mutations. Green vertical bars indicate the position of exon/exon boundaries.

These results suggest that Pipe-ST2 is specifically required in the ovary and that its loss does not affect viability. At least some of the other isoforms, however, are required for viability. In addition to the ovary, *pipe* is expressed in the embryonic salivary gland. Antibody staining and in situ hybridization demonstrated that in *pipe*^{C14} mutants, neither *pipe* RNA nor protein were detectable in either the ovary or the embryonic salivary gland (Fig. 2B,E,H). This finding is consistent with the idea that the *pipe*^{C14} mutation affects the transcription of all of the *pipe* isoforms. These results suggest that Pipe is required for salivary gland development or function, which may explain the effect of *pipe*^{C14} on viability.

Both *pipe*^{1} and *pipe*^{7} affect valine 123 (Fig. 1B), which is located within a stretch of amino acids extending from residues 120 to 127, PKG**V**SQTF, that is predicted to be within the binding site for PAPS, the high energy, small molecule donor in sulfation reactions. In the strong *pipe*^{1} allele, the nonconservative substitution of an aspartic acid residue for valine results in an apparently nonfunctional protein. Embryos from females carrying this allele are completely dorsalized (Fig. 3G,H). In *pipe*^{7}, however, the relatively conservative substitution of isoleucine for valine results in a hypomorphic allele. Females carrying *pipe*^{7} in trans to a deficiency uncovering *pipe* produce embryos that are only weakly dorsalized (Fig. 3D) and exhibit residual polarity during gastrulation (Fig. 3C), indicating that the protein retains considerable activity.

The location of the *pipe*^{7} mutant lesion within the putative PAPS-binding site suggested that its weak phenotype might result from a lowered affinity of the *pipe*^{7}-encoded protein for PAPS. To test this hypothesis, we fed *pipe*^{7}/*Df*(3L)*pipe*^{A13} flies yeast containing 1 M sodium chlorate, a compound known to inhibit the activity of PAPS synthetase (Lansdon et al., 2004; Baeuerle and Huttner, 1986; Greve et al., 1988). We reasoned that if the *pipe*^{7} mutant protein has reduced affinity for PAPS, then under conditions of decreased PAPS availability, *pipe*^{7}/*Df*(3L)*pipe*^{A13} females would be expected to produce relatively more dorsalized progeny than untreated *pipe*^{7}/*Df*(3L)*pipe*^{A13} females. Indeed, 94% of the cuticles (n=192) of the embryonic progeny of treated *pipe*^{7}/*Df*(3L)*pipe*^{A13} females exhibited a completely dorsalized D0 cuticular phenotype (Roth et al., 1991) and apolar gastrulation movements (Fig. 3E,F; Table 1). By contrast, the dorsalized D0 phenotype was exhibited by only 1% of the progeny of untreated *pipe*^{7}/*Df*(3L)*pipe*^{A13} females (Table 1). Wild-type flies fed sodium chlorate do not produce dorsalized progeny, which implies that the activity of the wild-type PipeST2 protein is not detectably affected by the sodium chlorate-induced decrease in PAPS availability, at least as measured by embryonic DV patterning. Our finding that the *pipe*^{7} mutant protein is sensitive to the concentration of PAPS, however, is consistent with our designation of Pipe-ST2 as a sulfotransferase.

Alcian Blue-staining material in embryonic salivary glands is dependent on the activities of Pipe and Windbeutel

To investigate whether sulfated molecules with the properties of heparan sulfate GAGs are present in the salivary gland, we carried out a histochemical analysis of embryos at various stages of development using the Alcian Blue histochemical stain. We observed intense staining associated with the lumen of the developing salivary glands (Fig. 4A,B), indicating that the staining material is likely to represent a secreted or membrane-bound sulfated molecule.

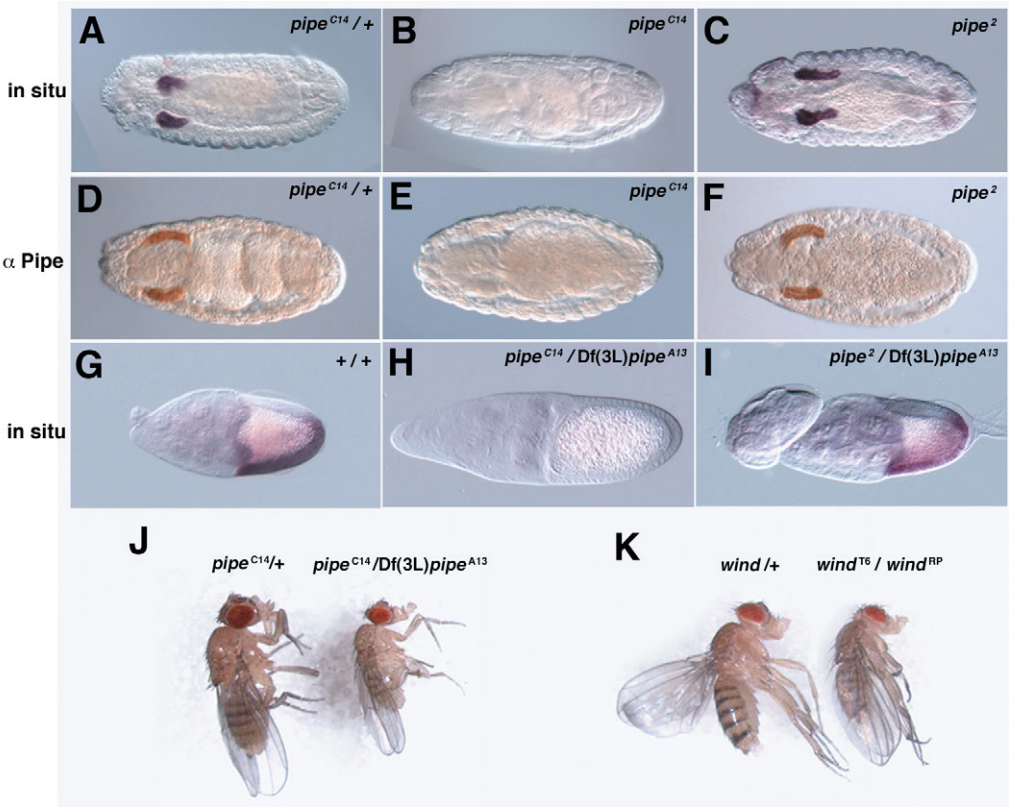

Fig. 2. The *pipe*^{C14} allele leads to the loss of *pipe* RNA and protein expression in the ovary and the embryo. (A-C) In situ hybridization to a wild-type embryo (A) and to embryos homozygous for *pipe*^{C14} (B) and *pipe*^{2} (C). A *pipe*-ST2-specific probe was used. (D-F) An antibody directed against a peptide sequence present in the N terminus of all Pipe isoforms was used for immunostaining of wild-type (D), *pipe*^{C14} homozygous (E) and *pipe*^{2} homozygous (F) embryos. (G-I) In situ hybridization to stage 10 egg chambers from wild-type (G), *pipe*^{C14}/*Df(3L)pipe*^{A13} (H) and *pipe*^{2}/*Df(3L)pipe*^{A13} (I) females using *pipe*-ST2-specific sequences as a probe. (J) *pipe*^{C14}/*Df(3L)pipe*^{A13} flies exhibit a growth defect relative to their heterozygous siblings. Similarly, *wind*^{T6}/*wind*^{RP} flies (K) are smaller than their *wind*/+ siblings. Both results suggest that zygotic expression of Pipe isoforms is required for normal growth.

Table 1. Cuticular phenotypes of embryos derived from untreated and sodium chlorate-treated *pip*^{7}*/Df* females

	D0	D1	D2	D3
pip^{7}*/Df* untreated (*n*=168)	28	94	41	5
pip^{7}*/Df* treated with sodium chlorate (*n*=192)	180	6	4	2

Embryonic phenotypes are scored according to Roth et al. (Roth et al., 1991): D0, completely dorsalized; D1, Filzkörper but no ventral denticles; D2, Fizkörper and ventral denticle bands of narrow width; D3, twisted or tail-up phenotype.

Although only one of the ten *pipe* isoforms, Pipe-ST2, appears to function in the follicle cell layer and to be required for embryonic DV patterning, multiple isoforms from the *pipe* locus are expressed in the salivary gland (Sergeev et al., 2001). As noted above, the *pipe*^{C14} and *pipe*^{3} mutations affect all of the Pipe isoforms, whereas the other 10 alleles examined, including the *pipe*^{2} allele, specifically affect the Pipe-ST2 isoform. We examined *pipe*^{C14}*/pipe*^{C14}, *pipe*^{3}*/pipe*^{3} and *pipe*^{2}*/pipe*^{2} mutant embryos for Alcian Blue staining in their salivary glands. We detected no Alcian Blue staining in the salivary glands of *pipe*^{C14}*/pipe*^{C14} and the *pipe*^{3}*/pipe*^{3} mutant embryos (Fig. 4D,F). By contrast, the salivary glands of *pipe*^{2}*/pipe*^{2} mutant embryos did exhibit Alcian Blue staining (Fig. 4G). These findings suggest that *pipe* activity is required for the presence of a sulfated molecule in the embryonic salivary glands. Furthermore, although the Pipe-ST2 isoform is required for the maternal function of *pipe*, our data suggest that the expression of other Pipe isoforms in the embryonic salivary gland is sufficient for the production of the Alcian Blue staining material.

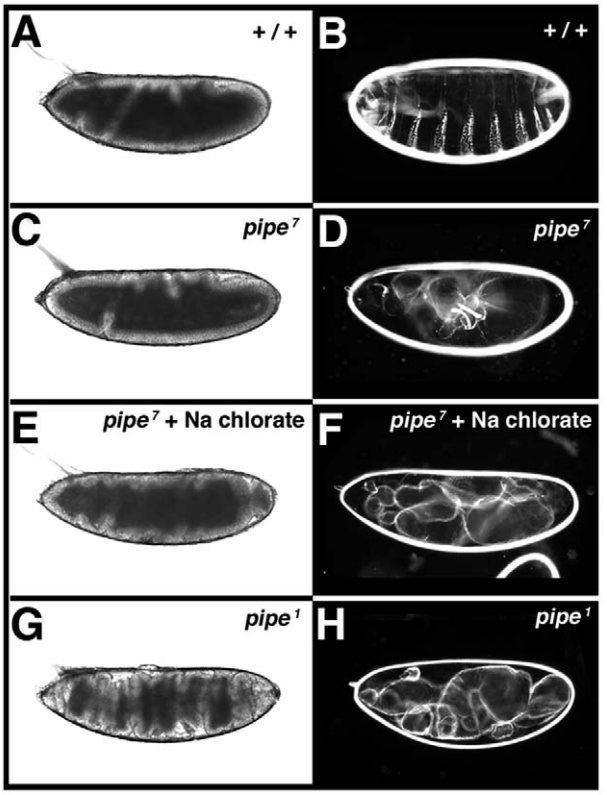

Fig. 3. Sodium chlorate influences embryonic dorsoventral patterning of the embryonic progeny of *pipe*^{7} mutant females Gastrulation patterns (A,C,E,G) and embryonic cuticles (B,D,F,H) of embryos produced by the following females: (A,B) Oregon R, (C,D) *pipe*^{7}*/Df(3L)*^{A13}untreated, (E,F) *pipe*^{7}*/Df(3L)*^{A13} treated with sodium chlorate, (G,H) *pipe*^{1}*/Df(3L)*^{A13}.

PAPS synthetase is required for the production of the Alcian Blue staining material in the salivary gland

To confirm that the Alcian Blue-stained material observed in the salivary gland represented a sulfated macromolecule, we investigated whether the presence of the material required the activity of PAPS synthetase. The gene encoding *Drosophila* PAPS synthetase (*papss*; *Paps* – FlyBase) has previously been cloned and mapped to polytene chromosomal interval 76C (Julien et al., 1997). We obtained mutant alleles representing 15 zygotically lethal complementation groups that mapped to the polytene interval 76B-D from Dr J. Kennison. We tested flies heterozygous for mutant alleles of each of the 15 complementation groups that had been identified within the

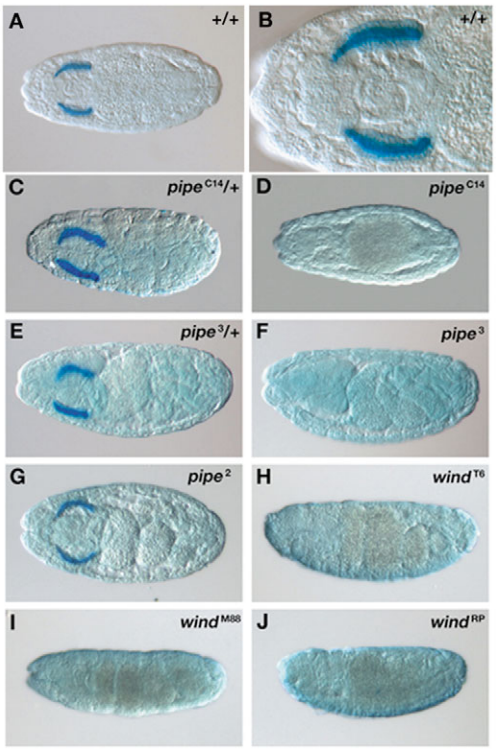

Fig. 4. Pipe and Windbeutel are required for Alcian Blue staining of embryonic salivary glands. (A) A stage 16 wild-type embryo stained with Alcian Blue. (B) The same embryo at higher magnification. Staging is according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985). Embryos heterozygous (C) and homozygous (D) for *pipe*^{C14} were stained with Alcian Blue, as were embryos heterozygous (E) and homozygous (F) for *pipe*^{3}. Embryos homozygous for the RNA null allele *pipe*^{C14} or for the protein null allele *pipe*^{3} failed to stain with Alcian Blue. By contrast, embryos homozygous for *pipe*^{2}, which affects only the Pipe-ST2 isoform, stain with Alcian Blue (G). Embryos homozygous for *wind*^{T6} (H), *wind*^{M88} (I) or *wind*^{RP} (J) do not produce Alcian Blue-staining material in their salivary glands.

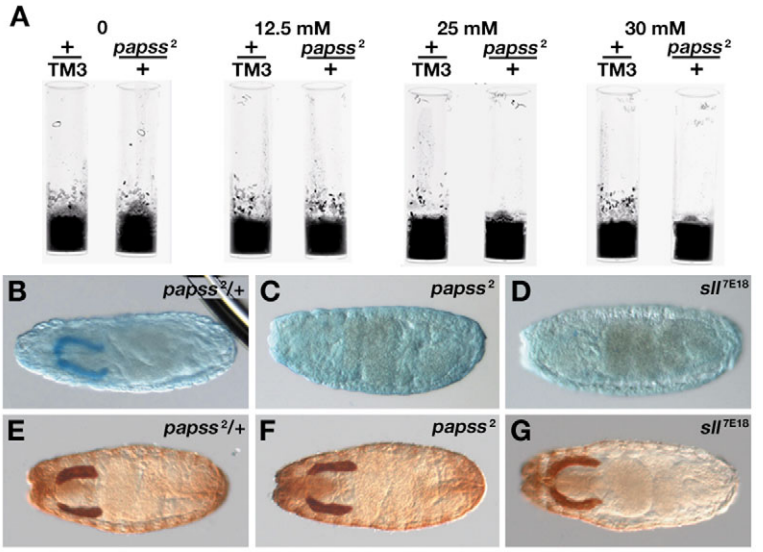

Fig. 5. Alcian Blue-staining in the embryonic salivary gland requires the activity of genes involved in biological sulfation. (A) Mutations affecting *papss* were identified by their increased sensitivity to the lethal effects of sodium chlorate. Concentrations of sodium chlorate are shown at top, as are the parental genotypes. At 25 mM and 30 mM sodium chlorate, few *papss*/+ pupae are present on the sides of the vials. (B-D) Embryos stained with Alcian Blue. Although *papss*^{2}/+ embryos stain with Alcian Blue (B), embryos homozygous for *papss*^{2} lack Alcian Blue salivary gland staining (C), as do embryos homozygous for *sll*^{7E18} (D). (E-G) Staining of embryonic salivary glands with an antibody against Windbeutel. The salivary glands of an embryo heterozygous for *papss*^{2} stain for Windbeutel (E), as do the salivary glands of *papss*^{2} (F) and *sll*^{7E18} (G) mutant embryos.

76B-D polytene chromosomal interval for their ability to produce progeny on food containing various concentrations (0-100 mM) of sodium chlorate. In comparison with wild-type flies, and with flies heterozygous for mutations in other genes in the interval, flies heterozygous for mutations in *l(3)76BDn* exhibited increased sensitivity to food containing sodium chlorate (Fig. 5A). To confirm that the *l(3)76BDn* locus corresponds to the *papss* gene, flies were transformed with a P-element vector carrying the *papss* cDNA (phs-CaSPeR-*papss*). This transgene rescued the lethality associated with homozygosity or transheterozygosity for alleles of *l(3L)76BDn*.

Embryos homozygous for mutations in *papss* did not exhibit Alcian Blue staining (Fig. 5C). We also examined embryos mutant for *sll*, which encodes the PAPS Golgi transporter, and demonstrated that they, too, also lack lumenal Alcian Blue-stained material in their salivary glands (Fig. 5D). These results support the idea that the stained material is sulfated, that it is generated in the Golgi, and that it is likely to correspond to a cell surface or secreted molecule. To ensure that our inability to detect Alcian Blue staining in these embryos resulted specifically from a failure to produce the material, and not from a lack of salivary glands altogether, we confirmed that the salivary glands of homozygous *papss*/*papss* and *sll*/*sll* embryos were present by visualizing them with immunostaining for Wind, which is expressed strongly in embryonic salivary glands (Fig. 5E-G).

wind encodes a homologue of the vertebrate endoplasmic reticulum protein Erp29 (Konsolaki and Schüpbach, 1998), and we have previously shown that Wind protein is required for the correct subcellular localization of the Pipe protein to the Golgi apparatus (Sen et al., 2000). As observed for the *pipe*^{C14}/*pipe*^{C14} and *pipe*^{3}/*pipe*^{3} mutant alleles, embryos homozygous for all three of the *wind* alleles tested lacked Alcian Blue staining (Fig. 4H-J). The most straightforward interpretation of these results is that the Pipe isoforms expressed in the salivary gland function as sulfotransferases that are directly involved in the formation of the Alcian Blue-staining material. Wind protein is likely to be required for the Golgi localization, and therefore the function, of all Pipe isoforms. Embryos homozygous for mutations in the dorsal group genes *nudel* (*ndl*), *gastrulation defective*, *snake*, *easter* and *spätzle* exhibited normal Alcian Blue staining, (data not shown), demonstrating that dorsal group genes other than Pipe and Wind are not required for the production of this material. These results are consistent with the idea that the staining material is a direct product of the catalytic activity of one or more of the Pipe isoforms.

Mutations in genes encoding GAG synthesis proteins do not affect Alcian Blue staining

The similarity between Pipe-ST2 and HS2ST suggested that the Alcian Blue-staining material in embryonic salivary glands might represent a heparan sulfate GAG. If so, we would expect that mutations in genes previously shown to be involved in the synthesis or modification of heparan sulfate would also affect Alcian Blue staining in the embryonic salivary glands. We therefore assayed for the presence of Alcian Blue-staining material in the salivary glands of embryos homozygous for mutations in the following genes: *sugarless* (*sgl*) (Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997) encodes the fly homologue of UDP-glucose-6 dehydrogenase, which converts UDP-glucose to UDP-glucuronic acid, a required step in the synthesis of the uronic acid residues present in heparan sulfate; *sulfateless* (*sfl*) encodes a protein with similarity to vertebrate N-deacetylase/N-sulfotransferases (Lin and Perrimon, 1999), which are known to mediate deacetylation and sulfation of the N-acetyl group on N-acetylglucosamine (GlcNac) of heparan sulfate; and *fringe connection* (*frc*), which encodes a Golgi transporter that is required for the Golgi uptake of nucleotide-sugars involved in the synthesis of heparan sulfate (Goto et al., 2001; Selva et al., 2001).

In contrast to embryos mutant for *papss*, Alcian Blue staining was clearly evident in embryos homozygous for mutations in *sgl*, *sfl* and *frc* (data not shown), suggesting that this stained substance does not represent a conventional heparan sulfate GAG. However, the segment polarity phenotypes that allowed the initial identification of mutations in *sgl, sfl* and *frc* are only observed in zygotically mutant embryos that are derived from mutant germline clones (Perrimon et al., 1994). This raised the

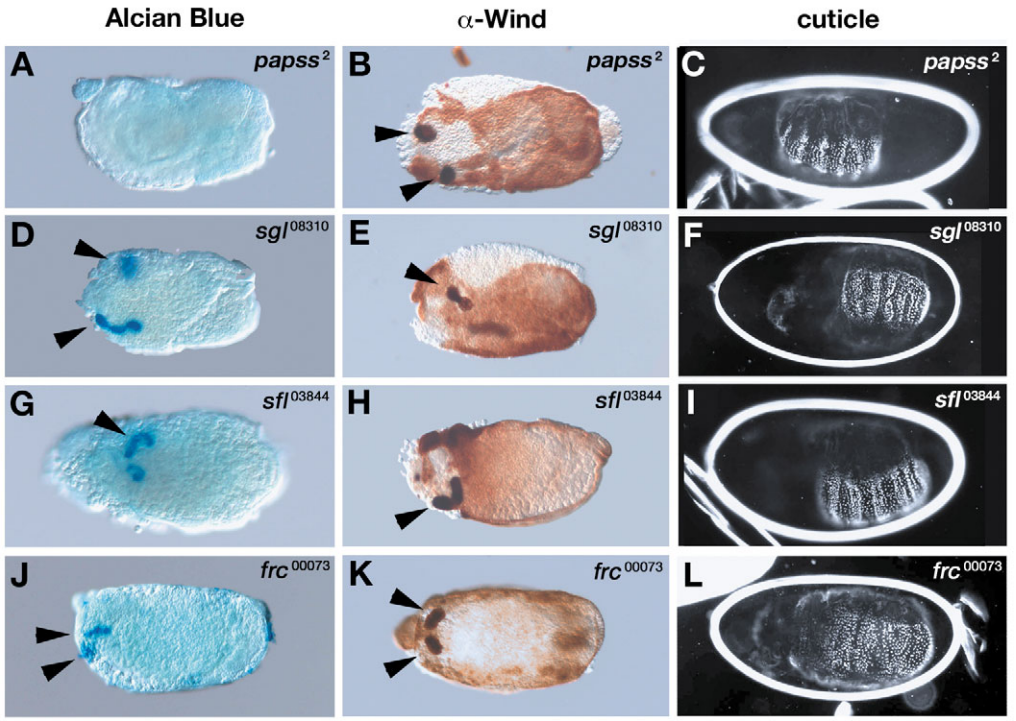

Fig. 6. The Alcian Blue-stained material in the embryonic salivary glands requires *papss* but not the expression of GAG-related genes. FLP-FRT-mediated recombination and the dominant female-sterile technique were used to generate adult females with germline clones homozygous for *papss* (A-C), *sgl* (D-F), *sfl* (G-I) and *frc* (J-L). For each of the four genes, embryos lacking both maternal and zygotic expression exhibited a typical segment polarity phenotype (C,F,I,L). Staining with an antibody directed against Windbeutel demonstrated that these embryos nevertheless developed salivary gland tissue (arrowheads in B,E,H,K). The salivary glands of embryos lacking both maternal and zygotic expression of *papss* failed to stain with Alcian Blue (A), but the salivary glands of embryos lacking maternal and zygotic expression of *sgl* (D), *sfl* (G) and *frc* (J) did stain.

possibility that maternal loading of transcripts might provide sufficient levels of protein expression to enable homozygous mutant embryos to produce Alcian Blue-stained material. To address this issue, we generated embryos lacking both maternal and zygotic expression of *sgl, sfl* and *frc*. Although cuticles of these embryos exhibited a typical segment polarity phenotype (Fig. 6F,I,L), embryos lacking both maternal and zygotic function of these three genes did exhibit Alcian Blue staining in structures that appeared to be salivary glands (Fig. 6D,G,J). Staining with an antibody against Wind confirmed that the Alcian Blue-stained structures corresponded to salivary glands (Fig. 6E,H,K). In contrast to *sgl*, *sfl* and *frc*, the salivary glands of embryos lacking both maternal and zygotic expression of *papss* failed to stain with Alcian Blue (Fig. 6A), even though Windbeutel staining demonstrated the presence of the salivary glands (Fig. 6B). Based on its central role in sulfation reactions, embryos derived from germline clones mutant for *papss* would also be expected to exhibit a segment polarity phenotype because of the loss of heparan sulfate, which was observed (Fig. 6C). Taken together, these observations indicate that the production of the Pipe-dependent Alcian Blue-stained material in the embryonic salivary glands does not require the function of genes known to be involved in heparan sulfate GAG synthesis.

Females carrying follicle cell clones mutant for genes involved in heparan sulfate synthesis do not produce dorsalized progeny

Our collective findings indicated that heparan sulfate GAGs do not represent the target of Pipe in the salivary glands. To extend these results, we tested whether heparan sulfate GAGs participate in the maternal function of *pipe*. If so, we would expect females carrying follicle cells mutant for genes involved in GAG synthesis to produce dorsalized embryos. As has been reported previously (Nilson and Schüpbach, 1998), we observed that the generation of ventral follicle cells mutant for *pipe* led to the production of dorsalized embryos (Fig. 7A,B).

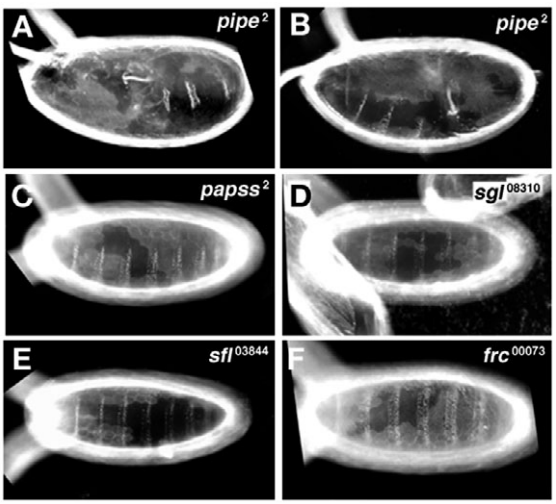

Fig. 7. GAGs are not required in the ovarian follicle cell layer for embryonic DV polarity. The technique of Nilson and Schüpbach (Nilson and Schüpbach, 1998) was used to generate marked mutant follicle cells whose position in the follicle layer could be determined by their altered chorion imprints. In all cases shown, mutant clones were located ventrally in the follicle cell layer, and are identifiable by the more transparent appearance of the chorion imprints in comparison to those made by wild-type cells. (A,B) Ventral clones of *pipe* mutant cells led to the production of embryos that were partially dorsalized, as indicated by the tail-up phenotype (A,B) and the narrow ventral denticle bands (A). By contrast, ventral follicle cells mutant for *papss* (C), *sgl* (D), *sfl* (E) and *frc* (F) did not lead to the production of dorsalized embryos.

In contrast to *pipe*, large ventral follicle cell clones homozygous for mutations in *papss* (Fig. 7C), *sgl* (Fig. 7D), *sfl* (Fig. 7E) and *frc* (Fig. 7F) did not lead to the production of dorsalized progeny.

Discussion

Although the *pipe* locus encodes ten different protein isoforms, our analysis of *pipe* mutant alleles indicates that the Pipe-ST2 isoform is uniquely required for embryonic DV patterning. Ten out of the 11 EMS-generated alleles that we characterized at the molecular level specifically affect the Pipe-ST2 isoform. Females homozygous for these mutations produce dorsalized embryos, implying that the function of Pipe-ST2 in the follicle cells is essential for the establishment of the DV axis in the embryo. Sergeev et al. (Sergeev et al., 2001) reported the expression of a second Pipe isoform (Box 7, according to their terminology) in ovarian follicle cells and proposed that several protein isoforms from the *pipe* locus contribute to DV patterning. Sergeev et al. (Sergeev et al., 2001) also carried out a nucleotide sequence analysis of genomic DNA isolated from flies carrying the *pipe* mutant alleles: $pipe^{1}$, $pipe^{2}$, $pipe^{3}$, $pipe^{4}$, $pipe^{5}$, $pipe^{6}$ and $pipe^{12}$. In contrast to our findings, their analysis failed to detect nucleotide sequence changes associated with *pipe*-coding regions in any of the *pipe* mutant backgrounds. Although we cannot explain the basis for this discrepancy, several observations suggest that we have indeed identified the nucleotide lesions responsible for the *pipe* mutant phenotypes. All of the identified mutations that affect only the Pipe-ST2 isoform are viable in trans to a deficiency. The $pipe^{3}$ mutation, which affects all Pipe isoforms, is semi-lethal, suggesting a distinct requirement for other Pipe isoforms. The hypomorphic $pipe^{7}$ mutation is associated with the relatively conservative change of valine to isoleucine within a domain that is predicted to be the binding site for the 5′ phosphosulfate of PAPS (Kakuta et al., 1998). This result is consistent with the prediction that the $pipe^{7}$ mutation alters the affinity of the mutant Pipe protein for PAPS and that this isoform alone is essential for embryonic DV polarity.

Our finding that the $pipe^{7}$ mutant phenotype is significantly enhanced by sodium chlorate treatment strongly supports the identification of Pipe as a sulfotransferase. This identification is further bolstered by our demonstration that the presence of a Pipe-dependent Alcian Blue-stained material in the embryonic salivary glands requires the function of two other genes essential for the sulfotransferase reaction: *slalom*, which encodes the *Drosophila* PAPS Golgi transporter; and *papss*, the PAPS synthetase gene. The finding that embryos mutant for *pipe*, *slalom* or *papss* all lack Alcian Blue staining in their salivary glands is strong evidence that the stained material represents a sulfated macromolecule.

The original molecular identification of Pipe as a putative sulfotransferase was made on the basis of its similarity to HS2ST. Consequently, it has been assumed that heparan sulfate is the likely substrate of Pipe activity. We reasoned that if Pipe acts as a heparan sulfate sulfotransferase, then the presence of the Alcian Blue-stained material in the embryonic salivary glands would be dependent upon the activity of genes whose products have been demonstrated to participate in heparan sulfate synthesis and modification in *Drosophila*. In contrast to this expectation, we found Alcian Blue staining to be present in the salivary glands of embryos mutant for *sgl*, *sfl* or *frc*.

We used a similar strategy to investigate the possibility that heparan sulfate is the target of Pipe activity in the ovary. We anticipated that genes encoding products involved in the sulfotransferase reaction, or in the synthesis of the Pipe substrate, would be required in the ventral follicle cells. Females carrying follicle cell clones mutant for *pipe* (Nilson and Schüpbach, 1998) (this work) or *slalom* (Lüders et al., 2003) produce embryos with a dorsalized phenotype. By contrast, embryos derived from females carrying ventral clones of follicle cells mutant for *sgl*, *sfl* or *frc* exhibited normal DV polarity. This suggests that like the Alcian Blue-stained material in the embryonic salivary glands, the target of Pipe function in the ovary does not correspond to heparan sulfate.

Surprisingly, females carrying *papss* mutant follicle cell clones did not produce dorsalized embryos. Although this result could be interpreted as an argument against Pipe acting as a sulfotransferase in the ovary, we do not believe this to be the explanation. Because PAPS, the product of PAPS synthetase activity, is a small molecule (507 Da), it may be able to pass through the gap junctions that exist between the oocyte and follicle cell layer (Giorgi and Postlethwait, 1985; Bohrmann and Haas-Assenbaum, 1993; Waksmonski and Woodruff, 2002). Gap junctions are known to allow passage of molecules of approximately 1 kDa in mass (Goldberg et al., 2004), which would permit passage of PAPS from a wild-type oocyte into mutant follicle cells. Another gene whose mutant alleles may behave nonautonomously for the same reason is *sgl*, which encodes UDP-glucose dehydrogenase. The product of Sugarless activity, UDP-glucuronic acid, is also a small molecule (577 Da) that may be capable of passing through gap junctions. Although the result for *sgl* mutant follicle cell clones may therefore be inconclusive, neither *sfl* nor *frc* mutations would be expected to exhibit nonautonomous behavior. *sfl* encodes N-deacetylase/N-sulfotransferase, a Golgi resident enzyme of Type II transmembrane topology. The product of Sfl activity, sulfated heparan sulfate, is too large to move between cells through gap junctions. The product of *frc* mediates the uptake into the Golgi of nucleotide sugars required for GAG synthesis and thus could not be rescued nonautonomously. Therefore, the finding that females carrying ventral follicle cell clones of *sfl* or *frc* did not give rise to dorsalized embryos provides the strongest evidence that heparan sulfate plays no role in the function of Pipe in embryonic DV patterning.

Although *sgl* mutations may behave nonautonomously in the ovary, this explanation cannot be invoked to explain the lack of effect of *sgl* mutations on the Alcian Blue staining in the embryonic salivary glands. Because these embryos were both maternally and zygotically mutant for *sgl*, there would be no wild-type cells present to supply UDP-glucuronic acid to the *sgl* mutant cells. By contrast, even though a role for *papss* could not be demonstrated in the ovary because of the possibility of nonautonomous rescue, its function was clearly necessary for the formation of the Pipe-dependent Alcian Blue-stained material in the embryonic salivary glands.

In addition to heparan sulfate, the ability of the Alcian Blue-stained material to form in the absence of *sgl* activity also rules out the possibility that Pipe is involved in the sulfation of dermatan/chondroitin sulfate, at least in that tissue. This is because UDP-glucuronic acid, the product of Sugarless

activity, is required not only for the synthesis of heparan sulfate, but also for the synthesis of dermatan/chondroitin sulfate polysaccharide chains. Two other pieces of evidence also argue against a role for Pipe in the sulfation of either heparan sulfate or dermatan/chondroitin sulfate GAGs. First, expression in the follicle cell layer of cDNAs corresponding to hamster HS2ST and the human dermatan/chondroitin sulfate 2-O-sulfotransferase failed to rescue the dorsalized phenotypes of the progeny of *pipe*/*pipe* mutant females (Z. Zhang and D.S., unpublished). The *Drosophila* genome contains another gene, CG10234, that encodes a protein that is much more similar to vertebrate HS2ST than are the Pipe isoforms (http://flybase.bio.indiana.edu/); the product of this gene is likely to represent the bona fide *Drosophila* heparan sulfate 2-O sulfotransferase. Second, we have not been able to detect heparan sulfate sulfotransferase or dermatan/chondroitin sulfate sulfotransferase activity in vitro using Pipe-ST2 protein expressed in cell culture (A. Amiri and D.S., unpublished). Although our data argue against a role for Pipe in the sulfation of uronic acid residues in heparan sulfate, the similarity of the Pipe isoforms to heparan sulfate 2-O sulfotransferase and dermatan/chondroitin sulfate 2-O-sulfotransferase suggests that Pipe acts on the 2-O position of a monosaccharide component of an as yet unidentified glycoprotein or glycolipid.

The existence of multiple Pipe isoforms is an intriguing feature of the *pipe* gene in *Drosophila melanogaster*. Blast analysis of the *D. pseudobscura* genome (http://flybase.net/blast/) indicates that multiple isoforms of Pipe exist in that species as well. By contrast, only a single Pipe isoform is encoded in the mosquito (http://www.anobase.org/cgi-bin/blast.pl) and flour beetle (http:bioinformatices.ksu.edu/blast/blast.html) genomes. Similarly, only a single Pipe isoform was detected in a database of silk moth ESTs (http://papilio.ab.a.u-tokyo.ac.jp/silkbase/index.html). In each of these three organisms, the single Pipe isoform exhibits strong sequence similarity to *Drosophila* Pipe-ST2. It therefore appears likely that only the Pipe-ST2 isoform was present in the common ancestor of true flies, mosquitoes, moths and beetles. This suggests that the ancestral role of the *pipe* gene was to act during oogenesis to regulate embryonic DV patterning. Multiple Pipe isoforms were probably generated via genomic duplication in *Drosophila*, where they appear to be required for salivary gland development and/or function. Lack of Pipe activity in the salivary gland may lead to a disruption of the feeding behavior of the larvae, which in turn reduces their growth rate and viability. The generation and expression of multiple protein isoforms may be a mechanism to produce extremely high levels of Pipe protein, if each isoform has a similar enzymatic specificity. Alternatively, each isoform may have a distinct substrate specificity that contributes uniquely to salivary gland development and/or function.

The elucidation of Pipe-ST2 function is crucial to understanding the spatial regulation of the serine protease cascade whose ventrally restricted activity defines embryonic DV polarity. The simplest model of Pipe action posits that Pipe-ST2 functions as a sulfotransferase, and that the target of Pipe must be sulfated in order to exert its function. Although the target of Pipe may be present throughout the follicle cell layer, it would be sulfated only in the ventral follicle cells and following its secretion it would be deposited into the ventral side of the egg. There, it would assemble or activate the dorsal group serine protease cascade, leading to ventrally restricted processing of the Spätzle ligand. Although the specific targets of Pipe action in the follicle cell layer and the salivary gland may not be the same molecule, the general class of glycan on which Pipe acts in the two tissues is likely to be related. Current efforts are directed towards identifying these molecules and defining their roles in DV patterning and salivary gland function.

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